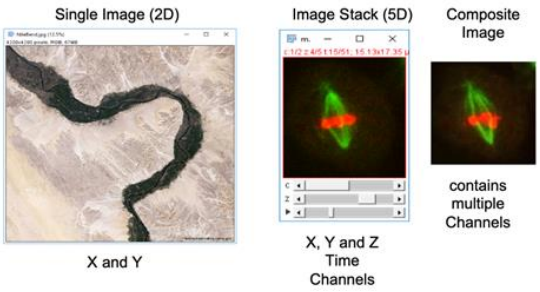
Image Processing

FIJI software

Introduction

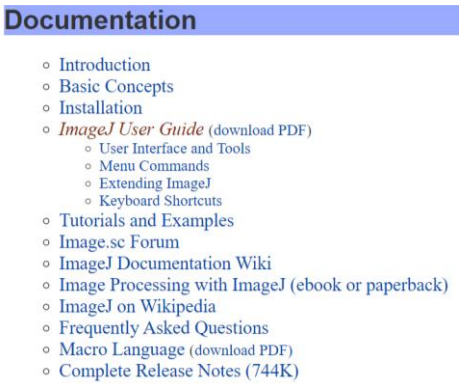
Image processing is the class of methods that deal with manipulating images through the use of computer algorithms. Bioimage analysis can help you to automatically analyze large amounts of image data (such as magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, hyperspectral images, optical microscopy and many more); to reproducibly extract quantitative information from images (to collect quantitative measurements in time and space), to quantify the form and structure of cells and organisms.

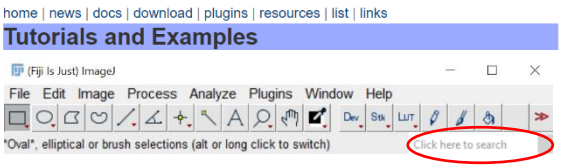
ImageJ is open source software for processing and analyzing scientific images. ImageJ facilitates image analysis techniques including image processing, colocalization, deconvolution, registration, segmentation, tracking, visualization, and more. fiji is an image processing package – a "batteries-included" distribution of ImageJ, bundling many plugins which facilitate scientific image analysis.



Beginning

Use Help! Most of the problems have already solutions. FIJI contains a vast amount of plugins. The *Documentation* option has a number of tutorials that guide you through a variety of typical tasks.





Use Search box with keywords to find the steps to solve your issue.

The most frequent types of images ImageJ deals with are

- 8–bit Images that can display 256 (2^8) gray levels (integers only), range of 0–255 (black, 254 grey shades, white).
- 16–bit Images that can display 65536 (2^{16}) gray levels (integers only).
- 32–bit Images that can display 4294967296 (2^{32}) gray levels (real numbers). In 32–bit images, pixels are described by floating point values and can have any intensity value including NaN (Not a Number).
- RGB Color Images that can display 256 values in the Red, Green and Blue channel. These are 24–bit ($2^{3\times8}$) images. RGB color images can also be 32–bit color images (24–bit color images with additional eight bits coding alpha blending values, i.e., transparency).

Opening images (Bio-Formats)

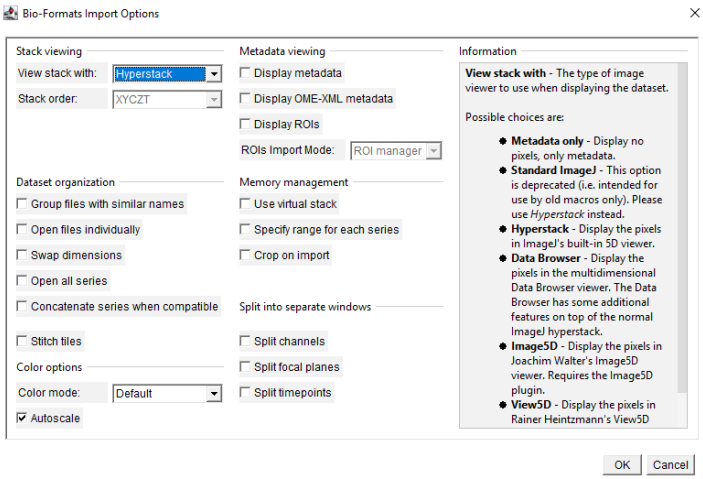
1. Drag an image to the FIJI tool bar or select *File>Open* from the top menu
2. If the image you are opening is a standard format e.g. TIFF, JPG, PNG it should open immediately. If the file type is more obscure you will see the Bio-Formats Import Options window which allows you to set how the image should be opened:

Stack Viewing Determines the window type used to display the image, choose Hyperstack.

Metadata Viewing Opens the standard metadata and/or OME-XML metadata, the latter can be useful when opening OME-Tiffs i.e. Micro-Manager files and will give details of how the image was captured.

Memory Management

1. For datasets too large to be loaded into memory, Virtual Stack is a good option as it only reads the image plane you are viewing into memory at a time.
2. *Specify Range for Each Series* opens only the specified range of image planes as selected by the user. A second dialog will open once the dimensions of the dataset have been analyzed by Bio-Formats. This allows you to select how much of the data you want to open.
3. Crop on import allows the XY dimensions of the image to be cropped to save on memory. In the dialog in the first two boxes you should input the top left corner pixel number of the rectangle you want to crop. The second two boxes tell's bioformats how big the cropped region should be.



Color Options

1. Color mode dictates how the channel colors are handled in the images. The most common option is *Composite*. The channels will be merged and open as a single plane, colored according to the image metadata.
2. Colorized will show a plane for each channel in a single window with a scroll bar at the bottom, so you will only be able to see one channel at a time.
3. If *Autoscale* is selected the image intensity display range will map to the min and max data values in the image rather than the bit depth limits of the file format (0-255, 0-4095 etc). Useful for 12- and 16-bit images as often the data is low intensity and is often only several thousand counts, mapping to the full range would make the image hard to see.

Common rules

1. Use tiff files or specific image files, use with cautions png files, do not use compressed jpeg.
2. ImageJ can help you to analyze large complex data, but it won't find more than you. Improve sample quality before image acquisition!
3. 8-bit images are displayed using a grayscale or color lookup table (LUT) => *Image* > *Color* > *Display LUT*)

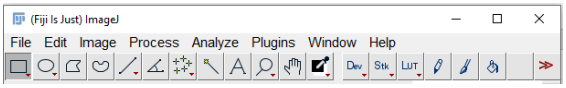
LUT describes the color to be used for each of 256 possible displayed pixel values (pseudo-color)

4. Button «*Undo*» is not like in Office => Use *ctrl+shift+D* to Duplicate image!

Use *Revert* function to return to original image.

Adjusting the image window size and magnification

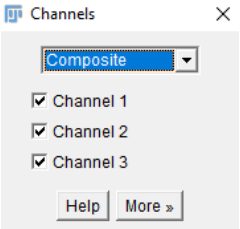
1. The size of the image window on the screen will depend on the physical pixel dimensions of the image itself and FIJI will try to optimize it.
2. You can increase/decrease the zoom and size of the image by selecting the *Magnifying Glass* icon. Left click to magnify, Ctrl-left click to de-magnify (% size shown next to the image title).



3. When you magnify you'll see two purple rectangles in the top left corner of the image. The outer rectangle represents the whole image and the inner rectangle is the part of the image you can see on the screen. If you want to see more of the image you can go to the bottom right corner of the image and drag it out. Alternatively, if you don't want to increase the amount of screen the image is occupying you can select the hand tool then left click and drag to make another part of the image visible.

Selecting a channel and changing colors

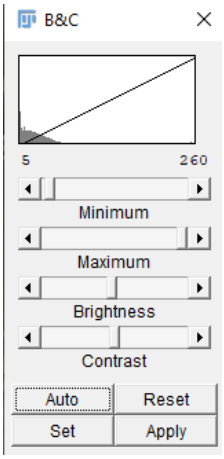
1. Most images will open with the channels merged and in Composite Image mode. Typically, there will be a horizontal scroll bar along the bottom of the image labelled "C" for Channel. Notice when you move this the image doesn't change apart from the color of the text at the top and the bounding box around the image. This is selecting a particular channel of the image for further manipulation e.g. to adjust the brightness of the selected channel only.



