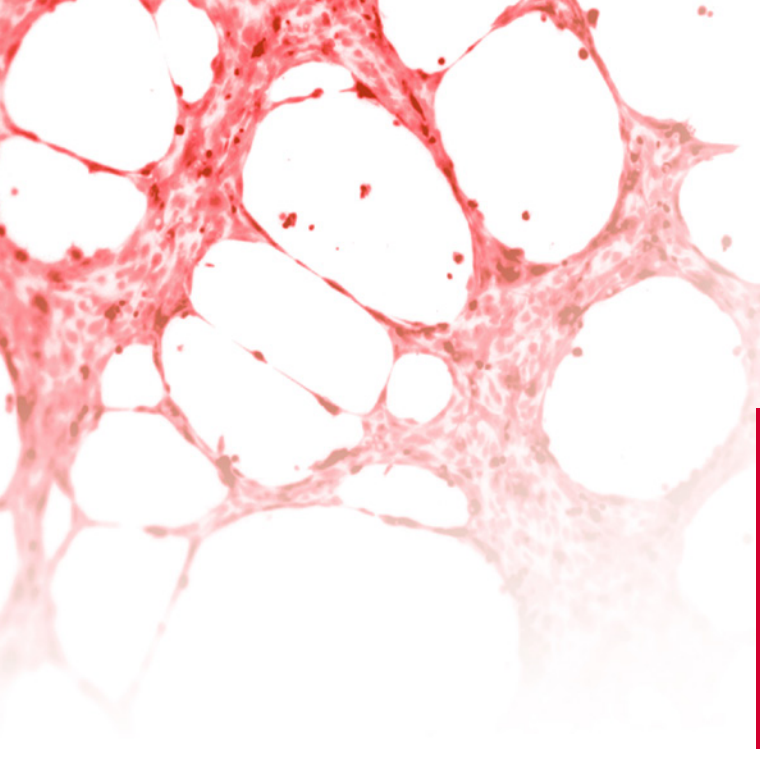


cells in focus ibidi®

ibidi Application Guide

Angiogenesis Assays

Selected Publications

Azad T, et al. (2018) A LATS biosensor screen identifies VEGFR as a regulator of the Hippo pathway in angiogenesis. Nat Commun 9(1):1061.
read abstract

Staresinic B, et al. (2018) Effect of calcium electroporation on tumour vasculature. Sci Rep 8(1):9412.
read abstract

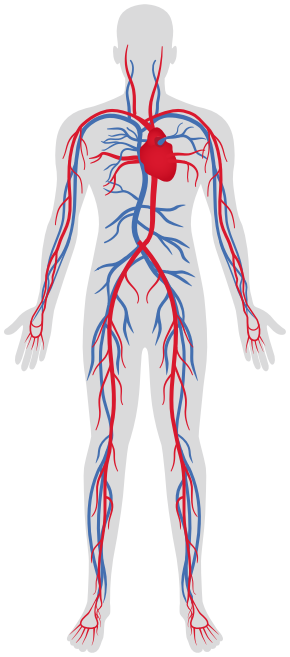
Yoshitomi Y, et al. (2017) JunB regulates angiogenesis and neurovascular parallel alignment in mouse embryonic skin. J Cell Sci 130(5):916–926.
read abstract

Angiogenesis in Development and Disease

The Blood Vessel Network

The blood vessel network is comprised of various functions in the mammalian organism. Vessels deliver fluids, gases, molecules, and cells to every part of the body. In addition, immune cells use the blood vessels to patrol through the body, protecting us against bacteria, viruses, and parasites.

Blood vessel formation is a crucial process during development, health, and disease. In adults, it occurs during wound healing, skeletal growth, pregnancy, and the menstrual cycle, for example. Dysregulated vessel growth is often involved in serious diseases, such as stroke, myocardial infarction, arteriosclerosis, and arthritis. Furthermore, tumor vascularization is one of the hallmarks of cancer.



Vasculogenesis and Angiogenesis

The blood vessel network is formed by two distinct mechanisms: vasculogenesis and angiogenesis.

