

The CuticleTrace

Image Analysis Toolkit

User Manual

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1. Introduction

Welcome to the instructional document for the CuticleTrace image analysis toolkit. To get started, download the contents of the CuticleTrace Github repository (<https://github.com/benjlloyd/CuticleTrace>).

After downloading, you should have four files:

- This document - *CuticleTrace_UserManual.pdf*
- *CuticleTrace_AllMacros.ijm*
- *CuticleTrace_DataFiltration.Rmd*
- *CuticleTrace_Example.zip*

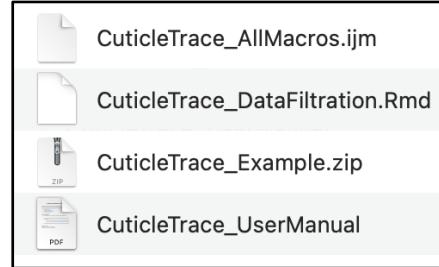


Figure 1. Contents of CuticleTrace GitHub repository.

The CuticleTrace toolkit consists of two files:

CuticleTrace_AllMacros.ijm and *CuticleTrace_DataFiltration.Rmd*. To learn how to use the CuticleTrace toolkit, use the tutorial in section 2 to analyze the images contained in *CuticleTrace_Example.zip*.

1.1. Toolkit contents

The CuticleTrace toolkit consists of four FIJI macros and an R notebook, all of which are described in detail in the main text of the CuticleTrace paper (Lloyd et al., 2023). The contents of the CuticleTrace toolkit are as follows:

1. Four FIJI Macros. All FIJI Macros are included in *CuticleTrace_AllMacros.ijm*, consisting of:
 - a. *CuticleTrace - Batch Generate ROIs*
 - b. *CuticleTrace - Single Image Processor*
 - c. *CuticleTrace - Batch Measure (Different Scales)*
 - d. *CuticleTrace - Batch Overlay*
2. One R Notebook. ROIs generated by the CuticleTrace FIJI macros are filtered with functions in *CuticleTrace_DataFiltration.Rmd*

1.2. Software requirements

CuticleTrace works on all operating systems that support FIJI and R. To install the necessary software and plugins, follow instructions below:

1. Install FIJI. FIJI is identical to ImageJ and ImageJ2, but with many plugins pre-installed. Download the software here: <https://fiji.sc>. This analysis pipeline was developed on FIJI running ImageJ2 version 2.9.0/1.54b.
2. Install the “Shape Smoothing” FIJI plugin from the BioMedGroup (Technical University of Dortmund, Germany) update site. Use the “Manage update sites” menu in the FIJI updater. Follow the instructions here: <https://imagej.net/update-sites/following>.
3. Install R and RStudio. Download both here: <https://posit.co/download/rstudio-desktop/>. This analysis pipeline was developed using RStudio version 2023.03.1+446, running R version 4.2.2.

1.3. FIJI macro installation

To begin analyzing images with CuticleTrace, install the FIJI Macros by following the steps below.

1. Open FIJI.
 - a. Install the shape smoothing plugin if not already installed (see above).
2. Open *CuticleTrace_AllMacros.ijm* in ImageJ (drag and drop).
3. Open your Startup Macros (Process -> Macros -> Startup Macros...).
4. Select the entire contents of *CuticleTrace_AllMacros.ijm*, copy and paste at the bottom of your Startup Macros.
5. Restart FIJI. There are now 4 new macros available (Process -> Macros) (Fig. 2):
 - a. *CuticleTrace - Batch Generate ROIs*
 - This macro is used for batch-processing whole image sets to create ROI sets from unprocessed images (Fig. 6). It may be used to measure the ROI sets it generates if all images are of the same scale.
 - b. *CuticleTrace - Single Image Processor*
 - This macro’s primary purpose is to visualize effects of CuticleTrace input parameters. It is ideal for determining ideal input parameters to use for *Batch Generate ROIs* (Fig. 17). It also may be used to analyze single images.
 - c. *CuticleTrace - Batch Measure (Different Scales)*
 - This macro allows for the measurement of large batches of images with different scales. It requires a CSV file with each image’s file name and corresponding scale.
 - d. *CuticleTrace - Batch Overlay*

- This macro allows for the visualization of ROI sets generated by *Batch Generate ROIs*. It creates batches of images with overlaid ROI sets (e.g., Fig. 6e).

Once FIJI has been restarted and the CuticleTrace macros appear in the Plugins menu (Fig. 2), CuticleTrace now ready for use. To learn how to use the macros, proceed to the tutorial below.

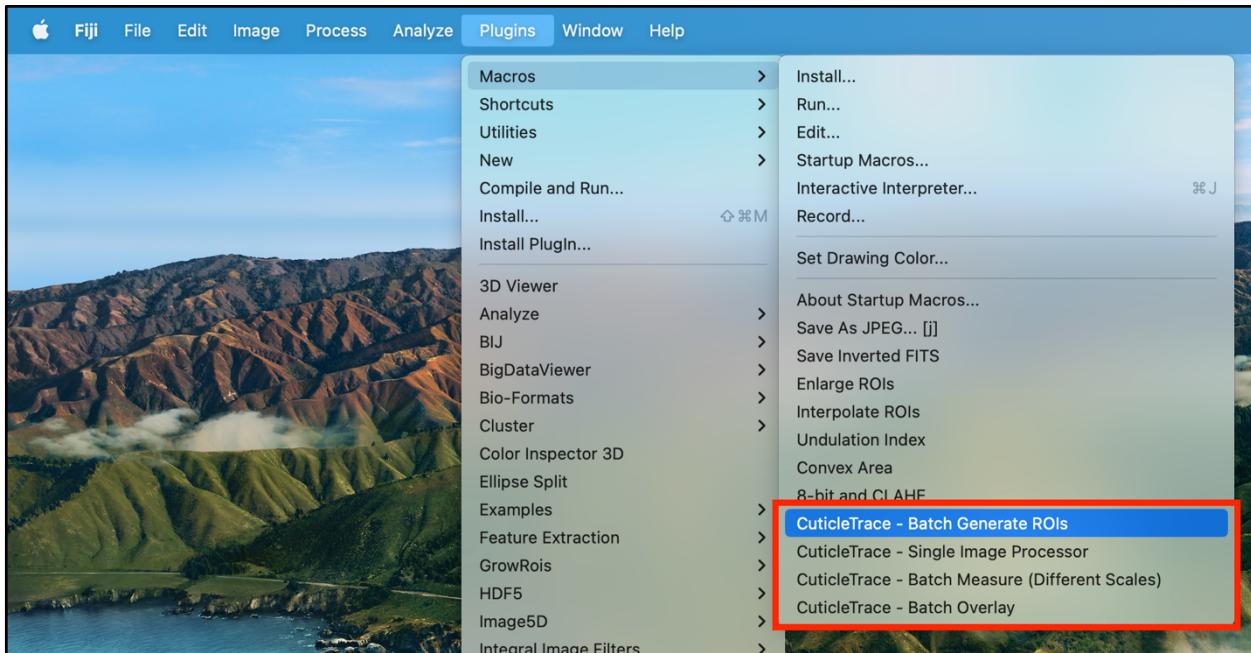


Figure 2. CuticleTrace FIJI macros after installation.

2. Tutorial - Analysis of example dataset

In this tutorial, we will generate final ROI sets and epidermal pavement cell measurements of the 10 images included in the CuticleTrace_Example.zip file.

