

A Heterogeneous Angiogenesis Assay using Primary Cell Co-culture in Microchannels

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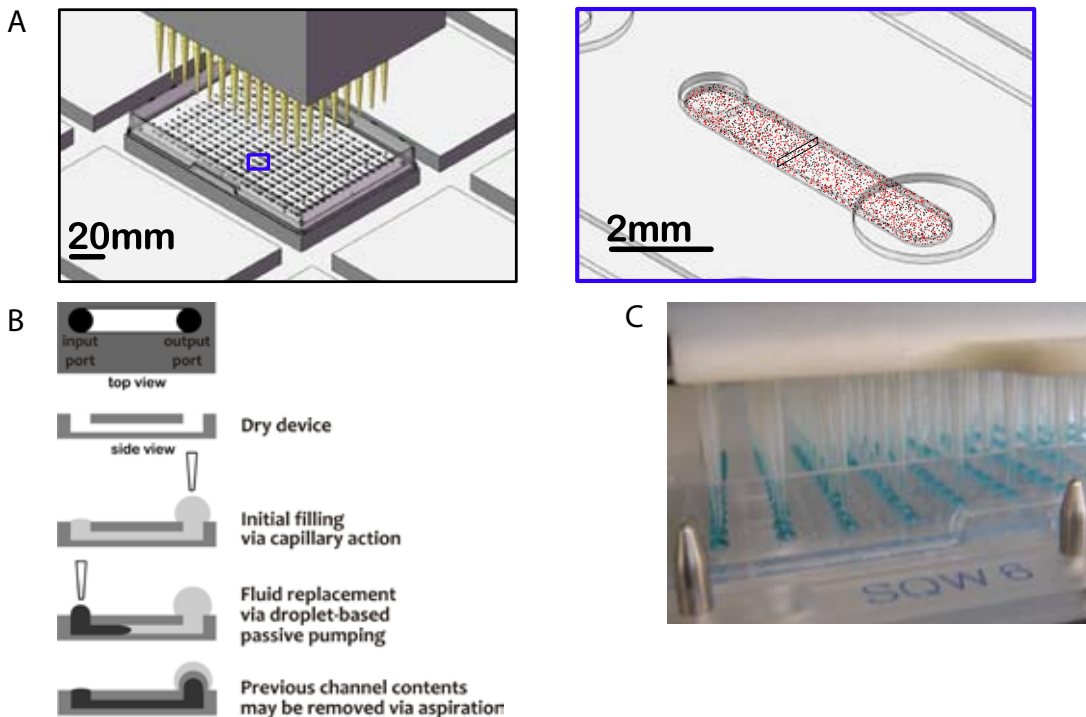
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Introduction

When primary endothelial cells are co-cultured with primary diploid fibroblasts, structures resembling a microvasculature bed can be obtained. This model recapitulates a wide array of processes important for angiogenesis including migration, proliferation, apoptosis, necrosis and deposition of an endothelial cell basement membrane¹. No exogenous extracellular matrix is required, and standard endothelial growth media containing low (2%) serum is sufficient. This cell model has been adapted for highly parallel operation in an array of microchannels. The microchannel arrays are designed to allow fully automated operation, including media changes and immunocytochemistry wash steps, all using standard liquid handling automation. Presented are results of validation using anti-angiogenic compounds.

Figure 1.