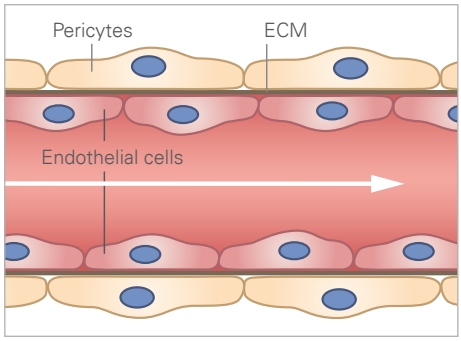
Vasculogenesis is the *de novo* formation of blood vessels. Mesodermal precursor cells (angioblasts) differentiate into endothelial cells, which then assemble to build new arteries and veins.

Angiogenesis is the expansion of the vascular network by sprouting from existing vessels.

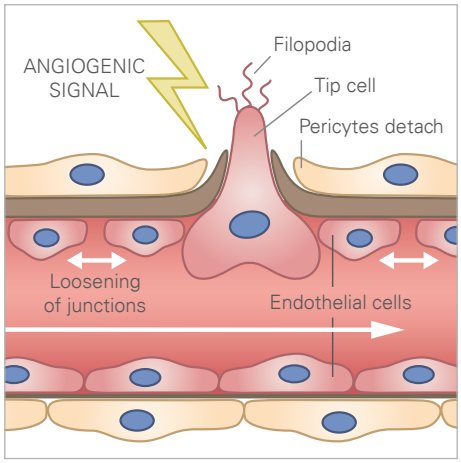
This sequence of cellular events includes breaking of cells through the basement membrane, cell migration, cell proliferation, and cell-cell communication.

One of the driving stimuli of this tightly regulated process is hypoxia, which induces the expression of several proangiogenic genes. Due to this, the inhibition or activation of angiogenesis offers many therapeutic opportunities.

For example, blocking the proangiogenic vascular endothelial growth factor (VEGF) with bevacizumab has been shown to improve the outcome of many patients with metastatic colorectal cancer (mCRC) and non-small-cell lung cancer (NSCLC), although some resistances remain. Angiogenesis assays give insight into the pro- or antiangiogenic potential of various agents, and thereby help to develop novel therapeutic approaches.



Quiescent Vessel



Branching Vessel

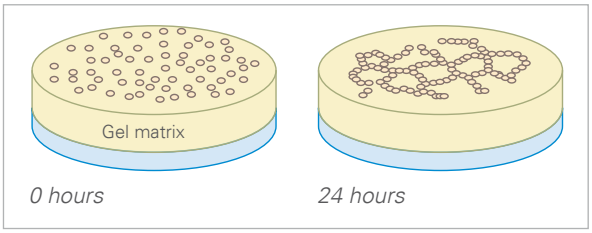
During angiogenesis, new blood vessels extend from existing vessels. Endothelial cells break through the basement membrane and migrate in the direction of an angiogenic stimulus that is produced by cancer cells, activated macrophages, or lymphocytes, for example. Behind this migrating front, cells proliferate and reorganize to form a new, three-dimensional blood vessel.

Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473(7347):298–307, 10.1038/nm0603-653.
read abstract

Rosen LS, Jacobs IA, Burkes RL (2017) Bevacizumab in Colorectal Cancer: Current Role in Treatment and the Potential of Biosimilars. Target Oncol 12(5):599–610, 10.1007/s11523-017-0518-1.
read abstract

Tube Formation Assay

In our body, endothelial cells are surrounded by the basement membrane, which is a thin and highly specialized extracellular matrix (ECM). When endothelial cells, such as human umbilical vein cells (HUVECs), are seeded onto a basement membrane-like surface (e.g., Matrigel®) they form capillary-like structures *in vitro*, which recapitulates angiogenesis.

