Shedding New Light On **MICROSCOPY**

APPLICATION NOTE

AX/AX R Confocal Microscope
Cell Observation Device BioStudio-T
General Analysis 3 Image analysis Software module

3D Angiogenesis Assay Utilizing Microphysiological Systems

Angiogenesis is associated with physiological processes such as reproduction, development, and wound healing. Simultaneously, it is intricately linked to diseases including cancer, autoimmune disorders, age-related macular degeneration, and atherosclerosis. Particularly in cancer, angiogenesis is a key factor behind tumor growth and metastasis, making it a hallmark of cancer and a vital target for drug discovery efforts. *In vitro* assays mimicking the angiogenesis process have been utilized for anti-cancer drug screening and other pharmaceutical developments. This application note introduces a validation study of a 3D angiogenesis assay using human umbilical vein endothelial cells (HUVECs) in AIM Biotech's idenTx series Microphysiological Systems (MPS).

Experimental Overview

The idenTx series enables enhanced physiological relevance in *in vitro* models by controlling shear stress on endothelial cells (ECs) through media perfusion, establishing concentration gradients of angiogenic factors, and inducing interstitial flow. The interstitial flow synergizes with the concentration gradient of angiogenic factors within the gel, promoting directed angiogenesis along the flow direction[1]. Compared to conventional 2D and 3D cell culture methods, this approach facilitates the implementation of angiogenesis models with higher physiological fidelity. In this study, AIM Biotech's idenTx3/9 (Fig. 1a, b) and idenTx40 (Fig. 1c) platforms were utilized. The MPS system employs gravity-driven perfusion facilitated by differences in media volume between medium ports, generating shear stress and interstitial flow within the gel (Fig. 1d, e, f, g). Collagen I was used to fill the hydrogel channel, and primary HUVECs were seeded only in one of the media channels (Fig. 1h, i). The opposing media channel was supplemented with medium containing angiogenic factors, replaced twice daily. This setup was designed to induce unidirectional interstitial flow and to drive angiogenesis over a four-day period.

