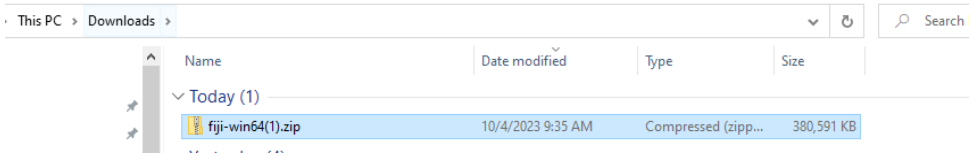
ImageJ Lab Walkthrough

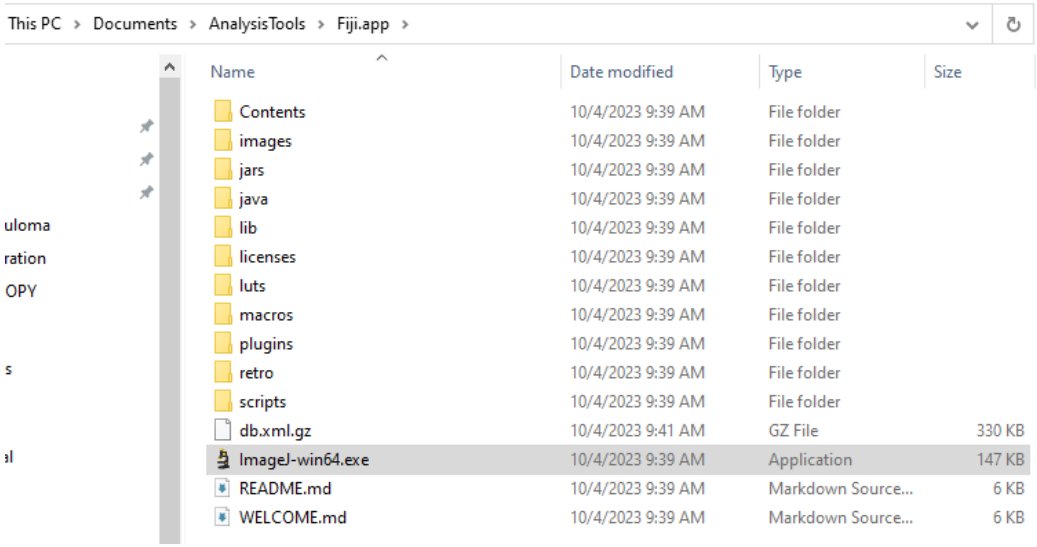
Information courtesy of Edward Evans, Michael Nelson, Ellen Dobson, as well as many others involved in ImageJ/FIJI development and production of educational materials, walkthroughs, and demonstrations.

Installing ImageJ/FIJI

1. Download FIJI package from the software site. FIJI has pre-installed plugins and is recommended for this lab.
    a. Fiji: https://imagej.net/software/fiji/downloads
        i. Look for the zip folder and extract the contents to the desired location on your computer. Keeping it in “Program files” is generally not recommended to avoid access/security issues.



        ii. Open the program with the “.exe” file



        iii. You may be prompted to start the updater after first opening the program

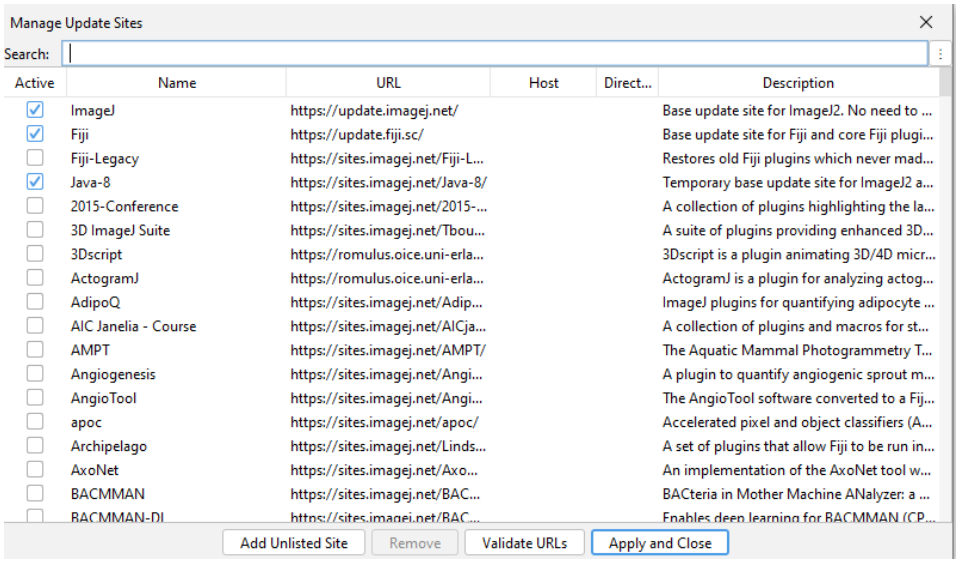
    b. Alternative to install ImageJ base package: https://imagej.net/ij/download.html

A Side Note on Updating

When using FIJI, it is recommended to use the **Help > Update** option rather than Help > Update ImageJ**.** Update ImageJ will specifically update only the base ImageJ package within FIJI, not the associated plugins. This makes it more likely that you will run into version issues and other update problems. Generally, updating when prompted upon opening FIJI will also prevent issues.

Install PlugIns with Update Sites

1. After running **Help > Update** there will be an option to **Manage Update Sites.** This provides access to common plugins that are compatible with FIJI. Use the checkbox to add a plugin, then click apply and close.



