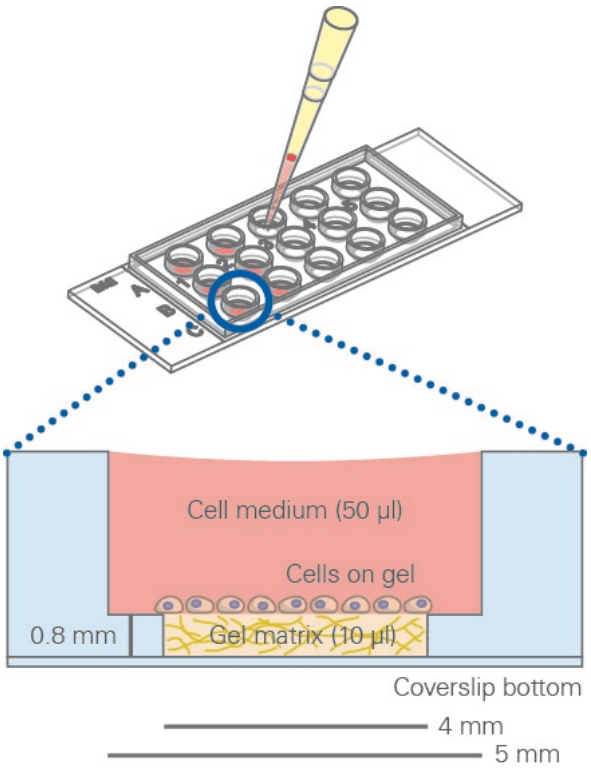
Tube Formation Assay With Laminin-Collagen I Gel in the µ-Slide 15 Well 3D

When seeding endothelial cells onto a basement membrane-like surface, they form capillary-like structures *in vitro*, recapitulating angiogenesis. This tube formation assay is used as an *in vitro* model system for angiogenesis to investigate pro- or anti-angiogenic effects of specific substances. The profound principle and a detailed protocol for this assay are described in Application Note 19: Tube Formation Assay in the µ-Slide 15 Well 3D (PDF).

Matrigel® is extracted from Engelbreth-Holm-Swarm mouse sarcomas and serves as the standard basement membrane-like (BM) surface. However, one disadvantage of Matrigel® is the poorly defined and variable composition of multiple BM proteins. This results in fluctuations of the mechanical and biochemical properties between different batches, hindering reliable reproducibility and comparability.

This Application Note describes a protocol for performing a tube formation assay with a Laminin-Collagen I gel (instead of Matrigel®) in the µ-Slide 15 Well 3D. The well-defined two-component Laminin-Collagen I gel enables reproducible and reliable formation of capillary-like structures and can serve as an alternative to Matrigel®. This application note does not discuss detailed analyses of the differences between the gels. For further information, please read the related documents and publications.



Schematic illustration of the µ-Slide 15 Well 3D. The ibidi "well-in-well" technology avoids gel meniscus formation resulting in a flat cell growth area.

Related Documents

- Application Note 5: 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 27: Optimizing Tube Formation Assays (PDF)
- Application Note 70: Data Analysis of Tube Formation Assays (PDF)
- Application Guide Angiogenesis Assays (PDF)
- Application Guide Live Cell Imaging (PDF)
- Instructions µ-Slide 15 Well 3D (PDF)
- Instructions Collagen Type I, Rat Tail, 5 mg/ml (PDF)
- Instructions Collagen Type I, Bovine, 5 mg/ml (PDF)

Related Software

- ibidi Collagen Calculator

Related Publication

- Rüdiger D, Kick K, Goychuk A, et al. Cell-Based Strain Remodeling of a Nonfibrous Matrix as an Organizing Principle for Vasculogenesis. Cell Rep. 2020 Aug 11; 32(6):108015. doi: 10.1016/j.celrep.2020.108015.

