A Guide to DiameterJ

Overview and Previous Literature (Hotaling, Bharti, Kriel, & Simon, 2015)..... 2

Downloading DiameterJ 3

Image Segmentation 5

PART 1: PREPARING SEGMENTATION 5

PART 2: OUTPUT 8

OPTIONAL: MANUALLY EDITING IMAGES 10

Image Analysis 17

PART 1: 17

OPTIONAL: PIXEL TO REAL UNIT CONVERSION 18

PART 2: OUTPUTS 21

Analyzing Results 24

Help (Troubleshooting) 25

References 26

Overview and Previous Literature (Hotaling, Bharti, Kriel, & Simon, 2015)

DiameterJ is an add-on for the program ImageJ and Fiji, which are open-source Java image processing programs. This add-on can analyze SEM micrographs, allowing for rapid image assessment in comparison to manual evaluation. Allowing for expedited image analysis with reduced systemic bias, providing an accessible and verified tool for efficient measurement of SEM micrographs.

DiameterJ utilizes two different algorithms to determine diameter: **Super Pixel Diameter Determination**, and **Fiber Diameter Histogram**.

Super Pixel Diameter Determination yields single fiber diameters for the inputted images, utilizing two different methods of centerline determination: the Thinning Algorithm (Zhang and Suen), and the Voronoi Spatial Tessellation. The former is also called the Sensitive Centerline Determination as it is sensitive to changes in the fiber surface, where the latter is called the Insensitive Centerline Determination as it is indifferent to fiber surface morphology. This method divides the area of all fibers by the length of all the centerlines, obtaining a mean fiber diameter. This method assumes rectangular fibers.

The **Fiber Diameter Histogram** determines the diameter at every pixel along the length, resulting in a histogram of values. This method utilizes Euclidean Distance Transformation algorithm by finding the distance to the closest mesh hole and transforming the fiber pixel to a grey scale value equal to the distance. The sensitive centerline is then utilized to determine the diameter.

Other tools include mesh hole analysis utilizing the Analyze Particle Algorithm, which allows users to determine percent mesh hole, mesh hole histogram, mean mesh hole area, as well as porosity and mesh hole size.

However, there holds some limitations and specifications to maximize results and to limit error.

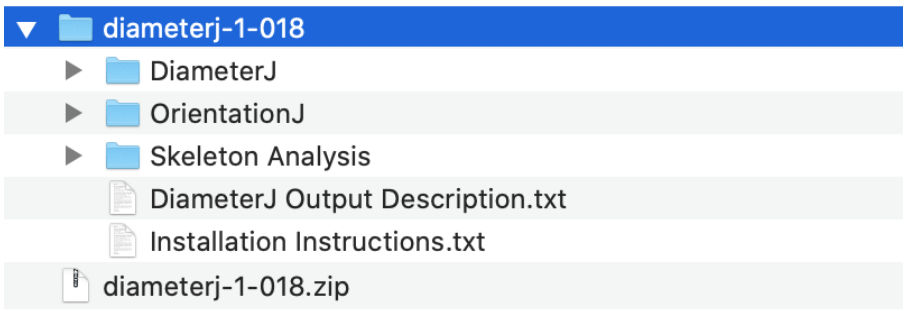
- Fibers should be at least 10px in diameter and should not be greater than 10% of the smallest dimension as it will result in errors over 10%
- Fibers with diameter greater than 512 px. cannot be analyzed (however, if the image does not violate the first specifications, it will work – image must be greater than 5120 px)
- Fibers that are closer in diameter than 3 pixels results in skewed values, must increase magnification of the image so that fibers have more than 3 pixels of difference between them.
- Images must be .tif, .jpeg, .png, .bmp, .gif

Downloading DiameterJ [1]

Step 1: Download and Install ImageJ (version 1.48 or newer) or FIJI.

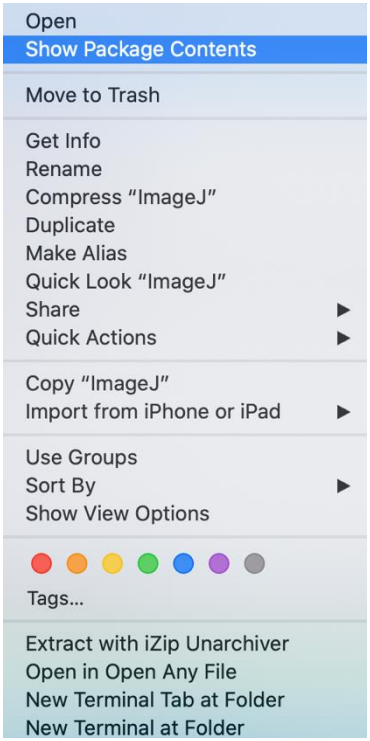
(1) Download the necessary package for Mac OS X, Linux, or Windows.

Step 2: Download plugin DiameterJ (For ImageJ, For FIJI) and unzip the files.



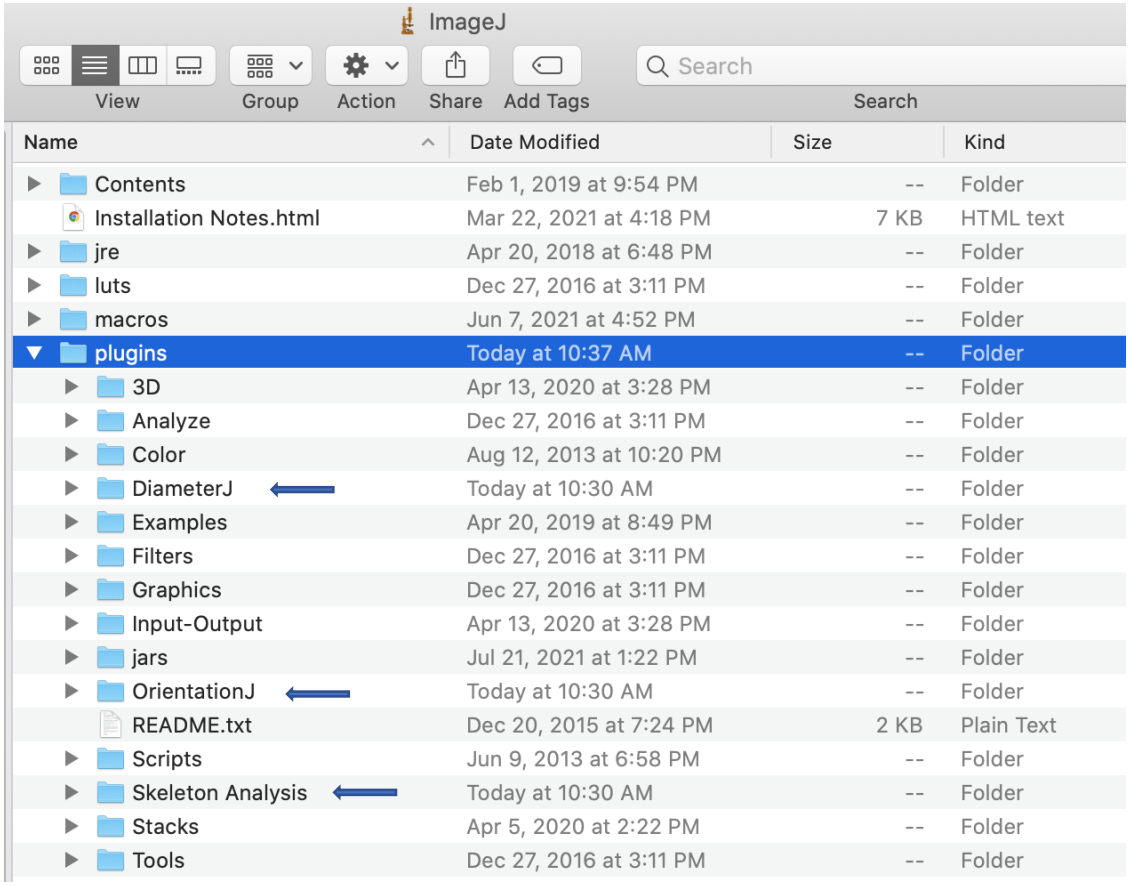
Step 3: Follow installation instructions within DiameterJ download folder.

(1) Right Click/control Click image J → Show Package Contents



[1] Steps are shown on Mac OS X and may appear differently on Windows/Linux

(2) Move or copy the three folders (DiameterJ, OrientationJ, Skeleton Analysis) into the plugins folder of ImageJ or FIJI



Step 4: Restart ImageJ/FIJI, DiameterJ should now be in the Plugin menu.