2. Click **Apply Changes,** then restart FIJI
3. You should now be able to search for and run the plugin

Downloading a Practice Dataset

The complete list of files may be downloaded at the following links:

Google Drive:
https://drive.google.com/drive/folders/1PI-BitlQb2j0bm2s35dp70WV9USqiT0_?usp=sharing

Github:
https://github.com/hwilson23/FIJI-ImageJ_Lab_Tutorial

Common Keyboard shortcuts

+ - zoom in
- - zoom out

Ctrl/Command + **S** - save
*no shortcut for “save as”

Ctrl/Command + **Shift** + **C** - brightness and contrast

Ctrl/Command + **Shift** +**Z** - color channel control

Ctrl/Command + **Z** - undo

Ctrl + **H** - histogram **just H for Mac*

Ctrl/Command + **K** - plot/line profile

Ctrl/Command + **L** - focus search bar

Ctrl/Command + **M** - measure

Ctrl/Command + **Shift** + **T** - threshold

Shift - to draw straight lines
Alt + **arrow keys** - to change current selection size

T - open ROI (region of interest) manager, and will add current selection as ROI

Ctrl/Command + **Shift** + **W** - close all

Also helpful:

Window > tile - will montage all open images/windows

Image> Transform - flip or rotate images

Help> Update - to update FIJI

Getting Started, Opening an Image

The easiest way to open a data set is to click-and-drag from the folder. However, some file types need to be specifically imported. To import a sequence of images that are saved within a folder, you can use the **File > Import > Image Sequence** option to open them as a stack or as separate images.
Additionally, using a **Virtual Stack** in the **Bio-Formats Importer** may be useful if trying to view large data files. This makes it easier to get a quick look at data when there is limited RAM. However, if you need to perform an image operation, such as background subtraction, then more memory will be required as the data needs to be loaded.

Starting with a basic image

1. Open “blobs.gif”
    a. Also available though **File > Open Samples > Blobs,** or **Ctrl + Shift + B**

Opening a Z-Stack

1. Open “t1-head.tif”
    a. Also available through **File > Open Samples > T1-Head (16-bits)**
    b. Use the bar to scroll through the stack of images
2. We can also display the orthogonal view by using **Image > Stacks > Orthogonal Views** or **Ctrl/Command + Shift + H**
    a. The yellow crosshairs can be used to change the display for each orthogonal view

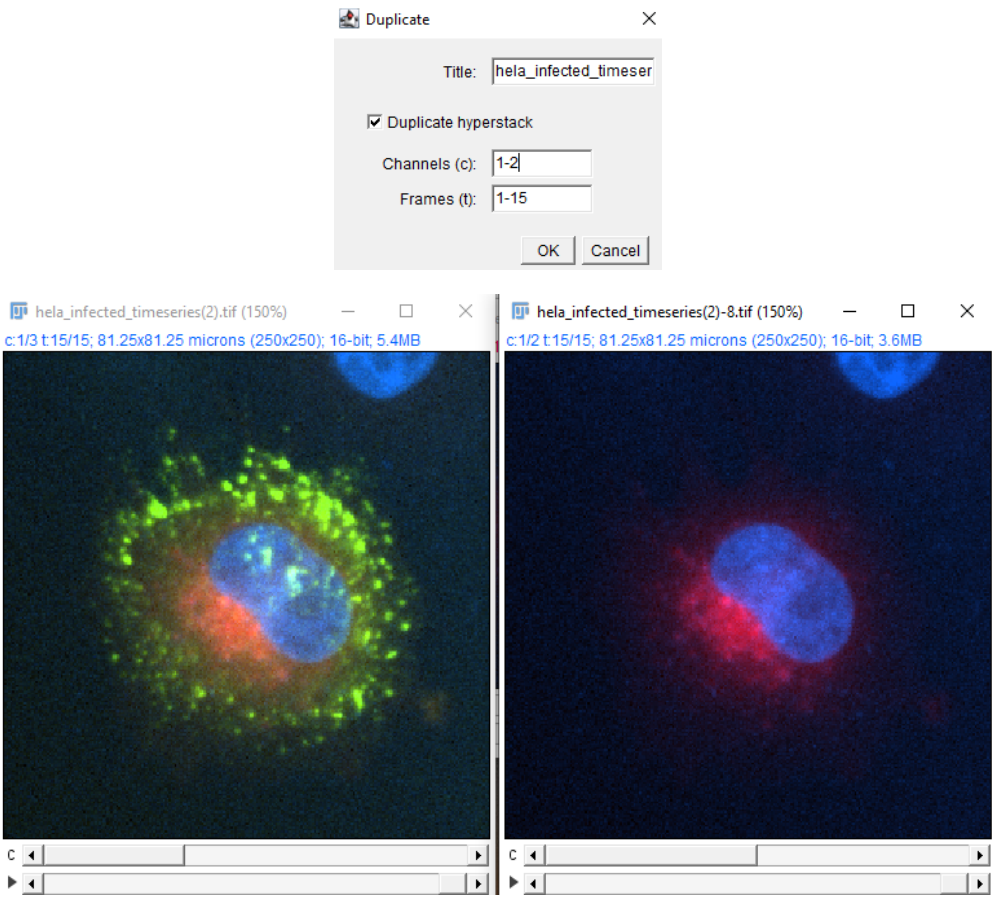
Hyperstacks

Hyperstacks are multidimensional data, such as a multichannel timeseries or z-stacks.

1. Start by opening the “hela_infected_timeseries.tif” image. This is available for download at: https://media.imagej.net/workshops/data/3d/

Duplicate A Channel Or Image

a. Use **Image > Duplicate** to bring up the duplication options window. This can also be done with **Ctrl + Shift + D** or **Right Click > Duplicate.** Here, we can specify which RGB channels and timepoints we want to separate. If we use 1-2 in the channels menu, it should produce an image of just the red and green channels. The duplicate hyperstack box should be checked.

