ImageJ Macro for Unbiased Stereology

Instructions for Use – Created/Updated 3/23/2018

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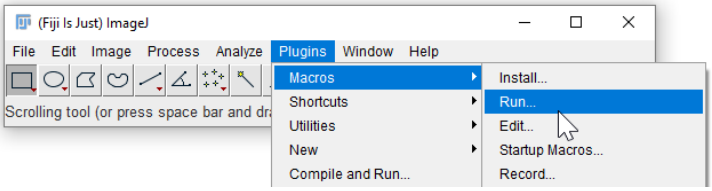
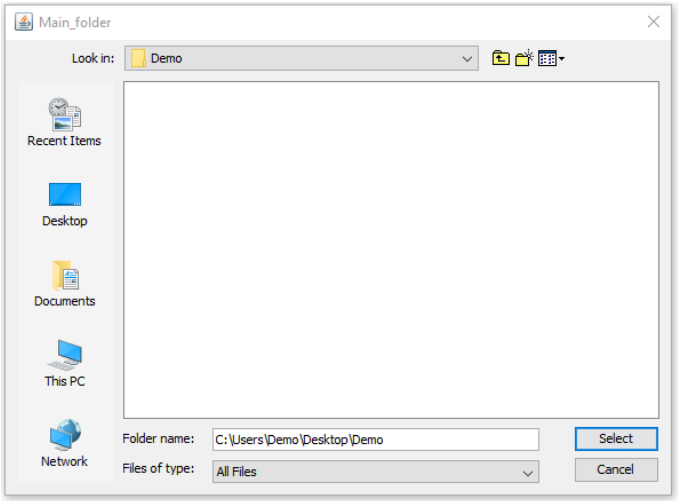
Processing Individual Images

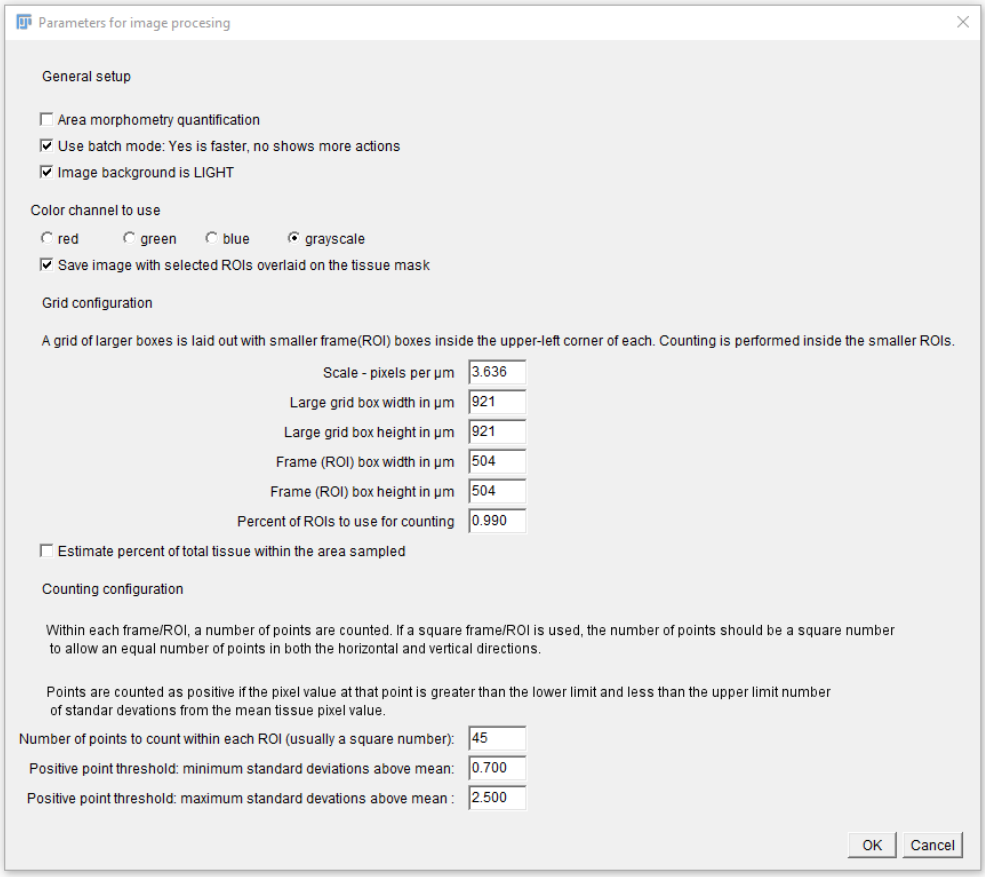
1. First, open the image to analyze in ImageJ.
2. Run the macro: Plugins->Macros->Run. Select the macro using the dialog.



