APPLICATION NOTE

3D image analysis and characterization of angiogenesis in organ-on-a-chip model

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Introduction

Angiogenesis is the physiological process of formation and remodeling of new blood vessels and capillaries from pre-existing blood vessels. This can be achieved through endothelial sprouting or splitting of the vessels and capillaries. Vascular cells respond to appropriate stimuli by degradation of the extracellular matrix, then proliferation and migration of endothelial cells[1,2].

Cells undergo these processes to create a tube containing a lumen, a dynamic space that facilitates blood flow and exchange of oxygen, carbon dioxide, nitric oxide and nutrients. Angiogenesis is a vital process in growth and development, as well as in wound healing and in the formation of granulation tissue. Angiogenic growth also supports the invasion of tumor cells in healthy tissue and is commonly measured in cancer research. When vascular sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in tandem, using adhesion molecules. These sprouts then form loops to become a full vessel lumen as cells migrate to the site of angiogenesis. Sprouting occurs at a rate of several millimeters per day *in vivo* and enables new vessels to grow across gaps.

Many anti-angiogenic drugs have been developed to use in cancer therapy, while pro-angiogenic molecules may hold potential in regenerative applications. *In vitro* experiments to date have modeled only some aspects of angiogenic mechanisms including scratch assays, Boyden chambers, and tube formation assays.

Benefits

- Visualize angiogenic sprouting and 3D reconstitution of structures
- Perform quantitative assessment of angiogenesis, including number of sprouts, total volume, and average intensity
- Generate physiologically-relevant results using the OrganoPlate® platform

MIMETAS scientists developed advanced and more physiologically relevant models that included the actual growth and sprouting of vessels from a main perfused vessel into a collagen extracellular matrix as directed by pro- or anti-angiogenic factors. Such cues can be added to either of the perfusion channels but can also be directly secreted by tissues in a co-culture setup.

High-content imaging allows visualization of angiogenic structures, 3D reconstitution, and complex analysis of angiogenesis and sprouting of new blood vessels. Here, we describe the imaging and analysis methods for obtaining multiple quantitative descriptors of angiogenesis that could be used for comparative research into disease phenotypes and compound effects.

Methods

Cell model

The 3D angiogenesis model was established in the MIMETAS OrganoPlate® 3-lane[3,4]. The design of the OrganoPlate 3-lane is based on a standard 384-well plate format with each microfluidic unit represented by 3x3 wells, totaling 40 units (Figure 1). Each microfluidic unit consists of three channels. Collagen-I extracellular matrix (ECM) gel was dispensed into the middle channel. Small pressure barriers called phaseguides pattern the ECM gel and prevent it from flowing into the adjacent perfusion channels. Next, endothelial cells (primary, cell line, or iPSC-derived) are seeded in the top perfusion channel and attach against the ECM gel. Perfusion is started by placing the OrganoPlate on a rocker platform and as cells proliferate, they form an endothelial microvessel. After the vessel has formed, a cocktail of pro-angiogenic factors is added to the bottom perfusion channel, on the opposite side of the parental endothelial vessel. The resulting gradient of angiogenic compounds results in the induction of angiogenic sprouts. Angiogenic sprouts were allowed to form for 0–4 days and were fixed for quantitative comparison. A schematic representation of the 3D angiogenesis model can be found in the addendum.

Imaging

Vascular cells and sprouts were fixed with 4% formaldehyde and stained with a primary antibody against VE-cadherin, followed by a secondary Alexa488 antibody (green). Actin filaments were stained with ActinRed™ ReadyProbes™ reagent (red) and nuclei were stained with Hoechst (blue). Cells were imaged with the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices). Images of cells were taken using confocal mode (60 µm pinhole spinning disc) and the 10X, or 20X water immersion objectives. For 20X magnification, z-stacks of 45-58 image planes were acquired at 2–4 µm intervals.

For 10X objective, z-stacks of 15–25 images were acquired using 4-6 µm intervals. Nuclei were imaged with the DAPI channel and angiogenic sprouts with the FITC channel, at 100 ms and 400 ms exposures respectively.

Image analysis

Images were analyzed using the Custom Module Editor in MetaXpress® High-Content Image Acquisition and Analysis Software. Details are described in the Results section. Briefly, a Neurite Outgrowth module was used to identify sprout extensions, and the Count Nuclei module for nuclei characterization. Then the objects were connected between z-planes in 3D space using "connect by best match" function. Secondary analysis was completed using Microsoft Excel software.