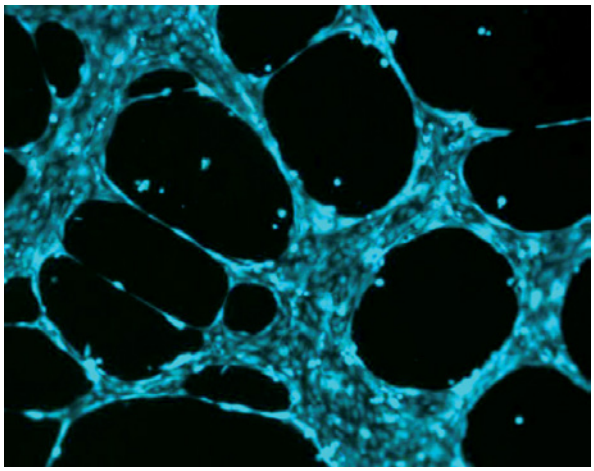


This so-called **tube formation assay** has been widely applied to solve a variety of experimental questions, such as:

- What is the pro- or anti-angiogenic potential of a specific drug?
- Which genes and pathways are involved in angiogenesis?
- What is the effect of inhibitors or enhancers on tube formation?
- How are signals transduced during angiogenesis?
- What are the cytoskeletal effects during angio-genesis?
- Which cells are endothelial progenitors?

Phase contrast (top) and fluorescence microscopy (bottom) of a single strand composed of HUVEC cells during a tube formation assay in the µ-Slide Angiogenesis. The F-actin cytoskeleton is stained green and the cell nuclei are stained blue.

The basement membrane surface and its components are the key to a successful tube formation assay. Different types of basement membranes can be applied (e.g., Matrigel®, collagen, or other hydrogel matrices), which can result in different tube formation rates. Endothelial cells differentiate morphologically to form tubes. The cells first attach to the matrix and migrate towards each other. Then, they attach to other cells, align, and form capillary-like tubes. The tubes maturate until the cells finally undergo apoptosis, resulting in detachment from the surface and tube destruction.



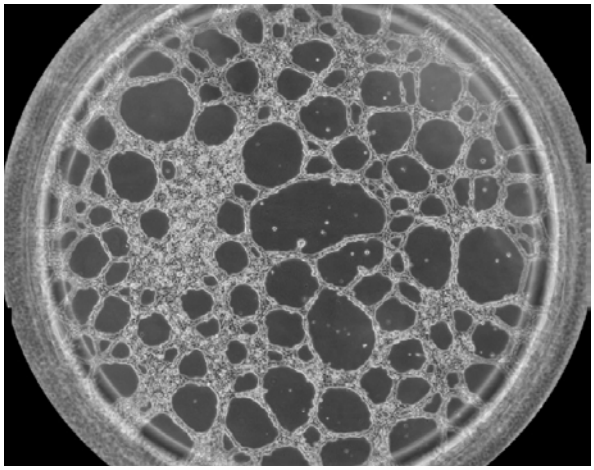
Characteristic pattern of human endothelial cells (HUVEC) stained with Calcein.

A tube formation assay is performed by first seeding single cells, and then observing and imaging the tube formation over time. Several readouts can be imaged and analyzed over time, such as tube length and the number of loops. Typically, tubes are formed within a few hours, making the tube formation assay a rapid tool for angiogenesis quantification in the research fields of embryonic development, cancer, wound healing, and tissue repair.

Tube formation assays are a widely used *in vitro* tool for accessing angiogenesis in an easy, cost-effective, and reproducible fashion. The use of only one cell type guarantees well-defined experimental parameters, which strongly facilitates assay analysis. Using the ibidi µ-Slide Angiogenesis or the µ-Plate Angiogenesis 96 Well for tube formation assays allows for live cell microscopy with all cells in focus on one 2D cell layer.

In our **Application Notes and movie**, you will find detailed information on the setup, optimization, data analysis, and interpretation of tube formation assays:

- AN 19: Tube Formation Assays in µ-Slide Angiogenesis (PDF)
- AN 05: Tube Formation in µ-Plate Angiogenesis 96 Well (PDF)
- AN 27: Experimental Setup Optimization and Data Analysis of Tube Formation Assays (PDF)
- Movie MV 15: Performing an Angiogenesis Assay



Phase contrast image showing one well of the µ-Slide Angiogenesis with HUVEC cells on Matrigel® after 12 hours of incubation during a tube formation assay.

