Millicell® µ-Angiogenesis Activation Assay

Catalogue No. MMA130

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Not for use in diagnostic procedures

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Introduction

Angiogenesis, the formation of new blood vessels from a pre-existing vascular network, occurs normally in development and is critical for a majority of the vessel formation that occurs during embryogenesis, tissue generation, and wound healing. However, abnormal blood vessel growth, either excessive or insufficient, can be the underlying cause of many deadly and debilitating diseases including cancer, cardiovascular disease, stroke, and diabetic and age-related blindness. Identification of specific compounds that promote or inhibit angiogenesis may provide promising treatments for these diseases.

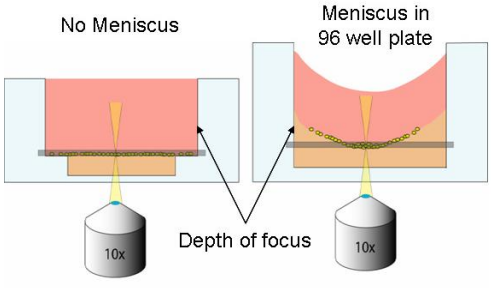
Tubular formation is a multi-step process involving cell adhesion, migration, differentiation, and growth. The formation of intercellular connections and lumina within endothelial cell networks in fibrin gels is dependent upon the actions of VE-cadherin, αvβ3 and α5β1 integrins, the cdc42 and Rac1 GTPases, and membrane-type matrix metalloproteinases (MT-MMPs). Angiogenesis within fibrin gels *in vitro* is regarded as an accurate model for studying wound healing and tumor angiogenesis, as tumor cell–derived vascular endothelial growth factor/vascular permeability factor promotes leakage of fibrinogen from the tumor vasculature and formation of a fibrin-rich pro-angiogenic provisional matrix.

Millipore's Millicell μ-Angiogenesis Activation Assay provides an efficient system for the rapid and accurate identification of factors that stimulate tube formation by endothelial cells. The Millicell μ-Angiogenesis Activation Assay takes advantage of the propensity of endothelial cells to rapidly align and form interconnecting networks that display patent lumina when cultured on top or within a fibrin gel. Included in the kit are the following components:

(1) Five μ-Angiogenesis Slides: Each chamber slide contains 15 wells for a total of 75 wells per kit. The μ-Angiogenesis Slides are based on the ibidi® technology platform, and possess enhanced optical imaging capabilities over the standard 96-well plate. The biocompatible plastic composition of the μ-Angiogenesis Slide provides an even, flat surface that eliminates the meniscus effect routinely observed in 96-well plates while providing similar high optical qualities observed in glass slides. As a result, all of the cells on the μ-Angiogenesis Slide are in focus; whereas, in a 96 well plate, only the few cells located at the center of the plate are in focus.



μ-Slide Angiogenesis Features	
Number of wells	15
Volume inner well	10 μL
Diameter inner well	4 mm
Depth inner well	0.8 mm
Volume upper well	50 μL
Diameter upper well	5 mm
Growth area inner well	0.12 cm^2
Coating area using 10 μL	0.23 cm^2



- All cells in focus
- 10μL gel per well
- No meniscus
- Excellent optics
- 15 wells
- Slide format

- Few cells in focus
- 100μL gel per well
- Meniscus formation
- Poor optics
- 96 wells
- Multi-well format

The μ-Angiogenesis Slide is compatible with multi-channel pipettes for easy filling and aspiration. It is also compatible with various fixation reagents such as methanol, paraformaldehyde, and other chemicals. Live cells can be observed directly or fixed and stained for further analysis.

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(2) 1 vial of Fibrinogen Solution, 1.5 mL: A soluble plasma protein that upon cleavage by thrombin forms a fibrin gel. The resulting cleaved fibrin molecules form regular, multi-molecular arrays that are highly translucent. The concentrations and formulations of the fibrinogen and thrombin in this kit have been optimized for maximal tube formation by HUVEC and easy visualization of these tubes.

(3) 1 vial of Thrombin Solution, 0.75 mL: A serine protease that enzymatically cleaves fibrinogen to form the fibrin gel.

(4) 1 vial of 100X ITS Media Supplement, 0.5 mL: A media supplement containing insulin, transferrin, and selenium that aid in general cell health and cell survival.

