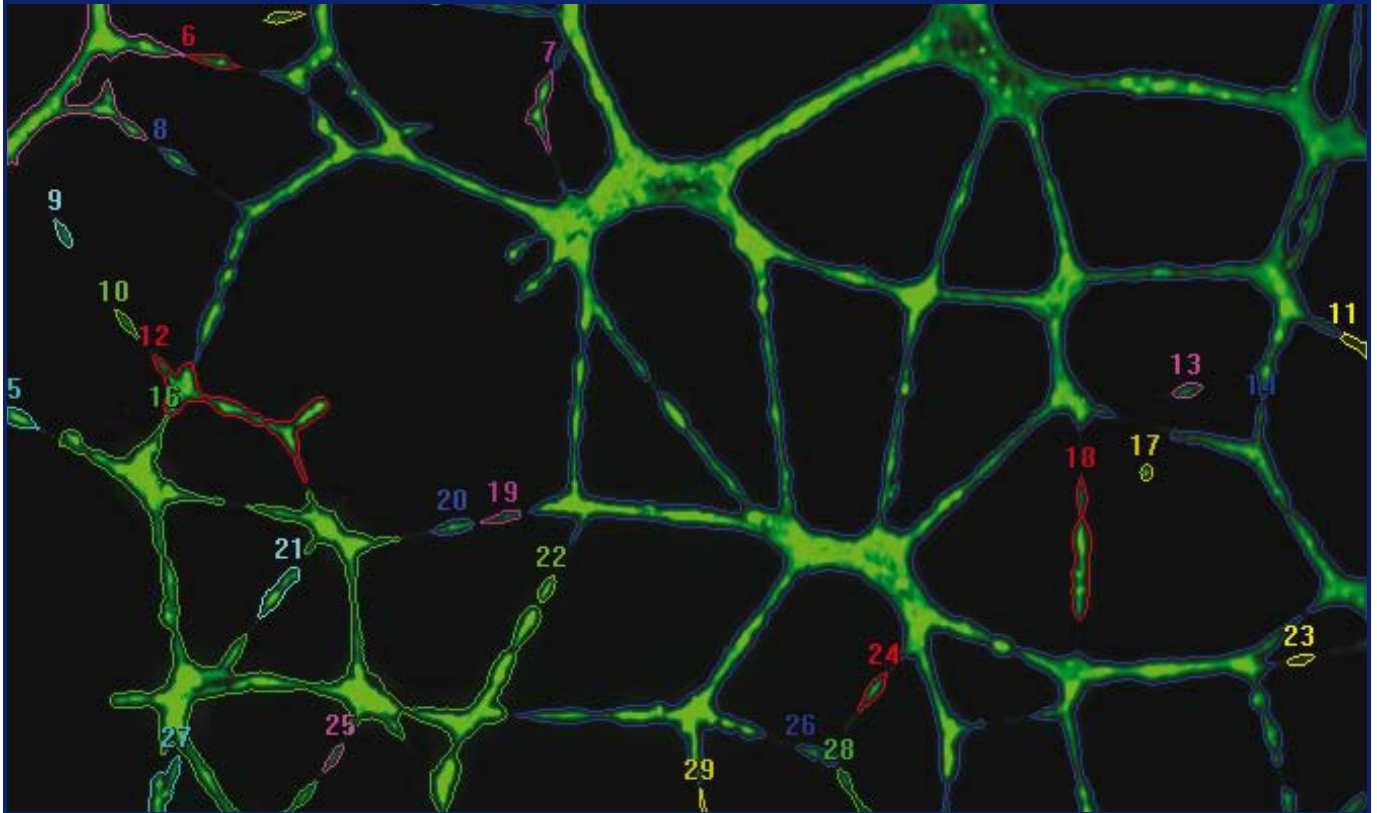


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An Image-Based Assay of Endothelial Cell Tube Formation as a Model of Angiogenesis



Introduction

Formation of new blood vessels is fundamental to the development of cancerous cell masses and has therefore been the focus of many drug screening and cell signaling research studies. Blood vessel development is a significant event in the development and growth of solid tumors, and is implicated in wound healing, retinopathy and macular degeneration.