2.1. Example dataset contents

The example dataset provided in the CuticleTrace GitHub repository is stored as a .zip file. Once expanded, it contains two folders:

1. *Example_Dataset* - This folder contains 10 cropped images from the Cuticle Database, and a CSV file with each image's scale. We will process them in the coming steps.
2. *Example_Dataset_COMPLETE_ForComparison* - This is a folder of all the results that can be generated with the images in the *Example_Dataset* folder. It is

there for comparison, to make sure that you know how things are supposed to look.



Figure 3. Contents of *CuticleTrace_Example*, showing a folder of 10 images and a CSV file with each image's scale.

2.2. FIJI macro input parameters

The first step in the generation of an epidermal pavement cell shape dataset with CuticleTrace is the determination of input parameters for batch image processing. **This is a non-trivial process and is necessary for the generation of accurate data.** For this tutorial, we have predetermined ideal input parameters for the example dataset (Table 1, Fig. 3). To determine the ideal input parameters for other datasets using *Single Image Processor*, see section 3.1 (Determination of Input Parameters).

Table 1. Ideal batch processing inputs for the example image set, as determined by Lloyd et al. (2023).

Input Parameter	Value
Cell Walls on Background	Dark on Light
Gaussian Blur	2 pixels
Thresholding Method	Sauvola
Initial Thresholding Radius	50 pixels
ROI Size Filter	500-50,000 pixels ²
Smoothing Value	5 %FDs retained
Scale	Various (see 2.3.2)

CUTICLETRACE INPUT PARAMETERS:

Cell Walls on Background:	Dark on Light
Gaussian Blur Sigma (pixels):	2
Thresholding Method:	Sauvola
Initial Threshold Radius (pixels):	50
ROI Size Filter (pixels ²):	500-50000
Smoothing Value (%FDs Retained):	5
Interpolate ROIs?	Yes

Figure 4. *CuticleTrace* Input Parameters with correct values for the example dataset, as they appear in the Batch Generate ROIs FIJI macro.

2.3. Batch processing in FIJI

The second step in the generation of an epidermal pavement cell shape dataset with CuticleTrace is the batch processing of images with the *Batch Generate ROIs* and *Batch Measure (Different Scales)* macros in FIJI. This step generates unfiltered ROI sets and results files that include accurate cell tracings as well as some undesired ROIs. Ideal batch processing inputs for the example dataset are below in Table 1 and Fig. 12.

2.3.1. *Batch Generate ROIs*

With your input parameters selected and your output directories created, you are ready to batch process the example image set with the *Batch Generate ROIs* macro.

1. In FIJI, with no images open, open the *Batch Generate ROIs* macro (Plugins > Macros > CuticleTrace - Batch Generate ROIs)
2. First, a “NOTICE” window will pop up, reiterating important points regarding the use of the macro. To continue, click OK.
3. The next window contains the input fields necessary to run the macro:
 - a. Select the folder containing the image set for the input directory (Fig. 5).
 - b. Double-check that the parameters in Table 1 match the default inputs in *Batch Generate ROIs* (Fig. 5).
 - c. As is evident in *Example_Dataset_Scales.csv*, not all these images have the same scale. **Therefore, do not select “generate results files”.**
 - d. Click OK.

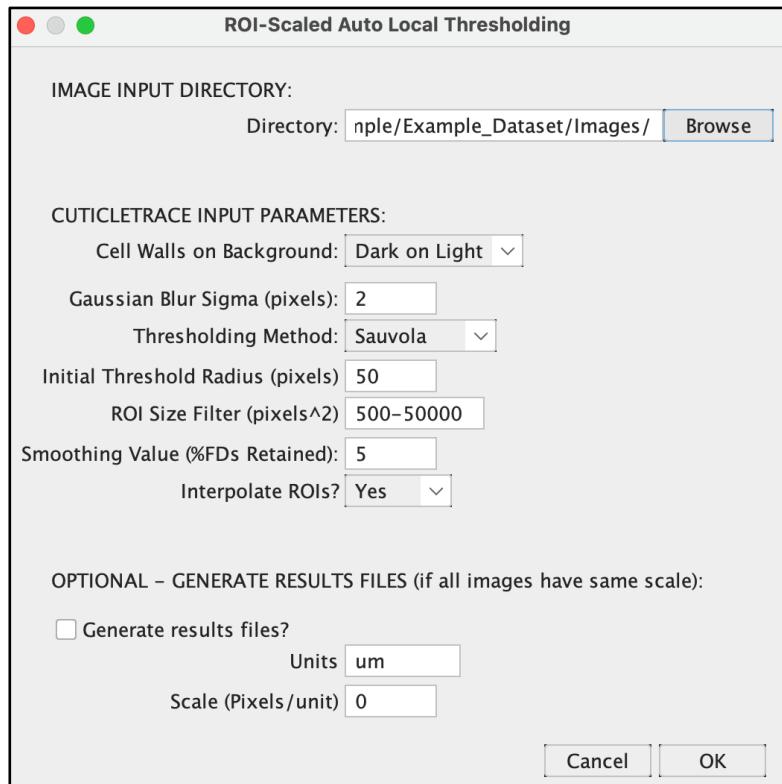


Figure 5. The input window of Batch Generate ROIs with the correct inputs to batch process the example dataset.

Batch Generate ROIs will run for a few minutes—you should see images popping up sequentially. Once it is finished, you will have new folders adjacent to your Image Folder, each containing the macro's outputs:

- *Thresholded_Images* should contain thresholded and smoothed binary images of each of the 10 input images (Fig. 6c).
- *Skeletonized_Images* should contain skeletonized versions of the thresholded images (Fig. 6d).
- *ROI_Sets* should include 10 zipped ROI Sets, 1 for each image (Fig. 6e).
- *Image_Metadata* should include 10 small CSV files with the thresholding radius and interpolation interval of each image (Fig. 6b), as well as 1 larger CSV file containing all the thresholding radii and interpolation intervals for the dataset. **We recommend adding these data into *Example_Dataset_Scales.csv*, to keep scales, thresholding radii, and interpolation intervals together in one dataset.**