(5) 1 vial of PMA (Phorbol 12-myristate 13-acetate), 1 mg: A biologically active compound that can induce tube formation in endothelial cells. The compound is provided as a useful positive control for activation studies.

(6) 1 vial of Calcein AM, 50 µg: A non-fluorescent, hydrophobic compound that can be readily transported across the membrane of intact, live cells. Calcein AM is provided as a simple, rapid, and accurate indicator of cell viability and cytotoxicity, and for short-term labeling of live cells. Once inside the cell, the acetoxymethyl (AM) ester group is hydrolyzed by intracellular esterases, yielding the free Calcein, a strongly fluorescent compound that is hydrophilic and thus well retained inside the cell cytoplasm. Apoptotic and dead cells are not labeled by Calcein AM, as they lack active esterases and also have compromised cell membranes which cannot retain the Calcein. Calcein is optimally excited at 495 nm and has a peak emission of 515 nm. For fluorescence microscopy, Calcein can be detected using filters for FITC.

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Kit Components

1. Millicell µ-Angiogenesis Slides: (Part No. CS203030) Five tissue culture treated slides. Each slide contains 15 wells. Store at room temperature.
2. Fibrinogen Solution: (Part No. CS203035) One 1.5 mL vial. Store at -20 °C.
3. Thrombin Solution: (Part No. CS203036) One 0.75 mL vial. Store at -20 °C.
4. ITS Media Supplement, 100X: (Part No. CS203037) One 0.5 mL vial. Store at -20 °C.
5. PMA (Phorbol 12-Myristate 13-Acetate): (Part No. CS203029) One 1 mg vial. Store at -20 °C.
6. Calcein-AM: (Part No. CS202541) One 50 µg vial. Store at -20 °C.

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Materials Required But Not Supplied

1. Inverted light microscope
2. Fluorescence microscope (if Calcein AM is used)
3. Precision pipettes
4. Accutase™ Cell Dissociation Solution (Cat. No. SCR005)
5. Human umbilical vein endothelial cells (HUVEC) (Cat. No. SCCE001) or any other experimental cell lines capable of tube formation.
6. EndoGRO™ LS Complete Medium (Cat. No. SCME001) or other endothelial cell basal medium.
7. Dulbecco's Phosphate Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
8. EmbryoMax® ES Cell Qualified Ultra Pure Water, sterile H_2O (Cat. No. TMS-006)
9. Low speed centrifuge
10. Sterile microcentrifuge tubes
11. Precision pipette tips. We recommend 0.1–10 mL TipOne® filter tips (USA Scientific® Cat. No. 1121-3810) or Rainin® 20 µL Precision Pipette Tips (Rainin Cat. No. RT-L10S)
12. 37 °C incubator with 5% CO_2
13. Hemacytometer
14. Trypan Blue
15. DMSO

Storage and Handling

1. Millicell µ-Angiogenesis Slides (Part No. CS203030) should be stored at room temperature until ready to use.
2. Fibrinogen Solution (Part No. CS203035) should be stored at -20 °C and used within six months from the date of receipt. For short-term storage, store at room temperature for up to 2 weeks. **Do not store at 4 °C**, as Fibrinogen Solutions tend to form aggregates when stored at 4 °C. For long-term storage, aliquot into sterile tubes in a sterile laminar flow hood, freeze on dry ice, and store at -20 °C.
3. Thrombin Solution (Part No. CS203036) should be stored at -20 °C and used within six months from the date of receipt. Unused Thrombin Solution can be stored at 4 °C for up to 1 month. For longer term storage, Thrombin Solution may be stored in aliquots at -20 °C.
4. ITS Media Supplement, 100X (Part No. CS203037) should be stored at -20 °C. Unused portions may also be stored at 4 °C for up to 1 year. Avoid freeze/thaw cycles.
5. PMA (Phorbol 12-Myristate 13-Acetate) (Part No. CS203029) is provided as a 1 mg lyophilized powder. To prepare a 1 mg/mL stock, add 1 mL sterile DMSO (not provided). Vortex vigorously. Centrifuge briefly to collect the solution. Unused PMA Solution may be stored at -20 °C in the dark for at least six months. ***WARNING: Highly irritating to skin and mucous membranes. TUMOR PROMOTER. Read the Material Safety Data Sheet (MSDS) prior to rehydration and use. Gloves and mask should be worn when using this compound.***
6. Calcein AM (Part No. CS202541) is provided as a 50 µg lyophilized powder. To prepare 1 mM Calcein AM Stock Solution, briefly centrifuge before opening the tube and then add 40 µL sterile DMSO (not provided). Vortex vigorously. Centrifuge briefly to collect the solution. Unused Calcein AM Solution may be stored in aliquots at -20 °C for up to 1 month.

