# Manual for image analysis workshop for the 2013 UCSF Microscopy Course

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Image analysis workshop will be carried out using Fiji and/or Micro-Manager. We've put Fiji for 32 bit Windows and the Mac installer on your USB sticks, so you should be able to use any computer for your analysis. If you want to get Fiji for another platform, you can download it here: http://fiji.sc/wiki/index.php/Downloads. The images mentioned here are also all on your USB stick, as are some example ImageJ macros.

**Section 0 - Information about ImageJ:**
Help on most commands in ImageJ can be found at the ImageJ website: http://imagej.nih.gov/ij/docs/index.html (or 'Help' -> 'ImageJ Website'). In particular http://imagej.nih.gov/ij/docs/guide/userguide-Part-V.html (Menu Commands) has documentation for each command in the ImageJ menus. Susanne Rafelski has written up a helpful introduction to ImageJ documentation here: http://nic.ucsf.edu/dokuwiki/doku.php?id=imagej_introduction

The help menu also has links to plugin and Fiji documentation that you may find helpful.

## Section 1 - Basic image operations

For this section, the students will work with images obtained from slides similar to the ones they have worked with (fluorescent staining of nuclei, mitochondria and actin).

1. Open the three tiff files in directory '3color' (use the Fiji 'File' -> 'Open' command). This is a 3-channel image saved in Micro-Manager format. Images from different software packages may require different importers to load. Fiji includes the 'BioFormats' importer that can read most microscopy file formats (but currently has a bug in its Micro-Manager importing code)..

2. Find out the intensity of a given pixel. (Hint: try mousing over a pixel.)

3. What is the bitdepth of the image you have opened? How can you tell? This data is converted to 8 bits for display (Why?). Note how the software chooses default settings to display this data.

4. Inspect the histogram of your image (Fiji menu: 'Image' -> 'Adjust' -> 'Brightness/Contrast', also look at 'Analyze' -> 'Histogram'). Notice the relation between setting minimum and maximum and brightness and contrast (describe this relation in words).

5. Cropping: Select a region of interest using the Fiji 'rectangular tool' and select 'Image' -> 'Crop' from the Fiji menu. You might want to duplicate your image before cropping ('Image' -> 'Duplicate').

6. Change color of the individual images by changing the Lookup Table (LUT): ‘Image’ -> ‘Lookup Tables’.

7. Overlay the three images into a single RGB image: ‘Image’ -> ‘’Color’ -> ‘Merge Channels...’

8. Add a scale bar: ‘Analyze’ -> ‘Set Scale...’ to set the relation between pixels and physical distance, ‘Analyze’ -> ‘Tools’ -> ‘Scale Bar’ to add the size bar to the image.

9. Export (save) resulting image. What format will you write this image out? Will you use any compression? What kind? Is it lossy? Lossless? Try saving both as a .jpg and a .tif. Reopen both files and compare the intensities of individual pixels. Zoom in to check for artifacts.

## Section 2 - Stacks

For this section, use the image ‘2color Z’.

1. Import the data set as a 2-channel Hyperstack. To do so, select ‘File’ -> ‘Import’ -> ‘Image Sequence...’. Open the directory ‘2color Z’. In the ‘File name Contains’ box enter ‘BF_’ and press OK. Repeat this, but use ‘RFP_new’ in the ‘File name Contains’ box. Choose ‘Image’ -> ‘Color’ -> ‘Merge Channels’, and select the two open stacks. In the resulting Hyperstack, select the Bright field channel, choose ‘Image’ -> ‘Hyperstacks’ ->’Channels Tool’, select ‘More’, and select ‘Grays’ to make the bright field channel Gray.

2. Z-project: ‘Image’ -> ‘Stacks’ -> ‘Z Project...’. There are a number of different kinds of projections. Try these to see which gives the best results for showing the brightfield and fluorescence channels.

3. Export the Z-stack as an AVI movie (‘File’ -> ‘Save As’ -> ‘AVI...’).

4. Explore 3D viewers: ‘Plugins’ -> ‘3D Viewer’, and ‘Plugins’ -> ‘Volume Viewer’. This may work best on a stack containing only the fluorescence channel. The 3D Viewer works better when you first convert the stack of a single channel to an RGB stack (‘Image’ -> ‘Type’ -> ‘RGB’), or display only a single channel. You may also need to adjust the voxel depth (under ‘Image’ -> ‘Properties’, 3 instead of 1 works quite well).

5. Use the 3D viewer to record a 360 degree rotation (3D Viewer menu -> ‘View’ -> ‘Record 360 deg rotation’). Save the resulting movie as an AVI file. Also try the 3D Project tool to generate a 3D rotation: ‘Image’ -> ‘Stacks’ -> ‘3D Project’.

6. There are also viewers that lets you see cross sections through the XY, XZ, and YZ planes of your data stack. They are located in ‘Image’ -> ‘Stacks’ -> ‘View 5D’ and ‘Image’ -> ‘Stacks’ -> ‘Orthogonal Views’. It works particularly well for looking at bead stacks - if you’ve acquired a PSF stack, try looking at it with this tool.

