Introduction to Fiji (ImageJ) for microscopy image analysis

2016 WIMM

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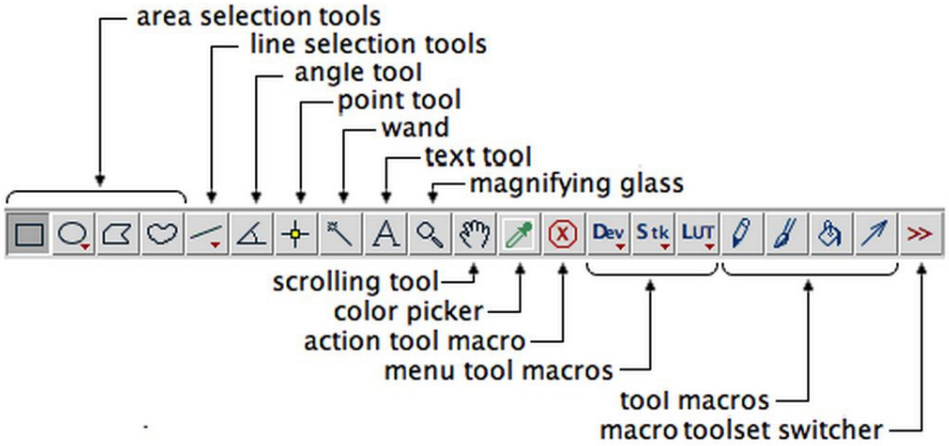
1. Basic Image Characteristics.



1a) Start by opening the Fiji application which you should have previously downloaded. This is achieved by either double-clicking the “Fiji.app” (mac) file or the “Fiji Image Win64.exe” file in the directory where you saved the files.

1b) Now we want to open an image. Before starting this course you should have downloaded and unzipped some image files. Go to this directory and drag the image named “Cell_Colony.tif” onto the Fiji toolbar (to the area just below the

icons). The image should then open. If this fails you can open the image using the Fiji menu system. Go to File, then Open. (*File->Open)*. From here find and open the images you downloaded before this practical.

1c) Try zooming in on the Cell Colony image (Magnifying glass tool button + mouse, or the +/- keys). Do you notice anything odd or inconsistent about the background? hint: this file was once a jpeg and the compression has affected the image. **Always keep your raw data and do not use image compression. Don't rely on compression formats for long-term storage. (There are some good examples, before and after compression: https://en.wikipedia.org/wiki/Compression_artifact).**

Invert the Cell Colony image (*Edit->Invert*). Imagine for a moment that the distorted background is noise: now find one of the faint "particles" (actually a colony), and draw a line through it, that is around 5-10x as long as the "particle" is wide. The line tool is the fifth one along the toolbar, left-click the line icon. You can plot a line profile using *Analyze->Plot Profile* (Ctrl-K (PC) and Cmd-K(mac)). Get a feel for what the intensity of the "signal" is compared to the random intensity variations in the background?

1d) Now take your inverted Cell Colony image and duplicate it two times (*Image->Duplicate* or Ctrl-Shift-D(PC), Cmd-Shift-D(mac)). Try multiplying one of the duplicate images by 8, and try dividing the other by 8 (use *Process->Math->Multiply...* and *Divide...*). Place the mouse cursor over the pixels in the results. On the toolbar you will see the pixel intensities, in the multiplied image do you notice anything strange about the numbers? Here we have artificially distorted the image intensities, but can you see why it is important to have appropriate image exposure and/or gain settings during your acquisition?

Note that *Image->Adjust->Brightness/Contrast* (Ctrl-Shift-C (PC), Cmd-Shift-C(mac)) cannot undo the damage of exceeding your detector's dynamic range. Ideally you want to just about fill the range available - no more, no less. To visualize whether there is saturation or clipping of the image intensities, change the Look-Up-Table of the image. If you choose the HiLo LUT you can view saturation as red pixels in the image and clipping of low intensities as blue pixels. *Image->Look Up Tables -> HiLo*. Now experiment with the Brightness/Contrast settings as before and see what happens.

2. Thresholding and binary operations.

2a) The image histogram is an important concept to understand. Open the image cell_fluorescent.tif. Select the whole image, *Edit->Selection->Select All* (Ctrl-A (PC), Cmd-A(mac)) and then go to *Analyze->Histogram*. The plot displays the number of pixels in each intensity bin (256 bins is the default). If you click the list button you can examine the raw numbers. Notice the statistics displayed

underneath, including minimum and maximum values, standard deviation, mean etc. Does the histogram make sense? Remember the peaks in the histogram represent the number of pixels of a certain intensity in the input image.

Select a dim region of the image using the 'Oval' selection tool and repeat the analysis. Now select a region entirely within a brighter area e.g. a cell and look at the histogram. Finally, examine the histogram for a region that is half cell body, half dim region. Can you explain what you see? This information can be used as the basis for distinguishing the two regions, using a thresholding method which analyses the histogram.

