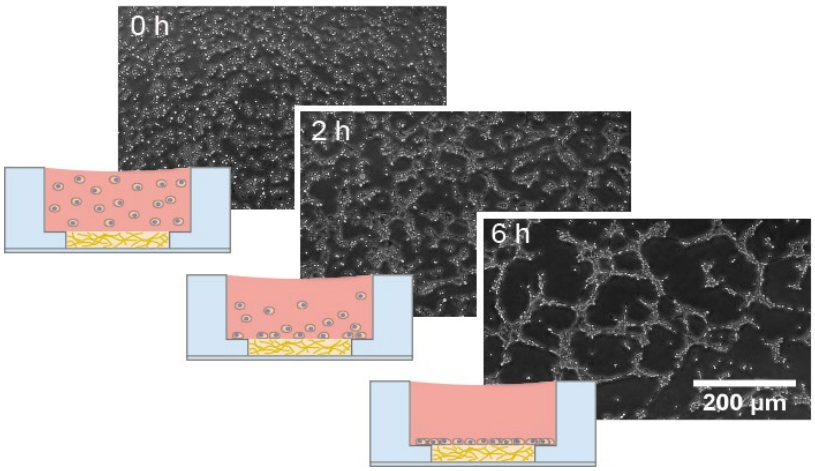
Optimizing Tube Formation Assays

The tube formation assay is a simple but powerful *in vitro* tool for screening substances for anti- or pro-angiogenic effects. These effects can be measured by different parameters, such as tube length or the number of loops formed on the gel surface.

The initial step usually involves creating a basement membrane-like gel matrix as a growth area (e.g., by using Laminin-Collagen I or Matrigel®). Next, the cells are seeded on the solidified gel matrix, followed by incubation and image data acquisition using a light microscope. Measuring tube formation parameters over time offers valuable data and can reveal changes in tube formation characteristics after adding anti- or pro-angiogenic substances.

Optimizing the experimental protocol and data acquisition method for tube formation assays is crucial for obtaining reliable and reproducible data, which is a prerequisite for proper analysis. This Application Note offers an overview of important assay parameters and guidelines for planning effective tube formation assays.



ibidi offers various solutions for tube formation assays:

- µ-Slide 15 Well 3D
- µ-Slide 15 Well 3D Glass Bottom
- µ-Plate 96 Well 3D
- µ-Plate 96 Well 3D Glass Bottom

Related Documents:

- Application Note 05: Tube Formation Assay in the µ-Plate 96 Well 3D (PDF)
- Application Note 19: Tube Formation Assay in the µ-Slide 15 Well 3D (PDF)
- Application Note 26: Preparation of Collagen I Gels (PDF)
- Application Note 66: Tube Formation Assay With Laminin-Collagen I Gel in the µ-Slide 15 Well 3D
- Application Note 70: Data Analysis of Tube Formation Assays (PDF)

1 Planning the Experiment

Before starting the tube formation and angiogenesis assays, it is essential to determine the optimal experimental setup through empirical testing. The chosen system should align closely with the research objectives and allow for feasible data collection. Therefore, establishing a strict protocol is crucial for generating reproducible and comparable data sets.

In tube formation and angiogenesis experiments, the critical factors include the types of cells, cell density, the growth medium, the gel matrix, and the data acquisition time points. These factors will be further discussed in the upcoming chapters.

Prior to conducting the experiment, calculate the required material, such as the amount of medium, gel matrix, substances, labware, and cell number. Other factors to consider are the laboratory equipment, space requirements, and time schedule. Every experiment should be carried out with the same cell culture and environmental parameters.

To perform a robust statistical analysis, we recommend at least four data points for each condition with a minimum of eight individual wells per condition. This results in a total minimum number of 32 individual experiments. The final number of experiments is dependent on the homogeneity of the data. This should be considered when calculating consumables, data acquisition, and data processing time.

1.1 Cell Type and Passage Number

Different cell types vary in size, growth behaviour (spreading, doubling rate), and in their demands for growth conditions. For example, the cell size determines the cell seeding number to adjust the cell density. Further, the composition of the growth medium can influence pro- or antiangiogenic effects.

