Automation of data extraction from primary/hPSEC Endothelial cells IF images - method to detect, count & measure protein marker fluorophore expression.

The purpose of this automation tool is to automate the non-qualitative process of data extraction from biology images. We are using this tool to extract data from endothelial cell IF images, however the tool is generic and can be easily adapted to use with any cell type/marker.

Automation Workflow:

Qualitative Analysis of Raw images by user/scientist

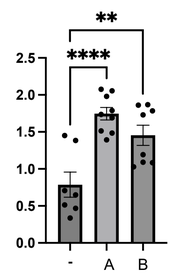
User provides image files (TIFF format) & qualitative information needed by the script

Script extracts data over a batch of images & outputs results and intermediate masks for the user to validate the results

Script outputs results of measurements in CSV files and intermediate masks for the user to validate the results

Results are validated before using for analysis

Quantitative Analysis



Script:

GITHUB: https://github.com/kelkarm/ec-data-extraction/blob/main/src/ec-data-extractor.ijm

Save the script file ‘DataExtractor.ijm’ in the Fiji/ImageJ application, Plugins->Scripts folder

- The script can be edited by navigating to: Plugins->Macro->Edit and opening the file.
- Test mode can be enabled by setting ‘TEST_MODE’ to 1 in the code.
- To test different threshold methods:

```
method = "Otsu"; // Otsu is one of the thresholding algorithms
setAutoThreshold(method);
call("ij.plugin.frame.ThresholdAdjuster.setMethod",method); setThreshold(minThreshold,maxThreshold);
```

- Script can be edited to take segmentation method as input parameter

Execute the Script:

Fiji/ImageJ Application->Plugins->Macro->Run-> ‘DataExtractor.ijm’.
For debugging, use Debug->Debug Macro from script editor menu.

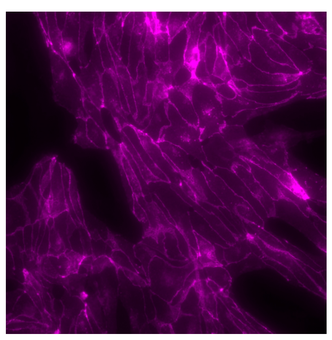
(A) To identify endothelial cells and count them from IF assay images of hPSEC cultures

(I) Create masks representing region of interest

NOTE: Compare different thresholding algorithms to choose the best one the accurately represents the region

Set Threshold *(White background, Default algorithm in Fiji),* ***Create Mask*** *(Dark region represents region of interest)*

CD144 channel

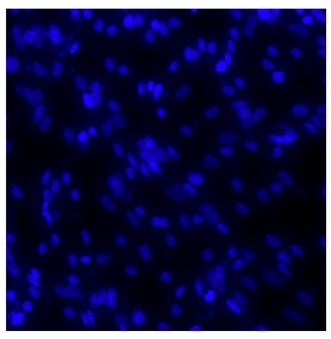


CD144 region mask

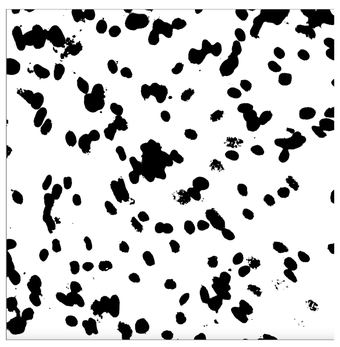
NOTE: Areas with nuclei clusters are connected. We need to separate them using a segmentation algorithm.

