# A Guide to SEM Imaging

## Overview

Traditional analysis of fibrin clot requires manual assessment of fiber count and width, each individual fiber counted and measured by the user. This process is extremely laborious and operator dependent, oftentimes taking days to weeks to accomplish. Thus, a program was developed in an effort to standardize the quantification process, to overall simplify and computerize the process

The structure of fibrin clots has been shown to be affected by the surrounding physiological milieu, including local thrombin concentrations and the presence or absence of anticoagulants. Scanning electron microscopy is a tool used to examine the surface structure of clots, the clot structure then quantitated by 3 parameters: porosity, fibrin diameter and the number of fibrin strand.

For this program, before users can determine these values, modification of the SEM images is required in photoshop, converting the fibers to black silhouettes. Further detail for image processing can be seen the following section.

For image analysis, this program scans the image horizontally multiple times, and for each fiber, follows the slope of the edge of the fiber, taking the traverse perpendicular across the edge as the length of the diameter, as seen in Figure 1. Working well for individual fibers, overlapping fibers have shown to cause issue, resulting in inflated measurements. To overcome this issue, multiple measurements would be taken, reporting only the smallest value as the diameter, seen in Figure 2.

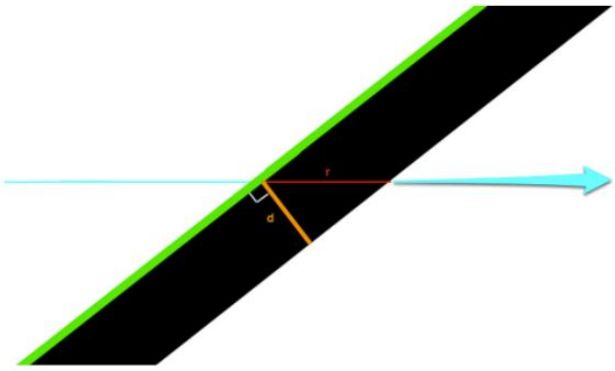


*Figure 1: Single Fiber Measurements*

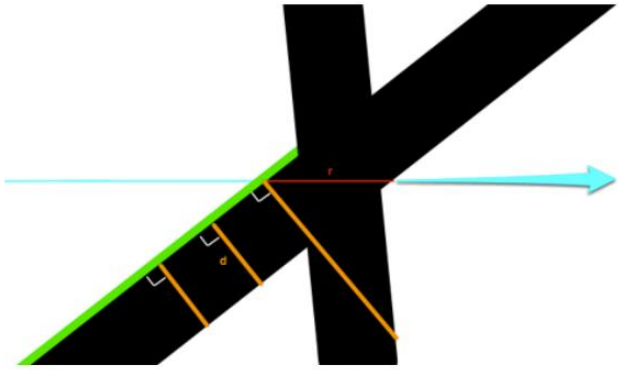


*Figure 2: Overlapping Fiber Measurements*

In order to differentiate single from multiple fibers, the image is processed to highlight the edges of the surface fiber (all done within Photoshop). The program utilizes this newly modified image as a reference by the program to clearly distinguish between single or multiple fibers. As the program determines that the measurement was taken across multiple fibers, an algorithm would take the subsection of the original measurement, reporting that as the final fiber width, seen in figure 3.

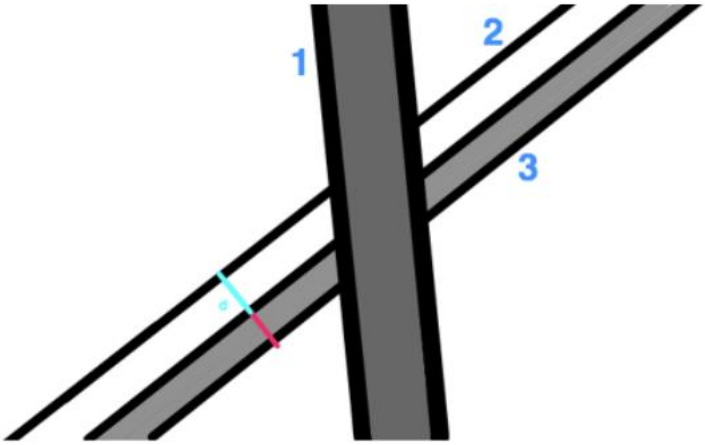



*Figure 3: Highlighted Edge Fibers*

Additional measures can also detect and remove measurements which may have the potential to produce inaccurate results or outliers. Through photoshop, the presence of surface fibers is accentuated, superficially removing non surface fibers. A sample of grey tone is taken over the fibers, only utilizing fibers that are considered to be dark enough as surface fibers, utilizing only these measurements. This process being considered the grey scale test.

With images of extremely dense fiber clusters or closely interwoven fibers, it is difficult to differentiate single fibers and make accurate measurements. To ensure that only an individual fiber is being measured, the smallest measurement taken must be repeated a certain number of times for the program to consider it to be a valid value.

For fiber count, the program scans vertically from within the fiber, and an array of the length of the lines are taken. The number of local maxima within the fiber is assumed to be the number of intersecting/branching fibers. The program takes multiple horizontal scans across the image and averages out the fiber count across the scans. Further information can be seen in Image Analysis.

## Download/Installation

Users are required to have the following software and files to complete image processing and analysis:

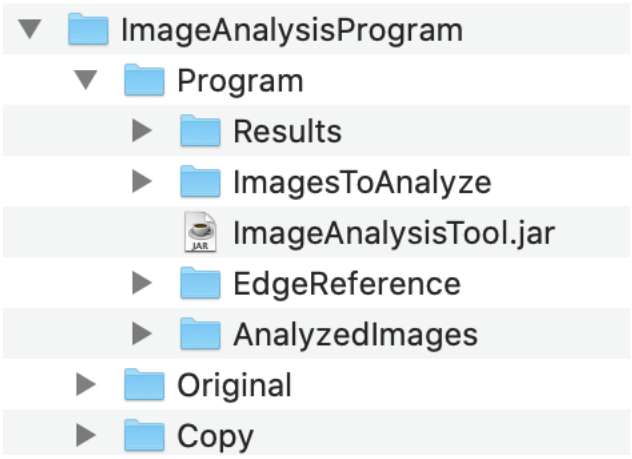
**For Image Processing:**

1. Users must have Adobe Photoshop downloaded. [1]
2. A download of the file "Photoshop Actions.atn"[2]

**For Image Analysis:**

1. A download of the file "ImageAnalysisTool.jar"[3]

**Set up a folder as follows:**




The parent folder will be called "ImageAnalysisProgram" with three sub folders: "Program", "Original", and "Copy".

"Program" will have four folders and the "ImageAnalysisTool.jar" file. The four folders will be labelled "Results", "ImagesToAnalyze", "EdgeReference", and "AnalyzedImages".

The folder "Original" will have the original SEM images.

The folder "Copy" will be a temporary folder with a copy of all the original images.

[1] Adobe Photoshop requires users to purchase a membership plan
[2] Users will be taken to a GitHub page; download will immediately commence for the file. Users can exit the page.
[3] Users will require Java to run this file.

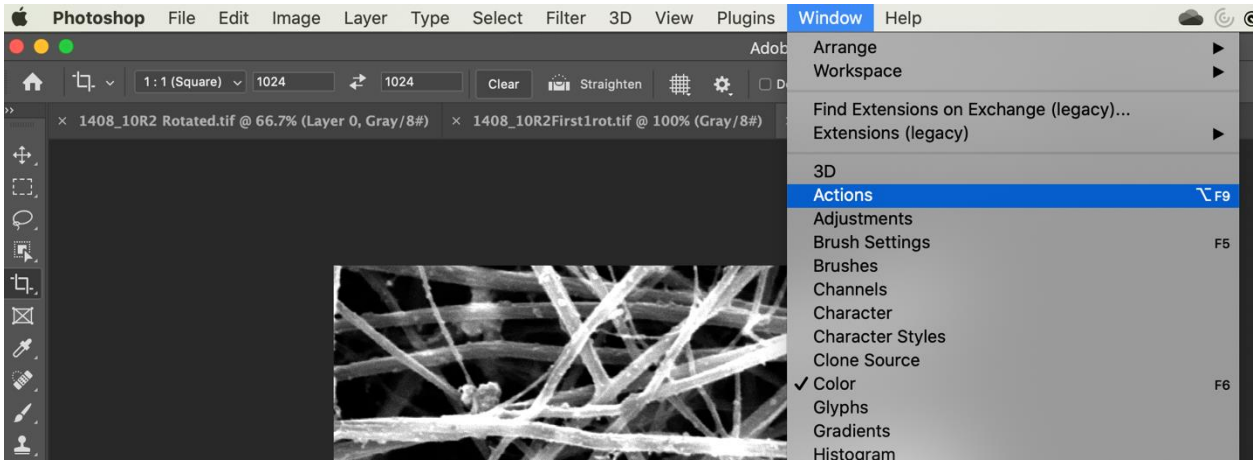
# Image Processing (with Photoshop)

Image processing[4] is required to be done prior to image analysis. Below details the steps to prepare the image for analysis:

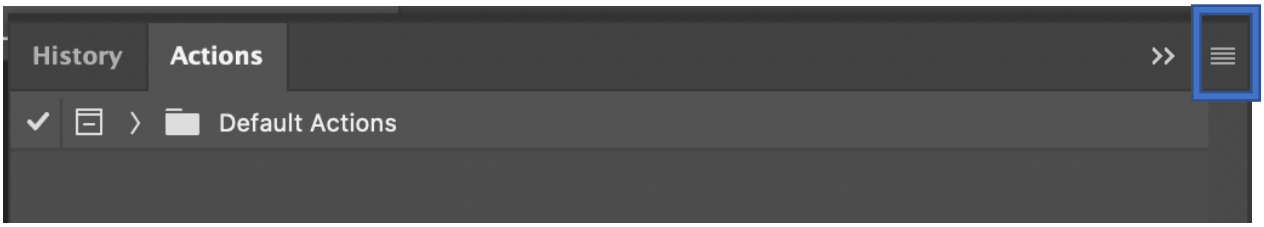
## Preparing Photoshop

Open Photoshop

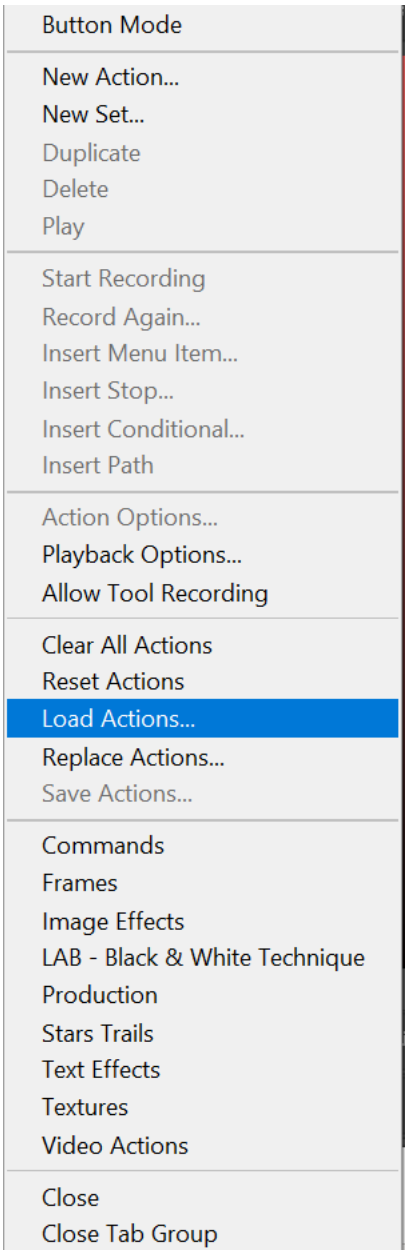
**Step 1:** Go to "Window" → "Actions". A pop-up should appear.



