Angiogenesis Analyzer for ImageJ

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ABSTRACT

Among the tools used to evaluate the anti-angiogenesis properties of drugs, the most used is the *in vitro* differentiation of primary endothelial cells culture in gel (Endothelial Tube Formation Assay (ETFA)). In suitable culture conditions these cells form structures that can branch and mimic a pseudo capillary *in vitro* formation. At later stage this differentiation can lead to a meshed network from different mesh sizes. Although widely used, the interpretation of this assay still remains a problem, especially to obtain a quantitative evaluation of the vessels-like net organization. We propose the *Angiogenesis Analyzer* as a simple tool to quantify the ETFA experiment images by extracting characteristic information of the network.

Keywords: angiogenesis, network analysis, HUVEC, endothelial tube formation assay

1. INTRODUCTION

In the field of cancer therapy, it is now well admitted that the use of anti-angiogenic molecules is a promising strategy to cure cancer. In this context, several *in vitro* as well as *in vivo* experimental models have been developed to select angiostatic molecules and further study their properties. Several of these tests have been developed and the most used of them is the *in vitro* differentiation of primary endothelial cells culture in biocompatible gel (Endothelial Tube Formation Assay (ETFA)).[1] The cells used in these assays, most often coming from the umbilical cord (Human Umbilical Vein Endothelial Cells, (HUVEC)) or from bovina aorta (adult bovine aortic endothelial cell (ABAE)), differentiate by presenting long extensions and cell alignments. These structures that can branch, mimic a pseudo capillary *in vitro* formation. At later stage, this differentiation leads to a formation of a meshed network from different mesh sizes. Although widely used, the interpretation of this assay still presents some problems, especially to obtain a quantitative evaluation of the vessels-like and their organisation. We propose the *Angiogenesis Analyzer*[2] as a simple and precise tool to quantify the ETFA experiment images. This program extracted characteristic points and elements of HUVEC network that were successfully used to characterize the differentiation level of HUVECs cultured in Matrigel^{TM}. The quantification was performed for two observation methods of the same field; pseudo phase contrast and image from the green fluorescent calcein staining. Both imaging methods gave some near similar results, with a tendency for the fluorescent labelling to return less artifactual elements.

2. MATERIALS AND METHODS

2.1 HUVECs culture:

Basement-membrane gels used for three-dimensional assays were prepared by Matrigel^{TM} (BD Biosciences (c.n: 356237)) polymerisation (10 mg/mL) for 30 min at 37°C. HUVECs (3x10^5 cells/cm^2) diluted in EBM-2 complete endothelial growth medium (Lonza Clonetics, c.n: CC-4176) containing 2% FCS were seeded and allowed to form pseudotubes for 9 h at 37°C with 5% CO_2. The cells were then kindly washed with PBS 1x and stained with 100 μg/ml CellTrace Calcein Green (Molecular Probes C34852) diluted in PBS 1x for 30 min at 37°C before image acquisition.

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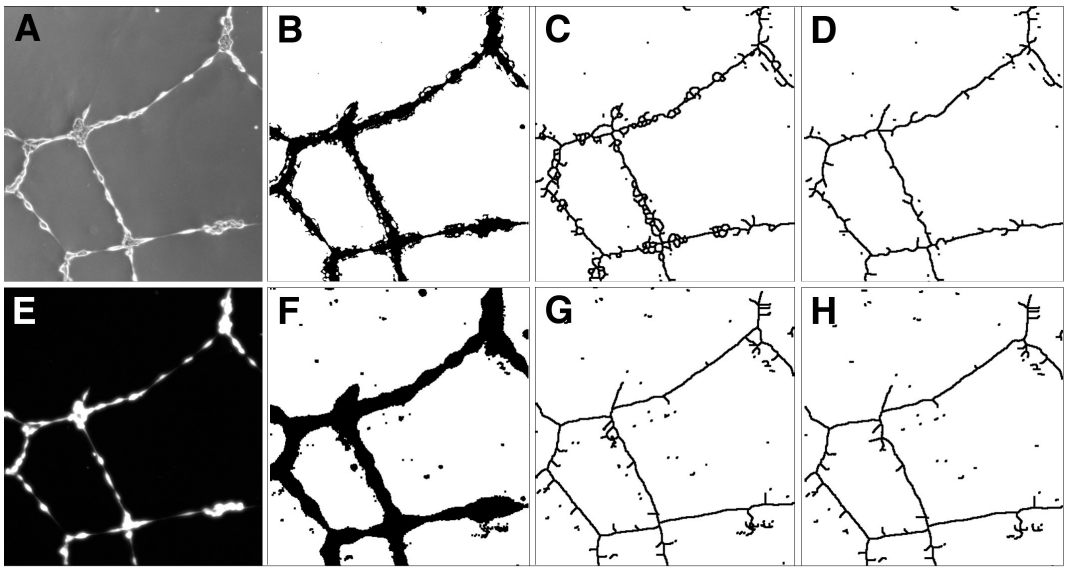

Figure 1. First steps of analysis: segmentation and skeletonization for pseudo phase contrast (first line) and fluorescence images (second line). A and E; sample of initial image in pseudo-phase contrast (A) and fluorescence (E). B and F; images after thresholding. C and G; skeletonization. D and H represent the final skeleton or tree after removal of the artifactual loops.

