ImageJ / Fiji Tutorials – Basics
Revised for 2018

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ImageJ and Fiji Citations and Sites

Sites to download ImageJ and related software

ImageJ download site: https://imagej.nih.gov/ij/download.html

Fiji download site: https://imagej.net/Fiji/Downloads

Cell Profiler: http://www.cellprofiler.org/

Icy: http://icy.bioimageanalysis.org/download

MicroManager: http://www.micro-manager.org/

A Note to Fiji Users

In this guide, I mostly refer to ImageJ and Fiji collectively as ImageJ. Most processes are the same across the two programs and typing ImageJ/Fiji got tiresome and used up space. If there is something different about or added to a process in Fiji (e.g. updating on pg. 3 or using a macro on pg. 11) I have highlighted that with a callout to Fiji users.

Citation for the Nature Methods paper on ImageJ

Schneider, CA, Rasband, WS, and Eliceiri KW. (2012) NIH Image to ImageJ: 25 Years of Image Analysis. Nature Methods 9(7): 671-5.

Places to go for expert help

The ImageJ Mailing List: https://imagej.nih.gov/ij/list.html

The ImageJ Forum: http://forum.imagej.net/

Both the Mailing list and the forum are monitored by a large community of ImageJ users and developers, including the creator of ImageJ Wayne Rasband and the Fiji development team, as well as a large number of plugin developers. Questions to the list and forum are usually answered within a day or two.

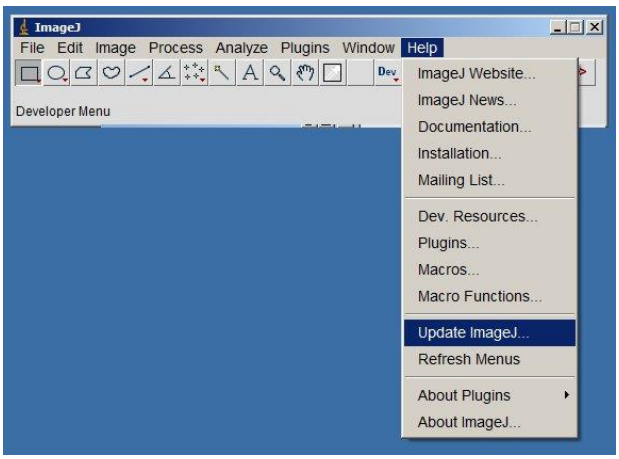
One Way to Site ImageJ in Your Manuscript

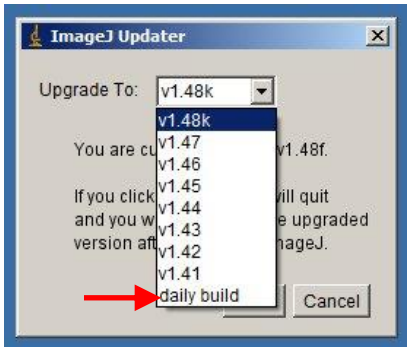
Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.

Updating the Software

Chances are good if you installed ImageJ from the Downloads page, you installed the latest *stable* version of ImageJ. This is not a bad thing, but it could be weeks or months behind the very newest build, which mostly likely came out within the last day or two. Yes, ImageJ is updated that often.

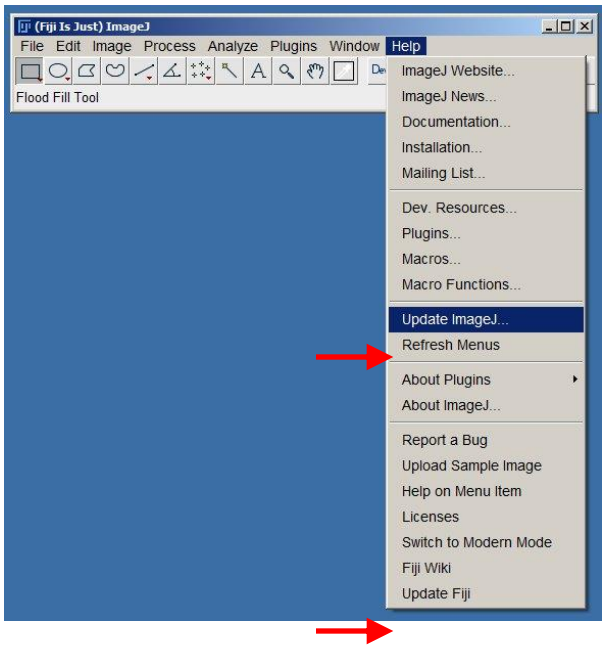
To update your copy of ImageJ, first make sure you have an internet connection. Then go to Help → Update ImageJ. The ImageJ Updater will pop up and tell you your current version of ImageJ. You can then choose to update to one of several builds, with the bleeding edge daily build at the bottom of the pull-down.



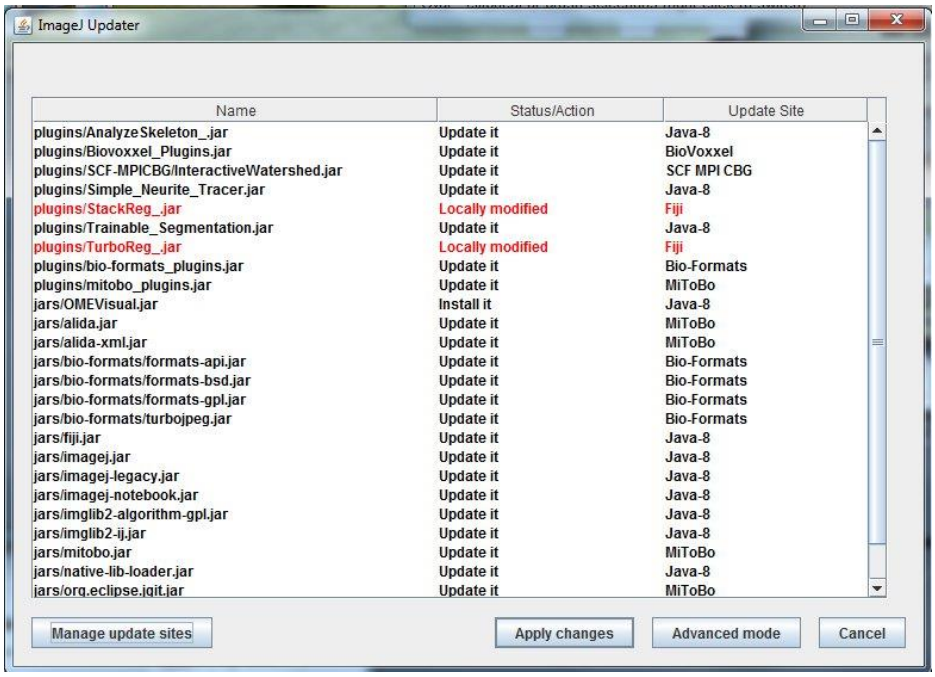


Use caution, the daily build may contain awesomeness, but it may also contain bugs! Choose the version you would like to install and it downloads and closes ImageJ automatically. Next time you open ImageJ you will have a shiny new version.

Fiji users: Fiji gives you two options for updates. One is to update just ImageJ, as we just did above (Help → Update ImageJ). The other is to update Fiji (Help → Update. . . or Help → Update Fiji depending on the version). If you choose to update Fiji, you update ImageJ and can also choose to update or install plugins from the Fiji Update Site.



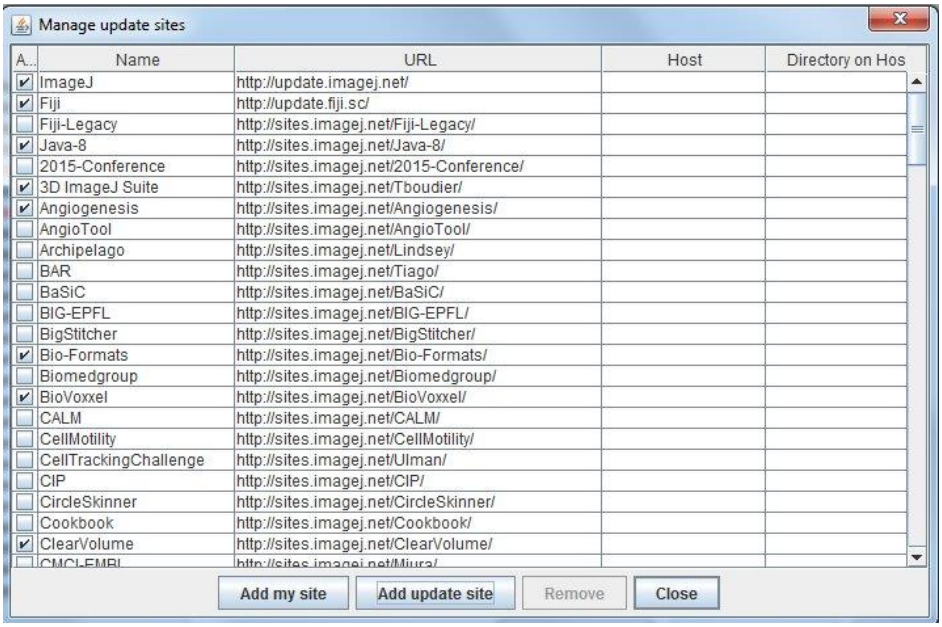
Updating / Installing Select Plugins (Fiji only)



Fiji users: If you choose the Help → Update. . . option, once the new version of ImageJ has been installed and you restart Fiji, you will have the option to install or update select plugins from the Fiji update site.

To do this, click "Manage Update Sites" at the bottom of the ImageJ Updater window.

This will take you to a new window called Manage Update Sites. To install a plugin, check the box next to that plugin. If a box is already checked, that plugin is installed in this version of Fiji. Any installed plugins will be updated automatically whenever a developer offers and update.



To un-install a previously installed plugin, uncheck the box.

Once you have selected the plugins you want, click "Close." You will now see a list of the files to be installed / updated / uninstalled in your ImageJ Updater Window (image see above). Any locally modified files (i.e. files you have updated manually and not thought the Updater) will appear in red to warn you that Updater will override the local changes.

Click "Apply Changes" and the files for the new and updated plugins will be installed automatically in their correct locations. You may need to restart Fiji a second time to see the files in your Plugins menu.

Installing a Plugin Manually (ImageJ or Fiji)

ImageJ is open source, so anyone can modify the Java-based code and create new tools. ImageJ is often extended with Plugins, which are additional tools that are added to ImageJ to extend its usefulness. Fiji comes with many of the most commonly used Plugins already installed and ready to use in the Plug-ins menu.

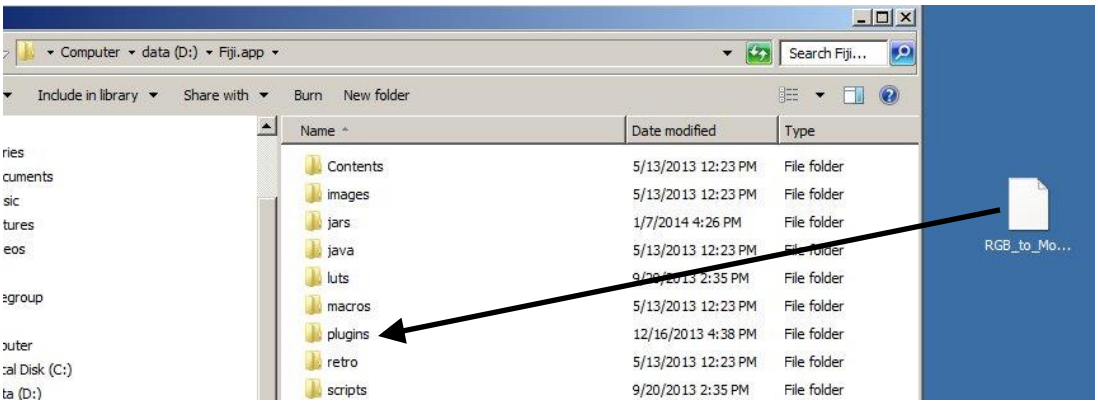
Plugins are different from macros (we learned about macros on page 11). Macros are scripts that make processes faster, but do not extend the function of ImageJ.

Installing a plugin is easy. In this example, we are going to install the RGB to Montage Plugin (with the RGB_to_Montage.class file).

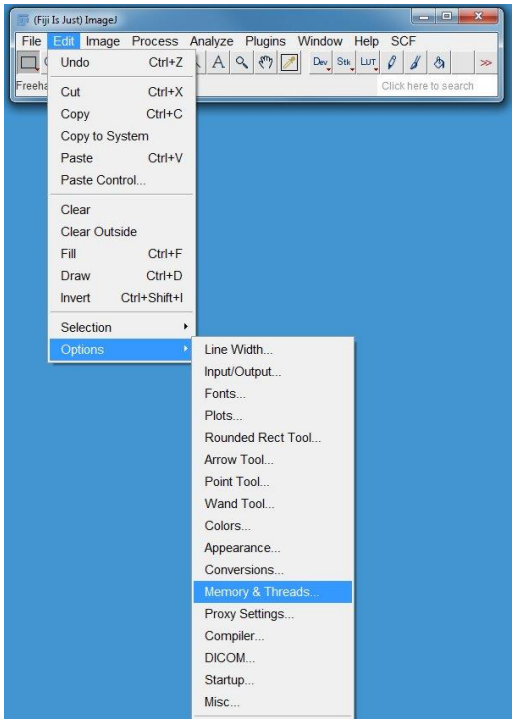
1) Download the .class or .jar file that is the plugin you want to install. The Core hosts a number of plugins in the ~ImageJ → Plugins folder on our servers. Other plugins, like the LOCI BioFormats plugin mentioned on page 26 are available free to download off the web.

2) Drag and drop the file into the plugins folder. You can make life easier by combining steps 1 and 2, downloading the plugin file directly into your ImageJ plugins folder.

3) If you have ImageJ or Fiji open, restart it. Check to see if your plugin appears in the Plugins menu. If not, choose Plugins → Install (for ImageJ) or Plugins → Install Plugin (for Fiji). Find your ImageJ → Plugins folder (where you installed the plugin file in step 2) and click on that file. Your plugin should extract / install and be visible in the Plugins menu without restarting ImageJ.



