ImageJ / Fiji Tutorials – Intermediate
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.

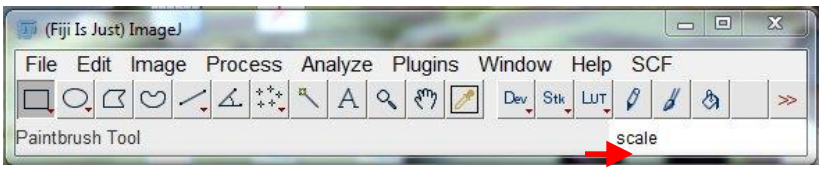
One Way to Site ImageJ in Your Manuscript

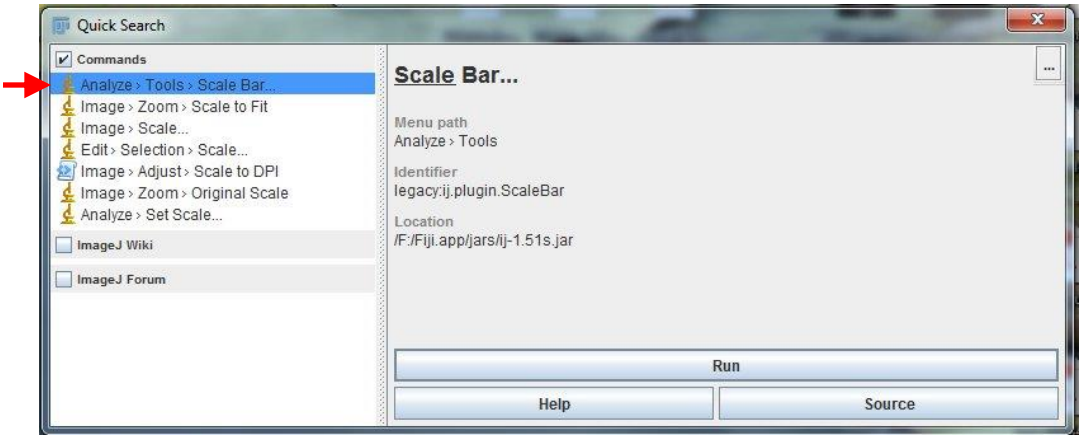
Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.

The Command Finder

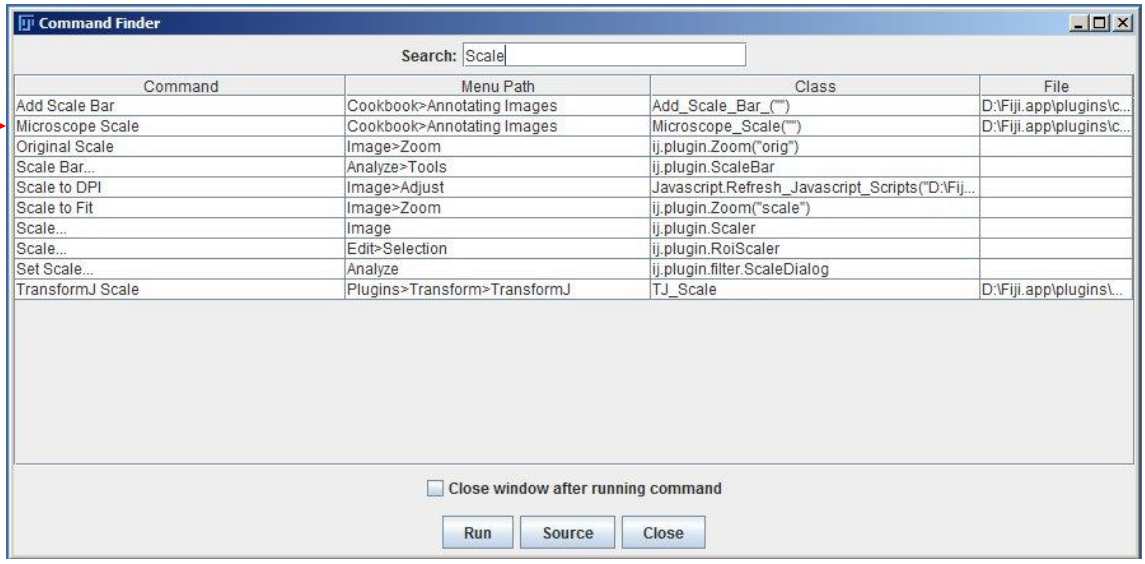
ImageJ has so many features it can be hard to keep track of all of them. Thankfully there is a guide to help you find the command you are looking for.

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





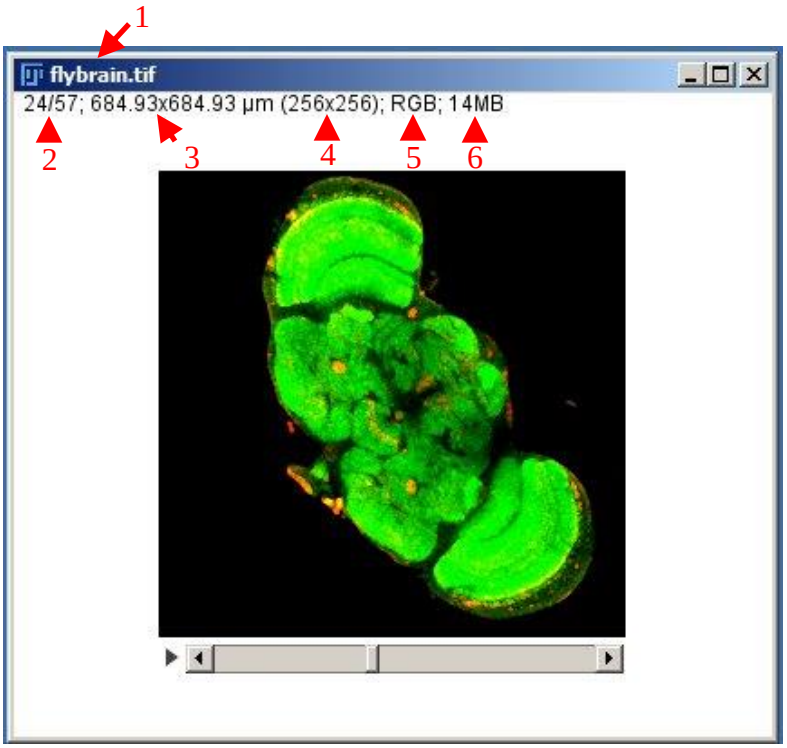
In ImageJ, The Command Finder can be brought up by pressing the letter L on your keyboard.



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.

The Information at the Top of your Image



This information is also found in our ImageJ Basics tutorial, but it is important (especially the highlighted section) so it bears repeating.

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.

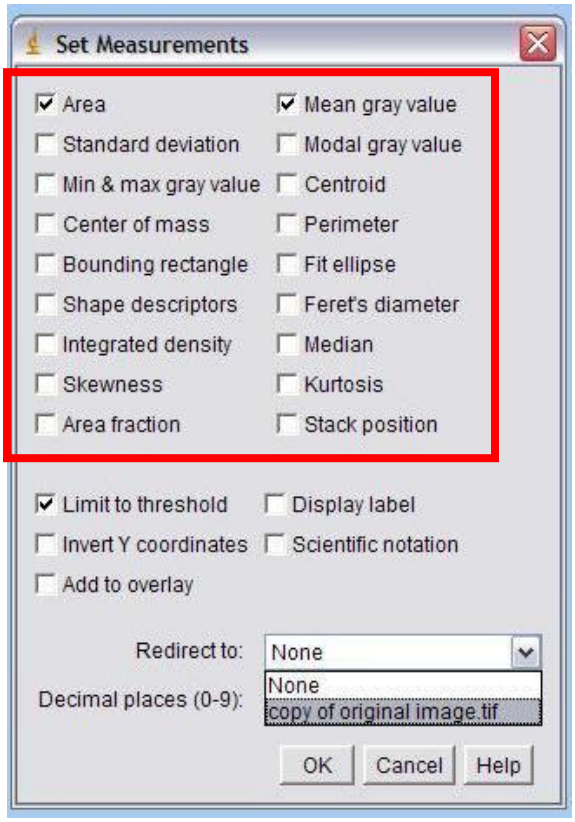
3) "684.93um x 684.93um" is the actual image size, or you can think of it as the size of the field of view. The units on this value depend on how the image is calibrated (see our ImageJ Basics tutorial pg. 19). In this case, the units are microns. When you make measurements that involve physical size (area, length, integrated density) the units on that value are THESE units. If the image is NOT calibrated, the units here and in your data table will be pixels.

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.

Set Measurements



Most of this tutorial is about getting quantitative data from your images. This is not an exhaustive tutorial on all the different kinds of data that can be obtained from ImageJ, that would take a book (or several)!

Mainly we will be looking at different ways to get data using the built in Analyze → Measure and Analyze → Analyze Particles commands. This satisfies many of the common requests for quantitative information that we get.

Before using these commands, go to Analyze → Set Measurements and choose the data you want listed in your data table.

The choices in the top portion of the "Set Measurements" window are described on the ImageJ Documentation page. Some of the more commonly used measurements are:

Area – The area of the selection in pixels or in units squared if the image is calibrated with Image → Properties or Analyze → Set Scale.

Mean Gray Value –The sum of the gray values of all the pixels in the selection divided by the number of pixels.

Centroid – The center point of the selection determined by averaging the X and Y coordiates of all the pixels in the selection. Given in X and Y coordiates (does not work in 3D). Center of mass is a brightness weighted version of centroid, with coordinates listed under XM and YM.