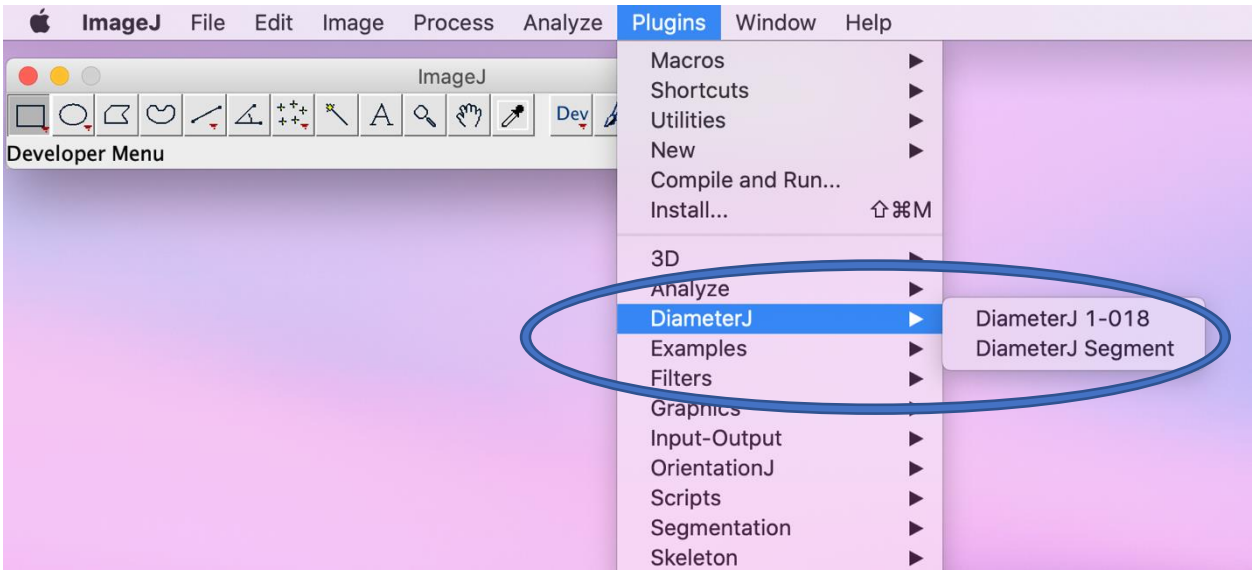
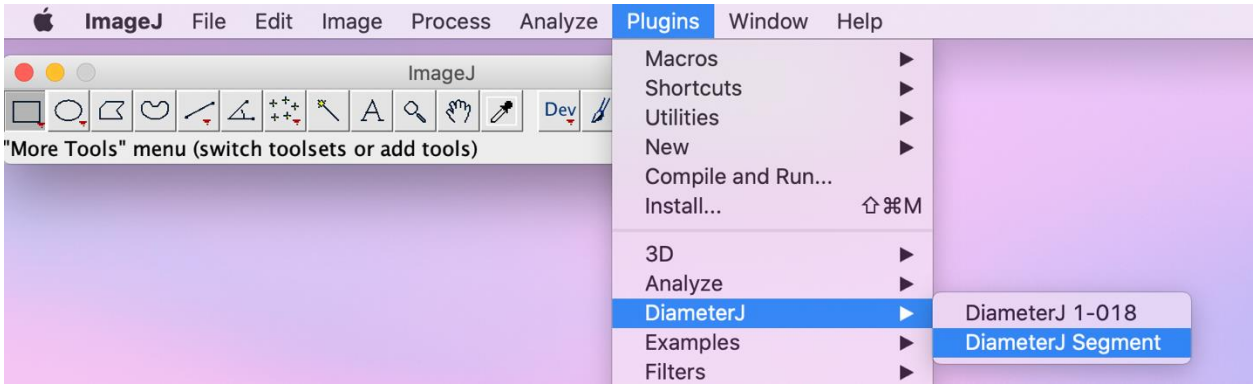


Image Segmentation

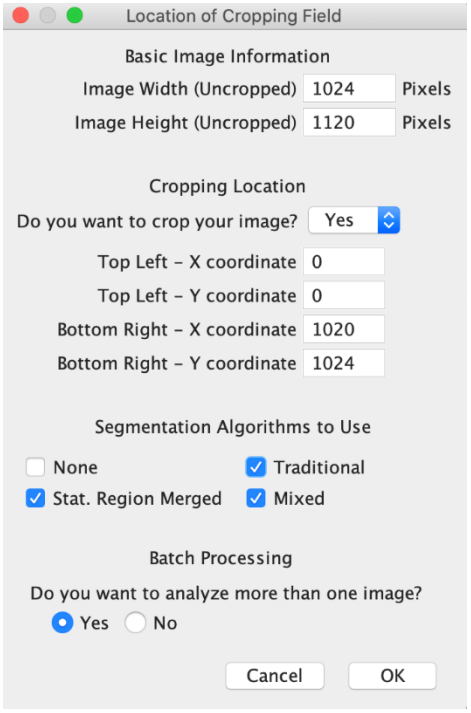
DiameterJ can only analyze binary images (black and white images). The image needs to undergo segmentation to be able to perform the analysis. The white pixels will represent the fibers (the portion of the image that will be measured), and the black pixels represent the background.

PART 1: PREPARING SEGMENTATION

Before segmentation occurs, ensure that the images are placed in a folder. To begin, open ImageJ and go to: Plugins → DiameterJ → DiameterJ Segment.

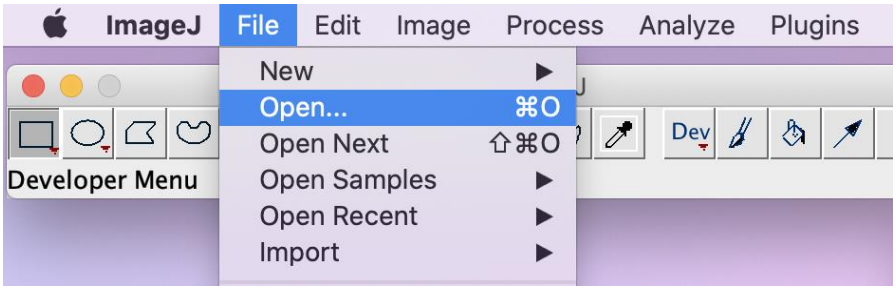


A window will appear asking for the user to input information. Input the information for the original image size and the region for cropping, this will vary based on the size of the original image.

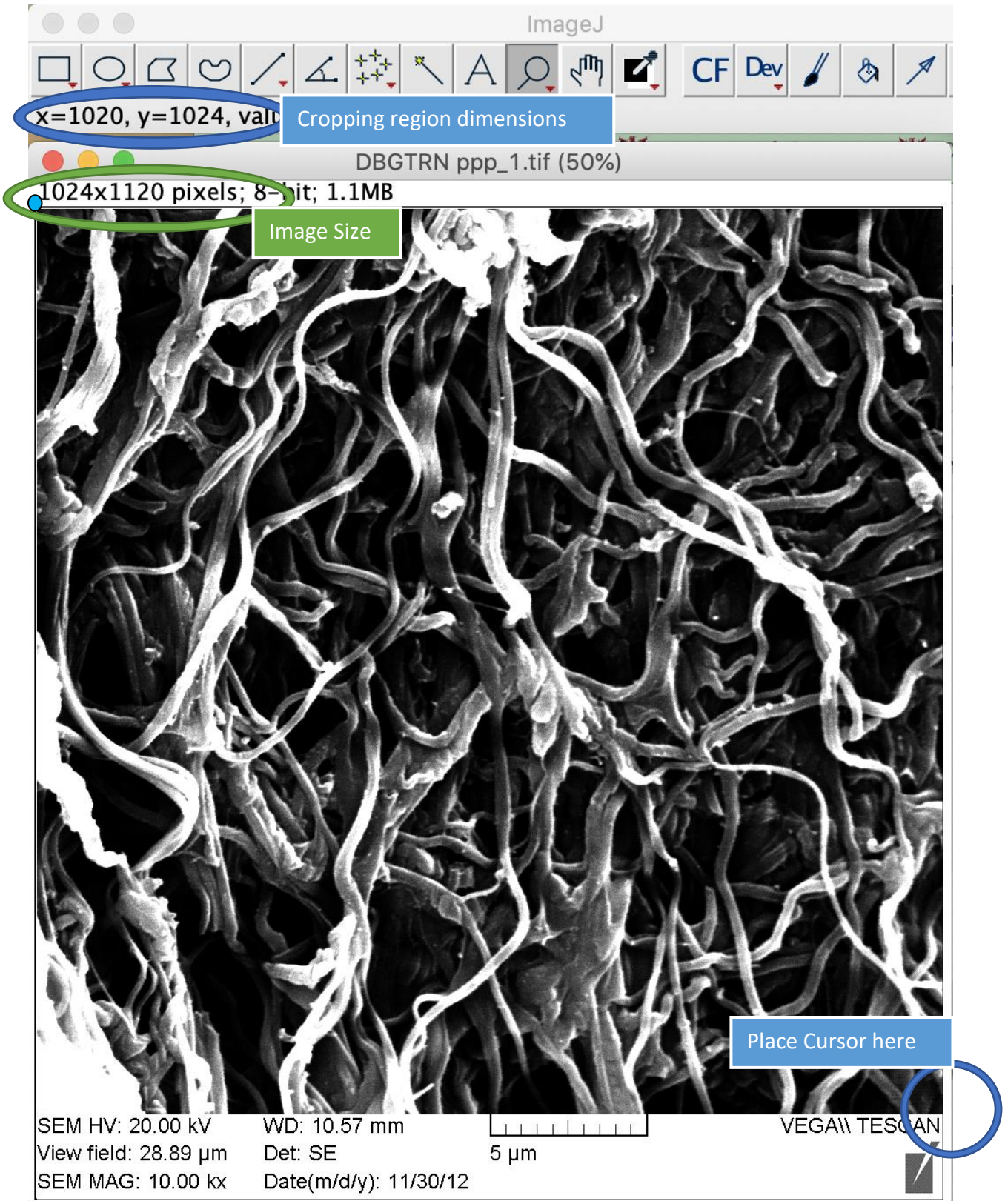


1. Basic image information & Cropping Location: Users can indicate the desired region for analysis, as well as the dimensions for cropping (crop out anything that will not be included in the analysis).

How to determine the size of image and cropping region: Open the image with ImageJ.



The dimensions will be at the top left of the same window of the image. To determine the cropping region, place the cursor at the bottom right of the image to obtain the dimensions, which will display on the ImageJ window, under the toolbar. For reference, the top left corner of the image is 0 x 0 (LIGHT BLUE DOT).