2b*)* Display settings allow the displayed values to be changed without altering the underlying pixel values in the raw data - a transfer function maps the raw pixel values to the displayed values. Open the 'blobs.tif' sample Image. *Image->Adjust->Brightness/Contrast* is the normal way to adjust display settings. There are buttons to *Auto*-adjust and 'Reset' to the original settings. The 'Set' button allows you to type in values manually, and even propagate the settings to other channels and images. For example, try setting the minimum and maximum values to 127 and 196, and reset to the original settings. You should be careful with the finalize button, 'Apply'. Set min/max to 127/196 again, but this time click 'Apply'. You should find that 'Reset' button no longer works, the changes have been permanently applied. Try *File->Save As->Text Image*, this is another way of inspecting the pixel values directly, can you see how your pixels have changed.

You may have used a gamma correction function in other image analysis packages - this maps the raw intensity data to displayed intensities in a non-linear way, emphasizing dim or bright features by assigning more levels to them. ImageJ does have a gamma correction function, but it is hidden in under *Process->Math->Gamma*. Note that this is a non-reversible transformation and so data is lost! If you click the Preview button you can see the effect of changing Gamma from a low value (emphasizing dim features) to a high value (emphasizing bright features). Use this only for illustration however and not for paper figures. Non-linear mappings must be avoided for analysis because they distort relationships in data.

2c) Segmentation is a means of separating image pixels into different subsets. The most common method for segmenting an image is to apply a threshold. Open the cell_fluorescent.tif sample image. Now we want to duplicate this image so we can experiment with different algorithms *Image->Duplicate*. Next open the threshold dialog *Image->Adjust->Threshold.* Move the threshold slide-bars under the histogram and notice the effect. Try and find a level with segments the blobs from their background and click apply. You have now segmented an image.

2d) Use a fresh copy of the cell_fluorescent.tif sample image. Now once again go to the threshold dialog *Image->Adjust->Threshold.* This time rather than manually changing the slider, click on the box which presently says 'Default' and then click

'Otsu'. This is an automatic thresholding algorithm which attempts to find a threshold value which best segments the image population in the intensity histogram. Click 'Apply' to see the output binary image. Notice now all the pixels are either of value '0' or '255'. Algorithms such as 'Otsu' can be used to automatically segment images without human interaction. More details about how the 'Otsu' algorithm works can be found here:
https://en.wikipedia.org/wiki/Otsu%27s_method

2e) Go back to the 8-bit version of the image and duplicate another fresh copy. Add some noise *Process->Noise->Add Noise.* Now once again apply a threshold and use the 'Otsu' algorithm. What you do you notice? Try this again on a fresh image but add noise to the same image three times. What do you notice as the noise gets worse? How do think having noisy data might affect your analysis?

2f) With a fresh version of the cell_fluorescent.tif image. Go to the threshold dialog *Image->Adjust->Threshold.* Choose another threshold method from the list and then click 'Apply'. Now we are going to manipulate the resultant binary image using binary morphological operations. Go to *Process->Binary* and click erode. What do you notice? Go to *Edit->Invert* and then click *Process->Binary->erode* once again. What do you notice? Create a fresh binary image. Try some of the other options. Invert the image and see how the operation changes its effect. These tools can be used as the basis of an image analysis pipeline. You could use these operators to split clumps of cells into individual cells or just to find the outline of a cell.

You have learnt a lot! Why not take a 5 min break to let all the information be absorbed.

3. More about image display

3a) In Fiji there are three main types of image: Single channel images, RGB images and multi-channel images. RGB images are those most commonly used in photography. Comprising of red, green and blue channels it is possible to create any natural image by combining these colours. RGB channels are not usually used in in microscopy as channels are generally used in isolation (one at a time), or in abundance (3+) and also generally have arbitrary wavelengths associated with them (not necessarily red green and blue). It is important therefore, to know the difference. You have already seen single channel images (e.g. cell_fluorescent.tif). An example of an RGB image is leaf.tif. Open this image and observe the pixel values as you move the mouse pointer around the image.

3b) Multi-channel images differ from an RGB image in that each channel is a completely separate image. Fiji has a number of tools to manipulate channels, their display, and conversion to and from RGB images.

Take a look at the neuron.tif sample image. You have a “c” slider which changes the channel (observe the fringe of the image changing colour). If you open *Image->Color->Channels Tool…* you can control which channels are on or off in this composite representation. Uncheck and re-check the channels to see how they contribute to the image. If you select ‘Color’ rather than ‘Composite’ from the drop-down menu you can view each colour channel in isolation. Look at the options in the *More >>* menu, and try changing the colour of the channel you are looking at. Note that the choice of colour in this visualization is up to you and not dependent on the wavelength of the channel for instance. Often grayscale is the best choice for analysis, and remember that the human eye is most sensitive to Green>Red>Blue in that order.