2.2 Image acquisition:

Images were acquired using an IX81 inverted Olympus microscope equipped with a DSU spinning disk confocal system (Olympus France, RUNGIS, France), coupled to an Orca R2 CCD camera (Hamamatsu Corporation, Japan). Observations were performed with the 4x objective (NA 0.13) equipped with a phase ring PH1, giving a pseudo phase contrast allowing highly contrasted images of the cell network. Fluorescence images were acquired using the spinning disc (DSU) confocal device to overcome the meniscus effect of the gel surface. Maximum projections of stacks of 6 slices, sampling a 300 μm thickness of sample were used for computer analysis.

2.3 Software programming and image analysis:

The *Angiogenesis Analyzer for ImageJ* project was programmed using ImageJs macro language.[3] Acquired images were analyzed by using this tool.

3. RESULTS

3.1 Segmentation and skeletonization

The first step of the analysis consisted in a segmentation of the cell areas. Fluorescent images were segmented by a *Percentile* threshold[4] and pseudo phase contrast images by a series of filtering operations ending by a *Min Error* threshold.[5] After skeletonization, small artifactual loops were removed to get the final skeleton (or tree) ready for further analysis (Fig. 1).

3.2 Remarkable points detection

The meshed network analysis consisted in junction detections associated to an iterative pruning of the tree. Different points and elements were detected to identify several structural characteristics: *Extremities* were defined by pixels of the tree that were in contact with only one other pixel (Fig. 2A-B). *Nodes* were identified as pixels that had at least 3 neighbours, corresponding to a bifurcation (Fig. 2A-B). *Junctions* corresponded to a node or a group of nodes that determined a bifurcation at the tree level. Indeed a real junction can content several node pixels as defined above (Fig. 2C-D).

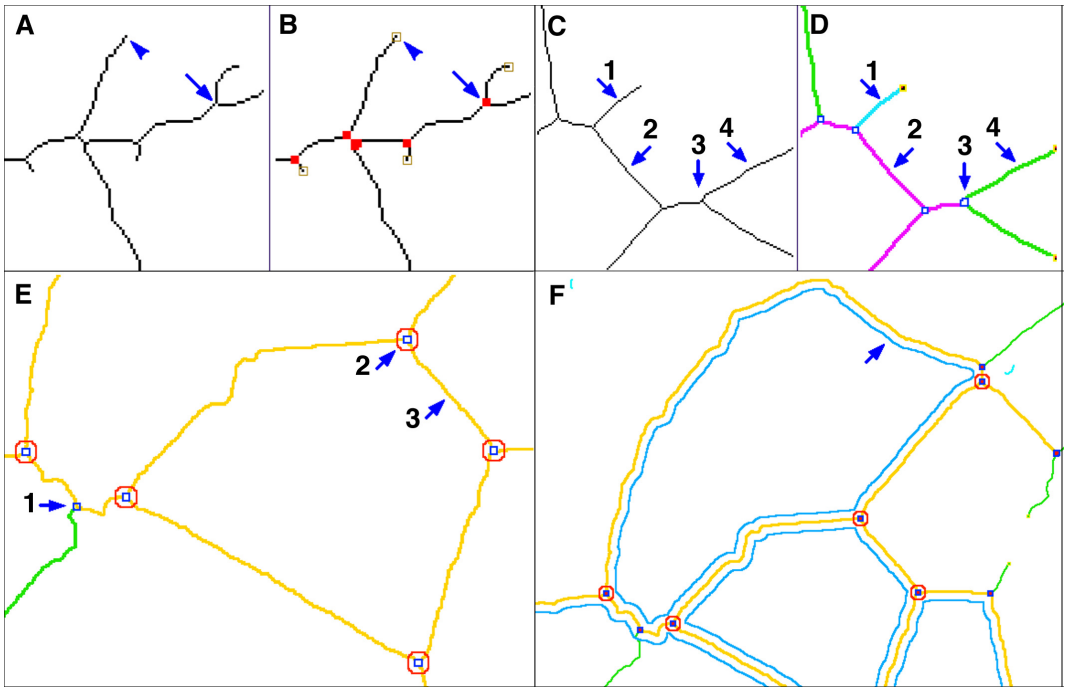

Figure 2. Detection of constitutive elements of the network as defined above in the results section: extremities (arrow head A-B), nodes (arrow A-B), twig (C1, D1), segment (C2, D2), junction (C3, D3) (note that this junction is composed by several nodes) and branch (C4, D4). E shows a junction implicated only in branch (E1) and master junctions like E2 delimiting master segments (E3). F shows the master tree composed from master segments associated by master junctions delimiting the meshes (arrow).