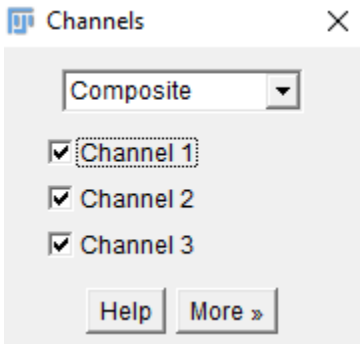


Adjusting Brightness and Contrast

a. Changing the contrast in an image can be a good visualization tool. Using **Ctrl + Shift + C** or **Image > Adjust > Brightness and Contrast** will display the B&C menu. Using Auto or the B&C sliders is okay for visualization, but **using the Apply button will change the pixel values, which generally should not be done.**

Changing Channel Colors

1. To change the display color of channels, use **Image > Color > Channels Tool** or **Ctrl + Shift + Z.**



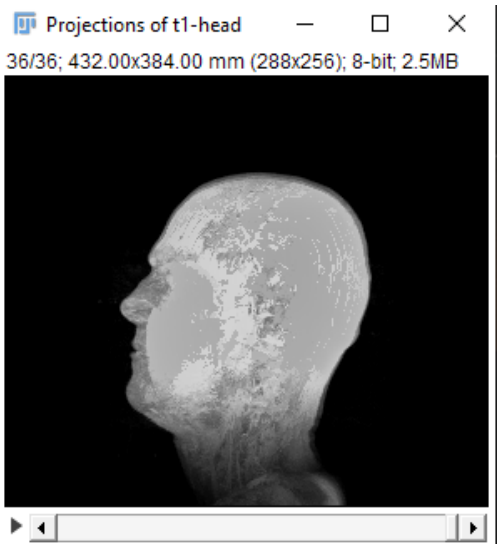
a. The “more” option can be used to apply colors to the selected channels.

Caution with Changing Image Types

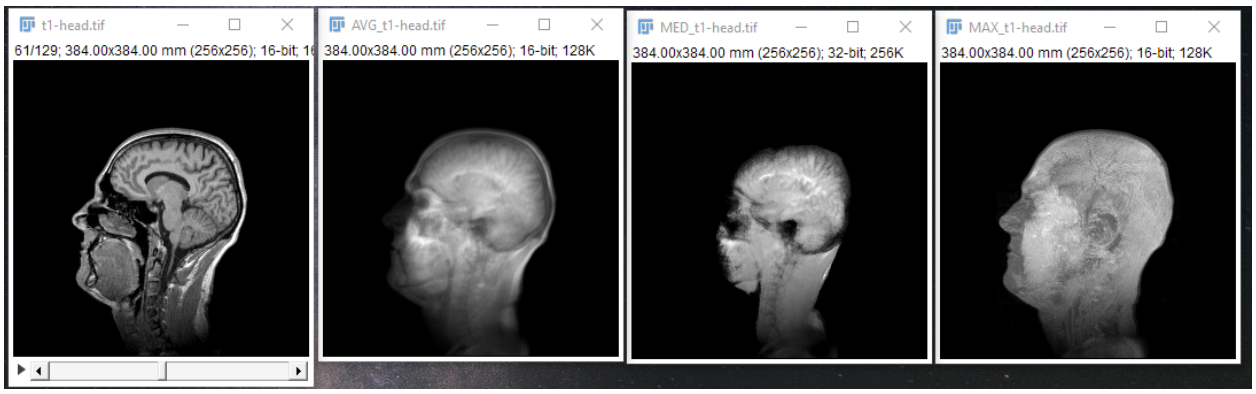
a. Although it may be useful in specific cases, changing the image type through **Image > Type** can result in the loss of information as we will see in a later example. Not all image types can be converted back to the original. The following error is likely to display if you are not able to complete a type conversion.

3D Volume and Projections

1. Open t1-head.tif, **File > Open Samples > T1 Head (16 bits)**
2. To display the 3D Volume, use **Image > Stack > 3D Projection > Click ok**
    a. In 3D projection dialogue box, there are various options for projection including axis of rotation and setting the slice spacing (step size) which may be useful for known parameters



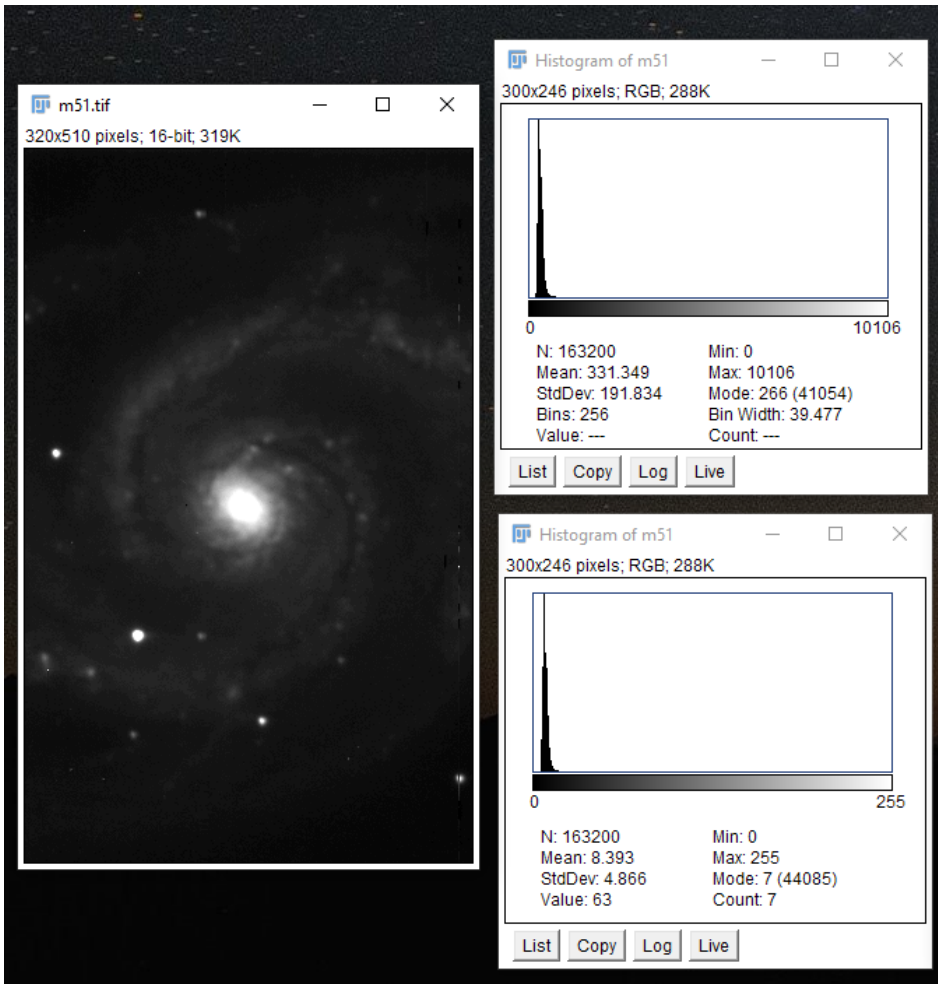
3. Additionally, the original image stack may be projected along one axis using **Image > Stacks > Z Project.** It is important to note that the information in the projection is highly dependent on the display parameter. A few examples are shown below (left to right: single slice of the stack, average projection, median projection, max projection).