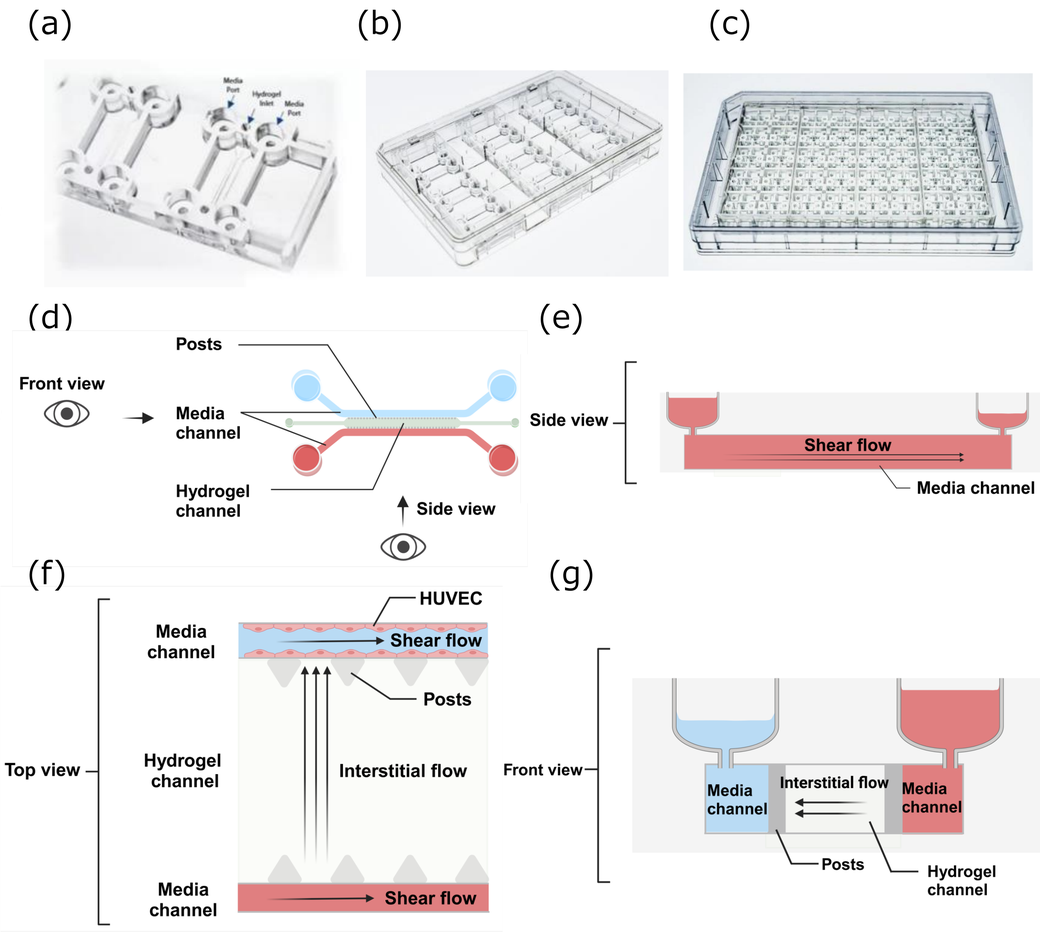

Figure 1. Schematic of the 3D Angiogenesis Assay
Schematic of the AIM Biotech MPS devices used: idenTx3 (a), idenTx9 (b), and idenTx40 (c) (URL: https://aimbiotech.com/). (d) Schematic of the microfluidic unit/chip in idenTx3/9. The three microfluidic channels are separated by triangular posts, with the central gel channel patterned with hydrogel. The gel channel is in contact with the media channels adjacent to the posts. (e) Side view of the idenTx3/9 channels. (f) Top view of the idenTx3/9 channels. (g) Front view of the idenTx3/9 channels. (h, i) Concentration gradients of angiogenic factors and interstitial flow promoting angiogenic sprout formation. Arrows in each panel indicate the direction of shear flow in the media channels and interstitial flow in the gel channel. The illustration was created using BioRender (https://www.biorender.com/).

Selection of Collagen Resistant to Excessive Gel Degradation During Angiogenesis

By day 3 of angiogenesis induction, gel degradation was observed around the posts (Fig. 2a). This phenomenon was consistent across collagen products from different manufacturers and was attributed to excessive activation of HUVECs, accompanied by protease secretion[2, 3]. In this 3D angiogenesis assay, measuring the length of angiogenic sprouts within the patterned collagen gel is one of the critical parameters. However, excessive gel degradation compromises the accuracy of measurement. Comparative analysis identified a collagen product from Manufacturer E that exhibited resistance to degradation during angiogenesis (Fig. 2b). Using this collagen gel, angiogenesis was successfully induced over four days without significant gel degradation, and sustained elongation of angiogenic sprouts was observed (Fig. 2c).