Now take a fresh image and make sure it is in ‘Composite’ mode. If you move the channel slider now, notice that the colour of the window border and info string (top-left of image) change to match the channel you have selected. Now try changing the second channel (“yellow”) to “green” and then adjust brightness/contrast (Ctrl-Shift-C(PC), Cmd-Shift-C(mac)). You should now, if you open the histogram as before, that the histogram is also coloured to reflect the channel colour you have set. Try ‘*Convert to RGB’* through using the ‘More’ menu in the Channels dialog box. Notice that the R,G,B values you see when you ‘mouseover’ the image are difficult to interpret.

In general, it is good practice to preserve separate channels for processing and analysis until the final stages of producing a figure.

3c) Look-Up Tables (LUTs) map intensity values to a displayed colour. Open the Blobs.tif image. Go to *Image->Lookup Tables->Invert LUT*. What values do you see when you mouse-over the dark and bright regions of the image? Is this what you expected? (i.e. are the values high when the image is bright?). Have a look at *Image->Color->Show LUT* and/or *Image->Color->Edit LUT* and you should be able to see what is going on (hint, press the Invert button).

The LUT for an image can be changed using *Image->Lookup Tables* - try the LUT named ‘Grays*’*. Now that you have the default LUT you should now find the intensity values in the image make more sense. Try some of the more colourful schemes: ‘Fire’, ‘Rainbow RGB’ etc. and examine how the colour changes with intensity using ‘Show LUT’ and/or ‘Edit LUT’ (hint: when I say change, I mean no change). LUTs can be helpful for visualizing subtle changes in intensity, and so are extremely useful for techniques such as FRET or fluorescence lifetime imaging. Using ‘Edit LUT*’* try making your own LUT that shows Blob centre, boundary and background in different colours.

4. Colocalization

This section is a bit more challenging and will require all the skills you have previously learnt. If you get stuck doesn't hesitate to ask for help.

1a) Open the image Healthy.lsm. Examine the image. You should notice that there are two channels. We want to establish the colocalization between the two channels using the Pearson's correlation coefficient. To do this you will first have to split the channels: *Image->Color->Split Channels*. Note: (Green channel is filipin III (binds cholesterol) magenta is Lysotracker (as name implies stains lysosomes). Niemann Pick C is a disease where cholesterol trafficking to plasma membrane is impaired and it accumulates in lysosomes). Inspect the images. From what you have learnt from the lecture on colocalization what do you think makes this image less than ideal for Pearson's colocalization analysis? Tip (try using the Hi-Lo LUT).

1b) Go, via the menu, to *Analyze->Colocalization-> Coloc 2* function. Add the two channels that you have just split, one from either channel, leave the 'ROI mask' unselected for now. Run the colocalization with all the parameters unchecked. Look for the output marked Pearson's R value (no threshold). What value do you get? Repeat this for the other image called Sick.lsm. How do the R- values differ for these two images? Do you think you could use this result as the first step towards distinguishing the visual phenotype of the two images?

1c) Open the image neuron.tif. We only want to work with the first and last channel. One way to separate them from the original image is to use image->Duplicate. Check the box marked 'Duplicate HyperStack' but type '1' in the channels box rather than having '1-4'. Click 'ok'. Repeat, but duplicate channel 4 instead. You should now have two images (one for each channel).

1d) Using the Fiji menu go to *Analyze->Colocalization->Coloc 2* function. Click the first two slices that you created, one from either channel, leave the 'ROI mask' unselected for now. Run the colocalization with all the parameters checked. Look for the output marked Pearson's R-value (no threshold). What value do you get?

1e) This time repeat the exercise but with an oval selection surrounding only the fluorescent region of the cell with minimal background. Next go to *Edit->Selection->Create Mask*. In the 'Coloc 2' dialog box you will have to select under 'mask or ROI' the newly created mask image entitled 'Mask'. Run the 'Coloc 2' plugin. What is the Pearson's R-value now? What is the effect of removing the background pixels on the R-value of the test and control condition? Why do you think this is the case?

1f) Repeat the previous step but this time create a mask from one of the

channels. To do this we are going to threshold the cyan channel. First, duplicate the cyan channel using *Image->Duplicate* command, as you do this rename the file by changeing the text 'neuron.tif' to 'mask' in the duplicate image dialog box. Then bring up the thresholding dialog by Image->Adjust->Threshold and then click 'set'. Type in '5' in the lower threshold box and '255' in the upper threshold box. Next, click 'ok', and then 'apply'. You will then produce a binary image of your thresholded image. The area within the cell should contain pixels of '255' and the are outside the cell should have pixel values of '0'. You may have to invert the threshold mask for it to work correctly *Edit->Invert*. Also ensure that the LUT is not inverted. *Image->Lookup Tables->Grays*. This can sometimes happen automatically depending on your Fiji settings. In the 'Coloc 2' dialog box you will have to select under 'mask or ROI' the newly created mask image entitled 'mask'. To establish what areas are being included in the colocalization analysis make sure the 'Display Images in Results' checkbox is positive. Once you have run the plugin, you can view the included pixels by selecting either 'Channel 1' or Channel 2' from the drop-down box at the top of the output dialog. Does the colocalization R-value change if you change the mask? What is the reason for this?