First tube formation assay:
Kubota Y, Kleinman HK, Martin GR, Lawley TJ (1988) Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107(4):1589–98.
read abstract

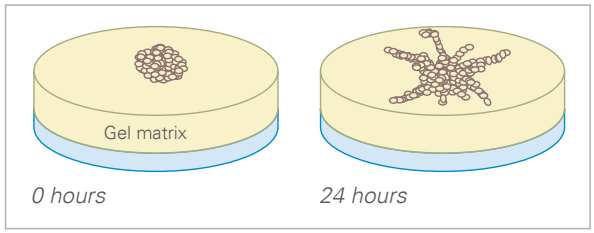
Review about angiogenesis assays:
Arnaoutova I, Jay G, Kleinman HK, Benton G (2009) The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. Angiogenesis 12:267–274. 10.1007/s10456-009-9146-4.
read abstract

Tube formation assay protocol:
Arnaoutova I, Kleinman HK (2010) In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 5(4):628–635. 10.1038/nprot.2010.6.
read abstract

Sprouting Assay

Similar to the tube formation assay, the sprouting assay can also be used to quantify angiogenesis *in vitro*.

Here, either multicellular spheroids or pieces of tissue (e.g., from the aorta) are placed into or onto a gel matrix (e.g., Matrigel®, collagen), where they then form sprouts. For analysis, the sprouting of the cell clusters is imaged at defined time points and the sprout length is measured. The sprouting assay, like the tube formation assay, needs a well-defined thickness of the gel layer, which is standardized when using the ibidi µ-Slide Angiogenesis or the µ-Plate Angiogenesis 96 Well.



Stahl A, Wu X, Wenger A, Klagsbrun M, Kurschat P (2005) Endothelial progenitor cell sprouting in spheroid cultures is resistant to inhibition by osteoblasts: A model for bone replacement grafts. FEBS Lett 579(24):5338–5342. 10.1016/J.FEBSLET.2005.09.005.
read abstract

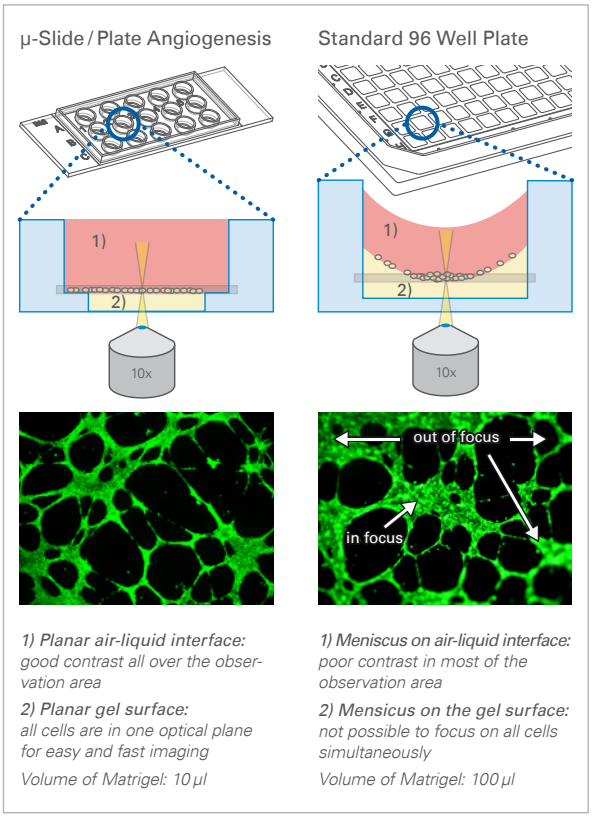
Every Cell in Focus: The Principle of the µ-Slide Angiogenesis

The µ-Slide Angiogenesis has a specialized geometry for the easy, convenient, and reproducible conduction of tube formation assays. It is also ideal for sprouting assays, immunofluorescence staining, and general 3D cell culture.