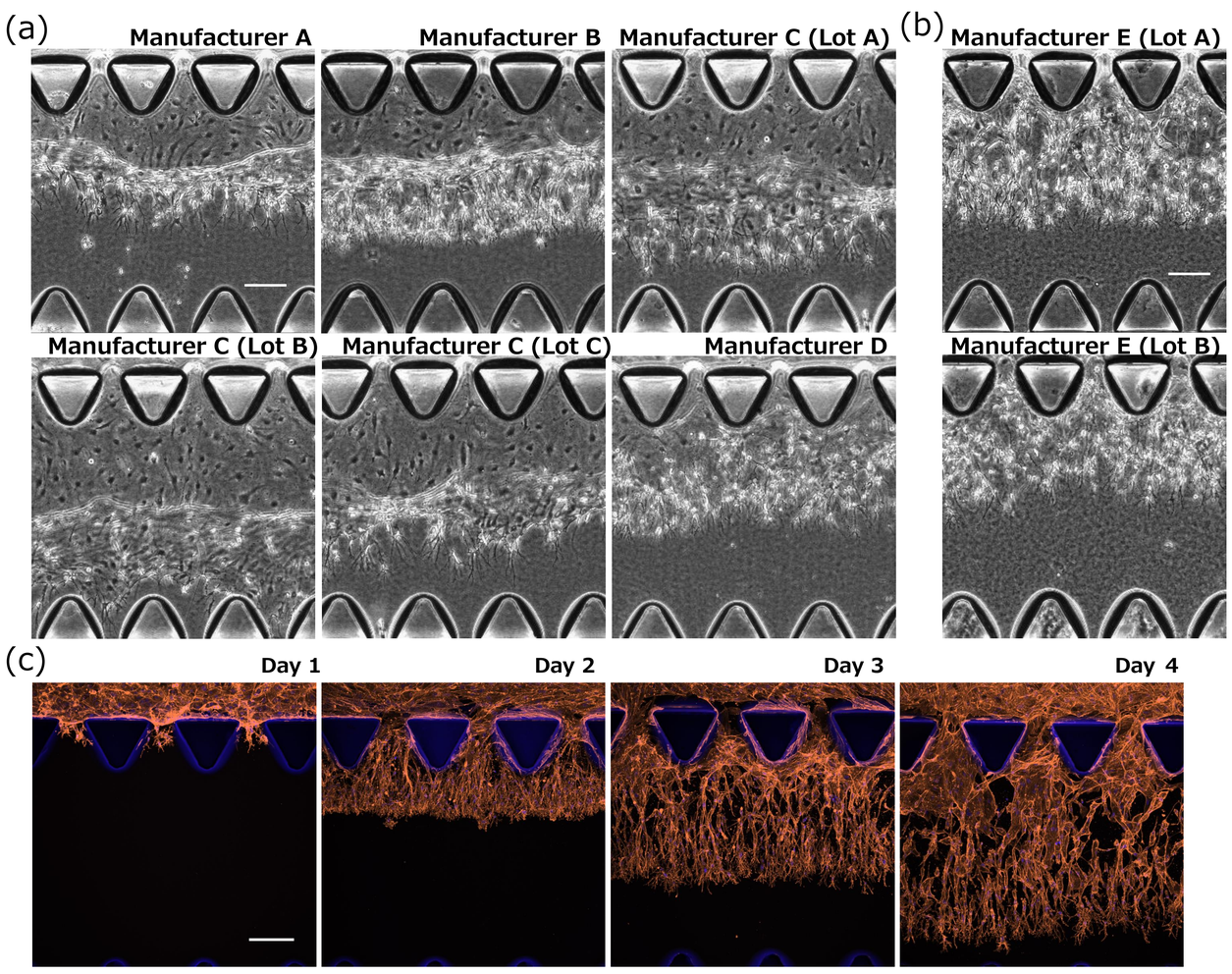

Figure 2. Selection of Collagen Resistant to Excessive Gel Degradation During Angiogenesis
(a) Phase-contrast images of angiogenesis in collagen gels from different manufacturers. (b) Phase-contrast images of angiogenesis in collagen gels from Manufacturer E. The idenTx3/9 MPS device was used, and all collagen gels were polymerized at a concentration of 2.5 mg/ml. HUVECs (PromoCell, Lot A) were seeded, and angiogenesis was induced by adding 50 ng/ml FGF-2, 100 nM PMA, 500 nM S1P, and 50 ng/ml VEGF. Phase-contrast images were captured on day 3 using the BioStudio-T imaging system (Nikon). (c) Confocal images of 3D angiogenesis. Fixed samples were stained with phalloidin (orange: F-actin) and Hoechst (blue: nuclei) at each time point, and confocal Z-stack images were acquired using the AX confocal laser microscope system (Nikon) with a 10x objective lens. Maximum Intensity Projection (MIP) images are shown. Scale bar = 200 µm.

Effects of the Anti-Cancer Drug Sunitinib on 3D Angiogenesis

To evaluate the influence of angiogenic factors on HUVEC-driven angiogenesis, treatments with and without angiogenic factors were compared on day 4 (Fig. 3a). Angiogenesis was promoted under treatment with angiogenic factors, and volumetric imaging followed by XZ cross-section analysis confirmed the formation of vascular lumens (Fig. 3b).

Next, the validity of drug efficacy evaluation was assessed using the known angiogenesis inhibitor sunitinib, a vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor (Fig. 3c). Fluorescent confocal images were analyzed using the General Analysis 3 (GA3) functionality of NIS-Elements (Nikon). Sunitinib significantly suppressed angiogenesis at a concentration of 10 µM (Fig. 3d, e, f, g).

To assess the reliability of the 3D angiogenesis assay, the conditions of solvent control (DMSO-treated) and sunitinib-treated (10 µM) samples were compared. Based on the maximum vertical length of angiogenesis, a Z'-factor of 0.49 was calculated, indicating that this MPS-based platform is suitable for evaluating the anti-angiogenic effects of anti-cancer drugs (Fig. 3d).

Robustness of the 3D Angiogenesis Assay

The robustness of the assay system was further validated using HUVECs derived from different donors. Using one lot from PromoCell (Lot B) and two lots from LONZA (Lots C and D), the anti-angiogenic effects of sunitinib were evaluated. The Z'-factor, based on the maximum vertical length, ranged from 0.39 to 0.76, demonstrating consistent results across multiple lots (Fig. 4a, b, c). These findings confirm that this 3D angiogenesis assay provides a robust and quantitative method for evaluating the efficacy of sunitinib.

