Tube Formation Assays with the µ-Plate Angiogenesis 96 well

1. Introduction

The proven, well-in-a-well format of µ-Slide Angiogenesis is now available in a 96 well format. The geometry provides a meniscus-free surface consisting of both gel and overlaid medium, which allows for high quality, phase contrast imaging, and a homogeneous cell distribution.

This Application Note describes the handling of the µ-Plate Angiogenesis 96 well with Matrigel™ and a multichannel pipette.

For detailed information about the Tube Formation Assay, please also refer to Application Note 19 "Tube Formation Assays in µ-Slide Angiogenesis".

The data analysis is discussed separately in Application Note 27 "Data Analysis of Tube Formation Assays".

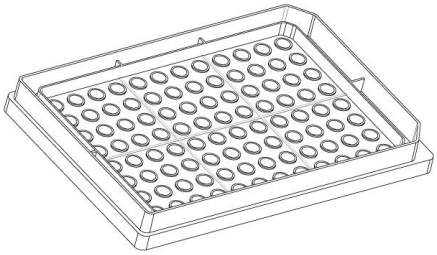

Fig.1: µ-Plate Angiogenesis 96 well

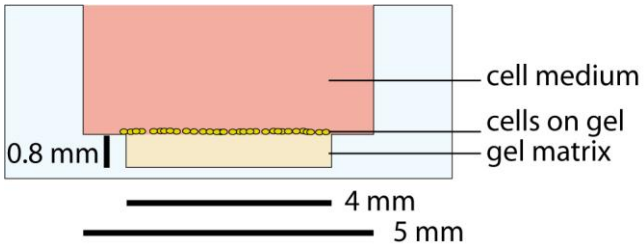

Fig.2: Cross section of a single well

2. Material

For a Tube Formation Assay with the µ-Plate Angiogenesis, the following material is needed:

Cells:	HUVEC (PromoCell, C-12200, C-12203)	1 x 10^4 per well
Medium:	Endothelial Cell Growth Medium (PromoCell, C-22010)	70 µl per well
Gel Matrix:	BD Matrigel™ (Growth Factor Reduced, #356231)	10 µl per well
Plate:	µ-Plate Angiogenesis 96 well, ibiTreat (ibidi, 89646)	1 Slide
Detach Reagent:	Accutase (PromoCell, C-41310)	8 ml per T75 flask

Further required equipment:

- Scale paper
- Cooling rack or crushed ice
- PCR stripe to make 8 aliquots for the multi-channel pipette
- Multi-channel pipette with tips

The volume of the medium and gel that is mentioned above is for one well only. You need to calculate the total amount of your required volume by multiplying it with the number of wells that will be used. Also, pipetting with the multi-channel pipette will recquire some additional liquid to ensure the correct uptake for all of the wells.

3. **Work Flow**

The protocol steps are the same as in Application Note 19 "Tube Formation in µ-Slide Angiogenesis":

4. **Preparation of Gel and Plate**

Gel Application

To use the gel with a multi-channel pipette, it must be distributed to the 8 small tubes of a PCR-stripe (alternatively in one column of wells of a 96-well plate with conical bottom) , and cooled on ice.
Add at least 10 µl more gel than you actually need to filleach of the wells.
Example: For pipetting the whole µ-Plate Angiogenesis 96 well (12 columns), add 130 µl to each of the 8 wells.

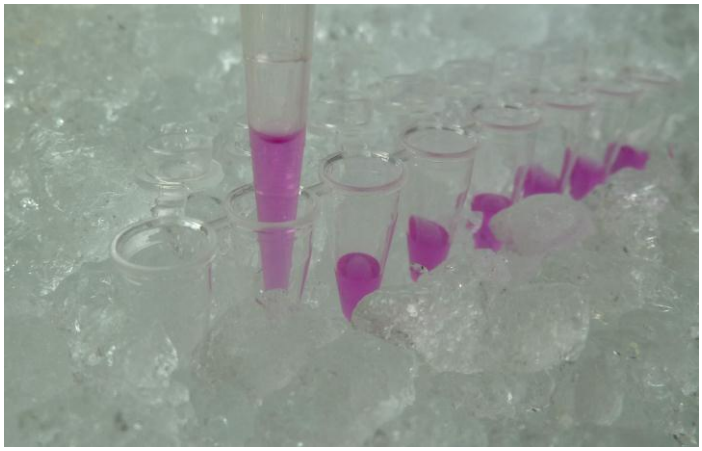

Fig.3: PCR-stripe with Matrigel on ice

Follow these steps:

1) The day before seeding the cells, place the Matrigel™ on ice in the refrigerator at 4°C. The gel can slowly thaw over night.