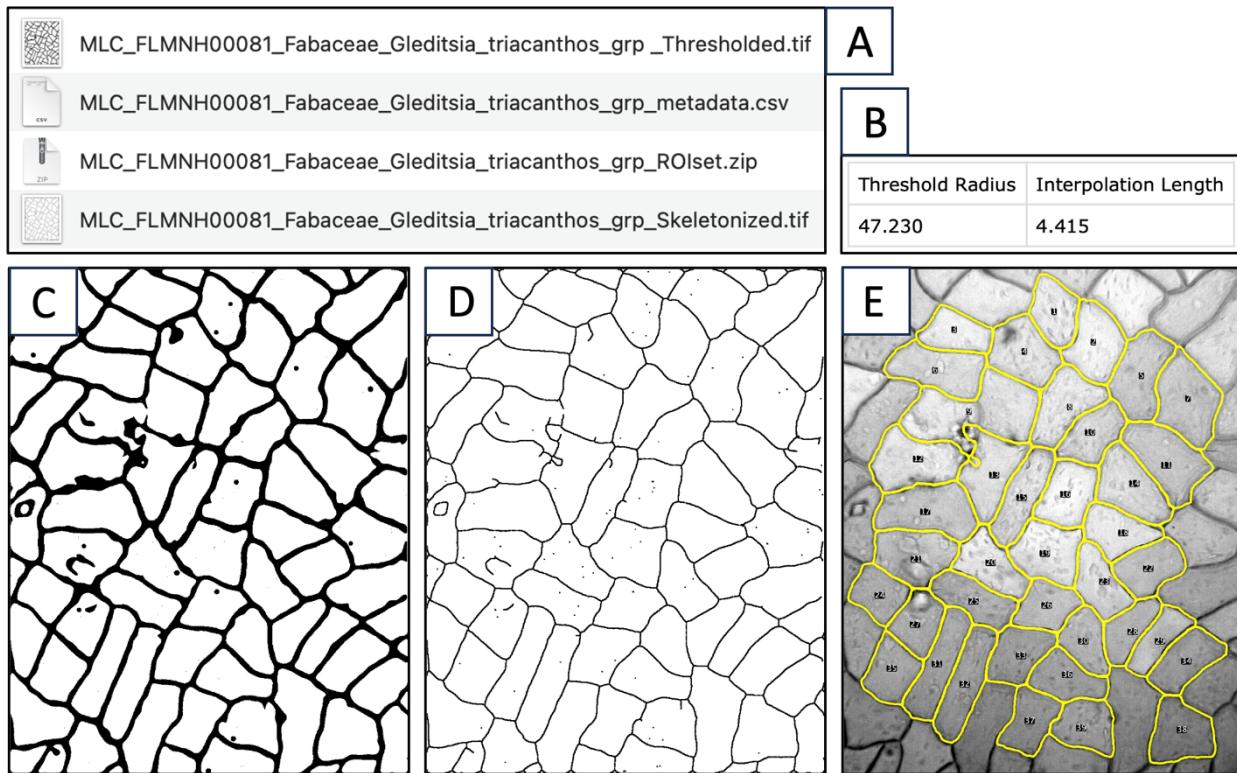


Figure 6. The outputs of Batch Generate ROIs from one image (FLMNH00081, *Gleditsia triacanthos*). A) The four files output by the macro, including B) the image metadata, C) the thresholded image, D) the skeletonized image, and E) the unfiltered ROI set (shown overlaying the original image).

2.3.2. Batch Measure (Different Scales)

Once you have verified that *CuticleTrace - Batch Generate ROIs* worked correctly, it is time to measure your ROI sets. Since not all these images are the same scale, we use *CuticleTrace - Batch Measure (Different Scales)*. This macro requires a CSV file with a column for the image names, and a column with the scale of each image. For the example dataset, this is *Example_Dataset_Scales.csv*.

1. In FIJI, with no images open, open the *Batch Measure (Different Scales)* macro (Plugins > Macros > CuticleTrace - Batch Measure (Different Scales))
2. Select *Example_Dataset_Scales.csv* as the CSV file, and double check that the column names match the default text (they should). Then select the folders containing your unprocessed images and your ROI sets folder.
3. Click OK.

Batch Measure (Different Scales) will run for a few minutes but should be much faster than *Batch Generate ROIs*. You should see images popping up in quick succession. Once it is finished, you will have a new folder, *Results_Files*, containing 10 new CSV files (Fig. 7). When you open these files, you should see measurements for all shape parameters discussed in Lloyd et al. (2023).

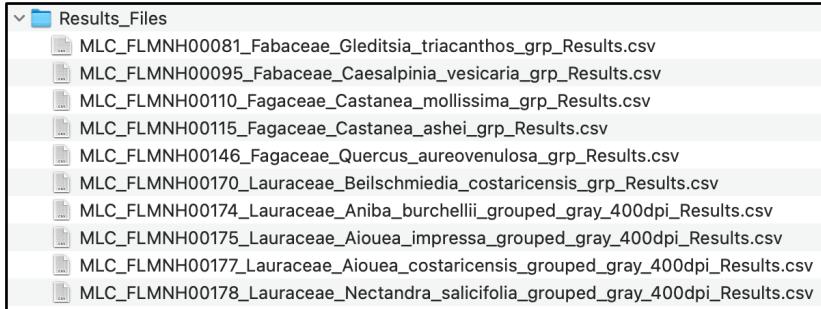


Figure 7. Results files as they appear after running Batch Measure (Different Scales).

2.4. Data filtration in R

Now that you have generated your unfiltered datasets, it is time to remove inaccurate and unwanted ROIs by filtering ROI sets with median statistics.

CuticleTrace_DataFiltration.Rmd creates new ROI sets and results files that have been filtered to ± 1 MAD and ± 2 MAD of the median of area, perimeter, circularity, Feret's diameter, aspect ratio, roundness, and solidity. For more detail on ROI filtering specifics, see the main text of Lloyd et al. (2023).

2.4.1. *CuticleTrace_DataFiltration.Rmd*

To begin the filtering process, copy *CuticleTrace_DataFiltration.Rmd* into the *Example_Dataset* folder. **To work correctly, *CuticleTrace_DataFiltration.Rmd* must be in the same directory as your *Results_Files* and *ROI_Sets* folders.** Follow these steps to filter your data and ROI sets:

1. Open *CuticleTrace_DataFiltration.Rmd* in RStudio. You will see sections of text detailing the filtering procedure broken up by code chunks. Code chunks may be collapsed for increased readability.
2. Read through the filtering procedure.
3. Make sure that the file paths that lead to your *Results_Files* folder (line 361) and *ROI_Sets* folder (line 367) are correct (Fig. 8). If your folders are named “Results_Files” and “ROI_Sets” and are in the same folder as *CuticleTrace_DataFiltration.Rmd* (as recommended), the default text will work.
4. The code can be run in two ways:
 - a. As you read through the filtering procedure, you may run each code chunk as you get to it by clicking the green triangle on the upper right corner of each code chunk (Fig. 8). This is a more transparent way to run the code and is recommended for a first-time user.
 - b. If you want to move quickly, click Knit (Knit to HTML) on the toolbar. This will run the entire code and create a nicely formatted HTML version of the notebook to be saved alongside the .Rmd file.

```

356 1. Make sure this .Rmd file is within the directory that contains your
Results folder and your ROI sets folder. .Rmd files automatically set the
working directory to the directory that contains the document. **If using
the example dataset, place this .Rmd file within the "Example_Dataset"
folder.**
357
358 2. Paste the directory path that contains your results files.
359
360 ```{r}
361 results_path <- "Results_Files/"
362 ```
363
364 3. Paste the directory path that contains your ROI sets.
365
366 ```{r}
367 ROIsets_path <- "ROI_Sets/"
368 ```

```

Figure 8. Line 361 and line 367 of CuticleTrace_DataFiltration.Rmd must lead to the Results and ROI Sets directories.

Now, you have generated your final, filtered datasets. Navigate to your *Results_Files* and *ROI_Sets* folders to see the results (Fig. 9).

- In your *Results* folder, you should see a new *Results_Reformatted* folder, which will contain a reformatted version of the unfiltered datasets. It will also contain 2 more folders:
 - *1MAD_Trimmed_Results* contains results files trimmed to within 1 MAD of the median of area, perimeter, circularity, Feret's diameter, aspect ratio, roundness, and solidity.
 - *2MAD_Trimmed_Results* contains results files trimmed to within 2 MAD of the median of area, perimeter, circularity, Feret's diameter, aspect ratio, roundness, and solidity.
- In your *ROI Sets* folder, you should see an unzipped version of all ROI sets, as well as a zipped and unzipped version of new ROI sets that have been filtered to ± 1 MAD and ± 2 MAD of the median of area, perimeter, circularity, Feret's diameter, aspect ratio, roundness, and solidity. These ROI sets correspond to each newly generated results file.

