Epithelial Thickness Barrier Assay Protocol

Summary

Epithelial barrier thickness assays can come in many forms, one of which is created in the model system consisting of red, green, and blue (RGB) colour staining to highlight different cell structures. Quantifying the presence of these colours manually on ImageJ can be tedious and labour intensive despite its' relatively simplistic procedure. This often consists of manually thresholding for the total area, as well as each of the colour channels after splitting the image, a process that must be repeated for each image. Therefore, we created a macro plugin that automates this process, and analyzes microbial/drug-VK2 co-culture images to permit higher throughput replication and unbiased quantification.

Statement of Need

Analyzing the epithelial barrier thickness assay is essential for biomedical researchers in understanding how microbes and drugs can affect vaginal epithelial barrier (VK2) cells. While there are various methods to measure these images, such as manually thresholding each image, they are labour intensive despite their simplicity. One of the many ways to perform analysis on Epithelial Barrier images can be completed using ImageJ, which is an open-source image processing software. However, the standard workflow in ImageJ requires a series of multiple steps that involves human intervention when thresholding the coloured area. Researchers will have to repeat these steps of analysis for total colour area and for each of the split channels. While creating the protocol, we realized that some of the tasks involved in the analysis could be automated by creating a macro plugin. Therefore, the *Epithelial_Barrier_Thickness_Tool* was designed to efficiently quantify these images and significantly reduce time and effort required.

The tool is user friendly and can be used with just a click of a button. This plugin could measure both total colour area and individual RGB channels, providing key measurements on the total area, area percentage, average height and standard deviation of the coloured regions.

Installation

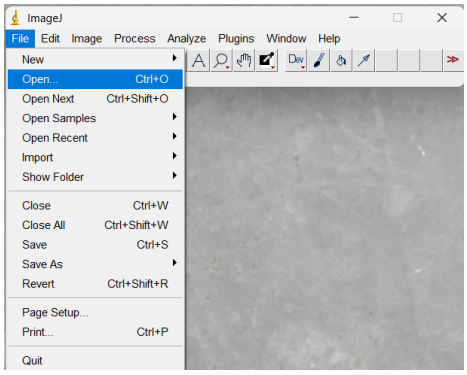
To use the plugin, ImageJ/FIJI is required and must be installed prior to starting.

The macro plugin can be downloaded from our GitHub page:
https://github.com/MicroStatsLab/Epithelial-Barrier-Thickness-Tools

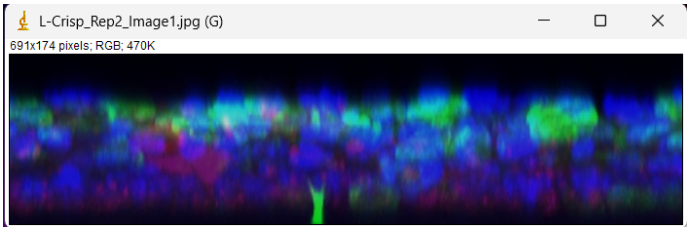
To install the plugin, first open ImageJ/FIJI and then select "*Plugins* > *Macros* > *Install…*". After that, navigate to where you have downloaded the plugin/ .ijm file, and once found, select the file and click "*Open*". The plugin will have been installed and can be accessed by using the "Thickness Tool" found in the control bar.

User Process

With the tool downloaded, the first step in preparing ImageJ for analysis is loading in an image. This can be done by going "*File > Open...*".



Note: Please make sure image is horizontal. See below for an example.



In the second step, it will prompt two values: "*Shaded Threshold Value*", which is the darkest value you are willing to include in your image; and "*Use last set scale?",* which allows you the option to adjust the scale being used. Once you click on the image, many tabs will open and close, and will even rotate 90 degrees. This is normal and necessary.

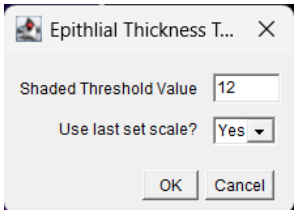


Figure 1: Main tool options

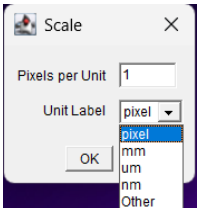


Figure 2: Scale menu options

The default values for these options are:

- For "*Shaded Threshold Value*": by default this value is 12.
- For "*Use last set scale?*": by default this is set to 'no'.

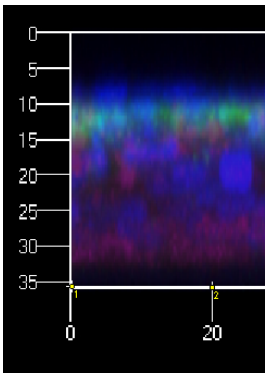
Note: If you are using scaling, it will not tell you whether images are scaled or not in the results tab. I recommend clearing results between scales in order to not mix up whether you have set the scale or not.

Finally, after submitting your options for the tool, the results will enter into the "*Results*" tab with the label (your image file name), the areas and area %, average height and standard deviation for each of the three colours (Red, green, blue) and the total area.

	Label	Area^2	Area %	Avg. Height	Std. Dev. Height
1	L-Crisp_Rep2_Image3-1-1.jpg (red)	48409	40.090	84.125	84.125
2	L-Crisp_Rep2_Image3-1-1.jpg (green)	41421	71.359	60.519	60.519
3	L-Crisp_Rep2_Image3-1-1.jpg (blue)	84734	202.917	122.113	122.113
4	L-Crisp_Rep2_Image3-1.jpg	85233	101.157	122.686	122.686

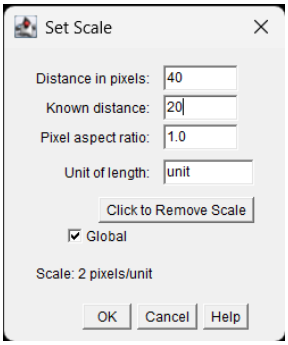
Finding your Scale

To learn which scale to use, first open an image with a known scale and the same scale as the images you will be using. Then use the multi-point tool to select along the edges of the known ruler. You will see a number beside the point.



Then use "*Analyze* → *Measure*". If you are using a horizontal scale, you will want to look at the difference in the x values. For vertical, the y. From here, simply divide the pixel distance by the known distance. For example, if in the above image the distance was 40 pixels, and the real distance is 20 µm, then the scale is 2 pixels/unit.

Alternatively, you can use "*Analyze* → *Set Scale*", and fill in the first box with the pixel distance, the second box with the known distance and it will give you scale at the bottom.



This value of "x pixels/unit" is your scale factor.

Finding the Threshold Value

To find the threshold value, open the first image for analysis and change the image type to an 8-bit by selecting "*Image > Type > 8-bit*". From here, hover over the darkest value you are wanting to include. Below the tool bar it will say "value x" and this is number that the threshold value should be.

Viewing the Individual Colours

Using the Region of Interests (ROI's) manager, you can view each of the colour's identified areas that is layered on top of the original image. The ROI's are coloured after their associated area's colour staining and in the order of red, green, blue, and total area.