Images were analyzed using a 3D custom module within the MetaXpress environment. Custom Module Editor (CME) and 3D image analysis capabilities are needed for described analysis method. The custom module contained several steps. First, angiogenic sprouts were defined and segmented in each image using Neurite Outgrowth module, then objects in different z-planes were connected in 3D space using the "connect by best match" option. Then the number of angiogenic sprouts, as well as their volumes and intensities were defined during analysis. Cell nuclei were defined as an optional step and either total number of nuclei per image, or number of nuclei per sprout was calculated. The region of interest mask was used during the analysis to include only objects that are located in the gel channel, but not in the endothelial tube channel. This way only angiogenic sprouts, not cells in the upper channel, were counted during analysis. The developed custom module was able to be used with both 20X and 10X images. Alternatively, image analysis can be performed in 2D by using a maximum projection image.

For evaluation of length of sprouts, a slightly different Custom Module Editor was also developed using a "Fibers" application module (not shown).

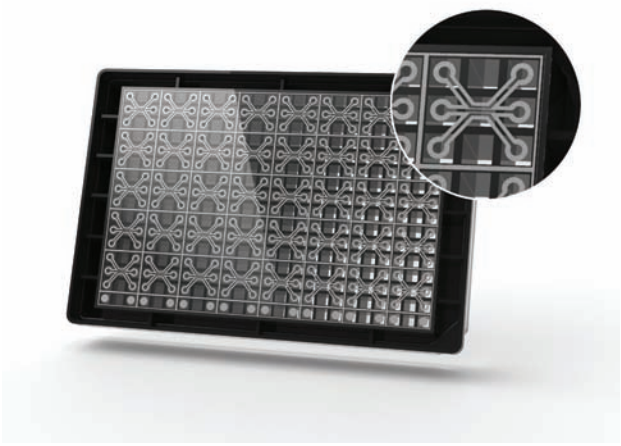


Figure 1. *Schematic presentation of OrganoPlate 3-lane.*

Results

Time dependence of the angiogenesis process was modeled in OrganoPlate 3-lane. Endothelial cells seeded in the upper channel formed a tube in three days. The model includes a tube of endothelial cells formed in the top channel line, or in both top and bottom channels, with collagen in the middle gel channel (Figure 1). Addition of growth factors to the bottom channel promoted formation of angiogenic sprouts through the collagen that could be imaged and analyzed (Figure 2).

Samples were imaged at 10X or 20X as depicted in Figure 2. A 20X objective with water immersion enabled sharp and precise resolution of cells inside a solid matrix. Using 10X objective resolved less details of the objects but acquisition was faster since only one site per well was imaged with fewer planes. Importantly, the region of interest was used to separate the area of sprouting of new vessels from the preexisting endothelial tube. Representative images of angiogenesis sprouts are shown in Figure 2.