3. The macro prompts for a main folder. This folder contains all output from the macro.



4. The macro then asks for parameters for image processing. See **Table 1** for descriptions.

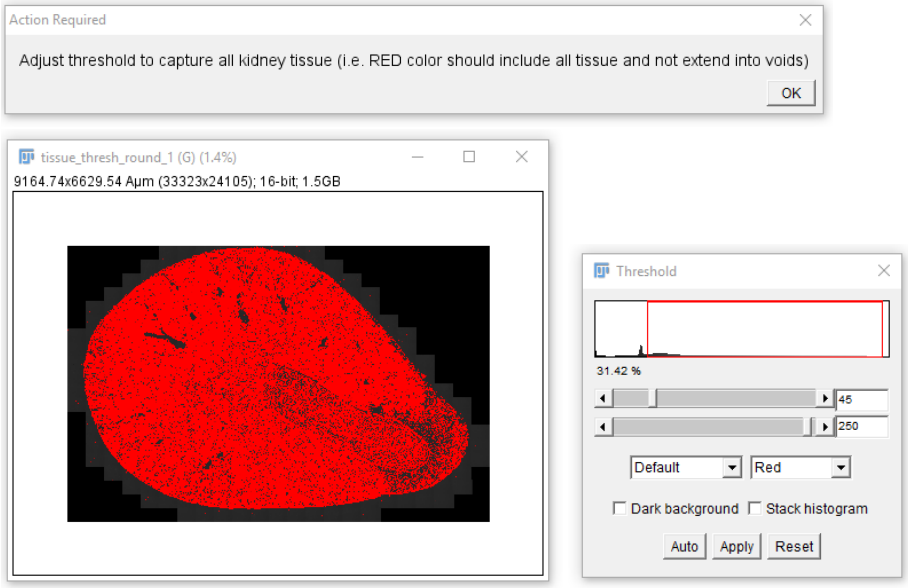


5. Press OK to begin processing. The macro begins working.

> **IMPORTANT**: DO NOT click anywhere, change the active window, or use other programs on the computer. Several macro commands are dependent on the expected window being in the foreground or for specific selections being made. Doing other work on the computer may result in the macro performing incorrect actions.

6. At this point the image on the screen changes as the macro performs various steps.

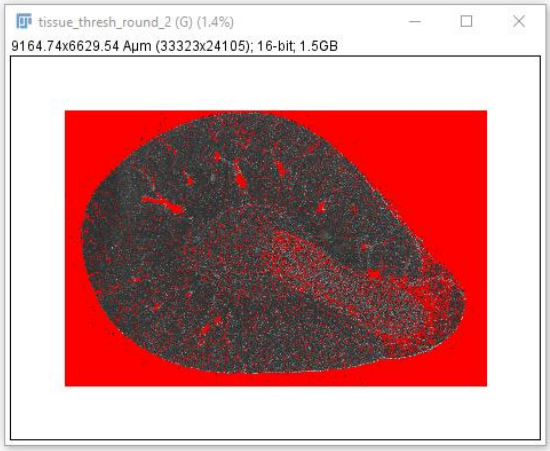
7. The macro prompts for selecting a threshold of tissue to include after initial preparatory work. Adjust the threshold so the red area includes tissue. A broader threshold, including some voids, will avoid tissue of interest being excluded but potentially includes some non-tissue in area calculations. Click OK in the "Action Required" text box when finished.