Traditional animal models to quantify the degree of blood vessel formation are being replaced by cell culture assays that are easier to set up, statistically reliable and can be automated in a drug screening laboratory.^{1,2}

These assays rely on the endothelial cells' ability to form distinct blood-vessel-like tubules in an extracellular matrix (BD Matrigel™ Matrix) where they can subsequently be visualized by fluorescence microscopy. Although quantification of the tubules can be performed by manual tracing, this method precludes the use of the assay in unbiased high-throughput applications. Much preferred is the use of an automated confocal imaging system such as the BD Pathway™ Bioimager with sophisticated image and data analysis algorithms. The assay is designed to work with live cells in order to avoid fixation artifacts such as the disruption of tubules.

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Methods

Human Umbilical Vein Endothelial Cells (BD™ HUVEC-2, Cat. No. 354151) were maintained in EGM-2 media (Cambrex, CC-3156). The cells were washed and trypsinized (trypsin-EDTA, Cambrex, CC-5012) then resuspended into basal media (Cambrex, CC-3162) containing 0.1% BSA (BD Cat. No. 354331) and seeded into BD BioCoat™ Angiogenesis System: Endothelial Cell Tube Formation plates (Cat. No. 354150) at 20,000 cells/well and incubated for 16 to 18h at 37°C (5% CO₂). These plates contain an optimized BD Matrigel™ Matrix which promotes endothelial cell tube formation. Suramin (Calbiochem, 574625) was used to generate dose response curves from 0.3 to 160 μM. Suramin was solubilized in H₂O and serially diluted into basal media containing 0.1% BSA.

After 16-18h, the media was discarded and cells were washed twice with Hank's Balanced Salt Solution (HBSS) and stained with Calcein AM (Invitrogen, C-3100MP), 8 μg/mL for 30 minutes at 37°C/ 5% CO₂. The dye was discarded and cells were washed twice to remove excess dye. Some preparations were fixed with warmed 1% paraformaldehyde (in HBSS), washed twice with PBS and labeled with Alexa 488 Phalloidin (Invitrogen, A12379, 1:200 dilution).

Cells were imaged on the BD Pathway™ Bioimager in confocal and non-confocal mode using the 4 (NA 0.13) objective for quantification of tubule formation and 10 (NA 0.3) to generate higher resolution images.

Results

Initial results suggested that the tube formation event occurs across several focal planes, even with the low magnification used. This is not surprising considering that the endothelial cells are known to actively migrate into the BD Matrigel Matrix. We therefore employed a new feature of the BD Pathway Bioimager to automatically generate a single image representing the in-focus portion of multiple Z sections across a plate. This “collapsed stack” feature was combined with the ability of the system to acquire confocal images, thus ensuring a high degree of optical sample flatness even before the image was analyzed. Image analysis was conducted using the new BD Tube Formation Module, an advanced development of the BD Neurite Outgrowth Module (Cat. No. 346203). The algorithm thresholds the cells from the background and identifies a number of features including, the total length of the tubules, the total area of the tubules and the total number of segments. The software allows both turn-key analysis using default values as well as adjustment of parameters for the development of specific assays. The software allows cellular debris or non-connected tube fragments to be removed prior to data analysis. The data generated is contained in text files and easily analyzed using BD Image Data Explorer (Cat. No. 341039).

Control images (*Figure 3*, left panel) obtained with the BD Pathway Bioimager show distinct development of tubes indicating that endothelial cells indeed do resemble blood vessels. The image in *Figure 1* was acquired using a 10 objective and shows very clear development of endothelial cell-derived tubules. These tubules mostly originate at aggregates of cells and appear to actively connect with other tubules or aggregates. The total number of tubes formed (*Figure 3* and *Figure 4*) is an important measurement parameter since the underlying signaling and gene expression processes which mediate tube formation may offer an attractive leverage point for drug development.