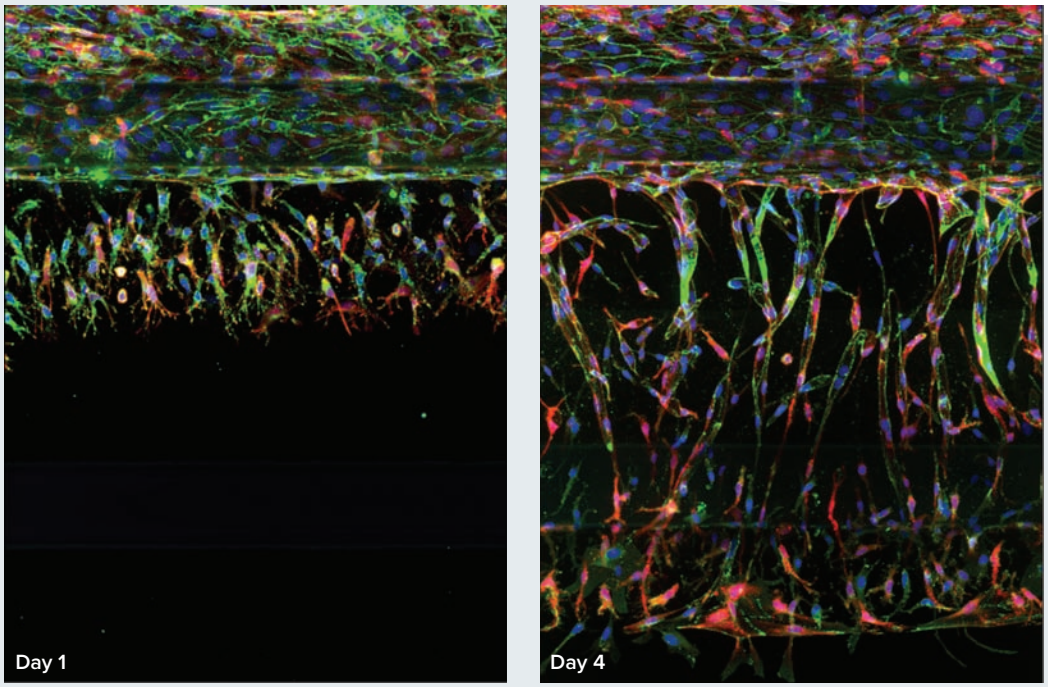


Figure 2. *Images of angiogenic sprouts in OrganoPlate. Maximum projection images presented for angiogenic sprouts formed after 1 day and 4 days in culture. Note the tube of vascular cells in the upper part of the images. Angiogenic sprouts coming from the upper tube into the lane with collagen, toward the lower line that contained growth factors. Nuclear stain (Hoechst) shown in blue, VE-cadherin in green, and Actin in red.*

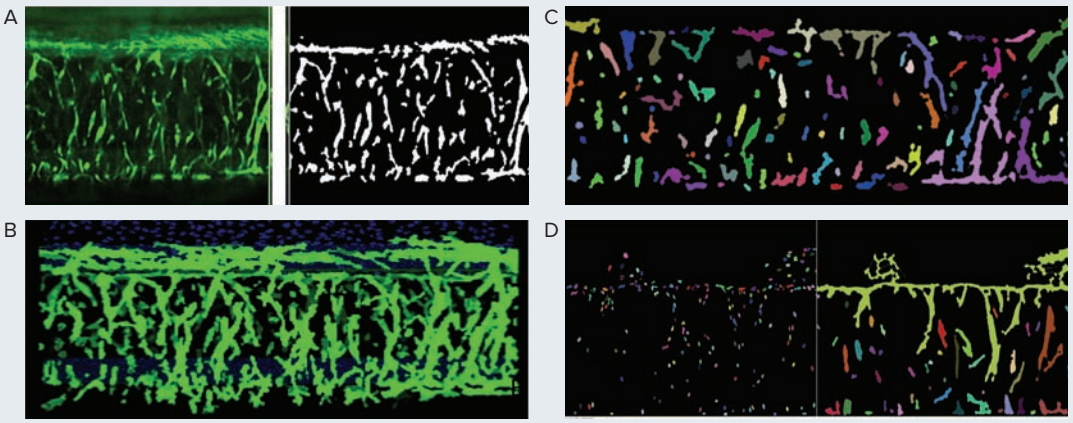


Figure 3. *A. Images and imaging masks using Custom Module Editor. Individual sprouts and nuclei identified in each z-image by using Neurite Outgrowth module, then objects in each image are connected in 3D using "connect by best match" function. Analysis allows for identifying total number of sprouts, total volume, average intensity, or nuclear counts. B. 3D visualization of angiogenic sprouts done by MetaXpress software. C. Objects in each image are connected in 3D using "connect by best match" function. Separate sprouts and nuclei shown as pseudo-colored. D. Nuclei were identified in each image using "Find Nuclei" module, then the objects were connected by using "connect by touching" option.*

Figure 3 depicts the process of image analysis including segmentation per plane and connecting features in 3D using the “connect by best match” function.

A time-dependent increase in the number and volume of sprouts was observed, as well as an increased number of cells or nuclei (Figures 4–5).

Analysis of the images of an entire plate performed automatically without user intervention. Additional adjustment of the image intensity thresholds might be needed between experiments if staining intensities vary significantly. Using Power Core is essential for analysis.

Figure 6 demonstrates the workflow of the Custom Module Editor used for analysis.