Microchannel Arrays used in this Study

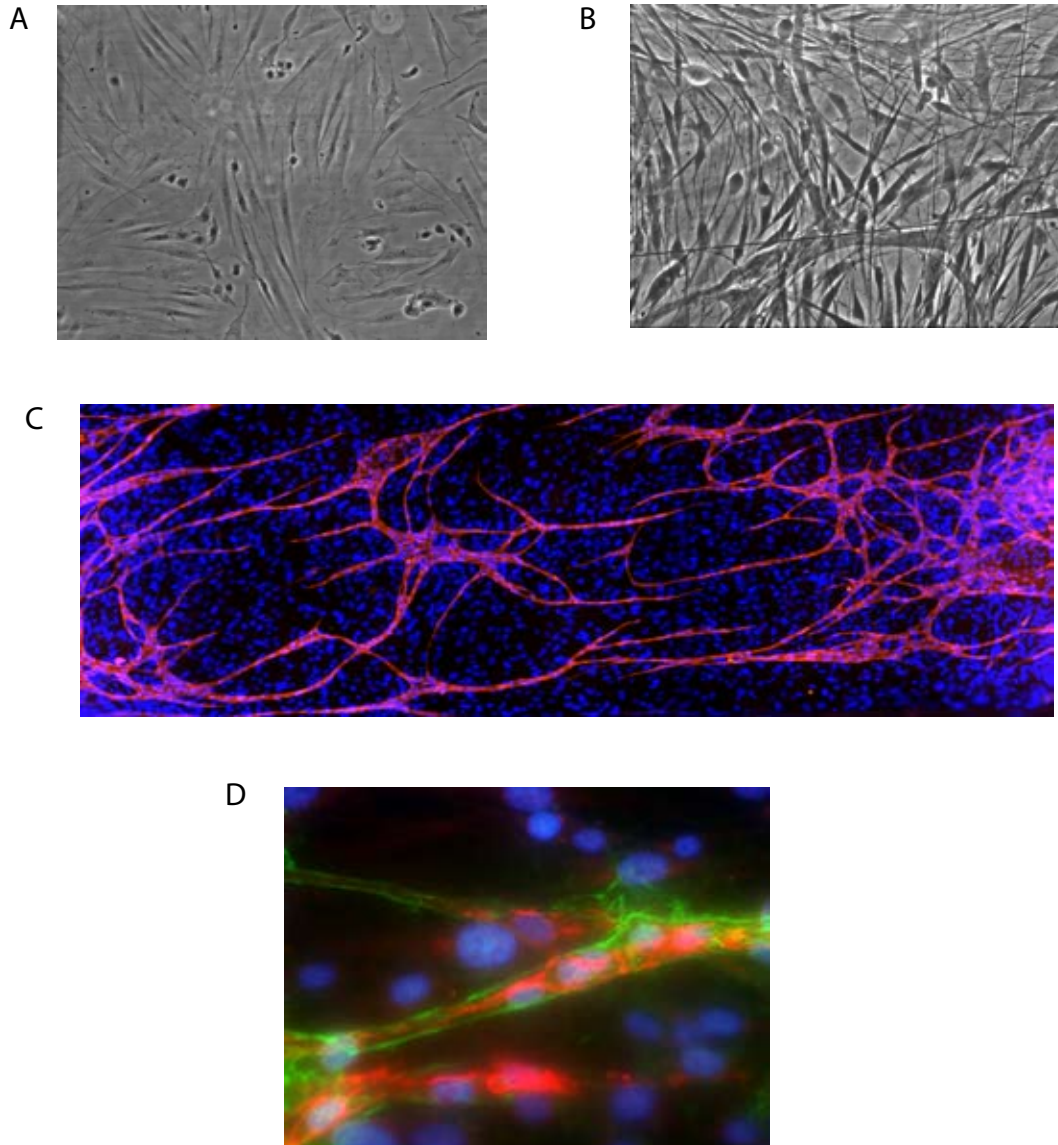


A) Schematics and dimensions of the iuvo™ microchannel arrays.

B) Droplet-based passive pumping is used for all assay related manipulations: (1) loading cells, (2) changing media, (3) treating cells with compounds, and (4) delivering assay reagents.

C) Operation of the microchannel arrays is compatible with existing liquid handling automation. Shown is transfer of a blue dye solution using the CyBi-Well® (CyBio).