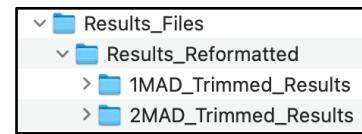


Figure 9. New *Results_Reformatted* folder within *Results_Files*, with directories containing ± 1 MAD and ± 2 MAD filtered datasets.

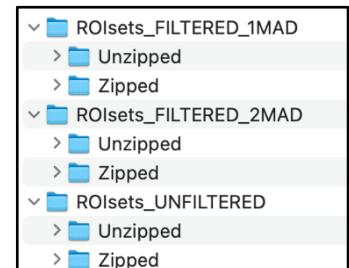


Figure 10. Recommended file organization for filtered ROI sets.

2.4.2. Organization of filtered ROI sets

We recommend reorganizing the ROI set outputs of *CuticleTrace_DataFiltration.Rmd* into 3 new folders within the *ROI Sets* folder, each with a *Zipped* and *Unzipped* folder within (Fig. 10). Refer to the folder *Example_Dataset_COMPLETE_ForComparison* for an example.

2.5. Visualize results in FIJI

To visually inspect the results of CuticleTrace ROI generation and filtering, it is important to create new image sets showing filtered and unfiltered ROI sets for each image. To do this, you may utilize the *CuticleTrace – Batch Overlay* macro in FIJI. Resulting images should look generally like the image in Fig. 6e.

2.5.1. Batch Overlay

Open FIJI and select the *Batch Overlay* macro (Plugins > Macros > CuticleTrace - Batch Overlay). Select the original, unprocessed image folder (*Images*) as the image input directory, and the Unfiltered ROI set folder as the ROI set directory. Since we are starting with the Unfiltered dataset, name the output folder “*Images_with_Overlays_Unfiltered*” or similar (Fig. 11).

Batch Overlay contains a lot of options for changing line thickness, fill color, and more, but we recommend leaving the inputs at their default values for the example dataset. Once you have selected the image directory, ROI set directory, and output folder name, click OK.

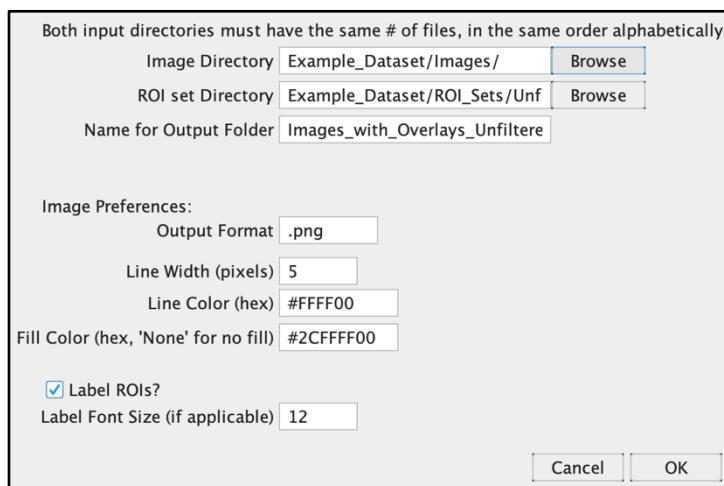


Figure 11. Correct inputs for processing Example Dataset images with the Batch Overlay FIJI macro.

Batch Overlay will run for a few minutes—you should see images flashing across the screen. Once it is complete, run it again with the ± 1 MAD and ± 2 MAD ROI set input and output directories. Once this is complete, you may navigate to these folders and view your final CuticleTrace-generated ROI sets overlaid on their source images. Good work! Since you now have 3 folders of Images with Overlays, we recommend reorganizing them into their own directory, containing 3 folders for images overlaid with

unfiltered, ± 1 MAD, and ± 2 MAD ROI sets (Fig. 12). You may now compare these images to those in the *Example_Dataset_COMPLETE_ForComparison* folder to verify that you generated the same results.

Congratulations! You have now used the full capability of the CuticleTrace toolkit.

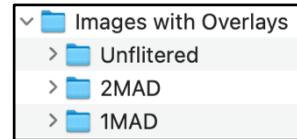


Figure 12. Recommended folders for Batch Overlay outputs.

3. Using CuticleTrace with new datasets

Once you have successfully generated and measured ROIs from our provided example dataset, it's time to move on to your own images. The level of customization available within CuticleTrace's input parameters allows this toolkit to be applied to a wide variety of cell morphologies and image qualities. However, the flexibility built into CuticleTrace means it is especially important to determine appropriate input parameters for new image sets. Below, we detail how to use FIJI's native functionality and the *CuticleTrace – Single Image Processor* macro to determine ideal input parameters for your dataset.

3.1. Determination of input parameters

The first step in the generation of an epidermal pavement cell shape dataset with CuticleTrace is the determination of input parameters for batch image processing.

3.1.1 Overview of input parameters

The input parameters required to batch process images are covered in depth in the main text of Lloyd et al. (2023). Input parameters may be seen as they appear in the CuticleTrace FIJI user interface in Table 2.

For images to be batch processed, all input parameters (except scale) must be identical. Images of different scales may be analyzed in the same batch by utilizing the *Batch Generate ROIs* macro to generate ROIs, followed by measurement with the *Batch Measure (Different Scales)* macro.

To efficiently and effectively batch process image sets, it is necessary to experiment with a subset of images to determine the ideal input parameters for the *Batch Generate ROIs* macro.

Table 2. Input parameters for CuticleTrace FIJI Macros.

Input Parameter	Options/Default Value	Method of Determination
Cell Walls on Background	<i>Dark on Light, Light on Dark</i>	Determined visually.
Gaussian Blur (pixels)	2 pixels	Determined experimentally on a subset of images with CuticleTrace - Single Image Processor . (See section 3.1.3)
Thresholding Method	Bernsen, Contrast, Mean, Median, MidGrey, Niblack, Otsu, Phansalker, Sauvola	Determined experimentally on a subset of images with CuticleTrace - Single Image Processor and Auto-Local-Threshold . (See section 3.1.3)
Initial Threshold Radius (pixels)	50 pixels	Determined by measuring a subset of approximate cell widths. (See section 3.1.2)
ROI Size Filter (pixels ²)	500-50,000 pixels ²	Determined by measuring a subset of approximate cell areas. (See section 3.1.3)
Smoothing Value (%FDs retained)	5% FDs retained	Determined experimentally on a subset of images with CuticleTrace - Single Image Processor (See section 3.1.3)
Scale (pixels/unit)	No default value. *	Should be known before processing.

* One single value may be entered in the *Batch Generate ROIs* macro if all images are the same scale, or a CSV file specifying the scale of each image may be imported with *Batch Measure (Different Scales)*.

3.1.2. Initial Thresholding Radius

The initial thresholding radius to apply to an image set is best determined by approximating a subset of cell widths. **The ideal initial thresholding radius is approximately half of the average pavement cell width in the whole dataset.** It is iterated upon for every image, so it does not have to be exact. To determine an ideal initial thresholding radius, we recommend measuring the approximate widths of ~10 cells across ~5 images, to get a reasonable average value to apply to the dataset (Fig. 13).

