

Scratch Assay Protocol

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July 2025

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Acknowledgments

The Scratch Wound Healing Tools a modified duplicate of the Wound Healing Size Tool created by Alejandra Suarez-Arnedo, Felipe Torres Figueroa, Camila Clavijo, Pablo Arbeláez, Juan C Cruz, and Carolina Muñoz-Camargo. Without their diligent work, our modification would not have been possible.

You can access their article on the plugin here:

<https://pmc.ncbi.nlm.nih.gov/articles/PMC7386569/>

and the GitHub repo associated with the plugin here:

<https://github.com/AlejandraArnedo/Wound-healing-size-tool/wiki>

Before you begin...

Before you begin your analysis, make sure your scratch wound contrast between wound edges, brightness of image, and clarity are as consistent as possible. This will save you time from having to make dramatic changes to the image analysis parameters between each image.

To use the plugin, you must have imageJ/fiji downloaded and installed on your device. You may download imageJ from the following link if you have not already: <https://imagej.net/ij/>.

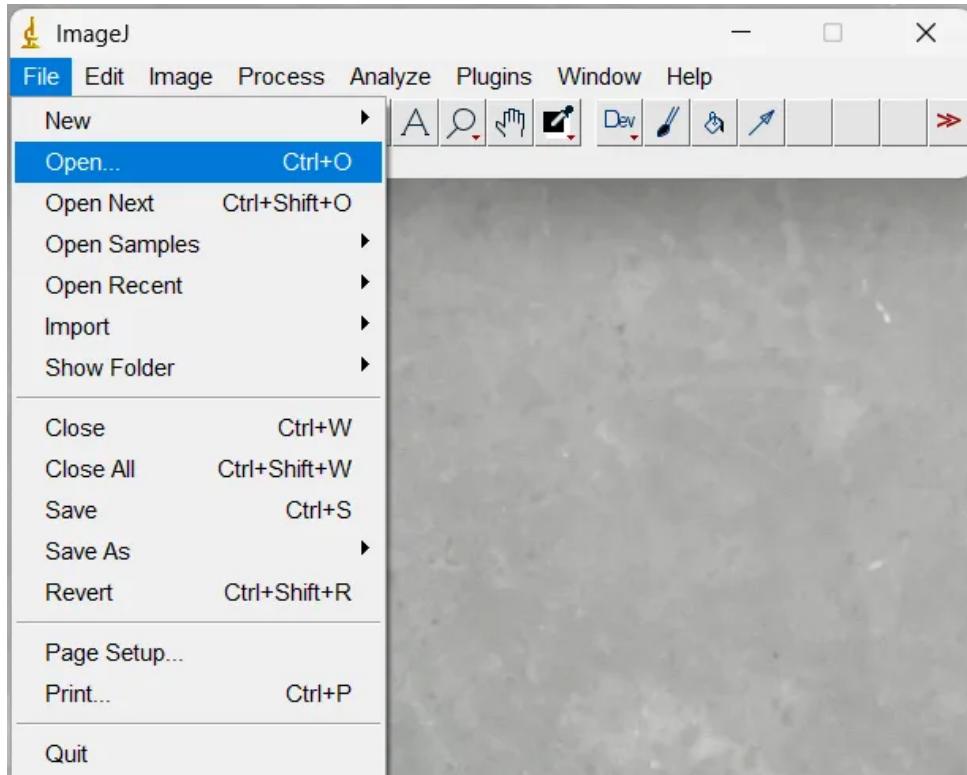
The most updated version of the Scratch_Wound_Healing_Tools can be found on the MicroStatsLab GitHub page in the Scratch Wound Healing Repository found here:

<https://github.com/MicroStatsLab/Scratch-Wound-Healing>.