Note: Always use precooled pipette tips (4°C) for pipetting the gel!

2) Place the PCR-strip (or 96-well plate) on ice or in any appropriately cooled stage. Place the vial with the thawed gel also on ice. Transfer the required volumes to the wells of the PCR-stripe with a cooled pipette tip.
3) Remove the µ-Plate Angiogenesis from the sterile packaging and pull off the protection foil (see Figure 4).
4) Adjust your multi-channel pipette to 10 µl and take up the gel (see Figure 5).
5) When applying the gel to the wells of the µ-Plate Angiogenesis, hold the pipette upright to prohibit the liquid from flowing into the edge of the upper well (see Figure 6).

Pipetting Tips

To avoid air bubbles, make three uptake and push-out movements (10 µl) with the pipette, while leaving the tip in the gel. Then transfer 10 µl aliquots to the wells.

Due to the high viscosity of Matrigel™, it might be necessary to adjust the pipet volume to slightly more or less than 10 µl (see Application Note 19 "Tube Formation Assays in µ-Slide Angiogenesis").

To control the right amount of gel, observe the gel in a phase contrast microscope with a low magnification (e.g., 4x).

Be sure to finish the whole pipetting procedure in a few minutes, so as to avoid evaporation effects.

Gelation

After filling all the required wells with Matrigel™, cover the µ-Plate Angiogenesis with the supplied lid and place it in the incubator to allow for the polymerization of the gel (30-60 minutes).

To help minimize evaporation, the side reservoirs can be filled with sterile water. Fill only half of the volume of the reservoirs to avoid any overflow of the liquid into the wells.

During the polymerization of the gel, prepare the cell suspension.

Fig.4: Pull off the protection foil.

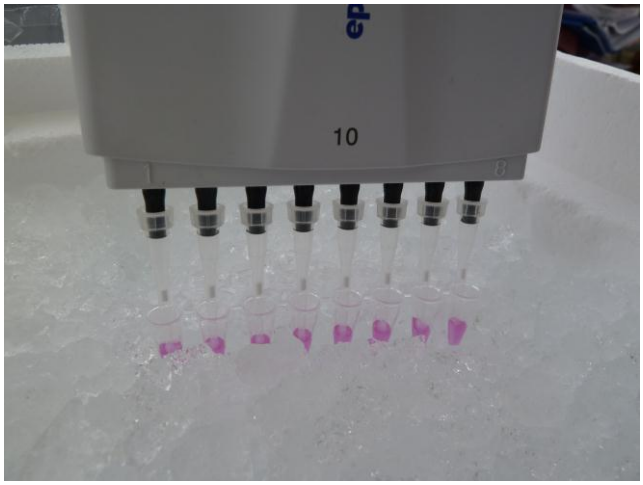

Fig.5: Take in 10 µl of gel from the PCR-stripe.

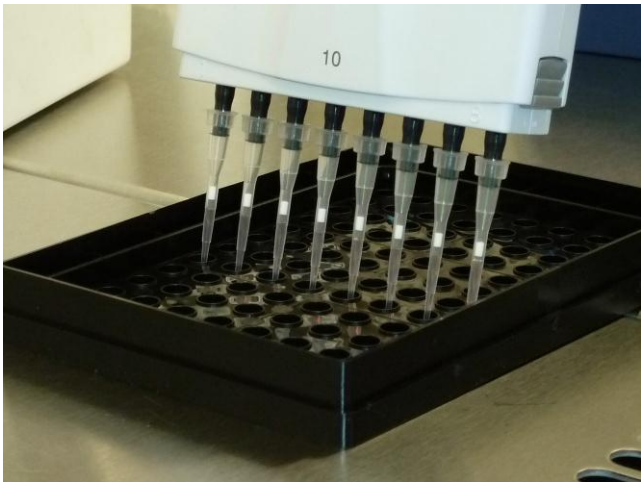

Fig.6: Apply 10 µl of Matrigel™ to the lower well.