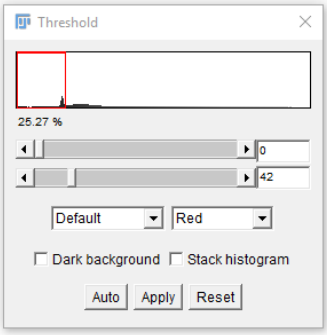


NOTE: The threshold tool can be difficult to use, especially on large images, as it is slow to respond. Wait for the display to update between changes.

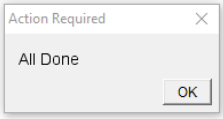
IMPORTANT: Only click to adjust the sliders or text boxes in the threshold tool or click OK in the "Action Required" message box when finished. *DO NOT* click in the image window, click Auto, Apply, or Reset in the threshold tool, or click OK in the action required window until

8. Similar to the prior step, the macro prompts for selecting a threshold of area to exclude after additional preparatory work. Adjust the threshold so the red area to include voids and non-tissue. A narrower threshold, not including some voids or non-tissue, will avoid tissue of interest being excluded but potentially includes some non-tissue in area calculations.





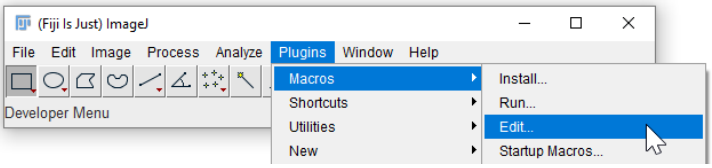
9. The script runs to completion at this point.



10. The resulting output is described in **Table 2**.

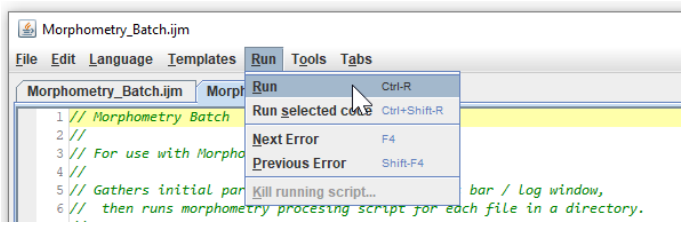
Processing Images as a Batch

1. First, open the image to analyze in ImageJ.
2. Run the macro: Plugins->Macros->Edit. Select the batch processing macro using the dialog.

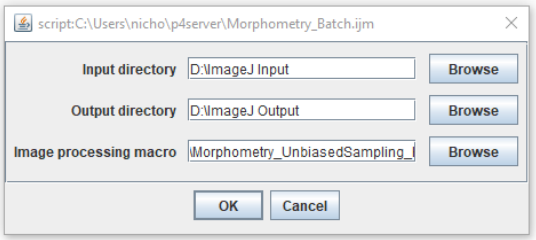


NOTE: Edit… must be selected rather than Run… at this point. ImageJ may present an error message if Run… is used. (ImageJ