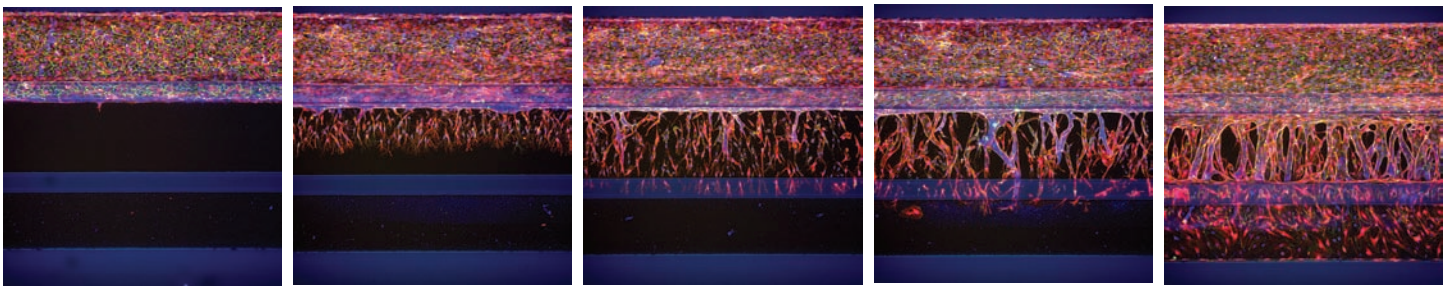


Figure 4. *Angiogenic sprouting in OrganoPlate 3-lane over time (RFP- HUVECs). From left to right, cultures were stimulated with an angiogenic cocktail in the bottom channel for 0, 1, 2, 3, and 4 days, respectively, resulting in the formation of angiogenic sprouts. Cultures were stained with for Actin (red) and VE-cadherin (green). Nuclei were stained with Hoechst.*