2. To physically toggle a channel off go to *Image>Color>Channels Tool*. Then uncheck the channels as required.
3. If you want to change the color of a particular channel, select the channel with the "C" scroll bar along the bottom then select *Image>Lookup Tables* and choose from the list.

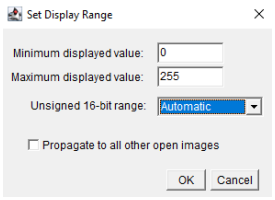
Brightness and Contrast

1. Select the channel first as described above then select *Image>Adjust>Brightness/Contrast*.
2. Where you see the histogram the two values on the x axis are the minimum and maximum displayed values. In simple terms moving the maximum scroll bar to the left will make the image brighter and to the left darker. Moving the minimum to the right will make the lower intensity pixels (background) appear to have a higher value or lower if moved to the left.
3. For an 8-bit image pixel intensity values are in the range 0-255 (0=black, 255=white). When you adjust the min and max you should consider these new values as if they were 0 and 255. For example, if the max is set to 208 any pixel with a value =>208 will appear to have an intensity equal to 255 on the display. Likewise, for the minimum, if it was set to 10 then any value <=10 will appear to have an intensity equal to 0.



4. It's important to note that this just changes the image display but does not change the underlying pixel values when you view a histogram or make intensity measurements. However, if you press the *Apply* button this will then set the underlying pixel values to the current display range and will affect intensity measurements but it will only do this for 8-bit images.
5. *Auto* automatically optimizes brightness and contrast based on the image's histogram. It makes a proportion of the pixels saturated. FIJI by default will run the Auto brightness/contrast function when you open an image as well.
6. Press *Reset* to set the display range e.g 0=208 to the full pixel value range e.g. for an 8-bit image 0-255.

7. How you set the brightness and contrast is important when you want to visually compare the intensity of several images captured with the same exposure time. You should either set them all to the full pixel value range by pressing *Reset* or use the *Set* button to manually set the display range. You can then propagate these values to the other open images.



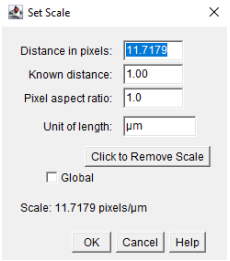
8. The Unsigned 16-bit range drop down menu allows you to scale the range of grey levels for 16-bit images. E.g. if your 16-bit image has a maximum of 3000 and the display range is set to the pixel value range 0-65535 the image will be very dark, but would be easier to see if you set it to the 12-bit 0-4095 range for example.

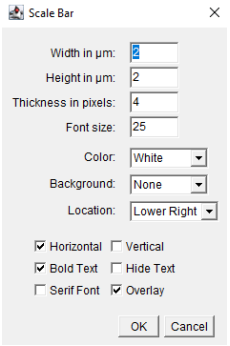
Multidimensional images

1. When you open an image that includes multiple dimensions e.g. Z, Multi-point and Time you can only look at one image frame at once. There should be horizontal scroll bars at the bottom of the image to allow you to move through each dimension e.g. C, Z, T.
2. Many of the function's relating to image stacks are found in *Image>Stacks.*