After the gel has been pipetted into the inner well and given time to solidify, the cells can be seeded on top of it for tube formation analysis. Due to the "well-in-a-well" technology, the amount of gel needed is reduced to only 10 µl per well, which is 10% of the amount used in regular multiwell plates.

Further, no meniscus is formed. This ensures the formation of a uniformly thick gel matrix on which all cells are in one optical plane, creating reproducible cell culture conditions. The µ-Slide Angiogenesis can be used with all common hydrogel matrices, such as Matrigel®, collagen gels, agarose gels, and hyaluronic acid gels.

With its 15 wells, the µ-Slide Angiogenesis is designed for low throughput assays. For large scale applications, ibidi provides the µ-Plate Angiogenesis 96 Well.



1) Planar air-liquid interface: good contrast all over the observation area

2) Planar gel surface: all cells are in one optical plane for easy and fast imaging

Volume of Matrigel: 10 µl

1) Meniscus on air-liquid interface: poor contrast in most of the observation area

2) Mensicus on the gel surface: not possible to focus on all cells simultaneously

Volume of Matrigel: 100 µl

Additional Angiogenesis Assays

Some additional *in vitro* angiogenicapproaches are the **rat/mouse aortic ring assay** and the **chick aortic arch assay**. With these assays, aortic rings from either rats or mice, or aortic arches from chick embryos are cultivated on a gel matrix (e.g., collagen or Matrigel®). Subsequently, the outgrowth of endothelial cells from these explants is monitored and quantified.

In vivo, angiogenesis can be studied in fertilized chick eggs with the **chorioallantoic membrane (CAM) assay**. A hole is cut into the shell of a developing chick egg, and the compound or graft of interest is placed on the CAM. The egg is incubated for several days and then the CAM angiogenesis is quantified.

In the **corneal angiogenesis assay**, vessel growth is induced *in vivo* in the normally avascular cornea of the rabbit or the mouse eye. After several days, the area of corneal neovascularization is assessed for quantification.

In the *in vivo* **Matrigel® plug assay**, a mixture of Matrigel® and cells, or pro-/antiangiogenic substances, is implanted subcutaneously into mice. The blood vessels that have entered the plug are quantified.

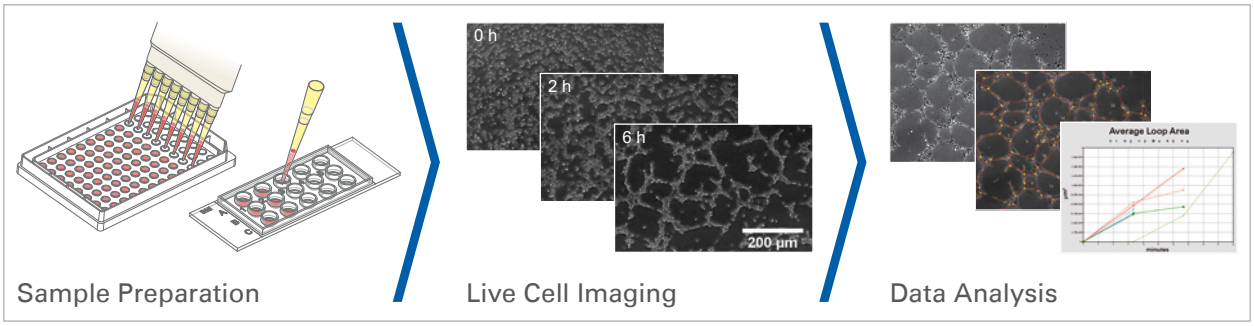
Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N (2003) Angiogenesis assays: a critical overview. Clin Chem 49(1):32–40. 10.1373/49.1.32.
read abstract

Masson V, et al. (2002) Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. Biol Proced Online 4:24–31. 10.1251/bpo30.
read abstract

Moreno-Jiménez I, et al. (2016) The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering. Sci Rep 6(1):32168. 10.1038/srep32168.
read abstract