ImageJ Introduction

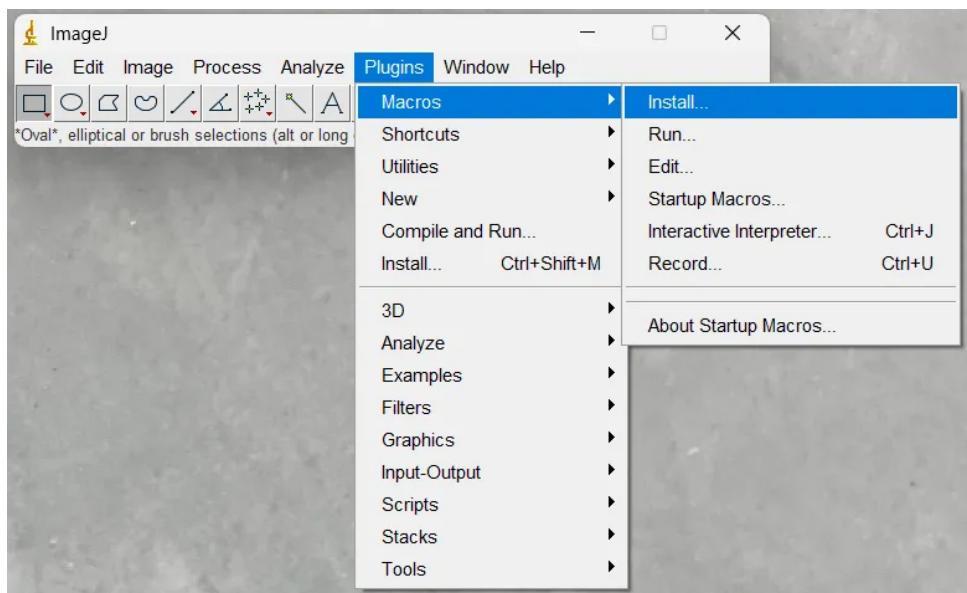
Step 1 - Opening an Image

Your first step in preparing ImageJ for analysis is loading in an image. This can be done by opening *File* and then *Open* or simply *ctrl/cmd + O* then just find and select the image you would like to start with.



Step 2 - Installing the Plugin

Your next step will be installing the plugin into imageJ. First hover over or click *Plugins*, then *Macros*, followed by *Install...* from here, just find where you have saved the txt file.



You can verify that the plug in has been successfully installed when the buttons on the right hand side of the tool bar have changed. See below for what this should look like.



Step 3 - How to Use a Macro Tool

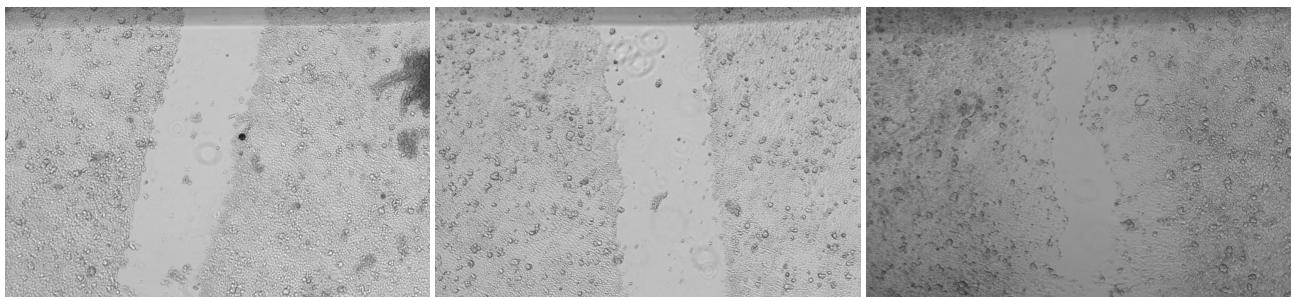
To use, simply click on the button and then click on the image. Depending on the tool, there may be a dialog box to set any parameters.

Analysis

Types of Images

Throughout this protocol you will see images referred to as "single wound images" or "multiple wound images". This represents whether a single image has one or more identifiable wound areas. Typically multiple wound images are those that have been taken later into the healing process, however this is not a rule. See below for examples of single and multiple wound images.

Examples of single wound images



Examples of multiple wound images



Button Review

The Scratch_Wound_Healing_Tools plugin consists of 6 main tools. Going from left to right, there are the Wound healing Size Stacks Tool, Wound healing size Tool, Wound healing size Manual Tool, Rotate 90° Tool, Multiple Wound Healing Size Tool, and Manual Measurements Tool. Below are the descriptions for each tool.