Conclusion

This application note demonstrates and validates a 3D angiogenesis assay using AIM Biotech's MPS. The assay successfully mimics the angiogenesis process with high physiological relevance and was shown to be effective in evaluating the known anti-angiogenic effects of sunitinib. Consistent results across HUVEC lots further show the robustness of this assay system. This assay has potential for applications beyond anti-cancer agents, extending to the development and evaluation of therapeutics for various angiogenesis-related diseases.

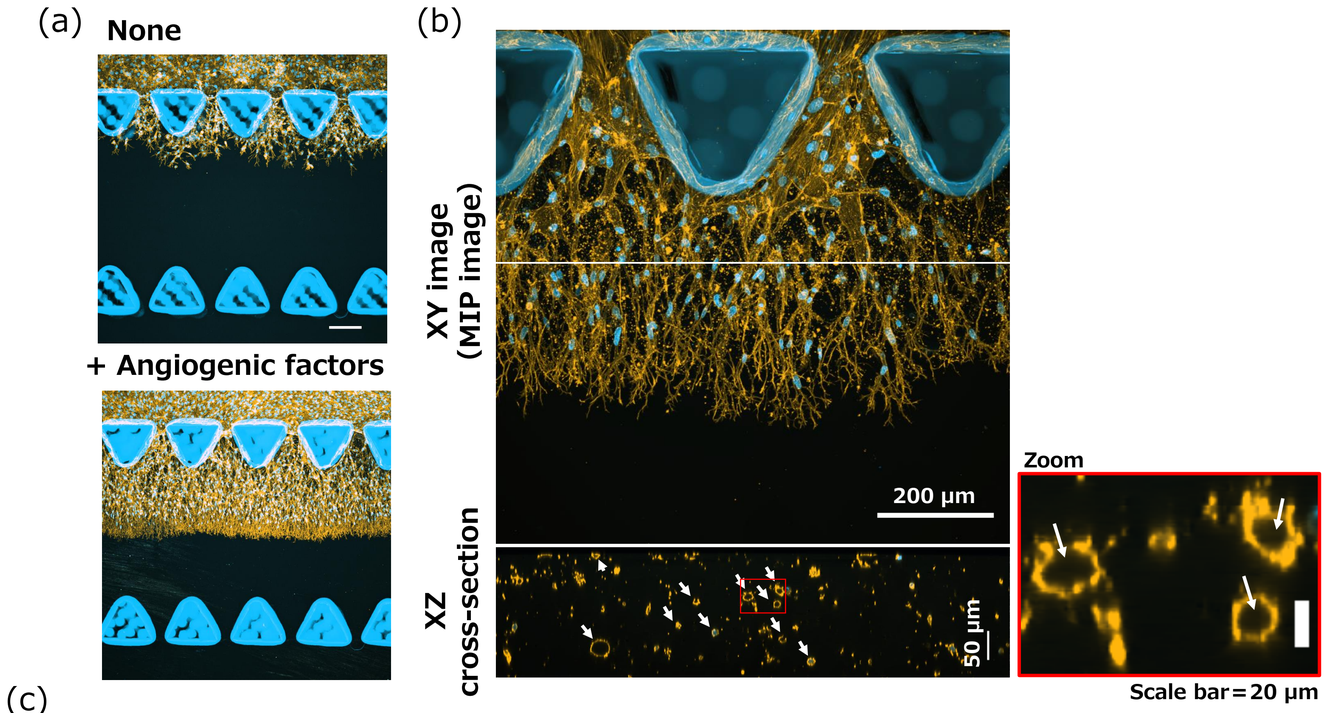

Figure 3. Effects of the Anti-Cancer Drug Sunitinib on 3D Angiogenesis in HUVECs
(a) Confocal images of angiogenesis. (b) XZ cross-sectional confocal images of angiogenesis along the white line. Arrows indicate the lumen structure of the angiogenesis. The bottom-right panel shows a magnified view of the red-framed area. (c) Confocal images of angiogenesis treated with sunitinib. (d-g) Quantitative image analysis of angiogenesis: Maximum Vertical Length (d), Branching Points (e), Total Area (f), and Nuclei Count (g). Error bars represent standard deviation (SD) with n = 5. * indicates $p < 0.05$ as determined by Dunnett's test compared to DMSO treatment. Angiogenesis was induced using HUVECs (PromoCell, Lot A) in idenTx40 and collagen from Manufacturer E, treated with 50 ng/ml FGF-2, 0.5 nM PMA, 500 nM S1P, 50 ng/ml VEGF, and various concentrations of sunitinib, and cultured for 4 days. Confocal Z-stack images of fixed samples were stained with phalloidin (orange: F-actin) and Hoechst (blue: nuclei) and acquired using the AX confocal microscope system (Nikon) with a 10x objective lens. The posts exhibit autofluorescence (blue) at Hoechst excitation wavelengths. Scale bar = 200 µm.

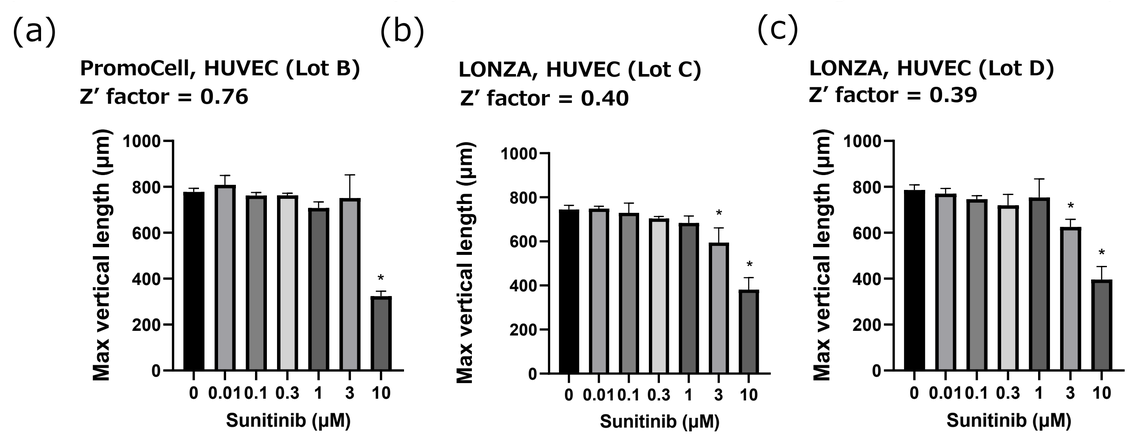

Figure 4. Lot-to-Lot Variation in 3D Angiogenesis Assay Using HUVECs