3. Now, in the edit window, select Run->Run.

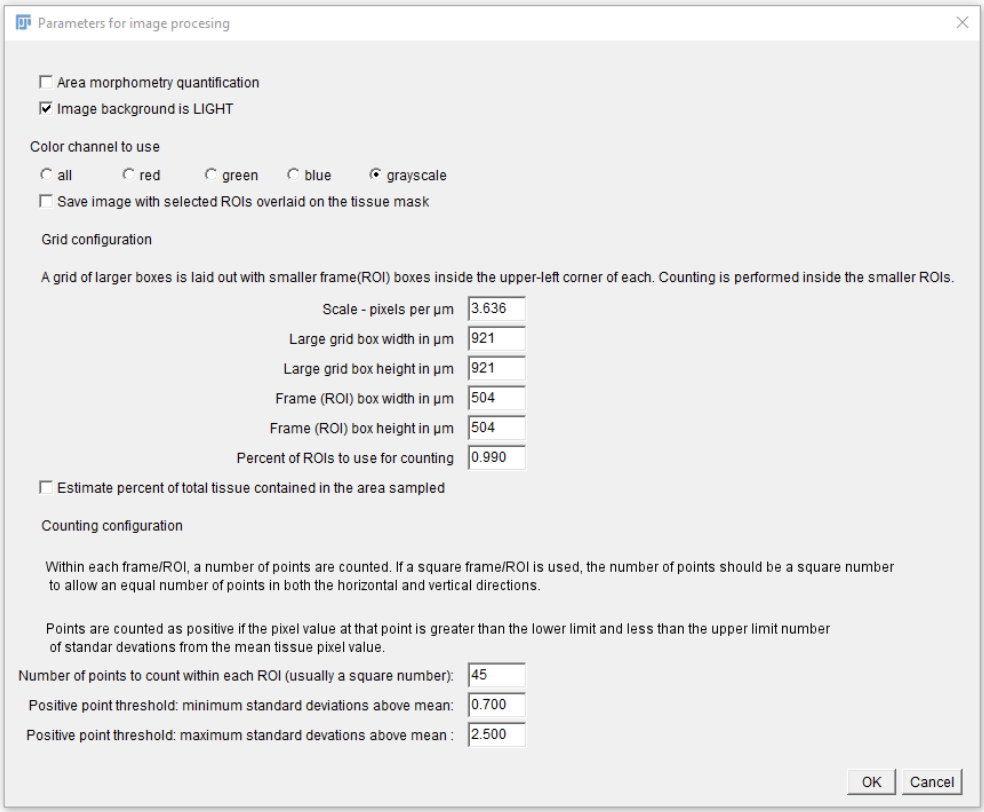


4. The macro prompts for the source (input) folder, the target (output) folder, and the image processing script. Select these, then press OK.



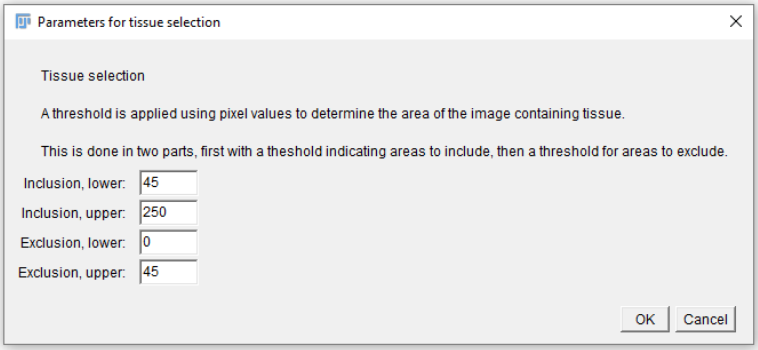
   a. The input folder contains the images to be processed,
   b. The output folder should be empty. After completion, it will contain subfolders for each processed image, each as described in **Table 2**.
   c. The image processing script is the same script as would be used directly for processing a single image.

5. The macro will prompt for parameters, as in the individual image processing script. The parameters selected will be used for processing all images. The parameters are described in **Table 1**.

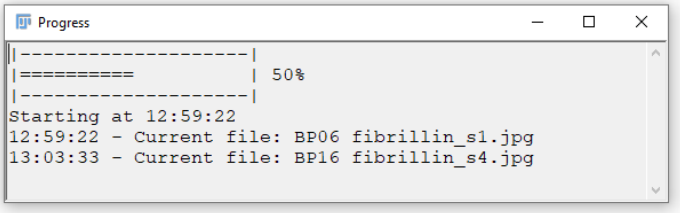


6. Now the macro will prompt for tissue mask values. The values are specified once and applied to all images, allowing the batch to run without user interaction. The parameters are described in **Table 1**.