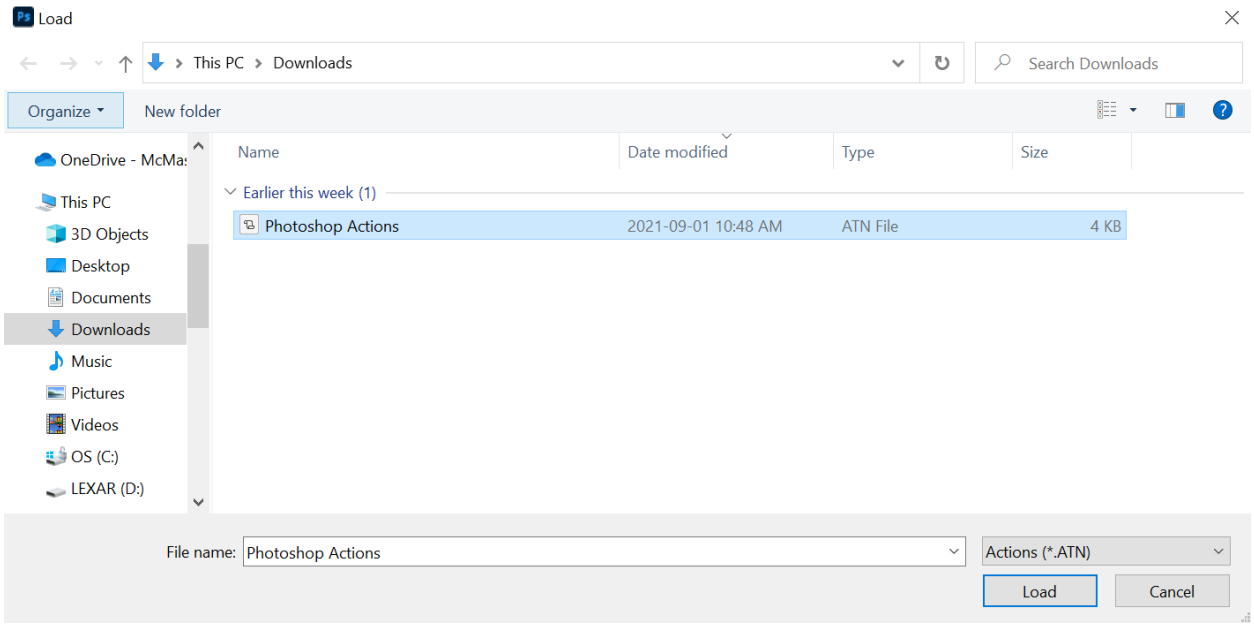

**Step 2:** Click the top right corner symbol with three horizontal lines and select "Load Actions"



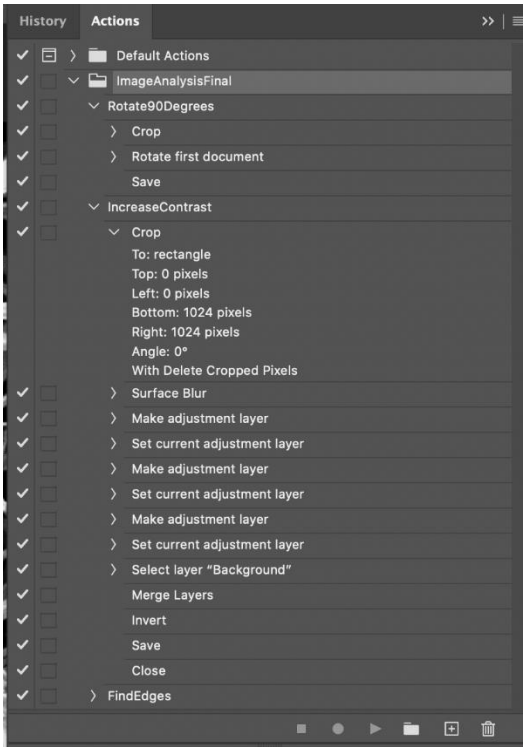

[4] A detailed explanation of the processes completed can be found in the "Manually Preparing Images" section




**Step 3:** Another popup window will appear prompting users to navigate to the file to import. In this case navigate to the file “Photoshop actions.atn”, downloaded in the previous section. Once the file is selected, select “Load”.



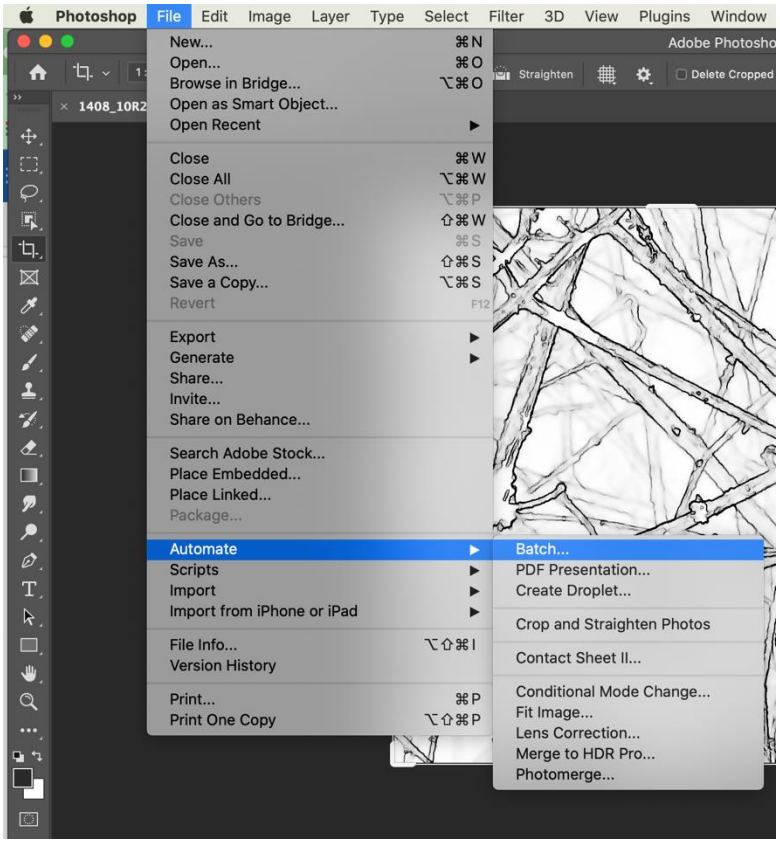

Users will now have the following displayed:



## Using Photoshop

Put all images to be analyzed into the folder “Original” and “Copy”. See Naming Convention prior to using photoshop.

**Step 1:** In Photoshop, go to File → Automate → Batch. A pop up window will appear.




**Step 2:** Fill in the window as follows:

Play

**i)** **Set:** ImageAnalysisFinal

**ii)** **Action:** Rotate90Degrees

Source

**i)** Select “Choose” and navigate to the temporary folder with a copy of the source images

Destination

**i)** **Folder:** Choose → select the folder with the original source images

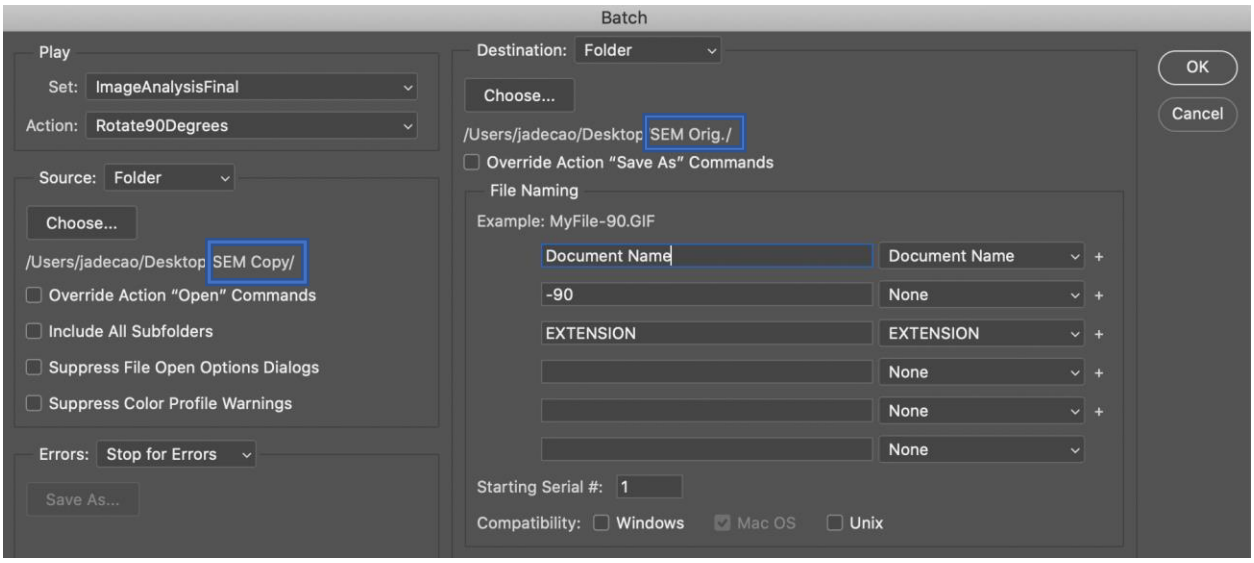
**ii)** File naming

a. “Document Name” should be in the first text field

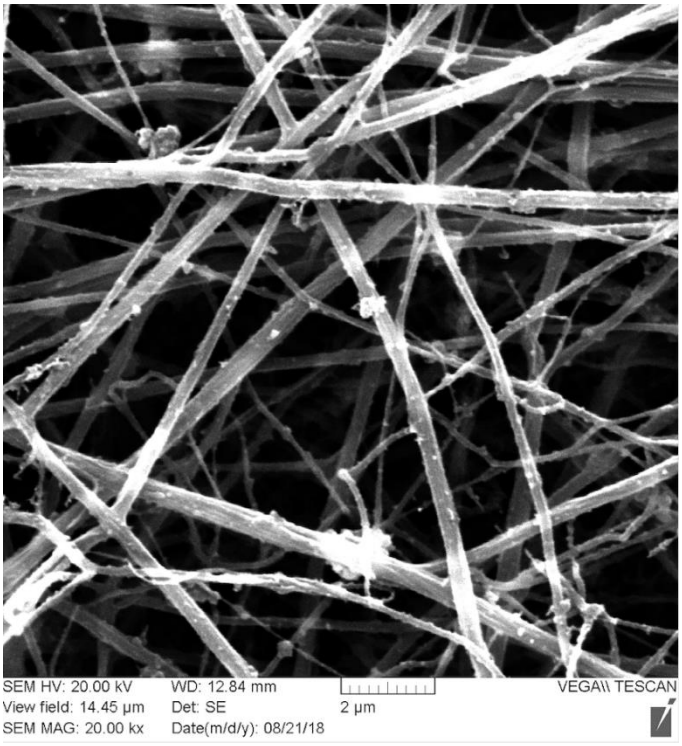
b. Set the next as “-90”

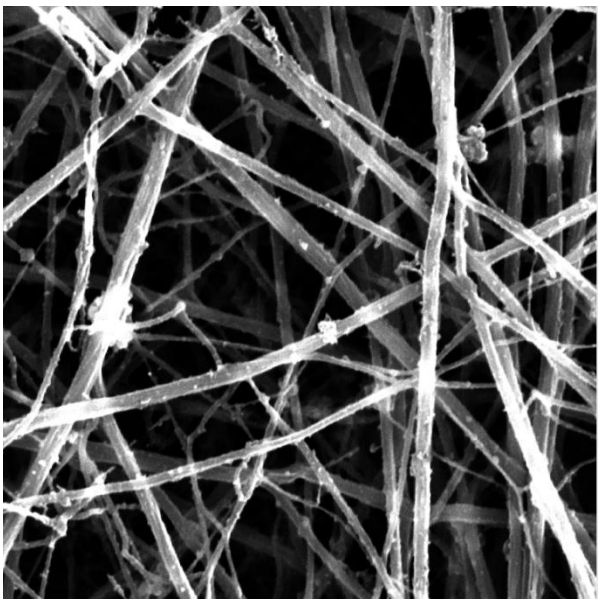
c. Set the third as EXTENSION (select with the drop-down menu)
    i. This just formats the new image (ex: .TIF)

**iii)** Compatibility should be set to the users operating system




**Step 3:** Delete the temporary folder. Ensure that the files in the original folder and rotated 90 degrees compared to the original. The original is seen on the left, and the processed image is seen on the right.