- To measure approximate cell width (in pixels), remove the image scale (Analyze > Set Scale... > “Click to Remove Scale”). Then select the straight-line tool from the toolbar and draw a line across the width of a cell (Fig. 13). Measure the line by pressing the M key. Measure ~2-5 more cells, and then repeat this process with ~3-5 other images. **Set the initial thresholding radius to approximately $\frac{1}{2}$ of the average of your length measurements.**

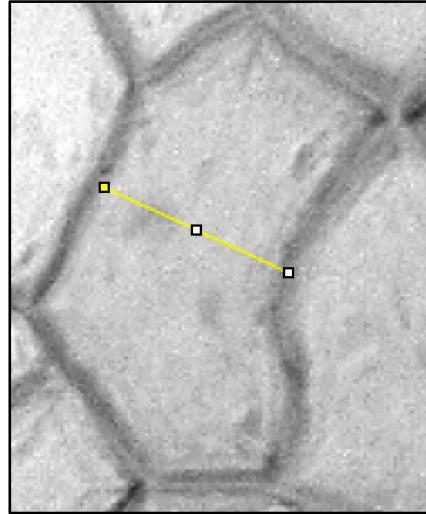


Figure 13. A cell from FLMNH00081 (*Gleditsia triacanthos*), approximately measured by drawing line across its width. This cell measured 95 pixels wide.

3.1.3. ROI Size Filter

The ROI size filter to apply to an image set is best determined by approximating the cell areas of the smallest and largest cells in the dataset (Fig. 14). **The ideal ROI size filter captures all cells in an image set, while removing ROIs that are much too small or large to consist of an accurately traced cell.** ROIs will be filtered further with the R notebook *CuticleTrace_DataFiltration.Rmd*, so some unwanted ROIs are expected to remain in the dataset at this step.

- To measure approximate cell area (in pixels), remove the image scale (Analyze > Set Scale... > “Click to Remove Scale”). Then select the elliptical selection tool from the toolbar and draw an oval around a cell, roughly approximating its area (Fig. 14). Measure the ellipse by pressing the M key. Measure ~2-5 more of the smallest and largest cells you observe, and then repeat this process with ~3-5 other images. **Set the ROI size filter to widely encompass the smallest and largest cells in your dataset, within an order of magnitude.**



Figure 14. A cell from FLMNH00081 (*Gleditsia triacanthos*), approximately measured by an ellipse of similar dimensions. This ellipse had an area of 20,080 pixels².

3.1.4. Thresholding Method

The easiest way to determine which local thresholding algorithm is likely to work best for your dataset is with FIJI's built-in *Auto Local Threshold* function. To use this function, first make your image 8-bit (Image > Type > 8-bit), enhance its local contrast (Process > Enhance Local Contrast (CLAHE) > OK), and apply a default Gaussian Blur of 2 pixels (Process > Filters > Gaussian Blur...). Then, proceed to the Auto Local Threshold menu (Image > Adjust > Auto Local Threshold). Input your previously measured thresholding radius and ROI size filter and select "Try All" for the thresholding method (Fig. 15).

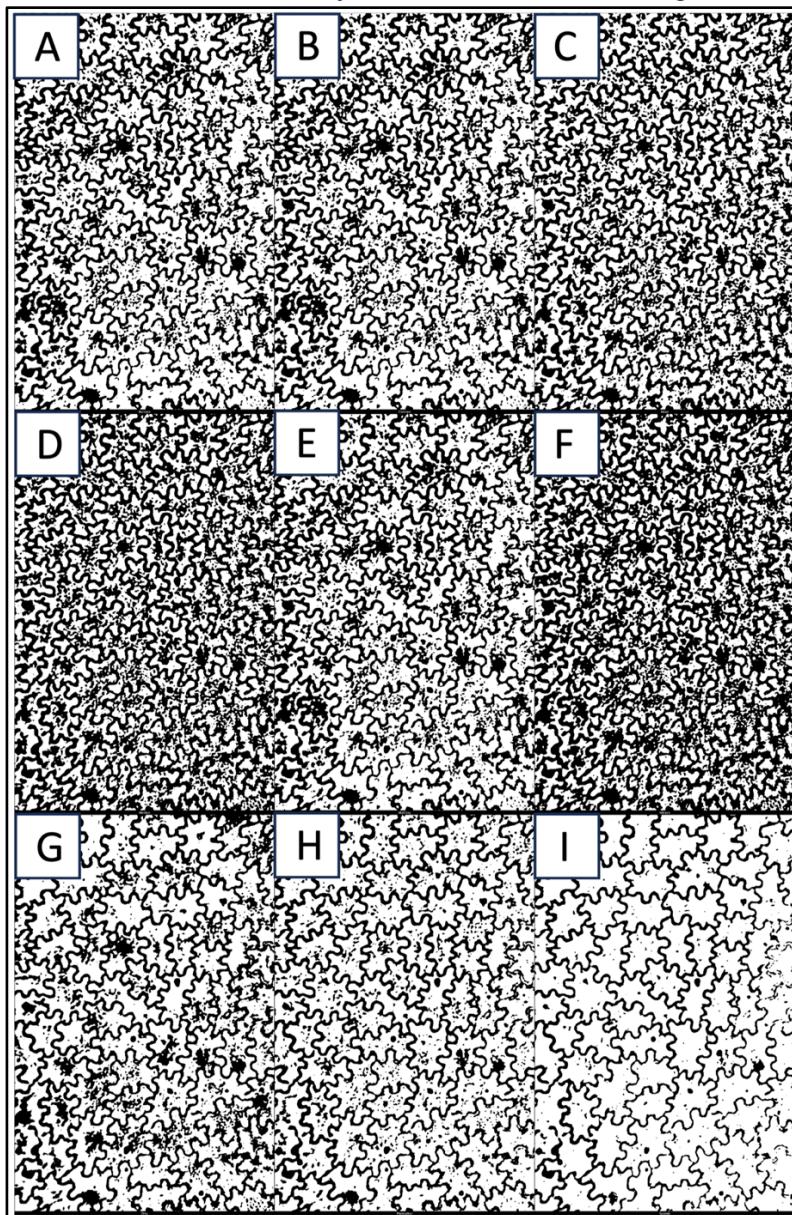


Figure 15. The output of FIJI's Auto Local Threshold "Try All" option on FLMNH00510 (Toxicodendron striatum). Thresholding methods are A) Bernsen, B) Contrast, C) Mean, D) Median, E) MidGrey, F) Niblack, G) Otsu , H) Phansalker, I) Sauvola (Ideal for this image).

Once you have an image montage with all local thresholding algorithms, note which methods work best for your image. These will be fine-tuned by *Batch Generate ROIs* and the *Single Image Processor*, so they do not have to be perfect. For the image in Fig. 15, Sauvola thresholding (Fig. 15i) works best, and Phansalker thresholding (Fig. 15h) does acceptably. Repeat this process with 3-5 images in your dataset to see which thresholding algorithms tend to work well for your images.

3.1.5. Thresholding Method, Gaussian Blur, and Smoothing Value with *Single Image Processor*

Once you have determined your initial thresholding radius and ROI size filter, and you have narrowed down your options for thresholding method, it is time to utilize the *CuticleTrace – Single Image Processor* macro to determine the ideal thresholding method, gaussian blur value, and smoothing value for your dataset. **It is necessary to experiment on and qualitatively evaluate a subset of images to determine these values.**

The *Single Image Processor* brings an open image in FIJI through all (or some of) the steps in the CuticleTrace analysis pipeline (see Fig. 1 of Lloyd et al., 2023). **It allows you to observe the combined effects of these inputs and to determine ideal inputs for your dataset.** It may also be used to individually analyze images instead of batch processing.