Moreover, the passage number (P) can affect the behavior and functionality of primary cells, which are commonly used for tube formation assays. Cells at lower passages (<P6) are more physiologically relevant. However, at higher passages, the cells may experience a loss of proliferation capacity, altered gene expression, and morphological changes, ultimately affecting the results.

A well-established and reliable experimental setup uses human umbilical vein endothelial cells (HUVECs) at a low passage (<P6) grown in endothelial cell medium with no growth factors and 2% serum or less.

1.2 Medium Composition

The cell culture medium is an important factor in tube formation assays, providing key nutrients and influencing cell behavior. Choosing the optimal medium ensures cell health and an accurate representation of the biological processes. Factors such as growth factor concentration and serum content are crucial in this selection.

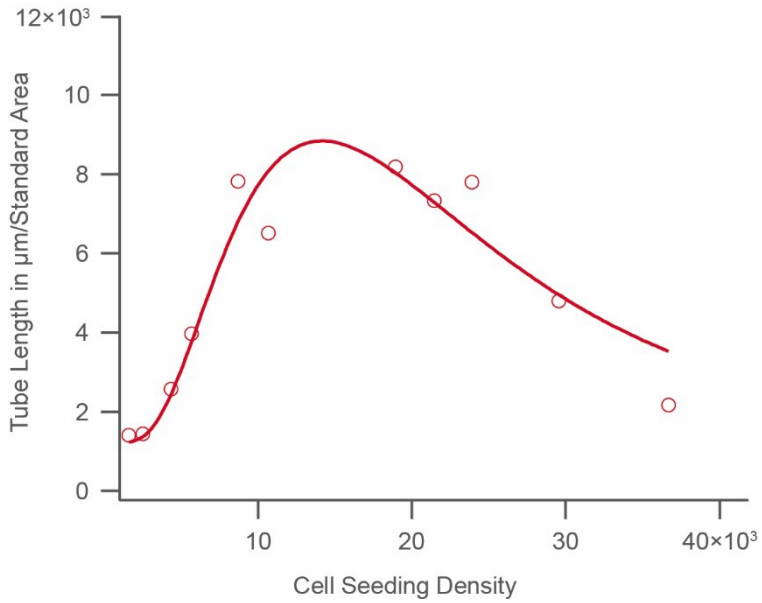
The addition of serum to the culture medium may influence tube formation behavior—in most cases, serum inhibits tube formation. To avoid this problem, different serum concentrations from 0–20% should be tested prior to the experiment, using the optimal cell density defined in Section 1.3. This will determine the optimal serum concentration for tube formation and cell survival.

1.3 Cell Density

The number of cells seeded on the gel surface is a crucial parameter for obtaining reliable results from a tube formation assay. 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 × 10^3 cells/ml. Then seed the cells onto the gel surface. After seeding, start recording phase contrast images with time intervals of 30 min. Ideally, use 3–5 replicates per cell concentration.

Evaluate the images as described section 3. Here, we recommend using the most common key parameter, the “total tube length”. Visualize the cell seeding density vs. tube length of one time point of your choice (e.g., after 4 hours) in a graph as shown below. The data will show a characteristic curve with a maximum value, which is the optimal seeding density for the used cell type. The example shown here uses HUVECs, for which the optimal seeding density was determined 10–20 × 10^3 cells/ml.



Exemplary seeding density response of HUVEC. The graph shows the analyzed tube length depending on the cell seeding density at 4 hours after seeding.

1.4 Positive and Negative Controls

Incorporating controls is crucial for meaningful, reliable results in tube formation experiments.

A positive control that stimulates tube formation should be used to establish a reference value for the response to your bioactive substances. To establish a positive control, choose an experimental setting where tube formation is assured (e.g., Matrigel® with reduced growth factors and serum-free medium for HUVECs). The positive control verifies the vital conditions of the cells and serves as a reference for any pro- or anti-angiogenetic substances.

The negative control ensures that tube formation can be inhibited in your cell culture experiment. To establish a negative control, choose a substance that is proven to have an inhibiting effect on tube formation development (e.g., suramin for HUVECs).

1.5 Gel Matrix

Gels used for tube formation assays serve as basement membrane-like (BM) surfaces to mimic native conditions *in vitro*. However, the biochemical and mechanical properties of the gels significantly influence cell behavior and ultimately the formation of the tubes.