3.3 Detection of network elements

From the remarkable points repartition, the program detected several elements that can be classified in *branches, segments and isolated elements* as follow: *Isolated elements* were peaces of tree delimited by two extremities. These elements were called isolated twig if their size was lower than an user defined threshold value (Fig. 2C-D). *Branches* were pieces delimited by a node and an extremity. These elements were called twigs when their size was lower than an user defined threshold value (Fig. 2C-D). *Segments* were elements whose limits were two junctions. These junctions can be implicated in a branch link and/or a segment bifurcation (Fig. 2C-D).

3.4 Detection of network master elements

The distinction was made between junctions implicated in branches and junctions between segments forming meshes: *Master segments* consisted in peaces of tree delimited by two junctions none exclusively implicated with one branch, called master junction (Fig. 2E). *Master junctions* were junctions linking at least three segments. They delimited the master segments (Fig. 2E).

3.5 Construction of master trees and detection of meshes

Master trees were defined as master segments linked by master junctions. The master trees were used for the final meshing analysis. *Meshes* were detected as closed structures composed from master segments linked by master junctions (Fig. 2F) in master trees.

3.6 Analysis of fluorescence and phase contrast HUVEC network

An iterative pruning of the tree allowed a characterization of the networks by analysing the above described elements. The sequence removed artifactual loops and twigs, revealing the master structure and its branching. The meshes were detected and measured, and global maps of elements were obtained (fig 3).

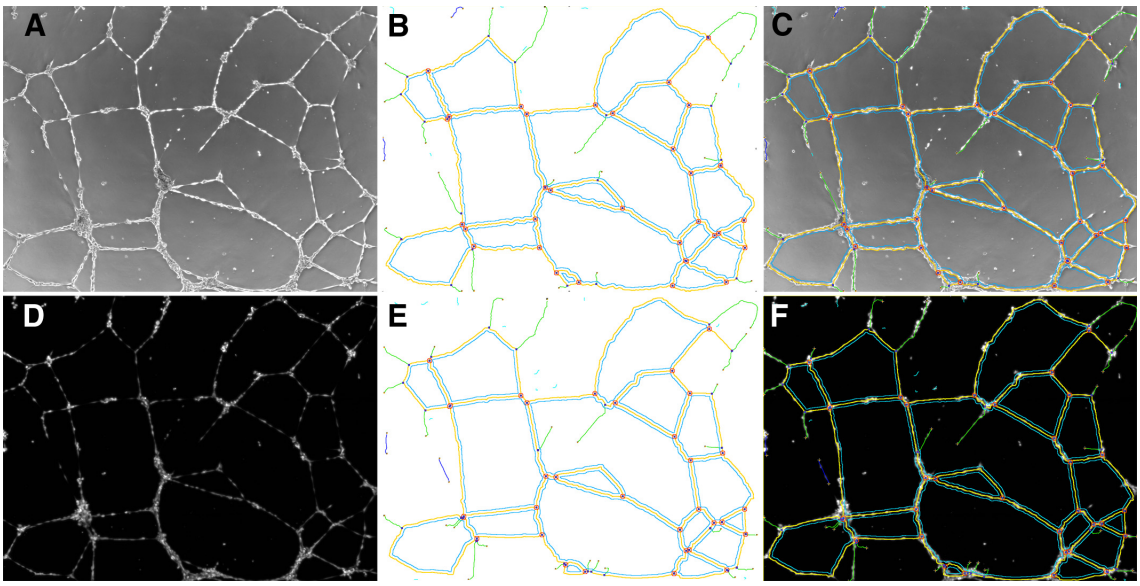

Figure 3. Final analysis of HUVEC network in pseudo phase contrast (first line) and fluorescence (second line) modes. A and D, initial images; B and E, the map of the detected elements; C and F the overlay of the detected elements on image. Note the similarity of the results.

4. DISCUSSION

After skeletonization, the tree analysis was performed through the detection of pixels corresponding to extremities (1 neighbor pixel), nodes (3 neighbor pixels), and groups of nodes forming real junctions (one or more nodes). From these connection elements, the tree analysis was continued by sorting the different peaces resulting from the cut off of the tree into branches (peaces connected to one extremity and one node or junction) and segments (peaces connecting to 2 nodes or junctions). The distinction was made between junctions implicated to branches, and junctions between segments forming meshes leading to the master tree identification and the final meshes detection. Finally, the tool that can manage batch of images returned an Excel like table containing the different extracted parameters among whom branches index and mesh index. The two methods of observation, pseudo phase contrast and fluorescence images, returned some near similar results with a good detection of meshes. The program contains the generally required online functionalities of software: documentation, demo images for training and update facilities which make a convenient tool for automatic analysis of ETFA networks.

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