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Assay Instructions

The following procedure is recommended for HUVEC cells and may be used as a reference point to further optimize specific cell type(s) of interest.

Day 0:

1. Culture HUVEC cells in a 10 cm tissue culture dish containing 10 mL EndoGRO LS Complete Medium (Cat. No. SCME001). Allow cells to grow until they reach 80–90% confluence.

   ***Note**: The EndoGRO LS Complete Medium contains 2% fetal bovine serum, 5 ng/mL EGF and other additional culture supplements. For optimal results, low passage (passages 1 through 8) HUVEC or other endothelial cells are recommended.*

2. Preparing and equilibrating µ-Angiogenesis Slides:

   Because of the microscale size of the wells in the µ-Angiogenesis Slides, a humidified environment is recommended to prevent evaporation and to obtain optimal results. All procedures should be performed in a biological hood utilizing aseptic techniques to prevent contamination.

   a. To create a humidified chamber, place 2 wet Kimwipes® wipers, folded in quarters, in a 10 cm Petri dish.

   b. Carefully open the µ-Angiogenesis Slide and remove the lid and slide from the packaging.

   c. Place the µ-Angiogenesis Slide in the humidified chamber (i.e., 10 cm Petri dish with wet Kimwipes wipers) and place the humidified chamber in a 37 °C, 5% CO_2 tissue culture incubator. *(Equilibrating the µ-Angiogenesis Slide inside the incubator overnight will help prevent air bubbles from becoming entrapped in the fibrin gel in downstream steps.)*

Day 1:

3. Thaw the Fibrinogen Solution rapidly and bring to approximately 37 °C by gently agitating the vial in a 37 °C water bath. Once thawed, set aside at room temperature until ready to use.
4. Thaw Thrombin Solution at room temperature.
5. Transport the humidified chamber containing the µ-Angiogenesis Slide (from Step 2) to the tissue culture hood.
6. Using a 10 or 20 µL precision pipette tip (USA Scientific Cat. No. 1121-3810 or Rainin Cat. No. RT-L10S), carefully and slowly aliquot 6.6 µL Fibrinogen Solution into the inner well of each of the 15 wells of the µ-Angiogenesis Slide. To avoid generating air bubbles, it is best to aliquot slowly. Gently spread the Fibrinogen Solution with the pipette tip to ensure that the solution is evenly distributed across each well.
7. Using a new 10 or 20 µL precision pipette tip (USA Scientific Cat. No. 1121-3810 or Rainin Cat. No. RT-L10S), carefully and slowly aliquot 4.4 µL Thrombin Solution into the center of each well. Immediately mix the Fibrinogen and Thrombin Solutions by repeat pipetting two to three times. Avoid generating bubbles by gentle pipetting.

   ***Note:** Thrombin acts as a catalyst, thus the order of addition is very important. Fibrinogen should always be added first followed by the addition of thrombin. The resulting fibrin gel forms rapidly within less than a minute after the addition of thrombin, thus it is important to mix thoroughly immediately upon the addition of the Thrombin Solution.*

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While a regular p10 or p20 yellow pipette tip can be used to pipette the Fibrinogen and Thrombin Solutions, we have found that the incidence of bubbles can be reduced with the use of pipette tips that are smaller than the standard. We recommend using either the 0.1–10 µL TipOne filter tip from USA Scientific (Cat. No. 1121-3810) or the p20 precision tip from Rainin (Cat. No. RT-L10S).