(a, b, c) Quantitative image analysis of 3D angiogenesis using HUVECs from different lots. Angiogenesis was induced using HUVECs from PromoCell (a) and Lonza (b, c), idenTx40, and collagen from Manufacturer E, treated with 50 ng/ml FGF-2, 0.5 nM PMA, 500 nM S1P, 50 ng/ml VEGF, and various concentrations of sunitinib, and cultured for 4 days. Each graph shows the max vertical length of angiogenesis analyzed from phalloidin-stained images. Z'-factors for lot-to-lot variation were calculated based on the max vertical length of angiogenesis under DMSO and 10 µM sunitinib treatments and are shown in each panel. Error bars represent SD with n = 5. * indicates $p < 0.05$ as determined by Dunnett's test compared to DMSO treatment.

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References

1. Synergy between interstitial flow and VEGF directs capillary morphogenesis *in vitro* through a gradient amplification mechanism, Cara-Lynn E Helm et. al., PNAS USA., 2005, 1;102(44):15779-84.
2. Cellular and molecular mechanisms of vascular lumen formation, M Luisa Iruela-Arispe et. al., Dev Cell., 2009, 16(2):222-31.
3. Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*., Duc-Huy T. Nguyen et. al., PNAS USA., 2013, 23;110(17):6712-7.

Product Information

AX/AX R Confocal Microscope:

- Low phototoxicity for live-cell imaging
- High-speed, high-resolution, and large FOV wide-field confocal imaging with minimal photobleaching
- High speed: Up to 720 frames per second (Resonant 2048 × 16 pixels)
- High resolution: Up to 8K (Galvano) / 2K (Resonant)
- High throughput: Ultra-wide field of view of 25 mm

Cell Observation Device: BioStudio-T

- Can be installed inside a CO_2 incubator
- Capable of capturing images of the whole well plate by moving the objective lens
- Enables stable, vibration-free time-lapse imaging
- Compatible with AIM Biotech's idenTx series gas-permeable laminate