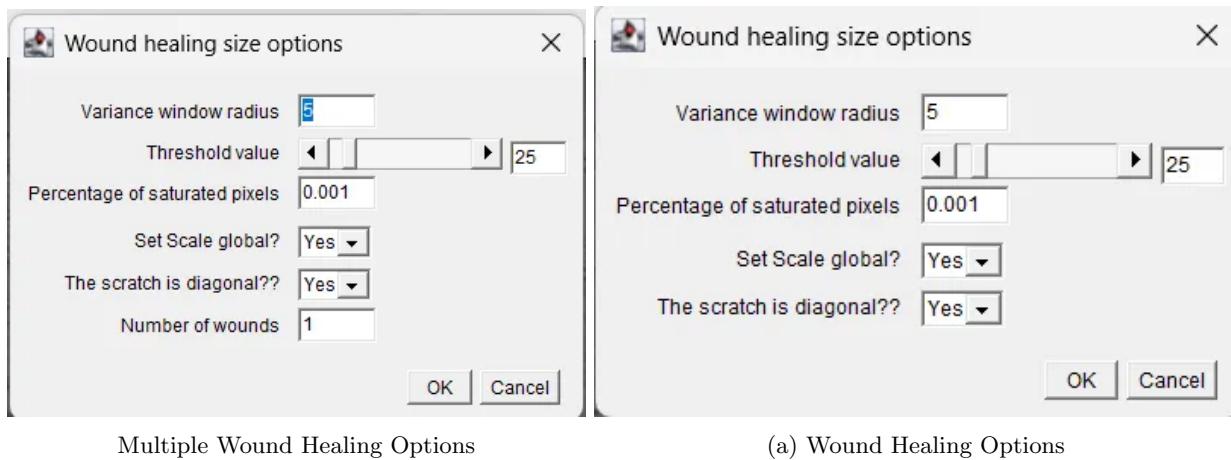
1. **Wound healing Size Stacks Tool** - this tool can take in a stack of single wound images whose parameter's settings will not need to be changed from image to image (see below for setting parameters). This would consist of images whose quality, wound edge clarity, and brightness are consistent.

It has been decided that stack analysis for wound images is not appropriate due to inconsistency and accuracy between images.

2. **Wound healing Size Tool** - this tool can take in a single image with a single wound. This tool (or the multiple wound tool below) will likely be used most often.
3. **Wound healing size Manual Tool** - this tool uses a single image. Manually, you must draw the outline of the wound (freehand selections works best, preferably with a stylus if you have). This tool may be necessary when dealing with images that the wound cannot be detected, on the incorrect part of the edge is being selected.
4. **Rotate 90° Tool** - Rotates an image 90° such that the scratch is mostly vertical.
5. **Multiple Wound Healing Size Tool** - This tool uses one either multiple or single wound image. It works by selecting the n largest wound areas it detects. Of course it may not select the ones you are wanting, in which case see below for how to deal with that.
6. **Manual Measure** - A short cut to modifying the "Set measurements...". This can be helpful when the above tools find the incorrect area.

Setting Values

Depending on which version of the size tool you use, there are 5-6 parameter settings.



Parameter Meanings

1. **Variance window radius** - Represent the radius of variance filter which is established to determine the empty or the occupied zones. The radius must be big enough, so the noise variance has no impact on tissue variance.
2. **Threshold** - The image resulting from the variance filter is converted to a mask by applying the given threshold.
3. **Percentage of saturated pixels** - Enhance the contrast of the image by determining the number of pixels in the image that can become saturated. Increasing this value increases contrast. This value should be greater than zero.
4. **Set Scale global?** - Select "Yes" if the scale calibration applies to all the images. Select "No" if the scale calibration only applies to the select image.
5. **The scratch is diagonal?** - Select "Yes" if you can observe that the scratch has an inclination differently of 0°. Select "No" if the scratch has not an inclination.
6. **Number of wounds** - As described, this is the number of wounds you want to select. Default value is 1. Cannot be above the number of areas identified by the program. This setting is exclusive to the Multiple Wound Healing Size Tool.

