**IMAGE PRE-PROCESSING PROGRAM**

**(performs a contrast-limited adaptive histogram equalization).**

1. In MATLAB, set the working directory to wherever you have put the ‘batchleaf\_grayscale.m’ file. Then at the command line, run

batchleaf\_grayscale(‘/path/to/myleafimages/', ‘extension’)

where /path/to/myleafimages/ (example: ‘C:/MyLeafImages/) indicates the folder where your JPG or TIF images from the camera or microscope have been stored. The path must have a trailing slash. Make sure the quotes are not ‘smart’ but straight. This script will work with any other image format, if the extension is declared in the command line.

2. The script will automatically contrast-enhance each image with name NAME and save a copy in the working directory as NAME-CLAHE.jpg.

3. If contrast is unsatisfactory increase the gain parameter in the script or change the claheRadius parameter (larger will reduce background artifacts; smaller will pull out more local contrast).

**VEIN RADIUS PROGRAM**

1. In MATLAB, set the working directory to wherever you have put the ‘thickness.m’ file.

2. Open the thickness.m file and change the wd=”…” line to the path to the contrast-enhanced venation images. These images are assumed to have filenames that end in –CLAHE.jpg. Also change the px\_per\_mm=179 line to whatever resolution the images are at. Finally change reps=50 to however many random analyses should be done.

3. At the command line, run

thickness

4. The script will find the first image that has not yet been processed for vein radius. The program will show randomly selected coordinates to the user. The user should find the vein segment that is closest to the random coordinate and draw a segment perpendicular to the segment representing the diameter of the vein. Once repssegments have been drawn the program continues to the next image, or stops when no more un-analyzed images exist.

The output of the program is a CSV file named after the input image and stored in the same directory. Each row is a unique vein segment; the first four values are the coordinates of the start and stop position of each vein segment (in pixels, relative to the cropped center region of the image given by 500:500+width, 500:500+width).

*The fifth column of the file is the most relevant and is the diameter of the vein segment in microns.*

At any point the program can be stopped by pressing Control-C and restarted at the last point it was used.

**IMAGE STATISTICS PROGRAM**

**1. Convert all XCF tracing images to PNG files by running the following command in the Terminal on a Mac system or the Command Prompt on Windows. Make sure you are already in a working directory that contains your XCF files.**

*If you can’t run this script then you can also convert each image file by hand to a PNG using File > Save As within GIMP.*

Note that if you are using a more recent version of GIMP than 2.6 (or are on Windows) you will need to edit the first word of the command, e.g. to ’C:/Program Files/GIMP/bin/gimp2.8.exe’

/Applications/Gimp.app/Contents/Resources/bin/gimp-2.6 -n -i -b - <<EOF

(let\* ( (file's (cadr (file-glob "\*.XCF" 1))) (filename "") (image 0) (layer 0) )

(while (pair? file's)

(set! image (car (gimp-file-load RUN-NONINTERACTIVE (car file's) (car file's))))

(set! layer (car (gimp-image-merge-visible-layers image CLIP-TO-IMAGE)))

(set! filename (string-append (substring (car file's) 0 (- (string-length (car file's)) 4)) ".png"))

(gimp-file-save RUN-NONINTERACTIVE image layer filename filename)

(gimp-image-delete image)

(set! file's (cdr file's))

)

(gimp-quit 0)

)

EOF

**2. Change the MATLAB working directory to wherever the batchvein file is. Then run at the command line**

batchvein(basedir, outdir, px\_per\_mm, medfilt, spur\_length\_max, color\_roi, color\_vein)

Here basedir is the folder enclosing the PNG images you wish to analyze, outdir the folder that will contain the program output, px\_per\_mm the image resolution in pixels per millimeter, medfilt the radius (in pixels) of a median smoothing filter to remove tracing uncertainty, spur\_length\_max (in pixels) of the length of the maximum short segment to be removed automatically, color\_roi a RGB triplet noting the color of the background for the traced image (probably [255 255 0] for yellow), and color\_vein a RGB triplet noting the color of the veins (probably [255 0 0] for red). *Note that the directories basedir and outdir must end in a trailing slash.* Smoothing values above 1.0 should only be used if the images result in analyses with many small spurs (i.e. affecting minimum spanning tree estimates).