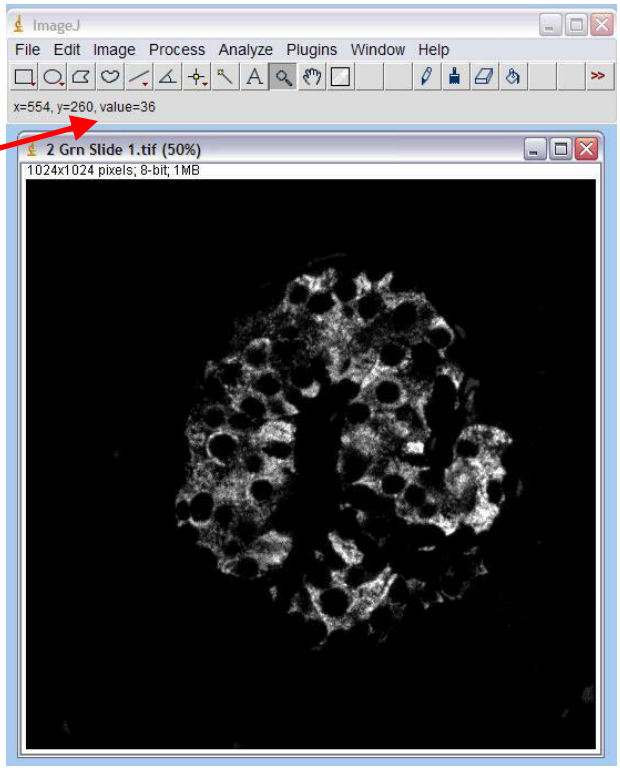
Feret's Diameter – The longest distance between any two points on a selection boundary. Choosing this option also gets you the smallest distance between any two points (MinFeret) as well as the Feret Angle and the X and Y coordinates of the starting point of the Feret diameter (FeretX and FeretY).

Integrated density – The product of the area and the mean gray value or the sum of all the pixel intensities in the selection. Often used to analyze dot blots.

Intensity Analysis

Hover and Read out Gray Levels Pixel by Pixel

There are many ways to read out the intensity information in an image or image stack. One of the easiest is to hover over an area of the image and view the readout of the gray level pixel-by-pixel on the ImageJ toolbar.

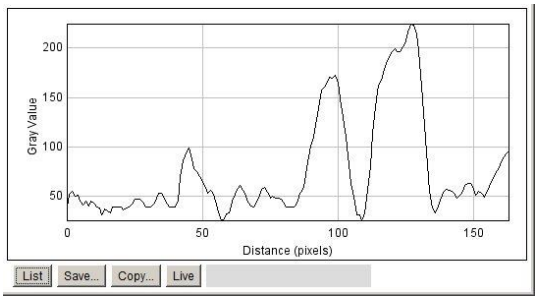


This method is useful for determining signal to noise in an image, estimating the gray value of your “background” pixels or other quick estimates of intensity.

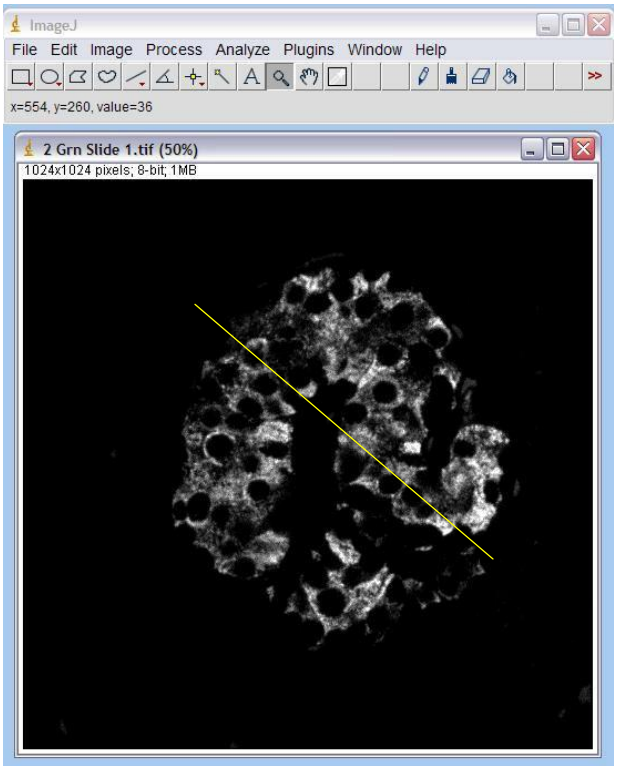
There are other methods better suited to measuring gray levels across an image or object.

Plot Profile

To plot the pixel intensities along a line, first draw a line on you image. Then choose Analyze → Plot Profile (keyboard shortcut Control + K).



To get a list of all the values in the plot, hit the “List” button at the bottom of the plot window. This list can be saved as an Excel compatible data table.

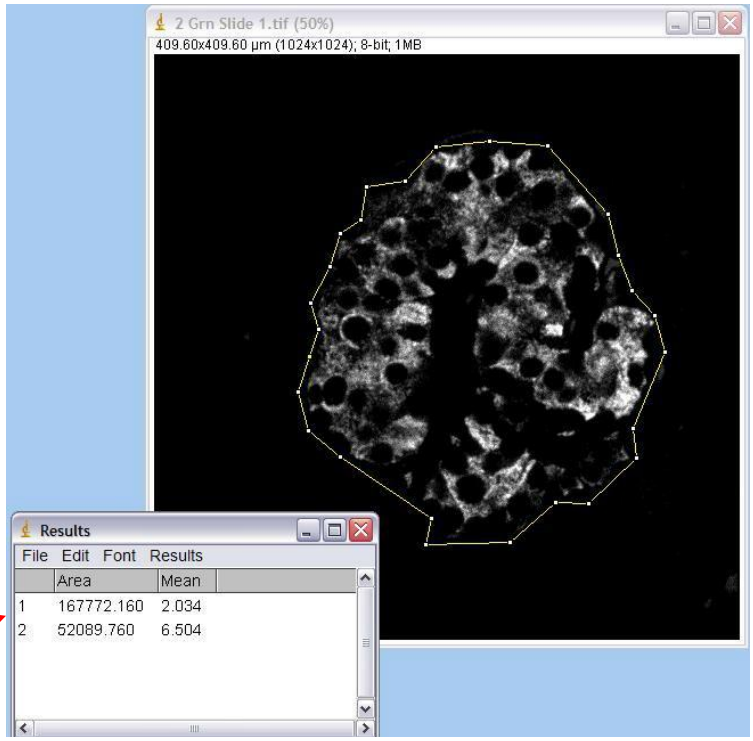


Using the Measure Tool with a Drawn Area

Analyze → Measure will give you statistics on the WHOLE IMAGE, including all the 0 and low intensity pixels around your object of interest.

Unless your object covers the entire area of the image, you are probably going to want to limit your measurements somehow.

Drawing an area around or within an object with a draw tool is one way to do this. Then Analyze → Measure will only consider what's inside the boundary.



"Mean" = mean gray level
"Area" is in um^2
1) Whole image
2) Within the yellow boundary line

If you would like to draw and measure more than one or two areas on a given image, consider storing your regions by adding them to the ROI manager (pg. 17).

Using the Measure Tool with a Threshold

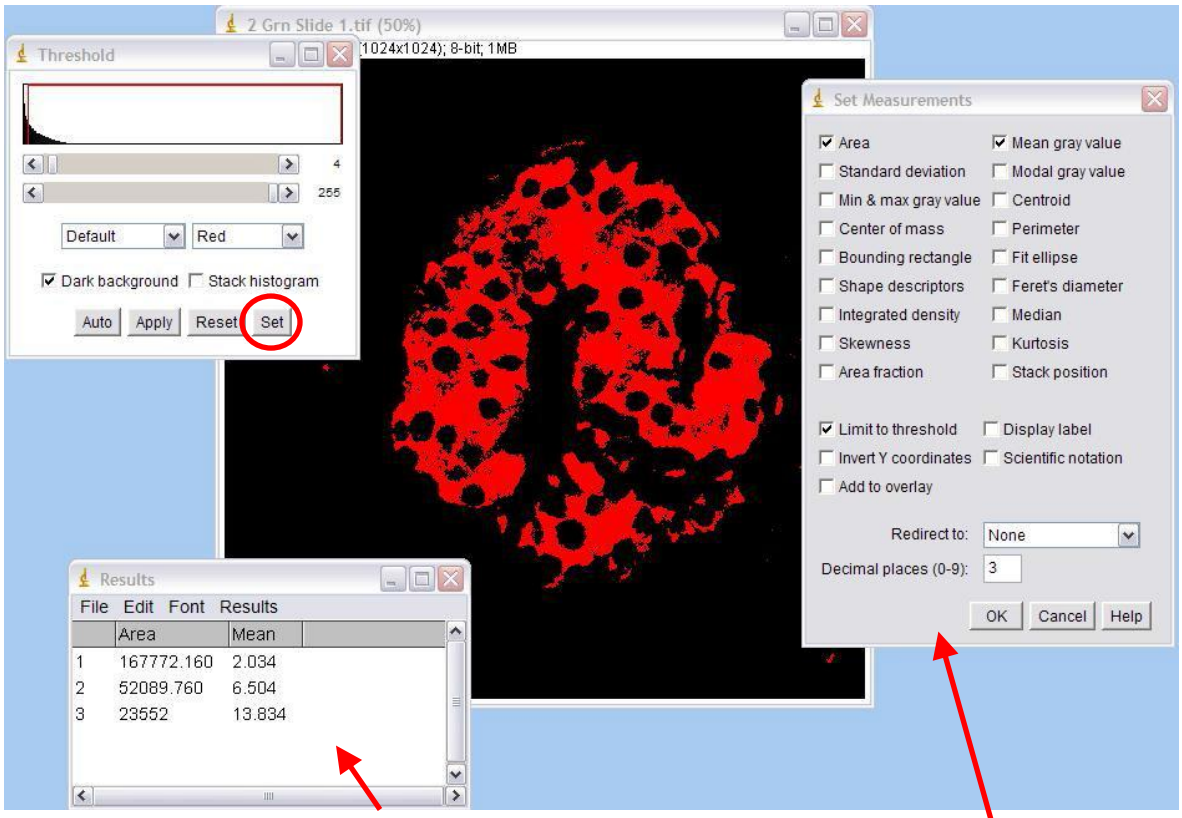
A threshold is another way to limit your measured area. Instead of a physical limit, like a line, this is an intensity limit. The threshold will only consider pixels with gray values within limits that you set.