Bit Depth

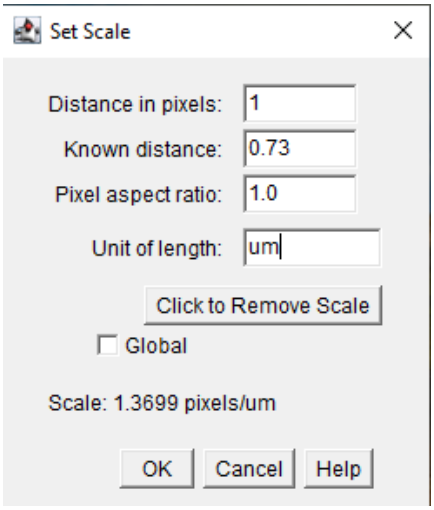
1. Open the “m51.tif” image, **Open > Open Samples > M51 Galaxy (16 Bits).**
2. Adjust the contrast so we can see the image a bit better. **Image > Adjust > Brightness and Contrast.**
3. Make a histogram of the image, **Ctrl + H,** and notice the scale of the histogram and the mean value. Keep this window open.
    a. 16-bit images have a maximum value of 65,536.
4. Convert the 16 bit image to an 8 bit image, **Image > Type > 8 bit.**
    a. 8-bit images have a maximum of 256 values. This means the data resolution and range is lower compared to a higher bit depth, so the data is compressed.
5. Make another histogram and keep the window open. How did the values change?

6. Convert the 8 bit image back into a 16 bit image and make another histogram. Do you still have the original data?



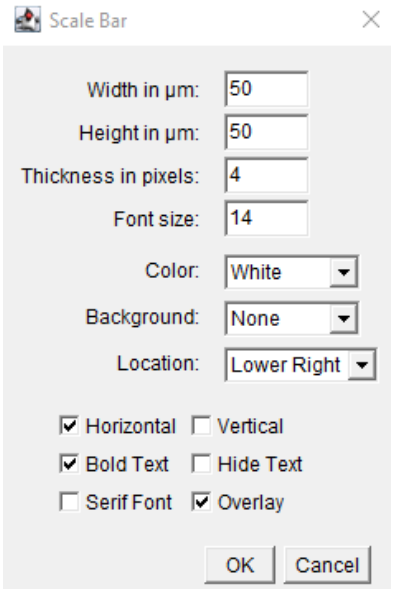
Set Scale and Scale Bars

1. If the size per pixel is known, such as from a microscope calibration, this can be used to change the image dimensions from pixels to the known field of view.
2. Use **Analyze > Set Scale** to enter the known distance. For example, an image with a pixel size of 0.73 pixels/micron would be entered as the following image.



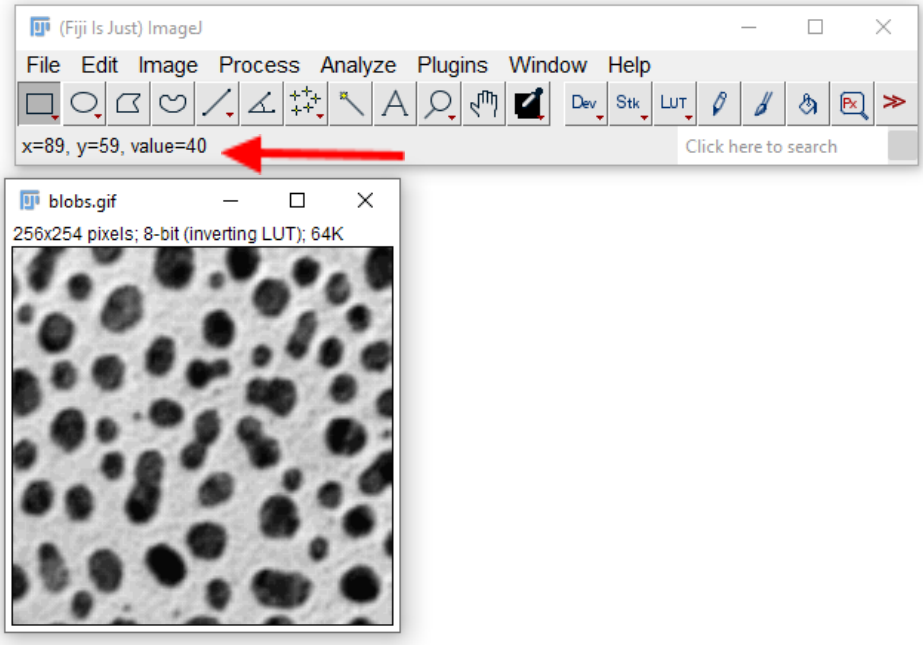
a. Further info for setting the scale based on a measurement in an image can be found here: https://serc.carleton.edu/eyesinthesky2/week2/get_to_know_imagej.html