8. Place the lid over the µ-Angiogenesis Slide. Close the humidified chamber.
9. Visually inspect each well under a light microscope to ensure that the fibrin gel is evenly distributed across the well.
10. Carefully transport the humidified chamber containing the µ-Angiogenesis Slide to a 37 °C, 5% CO_2 tissue culture incubator. Incubate at 37 °C for one hour to allow the fibrin gel to solidify.
11. While the fibrin gel is solidifying, harvest the HUVEC or other endothelial cells.
    a. Carefully remove the medium from the 10 cm tissue culture dish containing 80–90% confluent HUVEC cells or other endothelial cells.
    b. Wash the cells once with 10 mL sterile 1X PBS. Aspirate the 1X PBS.
    c. Add 3 mL Accutase Cell Dissociation Solution (Cat. No. SCR005) and incubate in a 37 °C incubator for 5–15 minutes.
    d. Inspect the dish and ensure the complete detachment of cells by gently tapping the side of the dish with the palm of your hand.
    e. Apply 10 mL EndoGRO LS Complete Medium (pre-warmed to 37 °C) to the dish.
    f. Gently rotate the dish to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
    g. Centrifuge the tube containing the cells at 1500 RPM for 5–10 minutes to pellet the cells.
    h. Aspirate and discard the supernatant. Be careful to not disturb the cell pellet.
    i. Apply 2 mL EndoGRO LS Complete Medium to the conical tube and resuspend the cells thoroughly. ***Note:*** *Do not vortex the cells.*
12. Count the number of cells using a hemacytometer. The total volume that each upper well in the µ-Angiogenesis Slide can accommodate is 50 µL. Depending upon the number of µ-Angiogenesis Slides and wells being used, adjust the total volume and the cell concentration with additional EndoGRO LS Complete Medium to obtain the following:
    a. For wells containing the cell suspension alone without any test compounds or the activation control (PMA) added, make up a 1–3 x 10^5 cells/mL cell suspension.
    b. For wells containing the cell suspension with the addition of test compounds (e.g., cytokines, pharmacological agents, etc.) or the activation control, PMA, make up a 2–6 x 10^5 cells/mL cell suspension.

Note: *It is recommended that at least 1 to 3 wells of each µ-Angiogenesis Slide contain the activation control, PMA, as a reference point for comparison.*

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13. **Optional: Preparation of the activation control (PMA and ITS)**

Because the total volume that the upper well of the µ-Angiogenesis Slide can accommodate is 50 µL, it is necessary to prepare the cell suspension and PMA or other test compounds in 2X concentrations separately before mixing in equal parts to obtain a final 1X concentration of 50 µL final volume. For HUVEC cells, 50 ng/mL PMA is sufficient to activate tube formation. For other cell types, some optimization may be required to determine the maximal level of activation. The following calculations are based upon the PMA Activation Control being performed in triplicate for one µ-Angiogenesis Slide. Scale up or down according to experimental design.

a. Dissolve 1 mg PMA with 1 mL DMSO (not provided) to make a 1 mg/mL PMA Stock Solution. Unused PMA Solution can be aliquoted and stored at -20 °C in the dark for at least six months.

b. Prepare a 1 µg/mL PMA Stock Solution by diluting 1 µL of the 1 mg/mL PMA Stock solution (from Step 13a) with 999 µL EndoGRO LS Complete Medium.

c. Make up the 2X Activation Control Mix. Assuming one µ-Angiogenesis Slide for which the user will perform the Activation Control in triplicate, make up a 75 µL stock solution of 2X PMA and 2X ITS Media Supplement by adding 7.5 µL of 1 µg/mL PMA Stock Solution (from Step 13b) and 1.5 µL of 100X ITS Media Supplement to 66 µL EndoGRO LS Complete Medium.

2X **Activation Control: (3 wells) (25 µL volume) = 75 µL total Volume**

Component	Stock Concentration	Amount	2X Concentration
EndoGRO LS Complete Medium		66 µL	
PMA Stock Solution	1 µg/mL	7.5 µL	100 ng/mL
ITS Supplements	100X	1.5 µL	2X
	Total Volume	**75 µL**	

d. For each activation control reaction, mix 25 µL of the 2X Activation Control (from Step 13c) with 25 µL cell suspension (from Step 12b) to make a total volume of 50 µL. Final PMA concentration should be 50 ng/mL.

14. Carefully add 50 µL of the appropriate cell suspension (from Steps 12a and 13d) into each of the upper wells of the µ-Angiogenesis Slide containing the polymerized fibrin gel (from Step 10). This is equivalent to approximately 5,000–15,000 cells total per well. Do not touch the gel matrix with the pipette tip.