ImageJ
x=1020, y=1024, valu
Cropping region dimensions
DBGTRN ppp_1.tif (50%)
1024x1120 pixels; 8-bit; 1.1MB
Image Size
Place Cursor here
SEM HV: 20.00 kV
WD: 10.57 mm
5 µm
VEGA\\ TESCAN
View field: 28.89 µm
Det: SE
SEM MAG: 10.00 kx
Date(m/d/y): 11/30/12

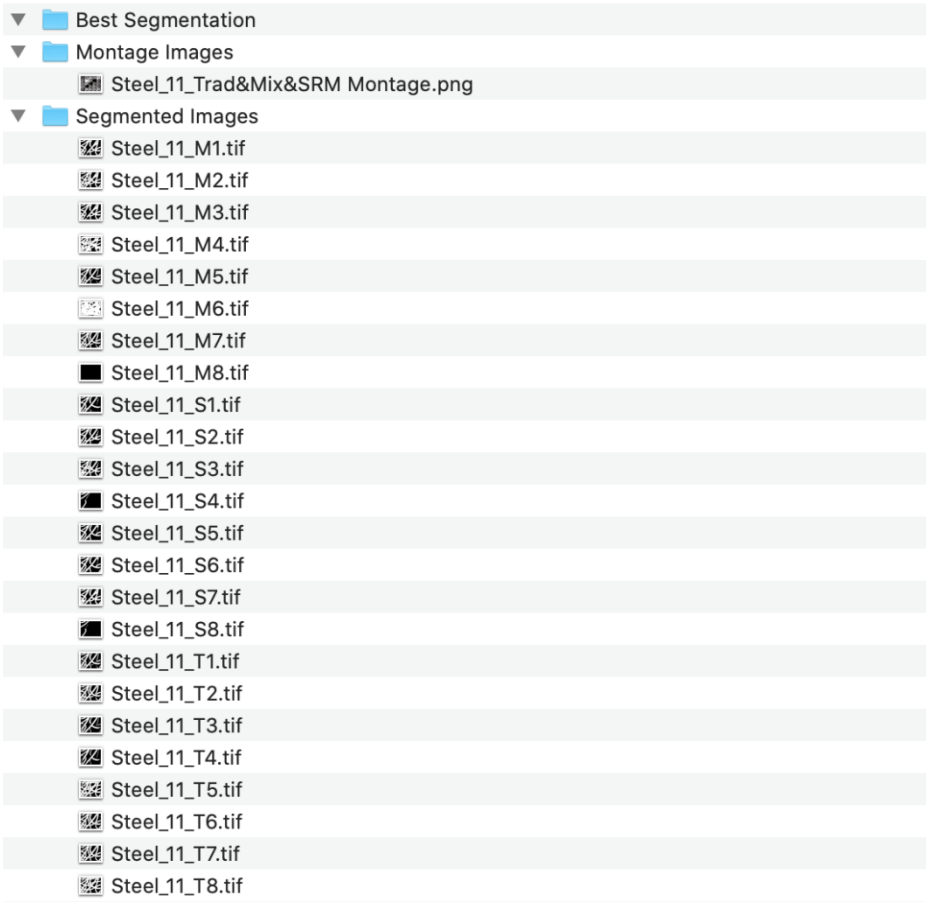
2. Segmentation Algorithms to Use: There are 3 different algorithms; *Traditional, Stat. Region Merged,* and *Mixed,* each producing 8 different images. Users can choose as many or as few methods as desired, though it is recommended to select all 3 to produce the greatest variety of options, which will be used in Part 2.

3. Batch Processing: Choose "Yes" (regardless of single or multiple image segmentation). A pop-up window will appear asking the user to navigate to the folder containing the images. All outputs will also be saved in the selected folder.

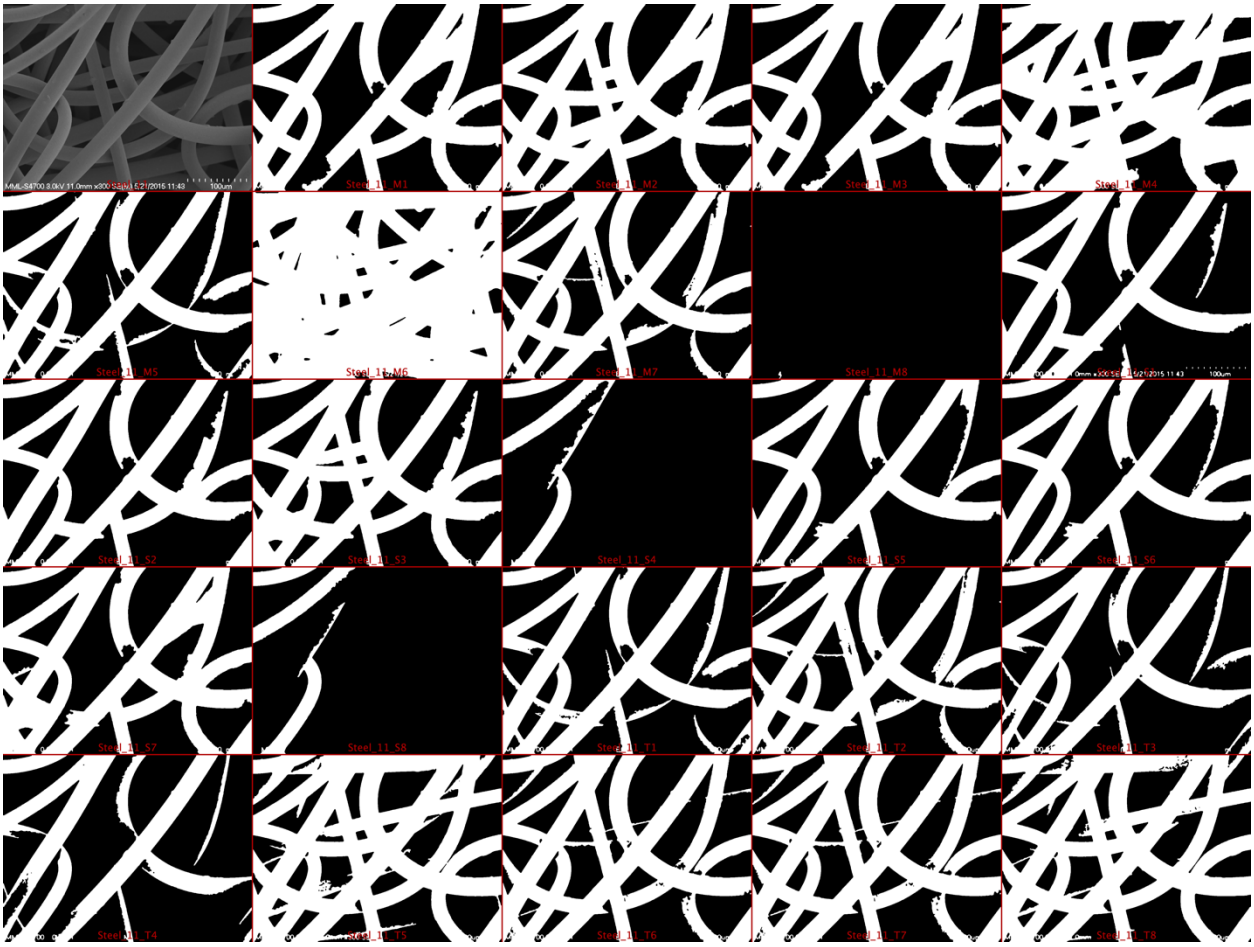
4. Select "OK" after all options have been specified to segment images. Part 2 will discuss the output of segmentation.

PART 2: OUTPUT

In the same folder as part 1, three new folders will be produced, labelled "Best Segmentation", "Montage Images", and "Segmented Images".



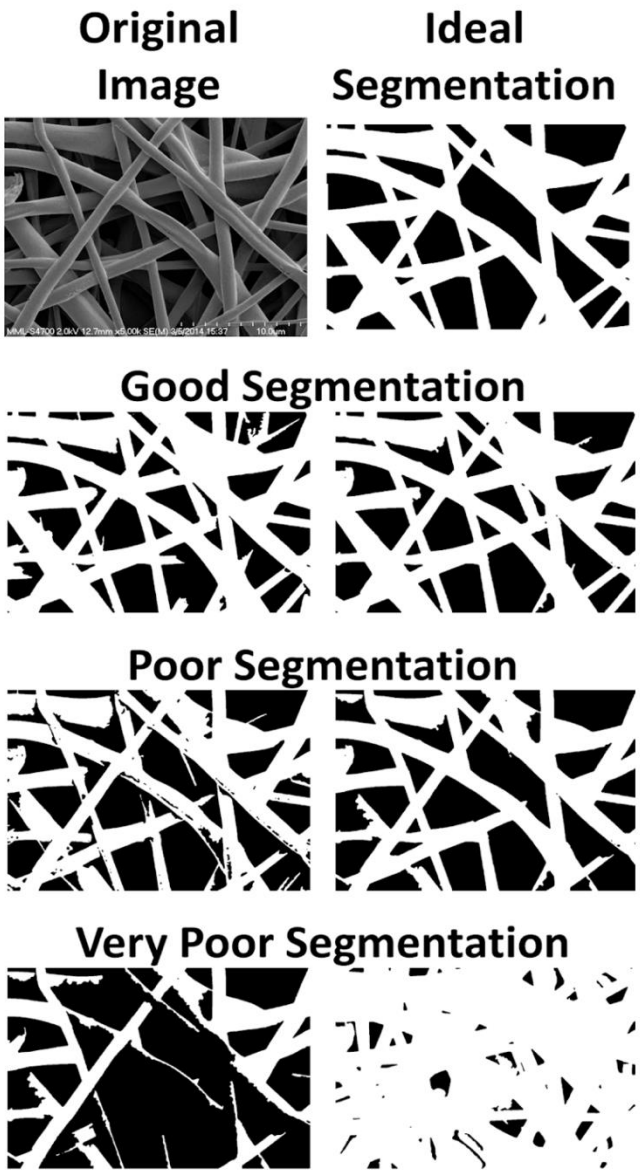
Montage Images: This folder will contain a collage of all the segmented images for each photo. Users will be able to compare the original image to the variety of segmented images. Choose the segmented image that best represents the original. However, further editing can be done (described below). Here is what the montage image should look like, the segmented image name is seen in red.



The criterion for determining the best segmentation is as follows:

(1) Many full fibers are present
(2) Intersection of fibers no dot contains black hole/minimal areas of overlap (as this will increase diameter size)
(3) Fibers are accurately depicted (segmented fibers are representative of actual fibers, and not the background)

Below is a visual representation of ideal, good, poor, and very poor segmentation. The segmentation expected to produce less than 5% error, over 10% error, over 30% error, and substantial error, respectively.



Segmented Images: This folder will have all the segmented images from the montage, select the best image and copy/drag the image to the "Best Segmentation" folder. The name of each photo can be seen at the bottom in red.