1 Material

1.1 Reagents and Buffers

- Human umbilical vein endothelial cells (HUVEC, C-12203, PromoCell)
- Endothelial Cell Growth Medium (C-22010, PromoCell)
- Collagen I, Rat Tail, non-pepsinized, 5 mg/ml (50201, ibidi)
- Collagen I, Bovine, non-pepsinized, 5 mg/ml (50301, ibidi)
- Cultrex 3-D Culture Matrix Laminin I 6 mg/ml (3446-005-01, R&D Systems)
- 10x PBS (70011-044, Gibco)
- NaOH in ultrapure H_2O, 1.25 M and 7 M
- Acetic acid, 0.1 M and 17.5 mM

1.2 Equipment

- µ-Slide 15 Well 3D, ibiTreat (81506, ibidi)
- µ-Slide Rack (80003, ibidi)
- Scale paper for checking the optimal gel volume
- Ice and cooling rack
- Standard cell culture equipment (pipettes, tubes, sterile working bench, cell culture incubator, culture flasks, hemocytometer, etc.)
- Inverted microscope
- Optional: Multichannel pipette

2 Preparation of the 3D Gel

Perform all the following protocol steps under sterile conditions.

Laminin-Collagen I gels can be prepared with Collagen Type I, Rat Tail or Collagen Type I, Bovine. For both, a 4 mg/ml Collagen I gel must be prepared first (Chapter 2.1). General information and tips for handling Collagen I gels can be found in Application Note 26: Preparation of Collagen I Gels (PDF).

Important Note for Pipetting the Gels

Always use precooled pipet tips (4°C) for pipetting the gels.

For the preparation of Laminin-Collagen I gels, reverse pipetting is recommended for all steps involving Laminin and Collagen I due to their high viscosity. Press the pipette to the second pressure point and fill the complete pipette tip with gel. Dispense the gel only until the first pressure point is reached. This leaves a residual amount of gel in the pipette tip to be discarded, but the volume is much more accurate. Alternatively, you can use pipettes designed for high viscosity solutions. Among others, we recommend Eppendorf Visco Tips or Gilson Microman E.

Note that even at 4°C, the gel mixture can be used for a maximum of 5 minutes before partial gelation occurs.

1. The day before the experiment, place the Laminin on ice in the fridge at 4°C to let it slowly thaw overnight. Prepare the Collagen I according to the instructions.
2. On the day of the experiment, place all materials for the gels and sterile tubes with sufficient capacity for the total gel volume in a cooling rack in the laminar flow hood. Always keep the materials and gels in the cooling rack during gel preparation.

2.1 Preparation of the Collagen I Gel

Important Note for Collagen I Concentration

Due to lot-specific deviations, the concentration of the Collagen I in the vials can vary.

Check the Certificate of Analysis (CoA) for the individual lot-specific Collagen concentration on the Collagen I product page in the CoA tab.

1. Before diluting, the original Collagen I gel in the vial must be actively mixed by pipetting up and down several times. This ensures that a homogeneous solution is created.

Note: Use the ibidi Collagen Calculator for variations of the protocols below, i.e. if different concentrations are needed of the collagen stock solution, the NaOH or the collagen gel.

Preparation of the Collagen I Stock Solution

2. Dilute the Collagen I to 4.5 mg/ml. For **Collagen Type I, Rat Tail**, use **17.5 mM acetic acid,** and for **Collagen Type I, Bovine**, use **0.1 M acetic acid**. Find the individual lot-specific Collagen I concentration in the Certificate of Analysis (CoA).

Preparation of the 4 mg/ml Collagen I Working Solution

3. Pipet all ingredients except for the Collagen I into a pre-cooled tube in the order listed in the table below. Mix thoroughly while keeping the tube in the cooling rack.
4. Add the Collagen stock solution to the mixture to get a final working solution of 4 mg/ml. Mix thoroughly by pipetting while keeping the tube in the cooling rack.

Collagen I, Rat Tail		**Collagen I, Bovine**	
10x PBS	16 µl	10x PBS	16 µl
NaOH **1.25 M**	2 µl	NaOH **7 M**	2 µl
Collagen I, Rat Tail, diluted to **4.5 mg/ml in 17.5 mM acetic acid**	142 µl	Collagen I, Bovine, diluted to **4.5 mg/ml in 0.1 M acetic acid**	142 µl
Total volume	160 µl	Total volume	160 µl