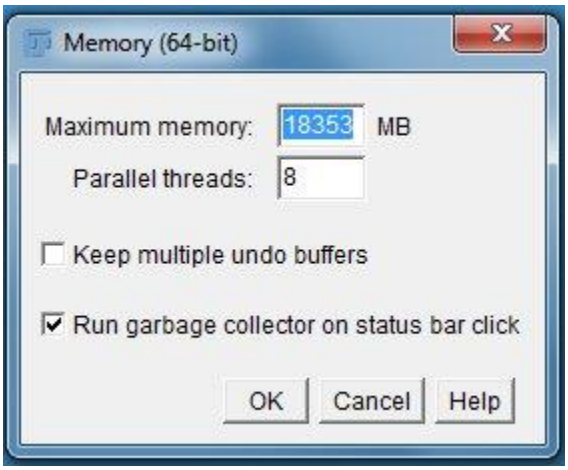
Increasing Available Memory



ImageJ automatically sets the maximum amount of memory available to 640MB. If you're working with large z or time lapse stacks, you should increase this, otherwise you may not be able to open and process your images. You can assign up to 75% of your computer's RAM for ImageJ, any more than that and you risk not having enough RAM for other programs / OS tasks.

Go to Edit → Options → Memory and Threads to set the amount of memory you would like to use for ImageJ.

If your computer has multiple processors or cores, you can take advantage of the "Parallel threads" option. A computer with 8 cores or processors will be able to use up to 8 parallel threads for image processing. This can speed up tasks if the routine or plugin you're using is programmed to take advantage of multiple cores (not all are).



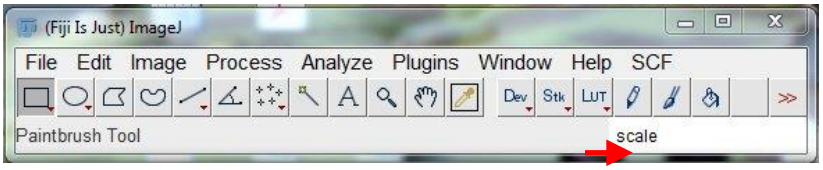
You will also see options to keep multiple undo buffers, which can increase the number of steps you can Edit → Undo (otherwise the default is ONE step back). This will still not make Fiji capable of undoing actions on stacks and the like, but will generally give you one or two more steps of Undo.

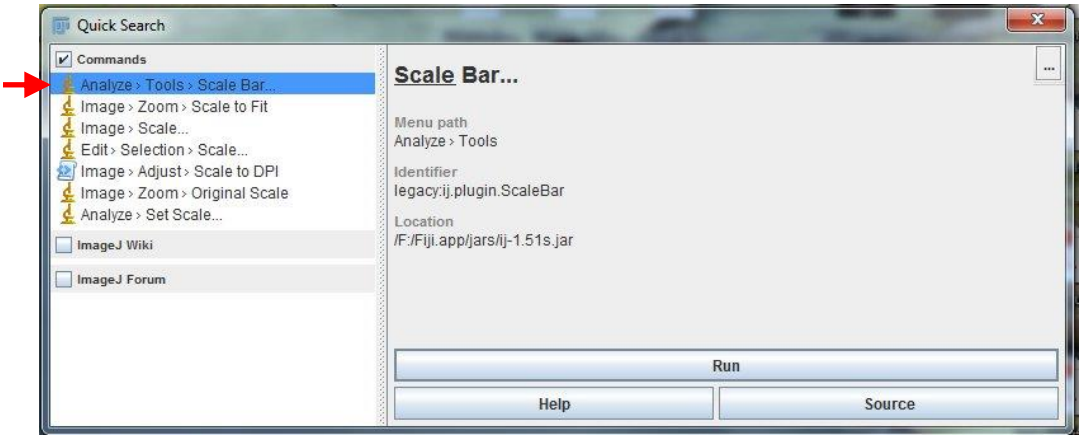
ImageJ runs on Java and is no efficient about releasing RAM once an image is closed or a process is finished. This can lead to low memory levels even when all images are closed and no processes are running. "Run garbage collector on status bar click" will sometimes release memory and keep tasks running efficiently, but sometimes restarting ImageJ is the only way to release memory and speed up processing.

The Command Finder

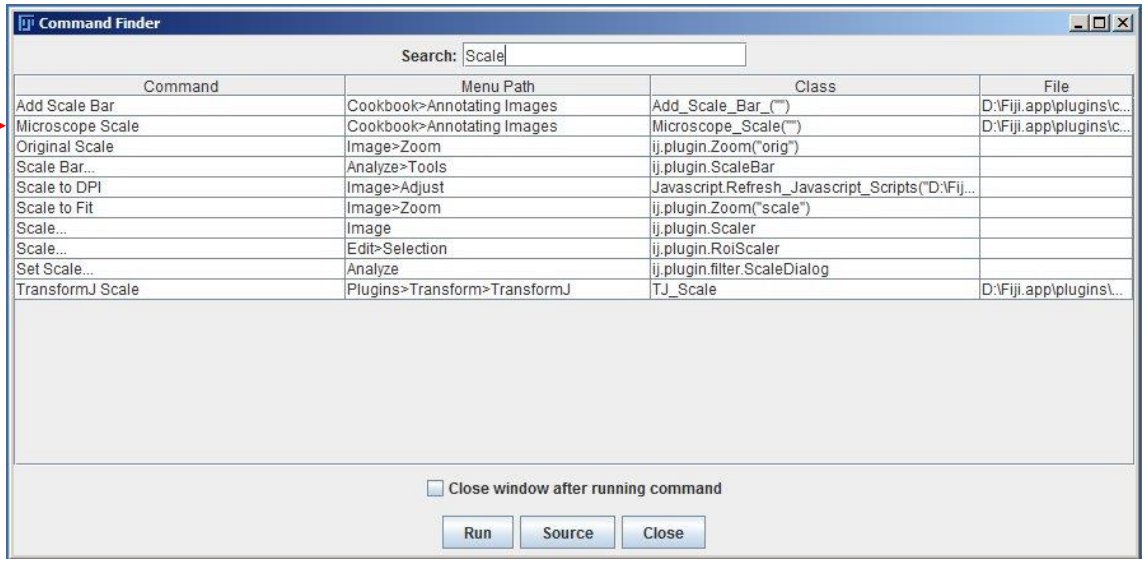
ImageJ has so many features it can be hard to keep track of all of them. Thankfully there is a guide to help you find the command you are looking for.

In Fiji version 1.51 and higher, type your search term in to the white box at the end of the taskbar and a new window will pop up with your search results





In ImageJ, The Command Finder can be brought up by pressing the letter L on your keyboard.

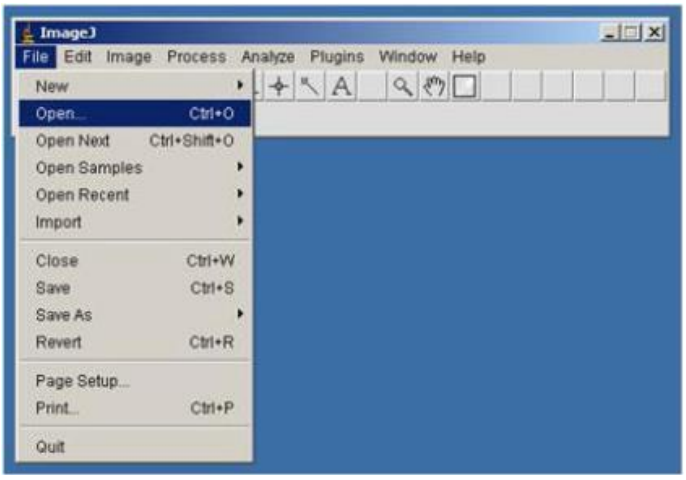


Command	Menu Path	Class	File
Add Scale Bar	Cookbook>Annotating Images	Add_Scale_Bar_("")	D:\Fiji.app\plugins\c...
Microscope Scale	Cookbook>Annotating Images	Microscope_Scale("")	D:\Fiji.app\plugins\c...
Original Scale	Image>Zoom	ij.plugin.Zoom("orig")	
Scale Bar...	Analyze>Tools	ij.plugin.ScaleBar	
Scale to DPI	Image>Adjust	Javascript.Refresh_Javascript_Scripts("D:\Fij...	
Scale to Fit	Image>Zoom	ij.plugin.Zoom("scale")	
Scale...	Image	ij.plugin.Scaler	
Scale...	Edit>Selection	ij.plugin.RoiScaler	
Set Scale...	Analyze	ij.plugin.filter.ScaleDialog	
TransformJ Scale	Plugins>Transform>TransformJ	TJ_Scale	D:\Fiji.app\plugins\...

For both Fiji and ImageJ, type the name of the command you are searching for in the Search box and ImageJ will list all of the commands with similar names. In this example, I was searching for the command to add a scale bar. The name of the command is in the far left column, followed by the menu path if you would like to run the command manually. You can also choose to highlight the command and then hit the "Run" button at the bottom of the screen to run the command.

Opening Standard File Types

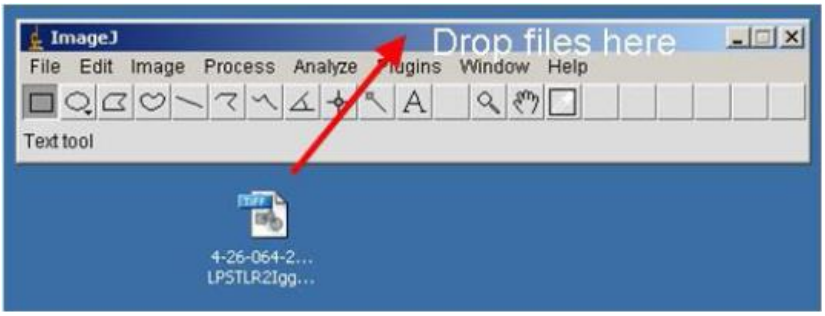
Standard file types include .tif, .tif stack, .jpeg, .gif or pretty much any other file type listed under the ImageJ File → Save As menu.



There are two ways to open standard file types: Select File → Open and choose the file to be opened (as with Photoshop or other software programs).

OR

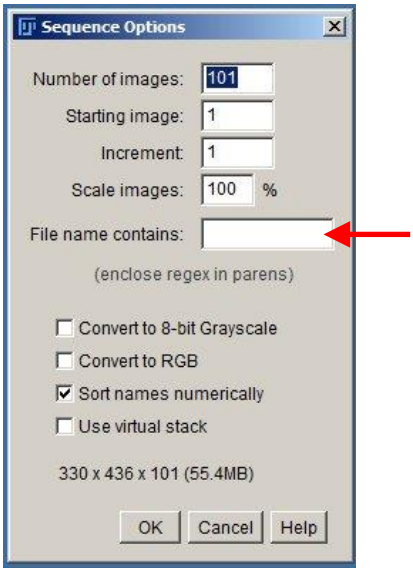
Drag and drop the file onto the task bar.



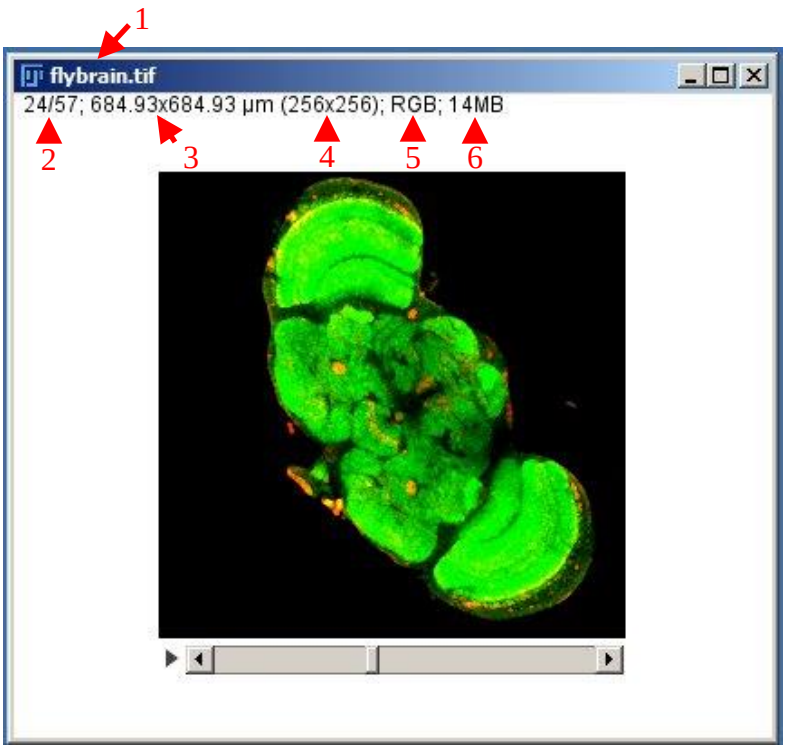
Import Image Sequence

If you have a group of single files that you would like to open in a stack, you also have two options. You can drag and drop the folder containing all of the files onto the taskbar, or, if there is more than one group of files per folder, you can use File → Import → Image Sequence. When you click on this option, go to the folder that contains your image and click on one of the images to be included in the stack. Click "Open" and a new "Sequence Options" window will appear.

In the section called "File name contains", type or paste in the part of the file name that is common to all files in your group (for example, if you have a group called CellsImage_ch00, CellsImage_ch01, etc., then type CellsImage into the box).



The Information at the Top of your Image



Once you open an image or stack of images, notice the information at the top of the image window.

1) The top line contains the name of the image. This is the “flybrain” sample image from the ImageJ library.

2) On the next line, you will see “24/57”. This means we are currently looking at slice 24 of a total of 57 slices in this stack. If we had a hyperstack (see pg. 28), there would be numbers for the color channel, z-slice and/or time point, depending on the information in our stack. If we had an image that was a single slice, these numbers would be missing and the information would start with image size.