3. To add a scale bar, use **Analyze > Tools > Scale Bar.**



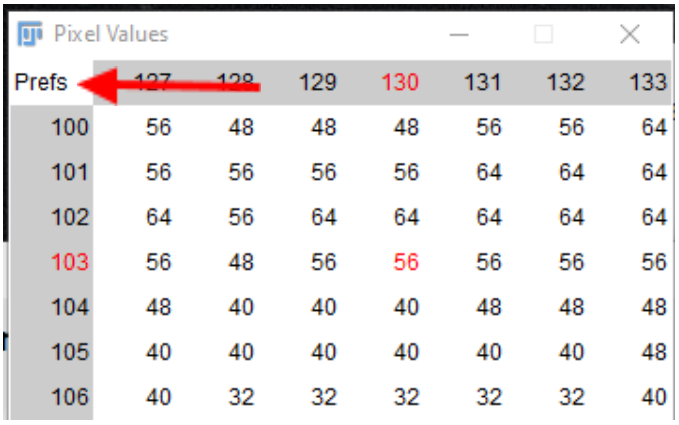
Inspecting Images

1. Hover over an area in the image. In the status bar of Fiji, you should see the pixel you are on, with xy coordinates, and its associated value.
    a. In an RGB type image, Fiji will also tell you the associated color values for the red, green and blue channels.



2. To see a larger area, use the **Pixel Inspection Tool**
    a. In the Fiji window, click the pixel inspection icon
    b. *If the icon is not present, you can use the double red arrows to add the icon, by opening the list and selecting "Pixel Inspector"*

    c. This brings up a window of pixel values that can be moved around the image. To adjust the parameters of this window, click **Prefs.** This can be used to adjust the size of the window.

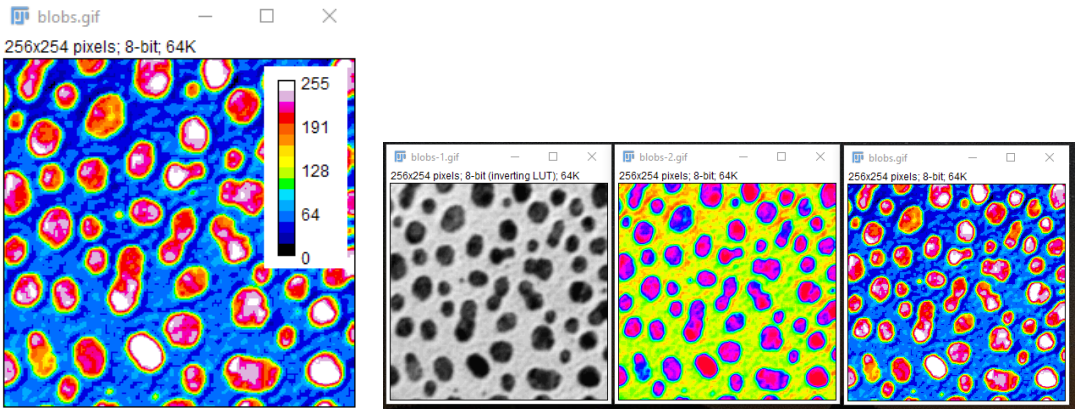


Inverting the background

1. Sometimes, the background may be white instead of black, depending on what you are interested in within the image. The color scheme can be inverted by selecting **Edit > Inverse.**

Changing the color scheme (LUT)

1. A lookup table, or LUT, control the colormap of the images. This can easily be changed using the **LUT** button. Try a few and see how the information in the background and foreground changes.
    a. Using **Image > Colors > Display LUTs** will display different types of LUTs.
    b. It is also helpful to include a scale/calibration bar when changing the LUTs. This can be added with **Analyze > Tools > Calibration Bar**



Colorblindness Consideration

To test how different images may appear to those with colorblindness, you can use the **Simulate Color Blindness** plugin if you have a RGB image. Mlp-viridis can be a good option as it is designed to be perceptually uniform (see https://imagej.net/imaging/visualization).

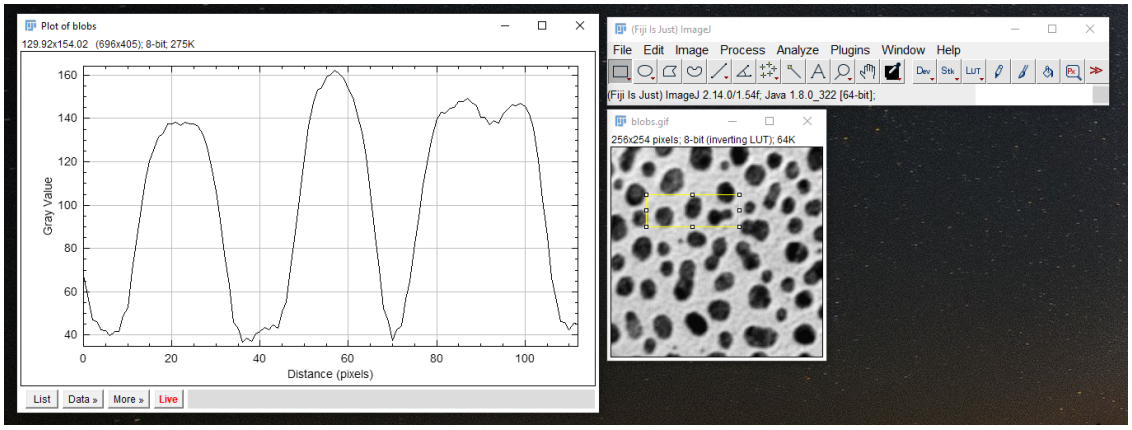
As a separate note, it may also be helpful to test any publication figures (images or otherwise) in grayscale, to see what color information may be lost if the paper is printed in grayscale. In many cases it may be more practical to develop images/figures in grayscale, especially for presentations, and it is still a valid representation of the data.