Image properties, setting scale and scale bars

1. Select *Image>Show info* and you will see the metadata of the image. If you need to find out a particular detail about the image or how it was taken this is where to do it.
2. Images with no scale set will only list dimensions in pixels at the top of the image. If a scale is set you will also see the dimensions in µm.
3. If you need to set or adjust the scale go to *Analyze>Set Scale*.
4. If a scale is set you can see it at the bottom of the window. To adjust the scale set the Distance in Pixels to 1. The known Distance is the number of µm 1 pixel represents. You need to know this figure in order to set the scale correctly.
5. Once the scale is set you can add a scale bar to the image through *Analyze>Tools>Scale Bar*.
6. You can change various aspects of the scale bars appearance most of which is self-explanatory. If Overlay is selected then the scale bar is added as an overlay. This is a non-permanent addition to the image which doesn't affect underlying pixel values. You can hide or show an overlay at any time using *Image>Overlay>Show/Hide Overlay*. It will only be accessible when you open the image in FIJI. If Overlay is not ticked then the scale bar is "stamped" onto the image and is permanent and cannot be removed. It also becomes part of the pixel data and will affect any intensity-based measurements.





Selections and ROIs (ROI Manager)

1. A Selection is essentially an ROI. They are used to isolate a part of the image e.g. if you want to crop the image or if you want to measure only within a defined area. You can use any of the selection tools to draw a selection on the image.
2. If you want to add multiple selections to the image and recall them at any point, use the ROI Manager (*Analyze>Tools>ROI Manager*).
3. Draw a selection then press Add to store it, a row will be added to the ROI Manager table. You can add multiple selections to the image in this way. Clicking on a row will make it active and show it on the image.
4. Change the color of selections using *Edit>Options>Colors*

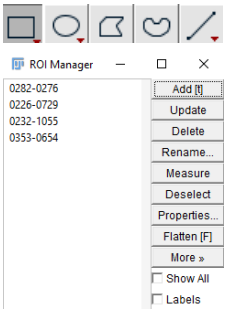
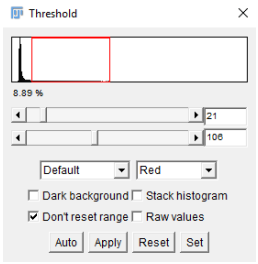


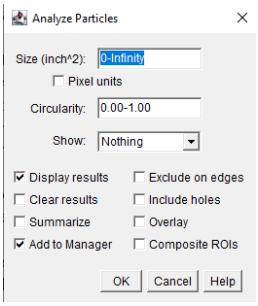
Image Wide and Selection Based Measurements

1. Select *Analyze>Set Measurements*, here you can decide what measurements you want to make.
2. Limit to threshold applies if a threshold has been applied to the image, see sections below.
3. Add to overlay creates a removable layer on the image which will highlight what has been measured, this can be toggled off/on via *Image>Overlay>Show/Hide Overlay*.
4. Redirect To enables selection of any open image and the measurements will be done on the image selected 5. To calculate these measurements, select *Analyze>Measure*.
6. If you draw a selection before pressing measure only the pixels within the selection will be measured.
7. A table of the results will open, save it using *File>Save As*.
8. To summarize the results and get the mean, SD, minimum and maximum of the values in each column select *Results>Summarize*.

Identification of Objects and Measurements (Thresholding)



1. Setting a threshold is a means to segment images into features of interest and background. It is intensity based and involves isolating intensities of interest using the image histogram.
2. Select *Image>Adjust>Threshold*. An automatic threshold will be calculated and any pixels included in the threshold will be colored red. Those pixels fall within the red box now on the histogram in the threshold window.
3. You can expand/reduce the limits of the threshold to include more or less intense pixels by moving the horizontal scroll bars. Press *Auto* to go back to the auto calculated threshold.
4. *Reset* will remove the threshold altogether.
5. *Apply* will binarize the image i.e. all pixels in the threshold become black, a value of 0 and all pixels in the background become white, a value of 255. Using *Apply* isn't necessary, just close the threshold window and the threshold will still be visible on the image.
6. Set the measurements you want to calculate as described earlier then select *Analyze>Analyze Particles*.