3) “684.93um x 684.93um” is the actual image size, or you can think of it as the size of the field of view. The units on this value depend on how the image is calibrated (see pg. 19). In this case, the units are microns. If the image is NOT calibrated, the units will be pixels and should match the next number.

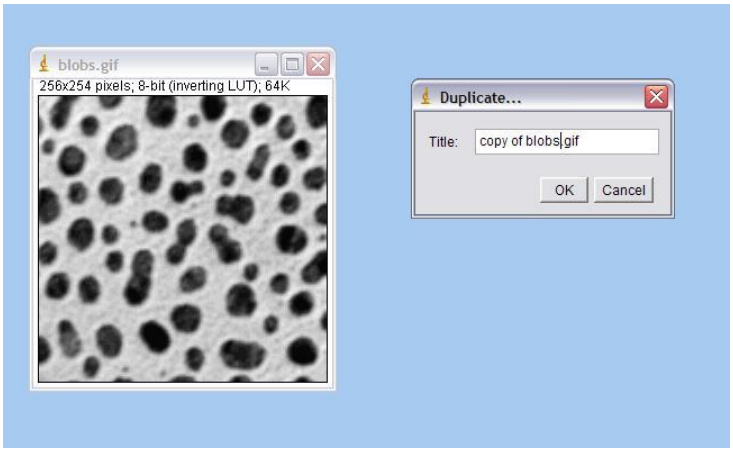
4) “(256x256)” is the image size in pixels. This number is always in parentheses and always has units in pixels, whether the image is calibrated or not. If the image is not calibrated, these numbers and the numbers before (#3) will match.

5) “RGB” is the image type. This image is an RGB color image. It is a composite of three 8-bit grayscale images, one for red, one for green and one for blue. Images can also be 8-, 12-, 16- or 32-bit grayscale. Note: if you have a 12-bit grayscale image, ImageJ will list it as a 16-bit grayscale image. I do not know why this happens.

6) “14MB” is the amount of memory your image takes up on your hard drive. You will notice that .tifs take up more memory than compressed file types like .jpgs and .gifs. .tifs are large, but they are the best file type for preserving your data, as they are non-lossy. They do not throw away any information from your image. With compressed file types, data is lost during the compression.

Duplicating All or Part of an Image or Stack

Creating a copy of an image is useful, especially if you plan to make changes to your image and want a way to compare back to (or just preserve) your original. Use Image → Duplicate to create a copy of your image. This copy can be named whatever you want in the "Title:" box, making it easy to keep track of potential changes.

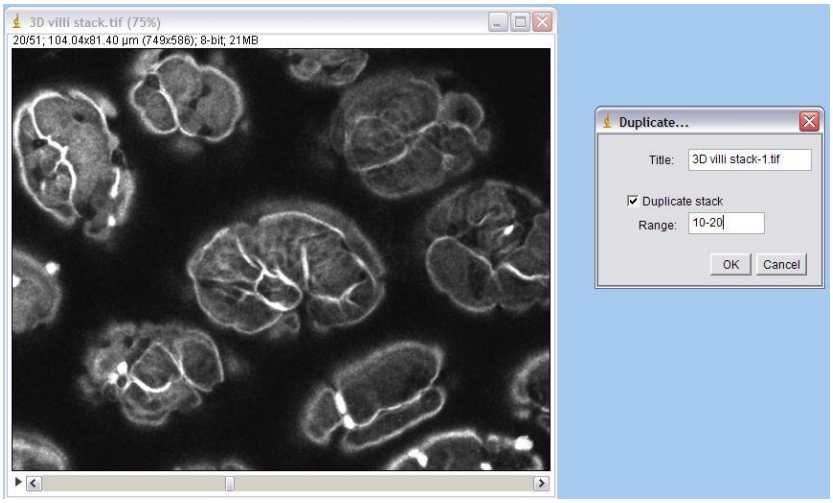


Hit "OK" and a new window will open containing a copy of your image.

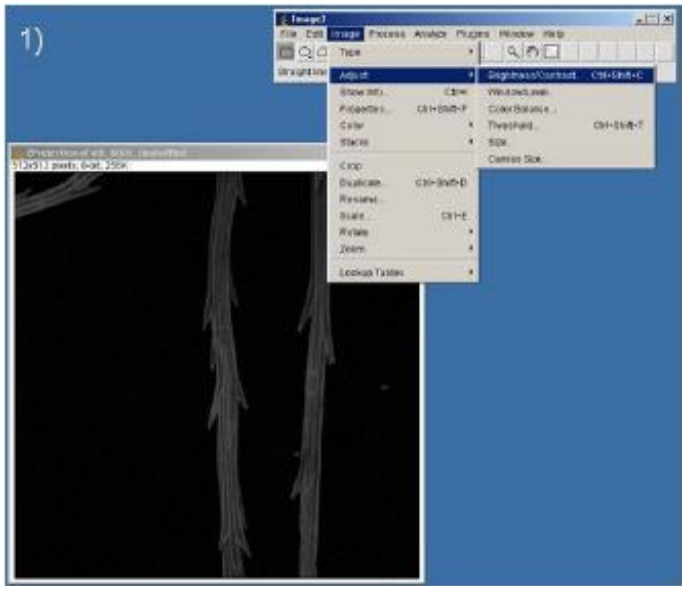
If you only want to copy part of your image, you can draw a region (pg. 11) and then Image → Duplicate will only copy out the portion of the image inside that region. For regions that are not rectangles, ImageJ will square off the image, giving you portions of your image that are not inside the region boundary. If it is critical to only have the area inside the region, you can use Edit → Clear Outside to set pixel values outside the region to 0.

If you are working with a stack, check the box next to "Duplicate stack" or you will only duplicate the current slice.

When duplicating stacks, you have the option of creating a substack by duplicating a range of slices. If you want the whole stack, it is not necessary to specify a range.



Adjusting Brightness and Contrast



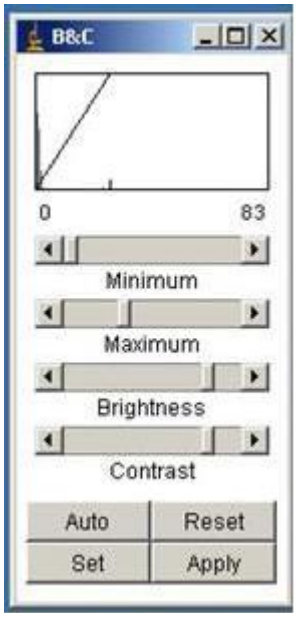
1) Choose Image → Adjust → Brightness / Contrast

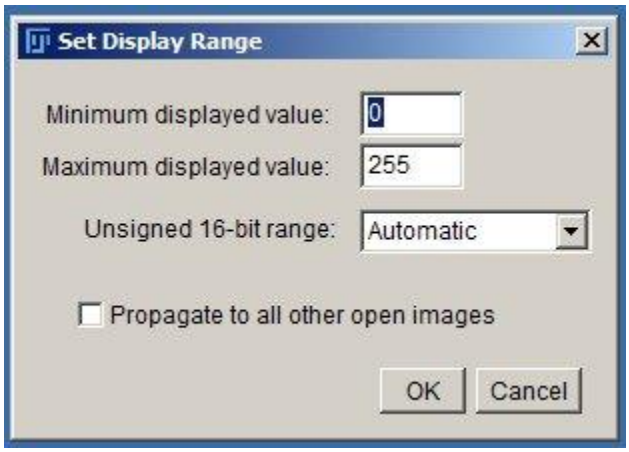
2) A new window will appear with four sliders for brightness and contrast.

3) To cut off low intensity pixels, slide the top slider to the right. To increase brightness, slide the second slider to the left. These two sliders set either the bottom or the top of the display range.

Adjusting the “Brightness” (third) slider adjusts both the top and bottom brightness values displayed without changing the size of the range displayed. The “Contrast” (fourth) slider also changes both the top and bottom brightness values, but in a way that increases or decreases the size of the displayed range. When using these two sliders, keep an eye out for saturation and clipping. You can easily push too many pixels to the top or bottom of the range.

The “Reset” button resets the image to its original (full) display range. It is effectively an undo button. Note this button does not undo if you have hit “Apply.”





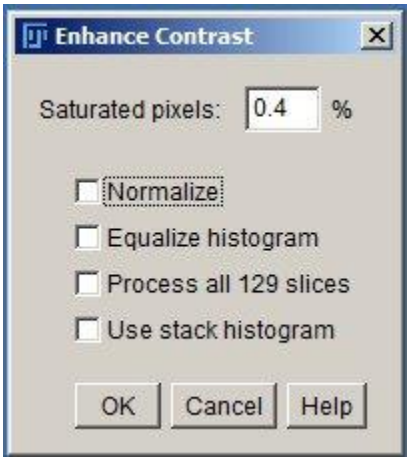
4) The “Set” button opens the “Set Display Range” window. This allows you to type in values for the top and bottom of the display range. This can be faster than using the sliders if you know the values you want and allows you to propagate these values to all of the other images open in ImageJ (a quick batch adjustment mode). However, with the sliders, you can preview how your image will change as you adjust. With the “Set” option, you have to hit “OK” before the image will change. The only way to go back after this is Edit → Undo.

IMPORTANT!! -- None of the changes made to the Brightness and Contrast of the image effect pixel intensity values UNLESS you hit the “Apply” button. Applying any change will alter the histogram and the pixel intensity values of your image. With stacks, a window will pop up where you can choose to apply the brightness and contrast to the whole stack or just to the current frame. There is NO UNDO if you choose to apply brightness / contrast adjustments to the whole stack, even with a multi-undo buffer.

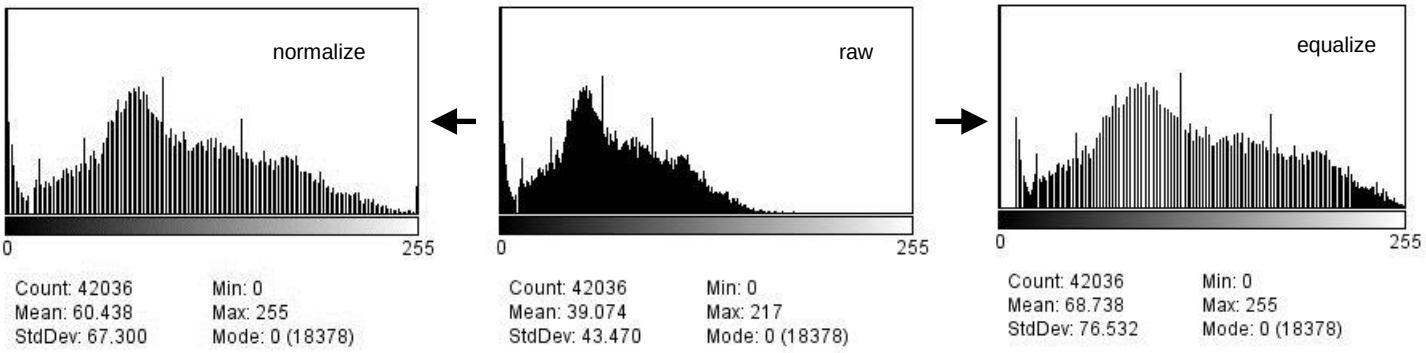
5) The “Auto” button chooses the best display range, based on saturating 0.35% of the pixels in your image. Again, this does not alter your pixel intensities unless you hit “Apply” after the auto adjustment.

If you want more control over the percent of pixels saturated, you can use the Process → Enhance Contrast option.

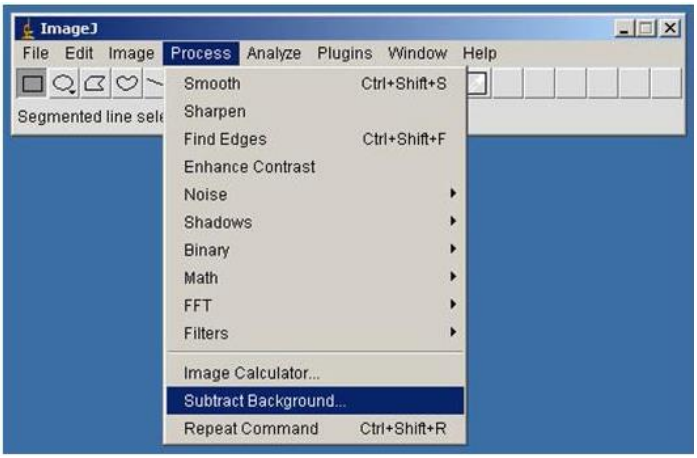
Using Enhance Contrast with stacks, you will get options to process all the slices in the stack with the values from the current slice or to process the stack with values from a histogram that takes into account values from all the slices in the stack.



In this option, both "Normalize" and "Equalize histogram" will stretch the histogram to the full range allowed by the image type (similar to the "Auto" adjustment under Brightness and Contrast). Normalize is a linear contrast stretch, which may distort values near the very top or very bottom of the histogram, but otherwise keeps the relationship of gray values relative to each other the way the raw data does. Equalize is a NON-linear method of stretching the histogram which can distort gray values across the histogram when compared to the raw data.



Subtracting Background with a Rolling Ball



If the pixel intensities in your image don't cover the whole display range, the brightness and contrast sliders can be a good way to change the display of your image and get it to look the way you want.

If, however, your image does cover the full display range and you have a smooth, continuous layer of low intensity values that interfere with your signal pixels, particularly if you are trying to threshold, use object segmentation or quantify your image in some way, you may want to consider background subtraction.

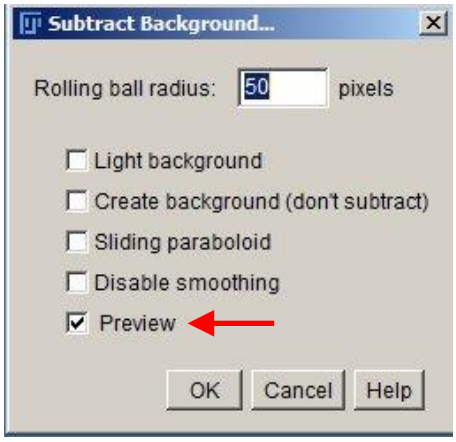
For a description of how the rolling ball method works for background subtraction, see the ImageJ documentation page at https://imagej.nih.gov/ij/docs/guide/146-29.html#sec:Process and scroll down to Subtract Background.