**Step 4:** Copy all the images from the original folder and place them into the folders: “ImagesToAnalyze” and “EdgeReference”:

**Step 5:** In Photoshop, go to File → Automate → Batch. A pop-up window will appear. Fill in the window as follows:

Play

**(i)** **Set:** ImageAnalysisFinal

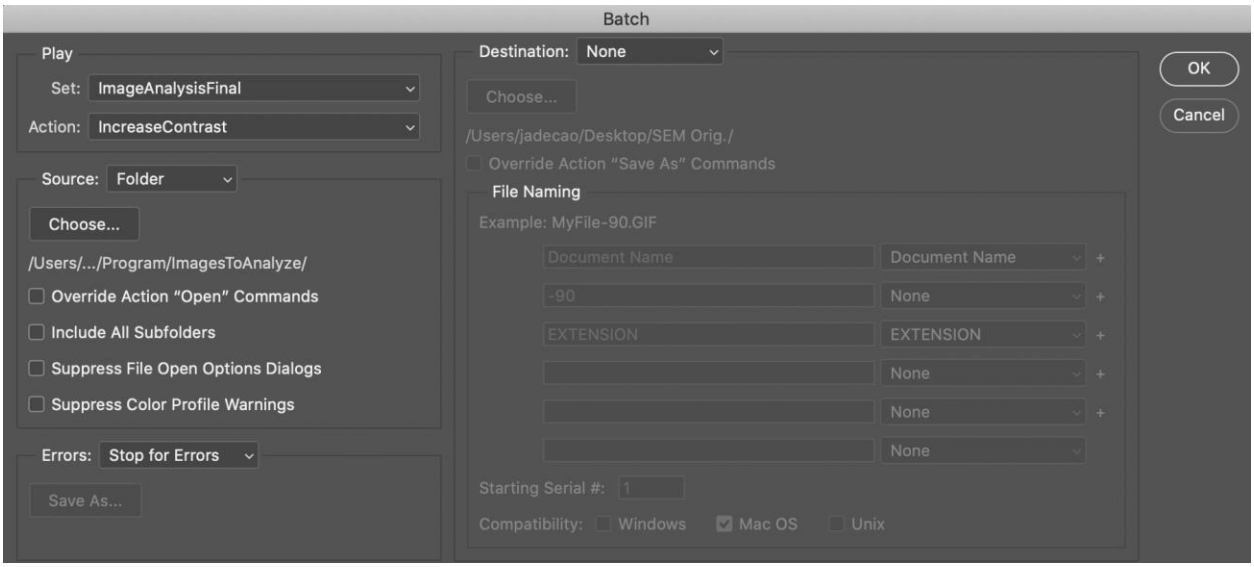
**(ii)** **Action:** IncreaseContrast

Source

**(i)** Select “Choose” and navigate to “ImagesToAnalyze”

Destination

**(i)** **Destination:** None



The files within the *ImageToAnalyze* folder will look like the following:

**Step 6:** In Photoshop, go to File → Automate → Batch. A pop up window will appear. Fill in the window as follows:

Play

**(i)** **Set:** ImageAnalysisFinal

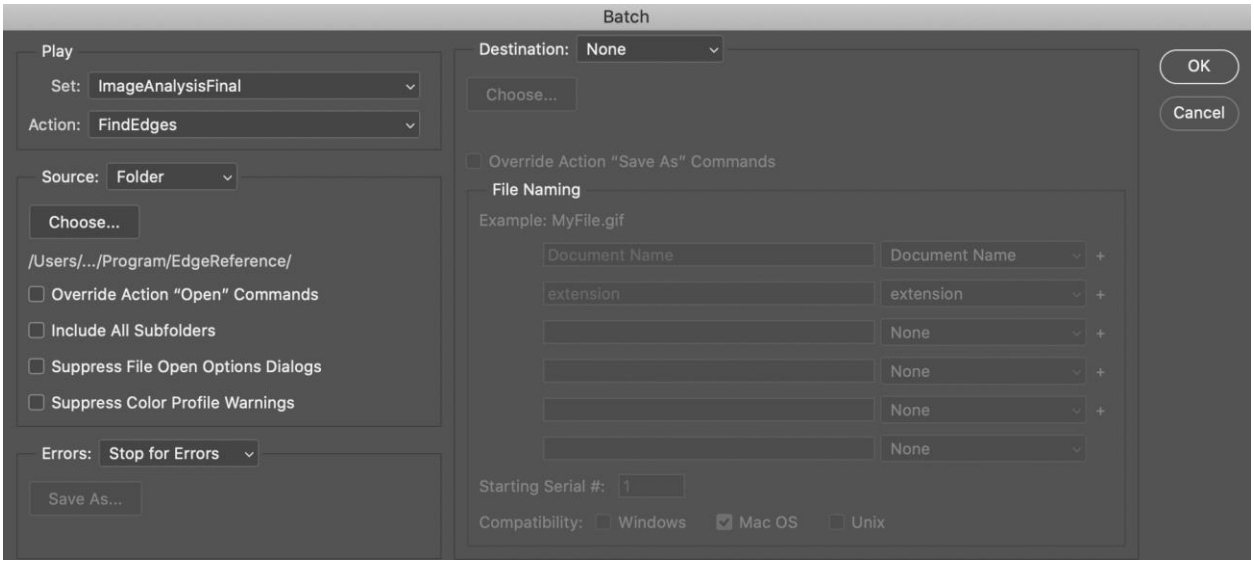
**(ii)** **Action:** FindEdges

Source

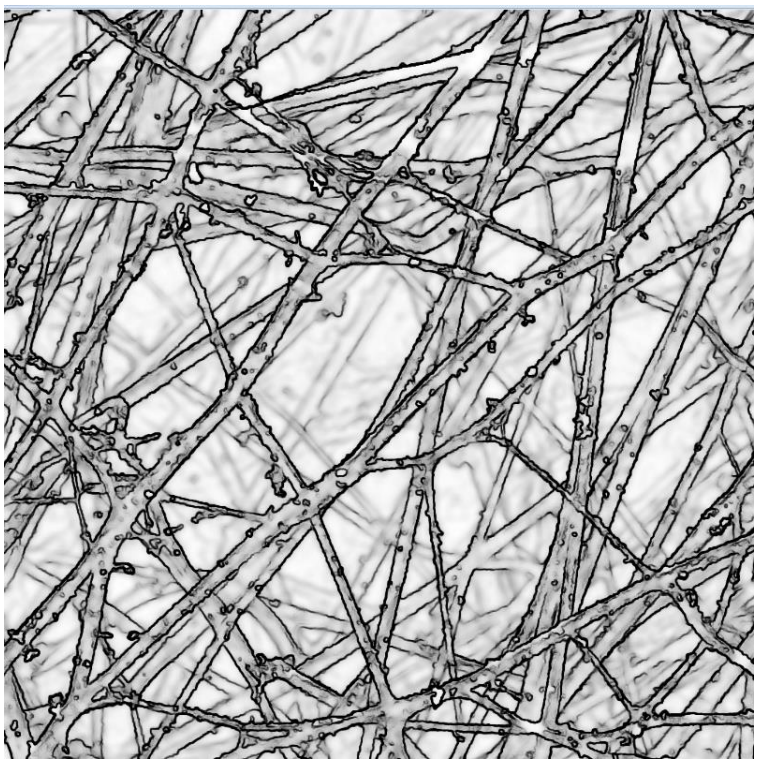
**(i)** Select "Choose" and navigate to "EdgeReference"

Destination

**(i)** **Destination:** None



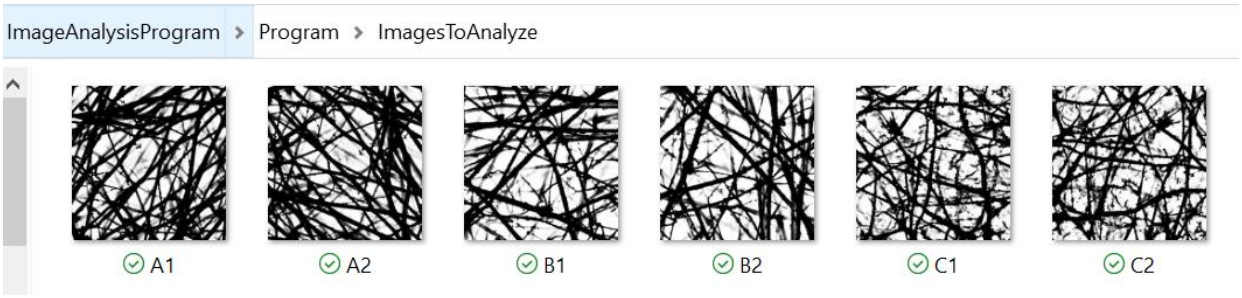

The files within the *EdgeReference* folder will look similar to the following:



## Image Naming Convention

It is recommended to have distinguishably named image files as image analysis will rename the results text files by the first three letters of the image name. For example, if the image name is named “1408_1A12.tif”, the produced file will be named “140-WidthResults.txt”.

Files with the same 3 letters will be merged into one text file (instead of an individual file for each image), making it extremely difficult to differentiate which results belong to which image. A naming convention which differentiates each original image, as well as the image rotated 90 degrees is recommended, as many SEM images typically have very similar naming convention. Ensure that the naming convention within the *ImagesToAnalyze*, and *EdgeReference* folder correspond for each image. An example naming convention is seen below, where each set of images is labelled alphabetically, and the original and rotated version is labelled numerically.

