3D imaging and analysis of angiogenesis in the organ-on-a-chip platform

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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.[1] This can be achieved through endothelial sprouting or splitting of the vessels and capillary. Vascular cells respond to appropriate stimuli by degradation of the extra cellular matrix, then proliferation and migration of endothelial cells. 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.[2]

Angiogenesis is a vital process in growth and development, as well as in wound healing. Angiogenesis also supports the invasion of tumor cells and is commonly measured in cancer research. 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. MIMETAS 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.

High-content imaging allows visualization of angiogenic sprouts and complex analysis of angiogenesis. Multiple quantitative descriptors of angiogenesis were characterized that could be used for comparative research for studying disease phenotypes and compound effects. We describe the methods for 3D imaging and analysis of the angiogenic growth in the OrganoPlate®[3] platform that allows visualization and quantitation of the number, volume, and length of angiogenic sprouts, as well as quantitation of cell proliferation and expression or angiogenic markers.

Cell models

3D angiogenesis model in OrganoPlate® 3-lane

A high-throughput microfluidic platform, called the OrganoPlate 3-lane, was employed to establish the angiogenesis model. The OrganoPlate 3-lane comprises 40 tissue culture chips, which each consist of three channels (Figure 1). A collagen-I extracellular matrix (ECM) gel was seeded in the middle channel of each chip and is patterned by phase-guides. Endothelial cells (primary, cell line, or iPSC-derived) are grown in the top channel of each chip and form an endothelial vessel under perfusion by placing the OrganoPlate on a rocker platform. Addition of a cocktail of angiogenic factors in the bottom channel, induces the directed formation of angiogenic sprouts from the parental vessel. Angiogenic sprouts were allowed to form for 0–4 days and then were fixed for quantitative comparison.

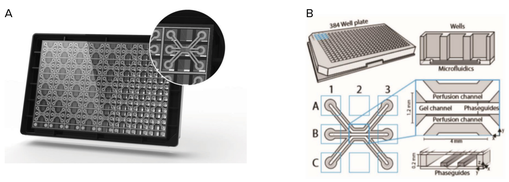
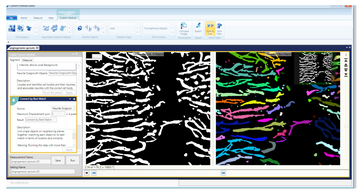


Figure 1. Formation of membrane-free angiogenic sprouts in the OrganoPlate 3-lane. A. Bottom of the OrganoPlate, a microfluidic device comprising 40 chips. B. Each chip consists of three channels: one gel channel for gel patterning and two adjacent channels. Phase-guides prevent the patterned gel from flowing into the adjacent channels.

Confocal imaging and analysis of angiogenic sprouts in 3D

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 disk) 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.

ImageXpress Micro Confocal High-Content Imaging System with water immersion option.



Custom Module Editor in MetaXpress High-Content Image Acquisition and Analysis Software.

Images were analyzed using the Custom Module Editor (CME) in MetaXpress® High-Content Image Acquisition and Analysis Software. Images were analyzed using a 3D custom module within the MetaXpress environment.

Results

Modeling angiogenesis

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).

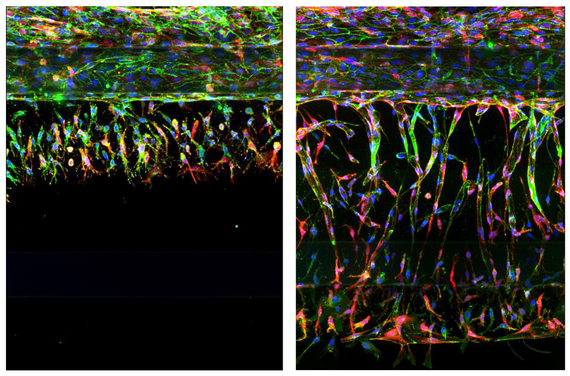


Figure 2. Images of angiogenic sprouts in OrganoPlate. Maximum projection images presented for angiogenic sprouts formed after 1 day and 4 days in culture. 20X magnification. Note the tube of vascular cells in the upper part of the images. Angiogenic sprouts coming from the upper tube into the lane with collagen, toward the lower line that contained growth factors. Nuclear stain (Hoechst) shown in blue, VE-cadherin in green, and Actin in red.