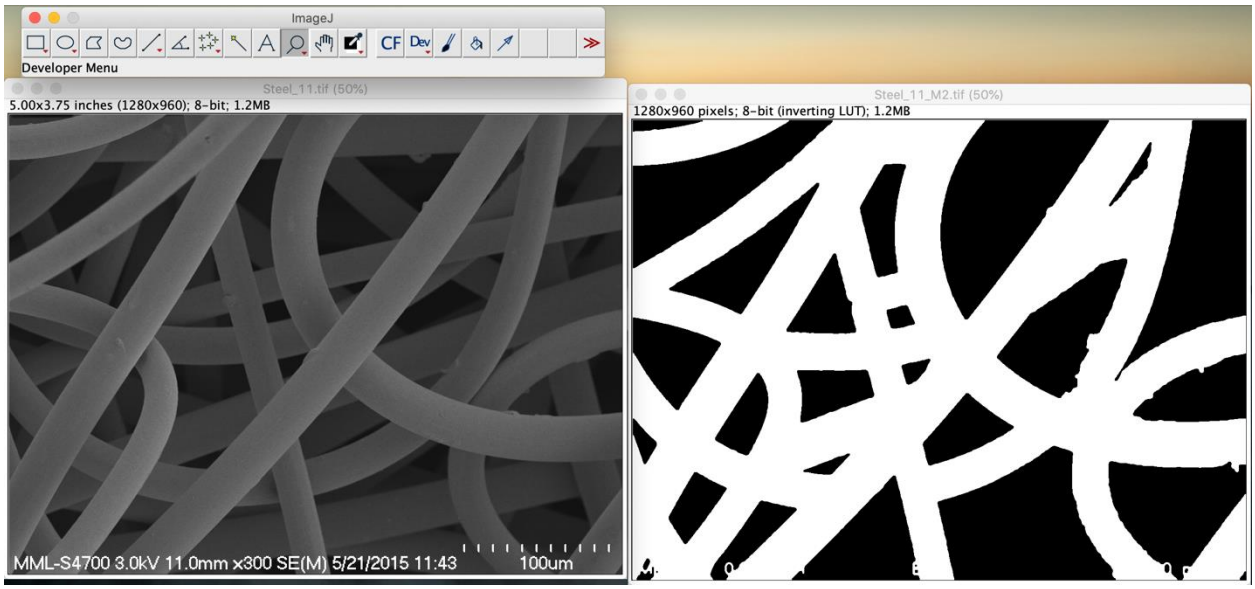
Best Segmentation: This is an empty folder which will hold the segmented images to be analyzed. Move the images to be analyzed into this folder, the image must be in black and white.

OPTIONAL: MANUALLY EDITING IMAGES

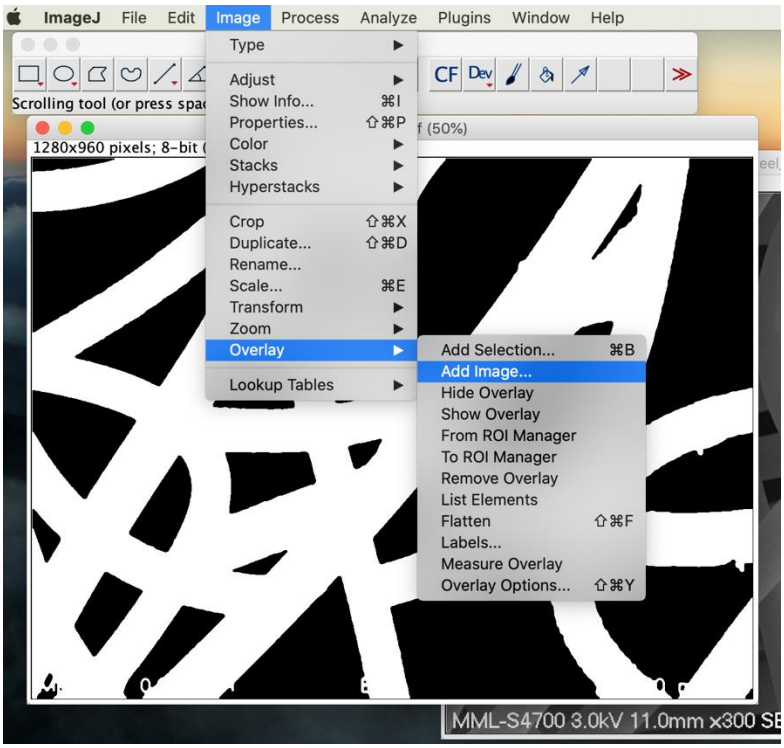
Images can be edited using any software of choosing (as long as the result produces a black and white binary image). However, editing can be done within ImageJ. This helps in "cleaning up"

the image and removing unnecessary white or black spots (edge defects, or densely packed areas)

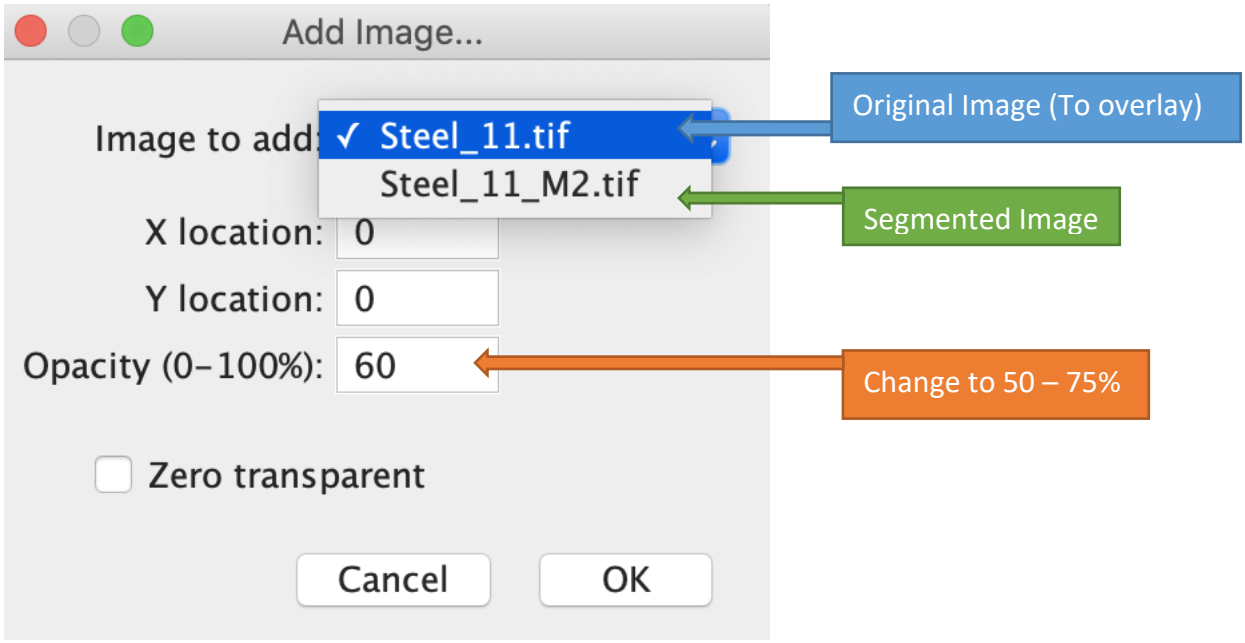
1. Open the “best” segmented image in ImageJ as well as the original image (ImageJ → File → Open)



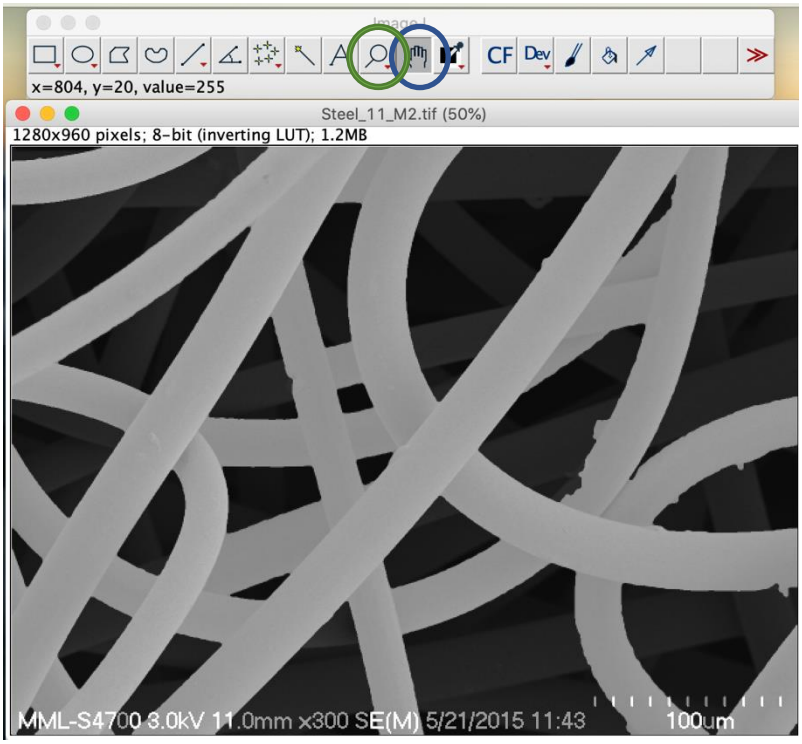
2. Click the segmented image → Image → Overlay → Add image.



