

Basic Image Processing with FIJI/ImageJ

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1 NO MATTER WHAT YOU ARE DOING, READ THIS FIRST

1.1 IMPORTANT CONSIDERATIONS

- Images are data. **NEVER overwrite an original image file.** If you make adjustments to the image, save it as a new file.
- **Keep track of the manipulations you've made** to each image, e.g. in a simple text file that you place in the same folder as the image.
- Because image processing changes an image's appearance, you must **carefully consider the ethical implications of what you are doing.** How do the changes you are making affect the interpretation and presentation of your data? A full discussion of the ethical implications of image processing is beyond the scope of this guide. See [this paper](#) from Douglas Cromeey at the University of Arizona for a brief overview. When in doubt, talk with your advisor and/or the light microscopy facility director.
- Except for ethically applied simple linear histogram (intensity) adjustments, reducing bit depth, and cropping, ALL image processing procedures MUST be disclosed in your Methods section and/or figure legend.

1.2 CONVENTIONS USED IN THIS DOCUMENT

- For a quick introduction to the layout of the ImageJ program, see [here](#).
- Navigation through menus and sub-menus is notated using '>'. For example, "Image > Type" tells you to go to access the Image menu and then the Type sub-menu.
- True-color images are images of specimens with color (e.g. histological stains). Fluorescence images are NOT color images; they are monochrome (black and white) and the color is added by the computer. (In rare cases, you may have taken a fluorescence image with a color camera; in this case, you should convert the image to monochrome. See [Section 3.](#))

2 DOWNLOADING FIJI/IMAGEJ

FIJI is a pre-packaged version of ImageJ that is pre-configured with numerous useful plugins. Most importantly, it allows you to open image files generated by nearly any microscope/camera software. The basic version of ImageJ cannot do this without loading additional plugins. *Although I usually refer to the program as “ImageJ” in the following instructions, I am assuming you are actually using the FIJI version of ImageJ and NOT the basic version.*

To install FIJI, download the version appropriate for your operating system [here](#). Save the “installation file” anywhere convenient (except in the Program Files folder if you are running Windows). Uncompress the file and the application is ready to use; there is no installation procedure. To start the program, double-click the *fiji.app* file.

3 GETTING STARTED

3.1 OPENING AN IMAGE FILE

Open an image file by dragging and dropping it onto the ImageJ main control panel.

- Most simple images (.jpg and .tif files) will open immediately.
- More complex files (.jpg2, .nd2, .zvi files) will display a large panel called “Bio-Formats Import Options.” Select **hyperstack** in the first drop-down and be sure that all boxes are **unchecked**.
 - If you have a true-color image (e.g. histology), once the image opens, select Image > Type > RGB Color. Close the old image and work with the new one.
 - See [Section 5](#) for how to work with multi-dimensional images (i.e. with >1 channel, z-slice, time-point, etc.)

If your image is a color image (RGB), but there is no important color information in the image (e.g. fluorescence; non-stained DIC), then it’s best to convert the file to monochrome (black and white): go to Image > Type > 8-bit. The color information is unnecessary.

Does the image look completely dark or very dim? You need to adjust the intensities. See Section 4.

3.2 NAVIGATING YOUR IMAGE

- **Zooming:** Press “+” to zoom-in; press “-” to zoom-out.
- **Panning** (moving the image around): hold-down the spacebar while clicking and dragging (or use the hand tool on the toolbar).

4 IMPROVING INTENSITY VISUALIZATION

This is most critical with fluorescence images. If you are adjusting a true-color image (e.g. histology), be sure that it is in RGB Color (go to Image > Type > RGB Color).

4.1 LINEAR HISTOGRAM ADJUSTMENTS

1. Go to Image > Adjust > Brightness/Contrast (or press SHIFT-C). You'll see a panel with a histogram of all the intensities in this image.
2. Dragging the "Maximum" slider to the left will increase the brightness of the image. Do not increase the brightness so much that you saturate (wash-out) features in your image.
3. Dragging the "Minimum" slide to the right will increase the contrast of the image. Do not increase the contrast so much that dim features in the background disappear.
4. All of your changes are temporary unless you click "Apply." Note that there is NO undo. Remember to save this image as a new file and DO NOT overwrite the original file.

4.2 GAMMA ADJUSTMENT

Sometimes an image contains a wide range of intensities and it is difficult to find a display range that shows the dimmest objects while not saturating the brightest. Adjusting the gamma setting may help.