State-of-the-art gels used for tube formation assays are Matrigel® or Laminin-Collagen I gels. Both gels have their advantages and disadvantages, which should be considered when planning the experiment.

Matrigel® is a native ready-to-use BM solution extracted from Engelbreth-Holm-Swarm mouse sarcomas. However, one disadvantage is the poorly defined and variable composition including growth factors of a cancerous environment. This results in fluctuations of the mechanical and biochemical properties between different batches, ultimately altering tube formation characteristics. Therefore, when using Matrigel®, all experiments should be conducted with the same Matrigel® batch.

In contrast, the two-component Laminin-Collagen I gels are considered to have a more defined matrix composition. However, it does not include all native components of a BM.

Detailed information about using Matrigel® and Laminin-Collagen I gels in tube formation assays are described in Application Note 19: Tube Formation Assay in the µ-Slide 15 Well 3D or Application Note 66: Tube Formation Assay With Laminin-Collagen I Gel in the µ-Slide 15 Well 3D.

2 Imaging

Imaging plays a pivotal role in tube formation assays, as it allows for the detailed observation and analysis of angiogenetic effects. Typically, phase contrast microscopy is employed to visualize cell dynamics without the need for staining. Tube formation assays can be either carried out as a time series to determine the dynamic behavior of formation or as an end-point experiment by fixing the cells after a certain time for imaging.

2.1 Choosing the Optimal Objectives

Since tube formation occurs over the entire surface area of the well, observing the largest possible field of view (FOV) is recommended to obtain the maximum amount of information. The FOV can be altered by changing the magnification of the objective—a low magnification using 4x to 10x objectives is adequate for most tube formation experiments.

Please keep in mind that the FOV correlates indirectly with the image resolution. Using a low magnification objective lens (2x–10x) will result in a large FOV with a low resolution. In contrast, using a high-magnification objective lens (20x or higher) will result in a lower FOV but with higher resolution.

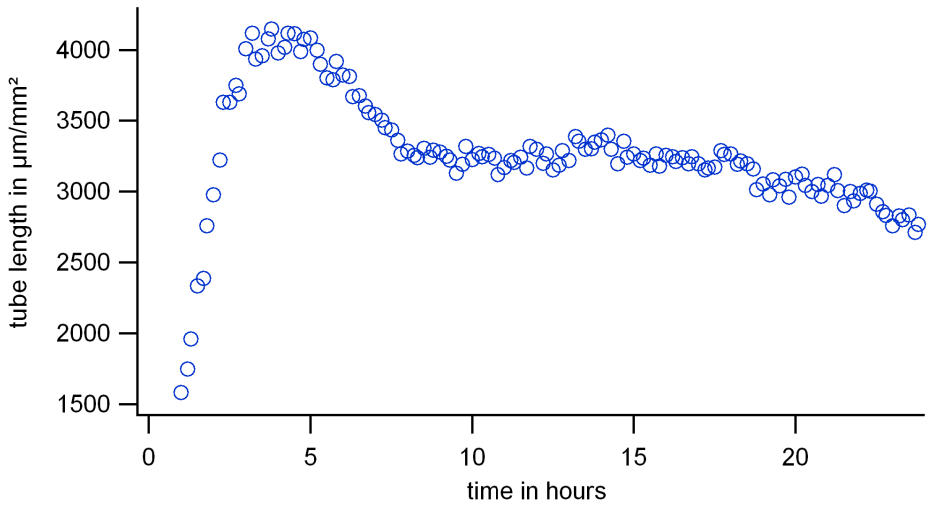
If a large FOV with high resolution is needed, we recommend doing mosaic scanning and stitching of the single images after acquisition.

2.2 Time-Lapse Imaging

For time-lapse and live-cell imaging, a stable and physiological environment should be maintained to ensure natural cell behavior, which can be achieved using the ibidi Stage Top Incubators. Immediately after seeding the cells on the gel surface, place the slide or dish into an appropriate incubation chamber on the microscope and start a time series. Focus adjusting for every time step might be needed, which can be done manually or by using a software-based tool.