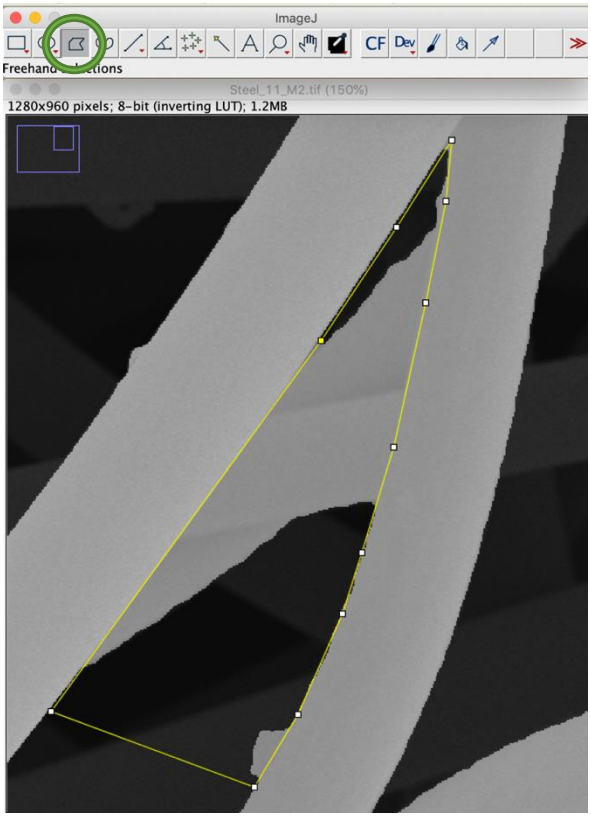
3. A pop-up window will appear, ensure that under “Image to add”, the original image is selected. Keep X and Y location as 0 to line up the two images. Opacity will then be changed to any value from 50 to 75%

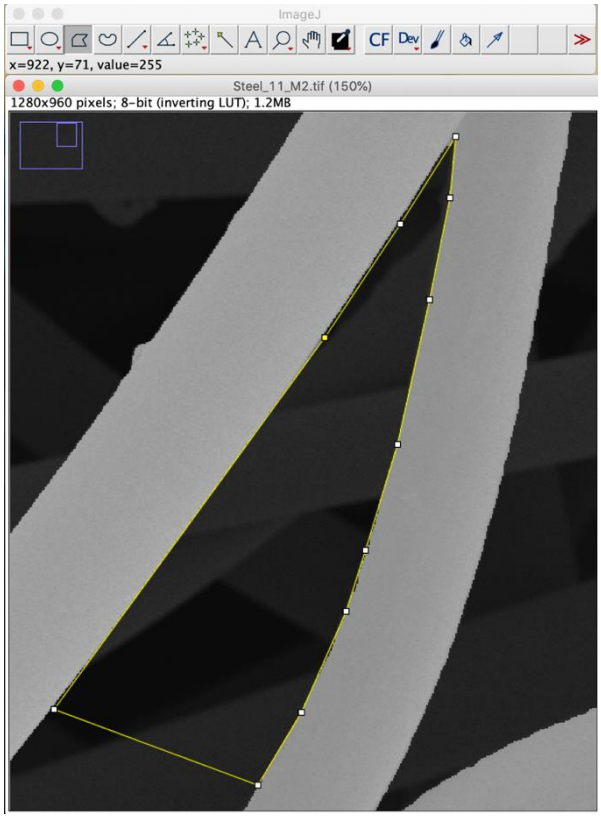


4. The images will now be overlayed on one another. Use the magnifying feature to zoom in to specific areas, and the hand to drag across the image

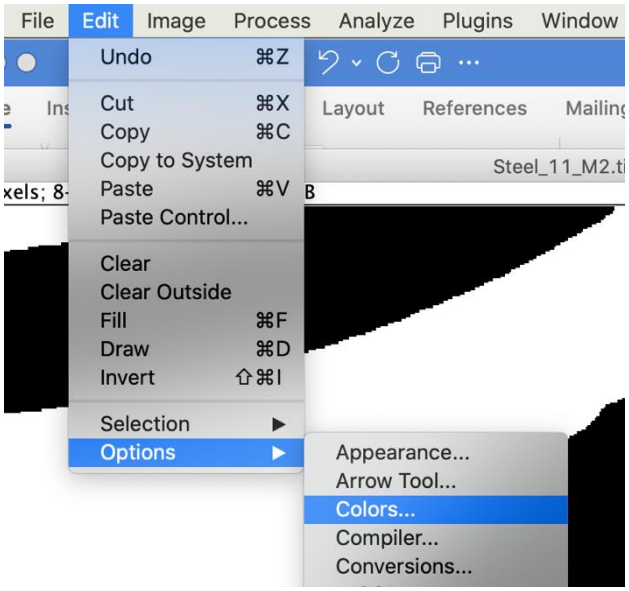


5. Using the polygon selection tool, users will outline areas for either the fibers, or the background. With this tool, all areas selected must produce closed shapes only. The default is set to black which can remove unwanted fibers or white spots. Backspace when satisfied to fill selected region.

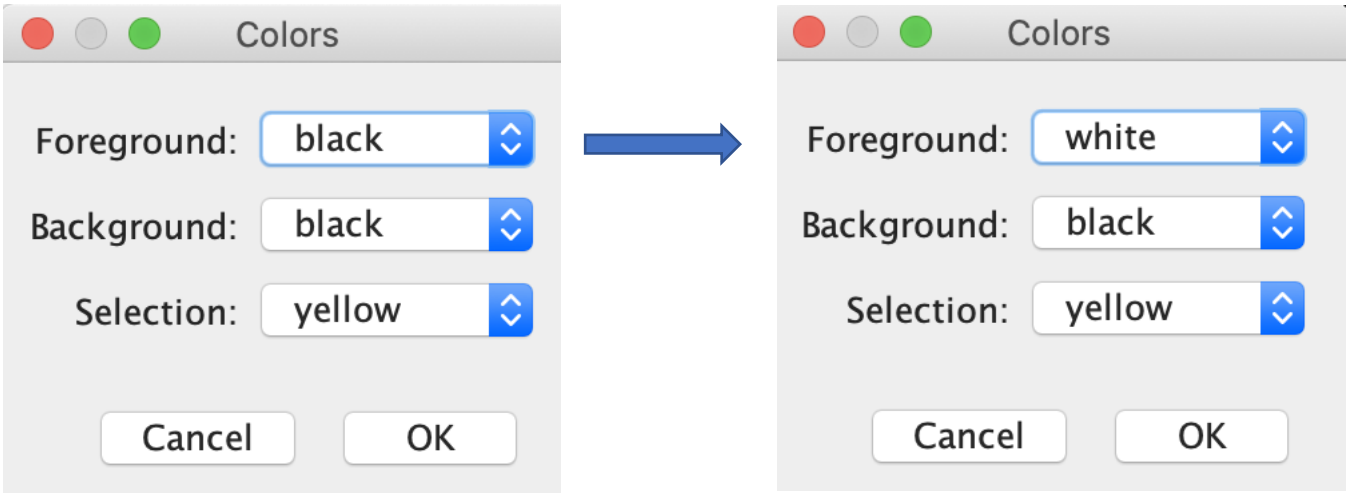




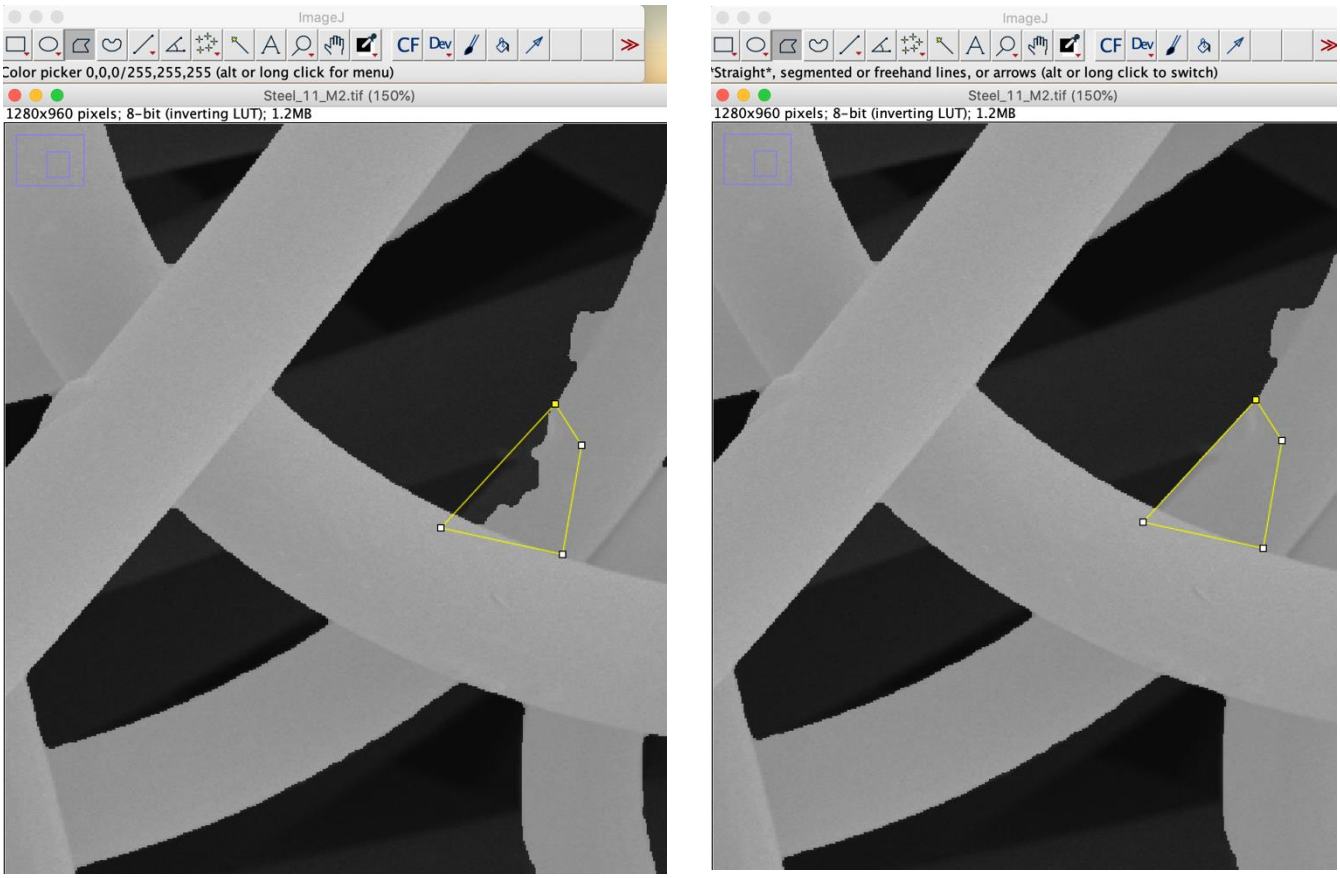
To change the fill colour (to outline parts of the fibers to include): Edit → Options → Colours.