Plot/Line Plot

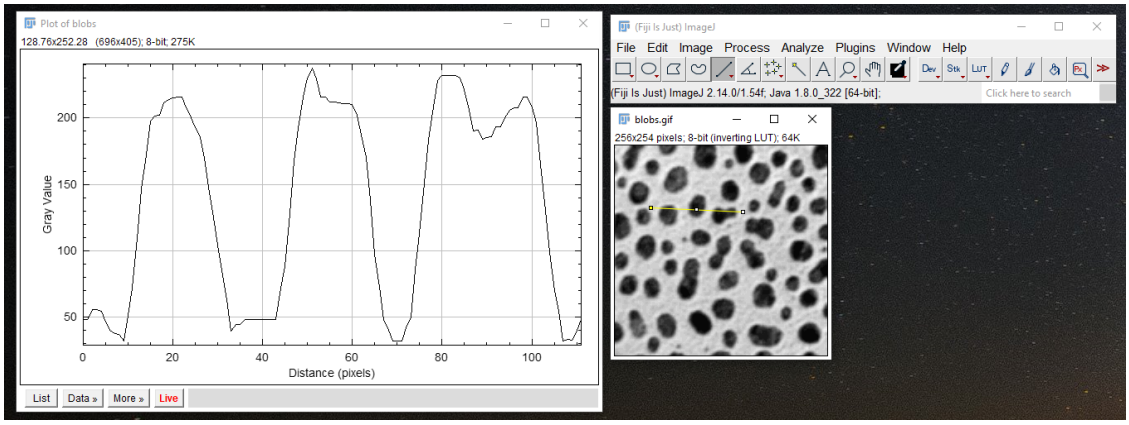
1. To see graphically how the pixel values vary across the image we can use the plot tool. This can be very useful for determining how much noise is in the background of an image, or for finding the width of an object, such as a point spread function.

    a. Draw a line or rectangle across the image and use **Ctrl + K** to display the plot. The **Live** button in the plot window can be used to create an active plot that changes when the selection in the image is changed.

    b. Here we can clearly see the increase in signal intensity across the three selected blobs. Since this is a rectangular selection, the values are averaged across the height of the rectangle.



    c. In comparison, the plot of a single line shows similar intensity changes when carefully drawn through the three blobs, but the plot is noisier and more sensitive to where the line is placed.



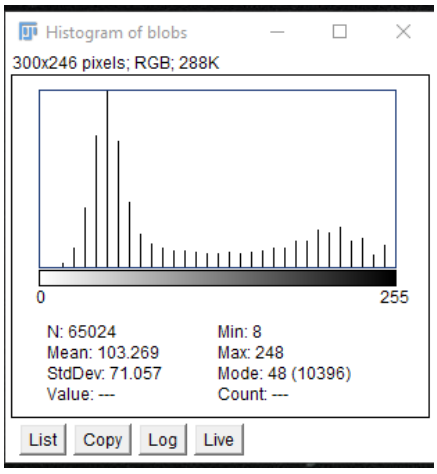
Background Subtraction

There are multiple methods to perform background subtraction in ImageJ, including **Subtract Background, Mean/Median Background Subtraction,** or **Gaussian Blur Subtraction.** ***However****,* any background subtraction used to process images should be carefully considered in relation to the original source of noise, how the image information is used, and any further processing or quantification of the image. In some cases the Subtract Background plugin, or other methods, can also introduce artifacts that are not present in the original image.

Some Imagesc forum discussion here:
https://forum.image.sc/t/consensus-on-subtract-background-built-in-or-other/7061

Segmentation/Thresholding

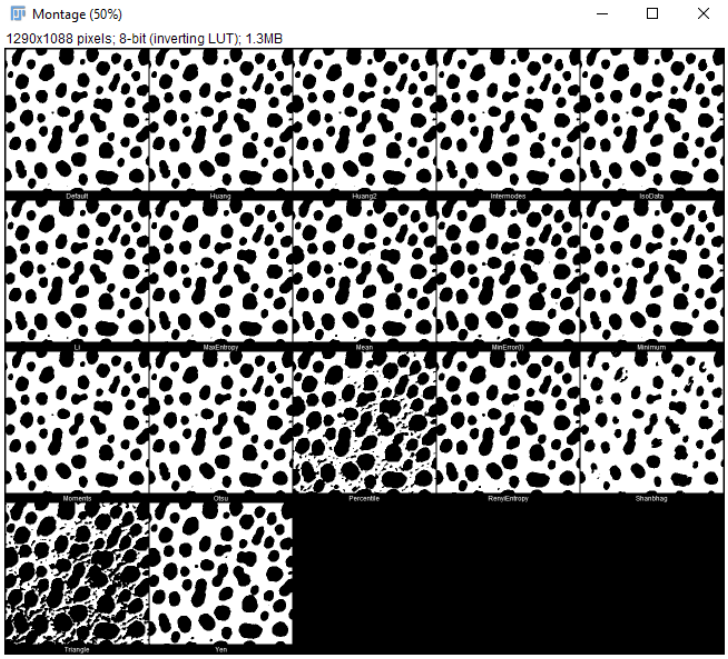
1. If we want to segment out the blobs we are interested in, one possible method is to use an intensity threshold. First, let's look at the histogram of the intensity values using **Ctrl + H.**



We can see that most of the background pixels have an intensity value around 50, whereas there is another grouping of pixel intensities around 210, which is likely the areas we are interested in because this image has a white background. Notice that the values are stretched outwards, this indicates that the original range was likely very small or somehow adjusted.

2. Use **Image > Adjust > Threshold** to see the automatic threshold applied based on the original histogram. This seems to segment the blobs pretty well. You can change the slider bars to see how the segmentation changes as the threshold moves. When ready, click **apply**, to create the binarized mask. Notice that the blobs now have a value of 255, and the background has been set to 0. This represents a **permanent change** in the image values, so going forward, most quantified analysis involving pixel values must be performed by applying the segmentation as a mask on the original image (see Masks section).

   a. There is also an auto threshold option, **Image > Adjust > Auto Threshold,** that you can use to compare different methods of thresholding. It is easiest to see the comparisons using the “Try All” method.