If your microscope does not have an incubation chamber, always place the sample in the cell culture incubator immediately after acquiring the image. In this case, try to keep the imaging time as short as possible (e.g., minimize the distance between the incubator and the microscope) to avoid temperature fluctuations in the sample.

When doing time-lapse imaging, the optimal time intervals as well as the total imaging time depend on the used cell line and the experimental conditions. For example, a first approach could be to image in 15–30 minutes time steps over 24 hours. Then, the time series parameters from this first experiment should be adjusted to find the optimal time steps. Therefore, analyze the time series by visualizing one key parameter (e.g., the total tube length, see Section 3) vs. time, as shown in the graph below. The time curve of this parameter is cell dependent and is showcased here exemplary for HUVEC cells grown on Matrigel®. Here, the time curve typically rises to a maximum (at ~4–5 hours), then declines to a plateau phase (beginning at ~7 hours), and finally slowly flattens (at >20 hours). A time point with a high and stable signal should be defined for analysis. In the example below, the recommended time point for measurement would be between four and five hours when using HUVEC cells grown on Matrigel®.



Time series over 24 hours of HUVEC cells grown on Matrigel® with 15 minutes time intervals. Here, the total tube length as representative parameter for tube formation was calculated from every time-step. The curve of this parameter typically rises to a maximum (here at ~4 hours), then declines to a plateau phase (here after ~7 hours), and finally slowly flattens (here at >20 hours).

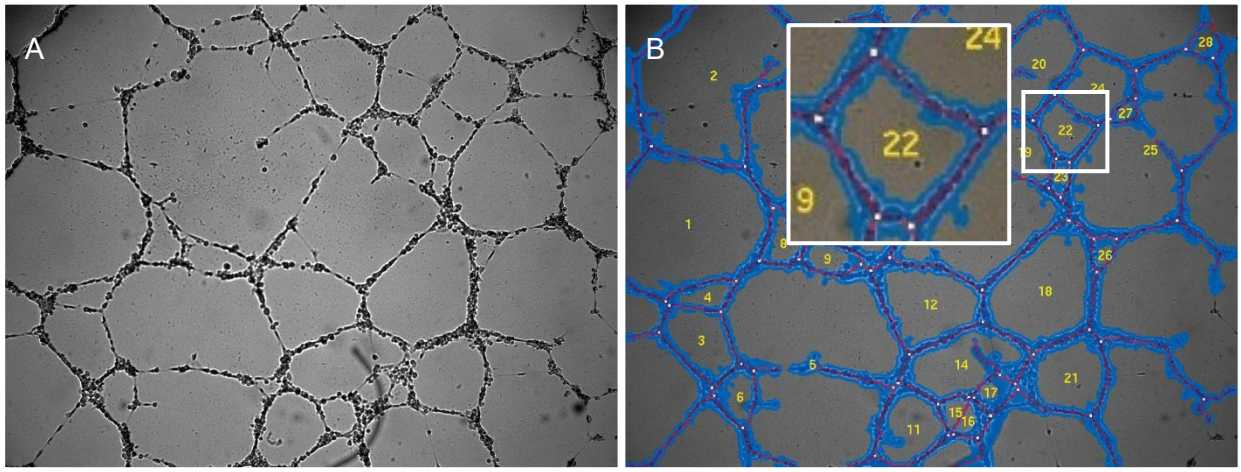
Learn more about data analysis of tube formation assays in Application Note 70: Data Analysis of Tube Formation Assays.

3 Output Parameters of Tube Formation and Angiogenesis Assays

The expression “tubes” describes the cords of cells that are visible in a 2D network. It does not mean, specifically, that the cords have a lumen. In tube formation assays, four key parameters can be determined from phase contrast images, as shown in the figure below.

- Cell-covered area [%] (blue area)
- Tube length [px] (red lines)
- Number of branching points (white dots)
- Number of loops (yellow number)

These key parameters show a consistent relationship to each other during the experiment. Therefore, it can be sufficient to determine only one feature: in this Application Note, only the length of the tube will be used as a representative value for tube formation.



Phase contrast image (A) and analyzed image (B) shows the key features of tube formation analysis: cell-covered area (blue), tubes (red), loops (yellow), and branching points (white).

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