Rogers MS, Birsner AE, D'Amato RJ (2007) The mouse cornea micropocket angiogenesis assay. Nat Protoc 2(10):2545–2550. 10.1038/nprot.2007.368.
read abstract

Experimental Workflow of a Tube Formation Assay



Before Starting

Necessary Equipment

Basic requirements

- Inverted phase contrast microscope (5x or 10x objective recommended)
- Camera for time lapse image acquisition
- Stage Top Incubator (especially required for assay optimization)

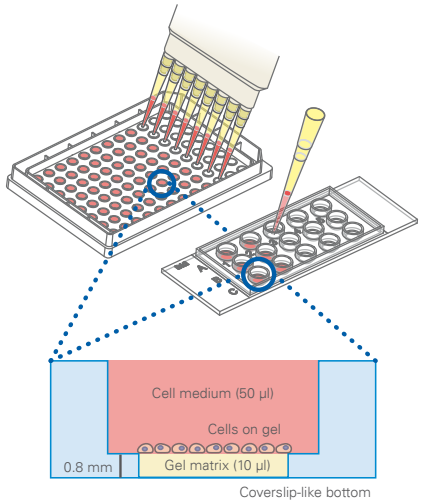
Recommended extensions

- Inverted fluorescence microscope with a higher magnification (20x or 40x objective recommended)
- Motorized stage for parallel image acquisition
- Autofocus

Questions to Ask

For a successful tube formation experiment, please address these questions before you begin.

Sample Preparation



The "well-in-a-well" feature avoids meniscus formation.

Principle

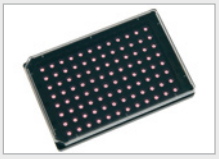
When using the µ-Slide Angiogenesis, the amount of gel matrix is reduced to 10 µl per well due to the well-in-a-well technology.

Pipet the gel matrix (e.g., Matrigel®, collagen gel, or hydrogel) into the lower well of the µ-Slide Angiogenesis. Incubate until the gel has polymerized, then seed the cells into the upper well. Shortly after seeding, the cells will sink down to the gel surface and then be ready for observation.

ibidi Solutions

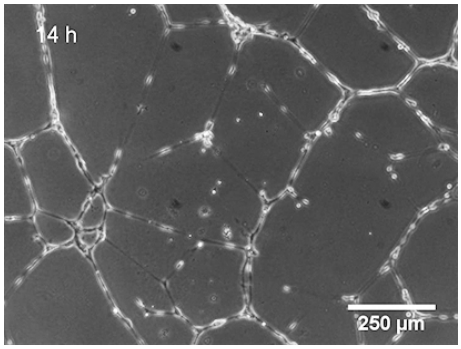
The µ-Slide Angiogenesis guarantees the convenient observation of tube formation on any inverted microscope without meniscus.

It can be used with all common 3D gel matrices, such as Matrigel®, collagen gels, and similar hydrogels. In addition, it helps to reduce costs drastically by minimizing the amount of gel needed to only 10 µl.



For large-scale angiogenesis experiments, we provide the µ-Plate Angiogenesis 96 Well.

Live Cell Imaging



Live cell imaging using the ibidi Stage Top Incubation System shows the tube formation of HUVECs in an angiogenesis assay.

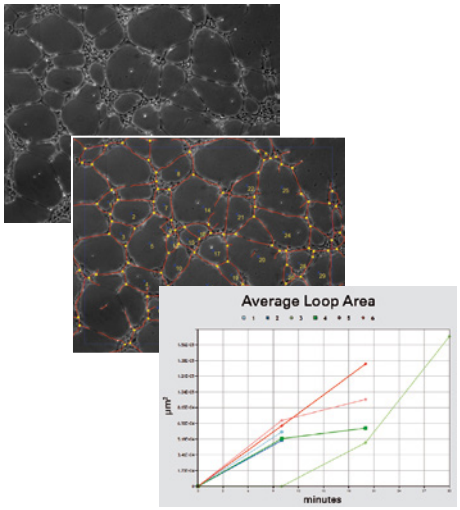
Principle

In most cases, phase contrast microscopy is used to monitor the tube formation, but fluorescence microscopy is also a possibility. Depending on your focus, this can be achieved with either live cell imaging or by taking images at distinct time points (e.g., after 6 and 12 hours).

ibidi Solution

The ibidi Stage Top Incubation System provides a physiological environment directly on the microscope, enabling live cell imaging during angiogenesis assays and tube formation assays.