To limit your measured area using an intensity threshold:

1) Go to Analyze → Set Measurements and check "Limit to Threshold". While you're here, consider checking "Display label" to have the name of your image listed in the Results window along with your measurements.

2) Use the Image →Adjust → Threshold tool to highlight the area(s) you wish to measure. You can either use the sliders or use the "Set" button to type in values. Set is useful when you want to quickly threshold a number of images using the same values.

3) Choose Analyze → Measure. If you keep your "Results" window open between measurements, you can add as many measurements from as many images as you want. This table can be saved as an Excel compatible table by choosing file → Save As from the menu on the Results window (NOT the main ImageJ window).

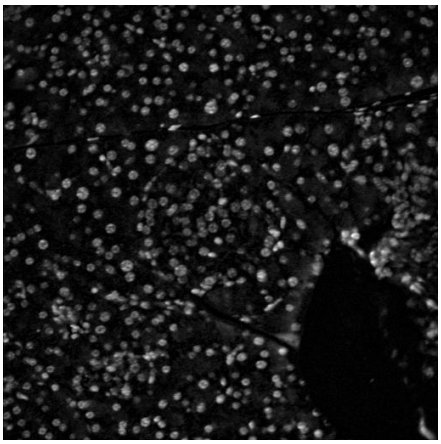


"Mean" = mean gray level
Area is in um^2
1) Whole image
2) Yellow area from pg. 3
3) Thresholded area

Information about the other options in this window can be found on the ImageJ website under Documentation, Menu Commands

Creating a Binary Image

What if we wanted to count the number of objects in an image, or know the mean gray value or area of each of those objects? We can use the Analyze Particles function to do both of these things.

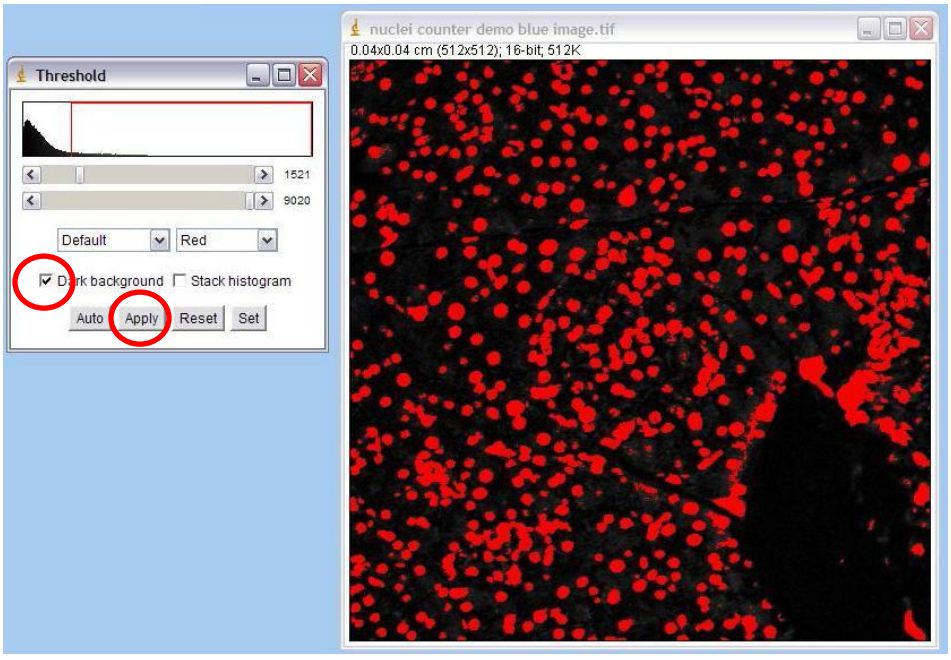


In this example, we are starting with a single channel, grayscale image.

1) If you just want an object count, or if the statistics you want for each particle (see pg. 5 for the list of possible stats) does not involve intensity (e.g. area, Feret's diameter) continue to step 2. If the statistics you want DO involve intensity information (e.g. mean gray value, integrated density) or you're not sure, first make a copy using Image → Duplicate or Cntl + Shift + D before continuing to the threshold step.

2) Use Image → Adjust → Threshold to highlight the objects to count and / or measure. A thresholded image is shown below.

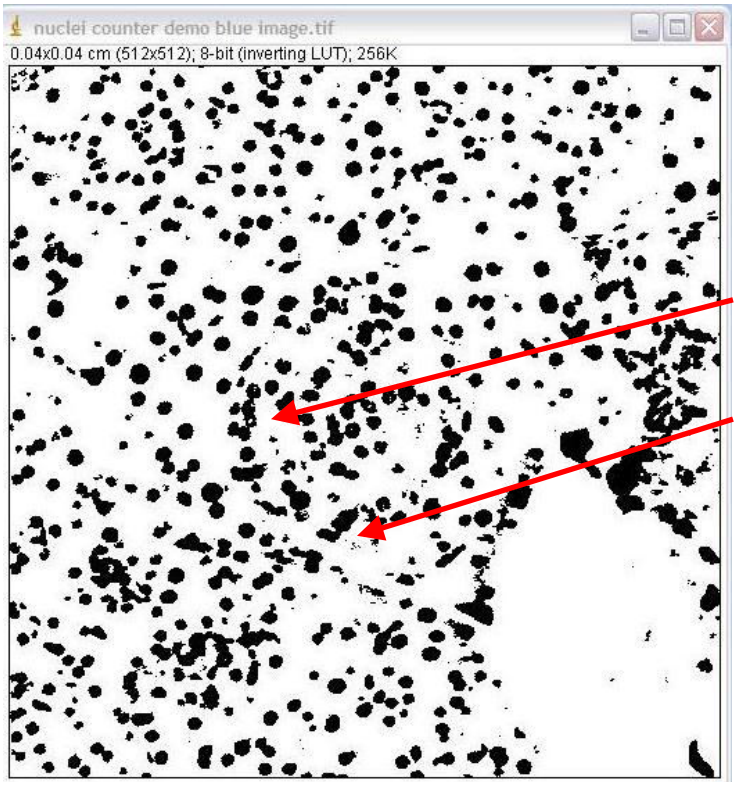
The Threshold Tool



3) In order to use the Analyze Particles feature, the image must be binary (contain only values that are 0 or 255).

To make this image binary, either use the "Apply" button on the "Threshold" window with "Dark background" checked, or choose Process → Binary → Make Binary.

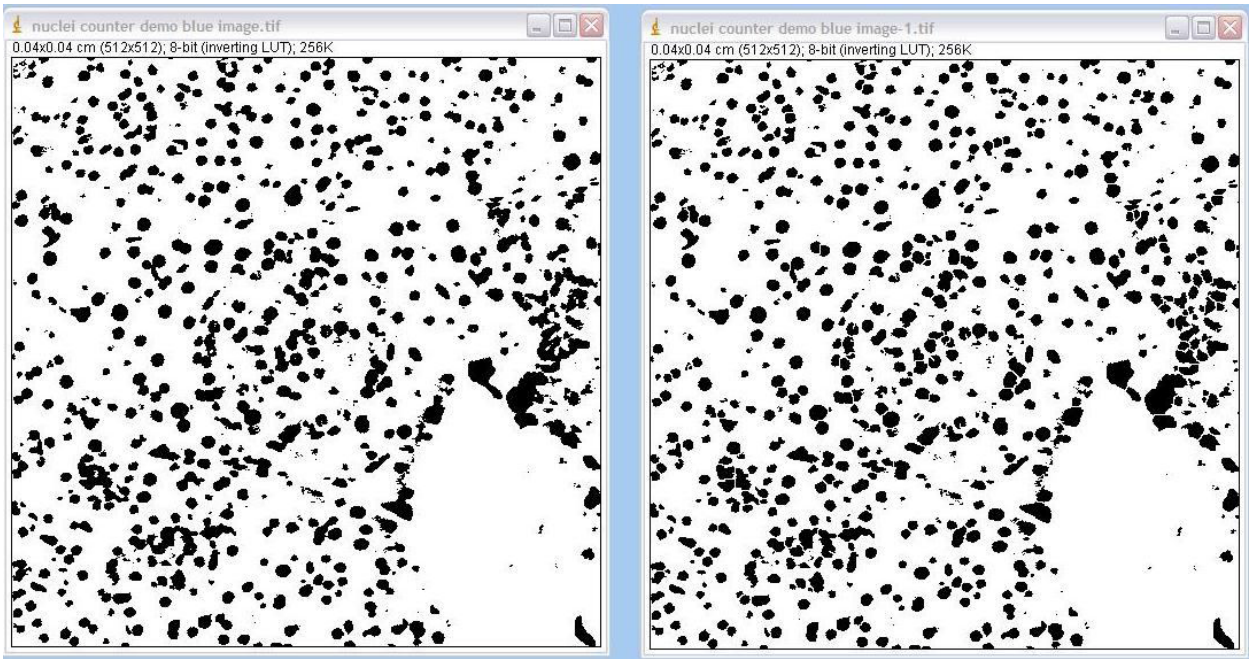
The Watershed Tool



4) This is a binary image. The pixel values in this image are either 0 (white) or 255 (black). Analyze Particles will only see this type of image.