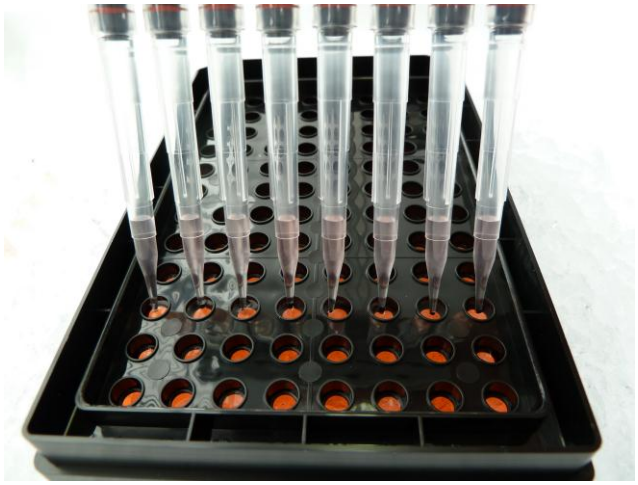

Fig.7: Apply 70 µl of cell suspension to the upper well.

5. Seeding Cells

The number of cells seeded onto the surface of the gel is a crucial parameter that is required to obtain reliable results. The cell type and size determines the number of cells that are needed. For the best results, optimize the cell seeding number before starting an experimental series. For a detailed description of the optimization, please see Application Note 27 "Data Analysis of Tube Formation Assays"

Follow these steps:

1) For a final cell number of 10.000 cells per well, adjust a cell suspension of $1.4 \cdot 10^5$ cells/ml. Then mix thoroughly.
2) Take the µ-Slide from the incubator and place it on the rack.
3) Apply 70 µl of cell suspension to each upper well. Keep the pipet tips upright and take care not to touch the gel with the pipet tip.
4) Close the µ-Plate Angiogenesis with the lid. The slide is now ready for observation.

5) After a few minutes, the cells will have sunk down to the gel surface and will be lying in one plane. However, due to the geometry of the wells, some cells will also be found on the plastic margins (not only on the gel).

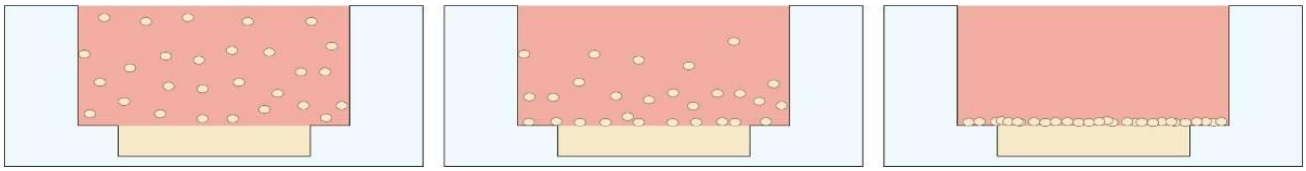

Fig.8: Sinking process of cells. After some minutes the cells will have fallen to the gel surface.

6. Observation on the Microscope

The network formation on top of the gel surface will follow a certain development characteristic, depending on your cell type. When observing the total tube length, for example, the parameters will rise to a maximum value during the first few hours after seeding, and then slowly decline in the following hours, until reaching a base value.
There is a detailed description on how to determine these characteristics in Application Note 27 "Data Analysis of Tube Formation Assays".

Immediately after seeding the cells, position the plate on an inverted microscope that is equipped with an incubation chamber. Program the positions you want to observe on your imaging system, and then start a time-lapse recording. For HUVEC, we recommend using a small magnification (4x or 10x) and a time interval of one hour in between the single images. Note: It is possible that cells will migrate into the gel and change the focal plane. Therefore, it is recommended to use a software autofocus program to get sharp pictures over an elapsed amount time.

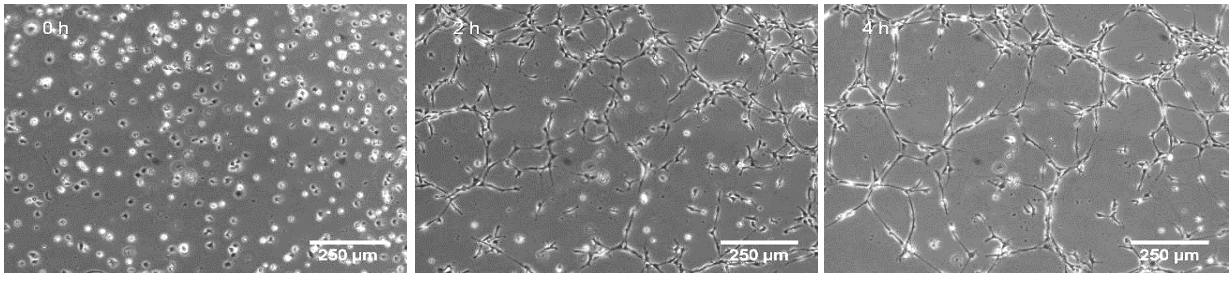

Fig.9: Time Lapse pictures with a 10x magnification at 0, 2, and 4 hours.