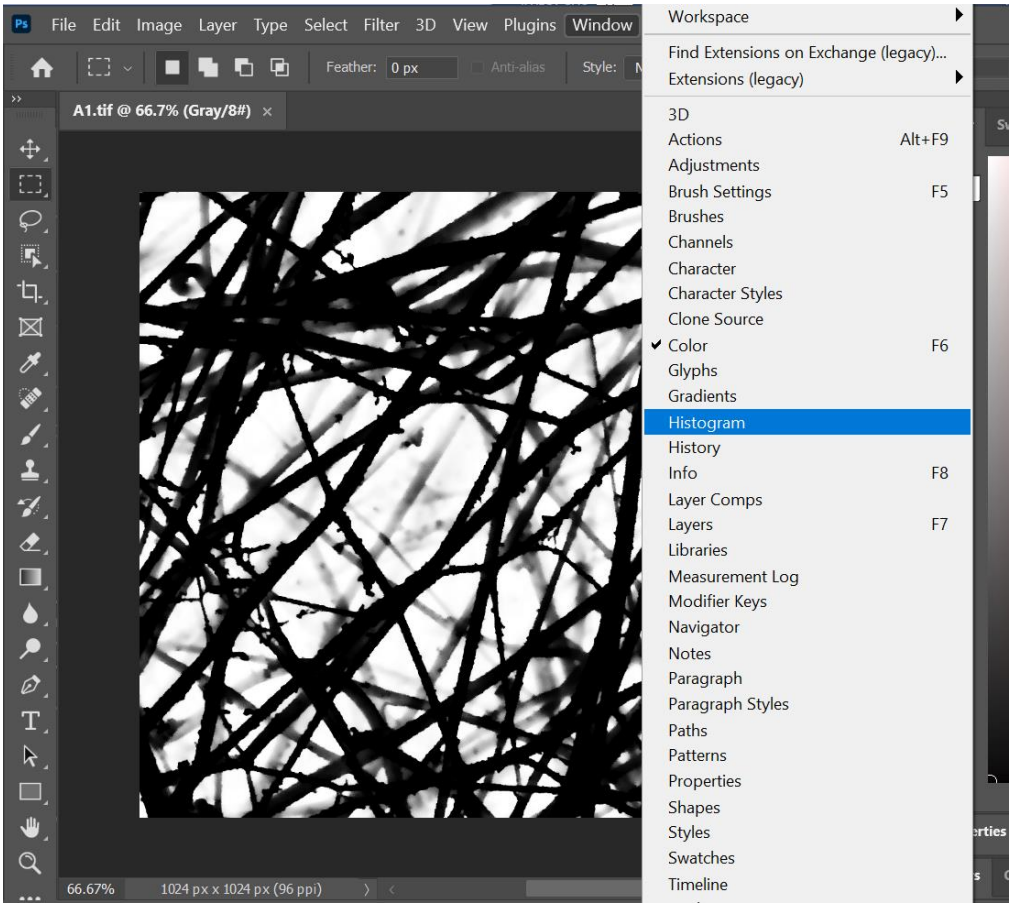



# Image Analysis

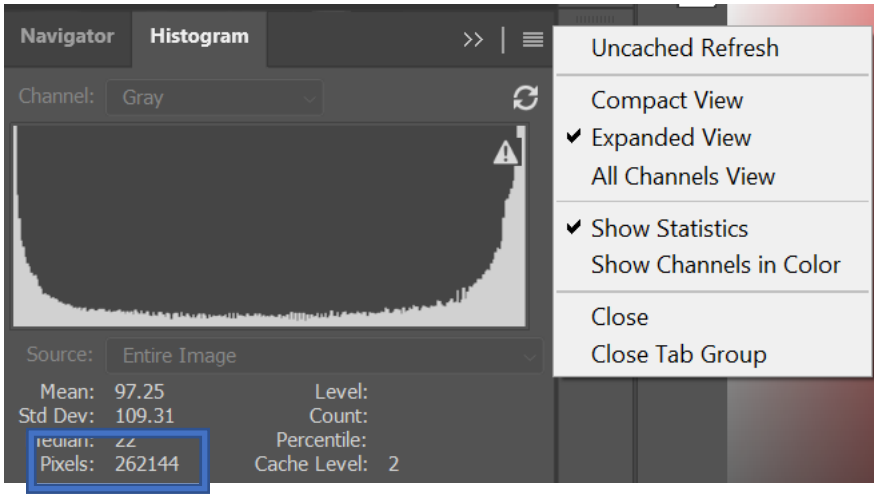
## Porosity

To determine the porosity of the image, users will utilize photoshop. Porosity is typically taken as the percentage of dark pixels compared to light pixels in the image.

**Step 1:** With photoshop, open the Image within the “ImagesToAnalyze” folder. Go to Window → Histogram. A Popup will appear on the right side.

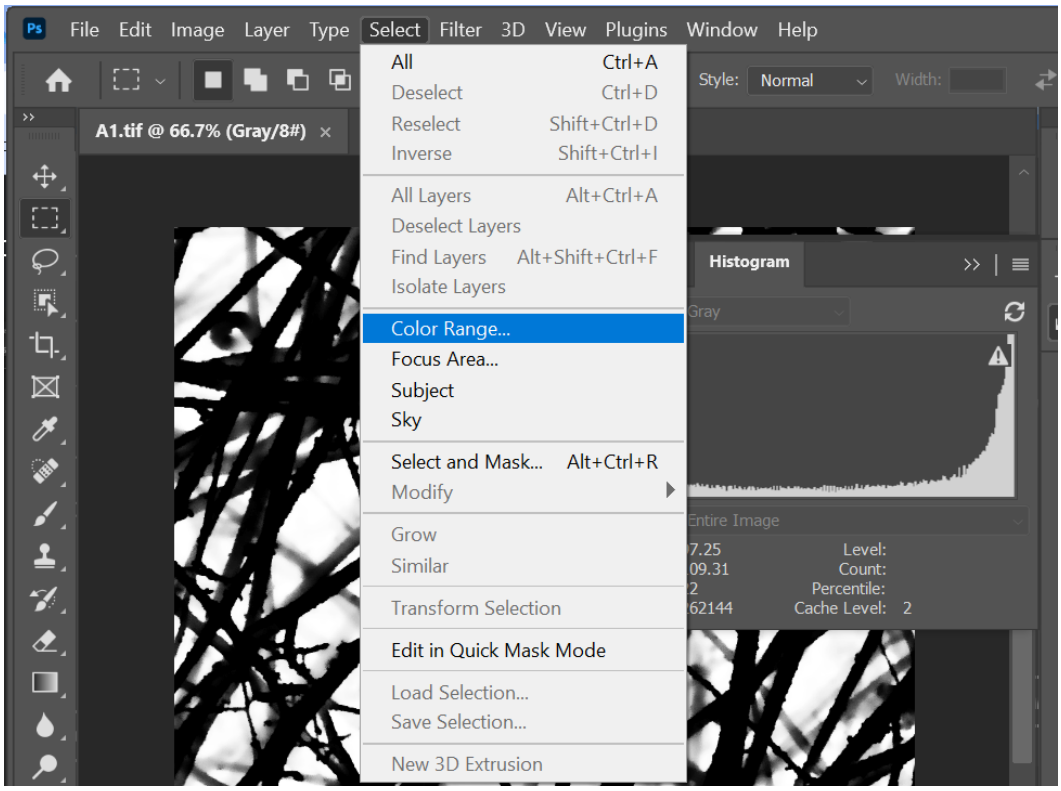


**Step 2:** Select the top right corner (three horizontal lines) and select “Expanded View”, and “Show Statistics”. The total number of pixels can be seen. Keep this number.

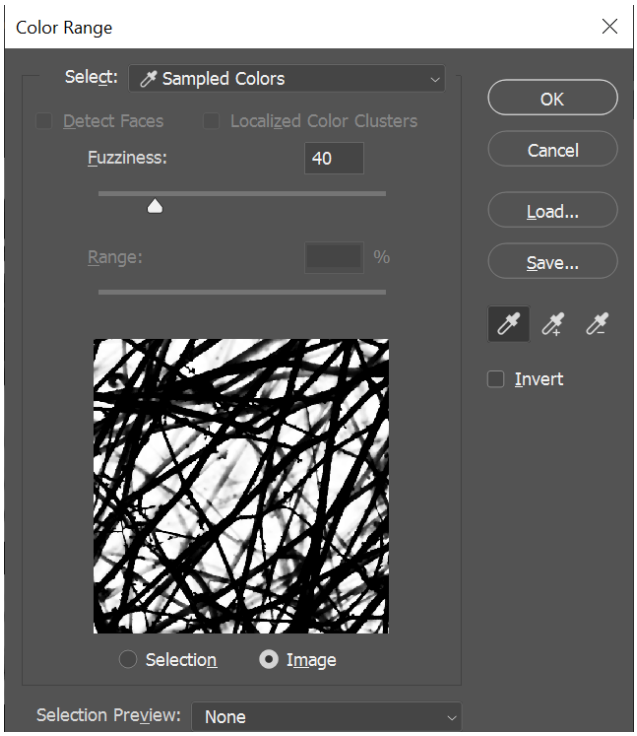



**Step 3:**

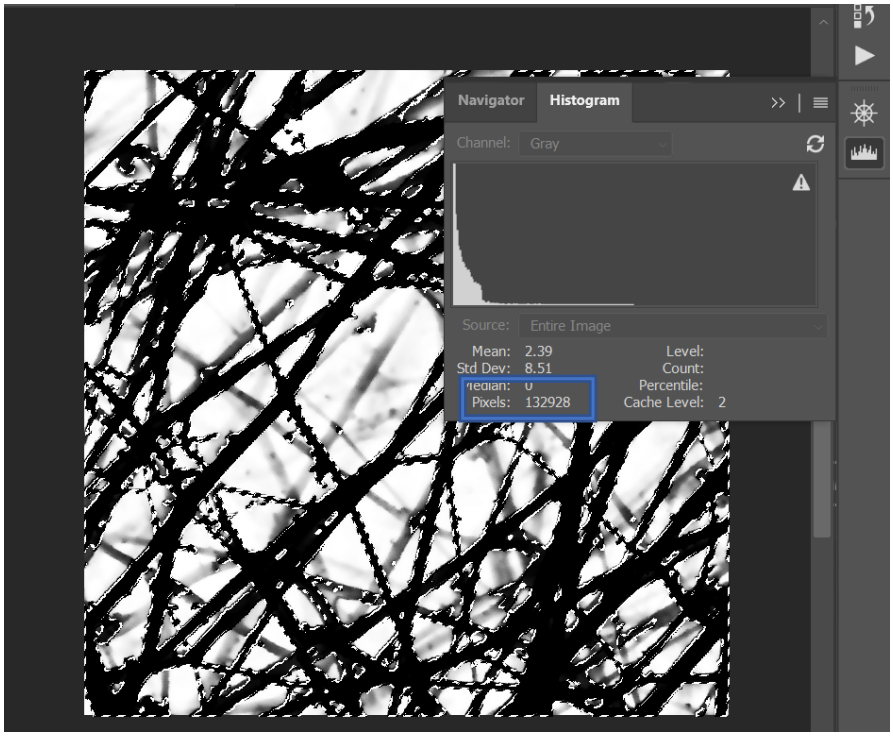
**(i)** Go to Select → Color Range…. A popup window will appear.



**(ii)** At the bottom of the window, select “Image”, with your mouse (which should appear to be a dropper), select any location on the image where the fiber is present. This will determine the number of pixels that appear the same color (selecting all of the pixels that are the fibers).



**(iii)** All of the fiber are should now be selected. With the histogram menu, the value should now represent the pixels that are the fibers. With this value in mind, users can determine the porosity.