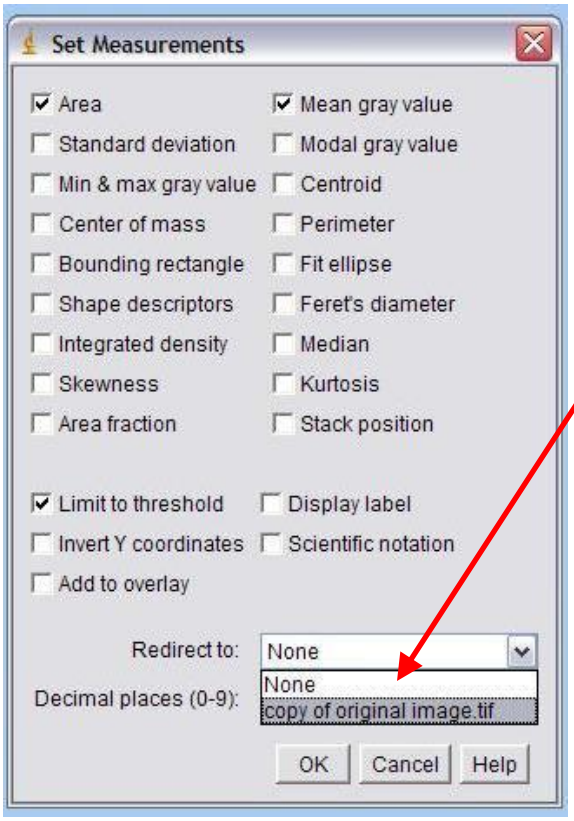
5) Holes in objects like this one can be filled with the Process → Binary → Fill Holes tool.

6) Before we count, notice that there are some areas where two or more objects are touching. We can use the Process → Binary → Watershed tool



Here is the image before (left) and after (right) the Watershed tool. Notice that most touching objects were cut at places where the diameter of the object changed. Some objects were cut perfectly, some were cut unnecessarily and some touching objects were missed. This is typical of the watershed tool. Different thresholds could give different results, so some experimentation with the Threshold and Fill Holes tools may be necessary to get the best results.

Redirecting to another image for gray values



7) At this point, if you choose any of the measurements that involve gray values, such as “Mean gray value” or “Integrated density,” go back to the “Set Measurements” window under Analyze → Set Measurements.

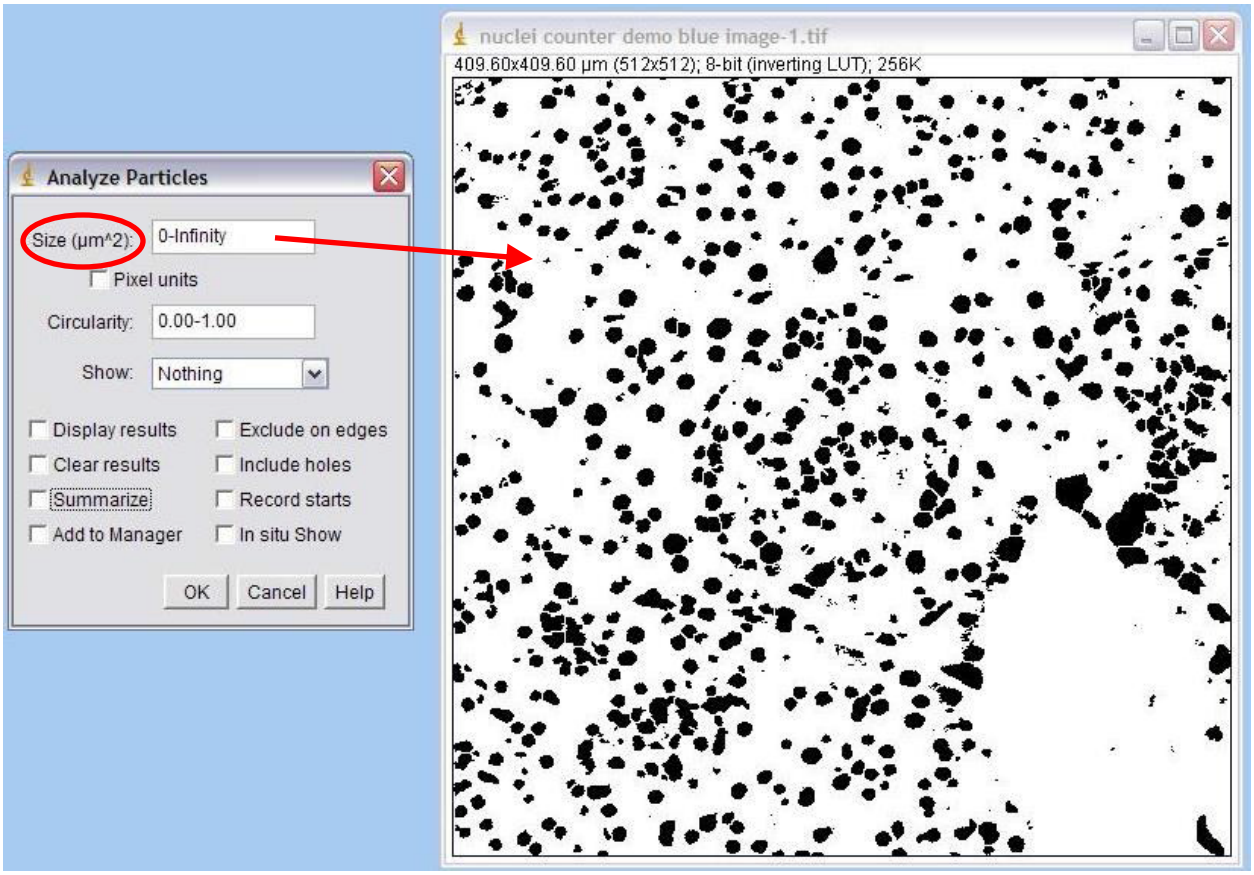
Use the Redirect to: pulldown at the bottom of the “Set Measurements” window to redirect to the copy of your original image (whatever its name may be). The image must be open in ImageJ to appear in the pulldown. If you don’t redirect, all of your values will be taken from the binary image, so they will be either 0 or 255, not the true values!

8) Once you have chosen the statistics you want, created a binary image and, if necessary, redirected to a copy of the original image for gray values, you are ready to choose Analyze → Analyze Particles. The next few pages will walk you through the Analyze Particles menu.

Analyze Particles

Analyze → Analyze Particles has lots of features that allow for fine tuning of object detection and documentation of counts. We will walk though each of these features in the next few slides.

The Help, Documentation, Menu Commands section on the ImageJ website also provides information on these features.