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 angiogenic sprouts are shown in Figures 2 and 3. A time-dependent increase in the number and volume of sprouts was observed, as well as an increased number of cells or nuclei.

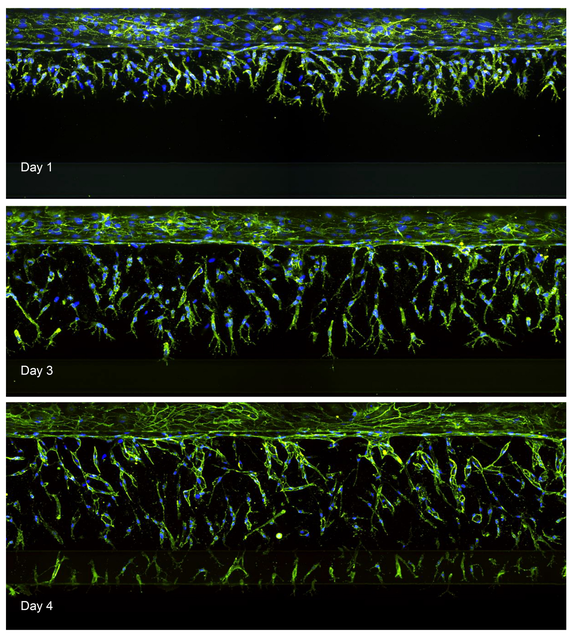


Figure 3. Images of angiogenic sprouts in OrganoPlate. Maximum projection images presented for angiogenic sprouts formed after one day and four days in culture. 20X magnification.

Results

3D analysis of angiogenesis

Images were analyzed using the Custom Module Editor (CME) in MetaXpress High-Content Image Acquisition and Analysis Software. The 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. Images were analyzed using a 3D custom module within the MetaXpress environment.

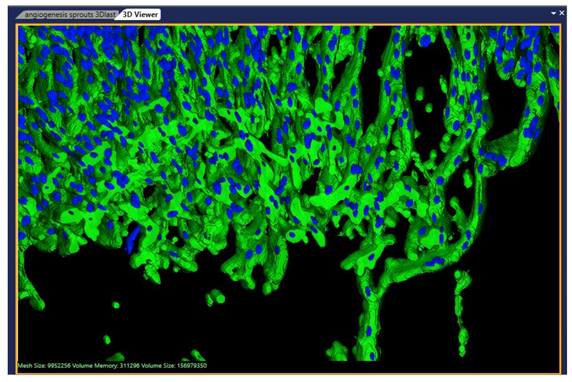


Figure 4. 3D visualization of analysis masks of angiogenesis in MetaXpress software. Nuclei presented in blue and angiogenic sprouts in green.

3D analysis protocol in CME

The CME and 3D image analysis capabilities are needed for the described analysis method. The custom module contained several steps. First, angiogenic sprouts were defined and segmented in each image using the 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.