Adding Selections to ROI Manager

1. Press **T** to open the ROI manager.
2. Use **Edit > Selection > Create Selection** to select all of the blobs based on the current threshold.
3. In the ROI Manager window, use **More > Split** to divide the selection into multiple ROIs.
    a. You can click on the regions to see the specific blob it corresponds to. If needed, the selected regions can be saved in a file and reopened later using **More > Save**

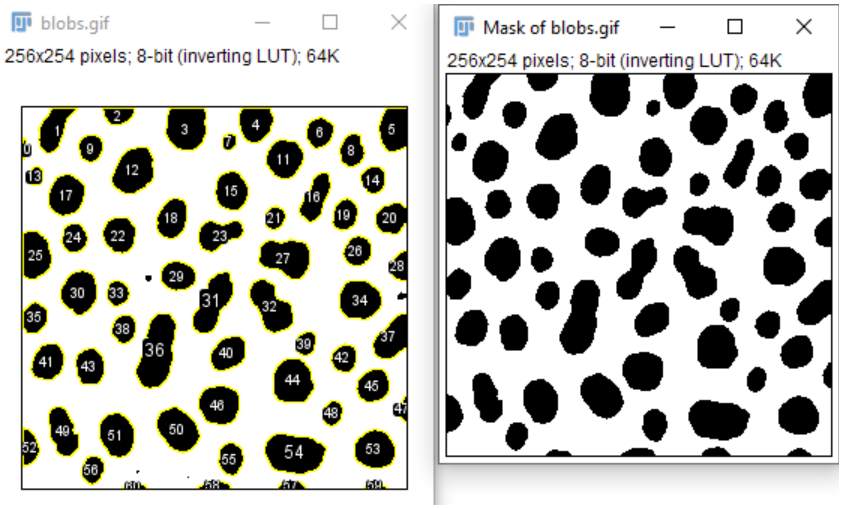
Analyze Particles

1. In this case, the Analyze Particles function can be useful for applying size exclusion, or removing any blobs that are below our desired size threshold. For example, we can see some smaller red circles in the image above that may represent noise rather than a full blob we are interested in. This can be especially useful if we have a biological problem, for example segmenting cells, where we know the cells must be above a certain size.
    a. Open the ROI manager by pressing **T**.
        i. If there are previous ROI selections that you would like to remove, click on the first one, hold shift, then click on the last one. This should be a simple way to select all of the ROIs at once. You can then press delete.
    b. Use **Analyze > Analyze Particles** to open the function window.
        i. The **Size** can be used to determine what size blobs are included. Here, we can specify **“30-Infinity”** to remove some of the smaller blobs in the mask. For many biological applications, it is best to know the size of the

pixels to know exactly how much area might be excluded, and if it corresponds to the correct size based on the experiment.

ii. Ensure the **Add to Manager** box is selected to add the ROIs to the ROI manager.

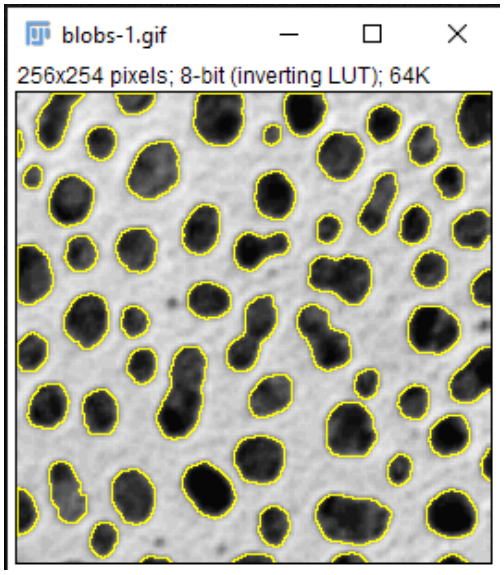
iii. For the **Show** dropdown, use **Masks** to display the following:



Notice that some of the blobs that are present in the binarized image, like the one next to number 29, are not included in the mask.

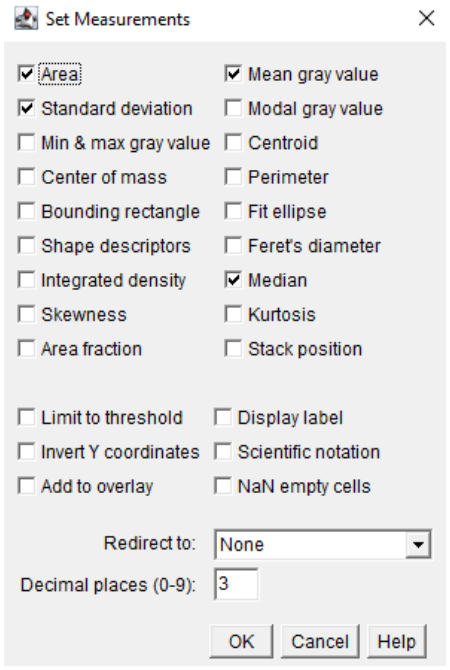
Masks For Measurement

1. Once you have a mask or the desired image, we can use the **Edit > Selection > Create Selection** tool to add the regions to the ROI manager.
    a. After selection, you can also use **Edit > Selection > Create Mask** to make a mask of the desired areas if the image is not already binarized.
2. To copy the ROI selection onto the original image, click on the mask while the selection is open, then click on the original image and press **Shift + E** or use **Edit > Selection > Restore Selection**



Making Measurements

1. To set the desired measurements to be collected use **Analyze > Set Measurements.** This window will determine what results are displayed or saved from the image.