What Values Should I Use?

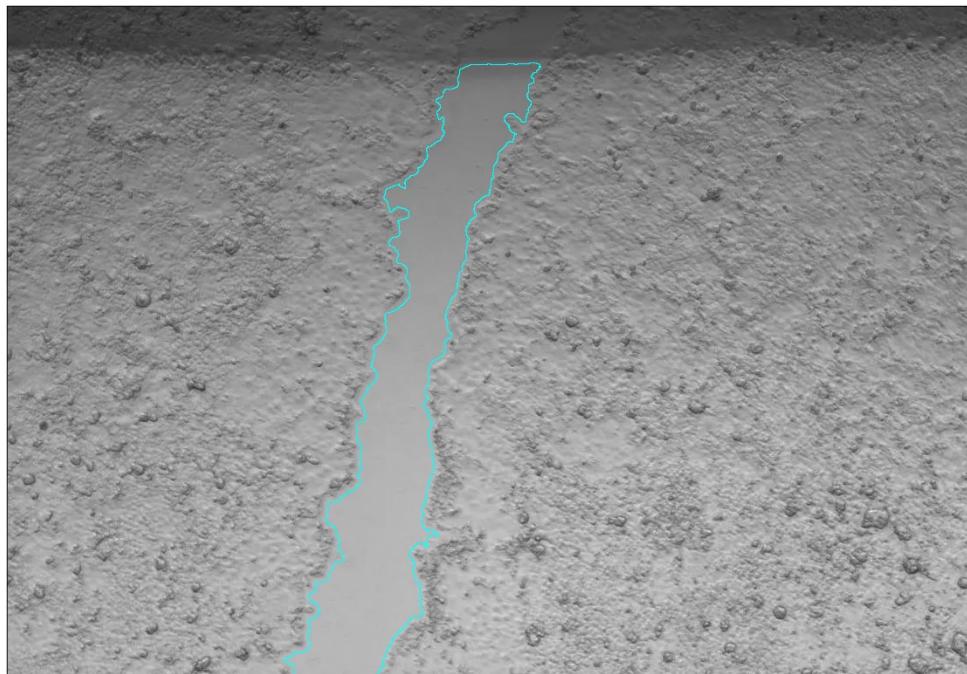
For wounds that have higher contrast in edges, or are brighter, I typically use a variance of 5. The lower the variance setting, the tighter to the edge the measurement gets. This is an issue when you have images with blurry, or harder to define edges.

For images with less clear edges, you need to work both the variance and the threshold to exclude any excess material. Typically I will first increase the threshold before deciding to increase the variance. When there is excess material being included, try first decreasing the threshold value. If there is not enough material, increase the threshold value. If the selection of the image area isn't selecting the correct area, despite increasing and decreasing the threshold, this is when I would typically increase the variance.

As an additional note, due to how variance and threshold are related, if you increase your variance (further from the edge of the wound) you may need to increase the threshold to push the selected area back towards edge.

The % pixels is a bit of a special case, for bright, well defined images, it doesn't make much difference. For dark images (or in our case, more healed wounds specifically), it made more impact. The typical settings I would test are 0.004, 0.04, 0.075, 0.09. Though some images did require testing outside those options.

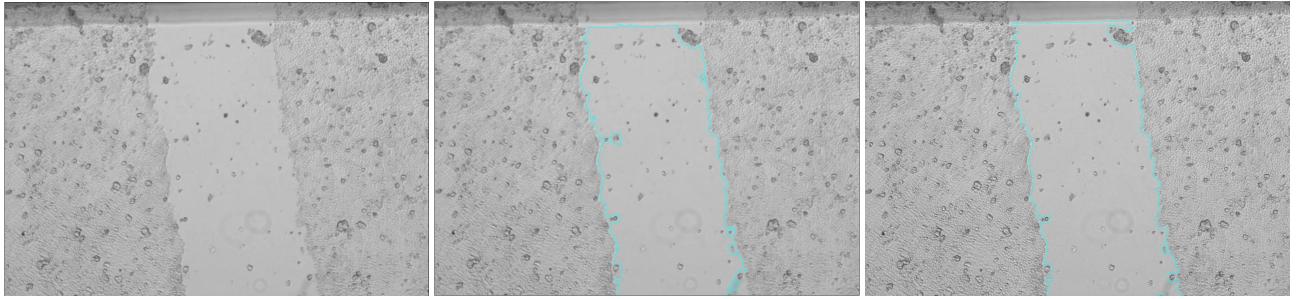
You will know you have the settings correct when the blue outline does not over or under estimate the wound area.