1. First, adjust the brightness and contrast (see Section 4.1, above).
2. Next, try adjusting the gamma setting. This setting basically changes how intensities are scaled between the two limits (min and max) that you set in the B&C panel. Gamma = 1.0 is normal. Values < 1.0 accentuate differences between low intensities while values > 1.0 accentuate differences between high intensities.
 - a. Go to Process > Math > Gamma...
 - b. Be sure that "Preview" is checked
 - c. Try adjusting the gamma value.
3. Hit "Apply" on the B&C panel and save this image. Note that there is NO undo. Remember to save this image as a new file and DO NOT overwrite the original file.

4.3 SINGLE-CHANNEL FLUORESCENCE IMAGES

In general, you should always display single-channel fluorescence images in black and white. If there is a large range of intensities in the image and it is difficult to find a display range that allows visualization of the dimmest objects while not saturating the brightest, you can consider applying a look-up-table (LUT). (See the above sub-section for an alternative strategy.) In addition, if you really need to color your single-channel image, you can also do this with a LUT.

To see a listing of the available LUTs, go to Image > Color > Display LUTs. The multi-color LUTs are to help you visualize a large range of intensities. The single-color LUTs are to color a monochrome image.

To apply a LUT to your black and white image (or to change you color image to monochrome):

1. Click on the LUT button on the main ImageJ toolbar
2. Select a LUT (choose "Grays" for black and white)

5 WORKING WITH MULTI-DIMENSIONAL IMAGES OPENED AS HYPERSTACKS

Multi-dimensional images will be displayed in a window with scroll bars at the bottom that allow you to scroll through each of the available dimensions (c = channel, z = z-slice, t = time-point).

How to extract a single image from a multi-dimensional series:

1. Be sure the image is displayed exactly as you desire.
2. Edit > Copy
3. File > New > Internal Clipboard
4. This procedure only works for single-channel images. If you have a multi-channel image, you must be in *Color* or *Grayscale* mode or convert it to RGB first (see Section 5.1, below).

5.1 IMAGES WITH MULTIPLE CHANNELS

Open the Channels Tool: press SHIFT-Z or go to Image > Color > Channels Tool...

How to control how the channels are displayed using the pull-down menu:

- *Composite* allows you to see all channels at the same time (overlaid). Use the check boxes to determine which channels are displayed.
- *Color* displays only one channel at a time. Use the check boxes to switch between channels (you can also use the scroll bar in the image window)
- *Grayscale* is the same as *Color* except it shows the channels in grayscale.

How to control the intensity and/or color of each channel:

1. Switch to *Color* mode using the pull-down menu.
2. Select the channel you want to change.
3. Change the brightness and contrast as described in [Section 4](#).
4. Change the color by clicking the “More >>” button and selecting the desired color from the drop-down menu.

How to convert a *Composite* (overlay) image to RGB for importing into PowerPoint, Photoshop, etc.:

- Click the “More >>” button and select “Convert to RGB”
- It is difficult to undo this process, so be sure to save your new image as a new document; do not overwrite the original.

5.2 IMAGES WITH MULTIPLE Z-SLICES (I.E. A Z-STACK)

There are a large number of z-stack manipulations that can be performed with ImageJ. The most common operation with z-stacks is to generate a z-projection:

1. Image > Stacks > Z-project...
2. Select the beginning and ending slices to be included in the projection (default is to use the entire stack).
3. Select the projection type. Maximum is commonly used for fluorescence images.

6 SHADING CORRECTION

This kind of correction is especially important for DIC but is also useful for any time when illumination is uneven or there is dirt in the optics that you want to remove. In the Flat-Field Correction process, a background image (i.e. nothing in the field-of-view) is divided from your image to “cancel-out” any variation in background intensity. What if you didn’t take any background images? Use the Pseudo-Flat-Field Correction – it works almost as well.

6.1 FLAT-FIELD CORRECTION

1. Take your image.
2. Take your background image: without changing any settings, move to a blank area of the slide and take an image. If you’re having trouble finding a completely empty area, try de-focusing to blur-out the objects. Alternatively, you can carefully remove your slide and put in an empty slide to get a background image.
3. Open both images in ImageJ.
4. Select the background image and hit Ctrl-A to select the entire image. Press “m” to measure the mean intensity of the image.
5. Go to Process > Calculator plus.
6. Set the following parameters, then hit OK:
 - a. $i1$ = the image
 - b. $i2$ = the background image
 - c. Operation = Divide ($k1*(i1/i2) + k2$)
 - d. $k1$ = the mean intensity you just measured
 - e. $k2 = 0$
7. A new window appears with you corrected image.

6.2 PSEUDO-FLAT-FIELD CORRECTION

If you didn’t take a background image.