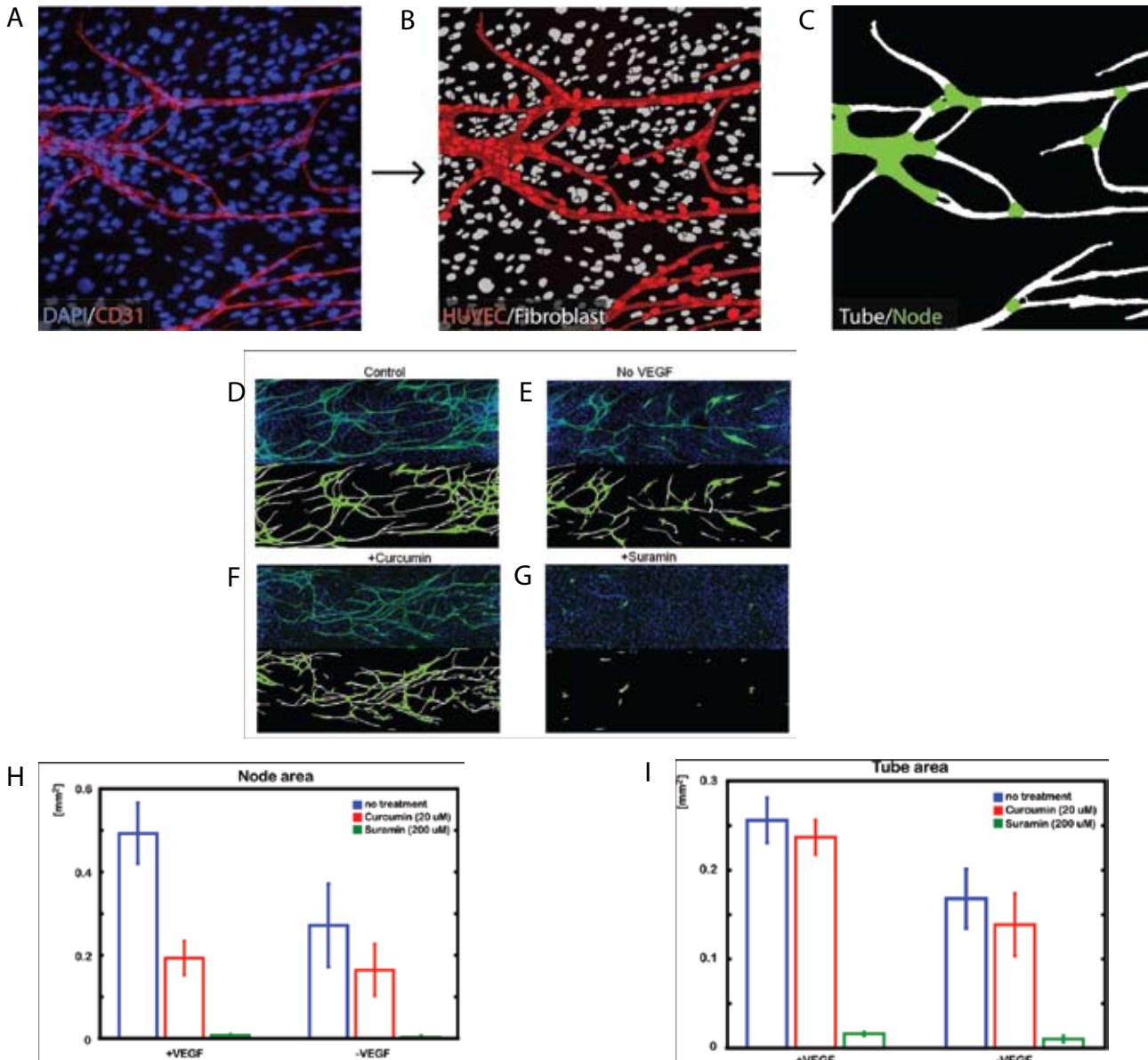
Figure 2.
Microvessel Formation in Microchannel Arrays



DAPI/CD-31/collagen IV

A mixture of 600 primary human umbilical vein endothelial cells (HUVEC) and 2400 primary normal human dermal fibroblasts (NHDF) were seeded into microchannels using 1.2 μ L cell suspension. Media was changed every day. A-B) Phase contrast images of cells at 1 day (A) and 5 days (B) post plating, 20X. With careful inspection, the endothelial tubes can be seen organizing amidst the fibroblasts. C) Immunocytochemistry for CD-31, an endothelial cell marker shown in red, and the nuclear counterstain DAPI (blue) was carried out in the channels using passive pumping based liquid handling. Shown is the entire channel region stitched together from images acquired at 10X. D) Closeup of microvessel stained with CD-31 (red), collagen IV (green) reveals deposition of endothelial cell basement membrane proteins imaged using a 40X objective.