Change “Foreground” from Black to White to fill in fibers.

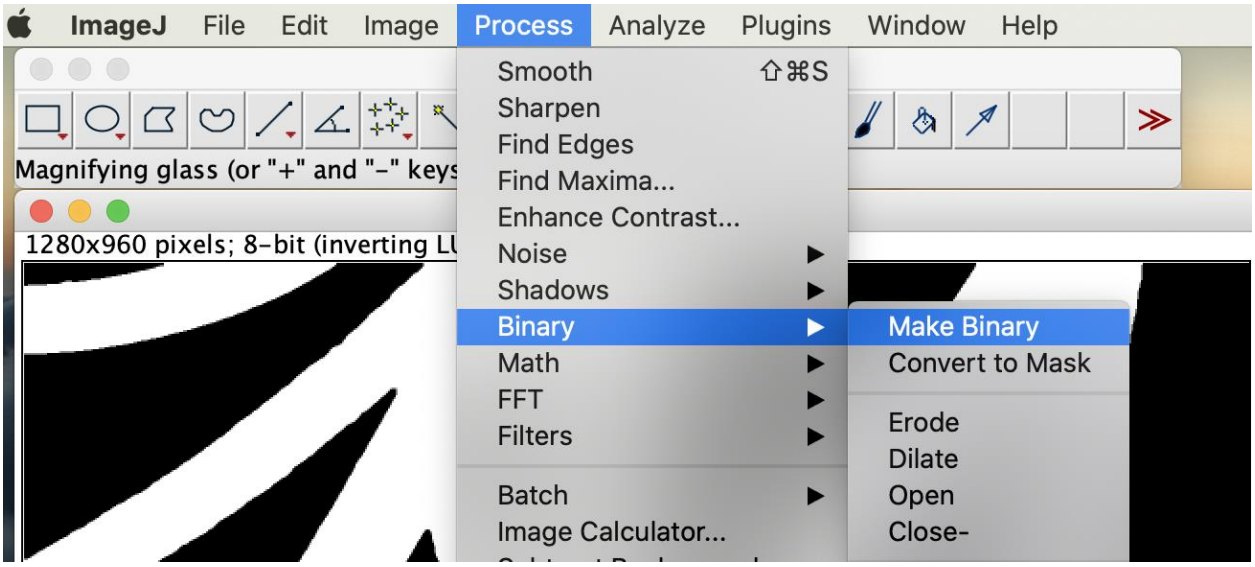


Users can now use the same polygon selection tool to fill in the fibers.



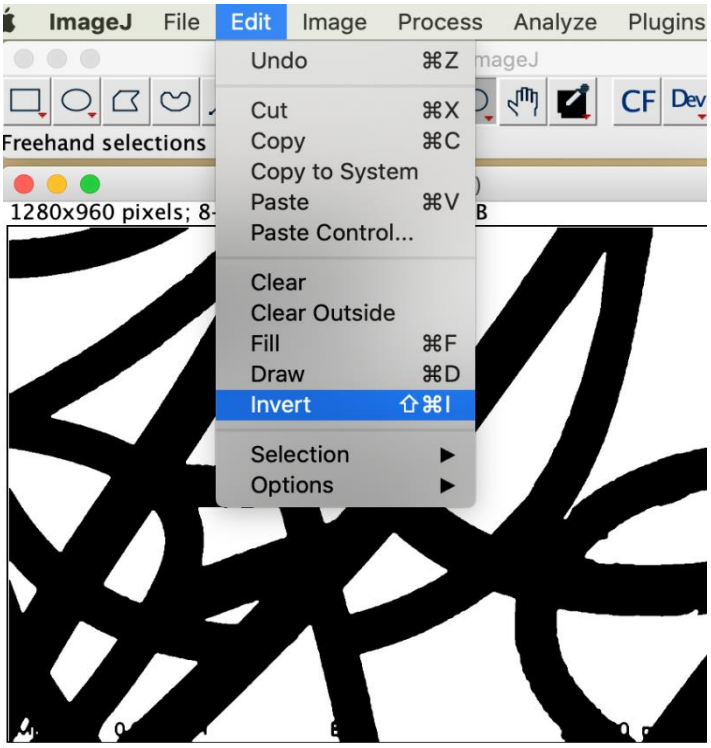
6. Image → Overlay → Remove overlay

7. Make the image Binary (black and white) → Process → Binary → Make Binary.

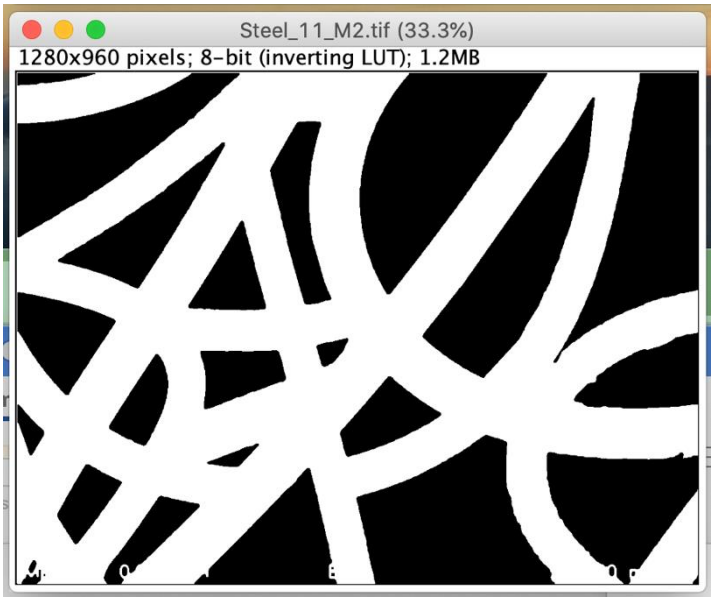


8. If the image is inverted go to Edit → Invert, to get white fibers on a black background

Before:



After[2]:

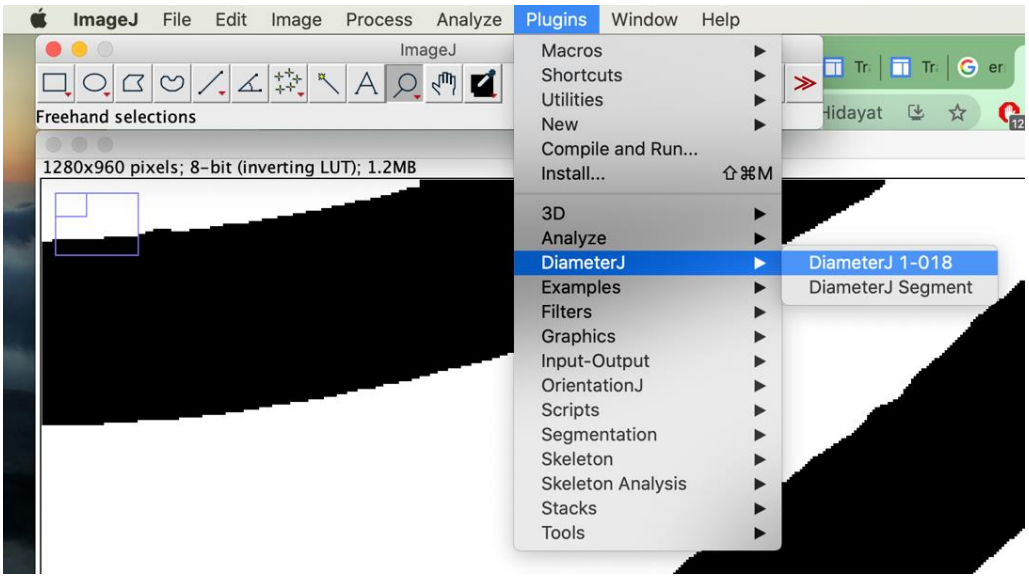


[2] The more fibers that are segmented out of an image result in larger pore sizes, lower intersection density, and higher characteristic fiber length

