Guide to FIJI / ImageJ

A public domain software for processing and analyzing scientific images.
FIJI (“FIJI is Just ImageJ”) builds on ImageJ with community-sourced plug-ins.
Download at https://imagej.net/software/fiji/

Updated for 2021 by AL

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Keyboard Shortcuts

Here's a list of some useful shortcuts in attempted alphabetical order.

- Brightness/contrast: ctrl + shift + c
- Copy ROI on each image: ctrl + shift + e
- Duplicate selected region (cropping): ctrl + shift + d
- Line scan: ctrl + k
- Measure (after drawing): m
- Orthogonal view: ctrl + shift + h

Z-Stack Analysis

This is how to measure the fluorescence of microtubules. Orthogonal view Z-stack analysis requires a few extra steps and has its own page below.

1. Open z-stack in FIJI.
2. Adjust brightness/contrast by dragging slider to ~middle and Ctrl + shift + c. Either adjust yourself or click auto.
3. Remove scale (microns -> pixels) by clicking through Analyze -> set scale -> remove scale.
4. Create a sum project by clicking Image -> stacks -> z project -> sum slices.
5. It may help you see the microtubules better to invert black and white. To do this, go to Image -> Lookup tables -> invert LUT.
6. Draw around the midzone area with the freehand select tool (bean-shaped symbol). Hit ‘m’ and FIJI will give you midzone area and mean intensity.
7. Try to drag drawn shape to background to measure the background; if there’s not enough space in the cell body then just draw a new area. Hit ‘m’ again to measure.

Z-Stack Analysis (Orthogonal View)

This is how to measure the fluorescence of microtubules from the orthogonal view. See above for non-orthogonal method.

1. Open z-stack in FIJI.
2. Adjust brightness/contrast by dragging slider to ~middle and Ctrl + shift + c. Either adjust yourself or click auto.
3. Remove scale (microns -> pixels) by clicking through Analyze -> set scale -> remove scale.
4. It may help you see the microtubules better to invert black and white. To do this, go to Image -> Lookup tables -> invert LUT.
5. Rotate if the cell is tilted through Transform, but try to avoid this as much as possible as the image processing results in some data loss.
6. Open orthogonal view through Image -> Stacks -> Orthogonal view.
7. Drag slider to the frame most in focus and adjust yellow crosshair to ~middle of the cell.
8. Save the popup window that shows microtubule bundles as dots. First convert to 8-bit through Image -> type -> 8-bit, then save as .tif.
9. Make sure to name this saved orthogonal view and to put it in a place you can find easily.
10. You can close the old windows and open your new orthogonal view.
11. It may help you differentiate microtubule bundles from background by setting a threshold. Click Image -> Adjust -> Threshold. Drag sliders to where only microtubules are highlighted.
12. Draw around the bundles with the freehand select tool (bean-shaped symbol). Hit ‘m’ and FIJI will give you the midzone area and mean intensity.
13. Draw around the whole cell to measure the background. Hit ‘m’ again to measure area and mean intensity.

Making Figures

This is how to make a figure, including how to crop and merge. Depending on what kind of figure you're trying to make, you may need to mix and match instructions. Make sure to save intermediate images and all your work in case you need to backtrack.

1. Cropping: To keep cropping consistent across several panels, use the ROI manager.
    - First draw a rectangle around your region of interest.
    - Go to Analyze -> tools -> ROI manager and add the ROI. Coordinates should show up.
        - If you write down the dimensions (in pixels) of the box you use, then you can easily make the scale bar by converting pixels to microns.
    - You can put this ROI on each image with shortcut ctrl + shift + E.
    - Duplicate your selected region (ctrl + shift + d) to get your image in a new window.
2. Saving your image: 16-bit doesn't work well with most computers (your image will appear to be a black box if you just click save). A little conversion is necessary to make it play nice.
    - Convert your image to 8-bit through Image -> type -> 8-bit.
    - Next, save as .tif to preserve metadata.
3. Setting a scale bar
    - Go to Analyze -> set scale/Tools
    - Set scale if necessary
        - The student scope at 100x is 21.4851 pixels/micrometer. You may need to find the correct conversions for other scopes/objectives.
        - Known dist = 1
    - To choose where you scale bar is on your image, draw a selection on the image, then set scale bar at the selection
    - To merge the scale bar onto the image (it's currently only a floating overlay), go to Image -> overlay -> flatten
4. Adjust brightness/contrast: Black background is optimal, but use your best judgement.

Selections/Projections with Z-stacks

A z-stack is a series of images from the bottom to the top of the cell. When you make a figure, here are just some of the things you can do: choose one slice from the stack, make a max intensity projection of the brightest pixels in all of the slices in the stack, make a sum projection of all the fluorescence across all the slices, or make a projection of just certain slices.