Example Images

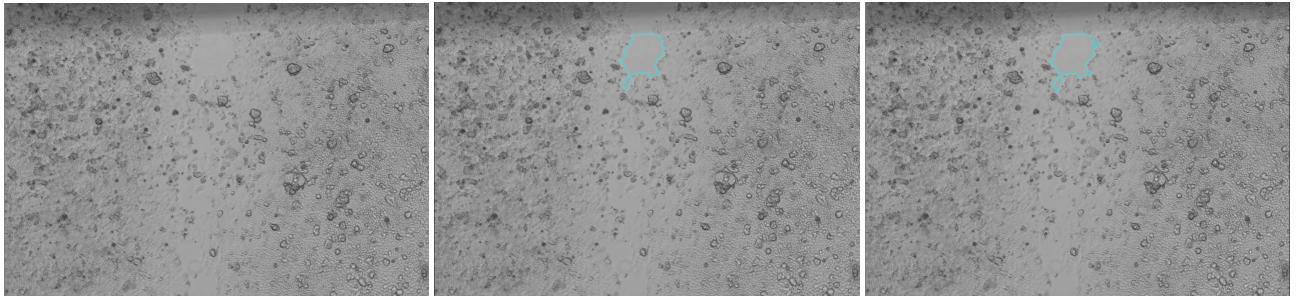
The following are examples of images and the parameters/process used.

In this image, because of the brightness and well defined edges, I choose a variance of 5. I also chose a higher threshold due to all the matter inside and near the edges of the scratch. I suggest picking a number and working your way up or down from that. For the following image, I may have picked 55 and incremented up or down by 10, then 2, then 1, until I was satisfied with amount of inclusion/exclusion. For this example, I've started at 25 in the threshold such that the difference in thresholding is noticeable.



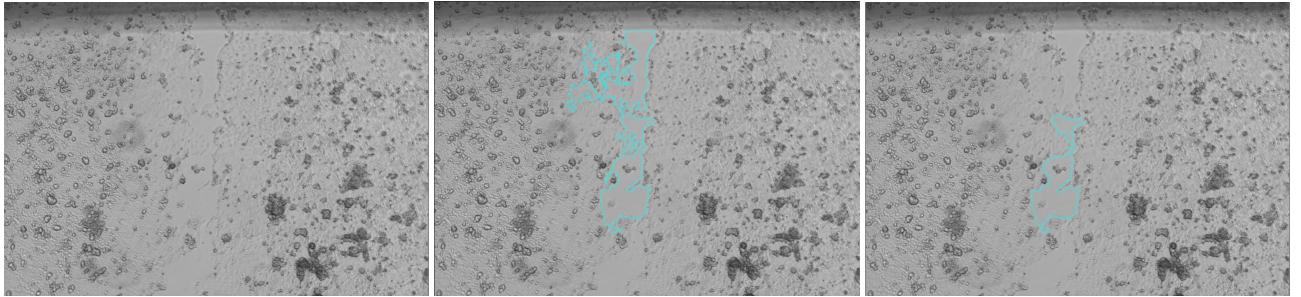
(a) Variance 5, threshold 25, % pixels
0.004 (b) Variance 5, threshold 80, % pixels
0.004

In darker images (or more healed images in our case), I recommend starting with a higher variance as the difference between wound and edge is less clear. That also is to say, they typically require a lower threshold.



(a) Variance 6, threshold 24, % pixels
0.09 (b) Variance 5, threshold 24, % pixels
0.09

In the following image, there is less contrast in the edges of the wound. I started with a variance of 5, threshold of 22, and % pixels of 0.04. A low threshold aids in selecting the wound area. However, the inclusions were just too extreme. Knowing that I am already as low as 22, and the edges found are overly complex in the upper half, I first increase the variance by 1, see the results, then try reducing the threshold by 5.