To use the *Single Image Processor*, open the macro (Plugins > Macros > CuticleTrace – Single Image Processor). The dialog box that appears will contain options to modify all CuticleTrace input parameters, and an option to select “desired outputs” (Fig. 15). The “desired outputs” menu allows you to decide what steps from the CuticleTrace pipeline you want to visualize (Fig. 16). The default option, “Thresholded and Skeletonized Image with ROIs and Results”, brings an unprocessed image through the entire pipeline (Fig. 17). Other options include fewer steps and/or allow for the processing of a previously thresholded or skeletonized image. In Fig. 17, we show the outputs of the *Single Image Processor* at two different smoothing values, to illustrate the macro’s ability to visualize outputs.

*To view each of these components individually, use FIJI’s native *Auto Local Threshold*, *Gaussian Blur*, and *Shape Smoothing* functions (Section 3.1.6).

Using the *Single Image Processor*, empirically determine the gaussian blur value, thresholding method, and smoothing value that most accurately characterize

cells across a subset of images. With these values (and your previously determined initial thresholding radius and ROI size filter) **you are ready to batch process your image set.**

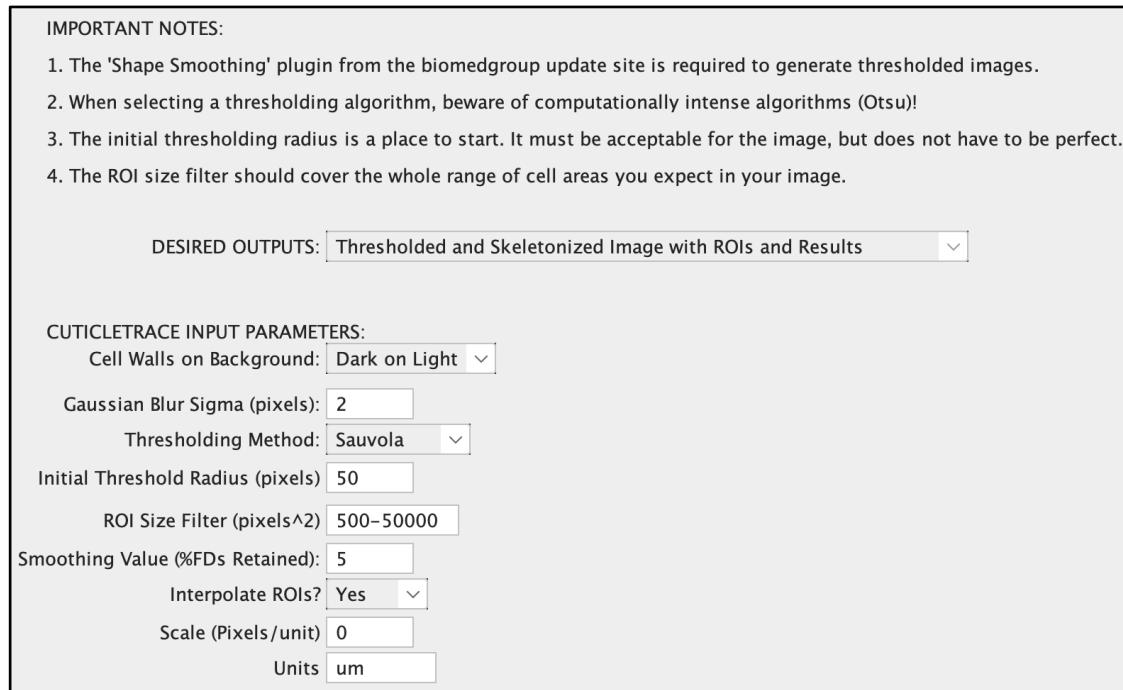


Figure 15. The input window for the Single Image Processor with default inputs for the example dataset.

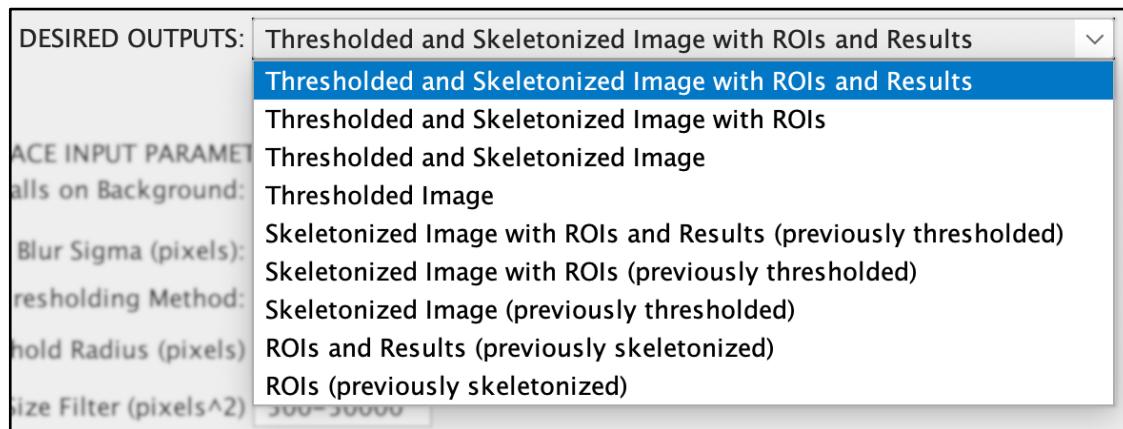


Figure 16. The "Desired Outputs" menu options within the Single Image Processor.

