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Quantification of tube topography on micrographs of fluorescent staining of multi-layers cell culture.

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Protocol status: Working
We use this protocol in our workspace and it is working.

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Abstract

The induction or inhibition of angiogenesis can be assessed in vitro by the differentiation of endothelial cells. Indeed, these cells are capable of forming structures resembling a capillary network in culture. This approach is known as Endothelial Tube Formation Assay (ETFA).

This type of analysis is traditionally performed on a low confluence monolayer of cells to assay the effect of soluble compounds on the formation of tubes. However, the modulation of tube formation by cell-cell interactions requires the co-culture of cells that leads to the creation of a multi-layer micro-tissue. Additionally, the identification of cells in the micro-tissue requires fluorescent staining resulting in discontinuities in the signal.

The present protocol allows to analyze the topography of tubes on micrographs of fluorescent staining of multi-layers cell culture.
The limit of this method resides in the fact that the 3D organization of the micro-tissue is not reconstructed and, thus, a part of the tube network is not analyzed.

Software

1 Install Icy (requires JDK 8+).

Software	
Icy	NAME
Windows/MacOS/Linux	OS
BioImage Analysis Lab (Institut Pasteur)	DEVELOPER
http://icy.bioimageanalysis.org	SOURCE LINK

1.1 Download the Icy script "ImageJ background subtraction." (file name: protocolfile-imagej-background-subtraction1)
See: **http://icy.bioimageanalysis.org/protocol/imagej-background-subtraction/**

1.2 Install the Icy plugin "Membrane Filter."
See: **http://icy.bioimageanalysis.org/plugin/membrane-filter/**

1.3 Install the ImageJ plugin "Luts Macros and Tools Updater."
See: http://image.bio.methods.free.fr/ImageJ/?Luts-Macros-and-Tools-Updater

1.4 Install the ImageJ plugin "Angiogenesis Analyzer for ImageJ."
See: http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ

Preprocessing

2 In Icy, open the image to be analyzed.

2.1 Extract the channel of interest (Channel → Extract → Channel x).
Save the new image as TIFF.

2.2 Subtract the background (Tools → Protocols → Load: protocolfile-imagej-background-subtraction1).
Settings: Rolling=20.

Save the new image as TIFF.

2.3 Reduce the discontinuities and noise on membranes (Plugin → Membrane Filter).
Settings: default parameters.
Save the new image as TIFF.

Definition of ROI

3 In ImageJ (in Icy), open the image obtained after preprocessing.

3.1 Enhance the contrast (Process → Enhance Contrast).
Settings: Saturated pixels: 0.0%; Normalize; Equalize histogram.

3.2 Subtract the background (Process → Subtract Background).
Settings: Rolling ball radius: 5.0 pixels; Sliding paraboloid.
Save the new image as TIFF.

Analysis of tube topography

4 In ImageJ (in Icy), open the image obtained after the definition of ROI.

4.1 Start Angiogenesis Analyzer.
Settings: select all options except "Suppress isolated elements."
Threshold values: Minimum object size: 10 pixels; Minimum branch size: 25 pixels; Artifactual loop size: 850 pixels; Isolated element size threshold: 50 pixels; Master segment size threshold: 30 pixels; Iteration number (advised 2 to 5): 3 iterations; Show iteration (for single analysis): 3 iterations.

4.2 Apply the Blurred Mask Tool (Angiogenesis Analyzer → Blurred Mask Tool) to the whole surface of the image.

4.3 Change the color encoding of the image to RGB color (Image → Type → RGB color).

4.4 Run the analysis (Angiogenesis Analyzer → Analyze HUVEC Phase contrast).

Save and analyze

5 Save the result files (Angiogenesis Analyzer → Save Current Analysis). Measurements are saved as a tab-separated values file (with "xls" extension) and can be further

analyzed in a spreadsheet.