**Step 4:** To determine the porosity, divide the number of fiber pixels by the total number of pixels in the image to obtain a fraction value.

$$Porosity = \frac{FiberPixels}{TotalPxels}$$

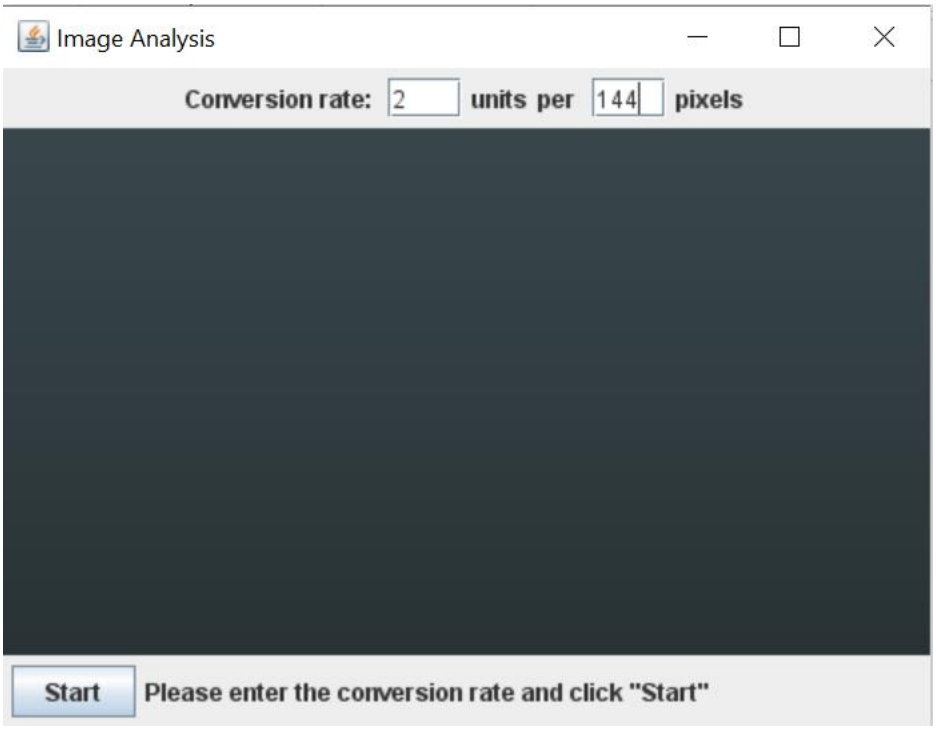
$$Porosity = \frac{132928}{262144}$$

$$Porosity = 0.507$$

## Fiber Diameter and Count

Image analysis was done on a Windows system. There proved to be some difficulty when attempting to run the program on a Mac OS.

**Step 1:** Launch the program: “ImageAnalysisTool.jar” within the “Program” Folder. A popup window will appear as seen below. Change the Conversion rate to 2 units (micrometers) per 144 pixels[5] and click Start.




**Step 2:** The results should appear in the Results Folder.

[5] This is the typical conversion rate but may vary dependent on the image and field of view. Users can easily determine the conversion rate in photoshop. Span the length of the measurement bar with the Rectangular Marquee Tool, the pixels will be the width.

## Results

Within the results folders, the program will produce one file labelled “FiberCountResults.txt”, which will display the number of gibers within each of the images analyzed.

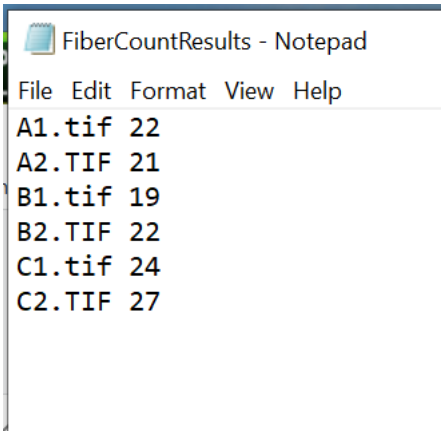



*Figure 4: Fiber Count Results*

Diameter results will be saved as a .txt file with the same name as the images. One will be created for the image with the original orientation (0 degrees), as well as one for the image rotated 90 degrees. The units of the values will be based on the original conversion rate. As seen below, files are named by the first three letters of the image name, in this case, as each image was only labelled with two, a period is in place of the third character.

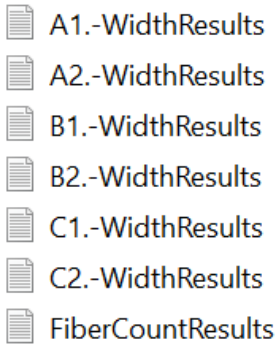



*Figure 5: Program Created Files*

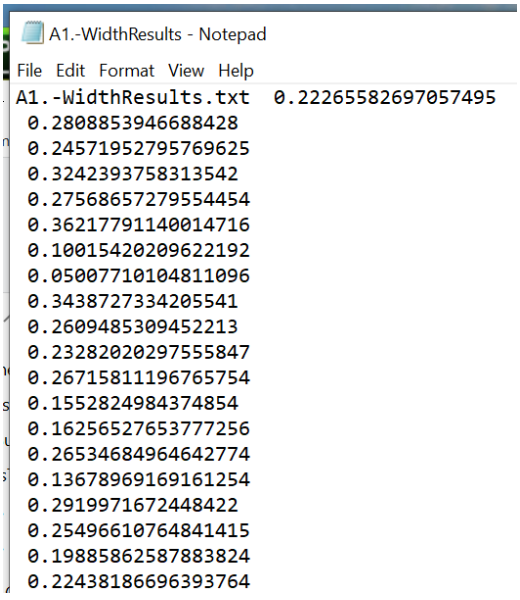



*Figure 6: Width Results File*

To run the program again, users will need an empty “Results” folder. Users can (1) delete all files in the folder, or (2) rename the folder and create a new “Results” folder for the new files. This will allow users to refresh results, or to analyze a new batch of images.

Within the “AnalyzedImages” folder, there will be two subfolders, holding the analyzed images. Users can see the mechanisms in which the diameter and fiber count values were determined.

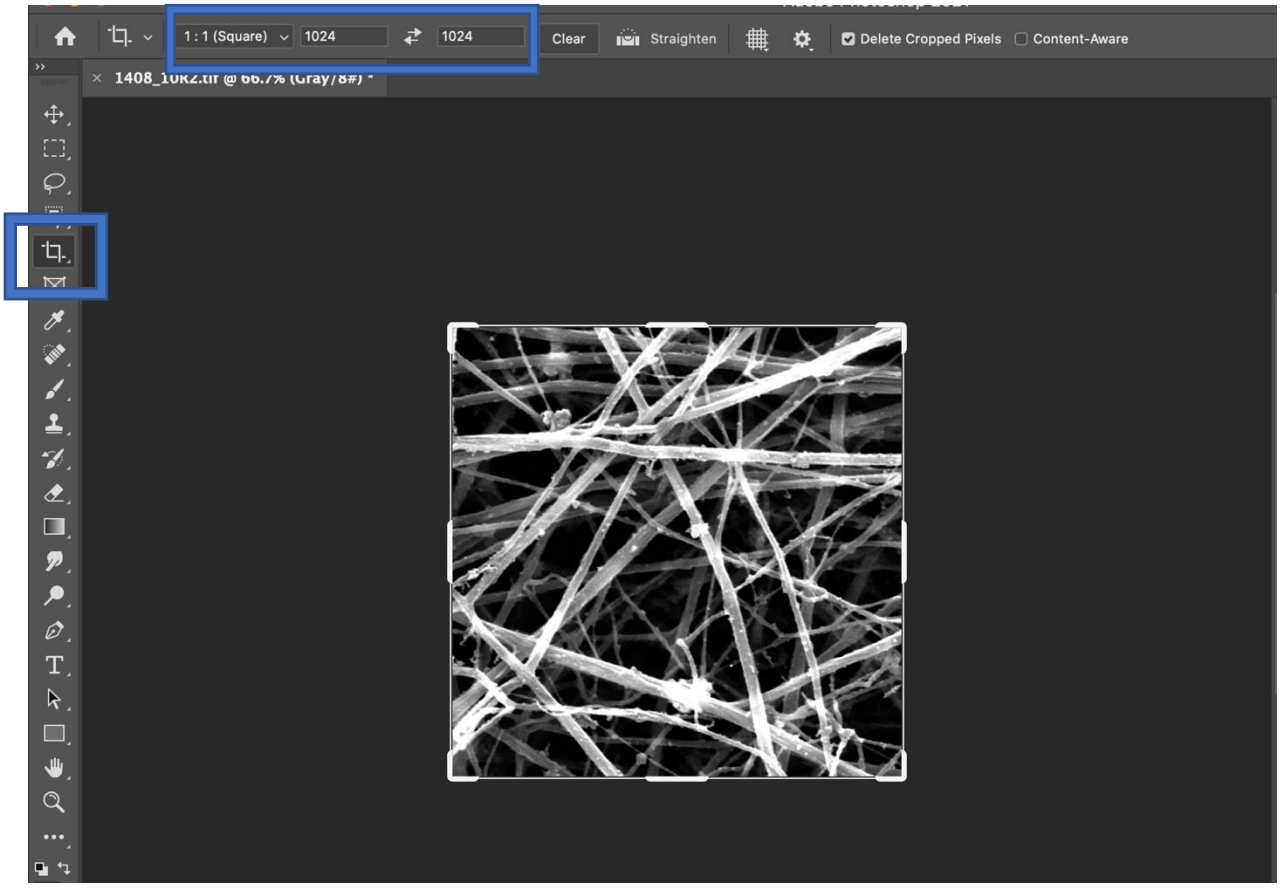
# Manually Preparing Images

Save image into two folders: *ImagetoAnalyze*, and *EdgeReference*.