Make Binary, Watershed, Create Mask

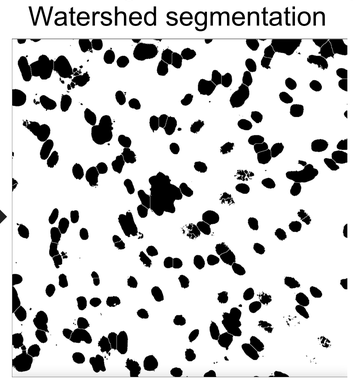
DAPI channel



DAPI region after thresholding



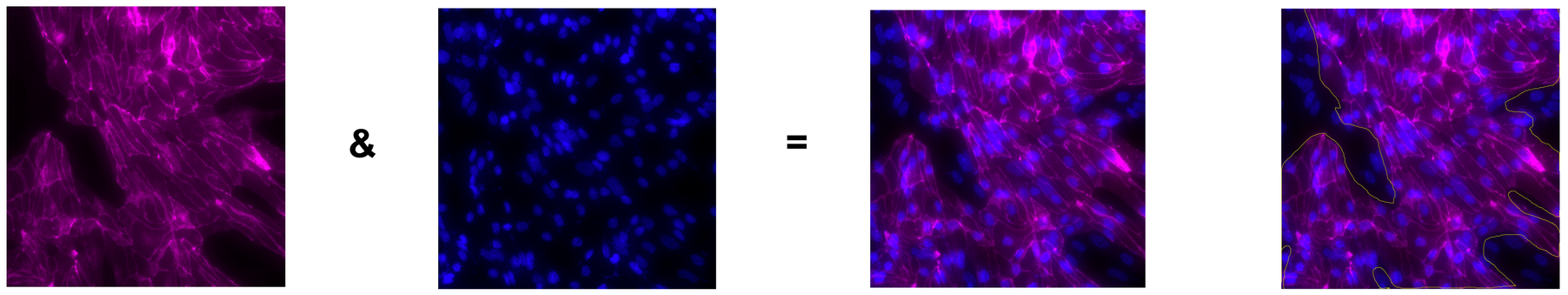
DAPI region mask created post Watershed segmentation



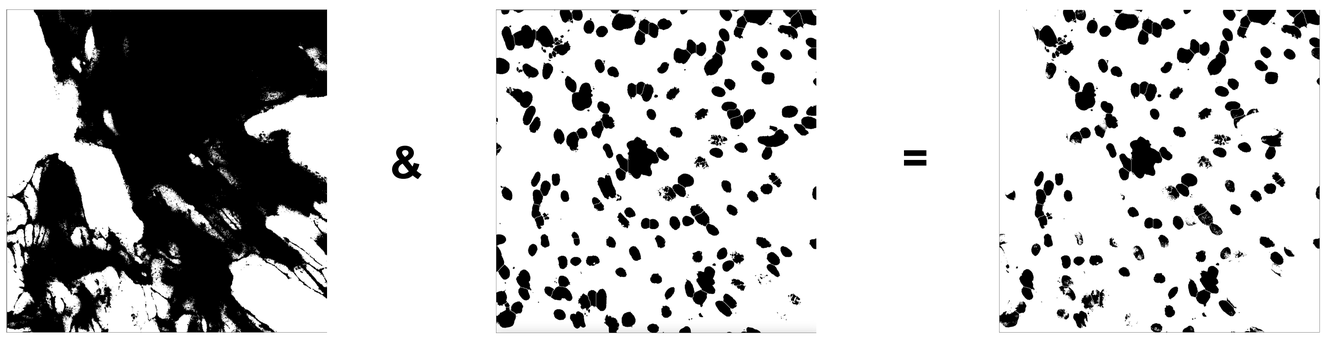
(II) Identify endothelial cell

ImageCalculator('AND create', DAPI_mask, CD144_mask)

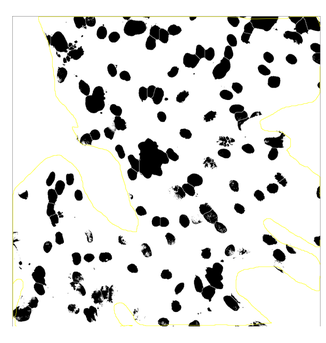
Overlap of nuclei region with endothelial region gives us endothelial nuclei



Observe ROI in yellow



Observe ROI in the above image fits with resulting overlap region generated below