15. Incubate at 37 °C in a 5% CO_2 incubator.

16. The formation of tubes can be monitored either at fixed intervals, or in real time using an inverted light microscope connected to a Heating Stage System. Cellular network structures are highly developed by 12–18 hours in the presence of PMA and the ITS Media Supplement (Figure 2) with the first visible signs apparent after 5–6 hours. However, cells will remain as monolayers when plated on top of the fibrin gels in the presence of bFGF or VEGF, and require the addition of another layer of fibrin gel for network formation to occur. The addition of an overlay also reduces the extent of cell death on the fibrin gels.

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17. **Optional: Preparation of fibrin gel sandwich**

a. Using a pipette tip oriented from the edge of the well, remove 50 µL of culture medium from each well. Avoid disturbing the cells or the bottom fibrin gel.

b. Carefully aliquot 6.6 µL Fibrinogen Solution on top of the cells in each well.

c. Carefully add 4.4 µL Thrombin Solution to each well. Immediately mix the Fibrinogen and Thrombin Solutions by repeat pipetting two to three times. Avoid generating bubbles by gentle pipetting.

d. Place the lid over the µ-Angiogenesis Slide. Close the humidified chamber.

e. Carefully transport the humidified chamber containing the µ-Angiogenesis Slide to 37 °C, 5% CO_2 tissue culture incubator. Incubate at 37 °C for 5 minutes to allow the fibrin gel to polymerize.

f. After the fibrin gel has polymerized, carefully add 40 µL EndoGRO LS Complete Medium containing the desired test compound to each well.

g. Incubate at 37 °C in a 5% CO_2 incubator for 24–48 hours to observe the tube formation. Tubular structures will remain stable in the fibrin sandwich for up to a week. Some cell death may be observed after 48 hours.

18. **Optional: Fluorescent monitoring of tube formation with Calcein AM staining**

a. Prepare a 1 mM Calcein AM stock solution by dissolving 1 vial of 50 µg Calcein AM (provided) with 50 µL of DMSO (not provided).

b. Vortex vigorously and centrifuge briefly to collect the solution. Unused Calcein AM solution should be aliquoted and stored at -20 °C and used within one month.

c. Dilute 1 µL of 1 mM Calcein AM stock solution with 19 µL EndoGRO LS Complete Medium to obtain a 50 µM Calcein AM stock solution.

d. Add 1 µL of the 50 µM Calcein AM stock solution to each well of the µ-Angiogenesis Slide.

e. Incubate at 37 °C for 15 minutes.

f. Replace the staining solution with 50 µL EndoGRO LS Complete Medium.

g. Observe the cells using a fluorescence microscope containing a FITC filter.

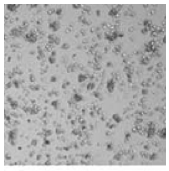
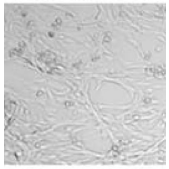
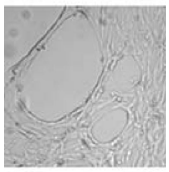
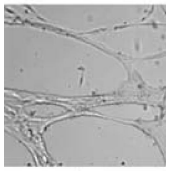
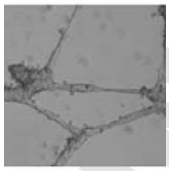
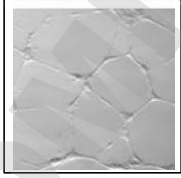
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Quantitation of Results

Activated endothelial cells form cellular networks (mesh-like structure) from capillary tubes sprouting into the stromal space (see Figure 3). The formation of these cellular networks is a dynamic process, starting with cell migration and alignment, followed by the development of capillary tubes, sprouting of new capillaries, and finally the formation of the cellular networks. The following are guidelines to provide quantitative assessment of these cellular networks.

A. Pattern recognition

Define visual patterns by looking at or photographing the cells with 5X–20X objectives at set time at 37 °C after seeding on the fibrin gel (end-point assay). Assign a numerical value to each pattern. This way a numerical value is associated with a degree of angiogenesis progression. An example is presented in the table below.

