Endothelial Tube Formation Assay in LunaX™ ECM

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Introduction

Angiogenesis and vasculogenesis are key processes involved in the formation of new blood vessels and rely on the coordinated migration, proliferation and differentiation of endothelial cells and supporting stromal cell types, such as pericytes and mesenchymal stromal cells (MSCs). Dysregulated angiogenesis plays an important role in the initiation and progression of a range of diseases, including cancer, autoimmune disease and rheumatoid arthritis.

In vitro endothelial tube formation assays provide a useful model for studying the cellular and molecular mechanisms that regulate new vessel formation, as well as for evaluating therapeutic strategies designed to modulate angiogenic processes.

This protocol describes optimised assay conditions for the formation of capillary-like endothelial networks by co-culturing human umbilical vein endothelial cells (HUVECs) with pericytes or MSCs within LunaX Photocrosslinkable Extracellular Matrix (ECM). The assay provides a physiologically relevant 3D model of angiogenesis and vasculogenesis for preclinical drug discovery, therapeutic screening and mechanistic studies of vascular network formation.

Expected Results

Capillary-like structures and highly interconnected cellular networks typically form within 2–3 days of culture, maturing into lumen-containing tubules with continued culture. Structures are readily observed by brightfield microscopy.

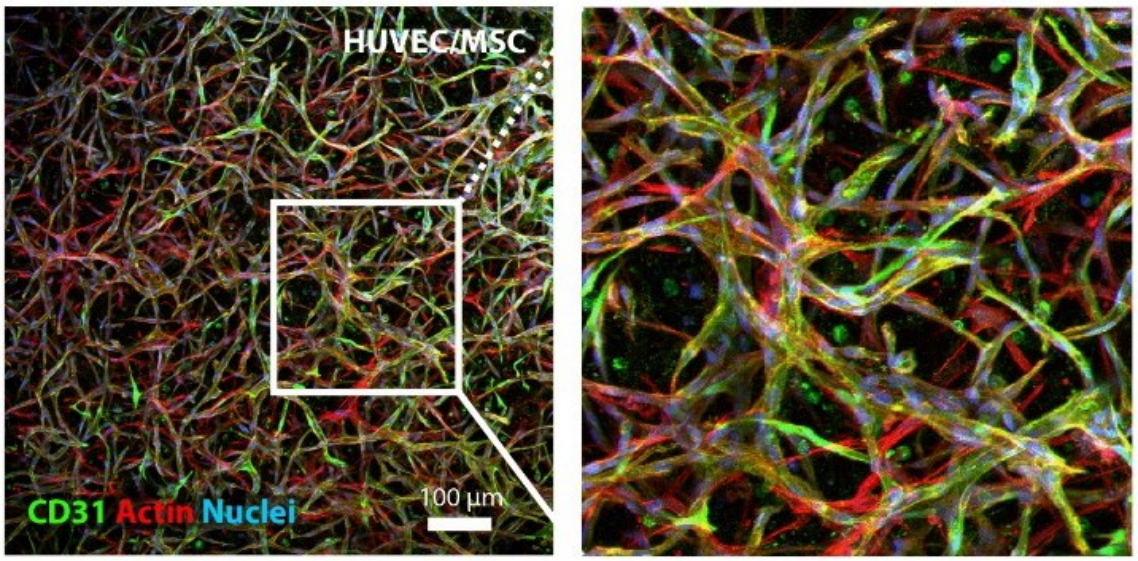

Figure 1. *Capillary-like network formation of HUVECs and MSCs in LunaX ECM, Day 7. Co-cultures were maintained with SDF-1, FGF-2, and VEGF, fixed with 4% PFA, and stained for the endothelial marker CD31 (green) and actin (red). Nuclei are shown in blue. Scale bar = 100 µm.*

Materials and Equipment

Reagent / Item	Specification / Concentration
LunaX MultiMatrix, Low Stiffness	Gelomics Cat. No. SKU0002, includes photoinitiator
LunaX Crosslinker	Gelomics Cat. No. SKU0028
24-well plate	Clear, flat bottom
Human umbilical vein endothelial cells (HUVECs)	Lower passage cells generally perform better, passage < 8; 70 – 80% confluency at harvest
Pericytes or mesenchymal stromal cells (MSCs)	70–80% confluency at harvest
Endothelial growth medium	e.g. EGM-2 BulletKit (Lonza Bioscience, Cat. No. CC-3162)
Stromal cell-derived factor-1 (SDF-1)	Final concentration of 50 ng/mL in media
Fibroblast growth factor 2 (FGF-2)	Final concentration of 50 ng/mL in media
Vascular endothelial growth factor (VEGF)	Final concentration of 50 ng/mL in media
Sterile PBS	1x concentration for photoinitiator reconstitution
Trypsin or dissociation reagent	As per cell-specific SOP
Water bath	Set at 37 °C
CO_2 incubator	37 °C, 5% CO_2, humidified
Benchtop centrifuge with tube carrier	-
Automated or manual cell counter	-
Calibrated pipettes and sterile tips	Suitable for reverse pipetting, or positive displacement