1. Open your image that you want to correct.
2. Duplicate it: Image > Duplicate...
3. Blur-out the duplicate image: Process > Filters > Gaussian blur...
 - a. Be sure “Preview” is checked
 - b. Enter a radius value of 5 and see how the image changes (but don’t hit OK yet!). You want the image to show the background but not the objects.
 - c. If you can still see the object you imaged, increase the radius value.
 - d. Once you’re satisfied, click OK.
4. Perform the same flat-field correction procedure outlined above, but using the blurred duplicate image instead of the background image you acquired.

7 COMMON MODIFICATIONS FOR PREPARING A FIGURE

7.1 REDUCING BIT-DEPTH

Our eyes are not sensitive to > 8-bit intensity levels. If your black and white image has a higher bit-depth, you will want to reduce it to 8-bit for publication to reduce your file size. (Note this does not apply to RGB images, which are already 8-bit.)

1. Go to Image > Type.
2. 16-bit is checked (even if your image is 12-bit or 14-bit, it is still “packaged” in a 16-bit file).
3. Click on 8-bit.
4. That’s it! Your eyes won’t notice a difference. Keep in mind, though, that the quantitative intensity data captured in your original image HAS changed. Be sure to keep the original image.

7.2 OVERLAY MULTIPLE FLUORESCENCE IMAGES

Basic rules for displaying fluorescence images:

- If you are only displaying one channel, display it in monochrome (black and white), NOT in color.
- If you are displaying a multi-channel overlay (in color), you should also display the individual channels in monochrome next to the overlay.
- Do not use green and red in the same image since it will be difficult for most colorblind individuals to differentiate the two channels. Use magenta instead of red.
- Blue is often difficult to see; try using cyan instead.

If you are working with a multi-channel image, you simply need to switch to *Composite* view and then convert the image to RGB (see [Section 5.1](#)).

If you are working with multiple, independent files...

1. Open all images that you want to combine.
2. Image > Color > Merge Channels...
3. Assign each image to the desired color
4. Be sure that all three boxes are checked
5. A multi-channel composite image is generated. You can now manipulate it as described in [Section 5.1](#).

7.3 SCALING/REDUCING THE IMAGE DIMENSIONS

Only do this inside the program you’re using to assemble your figure.

7.4 CROPPING

1. Use the box tool to draw a square around the area you want to isolate.
2. Image > Crop.

8 ADDING A SCALE BAR TO A FIGURE

8.1 SCALE BAR GUIDELINES

- Yes, you need a scale bar.
- If you feel like it's not necessary to include a scale bar, see the previous point.
- The length of the scale bar should be sized to provide a good reference for the features of interest in the image. Don't make the scale bar very short or very long.
- Scale bars should be white or black, depending on what stands-out the best in the image. Black with a white outline or white with a black outline are also appropriate.
- Scale bars should ordinarily be located in a bottom corner of the image, but can be located in other corners to avoid obstructing objects in the image.
- If there are multiple images with identical pixel sizes and image dimensions being displayed next to each other in a figure, you only need to include a scale bar in one of the images.
- According to long-standing tradition, you are not supposed to write the size of the scale bar (text) next to the scale bar. However, I feel like this is an antiquated practice and only serves to make the reader have to search your figure legend to find the size. My personal feeling is that you should always include the size in presentation visuals and, if you can get away with it, in your published figures.

8.2 DETERMINE THE IMAGE'S PIXEL SIZE

Before you can add a scale bar, you need to know your image's "pixel size" – the size of a single pixel, typically in microns (μm).

Sometimes the scale is automatically recorded in the image's metadata and is displayed in ImageJ. The confocal and several of the other microscopes in the facility do this. You can tell if your image is calibrated by checking the text at the top of the image window. Among other things, the text displays the dimensions of the image (e.g. 512x512). Check the units listed after the numbers: if the units are "pixels" or anything non-metric, then your image is not calibrated.

How to determine the pixel size in a calibrated image:

1. Open the image in ImageJ
2. Go to Image > Properties...
3. Set "Unit of length" to μm (micron; for most microscopes) or mm (for the Macroscope)
4. Read the pixel size from the "Pixel height" or "Pixel depth" boxes (they should be identical). This is the pixel size in units of microns/pixel (or mm/pixel if you set that).

How to determine the pixel size in a non-calibrated image:

- If you've done some image processing, it's possible that the calibration was lost along the way. Check to see if the original image is calibrated.
- Otherwise, if you used any other scope in the microscopy facility, you can check the specification sheet posted near the instrument. Note that the pixel sizes do NOT apply to confocal; you must check the image's metadata to determine the pixel size.