Figure 3.
Inhibition of Angiogenesis by Natural Products

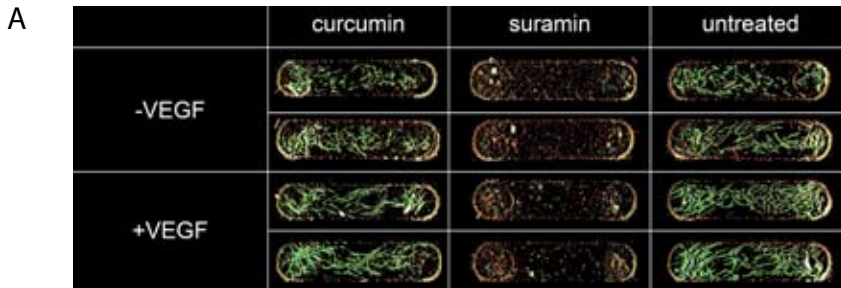


HUVEC and fibroblasts were seeded into microchannels as in figure 2. Cells were grown in Microvascular Endothelial media (Lonza) with and without VEGF and treated with either 20 μ M curcumin, or 200 μ M suramin, each day for 7 days. The angiogenesis assay was performed by fixing cells and then staining with CD-31 antibody followed by Alexa488 secondary antibody using standard immunocytochemistry procedures. Passive pumping was used for liquid handling transfers. A DAPI co-stain for visualization of nuclei for both fibroblast and endothelial cells was also performed. **A)** Images of both the CD-31 and DAPI stain were acquired with an automated microscope using a 10x objective. **B)** The two images were then analyzed using an algorithm that scores each cell as either fibroblast or endothelial based on co-staining of DAPI and CD-31. **C)** A tube formation algorithm was applied to the image of CD-31, which allows users to specify constraints on minimum and maximum tube width. Areas scored as tubes (between minimum and maximum width) are shown in white, and nodes (width above maximum constraint) in green.

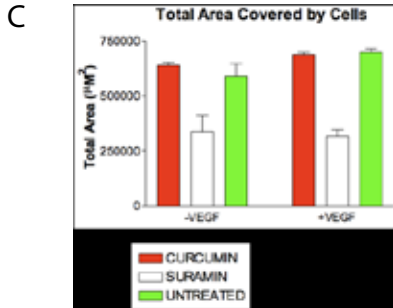
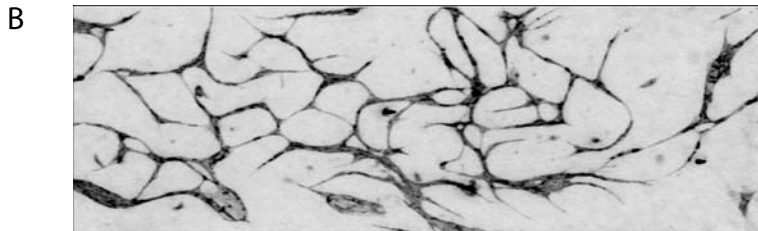
D-G) The top image of each panel represents a two color overlay of CD-31 and DAPI staining. The bottom image of each panel shows the result segmented image derived from application of the tube formation algorithm. **D)** Untreated control using only microvessel media (MV media, Lonza) containing VEGF. **E)** Media identical to **D)** except VEGF was not added. **F)** 20 μ M curcumin **G)** 200 μ M suramin.

H-I) Data analysis reveals that VEGF is important for tube and node formation. Suramin dramatically inhibited both tube and node formation, whereas curcumin led to a decrease in node area. Plotted are individual assessments of node area (**H)** and tube area (**I)** for each experimental condition.

Figure 4.
Imaging of iuvo Plate with TTP Labtech
Acumen ^eX3[®]



Imaging of iuvo plate with TTP Labtech Acumen ^eX3[®]. The iuvo plate from figure 3 was imaged using the laser-based plate scanner, Acumen ^eX3[®], with comparable image quality and resolution (A-B). Using the Acumen's algorithms for thresholding, analysis was performed looking at the total area stained positive for CD-31 (C). Using total area covered by HUVEC's as a measure of angiogenesis shows the dramatic effect of suramin on the endothelial cell population. However, more sophisticated algorithms are needed to investigate the effect of drug compounds on tube and node formation, but have not yet been tested on the ^eX3[®]. Reading of the microchannels in this experiment required device modification, including removal of the tray walls. Development is currently underway for an adaptor that will allow iuvo devices to be read in all Acumen Explorer instruments without modification.



Conclusions

- Passive pumping can support heterogeneous primary co-culture assays in micro-conduit arrays, operated by pipettors or standard liquid handling instrumentation.
- iuvo microchannels support an angiogenesis model using only co-culture of primary fibroblast and endothelial cells and does not require the addition of extracellular matrix proteins. Basement membrane deposition occurs at the basal surface of the microvessels.
- Small channel volume allows the use of only 600 HUVEC cells per data point.
- Data can be acquired using automated plate imaging platforms and analyzed using sophisticated tube formation algorithms which enables the use of cell morphology to be used as a metric for the effect of drugs treatment on angiogenesis.
- Based on this early data and looking only at area of tube and nodes, suramin treatment leads to a loss of both tube and node formation while curcumin treatment leads to a decrease in node area only.

References

1 Bishop, E. T. et al., *Angiogenesis* 3 (4), 335 (1999); Kunz-Schughart, L. A. et al., *Am J Physiol Cell Physiol* 290 (5), C1385 (2006); Phung, M. W. and Dass, C. R., *J Pharm Pharmacol* 58 (2), 153 (2006).

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