To use the rolling ball:

1) Choose Process → Subtract Background

2) A new window will appear with a place for a "Rolling ball radius." The radius will always be in pixels, whether your image is calibrated or not.

3) The smaller the radius, the greater the amount of intensity subtracted from the image. Try using the "Preview" option so you can see what's happening to your image. If you have histology or brightfield images, you will want the "light background" option. If you have fluorescence images, you do NOT want this option.

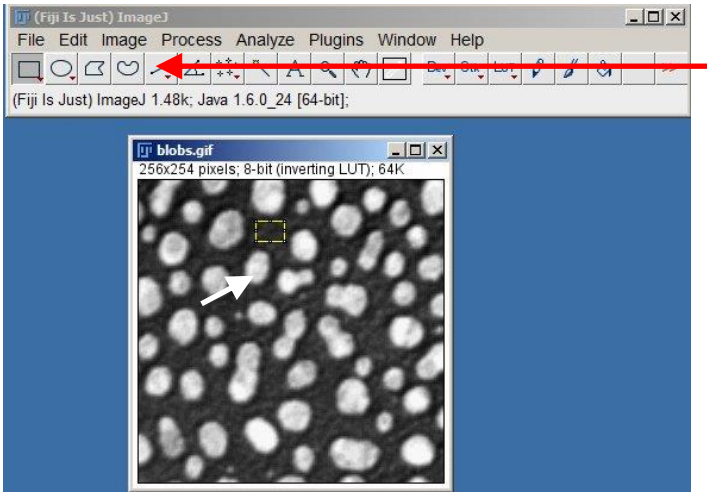


Subtracting Background with a Region of Interest

What if you simply want to select a region that contains your background and have ImageJ subtract the average value of that background region from the whole image?

There are two ways to subtract background using a region of interest: manually or with the BG_Subtraction form ROI macro. For single images, either will work. For stacks, the macro is much faster.

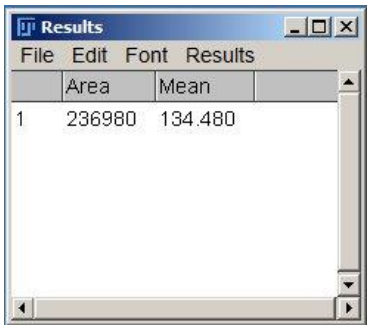
Manually subtracting background from an ROI



1) Using one of the four shape drawing tools from the taskbar, draw a region in an area of your slide you want to use as background.

2) Go to Analyze → Set Measurements and make sure "Mean gray value" is checked. For this exercise, none of the other checkboxes are necessary.

3) Go to Analyze → Measure. You should get a new window called Results that looks something like this:



4) Click back on your image window. Click in a part of the window that is away from your region to erase the region. If you don't do this, the background will only be subtracted from the region, not the whole image!

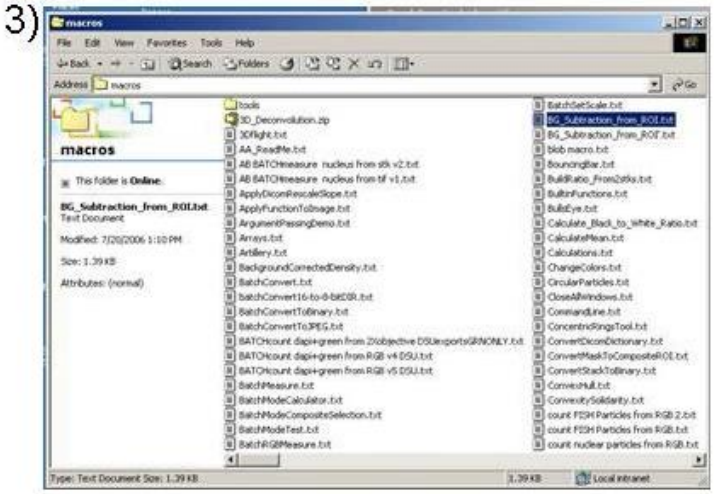
5) Go to Process → Math → Subtract and type in the value from the "Mean" column of your Results table. You can use the "Preview" checkbox to see what this will do to your image before making the change permanent.

Using the BG_Subtraction from ROI macro

A macro is a script that automates a process, making the process faster. There is a macro for automatic background subtraction from a region of interest written by Michael Cammer. Installing and using this macro (and macros in general) is easy.

1) 

2) 

3) 

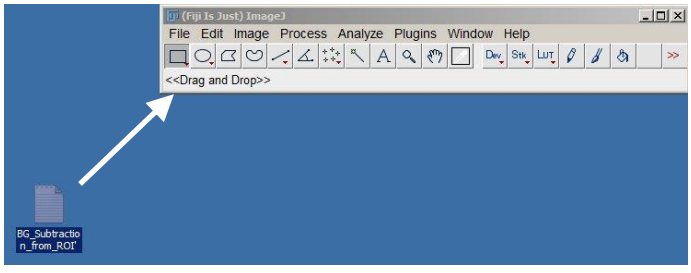
1) The macro file for this particular function can be found in the ~ImageJ folder on either of our data servers.

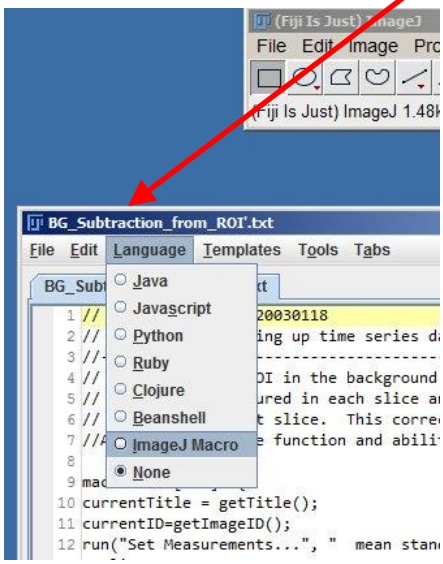
2) Go in the ~ImageJ folder (near the top of the list) and open the macros folder.

3) Find the macro BG_Subtraction_from_ROI.txt. Copy this text file to your computer.

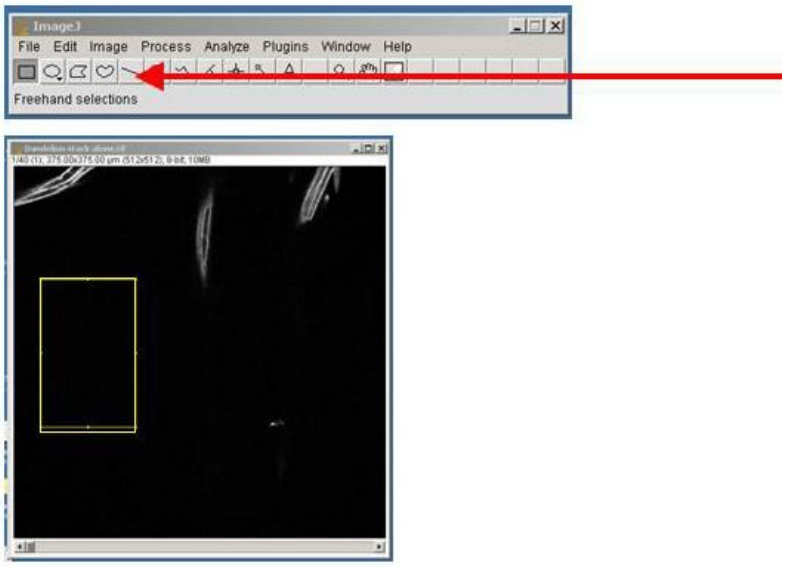
To open the text file, drag and drop the .txt file icon onto the ImageJ toolbar, as you would to open an image.

This should open a new window with the heading BG_Subtraction_from_ROI.txt. Fiji users: select Language → ImageJ macro from the menu on the macro window (this is not necessary in ImageJ).





4)

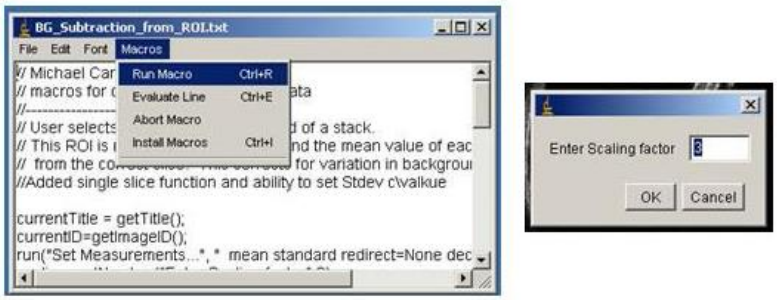


4) Use a shape drawing tool to draw a region of interest on your image, in the area you want to use as background. If you are subtracting background from a stack, make sure there are no objects in this ROI in any plane.

5) Once you have drawn your ROI and checked it for objects, go to the macro window and choose Macros → Run Macro.

6) The macro will determine the mean pixel intensity of the region and subtracts that value (multiplied by the value of the scaling factor) from every pixel in the image.

5)

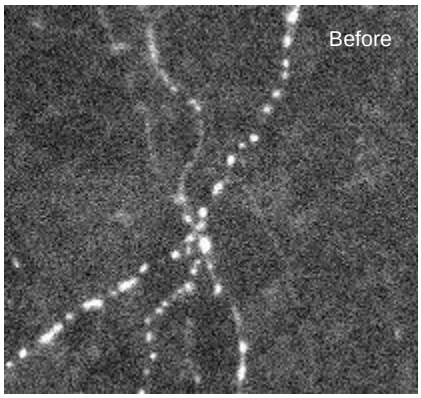


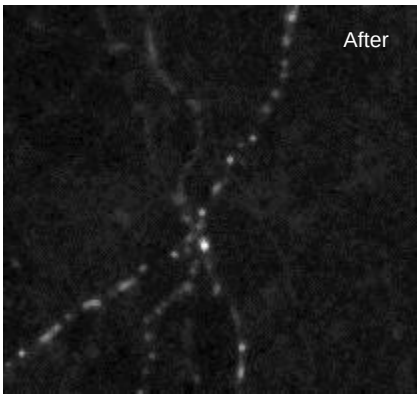
The default scaling factor is 3. If you do not want a scaling factor, use a value of 1. Note that for stacks, this macro also includes a correction factor for slice to slice variation, which determined by the standard deviation of the background value.

Filtering – Some Commonly Used Filters

Filtering can be a good way to smooth out unwanted pixel noise or highlight features of your image, and are typically used as part of a cell counting or signal quantification protocol. Here are some of the more commonly used filters, which work on single images or stacks.

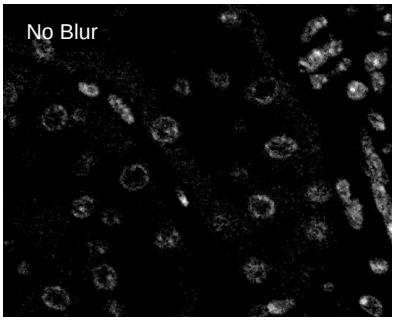
Process → Noise → Despeckle

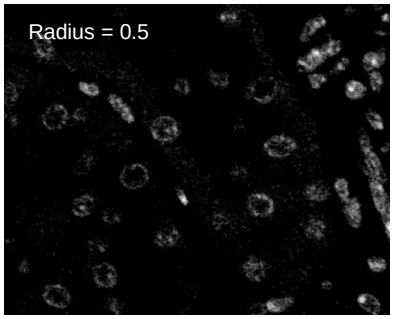


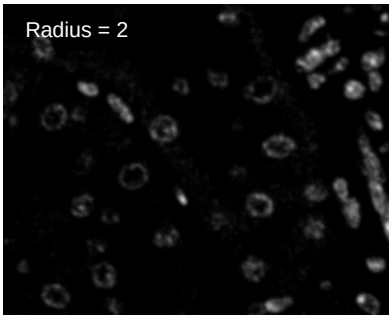


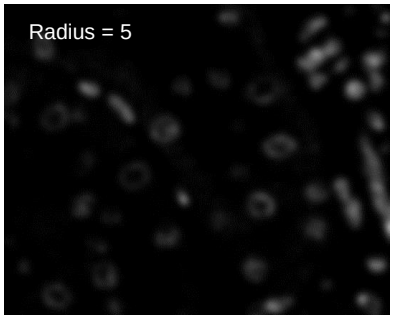
Good for getting rid of "salt and pepper" noise: i.e. single pixels that are bright but not part of your signal.

Despeckle is a hybrid median filter with a kernel of 3. For other kernels, you can use Process → Filters → Median. The larger the kernel of your filter, the more smoothing / blurring effect you will see. This action can be undone on single images but not stacks. There is no preview option.







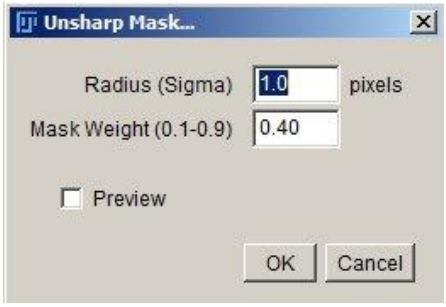


Process → Filters → Gaussian Blur

Blurs an image. Commonly used before setting a threshold when the objects being selected are not creating continuous surfaces. The larger the pixel radius, the more blurred the image gets. A pixel radius less than 1 can be used.

Checking the preview box allows you to see what will change before making the change permanent.