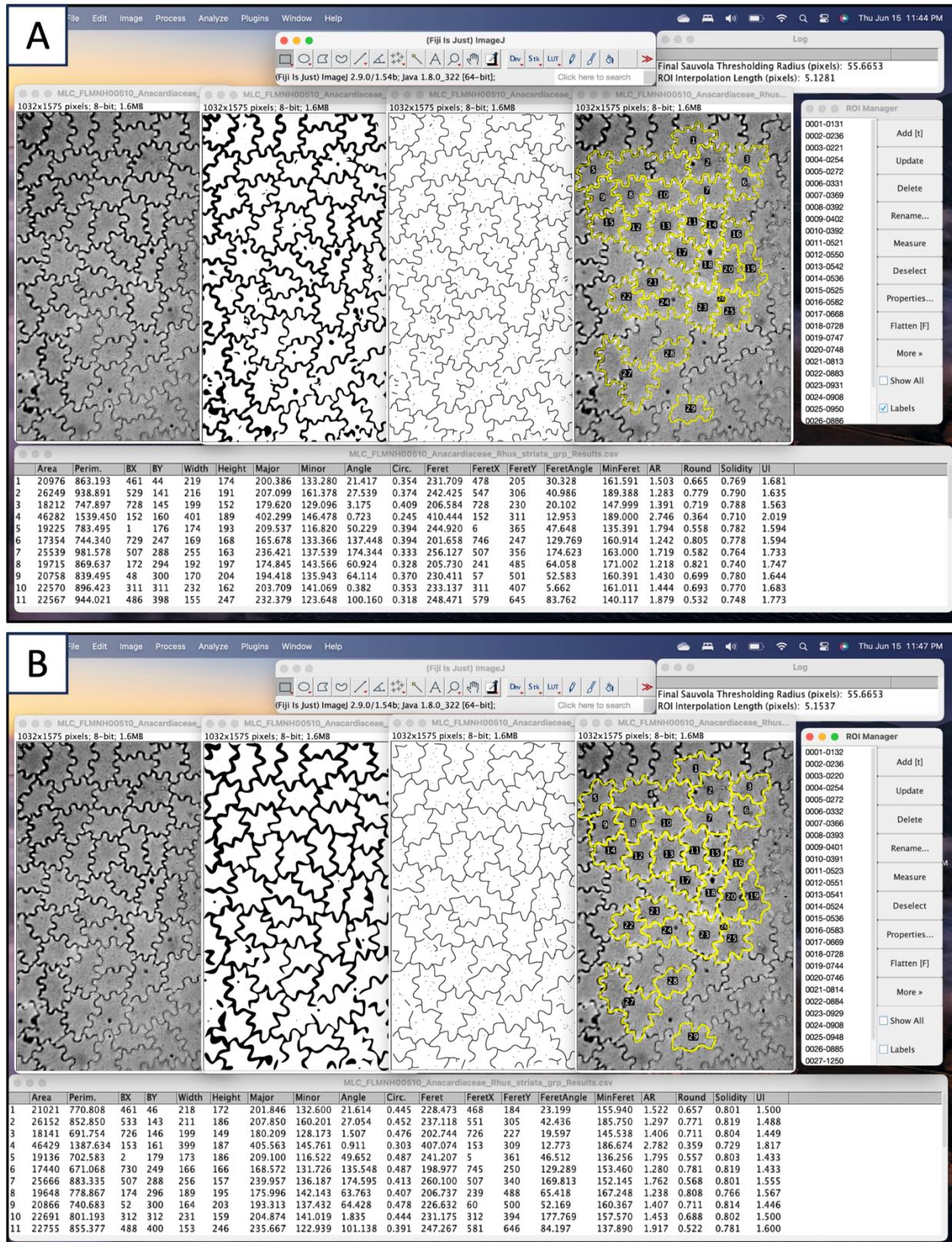


Figure 17. Outputs of the Single Image Processor on FLMNH00510 (*Toxicodendron striatum*) with A) all default input parameters (as seen in Fig. 14) and B) all default input parameters **except smoothing value** (2% FDs retained). The default smoothing value in A) (5% FDs retained) performs better for this image.

3.1.6. Optional - Manually replicate *Single Image Processor*

If you would like to further investigate input parameters at a more granular level of detail than what is supported in the *Single Image Processor*, follow these instructions to approximately replicate the steps of the CuticleTrace image analysis pipeline through FIJI's user interface. **This is not necessary to determine input parameters, but is provided for additional transparency.**

1. Make the image 8-bit (Image > Type > 8-bit)
2. Enhance local contrast (Process > Enhance Local Contrast (CLAHE) > OK)
3. Apply Gaussian Blur (Process > Filters > Gaussian Blur...)
 - a. In this step, you may experiment with different values to see how they influence downstream results. In general, the default value of 2 pixels works well with images of similar resolution of our example dataset. In general, increased blurring helps smooth out differences in contrast along cell walls but will erode details in cell shape at higher values.

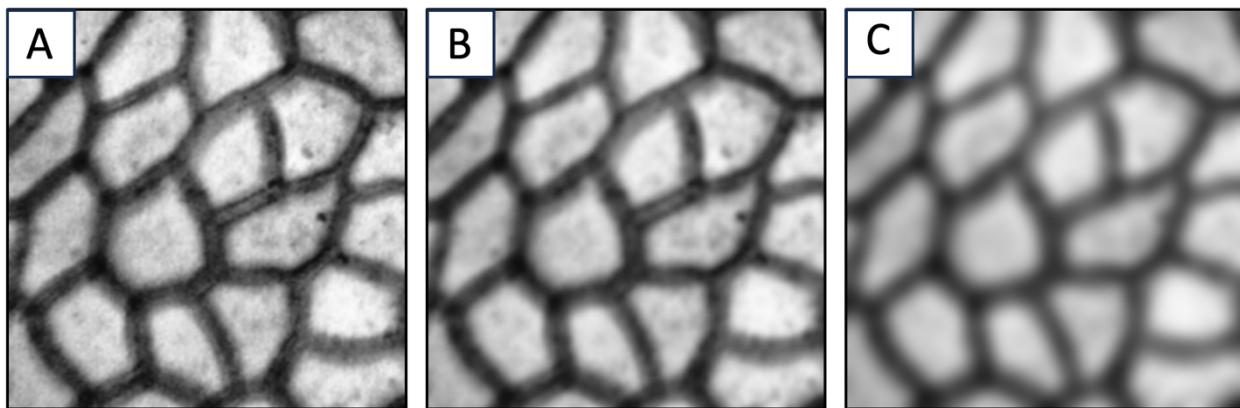


Figure 3. An image of *Castanea pumila* cuticle (FLMNH00115) after local contrast enhancement with gaussian blur set to (A) 1 pixel, (B) 2 pixels (ideal for this image), and (C) 5 pixels.

4. Apply Local Thresholding (Image > Adjust > Auto Local Threshold)
 - a. In this step, you may experiment with different thresholding algorithms, or select “Try All” for the method. This will result in a multi-paneled image showing the results of all thresholding algorithms for that image. The “Try All” option is recommended for determining which thresholding methods work well on your dataset.

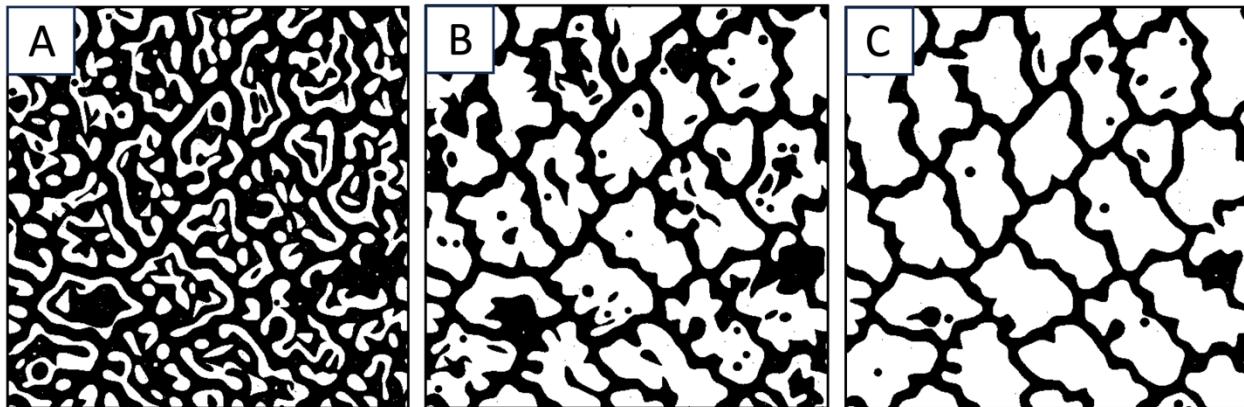


Figure 4. An image of *Talisia princeps* cuticle (FLMNH00443) after local contrast enhancement and blurring, thresholded by either the (A) NiBlack algorithm, (B) Bernsen algorithm, or (C) Sauvola algorithm (ideal for this image). See Lloyd et al. (2023) Fig. 2.