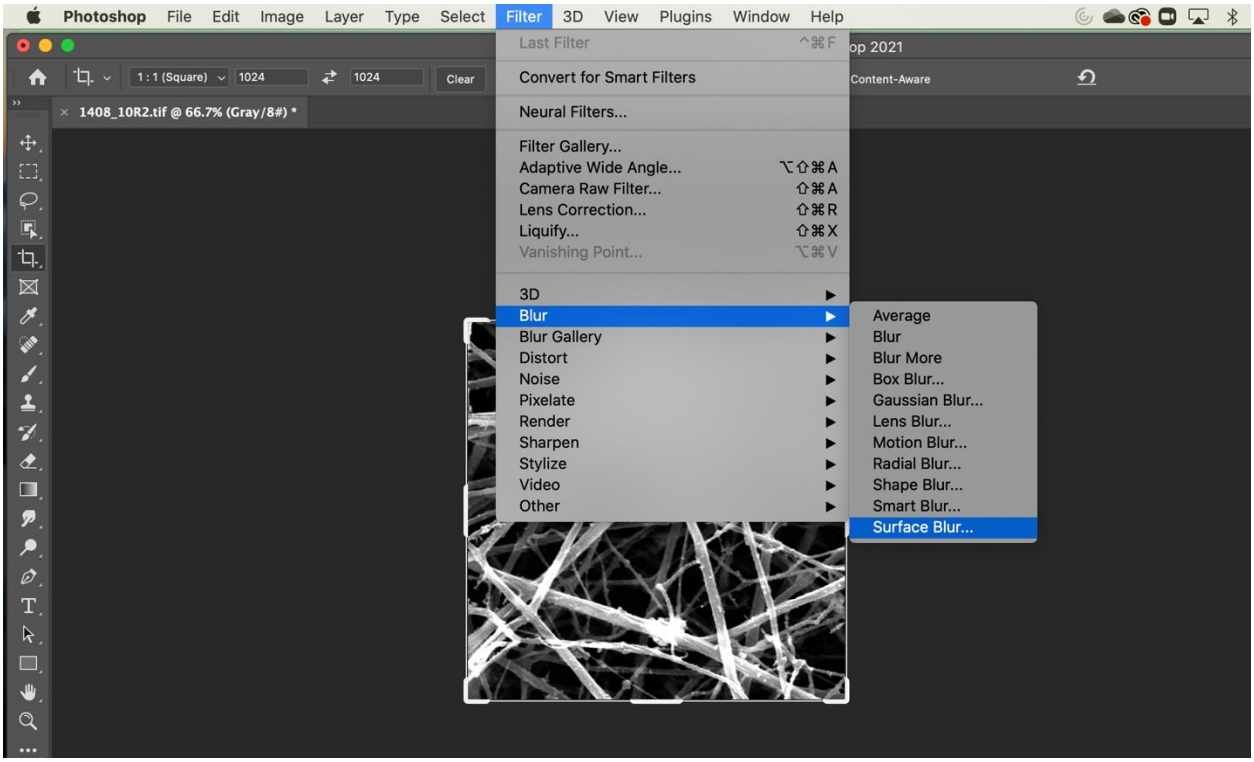
## ImagesToAnalyze

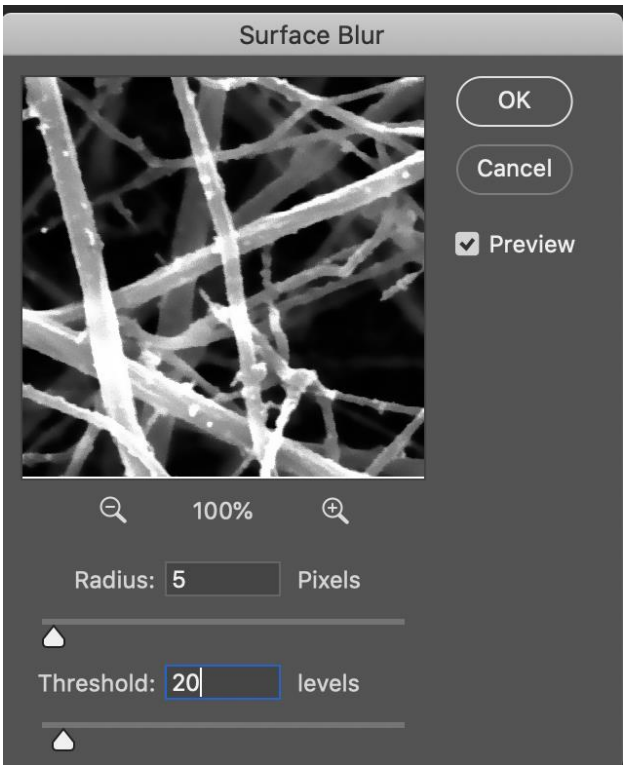
With photoshop. Open the image within the ImageToAnalyze Folder

1. **Crop the image:** Cropping out the bottom label, the final image is 1024 x 1024 pixel
   - The tool is on the left hand side: users can input the desired size of image and drag the original to fit within the box

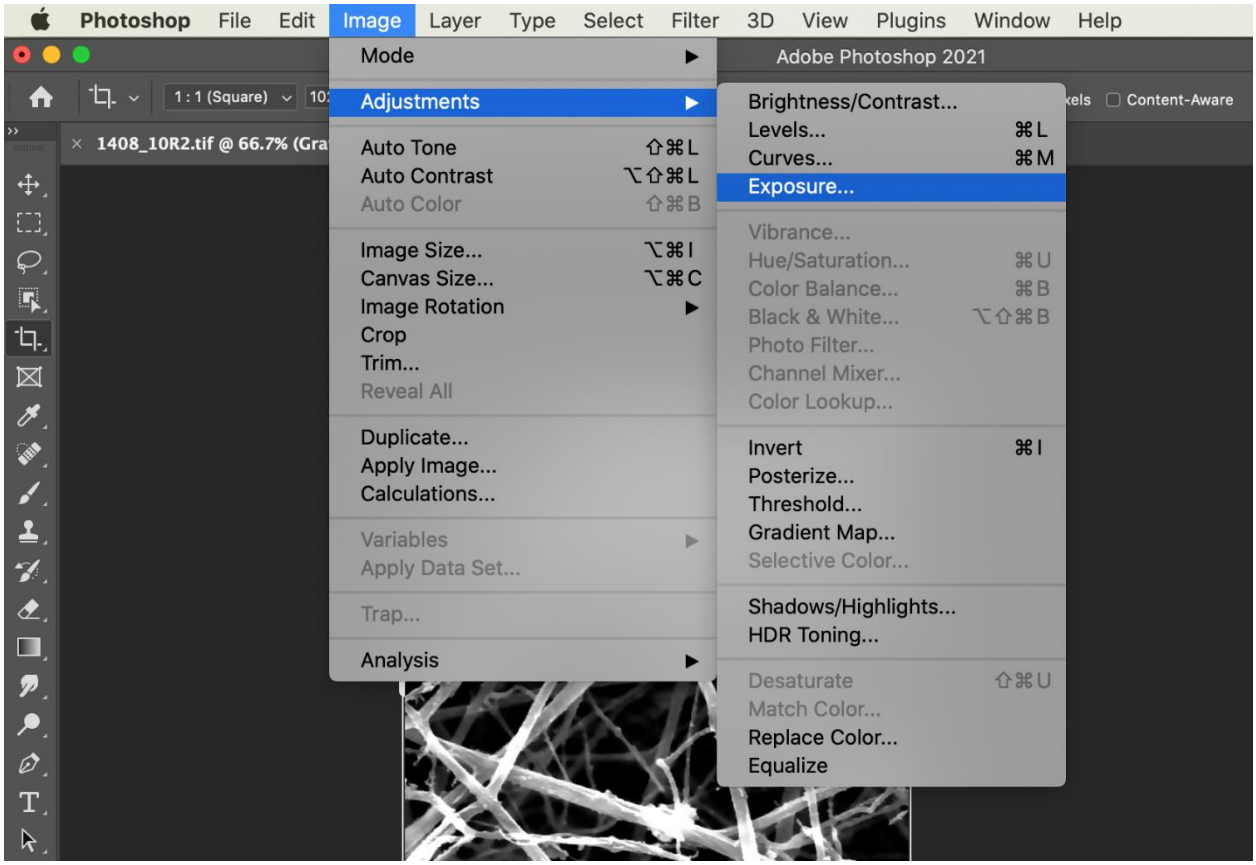


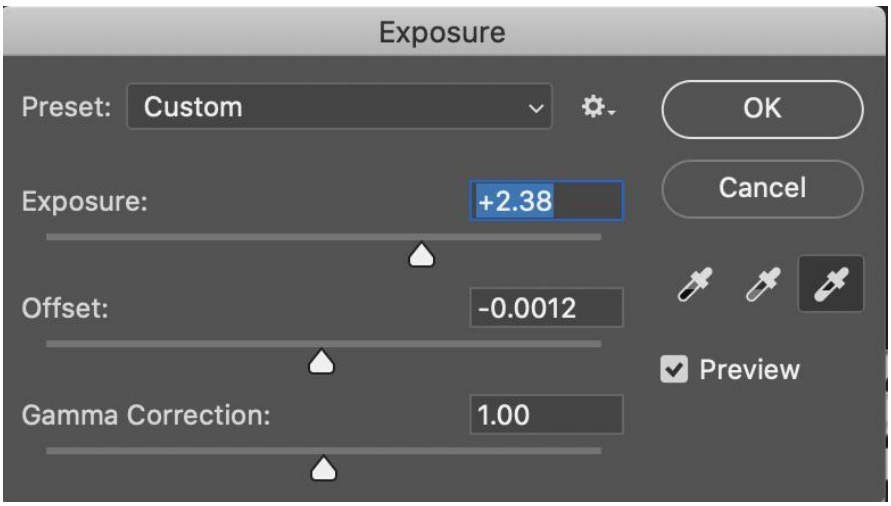
2. **Surface Blur**: radius 5 pixels, threshold 20



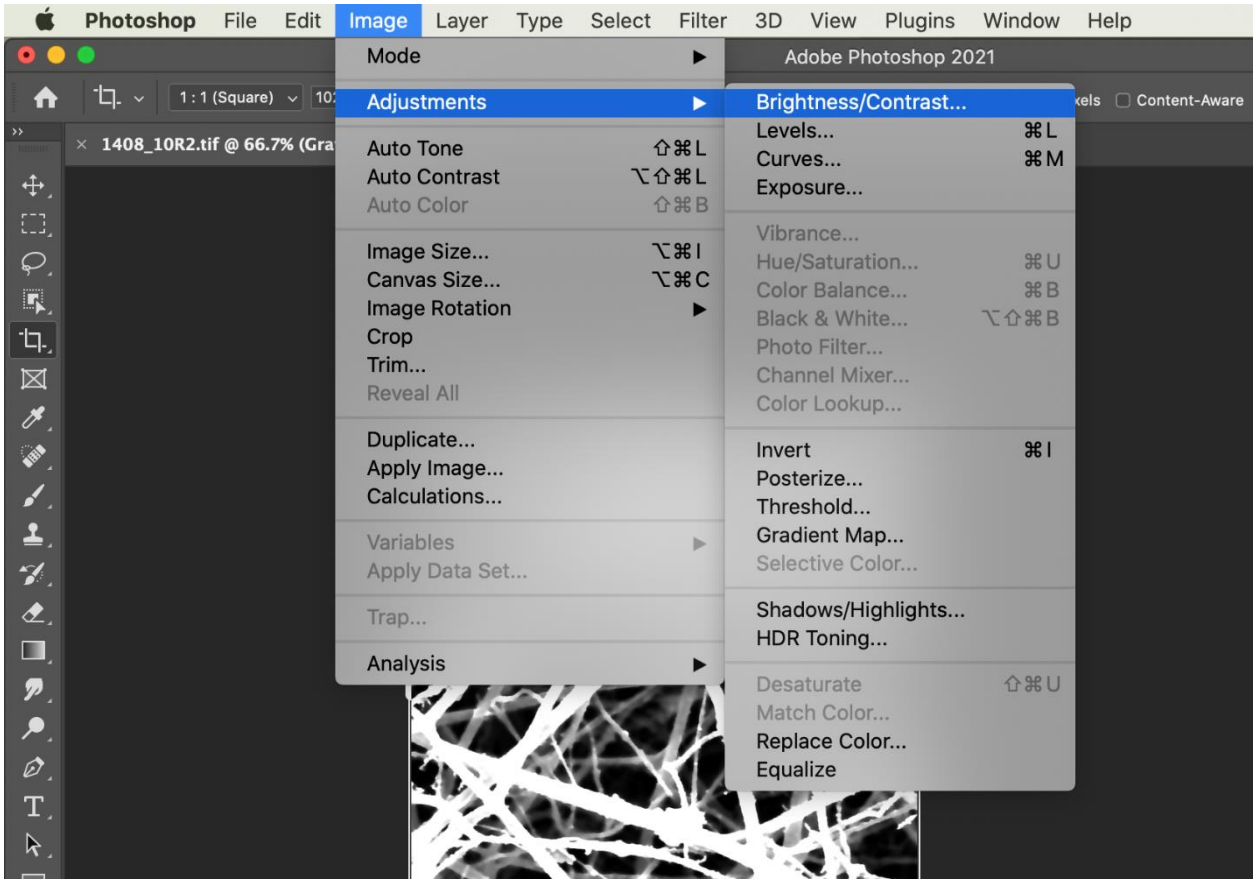



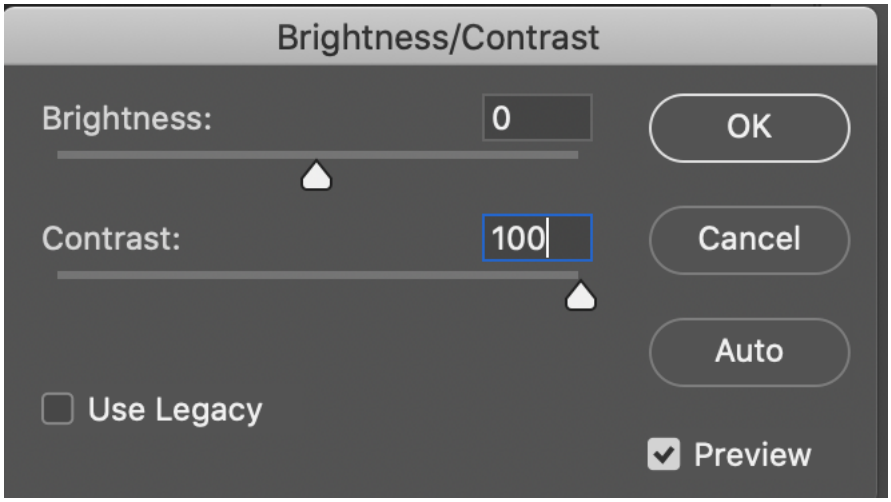
3. **Adjust exposure:** exposure 2.38, offset -0.012 (may use 0)