2. To create the measurements, use **Analyze > Measure** or **Ctrl + M,** while the desired image is active.
    a. This may produce one measurement for the whole image if an ROI is not active. To measure all ROIs at once, use **Measure** in the **ROI manager**. The

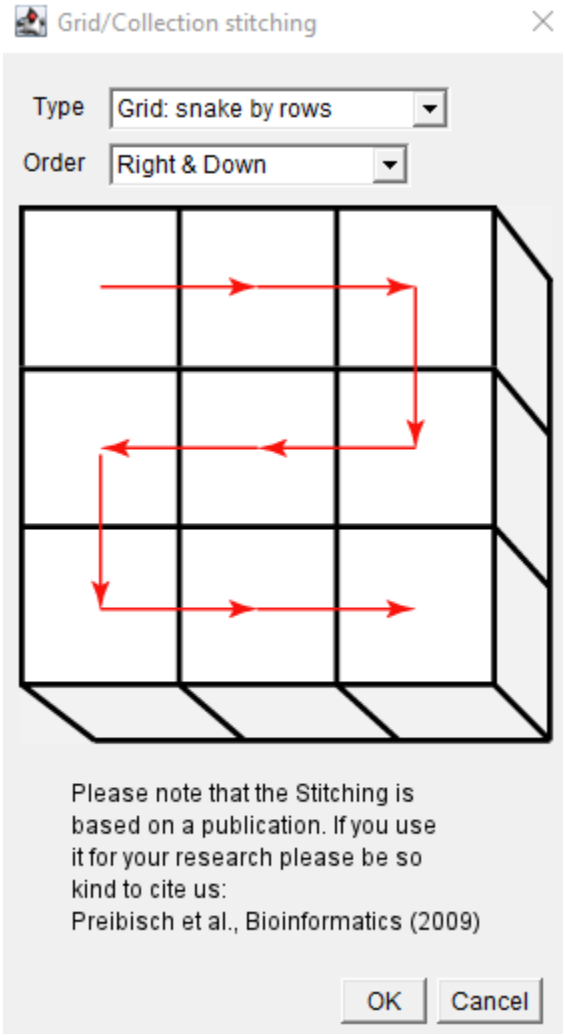
checkboxes for Show All and Labels can be useful to see what regions are being measured.

b. If you want, use **Edit > Selection > Make Inverse.** Notice this now selects the entire background. You can measure again to get the value for the background.

Stitching Images

There are multiple ways to stitch images using the **Grid/Stitching** plugin, including with metadata or file position.

1. Open the Grid/Stitching plugin. In this example, we will use “Grid: snake by rows” with the order of “Right & Down”



2. In the dialogue box, specify that the following parameters:
    a. Grid size is **x = 3 and y = 3**

b. **Tile overlap [%] = 0**. *Importantly,* this is a known value, as the image we are stitching was part of a larger image. In an experimental context, this may be part of the image acquisition settings during imaging.
c. The **first file index is 1**
d. Directory is to the **Leaf_stitch folder**
e. The file name is **leaf-{i}.tif**
    i. {i} specifies where in the filename to iterate through values. If this is written as {ii}, there will be an error because the first file is 1 and not 01.
f. **Uncheck the "Compute overlap" box** as there is a known overlap value.

3. Also try re-running the plugin with an overlap value of 10%. Notice how there are errors near the borders of the leaf and on the ruler (especially the faded 9 value), but errors at the center of the leaf may be harder to spot. This is why knowing the expected overlap value is very important, as spotting errors in experimental data may be even more difficult. In many cases, using stitching information from the image metadata is more helpful because the position information comes from the microscope.

Trackmate - Example from documentation

Ershov, D., Phan, M.-S., Pylvänäinen, J. W., Rigaud, S. U., Le Blanc, L., Charles-Orszag, A., … Tinevez, J.-Y. (2022). TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nature Methods*, *19*(7), 829–832. doi:10.1038/s41592-022-01507-1

Trackmate documentation and tutorials: https://imagej.net/plugins/trackmate/
The following demo can be accessed here:
https://napari.imagej.net/en/latest/examples/trackmate.html

1. Open the “trackmate_example_data.tif”
2. Run the Trackmate plugin **(Plugins > Tracking > Trackmate)**
3. Select “Next” as we don’t need to make any changes
4. Use the Laplacian of Gaussian filter (LoG)
5. Enter 17 as the estimated objet diameter and 0 as quality threshold, “ Next”
6. Select “Next”
7. For Initial Thresholding, verify you see 1496 spots selected, and select “Next”
8. Select “Next” as we do not need a filter
9. Use the “Simple LAP tracker”, then select “Next”
10. The linking max distance will be 8.3 microns, the gap-closing max distance is 5 microns, and the gap-closing max frame gap is 2 micron. Select “Next”
11. You can scroll through and see the proposed tracks for each cell. Select “Next”
12. Select “Next” again as we will not filter any of the tracks
13. Select “Next” twice more to finish the display, then select “Execute” for the final image.

Other Resources

Introduction to Bioimage Analysis (Pete Bankhead):
https://bioimagebook.github.io/README.html

ImageJ User Guide (Ferreira and Rasband):
https://imagej.net/ij/docs/guide/146.html

Introduction and Segmentation in FIJI:
https://www.youtube.com/watch?app=desktop&v=CZExS_mkGsQ

ImageJ Tutorials:
https://imagej.net/imaging/

COBA: Center for Open Bioimage Analysis (YouTube):
https://www.youtube.com/@cobacenterforopenbioimagea1864/featured

Scientific Figure Making with Fiji and Inkscape (Jan Brocher):
https://www.youtube.com/watch?v=F6ll37NOgXc

ImageJ Documentation: Built-in Macro Functions:
https://wsr.imagej.net/developer/macro/functions.html

ImageJ Macro Cheatsheet (Robert Haase, Github haesleinhuepf):
https://github.com/BiAPoL/imagej-macro-cheat-sheet/blob/master/ImageJ_macro_cheatsheet.pdf

Trackmate Information:
https://imagej.net/plugins/trackmate/

I2K Conference YouTube:
https://www.youtube.com/@I2KConference

Colocalization:

- NEUBIAS “Deconstructing Colocalization Workflows (video)
- A practical guide to evaluating colocalization in biological microscopy (publication)