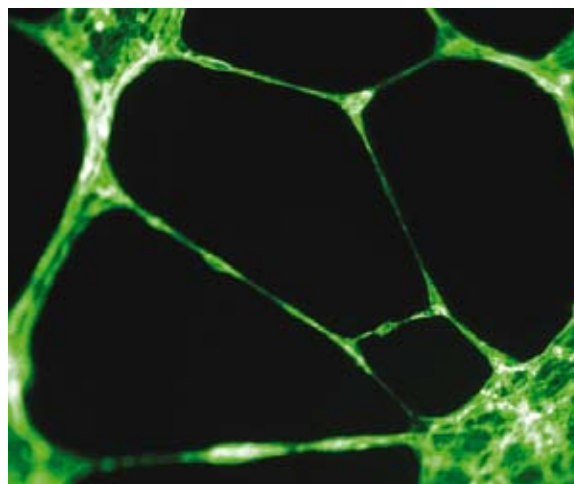


Figure 1. Endothelial cells form distinct tubules stretching between clumps of cells. The image was taken as a single non-confocal section using a 10x objective. Notice the out of focus areas indicating that the cells grow into the depth of the BD Matrigel™ Matrix.

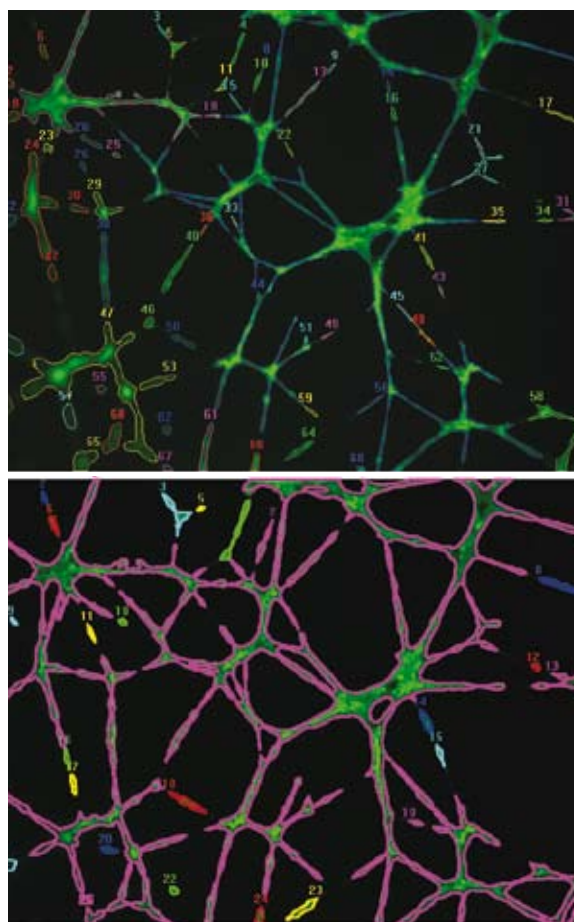


Figure 2. Confocal versus non-confocal imaging. The top panel shows control cells imaged in non-confocal mode and as a single optical slice. The bottom panel shows the same field of view but acquired in confocal mode and as a projection of 10 image planes (“collapsed stack”). The segmentation overlay indicates the tubules found. Notice that the confocal slicing and projection allow more precise identification of tubules. Non-confocal imaging tends to detect more disrupted tubules because they move in-and-out of focus, these lead to an overestimation of the number of tubules and an underestimation of total tube area and length. Numbers refer to identified tubes, the color for each is arbitrarily chosen.