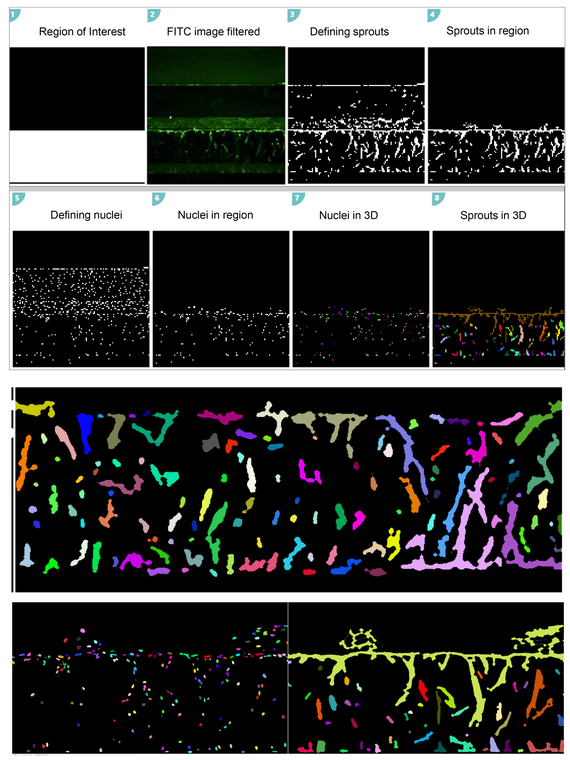


Figure 5. Upper panel: Step-cards for image analysis: 1. defining region of interest; 2. sharpening angiogenic structures; 3. defining angiogenic structures; 4. defining angiogenesis only in the intermediate space (region of interest); 5. defining nuclei; 6. defining nuclei in the region of interest; 7. & 8. counting nuclei and individual sprouts. Lower panel: masks of individual nuclei and sprouts in a single Z-step image. Separate sprouts and nuclei shown as pseudo-colored in various colors.

Results

Quantitative characterization of time-dependent growth

Time dependence of the angiogenesis process was modeled over four days. A time-dependent increase in the number and volume of sprouts was observed, as well as an increased number of cells or nuclei. Images were analyzed in 3D volume and defined sprouts and nuclei can be characterized by multiple readouts including total number of sprouts and nuclei, their intensities, volumes, distances between objects, also defining number of nuclei or cells per individual sprout, or average values. Secondary analysis was completed using Microsoft Excel.

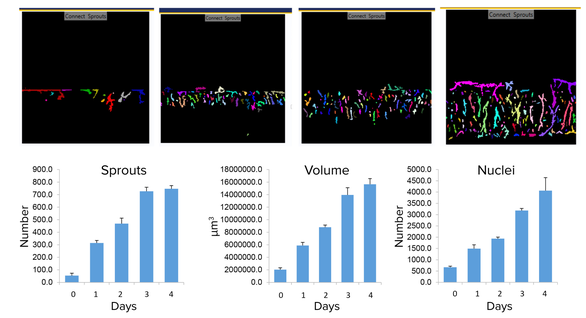


Figure 6. Quantitative assessment of angiogenesis. Examples represent growth of angiogenic sprouts in 3D collagen during four consecutive days. Individual sprouts shown in different colors. Bar graphs demonstrate quantitative measurements of angiogenic sprouts. Assay was performed in triplicates, error bars represent STDEV.

Analysis of the images of an entire plate was 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.

Variations of quantitative 3D analysis

Individual vascular cells can be defined using the cell scoring application module within the CME. In that approach, nuclei and stained positive cells were found in each z-plane, then objects were connected in 3D-space using "Connect by Best Match" function (shown below). Cell numbers, intensities, sizes, and numbers can be quantitated. In addition, to find the sprouting length, "Fibers" analysis protocol can be selected.

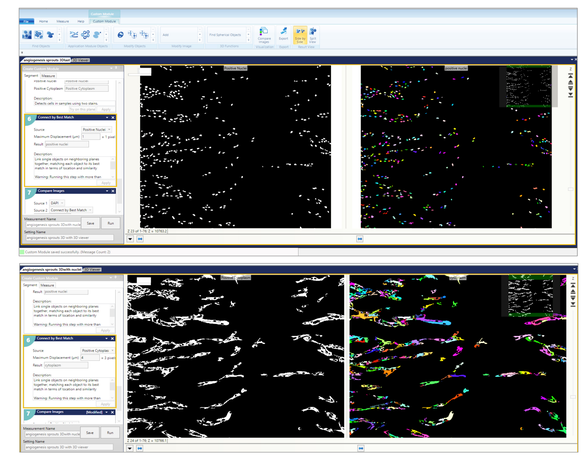


Figure 7. Upper panel: Defining nuclei and cell cytoplasm in 3D volume. Individual cells and nuclei shown in different pseudo-colors.

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.

References

1. Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O (2015). "Pericytes at the intersection between tissue regeneration and pathology". Clinical Science. 128 (2): 81–93.
2. van Duinen, V., Zhu, D., Ramakers, C. et al. Perfused 3D angiogenic sprouting in a high-throughput in vitro platform. Angiogenesis 22, 157–165 (2019).
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