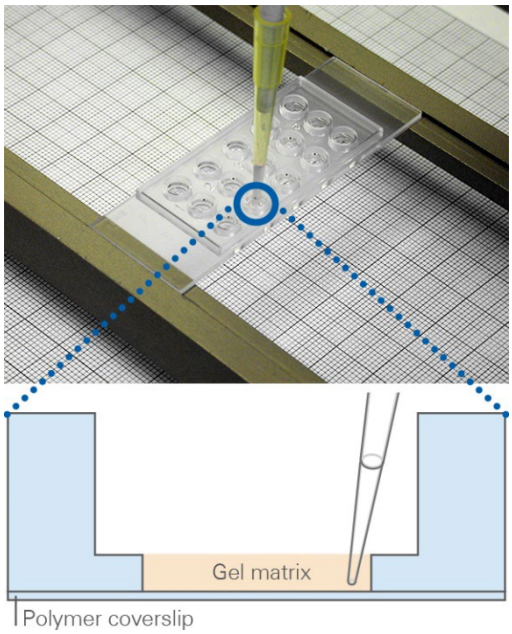
*Pipetting scheme for the final 4 mg/ml Collagen I working solution using the pre-diluted stock solution of 4.5 mg/ml Collagen I, Rat Tail (left) or Collagen I, Bovine (right). Note that to correctly set the pH value, use a 1.25 M NaOH solution for Collagen I, Rat Tail (left) and a 7 M NaOH solution for Collagen I, Bovine (right). All ingredients are listed in the order of pipetting. Differences in the concentrations are highlighted in **blue**.*

2.2 Preparation of the Laminin-Collagen I Gel

1. The mixing ratio of Laminin:Collagen I is 4:1 (e.g., mix 400 µl Laminin with 100 µl Collagen I working solution).
2. Mix thoroughly by pipetting while keeping the tube in the cooling rack. The gel is now prepared to be pipetted into the inner wells of the µ-Slide 15 Well 3D (Chapter 2.3).

2.3 Gel Application

1. Place a µ-Slide Rack in the flow hood. Remove the µ-Slide 15 Well 3D from the sterile packing and put it on the µ-Slide Rack.
2. Put a scale paper into the hood below the µ-Slide for volume adjustment. Make sure the distance between slide and scale paper is ca. 1–2 cm.
3. Always use precooled pipet tips (4°C) for pipetting the gel.
4. To avoid air bubbles in the gel, make sure the gel solution is properly mixed before filling it into the well. Therefore, make three up-and-down movements with the pipet (10 µl) while leaving the tip in the gel.
5. Apply 10 µl of gel to each inner well of the µ-Slide 15 Well 3D. Hold the pipet tip upright in the middle of the well to prevent gel leaking into the upper well.
6. Close the µ-Slide with the lid.



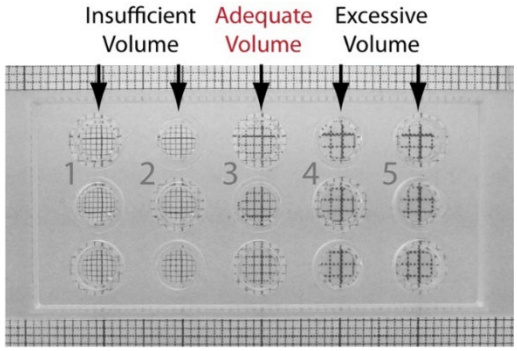
µ-Slide 15 Well 3D on µ-Slide Rack with scale paper for gel-volume adjustment.

Adjusting the Gel Volume

A meniscus-free gel with a plain surface is crucial for optimal imaging. To achieve this, the gel volume of each inner well needs to be precisely 10 µl. Due to the high viscosity of the gels, the pipet might need to be adjusted to more or less than 10 µl.

To adjust the gel volume accurately, place the slide 1–2 cm above a scale paper in the µ-Slide Rack. Now set your pipet to 10 µl and fill the gel into one inner well. Observe the markings of the paper through the filled well. If a magnification or demagnification effect is visible, change the volume in ±1 µl steps until you can no longer observe a magnification effect.

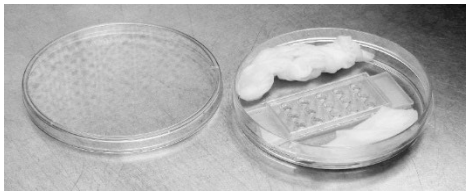
If the grid of the scale paper appears smaller, the pipetting volume must be increased. If the grid is enlarged, then the pipetting volume must be reduced.



Insufficient gel volume leads to a smaller appearance of the scale paper grid, whereas excessive gel volume leads to an enlarged appearance of the scale paper grid.

2.4 Gelation

1. Prepare a petri dish with water-soaked paper towels to serve as a humidity chamber.
2. Place the µ-Slide in the petri dish and close the lid.
3. For polymerization, place the whole assembly into the incubator for 60 min.
4. In the meantime, prepare the cell suspension (Chapter 3).