7. Data Analysis

For optimal results, and a fast and objective data analysis, we recommend using the WimTube Software. You can upload your images to the platform and your results will be ready for download within minutes. Follow this link to the Wimasis image analysis platform.

The images are analyzed detecting a variety of parameters, such as tube length, loops, or cell-covered area. There is a detailed description of data analysis and interpretation in Application Note 27 "Data Analysis of Tube Formation Assays".

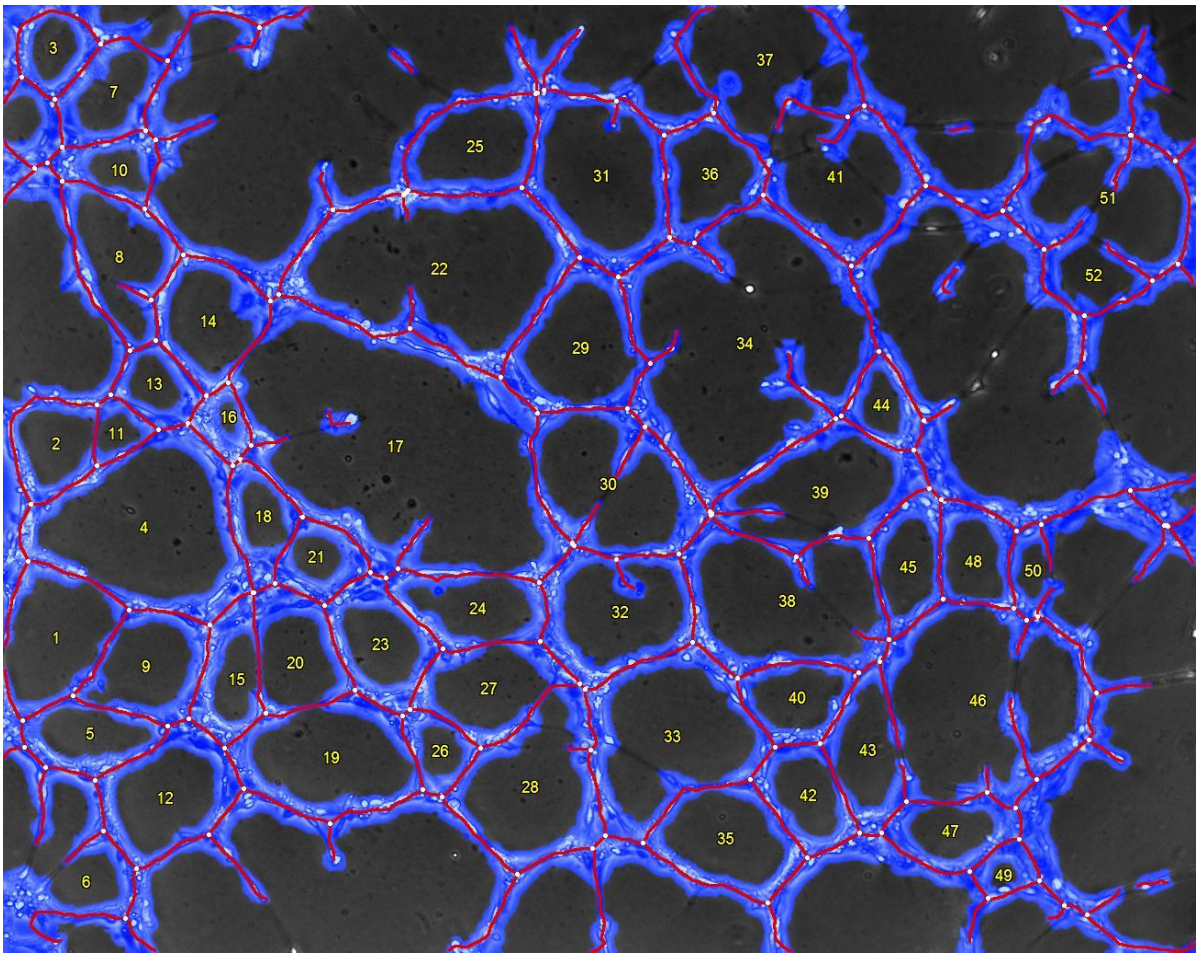

Fig.10: Data Analysis with the WimTube Module. The tubes are shown in red, the cell-covered area in blue. The branching points are shown as white dots. The numbers indicate a closed loop of tubes.