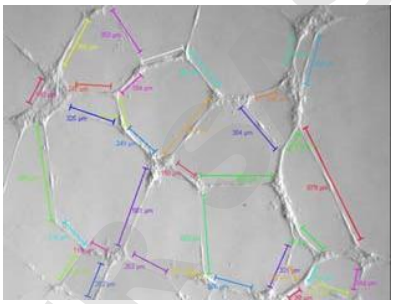
	Pattern	Value
	Individual cells, well separated	0
	Cells begin to migrate and align themselves	1
	Capillary tubes visible, no sprouting	2
	Sprouting of new capillary tubes visible	3
	Closed polygons begin to form	4
	Complex mesh-like structures develop	5

The pattern/value association criteria should be defined with the types of cells and experimental conditions that will be used in the angiogenesis assay. Several random fields-of-view (3–10) per well should be examined and the values averaged. This quantitation method will work best in assays involving potent inhibitors or activators of angiogenesis.

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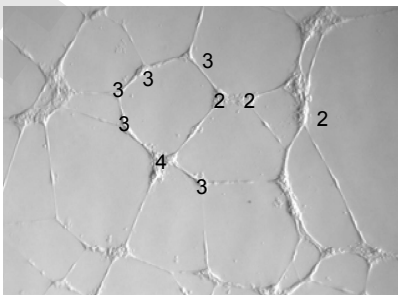
B. Branch Point Counting

A subtler, but more labor-intensive way to quantitate the progression of angiogenesis is to count the capillary tube branch points formed after a set amount of time (end-point assay). The length of the newly formed capillary tubes can also be taken into account when counting (do not count if shorter than an arbitrary predetermined length). Branch points in several random fields-of-view (3–10) per well should be counted and the values averaged.



C. Total Capillary Tube Length Measurement

An alternative method to branch point counting, suitable particularly for microscopes with imaging capabilities, is to measure the total length of all the capillary tubes in a field-of-view. The total capillary tube length in several random fields-of-view (3–10) per well should be examined and the values averaged.



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Results

The following images are typical results obtained using HUVEC cells and should be used for reference purposes only.

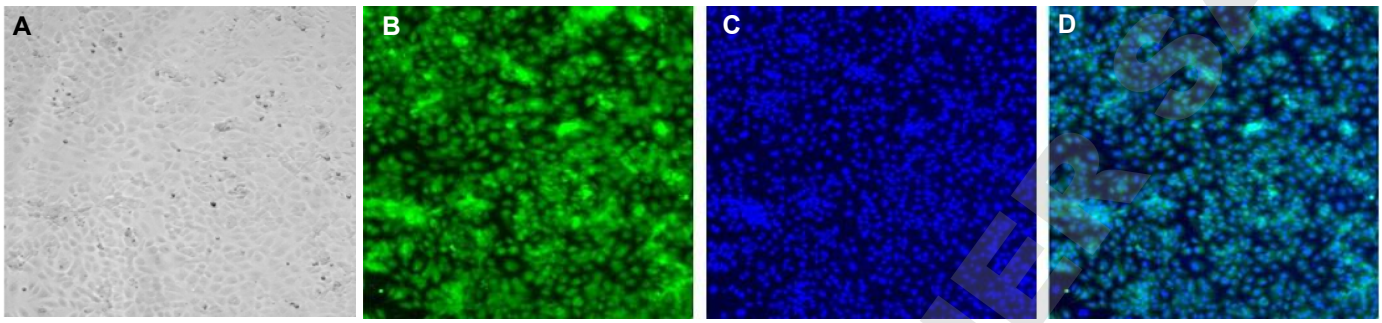


Figure 1. Unstimulated HUVEC cells form monolayers on fibrin gel. Passage 3 HUVEC cells (Cat. No. SCCE001) were cultured at 3 x 10^5 cell/mL per well on a fibrin-coated µ-Angiogenesis Slide without the benefit of a stimulant. Monolayer morphology was observed after 24 hour incubation at 37°C. Cells were stained with 1 µM Calcein AM and 1 µg/mL DAPI for 15 minutes in culture media. Staining reagent was removed and replaced with EndoGRO LS Complete Medium before imaging. (**A**) Bright field image, (**B**) Calcein AM stain, (**C**) DAPI stain, (**D**) Merged image of Calcein AM and DAPI. Images were taken with 5X objective.