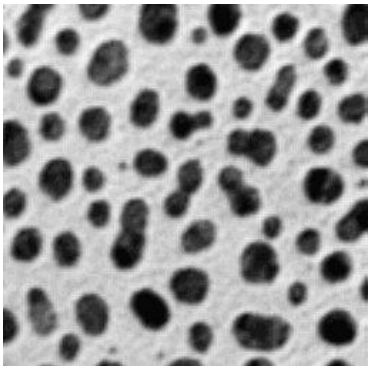
Process → Filters → Unsharp Mask



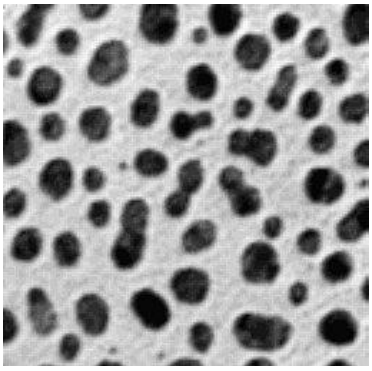
Used to highlight the edges of objects. Creates a blurred copy of the image and then subtracts the blurred copy from the original.

The radius of the mask is the radius of the Gaussian blur that is applied to create the blurred version.

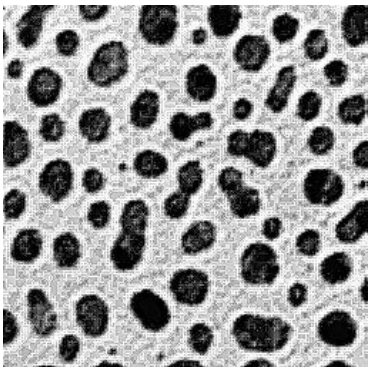
The mask weight determines the amount of subtraction, the higher the number, the greater the amount of subtraction and the greater the edge enhancement.



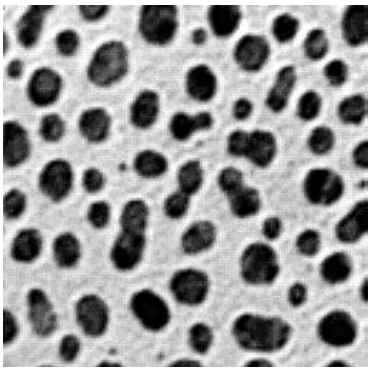
No unsharp



Radius 1, Weight 0.4



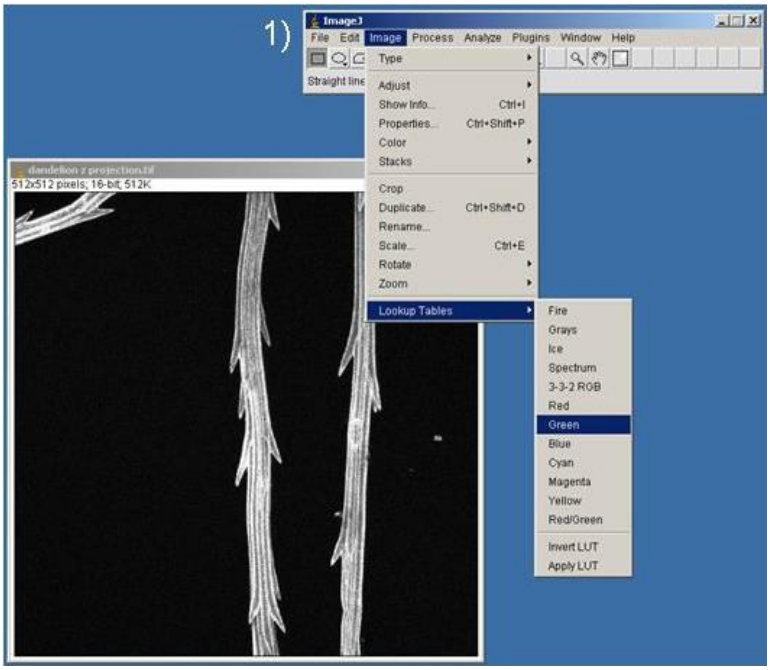
Radius 1, Weight 0.9



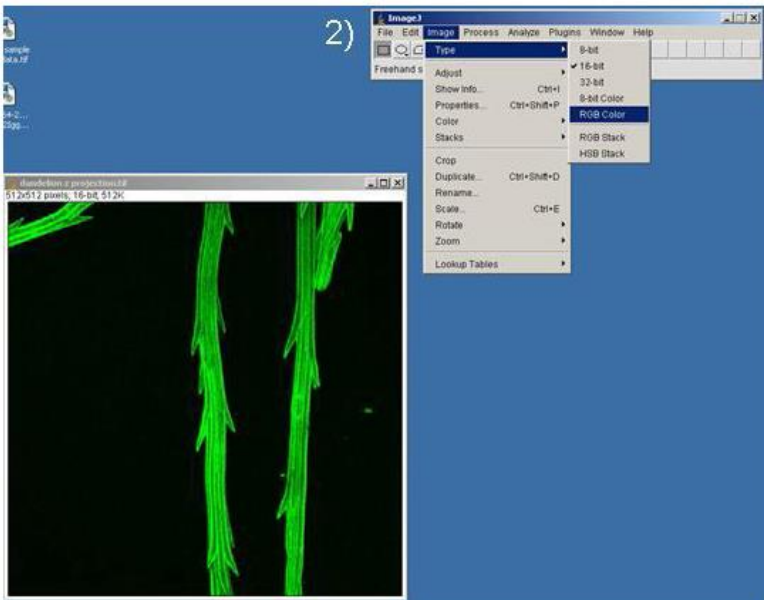
Radius 3, Weight 0.4

Applying Color to Single Color (Channel) Images

If your images have been saved in grayscale and you want them in color for inserting into a Photoshop or PowerPoint document, here's how to convert a single color (single channel) image or stack.

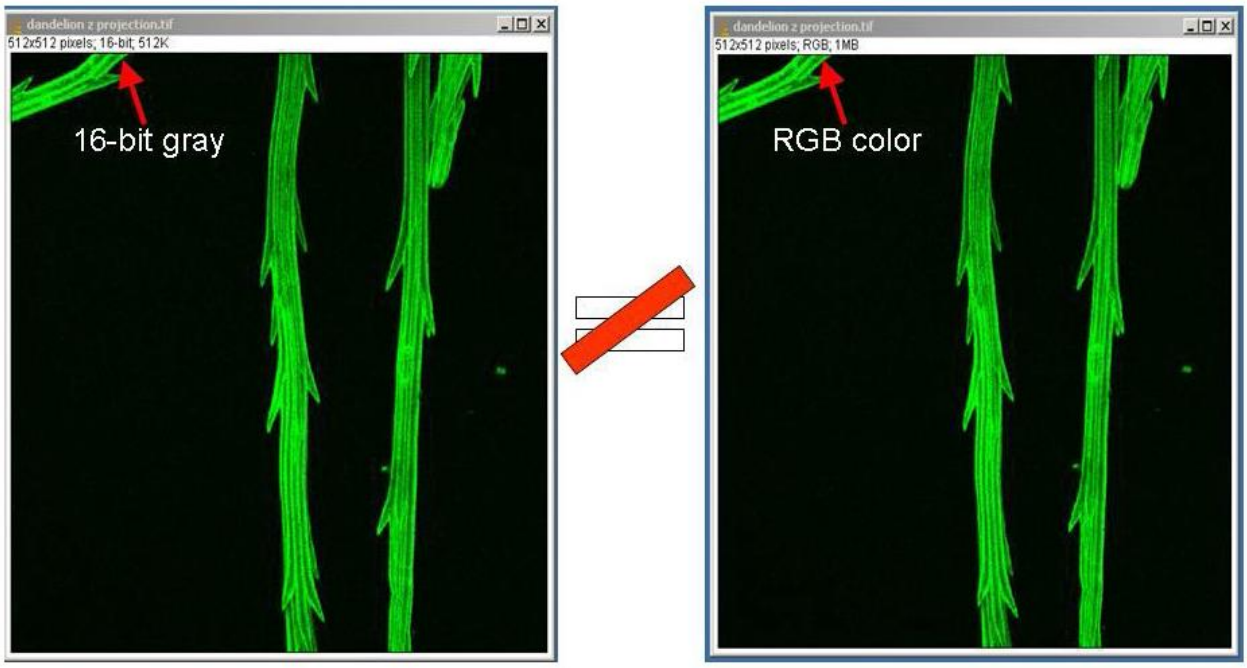


1) Choose Image → Lookup Tables → your color of choice (here it is Green). Fire, Ice, Spectrum and the choices below the second line are "hot" LUTs; they assign different colors to different pixel intensities and can be fun / informative. Image → Lookup Tables → Invert LUT flips the order of the colors in a hot LUT.



2) Once you have selected your color LUT, choose Image → Type → RGB Color. This will change the file type so that the color information goes along with the file. Now the file can be opened in color in any program.

If you open an image in ImageJ and it is already in color, take note of the information at the top of your image. An 8- 12- or 16- bit grayscale image with a color LUT is NOT the same as an RGB image! If you do not convert your colorized image to RGB it will not open in color in any other program.

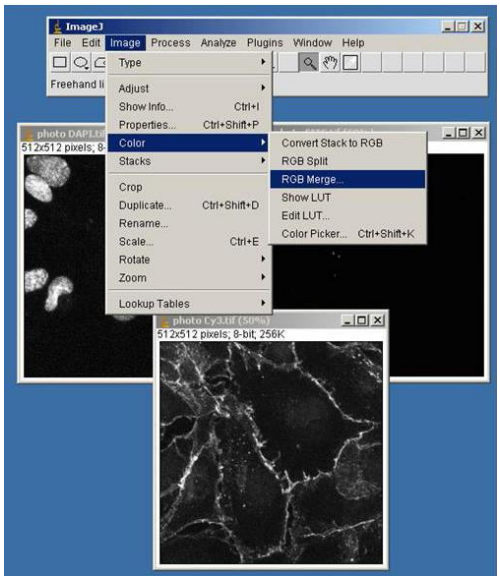


You can change the color of a grayscale image with a color LUT applied, just use the steps outlined above.

If your image is a single color channel but in RGB format and you must change the color, use Image → Type → 8-bit grayscale to convert it to grayscale first, and then add a different LUT.

Merging Multiple Color Channels

Many times we have images with multiple colors (multiple channels), but they are saved as single grayscale images. Merge them into multi-color images with these easy steps. This process also works to create stacks of multi-color images when each color is in its own stack.



1) Open the images or stacks you wish to merge. These files should be the same physical size (in this case 512x512), have the same number of slices per stack and be the same image type (in this case 8-bit grayscale).

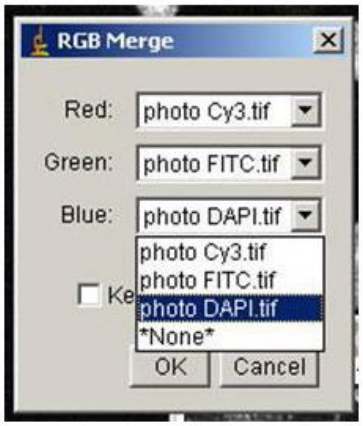
The original images do NOT need to be in RGB color or have a color LUT applied for this to work.

2) Choose Image → Color → Merge Channels

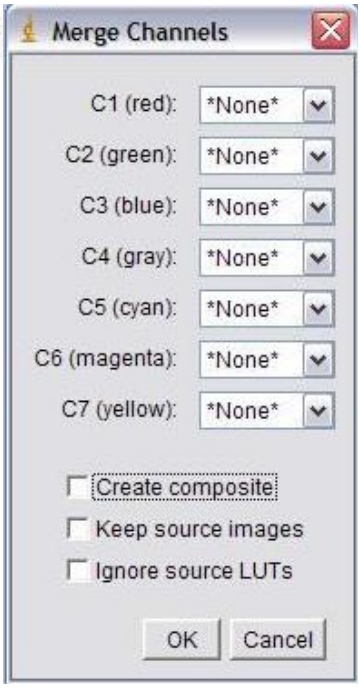
3) A new "Merge Channels" window will open. Use the pull-down menus to assign an image to each color. Newer versions of ImageJ and Fiji (shown in 4 below) have many more options than the old Red, Green and Blue (below, 3).

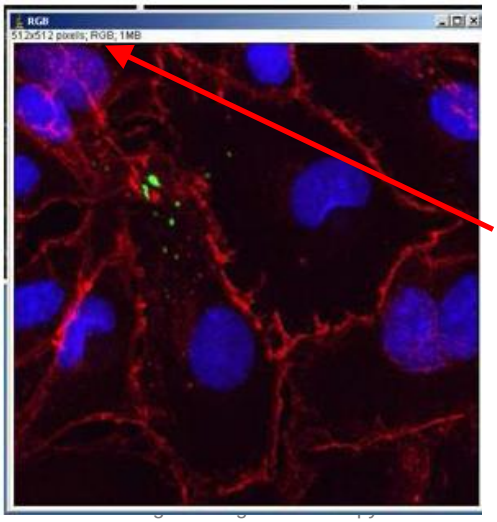
4) "Create Composite" will create a hyperstack (pg. 28). "Keep source images" keeps the original files open. Useful when making montages or if you have done a lot of work prepping your images and you haven't saved them yet. "Ignore source LUTs" works for images that have LUTs applied, but you want them in a different color in the merge.