Procedure

1. Prepare ECM and Photoinitiator

1. Warm the 1.5x LunaX ECM solution to 37 °C in a water bath for approx. 15 minutes.
2. Determine the volume of precursor solution required. Precursor solution can be prepared in 1.5 mL increments as follows:
   - Reconstitute one 2.7 mg vial of photoinitiator in 0.6 mL PBS. Protect from light.
   - In a sterile tube, combine 1 mL of 1.5x LunaX ECM solution with 0.5 mL of photoinitiator solution.
3. Reconstitute the required number of photoinitiator vials using 0.6 mL sterile PBS per vial. Protect from light.
4. Prepare the required volume of gel precursor solution by mixing the 1.5x LunaX ECM solution with photoinitiator solution as described above. Place the precursor solution in a water bath at 37 °C.
5. If you use part of a vial of photoinitiator, the remaining reconstituted photoinitiator can be stored at 2–8 °C, protected from light, for use on the same day. Only use photoinitiator

solutions prepared on the day of use. For experiments on subsequent days, prepare a fresh photoinitiator solution.

2. Cell Harvest and Encapsulation

1. Lift and count HUVECs and pericytes/MSCs according to your standard protocol. Important: Ensure you completely quench and remove the trypsin or other protease before proceeding.
2. For each 1 mL of final cell-laden LunaX ECM required, combine 6×10^6 HUVECs and 6×10^5 pericytes or MSCs (10:1 ratio) into a single tube and centrifuge to pellet. Important: Centrifuge under gentle conditions, according to the cell supplier's instructions or your protocol, to protect cell health and function.
3. Remove the supernatant completely, minimising residual liquid that could dilute the ECM.
4. Resuspend the cell pellet in the gel precursor solution by gentle pipetting until homogeneous. Mix thoroughly by pipetting to ensure a homogeneous suspension. Important: take care to avoid introducing air bubbles.

3. Dispensing and Crosslinking

1. Dispense 30 μL of the cell-laden LunaX ECM into the centre of each well of a 24-well plate to form dome-shaped samples.

   > ***Note:*** *Use reverse pipetting to avoid introducing air bubbles when dispensing into well plates.*

2. Carefully transfer the plate to the LunaX Crosslinker, taking care to avoid disturbing or smudging the domes.
3. Crosslink for 50 seconds at 10 mW/cm^2 to achieve ~1.5 kPa elastic modulus.

4. Culture and Maintenance

1. Add 1 mL of pre-warmed EGM-2 medium, supplemented with SDF-1, FGF-2, and VEGF to each well. Incubate at 37 °C, 5% CO_2.
2. Replace 70% of the culture medium every 2 days.

5. Imaging and Analytics

1. Gel constructs can be viewed using brightfield and fluorescent microscopy.
2. For imaging of antigens that are not fixation sensitive, fix your samples with 4% PFA, or your standard fixation protocol. For fixation sensitive antigens, image immediately without fixing.
3. For immunofluorescence, stain for markers such as CD31 and F-actin/cytoskeletal structures, with nuclear counterstaining, according to the antibody or reagent supplier's instructions. For crisp images of constructs, image using a confocal microscope.
4. Refer to separate protocol for paraffin embedding and sectioning.

Optional Control Groups to Consider

- Matrix-only wells (no cells) to identify gel artefacts.
- HUVEC monoculture wells (without pericytes/MSCs) to assess the contribution of co-culture to network formation and stability.
- A known pro- or anti-angiogenic compound as a pharmacological control, where applicable for your study design.

Troubleshooting

Problem	Solution
LunaX ECM solidifies during protocol	Return sealed ECM tube (with or without cells) to the 37 °C water bath until re-liquefied. Mix by gentle pipetting. Do not heat above 37 °C.
Air bubbles in the LunaX ECM suspension	Centrifuge the suspension (with or without cells) at 300 × *g* for 1 minute. If cells have been added, gently mix by pipetting to resuspend the cells.
LunaX ECM does not crosslink on light exposure	Confirm the photoinitiator was prepared fresh and reconstituted correctly.
Capillary-like structures not forming	Confirm HUVEC passage is below 8 and cells are healthy. Use fresh medium and growth factors at the specified concentrations. Use gentle centrifugation conditions.
LunaX ECM domes dissolve after encapsulation	Residual trypsin or protease may be degrading the hydrogel. Ensure complete removal by washing the cell pellet with serum-containing medium before encapsulation.
Cell outgrowth on the well surface around the gel dome	Use a non-tissue-culture-treated well plate where possible to minimise cell attachment and growth on the well surface.

References

- LunaX ECM and LunaX Crosslinker manufacturer's instructions (Gelomics)
- LX-APR-006 – Whole Mount Immunofluorescence (Gelomics)
- LX-APR-008 – Paraffin Embedding and Sectioning (Gelomics)
- EGM-2 BulletKit manufacturer's instructions (Lonza Bioscience).