7. The size box is a size filter, by default any object size is accepted so long as it falls within the threshold. Note the size is the area in pixels.
8. Circularity filters how close to a perfect circle the objects are, where 1 is a perfect circle. This is difficult to set without having a preview of how this affects the objects included in the threshold.
9. The *Show* drop down determines how the identified objects will be displayed, *Outlines* is useful as it creates a new image with no data other than the outline of each object a number to identify it.
10. Display *Results* will show the results table and associated values from all objects.
11. *Exclude on edges* and *Include holes* will exclude any objects touching the image boundary and will include any objects where the threshold encompasses the object but an area internally is not within the threshold respectively.
12. Selecting *Add to Manager* will add all the identified objects as separate rows in the *ROI Manager*. This allows selection of any of the objects for further individual or batch processing.
13. You could delete all objects not meeting your criteria by holding ctrl click to select them then press *Delete*. Then measure the remaining again by selecting *Show All* and press *Measure* from the *ROI Manager*.

Examples

A. Unmixing of objects with specific spectra

Nuance file **.im3** can't be opened in ImageJ using **BioFormat** plugin if reference cube was used. Therefore, cubes should be saved as a tiff cubes in Nuance.

1. *File > Import > Image Sequence > Select **your folder***.
2. *Image > Type* and select 8-bit to convert all slices in the stack (wavelengths are indicated)
3. *Plugin > Install > Depth Color Code 0.0.2*, MSA_514, and Compute. Select Plugins folder in Fiji.app
4. *Plugin > Stacks > Z stack > Depth Color Code 0.0.2*. Use Rainbow RGB
5. *Image > Stacks > Z project* (rendering)
6. *Image > Stacks > Plot Z-axis profile* (point, line, box) to see spectra of the objects, use ROI manager
7. *Plugin > MSA 514*. Number of factorial images 4 (For uniform background reference values of illumination should be acquired)
8. Multivariate Statistical Analysis of image series https://imagej.nih.gov/ij/plugins/inserm514/
   a) Principal Component Analysis (PCA) b) Correspondence Analysis (CA)
9. *Ctrl+Shift+D* to duplicate target component images and then make Composite image
10. *Image > Adjust > Threshold*; *Image > LUT > Red/Blue*; *Image > Color > Merge channels*

B. Quantification of area

1. *File > Open **your file***. *Ctrl+Shift+D* to duplicate image.
2. *Image > Type* and select 8-bit. Color values are averaged or *Image > Color > Split Channels*. Select the corresponding channel (8-bit).
3. Straight selection. *Analyze > Set scale (mm)*. Can be saved
4. *Image > Adjust > Threshold.* Use the same values in all images
5. Wand tool (Tolerance). *Analyze > Set Measurements*. Measure (see Help)
6. Copy results
7. *Analyze > Analyze Particles*. Select the 'Add to Manager'
8. **Create macros for batch analysis (Plugins > Macros > Record)**
   Repeat all the steps (*Image Type, Threshold, Analyze Particles)*. Do not close Threshold window. After press *Create*
9. **Use new macros in batch analysis (Process > Batch).** Choose the folder with your images, you want to analyze and your created macros.

C. Quantification of Live/Dead Staining

1. Split channels: live cells are in the Green Channel and dead cells are Red Channel. Discard the Blue Channel. Channels must be analyzed separately for live and dead fluorescent channels (*Image > Color > Split Channels*)
2. Work with one channel at a time. Convert the channel to 8-bit (if they are not yet in that color graphic format). This is done so that the images may be threshold based on intensity (*Image > Type > 8-bit*)
3. Select the *Find Maxima* function from each channel to count number of dead or live cells. Select the *Point Selection* output type and check the box for *Preview Point Selection*. Adjust the *Noise Tolerance* values by increments of 5 or 10 until background staining is excluded (Be mindful of adjusting the tolerance value so the red and the green "points" do not overlap. However, some overlap may occur and it is dependent on the user to determine how much overlap is acceptable). The number

of points will be the total number of cells positive for the stain of interest (numbers are based on user's definition of Live/Dead cells) (*Process > Find Maxima*)

Use the following formulas for quantification:

Total Cell Number = Live Cells + Dead Cells

Percentage of Live Cells = (Live Cells/Total Cell Number) *100

Percentage of Dead Cells = (Dead Cells/Total Cell Number) *100