**Troubleshooting**

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| **Problem** | **Possible Reason** | **Solution** |
| Spheres, balls, blobs, or other non-basement membrane visible in fluorescence image | Antibody aggregates or nonspecific antibody binding | Ensure all antibodies are spun down prior to use. Check blocking |
| Autofluorescence |  |
| Tile containing only background receives a non-zero score | Threshold is below “black point” | Increase threshold |
| Healthy region scored similarly to fibrotic region | Variable signal intensity across image (brighter healthy region) | Normalization, flattening across whole slide image |
| Insufficient interstitial space, crushed or compacted lung | Inflate lungs during processing and prevent crush damage/freeze damage to lungs |
| The pleura / perimeter of the lung scores differently than the proximal / center area | Uneven fixing or processing of lungs or drying out of tissue section during staining. | Ensure lungs are fixed/cryopreserved for sufficient time, ensure full deparaffinization of FFPE sections, ensure sections never dry out |
| Score heatmap has observable, artificial pattern | Signal intensity “tiling” effects, brightness is not level across tile scans | Shading/background correction in acquisition or stitching software |
| Correction post-imaging with QuPath of FIJI plugin (BaSiC, etc) |
| Images do not appear in search field | Not compatible file types | Change images to TIF or CZI |
| Script isn’t running after entering my desired parameters | Specifying tile size in microns without pixel pitch | Script only works with pixels, so it needs the micron/pixel ratio |
| Channel number doesn’t exist | Indexing starts at 0 when identifying channel number |
| Number of line profiles exceeds tile size | Profiles are one pixel thick, so the tile size is an upper limit |
| Program crashes during batch processing large image sets | Storage full | The tool saves a new image copy with a heatmap. It does not add a channel to your original image. Be sure to have plenty of available storage before large batch runs. |
| Healthy regions of separate lungs score very differently | Lungs differ in biologic or methodologic context and should not be compared | Lungs from separate animals must be processed consistently from euthanasia through staining and imaging. If methods are consistent, remaining differences are likely baseline biology, e.g. a constitutively expressing transgenic mouse may not share identical lung morphology to a wild-type mouse. |

**About**

The tool exists as a standalone Python application, where a user can search for compatible images through a graphical user interface (GUI) and run the scoring algorithm. Only CZI or TIFF images are currently supported. The user must input several important parameters in the GUI including tile size, the number of line profiles, the laminin channel number for multi-channeled images, a pixel intensity threshold, and an output file path. Using these parameters, the program first selects the laminin channel and subdivides the whole slide image (WSI) into tiles of desired size. Within each of these tiles, it draws a user-defined number of evenly spaced line profiles in both the X and Y dimensions. These line profiles are filtered with SciPy filt-filt and the smoothed plot is used to capture the width of the laminin network, which we observe to correlate highly with the progression of fibrosis as determined by modified Ashcroft score. At a user-defined or algorithmically identified threshold, we calculate the mean width under the peaks in the line profile, which is referred to as Mean Peak Width (MPW). The program classifies a point where the line profile crosses threshold as either crossing up or crossing down. It then correctly pairs the crossing points with either each other, the beginning, or the end of the tile depending on the sequence of up/down crossings. This ensures that it only calculates the width under the line profile (i.e. the width of true signal, not the width of background signal). The scores for each line profile are averaged into a score for the entire tile. There is an optional feature to then smooth the tile-map by taking an average with neighboring tiles in all directions. The tile-map is then saved as an additional channel on a copy of the input image, which can be viewed as a composite heatmap over other image channels. From here, various downstream analyses can take place, see figure. The tool is capable of batch processing large sets of images, exists as a standalone application (meaning all dependencies are included in the application), and transfers all image metadata between input and output images.

Graphical user interface, application

Description automatically generated

***Computational process outline. Images are selected and user-defined elements are set. The tool then divides the image into tiles and generates a heat map based on the MPW score for line profiles within that tile.***

**Using the Tool**

The tool exists as a standalone application. It can be downloaded and used without any external software, although some form of imaging software such as QuPath or ImageJ will be required to view the output image. Users will interact with the GUI to run the program and must understand what parameters to enter. Upon running the application, the user will be prompted with this screen:

A screenshot of a computer

Description automatically generated

First, click “Browse” and the program will open a file browser. Select the folder containing your images of interest. Note that these images may not appear in the file browser but will appear in the list in the GUI. When you have correctly selected the image folder, the listbox (left) will populate with any compatible images stored there. Only CZI of TIFF image types will appear in the listbox. Users can also manually copy a complete file-path into the Image Folder textbox.

A screenshot of a computer

Description automatically generated

Click on one or multiple images in the listbox to select them. Note that images appearing in the listbox are not automatically selected. Selected images will be highlighted black as shown below.

Next, a user will input a channel number to direct the program to the laminin channel. Note that even if the image has only one channel, the user still must specify “0” for channel number. It is also important to remember that the channel number is indexed from 0. So, a three channeled image would have channels 0, 1, and 2. In the example below, channel 1 is associated with laminin for each of the three selected images. For batch processing, the laminin channel must be the same index in each image, although other channels or the total number can vary.

A screenshot of a computer

Description automatically generated

The most challenging input is the threshold. Shown below as the green line, this can be understood as the user’s definition of where true signal begins. If implementing background subtraction or otherwise normalizing pixel values, this parameter must still be specified as “0”. Otherwise, it is recommended that the user use a histogram of pixel intensities across their image to select the threshold. The threshold will be the value at which the program measures the width, as shown below. In this plot, the threshold is drawn at an intensity value of 200 for a 120x120 pixel tile.

Chart, line chart

Description automatically generatedA picture containing text

Description automatically generated

The next step will be to specify the size of the tiles. Users can either enter a pixel value or a micron value and pixel pitch. If using microns, the program will not work unless you also specify pixel pitch. Users are allowed to enter both pixels and microns, but if the values do not match the program will prompt…

Graphical user interface, text, application

Description automatically generated

If the user closes this warning, the program will default to the given pixel value (not micron) and proceed. The user will have to end the program and reenter values to change them. Note that pixel pitch must only be entered if the user is specifying tile size in microns. It can otherwise be left blank. Please use decimal notation (ex. 0.321 um/px). In the example below, micron tile size and pixel pitch are left blank because tile size is given in pixels.

A screenshot of a computer

Description automatically generated

Next, the user will have to specify the number of line profiles to be drawn for each tile. Do not specify a number of profiles larger than the tile size (in pixels). The program will evenly space the number of line profiles requested across the tile. If the number of profiles is equal to the tile size, line profiles will be drawn at every pixel in the image. This is not recommended. Generally, one profile for every ten pixels is sufficient. For a 120-pixel tile size, this is 12 line profiles.

A screenshot of a computer

Description automatically generated

The smoothing checkbox is an optional feature that makes each tile’s score an average of the scores for all adjacent tiles. It creates a more appealing heatmap and can further mitigate random sources of error from anomalous tiles at the cost of spatial resolution. Another optional output is a CSV that gives the tile coordinates and their associated score to assist downstream analysis. An excerpt is shown below.

A screenshot of a spreadsheet

Description automatically generated

Finally, the user must specify an output directory. This is a local folder. Please enter the entire file path, not just the name of the folder. There is not a file browser associated with the output directory. Copy and paste the file path from File Explorer for your desired output location. The program saves a new copy of the entire image, including all channels, with an additional channel being the scored heatmap. It is therefore important to check that there is sufficient storage on the device, especially for large batch processing.

**A screenshot of a computer

Description automatically generated**