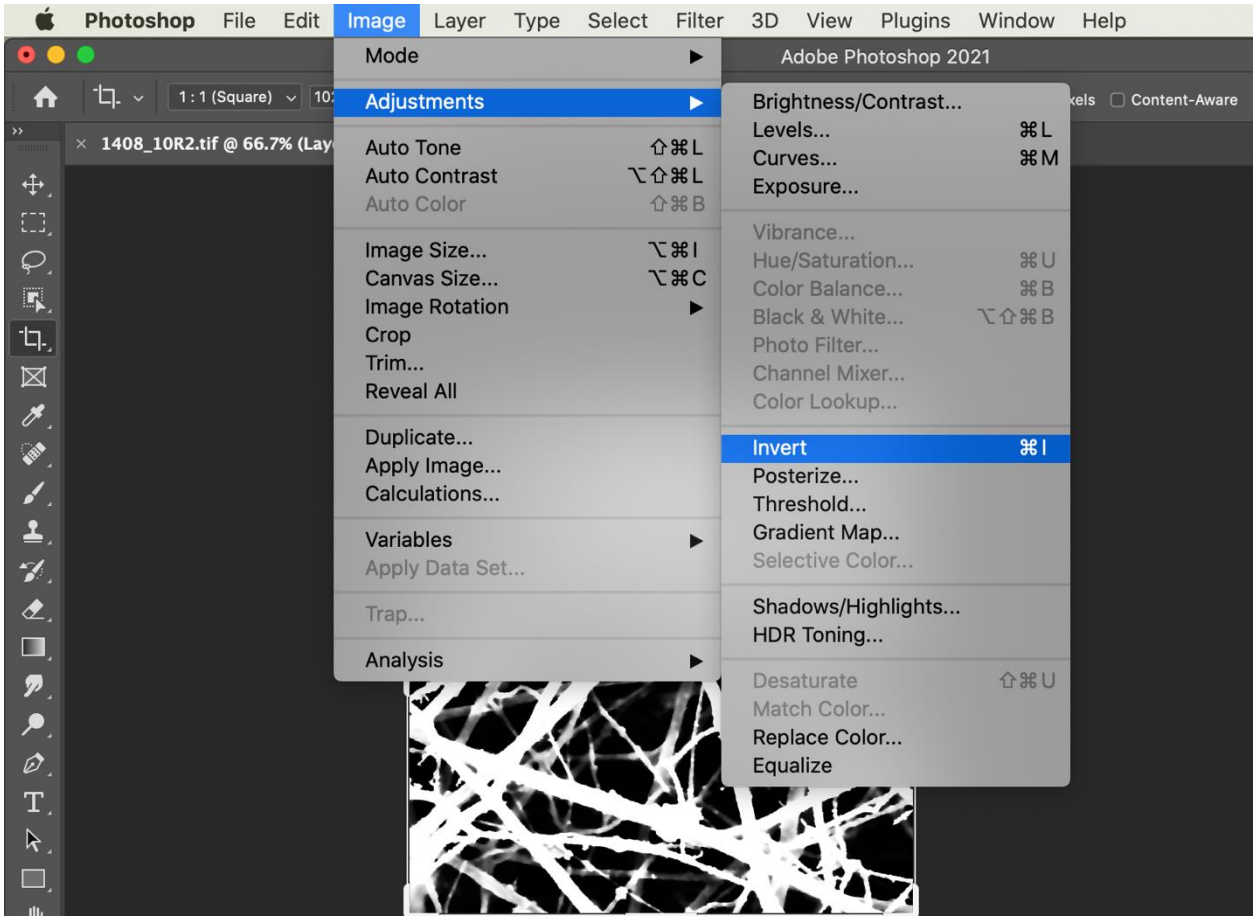
4. **Adjust brightness and contrast**: Brightness to 0, contrast to 100



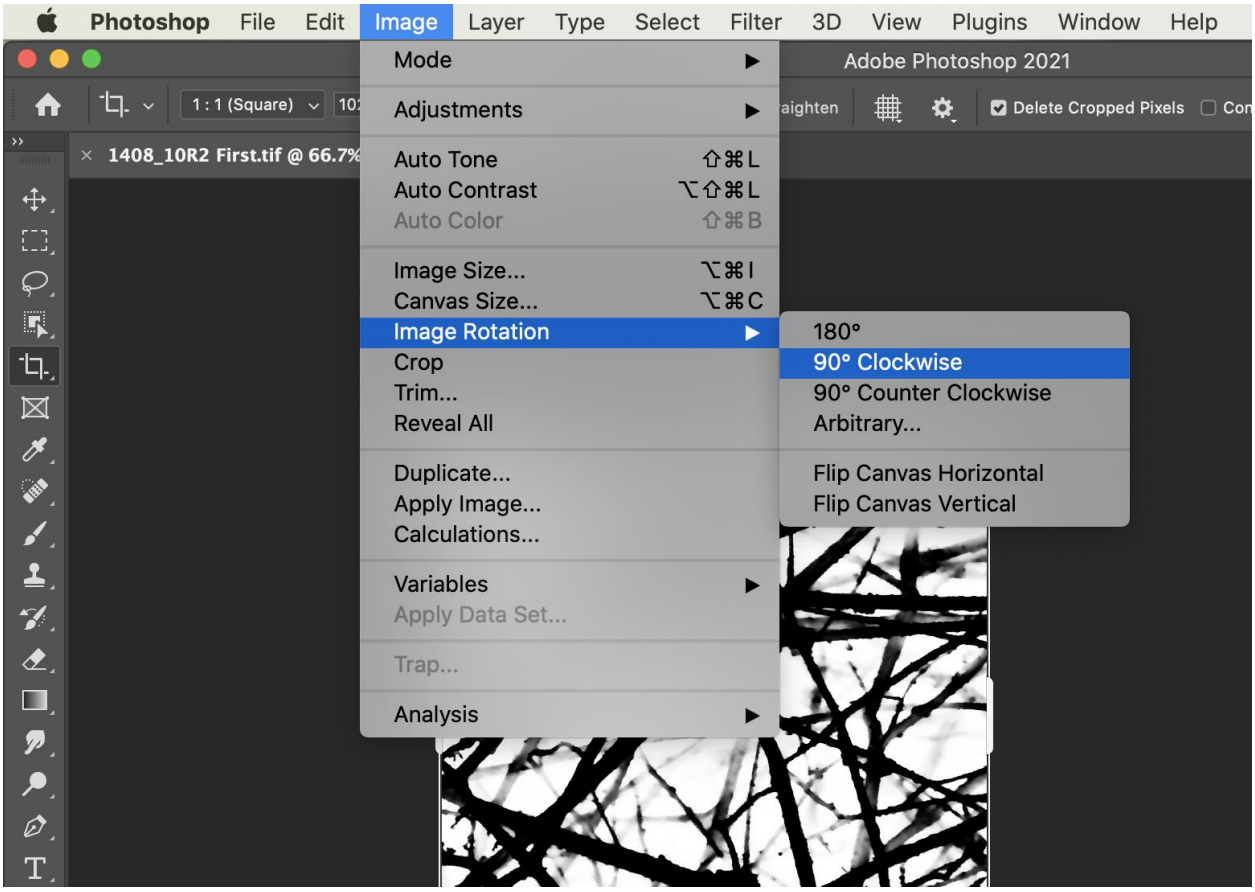




5. Repeat Adjust brightness and contrast step one more time (steps 4)

6. Invert pictures,  Save and close the file




7. (may require) Rotate the image by 90 degree and save as a different file in the same folder (2 files for each image)