The output of the program is a file called result\_veinstats.csv with the following fields:

|  |  |  |
| --- | --- | --- |
| variable | units | desc |
| filecode | NA | original file code for traced cleared leaf image |
| timestamp | datetime | date and time on which image analysis was run |
| px\_per\_mm | mm^-1 | traced image resolution |
| med\_filt | px | radius of median filtering on traced image before image analysis |
| spur\_length\_max | px | length of maximum vein spur removed before image analysis |
| stat\_area\_analyzed | mm^2 | area of region of interest traced |
| stat\_num\_areoles | dimensionless | number of areoles in region of interest |
| stat\_vein\_density | mm^-1 | length of veins per unit area |
| stat\_vein\_distance\_mean | mm | distance between veins (mean) |
| stat\_vein\_distance\_median | mm | distance between veins (median) |
| stat\_vein\_distance\_sd | mm | distance between veins (sd) |
| stat\_vein\_distance\_n | dimensionless | number of areoles (n) |
| stat\_vein\_length\_mean | mm | length of vein segments (mean) |
| stat\_vein\_length\_median | mm | length of vein segments (median) |
| stat\_vein\_length\_sd | mm | length of vein segments (sd) |
| stat\_vein\_length\_n | dimensionless | number of vein segments (n) |
| stat\_vein\_tortuosity\_mean | mm | tortuosity (perimeter of segment divided by length of straight-line segment connecting endpoints) of vein segments (mean) |
| stat\_vein\_tortuosity\_median | mm | tortuosity (perimeter of segment divided by length of straight-line segment connecting endpoints) of vein segments (median) |
| stat\_vein\_tortuosity\_sd | mm | tortuosity of vein segments (sd) |
| stat\_vein\_tortuosity\_n | dimensionless | number of vein segments (n) |
| stat\_mst\_ratio | dimensionless | minimum spanning tree ratio (number of vein segments in minimum spanning tree connecting all vein junctions divided by number of vein segments in observed network, weighted by segment length) |
| stat\_areole\_elongation\_mean | mm | areole elongation ratio (areole major axis length divided by minor axis length) (mean) |
| stat\_areole\_elongation\_median | mm | areole elongation ratio (areole major axis length divided by minor axis length) (median) |
| stat\_areole\_elongation\_sd | mm | areole elongation ratio (sd) |
| stat\_areole\_elongation\_n | dimensionless | number of areoles (n) |
| stat\_areole\_roughness\_mean | mm | areole roughness ratio (squared areole perimeter divided by areole area) (mean) |
| stat\_areole\_roughness\_median | mm | areole roughness ratio (squared areole perimeter divided by areole area) (median) |
| stat\_areole\_roughness\_sd | mm | areole roughness ratio (sd) |
| stat\_areole\_roughness\_n | dimensionless | number of areoles (n) |
| stat\_areole\_circularity\_mean | mm | areole circularity ratio (4\*pi\*areole area / squared areole perimeter) (mean) |
| stat\_areole\_circularity\_median | mm | areole circularity ratio (4\*pi\*areole area / squared areole perimeter) (median) |
| stat\_areole\_circularity\_sd | mm | areole circularity ratio (sd) |
| stat\_areole\_circularity\_n | dimensionless | number of areoles (n) |
| stat\_areole\_loopiness | mm^-2 | number of areoles per unit area |
| stat\_areole\_loop\_index | dimensionless | loopiness per squared vein density |
| stat\_meshedness | dimensionless | number of bounded faces of the network, as a fraction of the possible number of faces for other planar networks with the same number of vertices |
| stat\_fev\_ratio | dimensionless | fraction of vein segments that are freely ending |

Output also includes a further set of files for each image:

|  |  |
| --- | --- |
| image-VEINS.png | Segmented network of veins in various colors with region of interest in white |
| image-AREOLES.png | Segmented areoles in various colors with region of interest in white |
| image-GRAPH.png | Spatial graph representation of network in various colors with region of interest in white |
| image\_vertexlist.csv | Pixel coordinates of all vein junctions, with index corresponding to row |
| image\_edgelist.csv | Identities of edges, with each row indicating indices of two vein junctions |