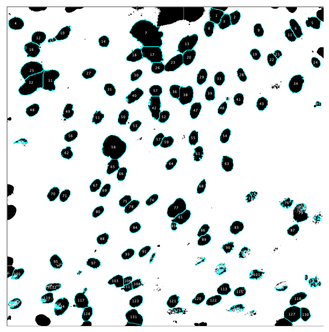
(III) Count endothelial cells

Method (1)

Using ‘Analyze Particles’. In this example, minimum Nuclei size is ‘10’ pts, so analyze particles 10-Infinity
**Result of dapi_mask is the overlap region from step (II)*

Summary

Slice	Count	Total Area	Average Size	%Area	Mean	IntDen
Result of dapi_mask	132	79050.314	144.321	15.056	255	36801.742

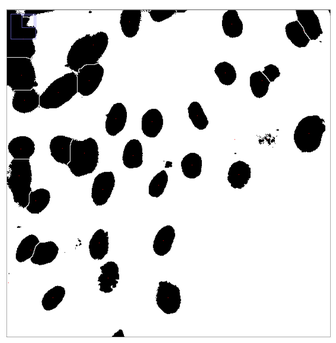


Method (2)

Using ITCN plugin.

Results

Image: Result of dapi_mask
Number of Cells: 132 in 526529.2438 square microns
Density: 0.0010 cells per square micron



Conclusion:

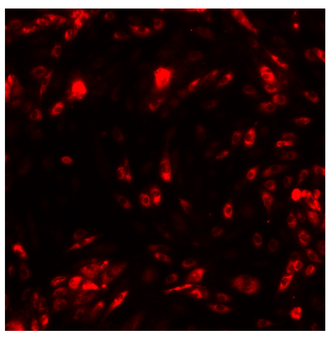
Of the two methods to count endothelial cells, Analyze Particles algorithm gives a better control over particle counting by allowing min-max particle size range setting and is not dependent on nuclei being evenly distributed. ITCN is no longer supported and has very high processing time to use in automation. ITCN is also highly sensitive to ‘width’ parameter set by user. If distance between nuclei has high variance in the image, the ITCN accuracy is low.

(B) Measure IF marker fluorophore levels in cells from IF assay images of hPSEC cultures

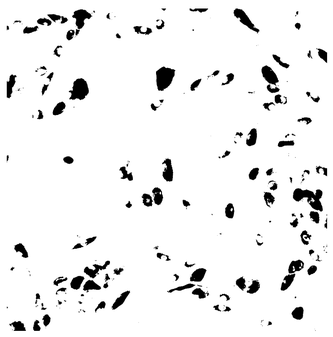
(I) Identifying ROI & Measure fluorophore intensity

Use thresholding (Default in Fiji tool) to identify regions of marker expression.

Fluorophore marker channel



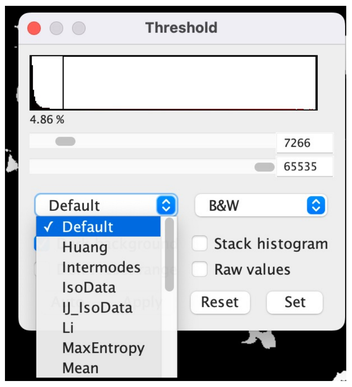
Thresholded Image representing expression region (dark areas)



Fluorophore intensity measurements

Results

	Area	Mean	IntDen	RawIntDen	MinThr	MaxThr
1	480240	866	416012012	984643127	0	7015



Several thresholding methods are available. A comparative study can be done on which one is best to use.

NOTE: hPSEC culture samples contain a mix of differentiated ECs and undifferentiated hPSCs. This method works for protein studies where the marker protein are specific to ECs. For study of non-specific markers expressed in ECs, EC regions first need to be identified using CD144/other endothelial marker. Then this EC mask can be overlayed on the marker image to measure flurophore expression in EC positive region.