5. Apply shape smoothing (Plugins > Shape Smoothing)

- a. In this step, you may experiment with different smoothing values. The shape smoothing plugin from the BioMedGroup (Wagner, 2016) smoothes binary images by filtering a relative proportion of Fourier Descriptors of each “blob” within the image. More information can be found on the ImageJ website (<https://imagej.net/plugins/shape-smoothing>). An ideal smoothing value minimizes thresholding artifacts without obscuring gross cell shape.

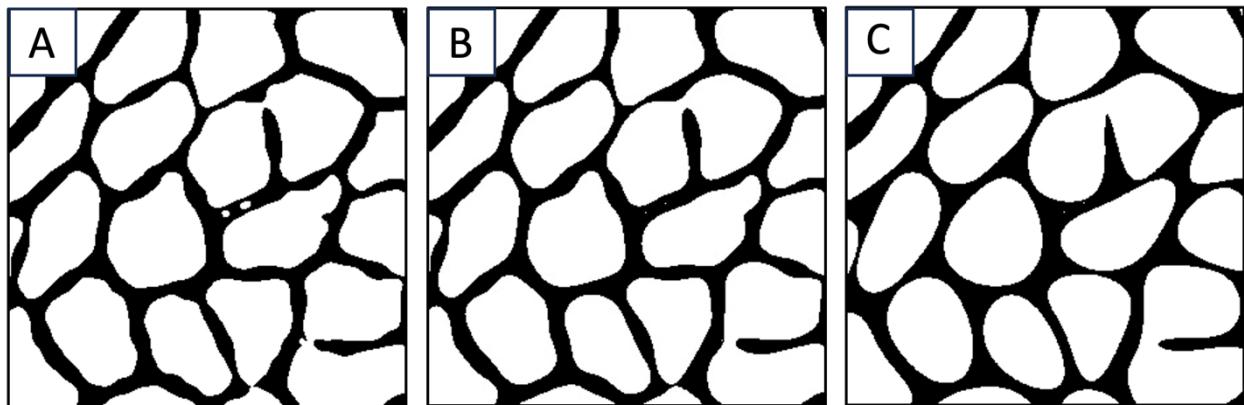


Figure 5. An image of *Castanea pumila* cuticle (FLMNH00115) after thresholding, with shape smoothing set to (A) 10% FDs retained, (B) 5% FDs retained (ideal for this image), and (C) 2% FDs retained.

6. As you experiment with these inputs, it is important to understand how a thresholded image will translate to a finalized ROI set.

- a. To take a thresholded image through the next steps performed by *Batch Generate ROIs*, select the *Single Image Processor* macro (Plugins > Macros > CuticleTrace – Single Image Processor). By selecting

“Skeletonized Image (previously thresholded)” from the “Desired Outputs” menu, this macro generates a skeletonized image with 3-pixel-wide cell walls, which can be directly analyzed to generate ROIs.

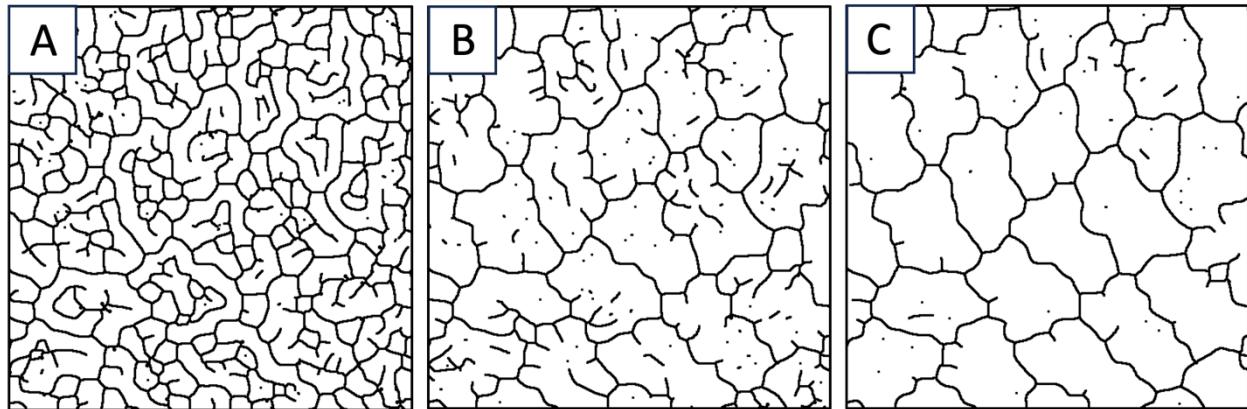


Figure 6. Skeletonized images of *Talisia princeps* cuticle (FLMNH00443) that has been thresholded by either the (A) NiBlack method, (B) Bernsen method, or (C) Sauvola method (ideal for this image). (See Lloyd et al., 2023 Fig. 2).

- b. To generate an unfiltered ROI set from a skeletonized image, continue to use the *Single Image Processor* macro (Plugins > Macros > CuticleTrace – Single Image Processor). By selecting “ROIs (previously skeletonized)” from the “Desired Outputs” menu, this macro generates an unfiltered refined and interpolated ROI set from a skeletonized image.

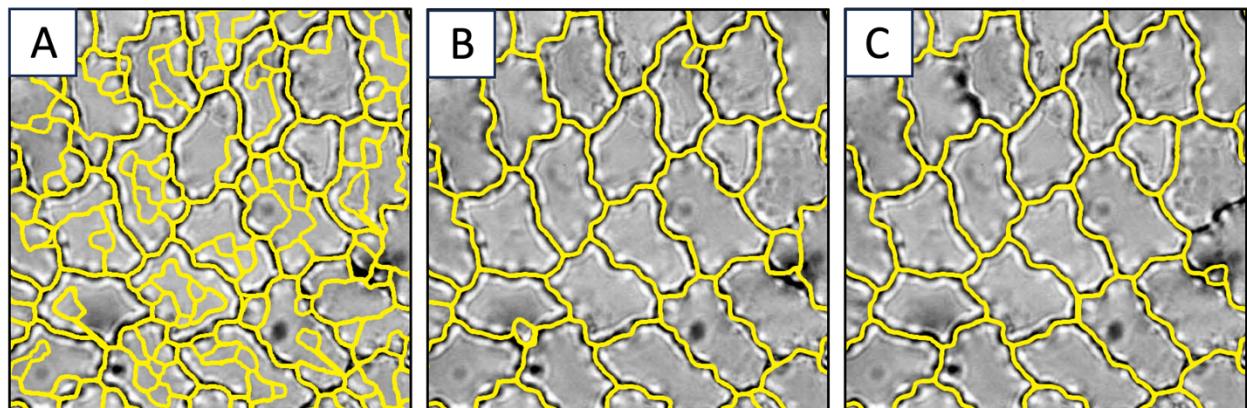


Figure 7. ROI sets generated from skeletonized images of *Talisia princeps* cuticle (FLMNH00443) that has been thresholded by either the (A) NiBlack method, (B) Bernsen method, or (C) Sauvola method (ideal for this image). See Lloyd et al. (2023) Fig. 2.

Once you have decided upon input parameters by visually vetting ROI sets from a subset of images, you are ready to batch process images.

3.2. Batch processing your own datasets

Now that you have an in-depth understanding of how to decide on your CuticleTrace input parameters, you are ready to apply CuticleTrace to your own images. **Batch processing your own images is no different than batch processing the example dataset**—just plug in your newly-determined input parameters and follow the tutorial from section 2.3.

With the CuticleTrace toolkit, we hope you generate increased sample sizes and more consistent measurements, all with significantly less time and effort in comparison to manual measurements. **Good luck!**