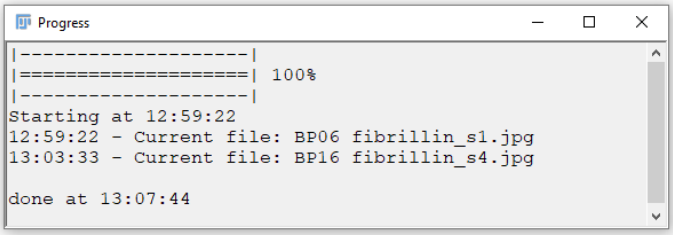
> **NOTE:** Because the tissue threshold is selected once and applied to all images, it is useful to manually find these threshold values first before using the batch macro. To do this, open a representative image, convert to grayscale (Image->Type->8-Bit) or individual channel (Image->Color->Split Channel) then use Image->Adjust->Threshold… to find values that, in the red selection:
>
> 1) include tissue, exclude voids
> 2) include voids, exclude tissue
>
> **IMPORTANT**: With a **light background** image, **first invert the colors** to set a dark background (Edit->Invert)



7. Press OK. The script will run to completion. A window displays the progress.



8. After completion, the progress window displays the message “done at [time].”



9. The contents of the progress window are saved in the output directory. This and the other contents of the output directory are described in **Table 2**.

Figure 1: Example grid layout and terminology

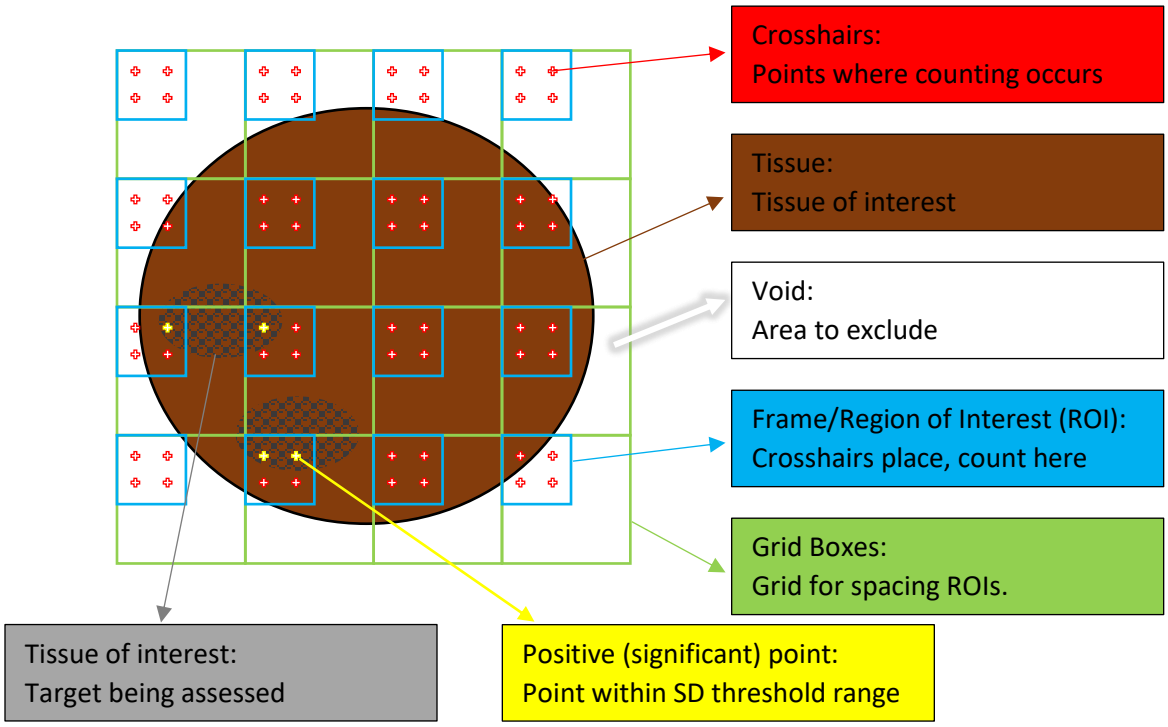


Figure 2: Counting direction and ROI placement

The macro lays out the final grid starting at a random ROI, then progressing from that point up and to the left until the top-left corner is reached, then down and to the right until the bottom right corner is reached. Each ROI will be tentatively created and assessed for whether tissue exists in the ROI. Areas without tissue will not have the ROI or crosshairs placed, but even a slight amount of tissue will result in ROI and crosshair placement.