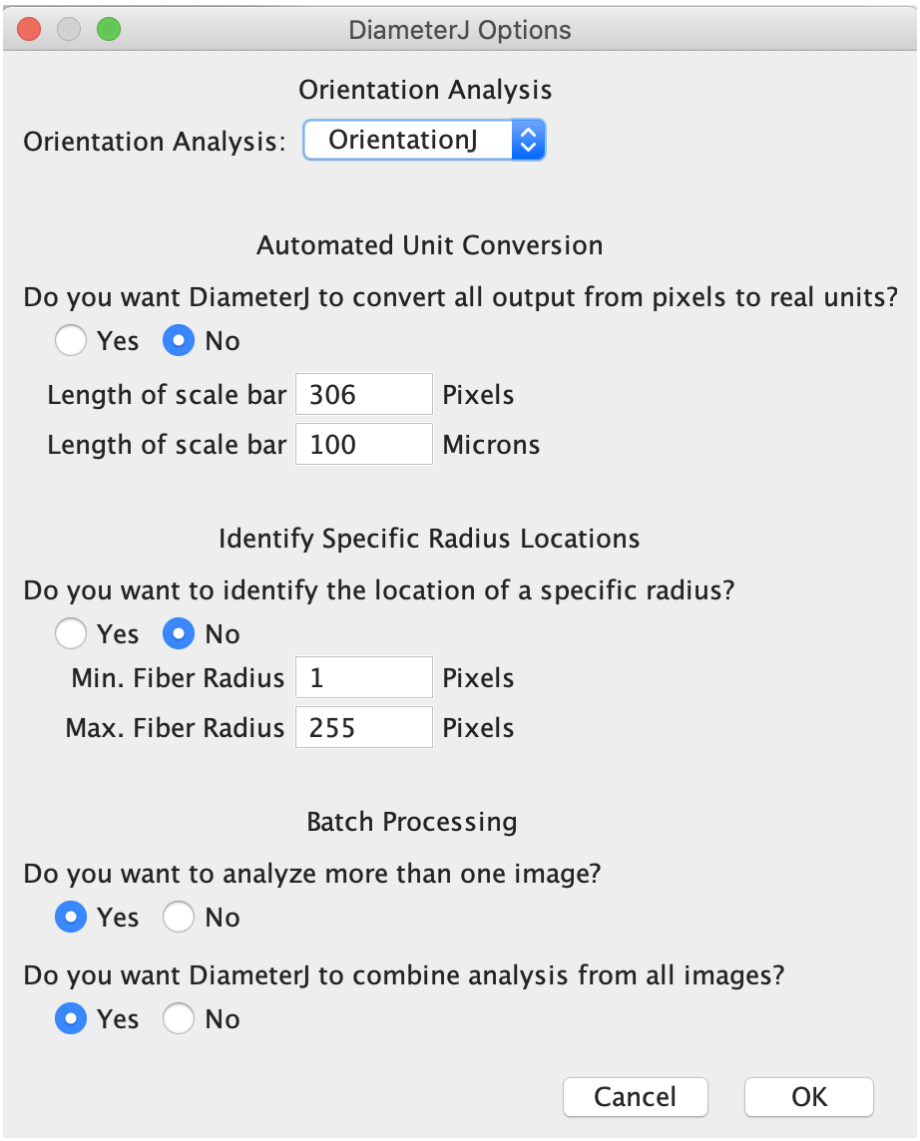
Image Analysis

PART 1:

Go to: ImageJ→ Plugins → DiameterJ → DiameterJ 1-XXX (based on the version of imageJ). A pop-up window will appear.



1. **Orientation Analysis:** Select Orientation analysis as OrientationJ. OrientationJ is the default and will give us the fiber orientation of the image.
2. **Automated Unit Conversion:** This allows users to report values in real units, rather than pixels. Input the length of scale bar (should be at bottom right of image) in pixels and microns for conversion. *See below
3. **Identify Specific Radius Locations:** Allows users to know where a particular radius of fiber is located, DiameterJ will create a graphic to show the location of the radii of the specified range – set the upper and lower bounds of the range. This can be used after an initial run if there are outliers or unexpected values to locate the discrepancies.
4. **Batch Processing:** Similarly, to Part 1, regardless of if analyzing a single image or multiple, a pop-up window will appear asking users to indicate the selected folder. DiameterJ is also able to combine results in a “Summary”.

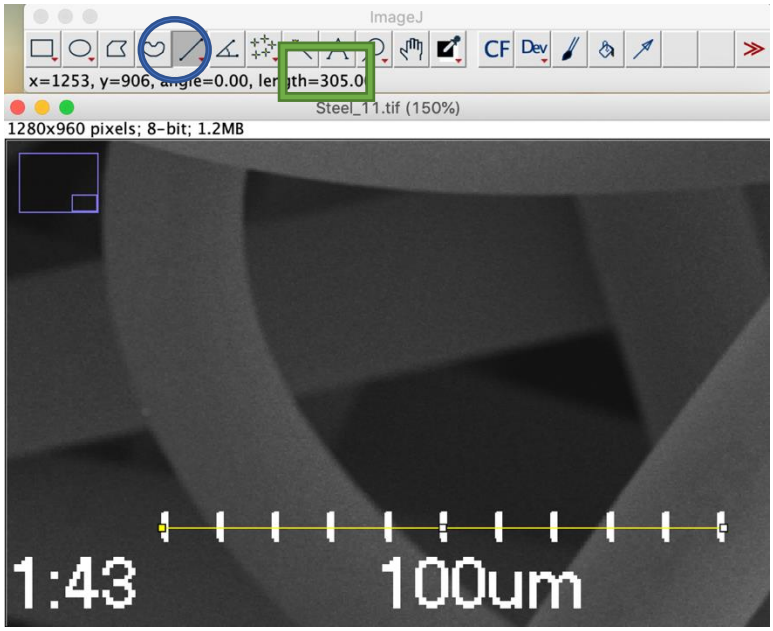


OPTIONAL: PIXEL TO REAL UNIT CONVERSION

All units produced by DiameterJ are given as pixels, but users can convert to any desired values.

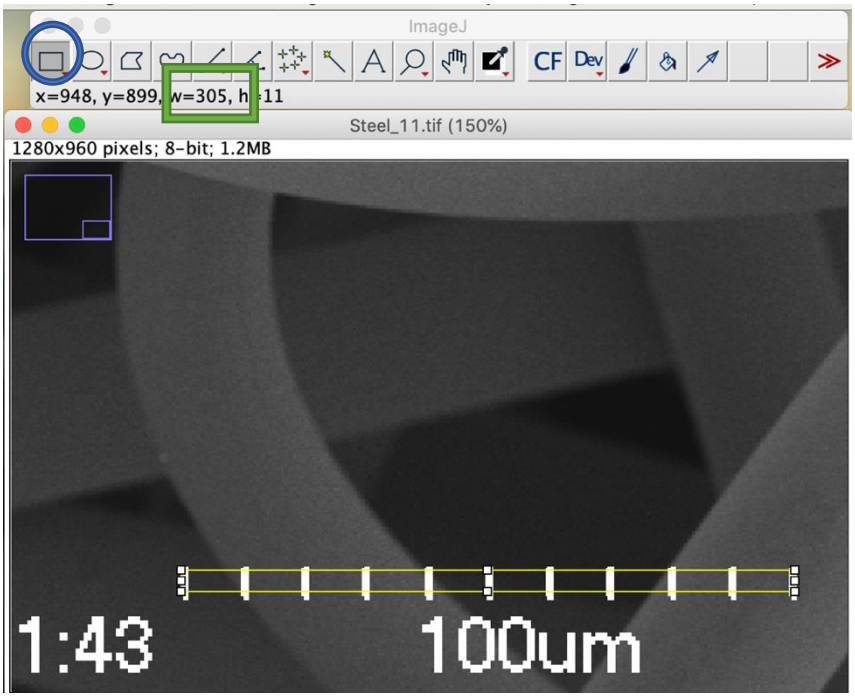
1. Open the original image with ImageJ. Use the magnifying glass to zoom in so the bar spans the width of the window.

2. Select the "Line Tool". Create a line spanning the measurement bar (bottom right). Ensure that the angle is 0.00 (creating a straight horizontal line). Length of the line is units of pixels is seen in the green box.



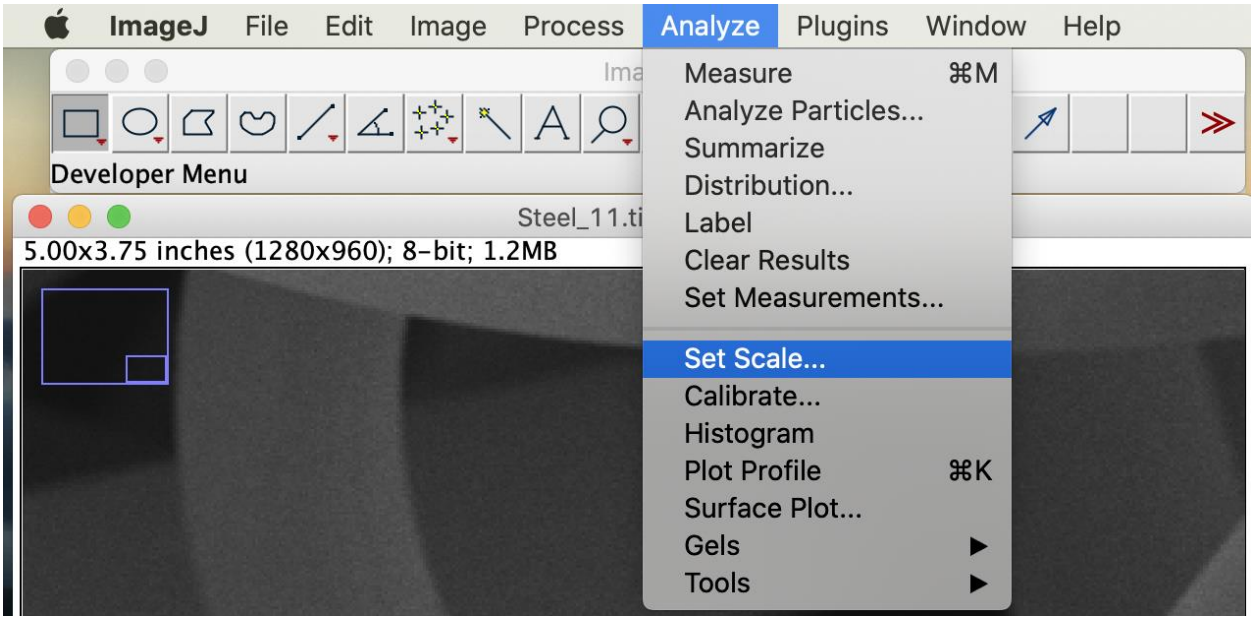
OR

2. Select the rectangle tool to easily create a rectangle spanning the length of the bar. The dimensions are displayed under the tool bar. The length of the bar is seen in the green box in units of pixels.