3)



4)

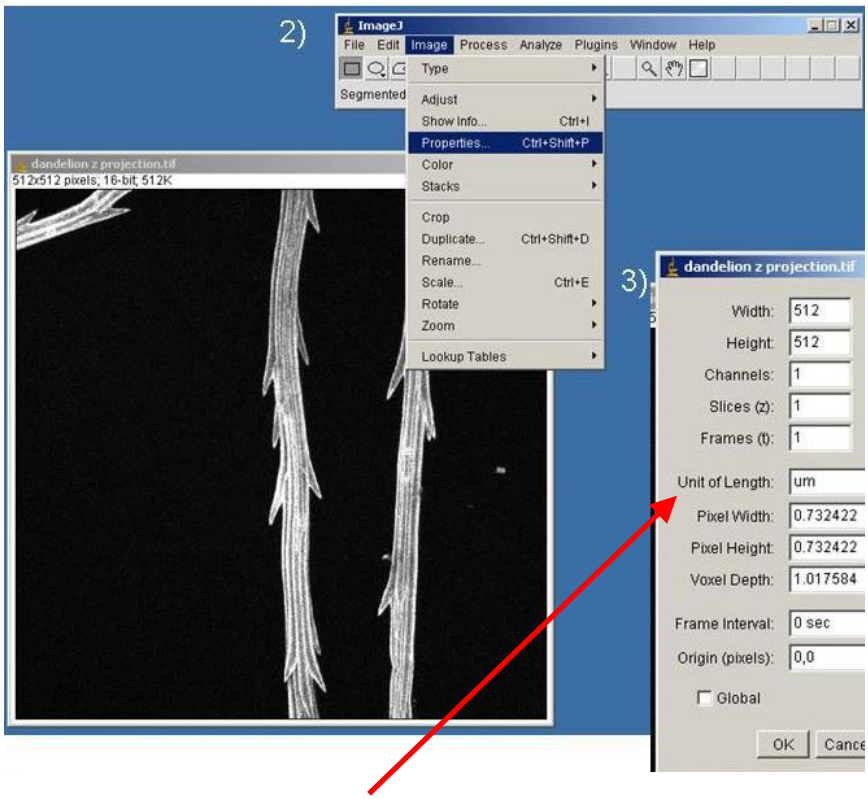




5) Click "OK" and your merged image will appear. This merged file is automatically an RGB type file and can be saved with File → Save as → file type of your choice without loss of color.

Calibrating an Image for Measurements or Scale Bars

If you know your pixel size (from a table or the software)



1) Open the image or stack

2) Choose Image → Properties

3) Input the correct pixel or voxel size information for your image.

Pixel size will depend on the microscope you are using and the magnification factor (objective plus any zoom applied during imaging). This information is sometimes found in a text or log file that is created by the capture software along with the image.

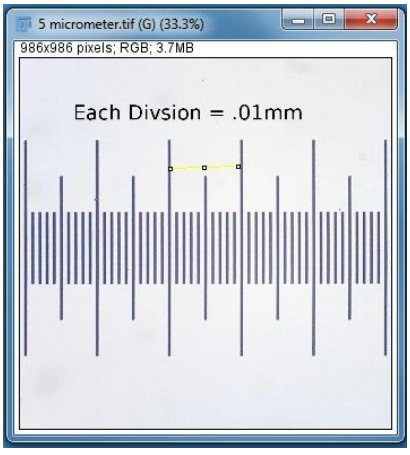
Saving the image as a .tif or .tif stack will save these values into the image metadata, so they will be there when you re-open the image in ImageJ.

Size, area, distance, etc. measurements made on a calibrated image will be given in units matching the unit of length you entered. Uncalibrated images give measurements in pixels.

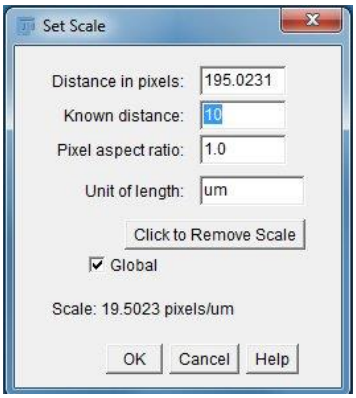
If you are measuring your pixel size with a micrometer

1) The image(s) of the micrometer and the image(s) of your sample should be taken with the same conditions (microscope, camera, objective magnification, zoom factor) otherwise your measurement will not be accurate.

2) Open the image of the micrometer. Use the line draw tool to draw across a known distance in the image (here each division is 1um and we drew across 10 for a total of 10um).



3) Choose Analyze → Set Scale. . .

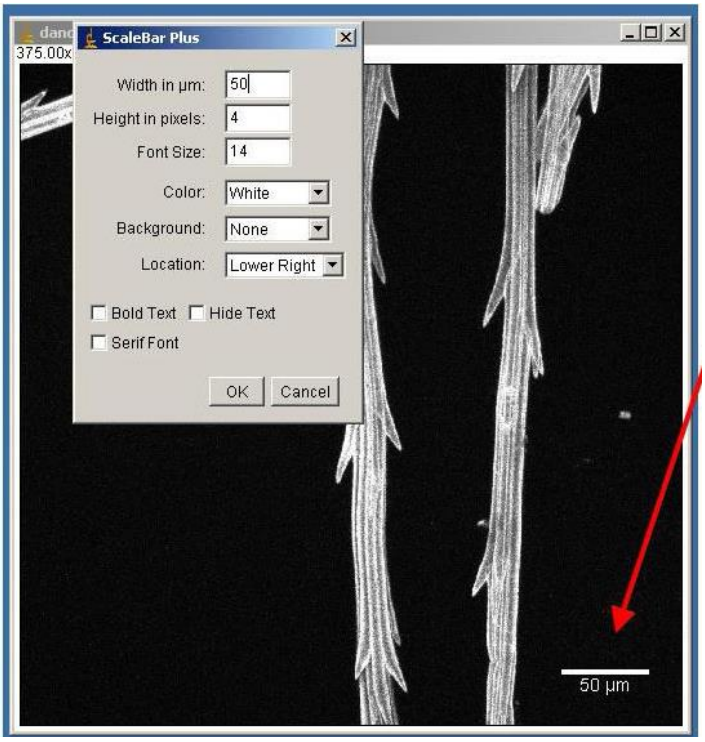


4) Do NOT change the distance in pixels. Input the known distance and unit of length for your micrometer (in our case 10 and um. Check the "global" box to have this calibration applied to all open images.

Adding a Scale Bar

1) Open your image or stack. Check your image calibration under Image → Properties to make sure it is correct and in the units you want. If not, calibrate your image with the steps on page 23.

2) Choose Analyze → Tools → Scale Bar.



3) Adjust the width, height (thickness) and other properties of the scale bar to your liking. You will be able to preview changes in the image window.

If you want the scale bar to be somewhere other than one of the corners, BEFORE you bring up the scale bar menu, use the rectangle draw tool (pg. 11) to draw a box on your image where you would like the scale bar positioned. Once that box is drawn, bring up the Analyze → Tools → Scale bar dialogue. Choose "At Selection" from the position pull-down menu. The bar width will fill the box, but you can make width, height and other adjustments to get the bar you like.

Scale bar color – If you have an RGB color image, you can have your scale bar in whatever color you want. If you have a grayscale image you can only have bars in white, black or shades of gray unless you choose the new "Overlay" feature (not shown in the image but in newer versions of ImageJ and Fiji it will appear with the boxes at the bottom). Overlay will write the scale bar in a layer on top of your image, instead of burning it into the image itself. This will give you a color scale bar on a grayscale image and also helps to keep the text from becoming distorted if you transfer the image to another program.

CAUTION – Once you click "OK" you can Edit → Undo right away, but if you don't, the scale bar becomes a permanent part of the image and cannot be erased, even using the "Overlay" mode.

Making Montages with ImageJ

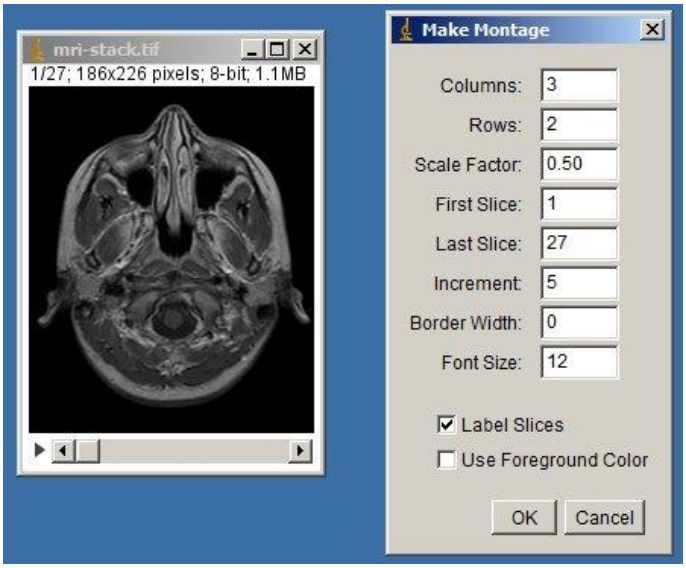
Montages are popular for presentations and papers, because they show a lot of information in a small amount of space. ImageJ does a good job of preparing images to be put into figures and making quick montages.

Make a Montage from a Stack of Images

This is easy if your images are already in a stack, such as a time lapse or Z (volume) stack. If your images are not in a stack, create one by opening the images you want to use for your montage and then choosing Image → Stacks → Images to Stack. Images will be put into the stack in the order that they are opened or created, and the order in the stack determines their placement in the montage with the Make Montage tool. To re-arrange your montage, see the link to instructions for the Magic Montage tool below.

1) Open or create your image stack.

2) Choose Image → Stacks → Make Montage. If you images are not in a stack you will get an error message at this point.

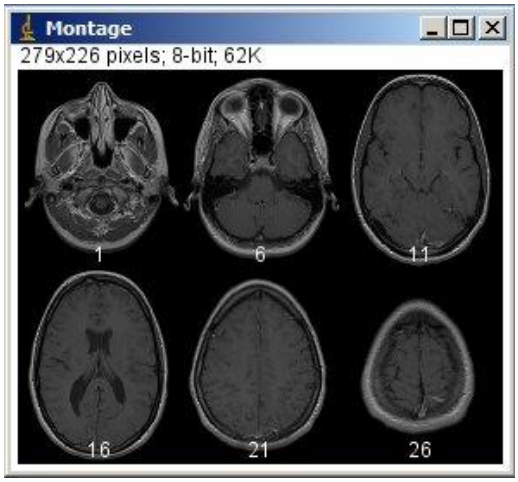


3) Specify how many columns and rows you want your montage to have. The product of rows and columns must be equal to or greater than the number of images you want to include; otherwise some images will be left off.

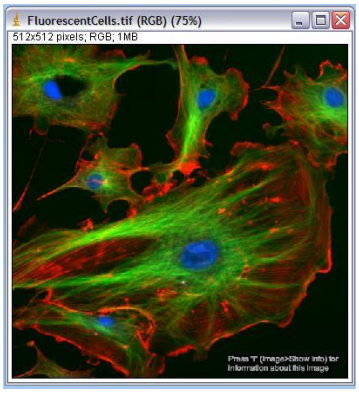
"Scale factor" determines the amount of shrinkage each picture will undergo. In this example, each image in the macro is half the size of the original. For the highest possible image quality -- and possibly a giant montage -- choose no scaling with 1.

"Increment" will allow you to choose every nth slice. This is particularly useful for long timelapses where it's not necessary to show every frame.

"Label slices" will add the slice number to the montage (see the image at right). If you created a stack and chose "Use Titles as Labels" in the "Images to Stack" window, your image titles will be the labels. Text is white unless you choose "Use Foreground Color," then the color is determined by the foreground color set under Edit → Options → Color.



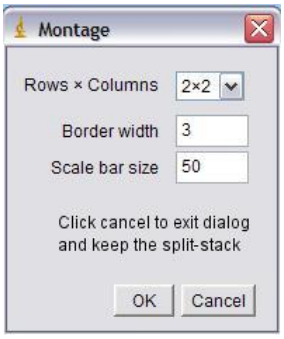
Quick Color Montage with RGB to Montage Plugin (plugin required)



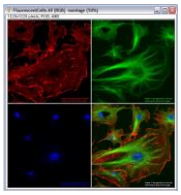
1) First, you must install the RGB to Montage plugin, which is available on the UChicago microscopy server under ~ImageJ → plugins → 0 RGB stuff or with the McMaster Biophotonics version of ImageJ (http://www.macbiophotonics.ca/downloads.htm).

To install the plugin, copy the RGB to Montage .jar file into the ImageJ plugins folder on your computer. You can also choose to install the entire RGB stuff folder. Either way, you must re-start ImageJ for the RGB to Montage plugin to appear under the Plugins menu (see pg. 21).

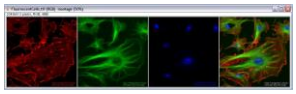
2) Open an RGB image that has multiple colors, such as the FluorescentCells.tif file shown here. This file comes from File → Open Samples. With the RGB image of your choice open, choose Plugins → RGB to Montage (or Plugins → 0 RGB stuff → RGB to Montage if you installed the whole folder).



3) A new menu window will open. From the pull-down, choose the format you want for your montage. A 2x2 selection will give you a square box with 4 panels, like this:



and a 1x4 selection will give you a linear montage, like this:



A 4x1 selection will give you a montage like the 1x4, just in a line going down instead of across. If you don't have an even number of images, in the square case there will be a blank box, in the linear case it will not matter, your line will just be shorter or longer.

4) Border width changes the thickness of the lines between the images, and scale bar size is the size of the scale bar that will be put in the merged image. NOTE: the original RGB image must be properly calibrated (Image → Properties) for this bar to be an accurate size. If you don't want a scale bar, type "0".

Make more sophisticated montages or re-arrange your panels with the Magic Montage toolset.