µ-Slide 15 Well 3D in humidity chamber for gel polymerization.

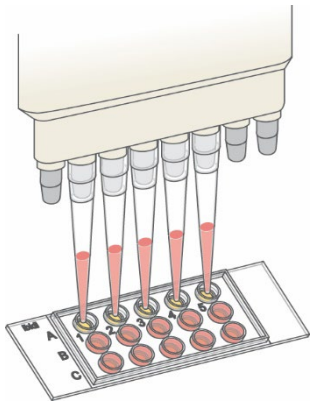
3 Cell Seeding

The number of cells seeded on the gel surface is a crucial parameter for obtaining reliable results from a tube formation assay. Deviation in the cell number can strongly influence the formation of the tubular network. The cell type and size determine the cell seeding number and must be optimized before starting an experimental series. HUVEC should be used in a low passage number (up to P6).

It is important to work quickly during the whole procedure to prevent the wells from drying out.

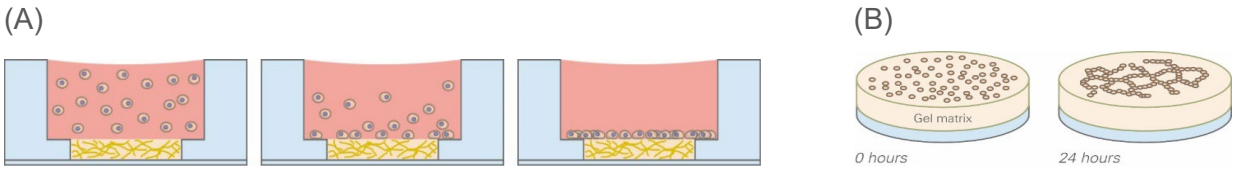
If not stated otherwise, all given volumes are per well, and all incubation steps are at room temperature.

1. Harvest the HUVEC, centrifuge, and dilute the cell pellet in a low amount of culture medium (depending on the required cell concentration) for counting.
2. Count the cells. Counting should be performed as accurately as possible and always in the same way to have the same number of cells in all wells. For a final cell number of 5,000 cells per well, adjust them to a final concentration of 1 x 10^5 cells/ml in culture medium.
3. Take the µ-Slide 15 Well 3D with the polymerized gel from the incubator and put it on a µ-Slide Rack under the flow hood.
4. Before adding the cell suspension to the wells, mix thoroughly by pipetting up and down several times. Apply 50 µl into each upper well. Keep the pipet tip upright, and do not touch the gel with the pipet tip. A multichannel pipet might be helpful for this step.



Pipetting cells into the µ-Slide 15 Well 3D using a multichannel pipette.

5. Cover the µ-Slide with the supplied lid. Optionally, ibiSeal 22 x 48 (10872), a self-adhesive cover film, can be used to cover the wells for improved phase contrast microscopy.
6. The µ-Slide 15 Well 3D is now ready for observation. The cells will settle to the top of the gel surface via a passive sedimentation process. Subsequently, tube formation begins and can be imaged in one focal plane without any disturbing gel meniscus.



(A) Passive sedimentation process of cells. After some minutes, all cells are located at the bottom. (B) Schematic of tube formation in the µ-Slide 15 Well 3D (only the inner well with the cells on the gels is shown).