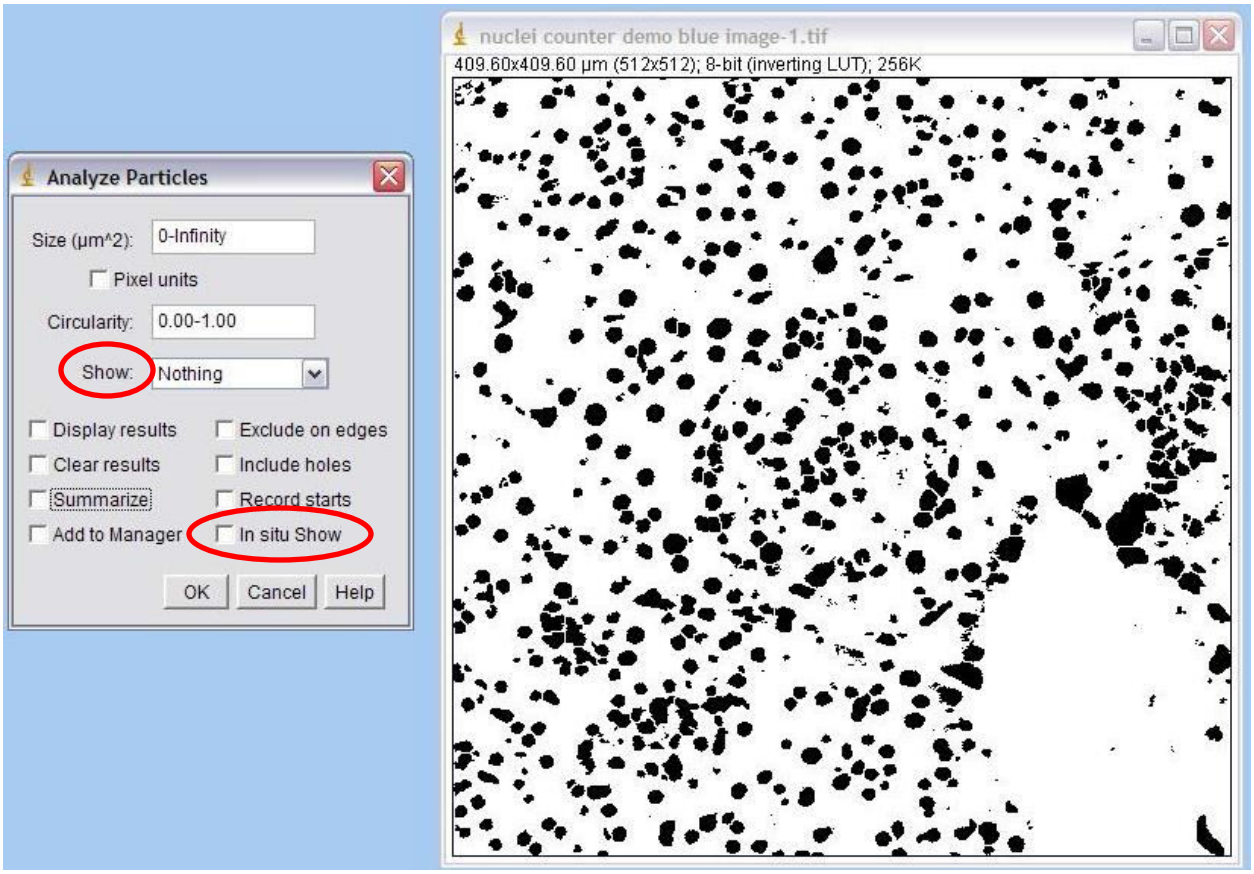


The first feature in the "Analyze Particles" window is a size threshold. The default (0-Infinity) is to include all objects no matter how large or small they are.

However, in this image, all of the nuclei should be roughly the same size. Other, smaller objects in the image are likely debris. If nuclei are approximately 100um^2 in size, a range of 80-120um would likely count all of the nuclei while excluding objects that are too large or too small to be considered.

IMPORTANT – check the units on your size threshold (circled above)! If you have not calibrated your image under Image → Properties, these units may not be correct and your size cutoff will be inaccurate.

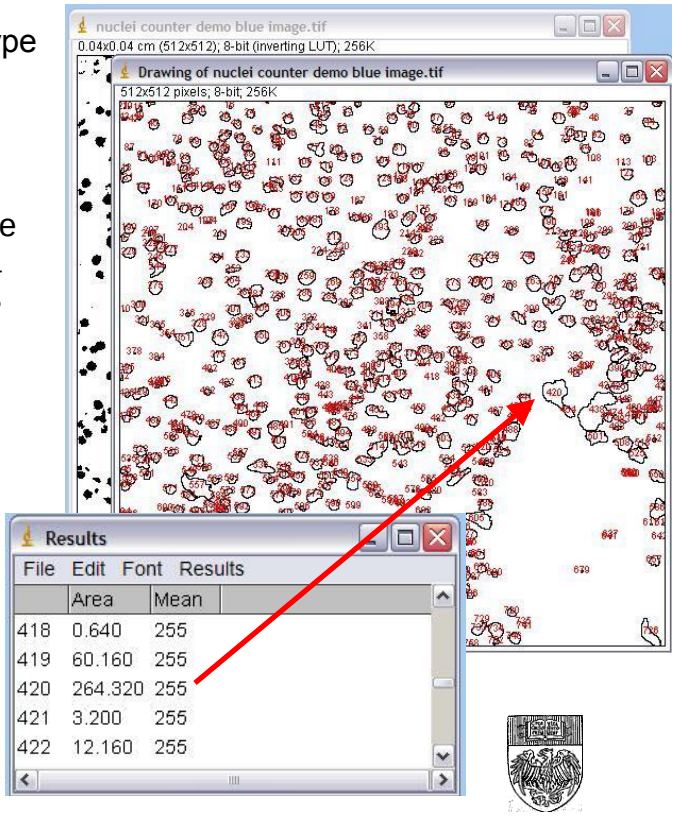
Checking the "Pixel units" box will cause the size threshold to ignore the units of scale and exclude objects based on how many pixels they contain. This is a good way to exclude single pixel noise spots from being counted as objects.



The second feature is a circularity threshold. The default (0.00-1.00) is to include all objects no matter how round they are. 0.00 is a straight line, 1.00 is a perfect circle. Most natural objects fall somewhere along this continuum, and often using the default full range is the best way to go. Some experimentation with size and circularity thresholds may be necessary.

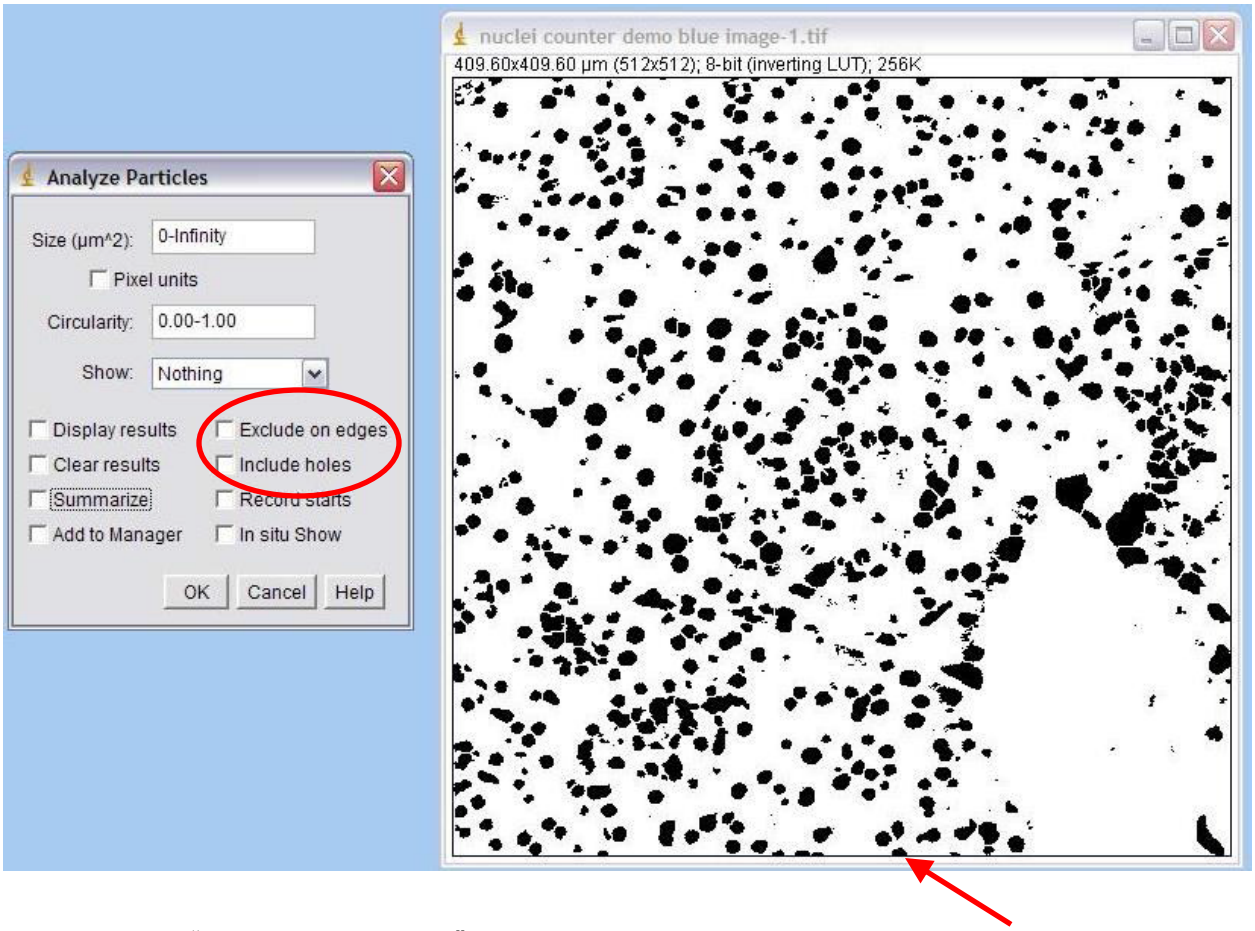
The “Show” feature of the box makes that type of experimentation easier. . .

The “Show” feature gives an image marking all of the counted / analyzed objects. “Outlines” (shown at right) creates a new image, draws the outline of the particle, and gives each particle a unique number that corresponds to the object’s number in the data table.



Other menu choices are described (with pictures) under Documentation → Menu Commands on the ImageJ website.
IMPORTANT -- Checking “In situ Show” writes the outlines /masks /particles directly onto the binary image, rather than creating a new window. DO NOT use this if you plan to re-do your analysis with new parameters!

“Exclude on edges” and “Include holes” are also used to exclude particles from analysis.