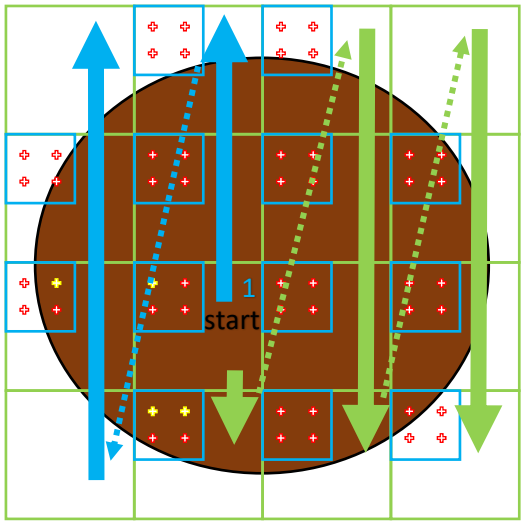


Table 1: Parameter Descriptions

General Setup

Parameter	Default	Description
Area morphometry quantification	Unselected (off)	If selected, maps location of RBCs and Injury along with tissue. Unselected, a simpler method is used to create a tissue mask to denote areas where tissue exists
Use batch mode	Selected (on)	If selected, ImageJ's batch mode will be used where possible. This increases performance by updating the display on the screen less frequently.
Image background is LIGHT	*Image-dependent*	If selected, the image pixel intensity will be inverted for some steps, and direction of "significant" pixel changes will be in the opposite direction
Color channel to use	*Image-dependent*	Selects which color channel to isolate. Grayscale will use a composite grayscale including all channels. Using a single channel allows for unidimensional evaluation of pixels.
Save image with selected ROIs overlaid on tissue mask	Selected (on)	At end of processing, generates an additional image showing entire tissue mask with ROIs overlaid, demonstrating where ROIs were placed

Grid configuration

Parameter	Default	Description
Scale – pixels per µm	*Image-dependent*	Number of pixels per physical µm, to relate distances on images to actual distance on physical tissue
Large grid box width in µm	*Image-dependent*	Width and height of large grid boxes. These boxes are used for laying out the spacing of the ROI/counting frame boxes. These boxes are contiguous (like graph paper). A grid of larger boxes is placed, and smaller ROI/frame boxes are placed at the upper-left corner of each box. These boxes are often square.
Large grid box height in µm	*Image-dependent*	
Frame (ROI) box width in µm	*Image-dependent*	Width and height of frame/ROI boxes. These boxes are where counting is performed. They are placed at the upper left corner of each grid box. These boxes are often square.
Frame (ROI) box width in µm	*Image-dependent*	
Percent of ROIs used for counting	*Image-dependent*	Percent of ROIs to use for counting. ROIs will be generated for each grid box with tissue inside. This percentage will limit the number of ROIs produced by determining an interval to sample. An ROI out of the complete set will be selected at random, and an ROI will be skipped at an interval which produces this percent.