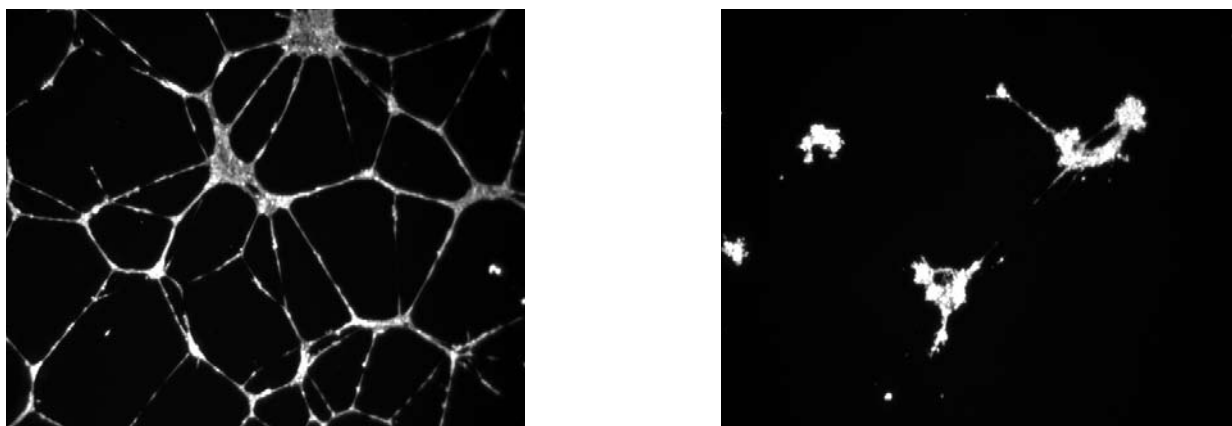


Figure 3. Effects of suramin on endothelial cell tubule formation. Left panel shows a full frame, confocal image stack projected into a single image (4x magnification) of control cells. Notice the distinct network of endothelial tubules formed. Right panel shows the inhibitory effects of suramin. At this concentration, suramin (40 μM) significantly impairs the formation of tubules.

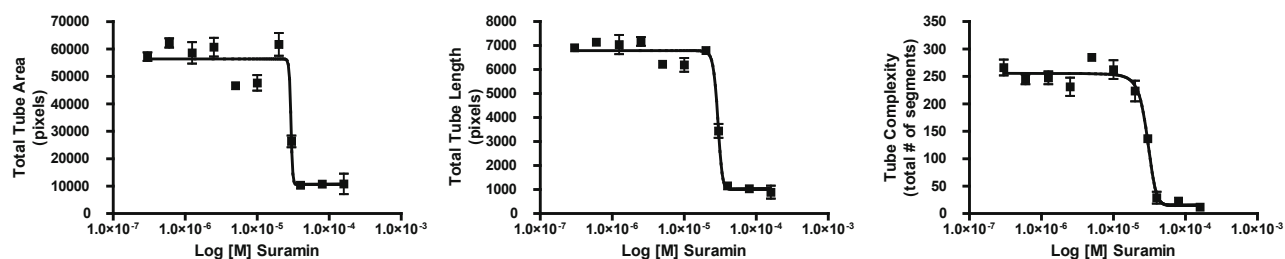


Figure 4. Dose response curves of suramin. Suramin inhibits tube formation in a dose-dependent manner. The effect was noticeable in the inhibition of the total tube area of the images (left panel), the total tube length (middle panel) and the complexity of the tubes which is a measure of the total number of tube segments (right panel). Data was acquired from $n=4$ wells and is shown as mean \pm S.E.M. For all three parameters, IC_{50} values of 29 μM were achieved, which is in agreement with published reports.³

Discussion

The advent of automated imaging platforms such as the BD Pathway™ Bioimager allow the migration of microscope-based assays into the drug discovery and systems biology arena. For this to succeed, images not only need to be acquired in a highly reproducible and hands-off manner but must also be analyzed with minimal user intervention. We present here such an assay consisting of human endothelial cells, 96-well coated plates, an automated imaging system and data analysis software. Confocal microscopy combined with the “collapsed stack” feature of the BD Pathway Bioimager enables analysis of angiogenic tubules in a 3 dimensional matrix that better resembles the native physiological environment. Alternative methods using non-confocal systems are being used where the matrix is dried out overnight to collapse the cells into a flat plane – this can not be done on live cells and may create significant distortions in the fragile branching structure of the sample.

Blood vessel formation is an important target in the treatment of multiple diseases including several cancer types. In the small molecule screening phase of drug discovery, animal experiments are low throughput and often technically too challenging, expensive and ambiguous to be used. Scoring the degree of blood vessel formation using an unbiased microscope approach allows this assay to be used in secondary screening applications against hundreds or thousands of putative drugs. Although cell culture experiments can currently not fully replace animal experiments, they aide in reducing the number of compounds to be tested and thus accelerate drug development and our understanding of cellular signaling events.

References:

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3. Fu, X.W, Nurse, C.A., Cutz, E. 2004. Expression of functional purinergic receptors in pulmonary neuroepithelial bodies and their role in hypoxia chemotransmission. *Biochem. J*. 385 (3-4), 275-284.

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