How to determine the pixel size in images acquired with the MacroScope or stereoscopes

1. You will need to have a ruler included alongside your specimens in your images. You don't need to actually display the ruler in your final images (you can crop it out), but you do need it to determine the scale.
2. Open the image in ImageJ
3. Use the line segment tool to draw a line of known length on the ruler (e.g. draw a line between the 0 mark and the 1 cm mark). Using longer lengths will improve the measurement precision (e.g. it's better to measure 20 mm than 1 mm).
4. Analyze > Set Scale...
5. The "Distance in pixels" will be automatically set to the length of the line you just drew.
6. Specify the "Known distance" and "Unit of length" (e.g. '10' and 'mm', respectively if you drew a 10 mm line).
7. "Pixel aspect ratio" should be 1.0 and the "Global" box should NOT be checked.

8.3 ADDING A SCALE BAR IN IMAGEJ

This is one way to add a scale bar. Basically, you make the scale bar in ImageJ and then cover it with a nicer-looking scale bar in whatever program you're using to assemble your figure. The reason for covering the original scale bar is a little technical and I don't want to explain it right now. Just trust me.

1. If the image is not already calibrated, determine the pixel size and calibrate the image.
 - a. Image > Properties
 - b. Set Units to "microns" or "mm" as applicable.
 - c. Set the width and height (numbers are expressed in $\mu\text{m}/\text{px}$ or mm/px depending on what units you set). The width and height should be identical.
2. Analyze > Tools > Scale Bar
 - a. Set the desired width (in microns or mm depending on your calibration)
 - b. Set the height in pixels so that the bar is skinny, but still visible.
 - c. Set "Color" and "Location" as desired (see guidelines in [Section 8.1](#)).
 - d. Set "Background" to none.
 - e. Select the "Hide Text" box. This will hide the scale bar size. Don't worry, you can add it back in the program you're using to assemble your figure.
 - f. Be sure that Overlay is unchecked.
3. This is a permanent change! When you save this image, do not overwrite the original! You might want to include the scale bar size in the file name.
4. When you import your image into your figure preparation program and resize it, the scale bar probably won't look very good anymore. You can solve this problem by drawing a new scale bar (a thin box) that covers-up the original.

8.4 ADDING A SCALE BAR USING A FIGURE-PREPARATION PROGRAM

This protocol is more involved, but gives you a bit more freedom to design your figure. You don't have to decide where to place the scale bar when you're working with the image in ImageJ, but instead can wait until you are finished assembling the figure. The protocol provided here for PowerPoint will be similar in other programs (e.g. Inkscape, GIMP), but the actual commands and menus will, of course, be different.

1. Determine the size of your image in pixels.
 - a. Often this is displayed in Windows Explorer or the Finder if you select the image file.
 - b. Otherwise, try right-clicking the image and choosing “Image Properties” or “Get Info.”
2. Calculate the image’s width in “real” space (i.e. the width of the imaged area in your sample, usually in μm or mm).
 - a. Multiply the width of the image in pixels by the pixel size of your image.
 - b. For example, if your image is 2000 pixels wide and the pixel size is $0.1 \mu\text{m}/\text{pixel}$, the width of your image in microns is $2000 \times 0.1 = 200 \mu\text{m}$.
3. Import the image into PowerPoint and resize it as desired, but DO NOT crop. You may crop the image after you’re finished with this procedure. Better yet, crop the image BEFORE importing it.
4. Determine the size of your image as it appears in your figure document.
 - a. For most versions of PowerPoint, if you click on the image, its height and width will appear on the Format Picture ribbon at the top-right of the screen.
 - b. Otherwise, right-click on the image, select “Format Picture” and look under the “Size” tab. You want to find the image’s size in inches (or cm if your computer uses metric).
5. Calculate the scale bar size.
 - a. Use the width of the image in “real” space (in μm) and the width of the image in the figure (in inches or cm) to set-up a proportion: “real” width / figure width.
 - b. For example, if the image is $200 \mu\text{m}$ wide in real life and 1 inch wide in the figure, your proportion will be $200 \mu\text{m} / 1 \text{ in.}$
 - c. Decide how big you want the scale bar to be.
 - d. Use the proportion to calculate how big your scale bar should be in the figure.
 - e. For example, if you want a $20 \mu\text{m}$ scale bar and your proportion is $200 \mu\text{m} / 1 \text{ in.}$, set-up and solve this equation: $200 \mu\text{m} / 1 \text{ in.} = 20 \mu\text{m} / ?$. Your answer will be 0.1 inches – that’s how long your scale bar needs to be.
6. Draw a thin box that has a width equal to what you just calculated.

9 SELECTED ADDITIONAL RESOURCES

- [ImageJ User Guide](#): comprehensive reference for all basic ImageJ functions
- [ImageJ Cookbook](#): selected basic and advanced topics
- [Peter Bankhead’s fluorescence microscopy in ImageJ book](#): basic to advanced topics, primarily geared toward fluorescence image analysis; also includes background information on nearly every topic discussed – very informative

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