## Section 3 - Quantitative analysis

Students will work with the images of nuclei (as in section 1)

1. Explore gray scale filters and understand how they work: using the software, process the image with the following:

- a mean filter (‘Process’ -> ‘Filters’ -> ‘Mean...’)
- a median filter (‘Process’ -> ‘Filters’ -> ‘Median...’)
- an edge detection kernel (‘Process’ -> ‘Find Edges’)
- Try designing your own filters. (‘Process’ -> ‘Filters’ -> ‘Convolve’) For starters, try making a mean filter of different sizes, or an edge detection or peak detection filter. Checking ‘preview’ makes this easy.

2. Measure intensity in regions of interest. Select ‘Analyze’ -> ‘Set Measurements’. Make sure that ‘Area’, ‘Mean’, ‘Standard Deviation’, ‘Min & max gray value’, and ‘Integrated density’ are checked. Use the ImageJ drawing tool to draw an ellipse around one of the nuclei. Select ‘Analyze’ -> ‘Measure’ (or press ‘m’ on the keyboard). Now move the ROI to an area outside of the nucleus and measure. The difference between the two measurements is the signal specific to the nucleus.

3. Explore manual thresholding and automated methods. For manual thresholding, select ‘Image’ -> ‘Adjust’ -> ‘Threshold’. Check ‘dark background’. The sliders determine the minimum and maximum pixel intensities that will be included in the ‘mask’. Move them such that the nuclei are ‘in’, and the rest is ‘out’. Look at the effect of pressing the ‘Auto’ button. Since setting threshold manually is highly subjective (and labor intensive!) automated methods are often preferred. Many automated methods are included in Fiji (‘Image’ -> ‘Adjust’ -> ‘Auto Threshold’). Try out the ‘Otsu’ method.

3. Binary operations: dilate/erode -> fill, skeletonize, etc..
Open the image of mitochondria (2color/img_00000000_Rhodamine.tif). Select ‘Image’->’Adjust’->’Threshold...’, make sure that ‘Dark background’ is selected, press ‘Auto’, and ‘Apply’. Duplicate the thresholded image (‘Image’ -> ‘Duplicate...’). Dilate the image (‘Process’->’Binary’->Dilate). What happened? Now erode the image (‘Process’->’Binary’->’Erode’). What happened? Compare the result to the original thresholded image.
Duplicate the original thresholded image. Use the binary ‘close’ command (‘Process’->’Binary’->’Close-’). Compare with the image that you previously dlilated and eroded. What does the binary method ‘Open’ do and how does it work?
Go back to the original thresholded image of mitochondria. Skeletonize the image (‘Process’->’Binary’->’Skeletonize’).

4. Object counting and measurements
Open the image of nuclei. Open the Threshold tool, click ‘Auto’ but do not apply the theshold.

Select ‘Analyze’ -> ‘Analyze Particles’. Check ‘display results’, and check ‘Add to manager’. Check the results table and hypothesize which of the nuclei are in G1, S and G2 phase of the cell cycle. Open the Rhodamine channel. In the ROI manager, you can now click on the ROIs corresponding to a nucleus and see where it is located in the mitochondria image, and you can measure the intensity under this mask (click ‘Measure’).

## Section 4 - Fourier transforms and filtering

Low pass, Hi Pass, and Bandpass Fourier filters.Use ‘tubulin field.tif’

In this exercise, we want to use the Fourier transform of the images for filtering.

1. Filter the images with a low pass and hi pass filters, and two different bandpass filters, designed to select low and high spatial frequencies. For each filter you can use ‘Process’ -> ‘FFT’ -> ‘Bandpass Filter’. Save each of these.
2. In constructing these filters, how did you decide the ranges to use for low pass or high pass filters? Can you construct these filters based on an examination of the images?
3. Pick a region defined in one of these images and measure the sum of the intensity in this region in all of these images. How does the intensity change? What happens if you compare the intensity this region with another region (i.e., calculate the relative intensity).
4. FFTs implicitly calculate the transform of an infinitely tiled version of your image (why?), so they are sensitive to the structure at the edge of your image. Try smoothing any structures cut off at the boundary of the image and see how this affects the FFT. The ‘Fast’ part of the FFT also requires that your image be a size that is a power of two, so ImageJ will pad your image with zeros to make its size a power of two. Therefore, the FFT may change when cropping your image to different sizes. Experiment with cropping your image to different sizes (some a power of two, some not) and see how that affects the FFT.

## Section 5 - Scripting and Macros

ImageJ has an extensive macro language that can be used to automate image analysis. It’s extensively documented at http://rsb.info.nih.gov/ij/developer/macro/macros.html and we’ve provided a set of macros, written by Susanne Rafelski, that introduce you to it. These are located in the Image Analysis/Macros folder on your USB stick. These are designed to work on the data in the Image Analysis/Raw Data folder on the USB stick. Work through these macros to see what they do. You’ll have to change the paths in the macros to point to the folder on your USB stick.

The folder ‘multiposition’ has three-color images taken at ten different positions on a slide that you can use to automate analysis of. For example, write a macro to produce 3-color overlays of each position, or quantitate the nuclear intensity as in part 3.4 to try and determine cell cycle state for each cell in all ten images. Macro3 will be helpful to see how to automatically iterate over files.