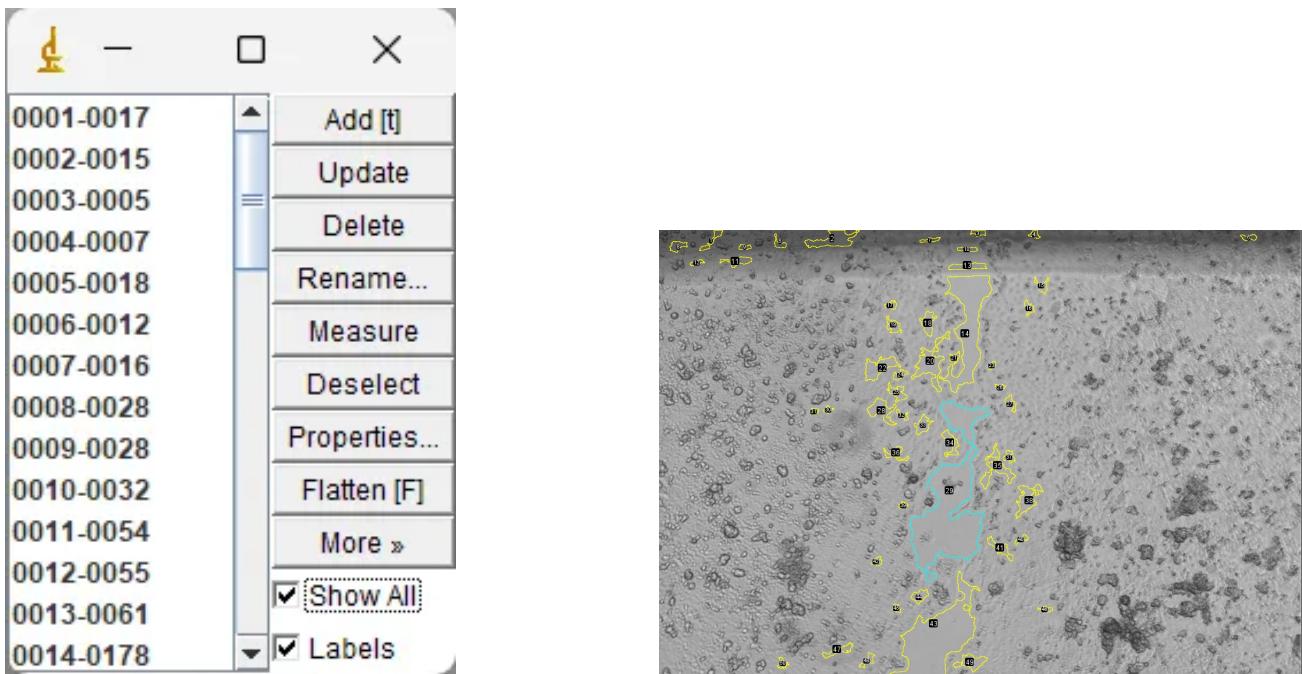
(a) Variance 5, threshold 22, % pixels
0.04 (b) Variance 6, threshold 17, % pixels
0.04

Potential Issues with Image Analysis

Images with Multiple Wounds, but Incorrect Selection of Wound Areas

If you have used the multiple wound tool, but the areas selected by the tool are not those which you were looking for, there is a relatively simple fix. First, click “show all” on the ROI manager if not already selected. This shows all wound areas it has detected. Using this, look to see which sections your wound is made up by.

For example, I can see that this wound consists of sections 14, 29, and 43. I would continue to tweak at the threshold for section 14.



Using button number 6, the Manual Measure, click on the image. Then, in the ROI manager, hold control and select the areas you want. Then click on the “Measure” button.

Manual Wound Measurements

If you still cannot get the edges of the image to line up with the wound no matter what, you can use the manual tool (the third box). To do this, first use the “Freehand selections” tool. Then outline your wounds by drawing the edges. Finally, click on the “Wound Healing size Manual tool”. (This image does not include our extra buttons, but they do not make any impact on this function).