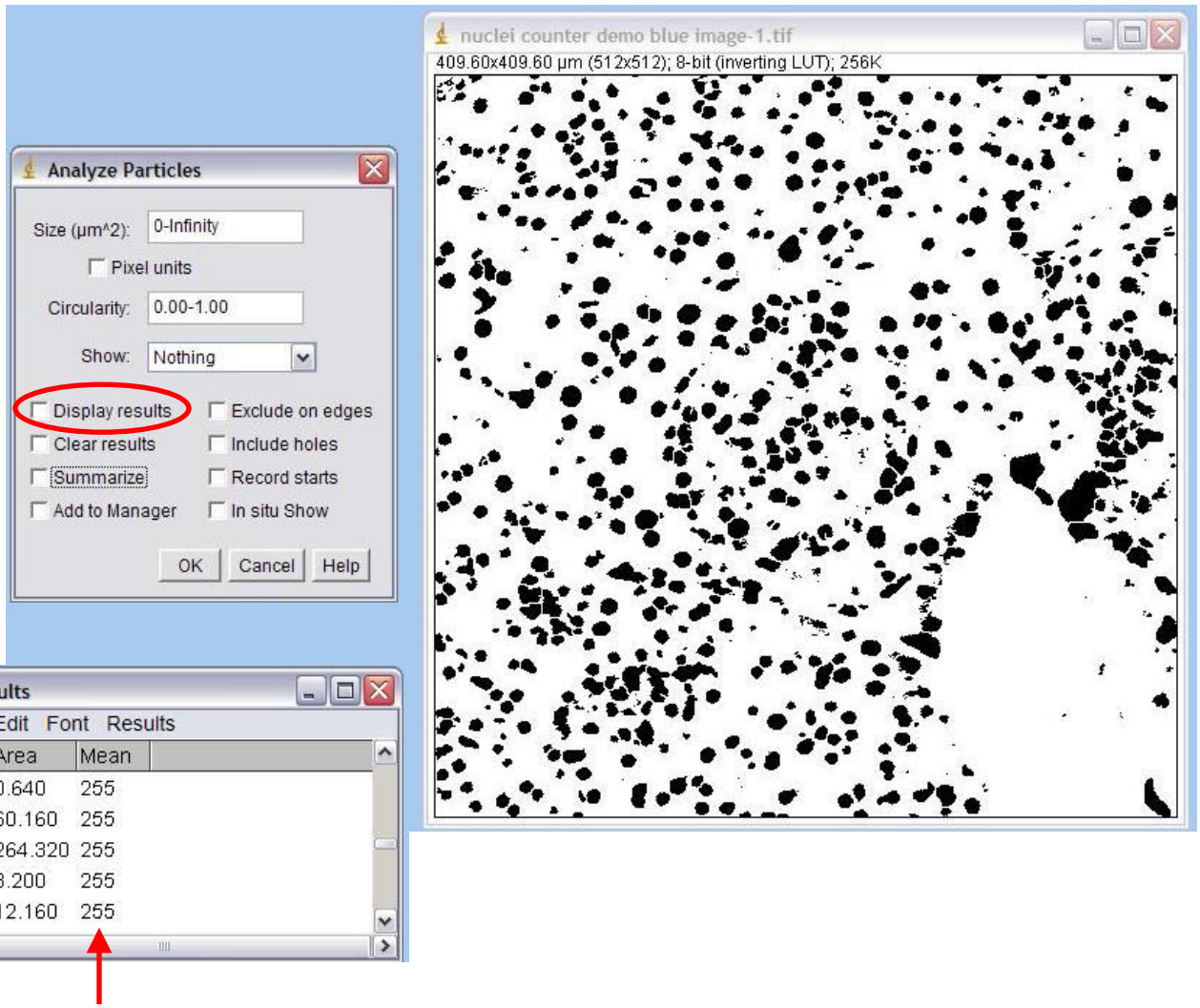


Checking “Exclude on edges” will exclude any particle touching the frame of the image from being counted / analyzed.

Enabling “Include holes” will NOT count objects enclosed within other objects and will measure objects with inclusions as though they were solid (without inclusions).

The rest of the “Analyze Particles” box deals with data and region of interest (ROI) management.

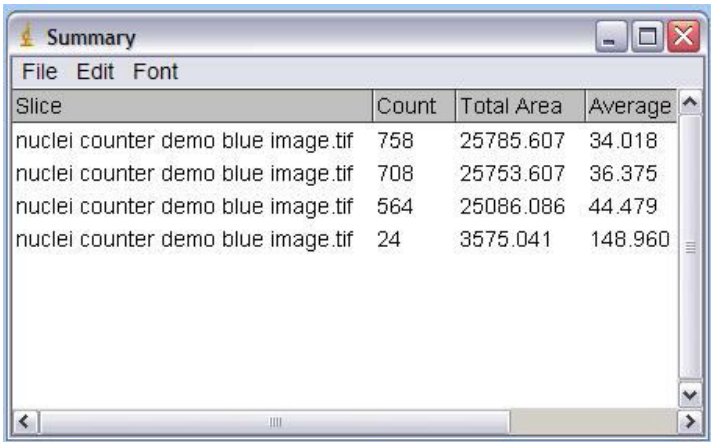
“Display results” will create a data table listing all of the objects in the image with the statistics you chose under Analyze → Set Measurements for each object.



This is a partial data table for the image above. Notice that all of the mean gray levels listed are 255. This is because Set Measurements was not set to “redirect” to the original, non-binary copy of the image!

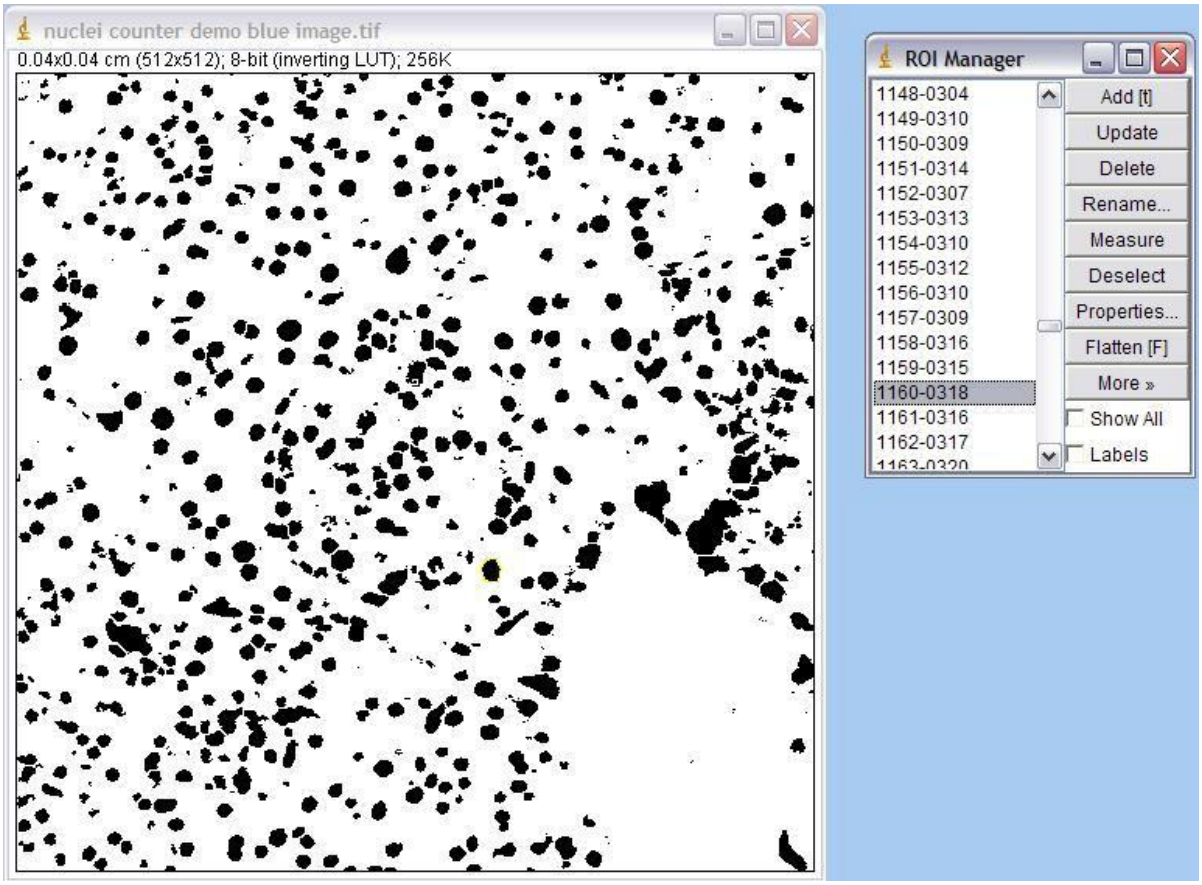
If you find yourself in this situation, start back at the beginning. Create a copy of the image with Image → Duplicate, then use the threshold tool to make your copy into a binary image (pg. 9). Set up your redirect to the original (pg. 11), click back on the binary image and run Analyze → Analyze Particles again with “Clear results” checked so you *delete* all of the previous results out of the table and restart the particle numbering from 1. Leaving “Clear results” unchecked will *add* the results of the current analysis to any existing data in the “Results” table. Particle numbering will start from the last number in the previous dataset (423 in this example).

“Summarize” will give a separate data table listing the name of the image being analyzed, the TOTAL object count and the MEAN value for all other statistics.