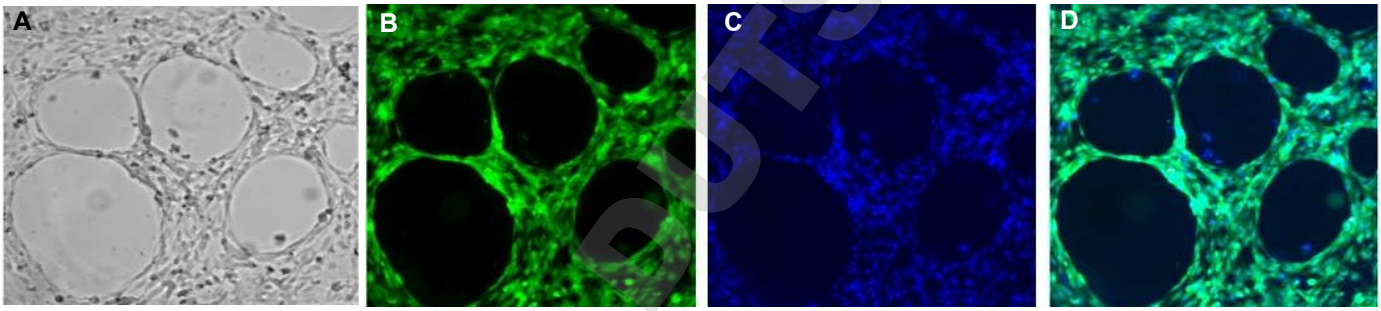


Figure 2. HUVEC cells form tubular structures on fibrin gel when stimulated with PMA and 1X ITS Media Supplement. Passage 3 HUVEC cells were cultured at 3 x 10^5 cells per well on a fibrin-coated µ-Angiogenesis Slide in the presence of 50 ng/mL PMA and 1X ITS media supplements in EndoGRO LS Complete Medium. Capillary tubes appear after 24 hour incubation at 37 °C. Cells were stained with 1 µM Calcein AM and 1 µg/mL DAPI for 15 minutes in culture media. Staining reagent was removed and replaced with EndoGRO LS Complete Medium before imaging. (**A**) Bright field image, (**B**) Calcein AM stain. (**C**) DAPI stain. (**D**) Merged image of Calcein AM and DAPI. Images were taken with 5X objective.

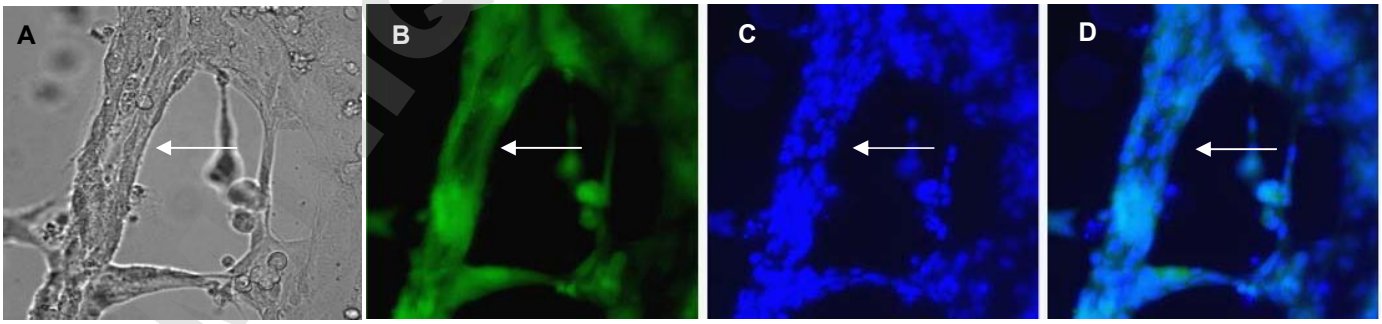


Figure 3. Lumen formation of HUVEC cells on fibrin gel in the presence PMA and 1X ITS Media Supplement. Lumens (A, closed arrow) are observed after 24 hour incubation with 50 ng/mL PMA and 1X ITS Media Supplements. Cells were stained with 1 µM Calcein AM and 1 µg/mL DAPI for 15 minutes in culture media. Staining reagent was removed and replaced with EndoGRO LS Complete Medium before imaging. (**A**) Bright field image, (**B**) Calcein AM stain, (**C**) DAPI stain, (**D**) Merged image of Calcein AM and DAPI. Images were taken with 20X objective.

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