Counting configuration

Parameter	Default	Description
Number of points to count within each ROI	*Image-dependent*	A grid of equidistant points will be laid out in each ROI, to be used for counting. The actual number of points used is a square number, generated by taking the square root of the requested number and counting that many points in the x and y dimensions. Formulas for point spacing: $$point\ spacing\ X = \frac{image\ width}{\sqrt{requested\ points}}$$ $$point\ spacing\ Y = \frac{image\ height}{\sqrt{requested\ points}}$$
Positive point threshold: minimum standard deviations about mean	*Image-dependent*	A mean and associated standard deviation is calculated from all points on tissue. Then, each point is counted as "positive" (significant) if it falls on tissue, is above the minimum SD above mean, and below the maximum SD above mean.
Positive point threshold: maximum standard deviations about mean	*Image-dependent*	

Tissue selection (only visible when processing images as a batch)

Parameter	Default	Description
Inclusion, lower	*Image-dependent*	Inclusion is for the area to include in the tissue mask. These threshold values should select for pixels falling on tissue. If it is difficult to select only tissue, a wider threshold will avoid unintentionally removing tissue by the mask
Inclusion, upper	*Image-dependent*	
Exclusion, lower	*Image-dependent*	Exclusion is the area to exclude from the tissue mask. These threshold values should select points *not* falling on tissue. If it is difficult to select only voids, a narrower threshold will avoid unintentionally removing tissue by the mask.
Exclusion, upper	*Image-dependent*	

Table 2: Output

Directory	Files	Description
Output directory (batch only)	batch_log.txt	Only created by batch script. This contains the contents of the progress window so files processed and processing time is recorded.
Main directory (Top-level directory for individual image macro, or a directory named for each image processed placed under the output directory in batch macro.)	statistics.csv	Image statistics in CSV (spreadsheet) format. If images processed as batch, will include statistics from each image processed.
1_RESULTS & OUTPUT IMAGES	ALT_GRID_BOXES.zip	Regions for grid boxes (spacing containers for sample frames/ROIs). View by opening TISSUE_MASK.png, then opening file in the "ROI Manager" (Analyze->Tools->ROI Manager, then Open).
	ALT_SAMPLE_BOXES.zip	Regions for frames/ROIs. Open in "ROI Manager, View against TISSUE_MASK.png.
	FINAL_STATS.txt	Statistics for image with description of each value
	GRID_POINTS.zip	Points sampled. View against TISSUE_MASK.png, open in "ROI Manager".
	GRID_VALUES.csv	Coordinates of points in pixels relative to TISSUE_MASK.png.
	PARAMETERS.txt	Record of parameters selected for image processing
	PERCENT_TISSUE_SAMPLED.txt	Created if option to estimate percent of total tissue contained in the sampled area is selected. Contains the estimated percent.
	RESULTS_45_CROSS_HAIRS.txt	Contains pixel value with X and Y pixel coordinates for each point sampled, relative to each frame/ROI in which they are contained.
	ROI_OVERLAY.png	TISSUE_MASK.png with ROIs overlaid.
	TISSUE_MASK.png	Mask used to define tissue area

<table>
<tr><td>2_FRAMES</td><td>B-n.jpg</td><td>Images created from each ROI, with -n being the ROI number assigned in the ROI manager.</td></tr>
<tr><td>3_NTH_TILES_SUBSET</td><td>B-n.jpg</td><td>Subset of images created, by including only the number of images required to obtain the requested percent of ROIs to use for counting.</td></tr>
<tr><td>4_CROSSHAIR_IMAGES_SUBSET</td><td>B-n.jpg</td><td>Images from NTH_TILES_SUBSET, with crosshairs placed for counting.</td></tr>
<tr><td>5_THRESHOLD_POINTS</td><td>B-n.jpg</td><td>Images from NTH_TILES_SUBSET, with circles at each point colored by how far the point’s value is in standard deviations from the mean. Crosshairs are drawn at the points within the SD threshold.
Color key:
<table><tr><td></td><td>< mean</td></tr><tr><td></td><td>0.0 – 0.5 SD</td></tr><tr><td></td><td>0.5 – 1.0 SD</td></tr><tr><td></td><td>1.0 – 2.0 SD</td></tr><tr><td></td><td>2.0 – 3.0 SD</td></tr><tr><td></td><td>> 3.0 SD</td></tr></table></td></tr>
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