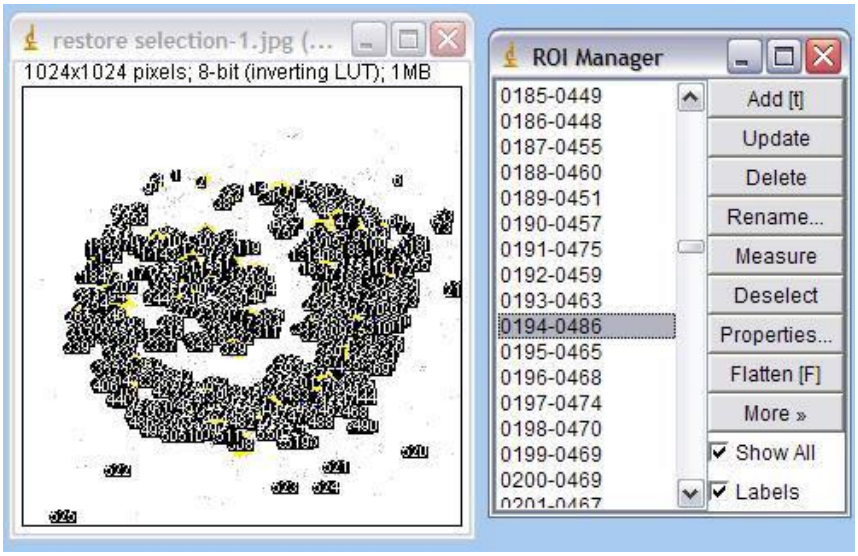


The “Summary” window can also be left open through several rounds or images worth of analysis to build a dataset. This table shows the results of four rounds of analysis on the image above, with particle size cutoffs of 2, 10 and 100 um^2.

“Add to Manager” will add outlines of all of the particles to the ROI (Region of Interest) Manager.



The ROI manager holds the shape and position of each region and can be used to transfer regions created on one image to make measurements in the same area on one or more other images.



This is the ROI Manager

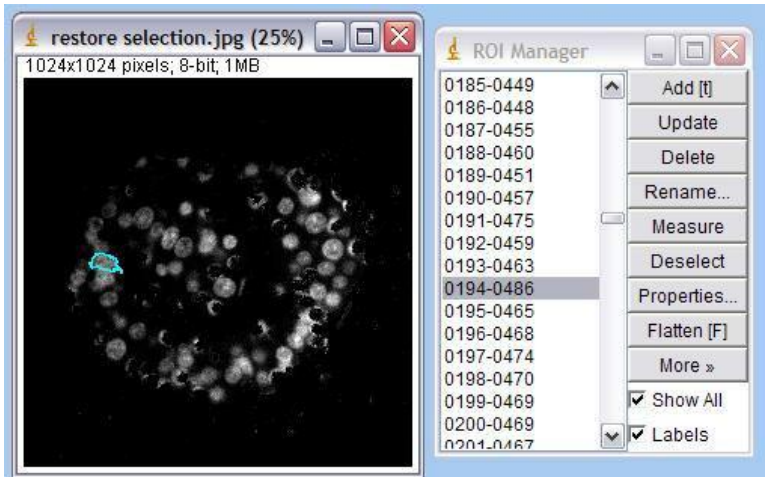
You can get to this tool either by opening Analyze → Tools → ROI Manager and adding regions by hand, or by checking the "Add to Manager" box in the Analyze Particles Window and automatically adding all ROIs to the Manager.

Notice each region is individually numbered, both in the manager and on the binary image.

Unchecking "show all" will allow you to see one region at a time, for easier viewing.

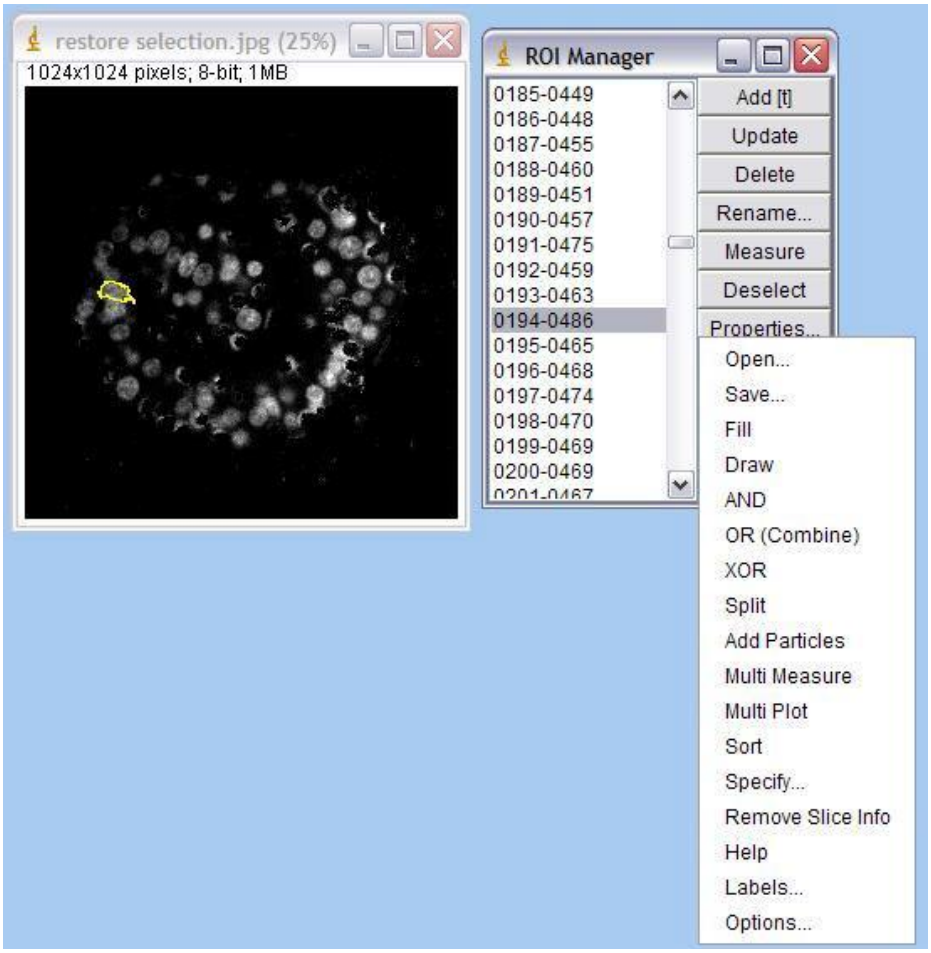
Unchecking "labels" will turn off the numbering on the image.

If we click on the original image and then on one of the region names in the ROI manager list, we can restore the region to the original image (blue outline shown at right).



Multiple regions can be selected by holding down the "shift" or "control" keys.

Hitting the "Measure" button in the ROI Manager window will measure the region(s) selected, giving you a Results table with all the measurements you selected under Analyze → Set Measurements. "Delete" will remove an ROI from the list, "Update" will save any changes (movement, resize) done by hand to an ROI. "Add" or keyboard shortcut "t" will add a hand-drawn region to the list.



The “More” button at the bottom of the list will give even more tools.

The “Multi Measure” option is particularly useful, it will give whatever measurements you selected under the Analyze → Set Measurements menu for all regions in the ROI manager. Regions do not have to be highlighted (selected) to be measured.

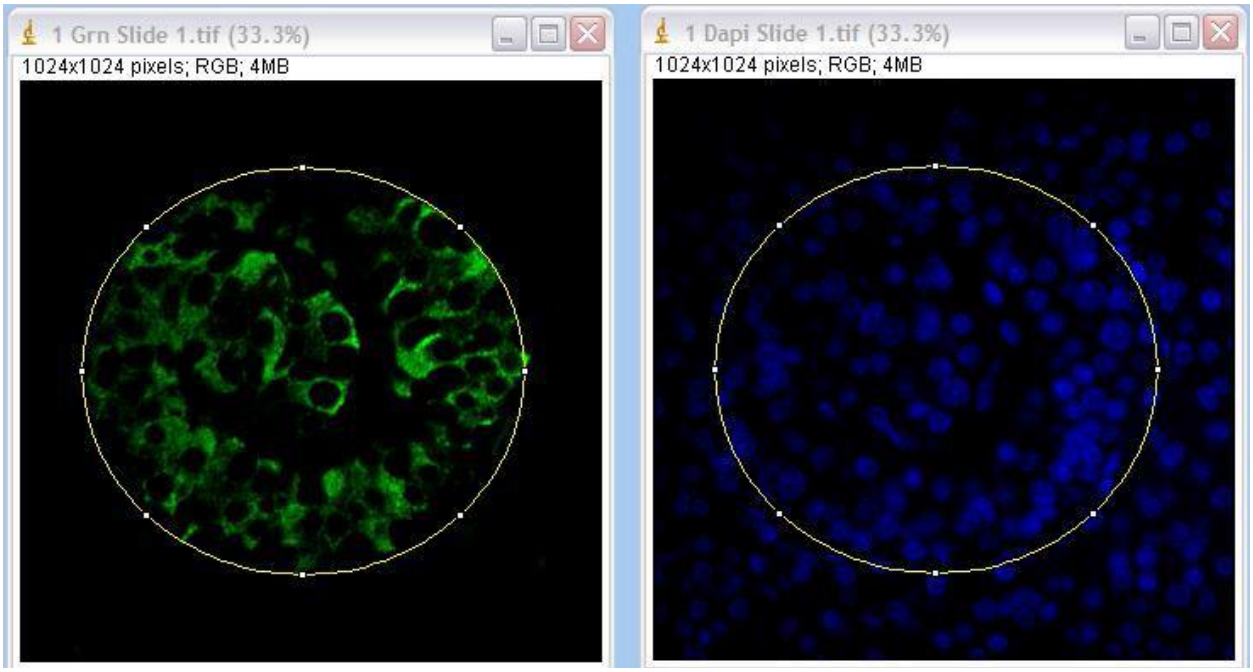
“Save” will allow you to save your list of ROIs for future use. The list gets saved in a zipped folder. To reuse, open the “Open” Analyze → Tools → ROI manager and go to More → Open. Choose the zipped folder with your ROI set in it and the regions will appear back in the list.