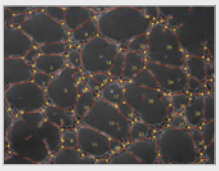
Data Analysis



Principle

After image acquisition, parameters such as tube area, tube length, tube number, loop area, loop number, and the number of sprouts can be analyzed. This can be performed manually or by using automated software. The statistical comparison of the parameters (either alone or in combination) will give an overview of the extent of tube formation under different conditions.

ibidi Solution



With the Tube Formation ACAS Image Analysis software, ibidi provides a time-saving, fully-automated solution for the quantification of tube formation assays. It includes analyses of the loop count, average loop area, average tube length (per loop), total tube length, and branch count.

Questions to Ask Before Starting an Experiment

In order to set up a chemotaxis assay correctly, it is crucial to answer the following questions first:

Which endothelial cells should I use?

Endothelial cells are highly organ- and tissue-specific. Due to this heterogeneity, the time for tube formation varies among the different endothelial cell types. This must be considered when planning the experiment. Typically, low-passaged human umbilical vein cells (HUVECs) are used for a tube formation assay.

Further examples of cell types that can be applied are:

- Primary bovine aortic endothelial cells (BAOEC)
- Porcine bone marrow-derived endothelial progenitor cells (EPCs)
- Human aortic endothelial cells (HAEC)
- Human pulmonary artery endothelial cells (HPAEC)
- Human coronary artery endothelial cells (HCAEC)

Which cell density is optimal for my experiment?

The determination of the correct cell number is crucial in obtaining optimal results from a tube formation assay. The cell density is dependent on the cell type and cell size. Before starting the actual experiment, we recommend seeding several cell numbers under non-inhibited conditions and imaging the tube formation. The cell density with the highest number of tubes created will show the most distinct effects of substances, and therefore should be used for the experiment. Remember that the cells typically do not proliferate on the gel matrix. Generally, we recommend the use of 5,000–10,000 HUVECs per well.

Which gel matrix should I use?

The tube formation rate of endothelial cells depends on the type and the components of the gel that forms the basement membrane-like extracellular matrix (ECM) surface, on which the cells are seeded. Several basement membrane extracts are commercially available, such as Matrigel® and Cultrex®., both of which are secreted by Engelbreth-Holm-Swarm (EHS) murine sarcoma cells. Before starting the experiment with a particular endothelial cell type, we advise testing several basement membrane substances and following the published protocols. If pro-angiogenic agents will be tested in your assay, a growth factor-reduced gel matrix should be used. Also, if possible, use the same lot for the whole experiment.

Which controls should be included in the experiment?

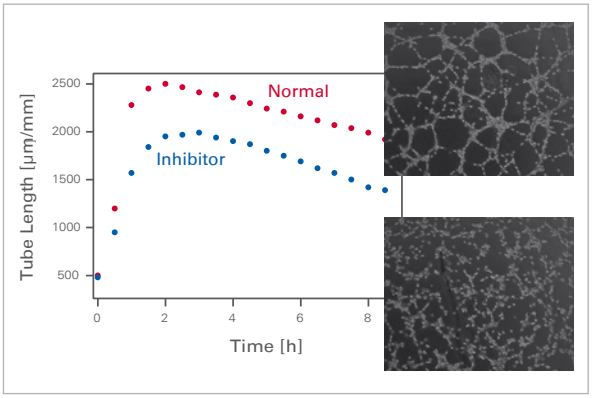
For the proper statistical analysis of your results, it is important to include a positive and a negative control. As a positive control, perform a tube formation assay with your cell line of interest without using any inhibitor. When cultivated on growth factor-reduced Matrigel® with starving medium, endothelial cells should show formation of tubes within a few hours. As a negative control, a tube formation inhibitor that does not affect cell viability can be used.