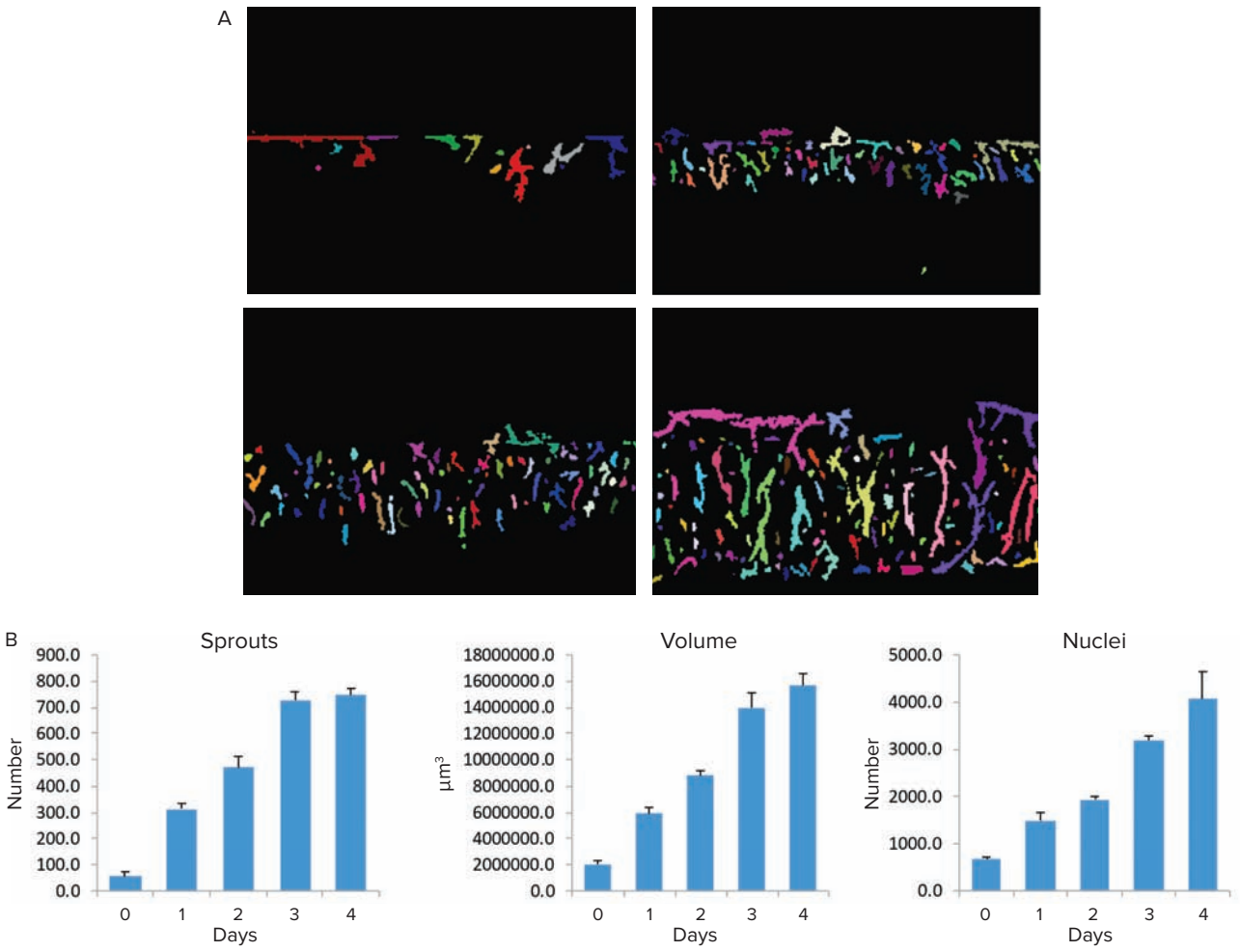


Figure 5. *Quantitative assessment of angiogenesis. Examples represent growth of angiogenic sprouts in 3D collagen during four consecutive days. Bar graphs demonstrate quantitative measurements of angiogenic sprouts. Assay was performed in triplicates, error bars represent STDEV.*

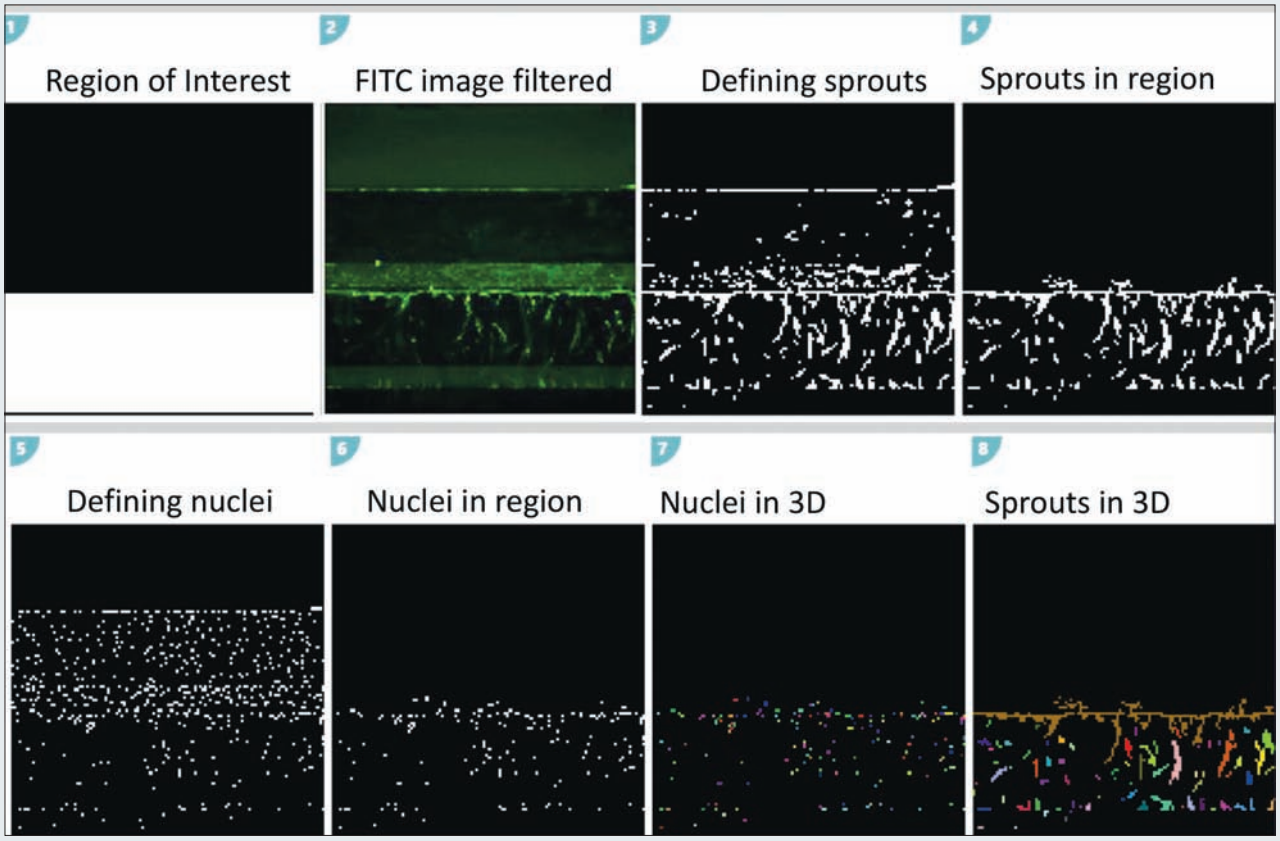


Figure 6. *Custom Module Editor. Step cards shown for the Custom Module Editor.*

Conclusion

It is essential to derive quantitative data from phenotypic changes of complex biological processes like angiogenesis. While 3D biological models offer a better representation of the complexity of human biology, image analysis of convoluted 3D structures can be challenging.