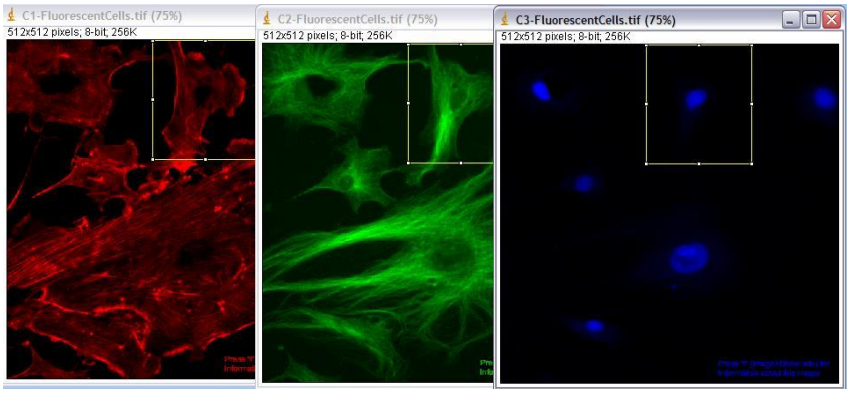
Download and tutorial links are on the ImageJ Macro Toolsets page at http://rsbweb.nih.gov/ij/macros/tools/MontageShufflerTool.txt

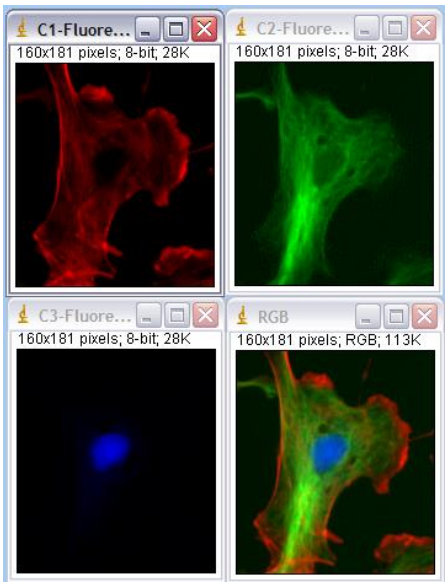
Cropping Multiple Images with the Same Selection Boundary

Figures for manuscripts are often made in other software programs (Photoshop, Illustrator, etc.) because these allow for multi-panel figures, and for text, graphs and other objects to be added to the final image. However, there is a cropping trick in ImageJ that is very useful for making figure montages. It will crop all images in a group to the same size and from the same place on the image.

1) First open all the images in the set to be cropped. This can be a red, green and blue set, CFP/YFP, fluorescence stack plus DIC image, whatever. It will work on .tif, .jpg, .gif and .tif stacks.

2) Select the area you want to crop on one image using one of the selection tools (rectangle, circle, polygon, freehand, see pg. 11). Click on the name bar of another image to select that image. Choose Edit → Selection → Restore Selection (Ctrl + Shift + E). The selection should appear on the second window. Continue selecting and restoring until all of your images have the bounding box drawn on them.





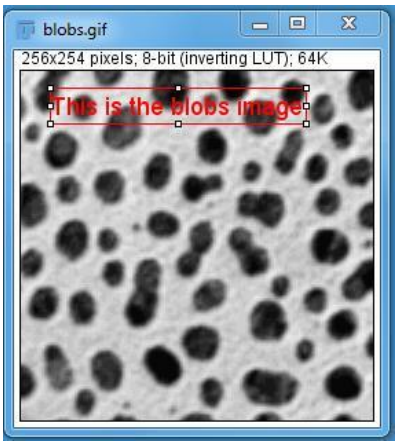
3) Crop the images by selecting either Image → Crop (Ctrl + Shift + X) which will crop the original image, or Image → Duplicate (Ctrl + Shift + D) which will create a new window with the cropped image and leave the original intact.

Cropping does not change your pixel size, so if you have calibrated your image (pg. 23) this pixel size is still correct for the cropped image.

Adding Text

1) Open the image

2) Click the text button on the task bar. It's the one in the middle with an "A" on it.



3) Drag to draw a box on your image. Don't worry if it's not exactly where you want your text to end up, you can move it before you draw on your text. Notice there are some instructions in the box. Make sure you do all your formatting before you draw or overlay your text.

4) Type your desired text into the box (like in the image at left). Now you can format your text.

5) To change the font type or font size, go to Edit → Options → Fonts (image below). The text on your image will change to give you a preview of the final product.

6) To change the font color, go to Edit → Options → Colors and change the foreground color (image right).

7) To move the text box, hover over the box until the cross becomes an arrow. The hold down the right mouse button and drag the box where you want it. The box can also be dragged to resize.

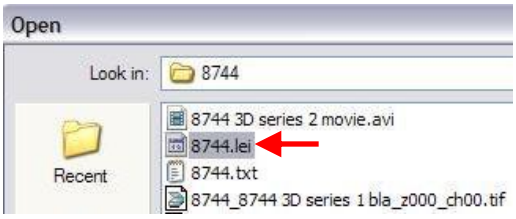
8) Once you have your text formatted the way you want, you can use Ctrl + b to overlay or Ctrl + d to draw the text onto the image. Draw burns the text into the image, which will cause colored text to turn gray on a grayscale image and can cause pixilation. Overlay keeps the font color no matter the image type, and will prevent pixilation.

Opening Files with the Bio-Formats Plugin

Non-standard file types, particularly proprietary formats that contain batches of images such as OpenLab .LIFs, Leica .LEI and .LIF and (sometimes) SlideBook .SLD files, can be opened using the Bio-Formats plugin.

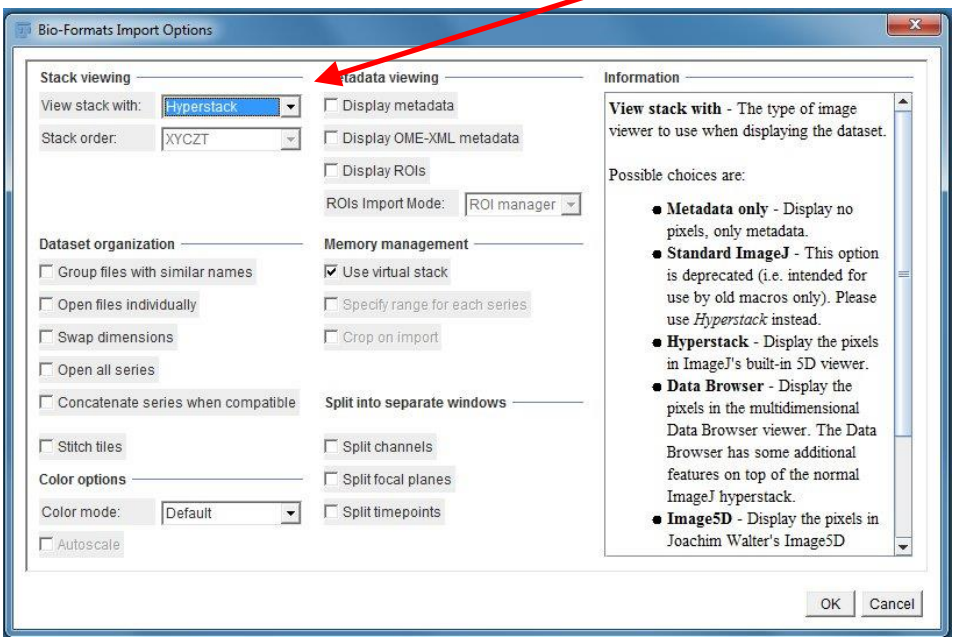
1) To use Bio-Formats, you must first install it in your ImageJ plugins folder. If you are using Fiji, you can simply use the Manage Update Sites tool (pg. 4) to install Bio-Formats into your Plugins menu. If you're using ImageJ, download the *stable build* from the OME website: https://www.openmicroscopy.org/bio-formats/ and install into your ImageJ → plugins folder manually (pg. 5). Either way, you must restart your ImageJ to complete the installation of Bio-Formats and see it in the Plugins menu.

2) Once Bio-Formats is installed, start ImageJ. Choose Plugins → Bio-Formats → Bio-Formats Importer. A new window will appear, prompting you to point to the file to be opened. In this example, we are opening a Leica .lei file.



3) Once you have selected the file to be opened, you may have to wait a few seconds while the software reads the data. You may also be asked to confirm the name and filepath of the file you are opening.

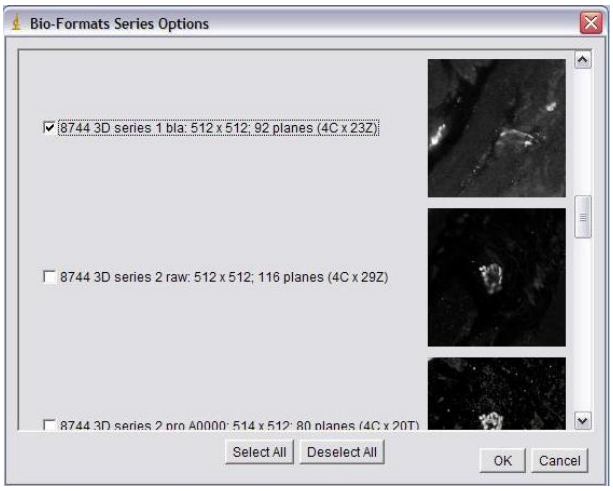
4) When the "Import Options" window opens, you can choose how you want your data to be displayed. The safest option is to choose "Hyperstack" and leave all other options unchecked. If you want to know what an option will do, hover over it and read the description on the righthand side of the Bio-Formats Import Options window.



If you can't see an image in your file when it opens, try checking "autoscale." To learn more about the other options, hover your mouse over any option and information about that option will be displayed at the bottom of the window.

When you have set the display the way you want it, click OK.

5) After a few more seconds, a new window will appear with the names and thumbnails of all the images/image stacks in the file.

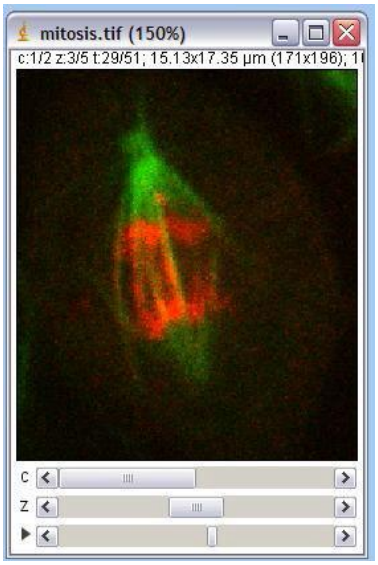


Choose which image(s) or stack(s) to open by clicking on the checkbox next to the name. The first image is always set to open, but you can deselect that image and open others instead.

Keep in mind, the more images you set to open, the longer it will take to import all the data.

Your data will then open as images or stacks that can be modified and saved as individual .TIF or .TIF stack files.

Hyperstacks

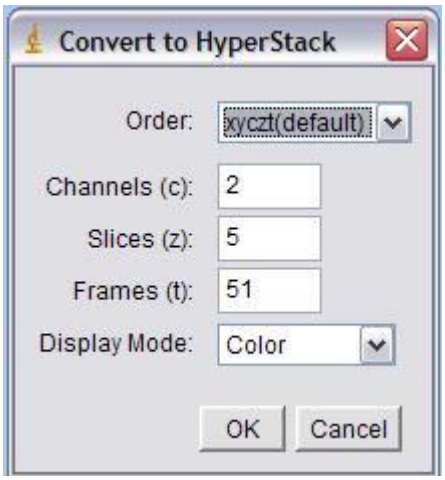


A hyperstack is not a stack that has had too much coffee. It is a way to display your image data in a more interactive way. Hyperstacks are most often used for .tif series that have more than one multi-channel feature (i.e. multi-color z stacks or Z over time lapse stacks).

Notice that unlike a regular stack, there are multiple sliders under the image with a hyperstack. These sliders allow you to move through one dimension at a time. This is the Mitosis example under File → Open Samples.

LOCI BioFormats can create hyperstacks from stacks saved in many different formats. You can also create a hyperstack from a regular .tif stack. Open your stack and then use Image → Hyperstacks → Stack to Hyperstack.

You can determine the order of your stack by running though it and looking at what changes first. For example: if your images flip between colors, then the z-plane changes, and then the time point changes, you have the default stack (xyczt). c = channel, z = z plane and t = time. Otherwise, choose the order that best fits your data.



Check that the numbers for channels, slices and frames fit your dataset. Also check that these numbers multiply to equal the total number of slices in your original stack. ImageJ will not render and will give you an error message if your numbers are not correct.

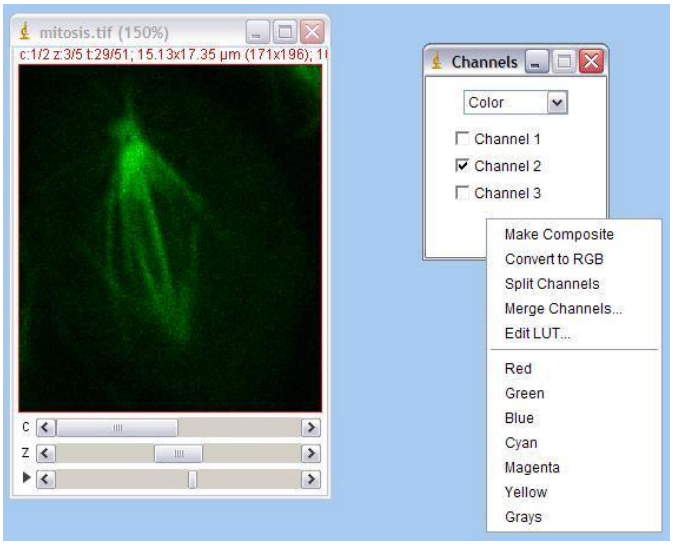
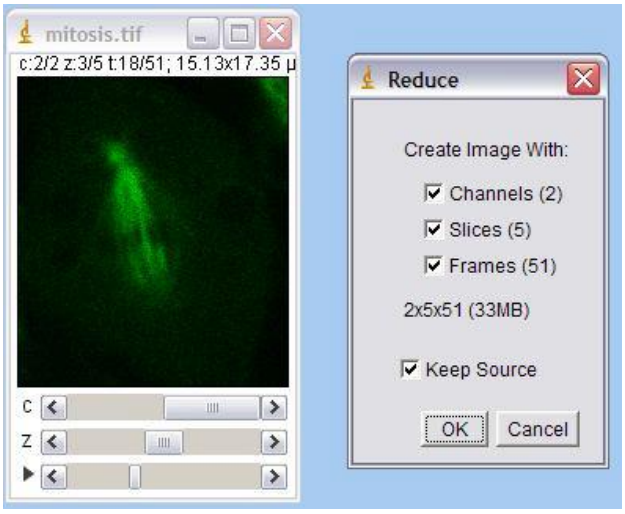


Image → Hyperstacks → Channels Tool brings up a menu for working with the colors in your image. You can change the lookup table (false color) of a channel, turn channels on and off in the display, split the colors into different stacks, merge channels together, or convert the whole stack to an RGB image.