4 Image Acquisition

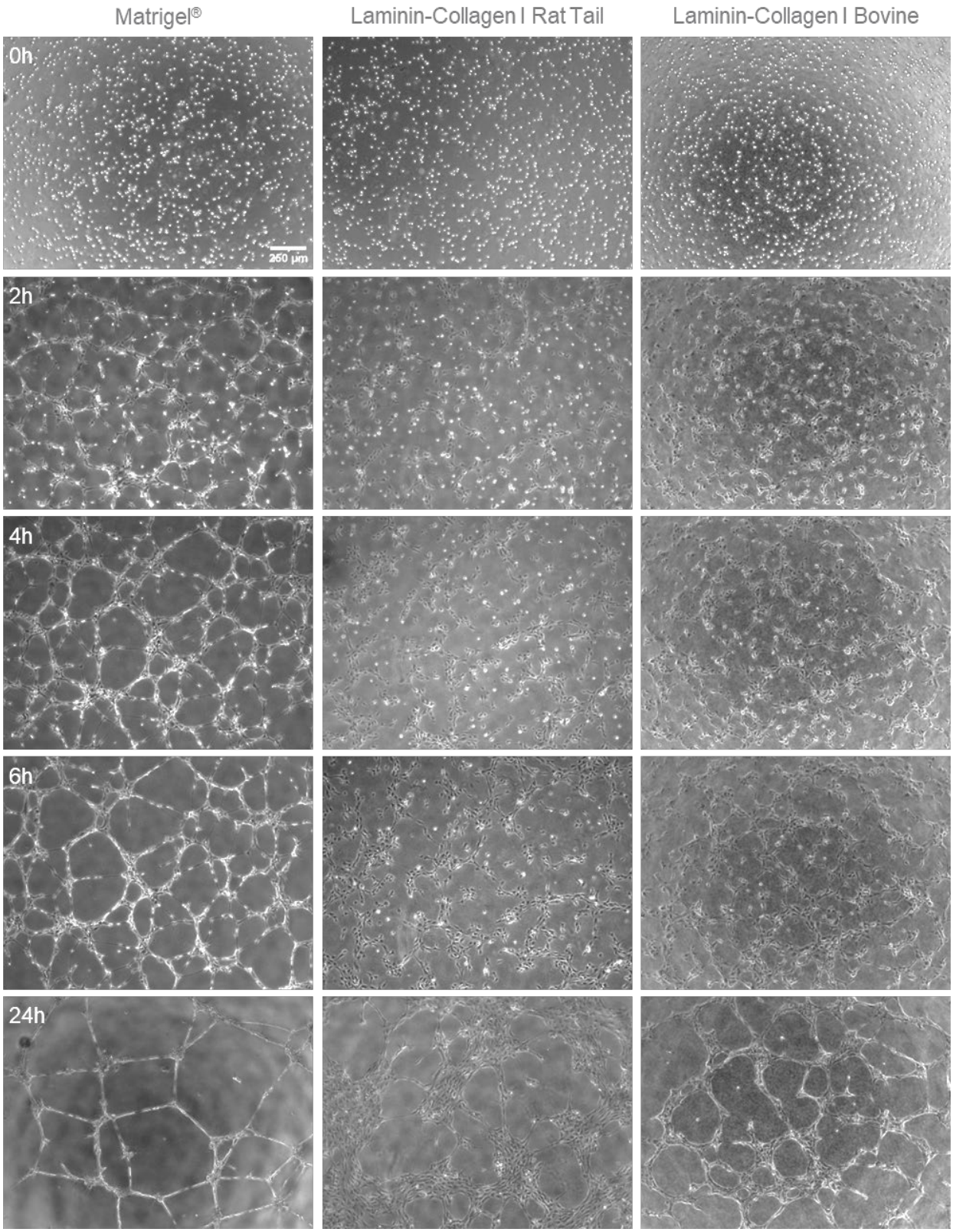
Tube formation can be imaged using brightfield, phase contrast, or fluorescence microscopy (e.g., when staining with a live cell dye such as calcein), see Application Note 19: Tube Formation Assay in the µ-Slide 15 Well 3D (PDF).

Data collection on the microscope can be performed manually or automatically. Especially when performing a tube formation assay experiment for the first time, we recommend automatic data acquisition by recording a time-lapse video to determine the time dependency and the characteristics of the curve (e.g., maximum and stable phase). In subsequential experiments, single manual measurements are usually sufficient for investigating the effects of substances on tube formation.

For HUVECs, we recommend a 4x or 10x magnification and a time interval of 5 minutes between the single frames for at least 5 hours. To quantify tube formation, the images can be analyzed based on different parameters, such as tube length, loops, cell-covered area, or branching points.

5 Results

Both the rat tail and bovine Laminin-Collagen I gels induce the formation of a tubular network. After 2 hours, the tubes, loops, and branching points will be visible. The structure of the network and the dynamics of network formation differ in comparison to Matrigel®. Further detailed analyses of differences between the gels and their causes are not discussed in this Application Note. However, both gels can be used to investigate the influence of pro- or anti-angiogenic effects of specific substances on the detectable key parameters of the tube formation network.



Time lapse images of a tube formation assay using HUVECs in the µ-Slide 15 Well 3D. Phase contrast microscopy with a 4x objective at 0 h, 2 h, 4 h, 6 h, and 24 h after cell seeding on Matrigel® (left), Laminin-Collagen I Rat Tail gel (middle) and Laminin-Collagen I Bovine gel (right).