We developed and optimized imaging and analysis protocols that allow capturing, visualization, and quantitative analysis of angiogenic sprouts in MIMETAS assay. The imaging protocols were developed for ImageXpress Micro Confocal system and MetaXpress software to offer an integrated workflow for imaging and analysis. Combining the system with the strength of a scalable organ-on-a-chip platform unlock quantitative characterization of phenotypic effects for disease modeling and compound screening.

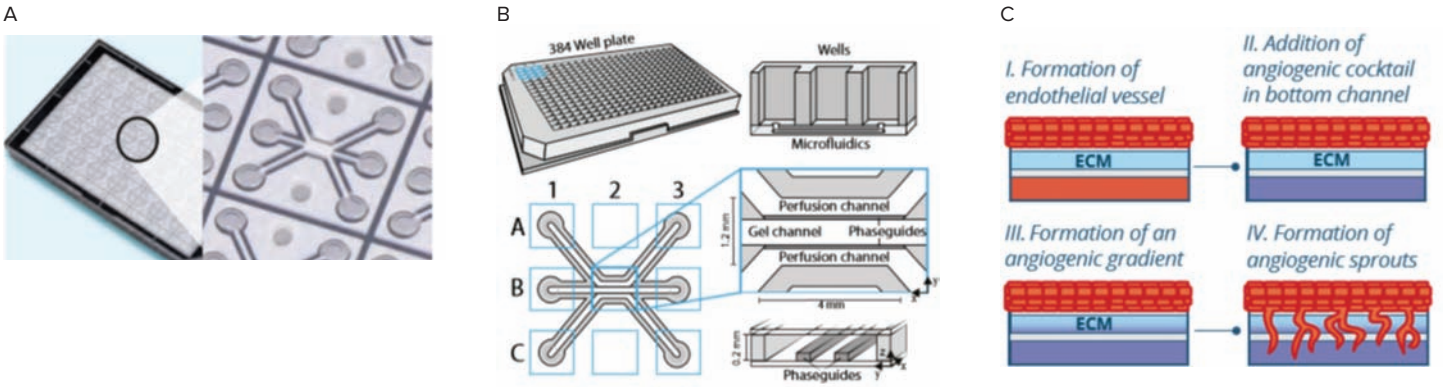
Addendum

Description of the Custom Module Editor

Images were analyzed in 3D using MetaXpress 6.6 software. To quantify the number of sprouts in a 3D volume, a customized analysis was set using the MetaXpress Custom Module Editor (CME). Briefly, for each plane, the FITC channel representing the sprouts were processed with a Gaussian filter. Sprouts were then segmented using Neurite Outgrowth objects analysis module. The Region mask* created during acquisition was used to select sprouts growing into the middle gel channel using Keep Marked Objects. Because Keep Marked Objects is an object based selection, sprout objects with areas outside the Region mask will also be selected. Alternatively, instead of using Keep Marked Objects, sprouts can be selected using the Logical Operation AND to keep sprout regions that are inside the Region mask (Region Mask AND Neurite Outgrowth Objects). Lastly, sprouts from each plane were connected in 3D using the Connect by Best Match algorithm. Output measurements included volume, diameter and intensity. To increase the analysis speed, MetaXpress® PowerCore™ High-Content Distributed Image Analysis Software was used to run the analysis for the entire plate.

*A custom Create Region journal was used during acquisition to create a user-defined region that includes only the middle gel channel of the microfluidic unit. The journal converts the defined region into an image mask for downstream analysis.

Description of 3D Angiogenesis Model



Formation of membrane-free angiogenic sprouts in the OrganoPlate 3-lane. A. Bottom of the OrganoPlate, a microfluidic device comprising 40 chips. B. Each chip consists of three channels: one 'gel' channel for gel patterning, and two adjacent channels. Phaseguides prevent the patterned gel from flowing into the adjacent channels. C. Schematic representation of the formation of angiogenic sprouts.

References

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