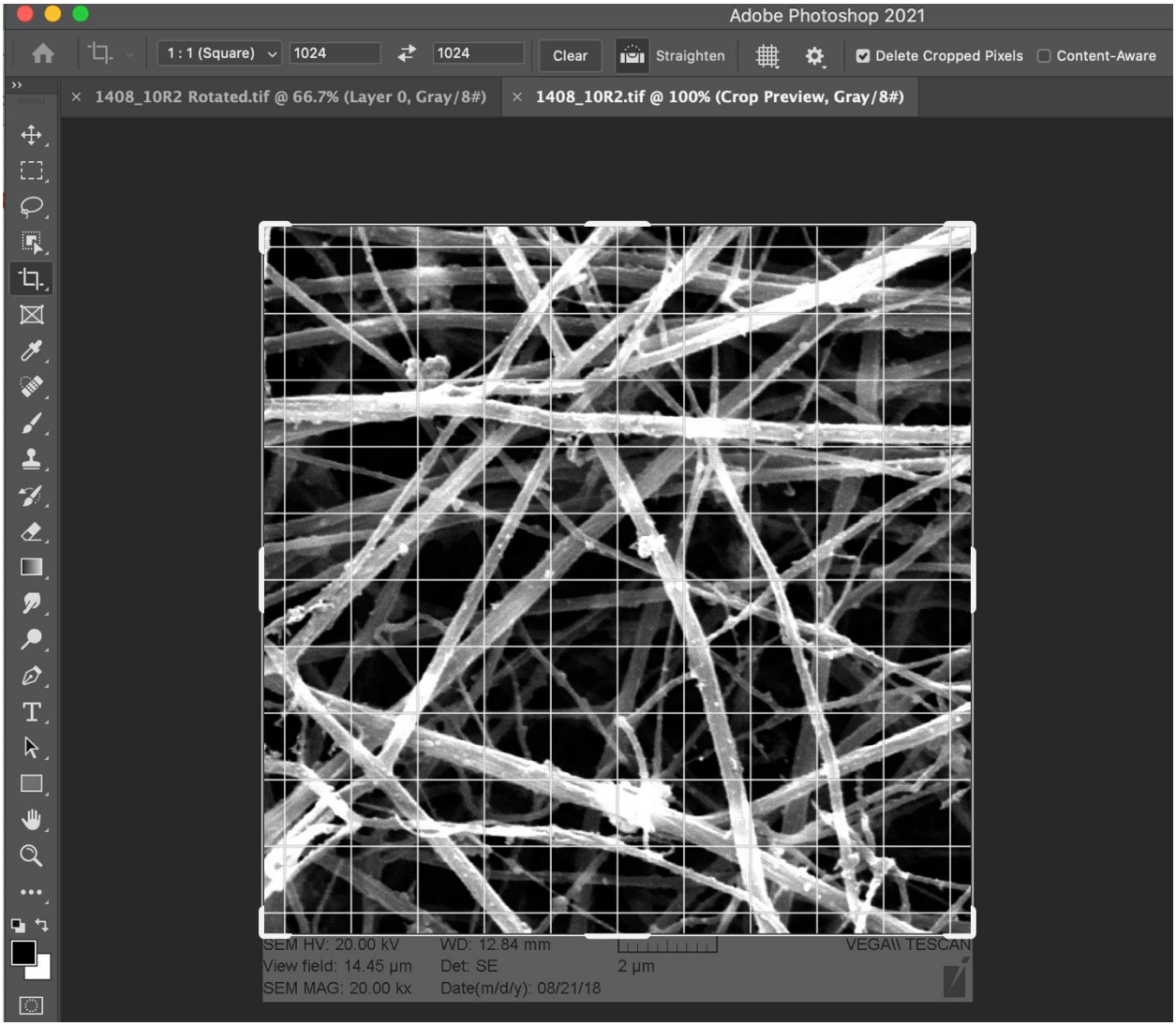

Photoshop
File
Edit
Image
Layer
Type
Select
Filter
3D
View
Plugins
Window
Help
Adobe Photoshop 2021
1 : 1 (Square)
Delete Cropped Pixels
1408_10R2 First.tif @ 66.7%
Mode
Adjustments
Auto Tone ⇧⌘L
Auto Contrast ⌥⇧⌘L
Auto Color ⇧⌘B
Image Size... ⌥⌘I
Canvas Size... ⌥⌘C
Image Rotation
Crop
Trim...
Reveal All
Duplicate...
Apply Image...
Calculations...
Variables
Apply Data Set...
Trap...
Analysis
180°
90° Clockwise
90° Counter Clockwise
Arbitrary...
Flip Canvas Horizontal
Flip Canvas Vertical

Final Image:

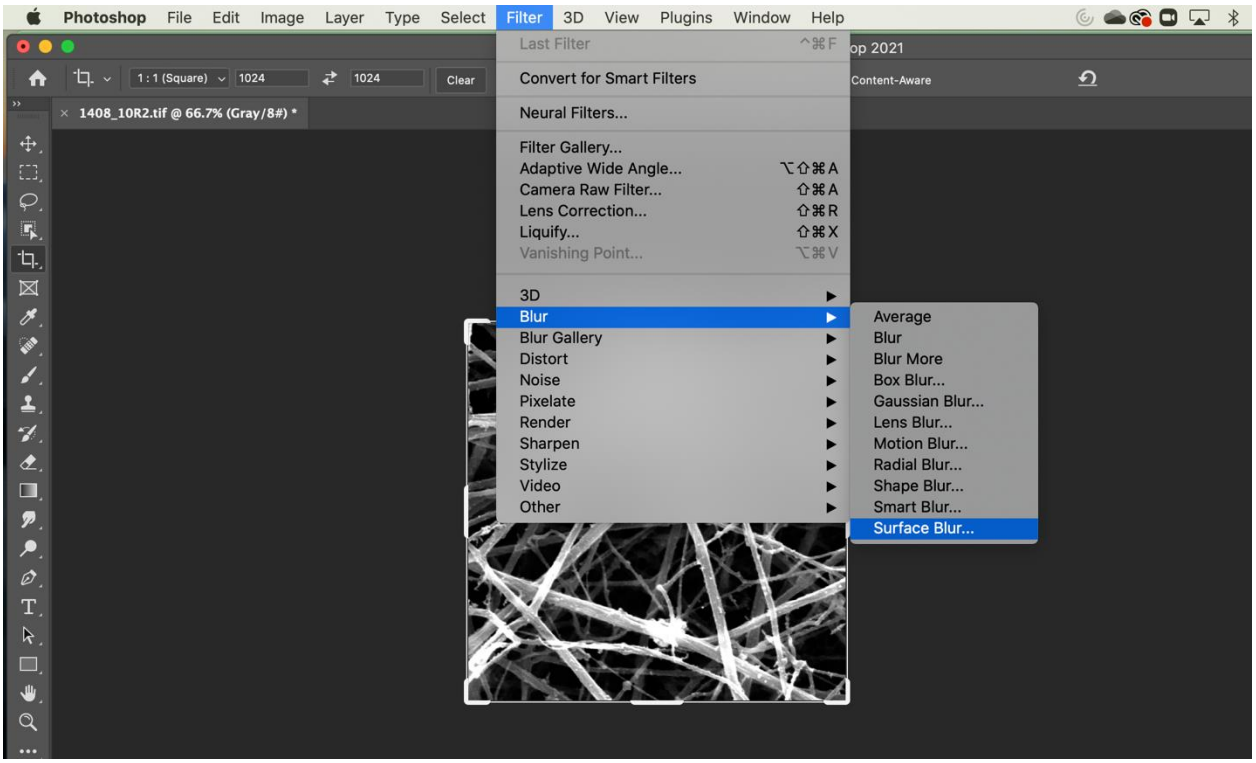
## EdgeReference

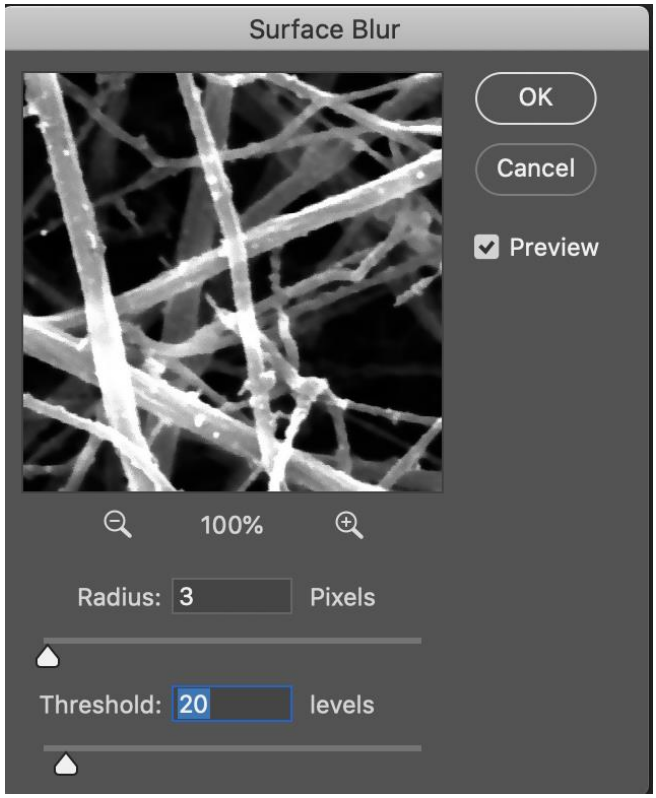
Open the image saved in the folder EdgeReference

1. Crop the bottom label of the image, final image size 1024 pixel x 1024 pixel

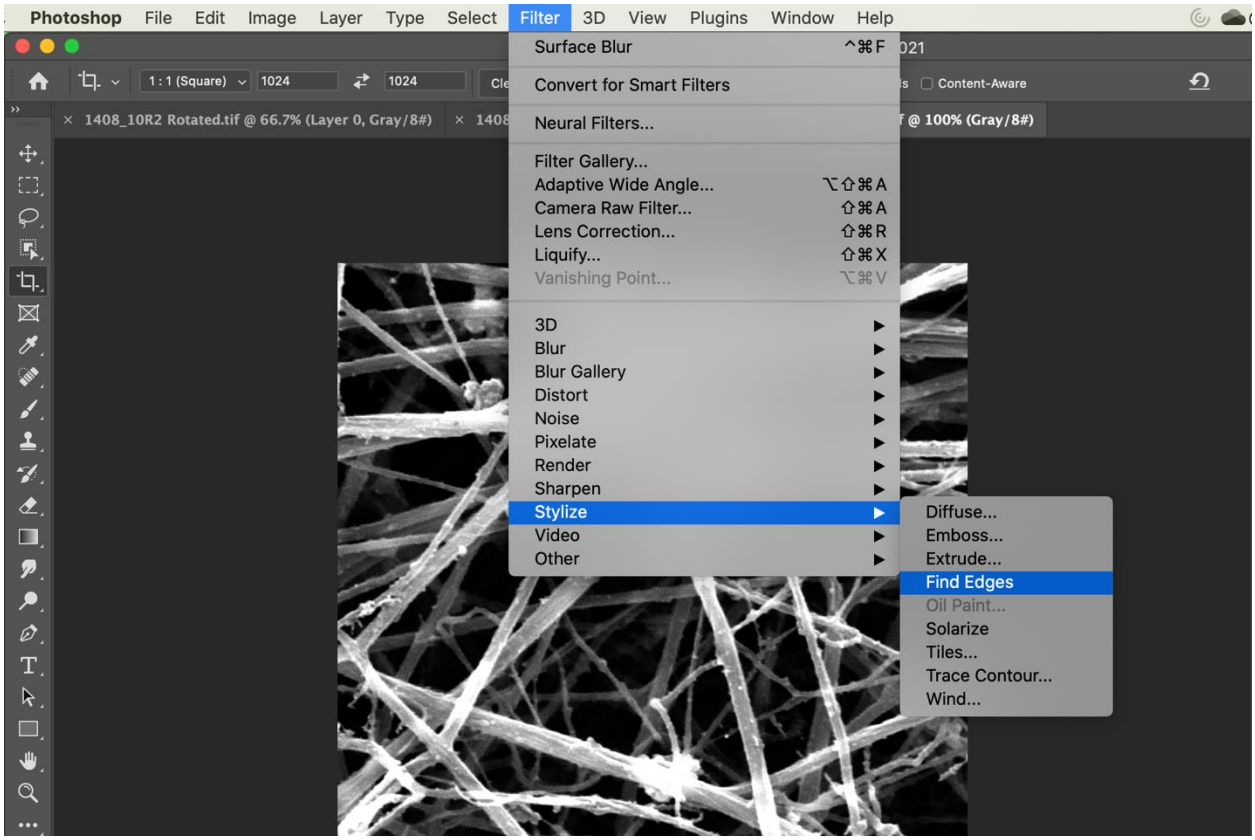



2. **Surface Blur:** radius 3 pixels, threshold 20

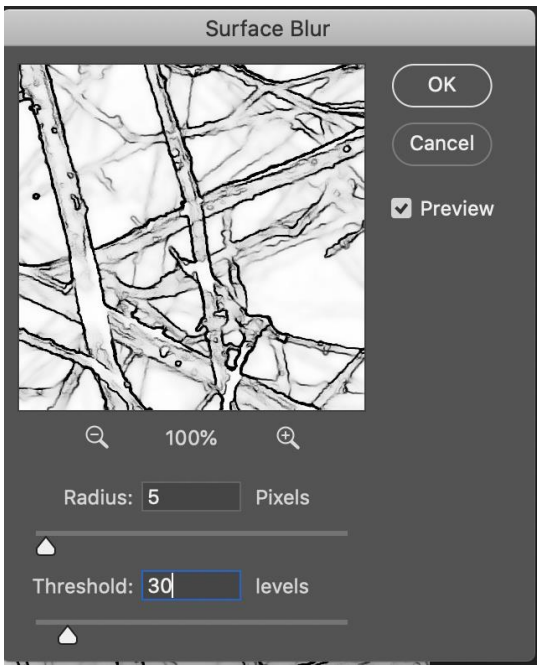



3. **Apply filter**: Find Edge




4. **Repeat Surface Blur:** radius 5 pixels, threshold 30



5. Save and close the file. (may require) Rotate the image by 90 degree and save as a different file in the same folder (2 files for each image)