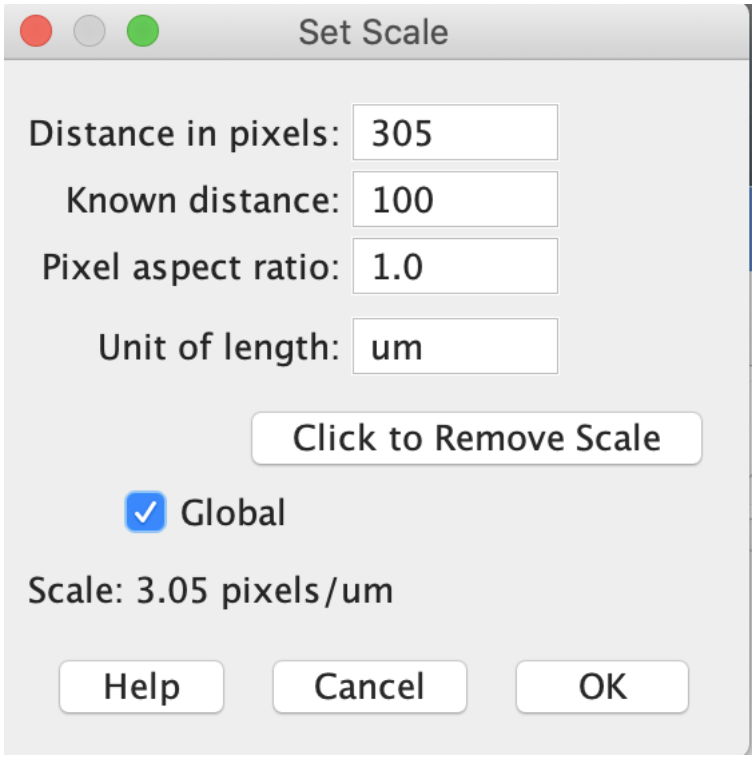


Using the determined length of the scale bar, this can now be used in the "Automated Unit Conversion" portion of the Image Analysis.

Optional: 3. Go to "Analyze" → "Set Scale"



4. The pixels should be displayed/automatically set, input the indicated length of the bar (should be given) as the known distance. This will show the number of pixels per units.



PART 2: OUTPUTS

When all options are specified, a pop-up window will appear once more after pressing "OK", users then will indicate the specified folder for image analysis.

Diameter Analysis Images

Imagename_Compare.png

- A montage image will be produced with the original segmented image (top left), the centerline of the fibers per the Voronoi Tessellation (top right), an overlay of a yellow line at the locations where fiber diameters were measured in the image on top of the Euclidean distance transform of the segmented image (bottom left), and all pores measured (bottom right)

Histograms

Imagename_Radius Histo.csv

- ***Radius Value:*** Radius length
- ***Radius count:*** Number of times the radius value occurred (the frequency). The radius values are on the x axis, and the frequency of occurrence on the y axis.

Imagename_Char Lengths.csv

- *Skeleton ID*
- *Calibrated branch length*
- *3D coordinates of the extremes of the branch (V1x, V1y, V1z, V2x, V2y, V2z)*
- *Euclidean distance between extreme points (indicator of the tortuosity of the 3D object when compared to the calibrated branch length)*
- *Branches sorted by decreasing length*
- *Bottom of the table has the average, SD, min and max*

Imagename_Pore Date.csv

- ***Column 1***: Number identifier for each pore counted in the image
- ***Label:*** Mean, SD, Min, and Max labels at the bottom of every list. Rows containing the summary metrics for the entire column above
- ***Area:*** List of all the black pixel clusters' numbers of black pixels that are not in a pore that touches the side of the image
- ***Perim.:*** The number of pixels in the perimeter of each pore
- ***Major:*** Length of the primary axis of the best fitting ellipse for each pore
- ***Minor:*** Length of the secondary axis of the best fitting ellipse for each pore
- ***Angle:*** Angle between the major axis of the pore and a line parallel to the x-axis of the image
- ***Circ:*** $([4\pi \bullet \text{Area}] / [\text{Perimeter}^2])$. A value of 1 indicates a perfect circle, values approaching 0 indicate an increasingly elongates shape.
- ***AR:*** The aspect ratio of the particles' fitted ellipse: [Major Axis] / [Minor Axis]
- ***Round:*** $([4 \bullet \text{Pore Area}] / [\pi \bullet \text{Major Axis}^2])$. This value can also be taken as the inverse of the aspect ratio

- ***Solidity:*** [Pore Area]/[Convex area of pore]

Imagename_RadiusPlot.tif

- Output image of the histogram of all measured fiber radii from the original image

Summaries

Imagename_TotalSummary.csv: A file will be created for each image analyzed

Under "*Diameter Metrics*"

- ***Super Pixel:*** As mentioned in the Overview section. This value is calculated via the super pixel determination and provides a global average of fiber diameters that are extremely diverse.
- ***Histogram Mean:*** The mean value of the Gaussian Curve fitted to the radius data. Provides a more accurate global average of fiber diameter with very similar values.
- ***Histogram SD:*** The standard deviation of the Gaussian curve to the radius histogram values.
- ***Histogram Mode:*** Most frequency value of fiber diameter
- ***Histogram Median:*** The middle fiber diameter value
- ***Histogram Min Diam:*** Smallest fiber diameter
- ***Histogram Max Diam:*** Largest fiber diameter
- ***Histogram Integrated Density:*** The produce of length of fibers and the average radius
- ***Histogram Raw Integrated Density:*** Sum of the radii at all pixels in the image (or the selection)
- ***Diameter Skewness:*** Third order moment about the mean.
- ***Diameter Kurtosis:*** Fourth order moment about the mean
    - Skewness and Kurtosis are good indicators of how normal your radius histogram is. If both Skewness and Kurtosis are <1, the radius histogram van be considered normal.
- ***Fiber Length:*** Total length of fiber centerlines in the segmented image.

Under "*Other Metrics*"

- ***Mean Pore Area****:* (Total black pixels in pores) / (Total number of pores)
- ***Pore Area SD:*** Standard deviation of all pore areas measured
- ***Min. Pore Area:*** Minimum pore area measured
- ***Max. Pore Area:*** Maximum pore area measured
- ***Percent Porosity:*** (Total number of black pixels) / (Total number of pixels)
- ***Number of Pores:*** Total number of pores counted by DiameterJ.
- ***# of Intersections:*** Total number of intersections in image.
- ***Intersection Density (100x100px):*** (Number of fiber overlaps) * 1000 / (Total pixels)
- ***Char. Length:*** Mean length between fiber intersections
- ***SD. Char. Length:*** Standard deviation of fiber intersection / overlap lengths
- ***Max Span Length:*** Maximum length between any two intersections
- ***Old Char. Length:*** (Tot. Fiber Len. /# Intersections)

Diameter Location Folder (ONLY IF SELECTED)

Imagename_Radius Location.csv

- A file will be produced indicating the location on the image of the indicated radius, specified previously.

Combined Files (ONLY IF SELECTED):

All Char Length Values. csv

- The file contains a combination of all characteristic length values and the Euclidean distance between the two terminal points of each of those characteristic length segments from all files analyzed
    - **Characteristic length:** The branch length between two intersections of fibers
    - **Euclidean Length:** The shortest distance between the two terminal points of the fiber segment

All Pore Area Values.csv

- The file contains all pore area values from all the images, as well as major and minor axis length for each pore. Column one is a counter starting from 0 to 180.
    - **Pore area:** Number of pixels in the pore. Number corresponding to the pore as seen in *Imagename_Compare.png* in the "Diameter Analysis Images" folder
    - **Major Pore Axis:** The length of the primary axis of the best fitting ellipse for each pore
    - **Minor Pore Axis:** The length of the secondary axis of the best fitting ellipse for each pore

All Summary File Values.csv

- Contains combination of all summary metrics from all images

All_Radius_Values.csv

- Contains combination of radius value frequencies and the sum at each radius value

Analyzing Results

The output of DiameterJ can be used to determine averages. The mean of the averages average should only be used for percent porosity, number of pores, number of intersections, and intersection density. The formula can be seen below:

$$Mean = \frac{\sum Average\ Values}{\#\ of\ values}$$

To determine the average of all values, use values from the Combined Files Folder.

$$Average = \frac{\sum Values}{\#\ of\ values}$$

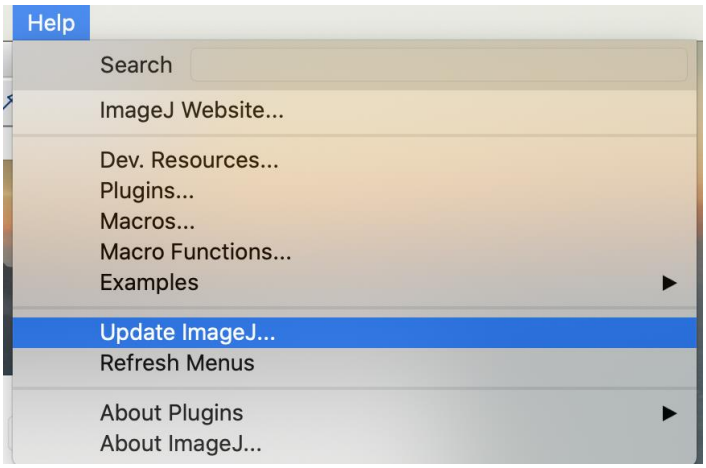
$$\sigma = \sqrt{\frac{\Sigma|mean - value|^2}{\#\ of\ values}}$$

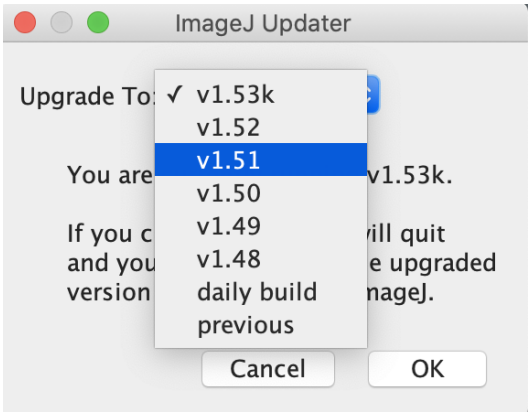
When values are in px^2, user may need to be conversed to μm^2 (multiplying by conversion factor squared)

Help (Troubleshooting)

If the user is facing errors when attempting to analyze the images, users are encouraged to change the version of ImageJ being used.

Help → Update Image J





A pop-up window will appear, and users would be able to go to previous versions of the programs. Using version 1.51 should resolve this problem. ImageJ should restart and when opened again be on the indicated version.

It is also recommended to analyze 2+ images, rather than single images. For more information, go to: https://sites.google.com/site/diameterj/home

References

Hotaling, N. A., Bharti, K., Kriel, H., & Simon, C. G. (2015). DiameterJ: A validated open source nanofiber diameter measurement tool. *Biomaterials*, 327-338. https://doi.org/10.1016/j.biomaterials.2015.05.015.