Image → Hyperstacks → Reduce Dimensionality works to reduce the amount of information in the hyperstack. If we reduce the channel dimension, we can see just one color in the whole volume over all timepoints. If we reduce the volume, we see one just one focal plane in two colors over time.

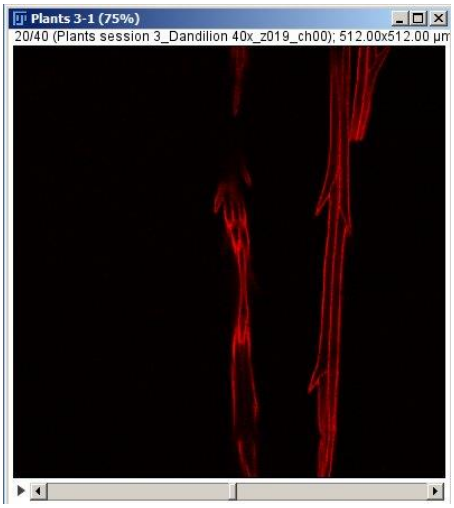
To delete JUST ONE of the time points across all volumes, use the Image → Stacks → Delete Slice tool. Most of the tools in the Stacks submenu work on hyperstacks, except Make Substack.

Image → Hyperstacks → Reduce Dimensionality can also be used to create a substack from a hyperstack. If we were to remove the checkmark from the "Slices" box, we could generate a new stack with both red and green channels and all time points, but only the middle (current) of the five focal planes. Checking "Keep Source" allows you to keep the full hyperstack, in case you make a mistake and want to go back.



Creating Intensity, Orthogonal and 3D Projections

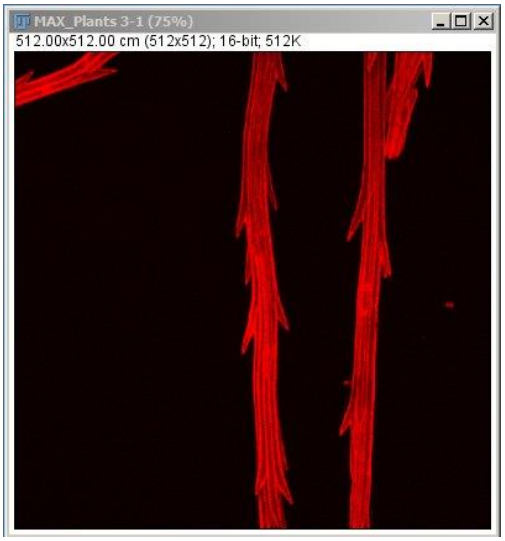
.Tif stacks can be viewed in a variety of ways, depending on the type of information you want from the stack. These techniques work for single (grayscale) and multi-color (RGB) images.



IMPORTANT - Before doing an orthogonal or 3D projection, make sure your image properties are correct (see pg. 23). This is particularly important when it comes to voxel depth (which is equal to your z step size). If this is not correct with respect to your pixel size in XY your projections will be distorted.

Intensity projections flatten an image

Z Projections bring all of your Z planes together into one plane to "flatten" a stack of objects. The most commonly used is the maximum intensity projection, which finds the brightest pixel in the stack for each XY position and projects those into a single XY plane.



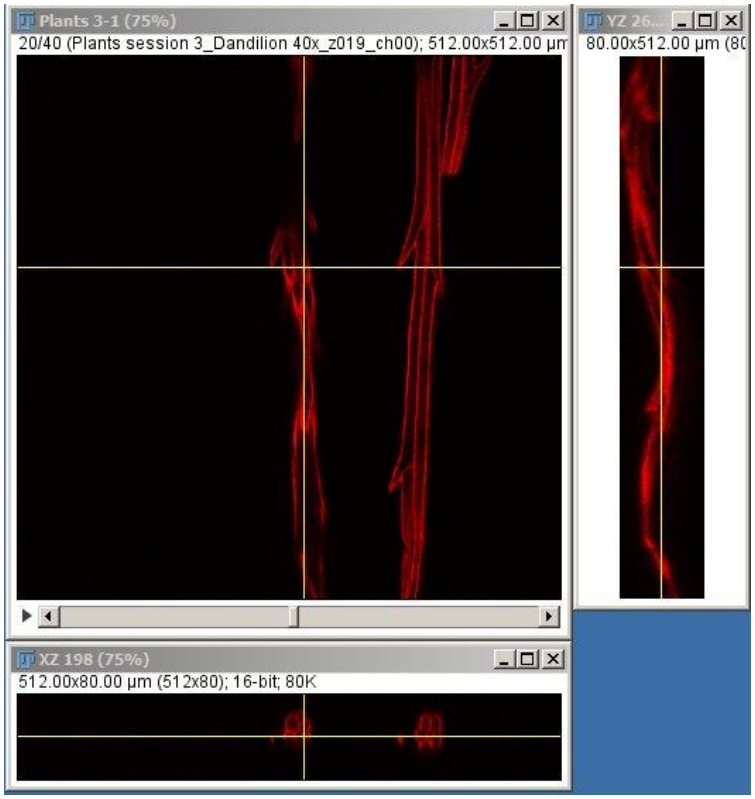
1) Open the stack(s) you want to use. You can add color with a LUT (pg. 20) or color merge (pg. 22) now or after the projection.

2) Choose Image → Stacks → Z Project. You can choose to project every image in the stack, or just a subset. You can also choose the type of projection to do. This is a max intensity projection. Descriptions of the other projections can be found on the ImageJ documentation page.

3) A new window will appear with your projected image. This can be saved as you would any other image.

Orthogonal projections provide cross sections

Orthogonal projections cut though the stack along two lines and lay out the XZ and YZ cross sections of the stack. The projection is made in real time, allowing you to drag the lines around the image to see different views.



1) Open the stack(s) you wish to use. In this case, it is best to apply color (pg. 20) or merge color channels (pg. 22) before doing the projection.

2) Check your pixel size calibration, otherwise you may get a distorted cross section (pg. 23).

3) Choose Image → Stacks → Orthogonal Views. Drag the line around your image to view different cross sections.

4) Image → Stacks → Reslice is another way of getting cross sections from a stack of images. Reslice only works in X OR Y, not both at the same time, and is not real time. It will, however, provide you with a full stack of XZ or YZ cross sections through your sample.

3D Projections provide a full rendering

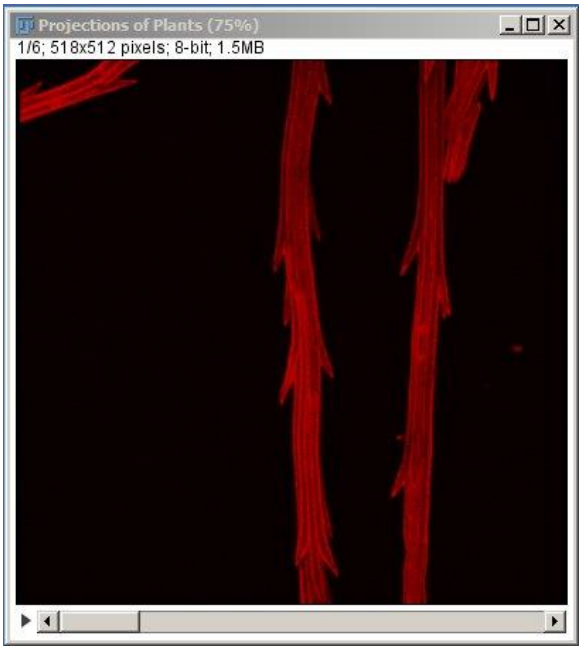
3D rendering can provide the full 360 degree view of the object(s) in your stack.

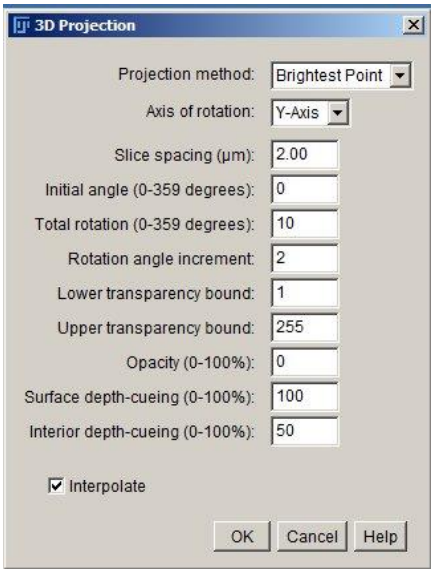
1) This option can take a lot of memory, so be sure to check that you have assigned as much memory as possible to your ImageJ (pg. 6).

2) Open the stack(s) you wish to use. In this case, it is best to apply color (pg. 20) or merge color channels (pg. 22) before doing the projection.

3) Check your pixel size calibration, otherwise you may get a strange looking rendering (pg. 23).

4) Choose Image → Stacks → 3D Project….





5) "Projection method" Brightest point will give you a maximum intensity projection at every angle. Nearest point will give you something like a surface rendering.

You can rotate about the X, Y, or Z axis, although generally X or Y is the most useful.

"Slice spacing" should match your step size and will be brought in from Image → Properties. You can cheat a little bit here, making the slightly smaller or larger to get a more compact or expanded view, but I would start with the correct step size and go from there.

An "initial angle" of 0 will start you off with a front view, similar to a Z projection. "Total rotation" will determine how much of a 360 degree view you get. Most of the time rotating all the way around is not necessary. Experiment with different amounts of rotation until you get something that you like.

"Rotation angle increment" determines the jump between frames and is measured in degrees. For example, if you put in an initial angle of 0 and a total rotation of 50 with a rotation angle increment of 5, you will get a projection with 11 planes, each 5 degrees apart. The more planes you create, the more memory the projection will take to create, especially with RGB images (which are projected one channel at a time and then automatically merged together). Be aware of your memory limits.

Checking "Interpolate" will fill in any gaps you have between slices and get rid of the venetian blind effect that can happen when you turn a stack of images to 90 degrees or so. Interpolation is memory and time intensive, so if you are experimenting with different types of rotation and don't have a very powerful computer, you may want to hold off on interpolation until you have the parameters you want.

I usually leave the other parameters in the default state -- most of the time I think this looks best, but occasionally with very dense samples I will experiment with different amounts of transparency in order to see thought some of the layers.

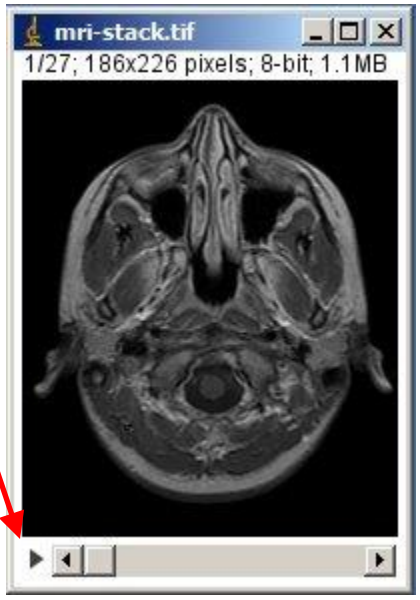
6) When you hit OK you will get a stack of images, each of a projection at a different angle. These can be saved as a .tif stack or as a movie with File → Save As → .avi. Image → Stacks → Tools → Animation options will let you preview your stack as an animated movie. This feature also works for time lapse.

More Rendering Tools

For more control over the movement of your 3D rendering, I like Volume Viewer by Kai Uwe Barthel (built into Fiji plugins or install at https://imagej.nih.gov/ij/plugins/volume-viewer.html), Clear Volume by Florian Jug (https://imagej.net/ClearVolume or install through Fiji Manage Update Sites (pg. 4)), or ImageJ 3D Viewer by Benjamin Schmidt (http://132.187.25.13/home/?category=Download&page=Viewer3D).

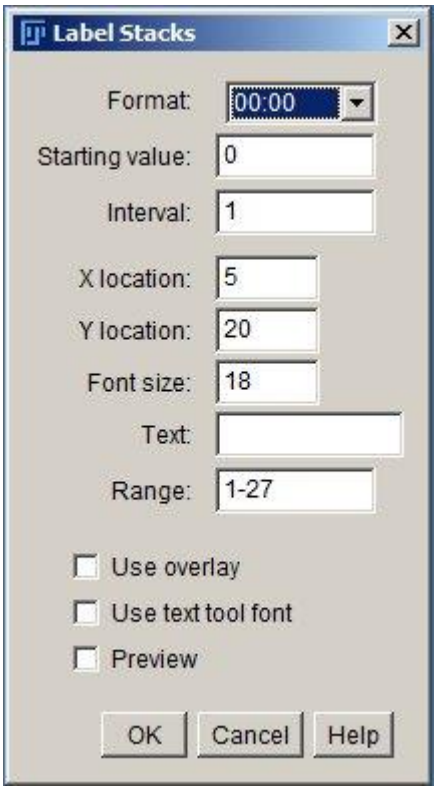
Making a movie from a .tif stack

Creating a movie from a stack of .tifs is easy. You can even dress up your movie with a time stamp if it is a time lapse.



1) All image stacks have a play button on them. Hit this play button again to have your stack play through like a movie. Hit it again to stop.

2) To adjust the playback speed and other animation parameters, choose Image → Stacks → Tools → Animation Options.



3) You can annotate your movie with text using Image → Stacks → Label. There are multiple formats, including two different time formats (shown, left).

4) For X and Y location, you can try to guess where you would like your text to go, or you can make it easy on yourself by drawing a box with the draw tools (pg. 11) roughly where you want your text to go on your image. The "Preview" feature helps with good text placement and font size.

"Text" will add text to the right of your stamp, so you could use this to add units to a time stamp, for example.

"Range" determines which slices of the stack get the stamp. The default is to stamp all images in the stack.

"Use overlay" will make your text into an overlay instead of burning it in to the image. "Use text tool font" will use the font set under Edit → Options → Fonts for your stamp.