Are my pipettes working precisely and are they calibrated frequently?

To avoid meniscus formation of the gel matrix when using the µ-Slide Angiogenesis, it is crucial to pipet an exact amount of 10 µl into the inner well. In addition, seeding of the right cell number is extremely important. It is therefore necessary to use calibrated pipettes that are in perfect working order. We recommend to wet the pipet tip with the respective gel and then to fill the wells using this one tip.

During which timeframe should I observe the cells?

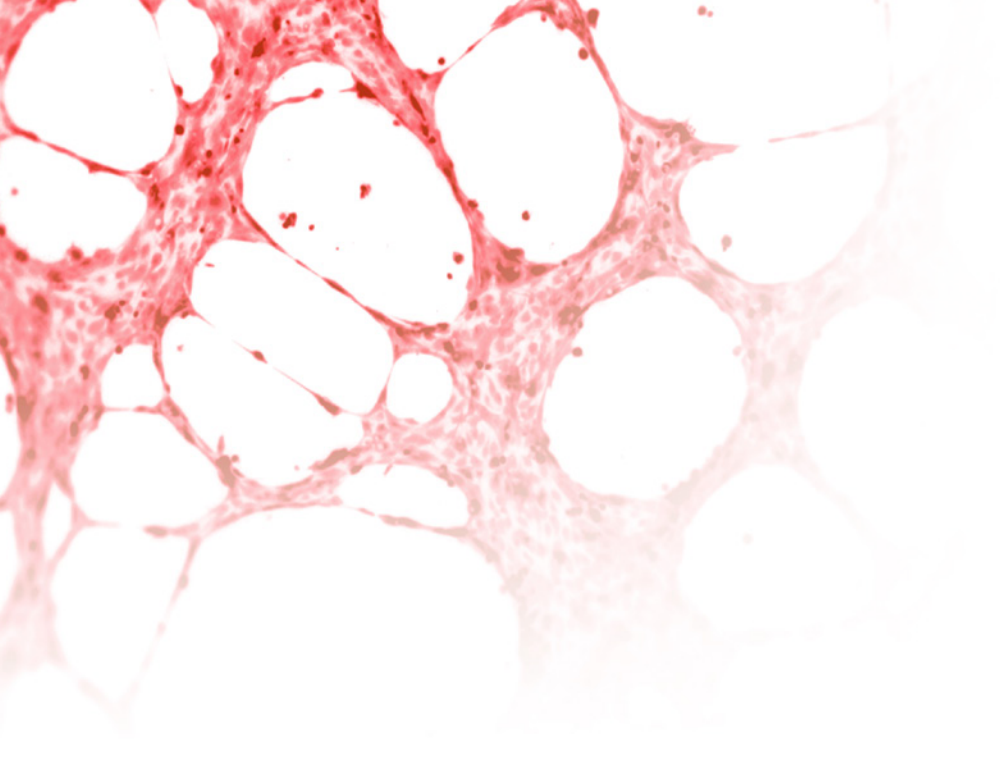
The duration of tube formation depends on the cell type and on the extracellular matrix being used, and should be determined individually. Typically, HUVECs already form tubes after 2–4 hours. After 24 hours, the cells start to undergo apoptosis, and this leads to detachment from the matrix and the breaking of the tubes.



Influence of inhibitors on tube formation over time.

Should I monitor the tube formation manually or automatically?

If live cell imaging equipment is available, we recommend using time lapse video imaging—especially, if you are not familiar with the angiogenetic behavior of the used cell type. In a typical setup, a photo is taken automatically every 5–15 minutes within the timeframe of 24 hours. This enables the precise monitoring of the progress of tube formation, e.g., after the addition of a special substance. If you already know the approximate time frame of the tube formation, it is also possible to manually take pictures at the expected time points of interest.



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