Transferring Regions

Restore Selection

The Redirect and ROI Manager features are good ways for generating a lot of regions (e.g. outlines of nuclei) and then using those regions to get information from multiple images (e.g. multiple color channels, to determine something like how many nuclei are red, how many are green and how many are both).

But what if we wanted to know how many small particles were inside a larger object? For example, in the image below, we have one channel representing a pancreatic islet (green, left) and one channel representing nuclei (blue, right). What if we need to know how many nuclei were in the islet vs. in the whole image?



Edit → Selection → Restore Selection (keyboard shortcut Ctrl + Shift + E) can be used to copy any object drawn on one image to other images of the same size. The region/line/arrow will be copied with the same xy position and size.

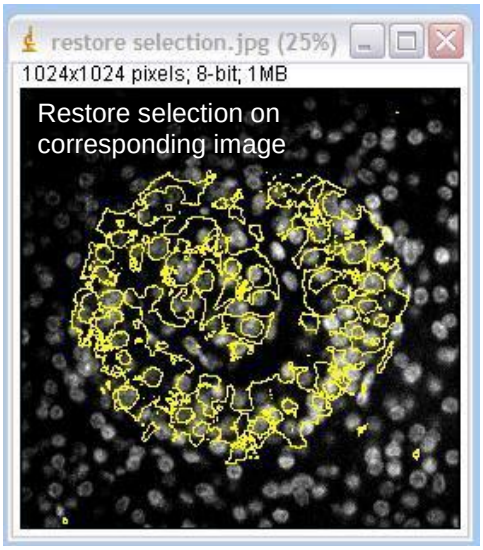
Regions can be moved or resized once they are transferred to the new image. In this example, the islet in the green image was circled using the oval draw tool. Then the DAPI blue image was selected and the selection was restored with the Restore Selection command. The “clear outside” tool could be used to eliminate non-islet nuclei from an object count.

Restore selection is also useful for cropping multiple images to the same size and shape for figure creation. You can use Edit → Selection → Specify if you need to draw an object with a defined size.

Creating and Transferring a Selection with a Threshold

Drawn regions have their place, but often an image has objects with irregular shapes, multiple objects, or we have a dataset with too many images to hand-draw regions. In these cases, a more exact way to transfer the location of regions is to create a mask. Using the green islet image from the previous example, first use Image → Adjust → Threshold to threshold the object(s) to be outlined. Remember threshold only works on grayscale images, which is why this image was converted from RGB color to 8-bit gray.

Once the object is thresholded, use Edit → Selection → Create Selection to outline the thresholded area. Finally, click the image you want to transfer the region to and use Edit → Selection → Restore Selection to create the region on the new image(s).



Let's say we now wanted to count the number of nuclei and know the average pixel intensity of each nucleus inside the region defined by the islet (nuclei inside the yellow border (right).

1) Once you have restored your outline onto the nuclei image, use Edit → Clear Outside to remove nuclei outside the region of interest.

2) Create a copy of the cleared image and threshold / apply the remaining nuclei to create a binary mask image (pg. 11).

3) Redirect to the non-binary copy under Analyze → Set Measurements (pg. 5).

4) Click on the cleared, binary copy of the nuclei image. Use Analyze → Analyze Particles to get statistics for all nuclei in your image (pg. 12).

Installing and Using Toolsets

Toolsets are another type of addition for ImageJ. They add on to the functions of the main task bar, and are written like macro code but installed in a way that is similar to installing a plug-in. We will see two different toolsets in this tutorial which enhance skills that we have from the Basics and Intermediate classes, but there are other toolsets out there. Remember, all ImageJ resources are free to anyone, so don't be afraid to download new tools and explore their functions!

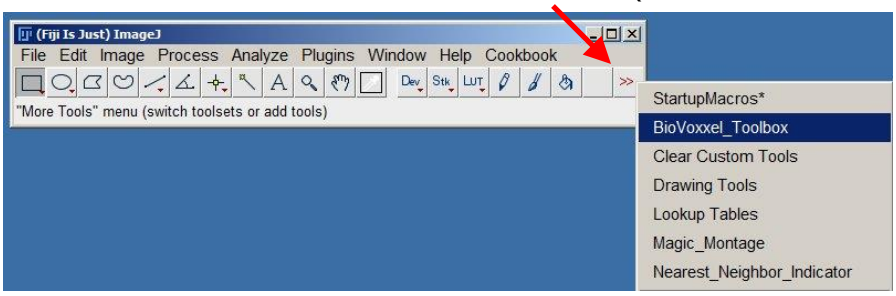
BioVoxxel_Toolbox

The BioVoxxel Toolbox was developed by Dr. Jan Brocher, a member of the ImageJ/Fiji community with extensive experience in collecting and processing images.

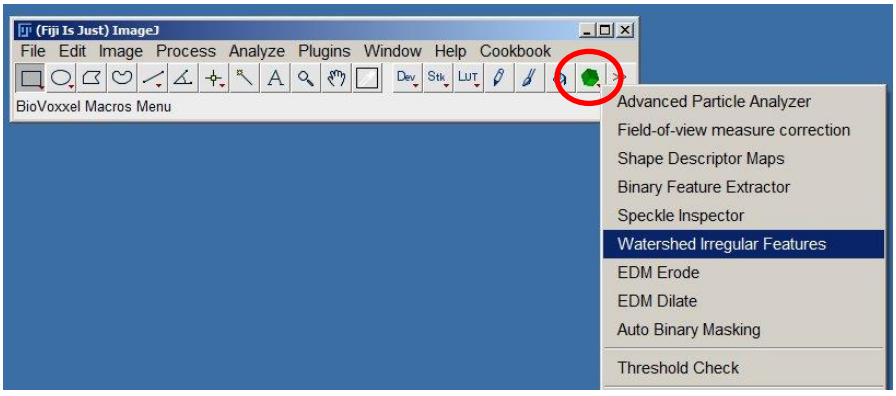
His Toolbox is available (with descriptions of the functions) on the Fiji site: http://fiji.sc/BioVoxxel_Toolbox or without descriptions from his own site at http://www.biovoxxel.de/macros.html. Either way, to install, right click on the link and choose the "Save link as" option, saving the link into your ImageJ → Macros → Toolsets folder.

If you are using Fiji, you can set up automatic updates for the BioVoxxel_Toolbox under Help → Update Fiji. Click the "Manage Update sites" button (after the Checksummer) and check the box next to "BioVoxxel."

Once installed, click the More Tools Icon (the red double arrow) on the taskbar



Selecting the BioVoxxel_Taskbar will add a new icon to your taskbar. The Bio_Voxxel icon is a green cube with a red arrow. Click this icon to see the Toolbox dropdown menu.

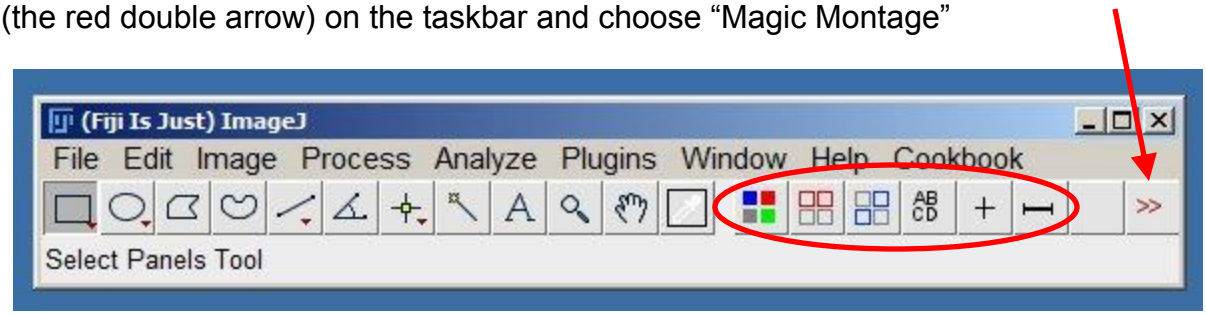


My two favorite tools are Watershed Irregular Features, which allows tuning of the watershed function for better separation of non-round objects, and the Threshold Check, which allows you to try all of the autothresholds at once and can help with choosing a suitable autothreshold function. Click the Fiji link above for more documentation on these tools and all others in the Toolbox.

Magic Montage

Magic Montage was created by Jerome Mutterer. The code for the Magic Montage tool can be found on the ImageJ main page at http://rsbweb.nih.gov/ij/macros/toolsets/Magic%20Montage.txt.

To install, copy/paste the text into a .txt file and save it to your ImageJ → Macros → Toolsets folder. Once installed, restart ImageJ/Fiji and then click the More Tools Icon (the red double arrow) on the taskbar and choose "Magic Montage"



You will see some additional icons added to your toolbar:

The first icon will create a montage from an RGB image (splitting out one panel per color channel, similar to the RGB to Montage plugin).

The second button allows you to right click on and manipulate images within the montage (copy, paste, add images, etc).

The third button allows you to re-arrange panels within the montage, and dragging the line outside the border of the montage allows you to add panels.

The fourth button allows you to add text (right click on the button to see options).

For more information, there is a great, short video tutorial by Mathieu Erhardt on the ImageJ / Fiji documentation wiki:

http://imagejdocu.tudor.lu/doku.php?id=video:utilities:creating_montages_with_magic_montage