1. Taking a single slice from a stack
    - Open the .tif stack in FIJI
    - There is a slider at the bottom of the stack. Scroll to the slice you want to use.
    - Duplicate it (ctrl + shift + d) to get it in a new window. If it prompts you in a window to fill in the number of the slice you want to duplicate, fill in the number, uncheck “entire stack,” and click ok. Save this slice as 8-bit through Image -> type -> 8-bit.
2. Making a z-projection from the slices
    - Go to Images -> Stacks -> Z-project
    - Select the kind you want (max intensity, sum, etc.). Max usually results in pretty pictures.
    - Save as 8-bit through Image -> type -> 8-bit.
3. Adding pseudo color to grayscale image
    - Image -> Lookup tables
4. Inverting values (useful for things with dark background if you plan to print)
    - Edit -> Invert

Merging Color Z-Stacks

This is how to color your z-stacks and merge 2 stacks of different colors into the same image. This is useful for visualizing 2 different kinds of staining at the same time. If you want overlapping structures to be highlighted in a different color after the merge, see the next page on co-localization.

1. Open the two stacks.
    - They must have the same number of slices and image size.
2. For each stack, change from B&W to color using Image -> Lookup tables.
    - Traditionally actin is red, microtubule is green
    - To make them color-blind friendly, use magenta and green (and blue, if three colors)
    - Another alternative is red, cyan, yellow.
3. Merge the stacks via Image -> color -> merge.
    - Make sure both source images and combined images are saved.
4. Adjusting intensity of each color channel
    - You will see 2 sliders below your merged image. One navigates through the planes in the stack, and the other switches between color channels. Slide the latter to the color you want to adjust and hit ctrl + shift + c for brightness/contrast.
    - If you want to save the image without the two channel slider, then go to Image -> type -> RGB color. Now you have one slider, but you won't be able to adjust the colors separately anymore.

Co-localization

This is a method to merge images and highlight overlapping structures in a different color. This is useful for visualizing 2 different kinds of staining at the same time. While it's not a perfect technique, some labs use it.

1. Open your 2 cropped images with the area of interest selected.
    - Images must have the same bit depth (ex. 8-bit, 16-bit)
2. Go to Analyze -> Colocalization -> Coloc 2.
3. In the Coloc 2 window, select each image, then select "no mask" and "Costes regression."
4. Select show pdf, and make sure all algorithms are checked.
5. In the results box, use the Pearsons coefficient value
    - 1 -> 100% colocalization
    - 0 -> no correlation
    - -1 -> 100% anti-colocalization
    - Any positive coefficient value indicates some colocalization
    - The closer the number is to 1, the better the 2 images colocalize.

Line Scan Analysis

This is how to measure fluorescence along the length of a line of your desired length and width.

1. For each z, create a sum project via Image -> stacks -> z-project -> sum project.
2. Draw a line across the midzone (not pole to pole). Doubleclick on the line tool to set line width.
    - Keep track of the width you use, and keep it consistent across all the z's
    - Analyze each cell twice, once with the default width of 1, and once with a thicker line
        - When choosing thickness, use your best judgement to make sure it doesn't go outside the midzone. Expect something around 25.
3. Go to Analyze -> plot profile (ctrl + k) and a new window will open. At the bottom, if you click Data -> copy 1st dataset, you can copy the data into a spreadsheet of your choice.

Changing FIJI's Default Cursor

Here is how to change FIJI's default cross cursor to your computer's default pointer which is useful if you find FIJI's cursor too large or distracting to draw accurately. It is possible to change the cursor to something of your choosing via macros if you have a .gif image (see "25.2 Pointer" at this FIJI guide: https://imagej.nih.gov/ij/docs/guide/146-25.html), but this is a quick fix.

1. Go to Edit -> Options -> Misc… -> "Use pointer cursor".
2. That's it!

Correcting for Photobleaching

Fluorescence is lost whenever the cells are exposed to light for photography. This is how to take photobleaching into account when you take measurements of cell intensity. Generally you only need to calculate the photobleaching factor once for a certain day and apply it to all cell z-stacks taken that day, assuming that all environmental factors are constant within one photography session.

1. Make sum projects of each z-stack at Images -> Stacks -> Z-project -> Sum.
2. Draw along the entire cell for each z-stack, each representing a different time frame.
3. Hit m to measure the area and mean intensity of the whole cell over time.
4. Find the photobleaching factor. It is recommended to do this in a spreadsheet.
    - Create a column for area, and another for whole cell intensity
    - In a third column, get the photobleaching factor by dividing all whole cell intensities by the first one. This can be done by putting a formula such as “=A1/A1” in the first cell, then drag down to propagate the formula.
5. Go back to the data you’re trying to correct for photobleaching. Divide each total intensity of the midzone by its photobleaching factor.