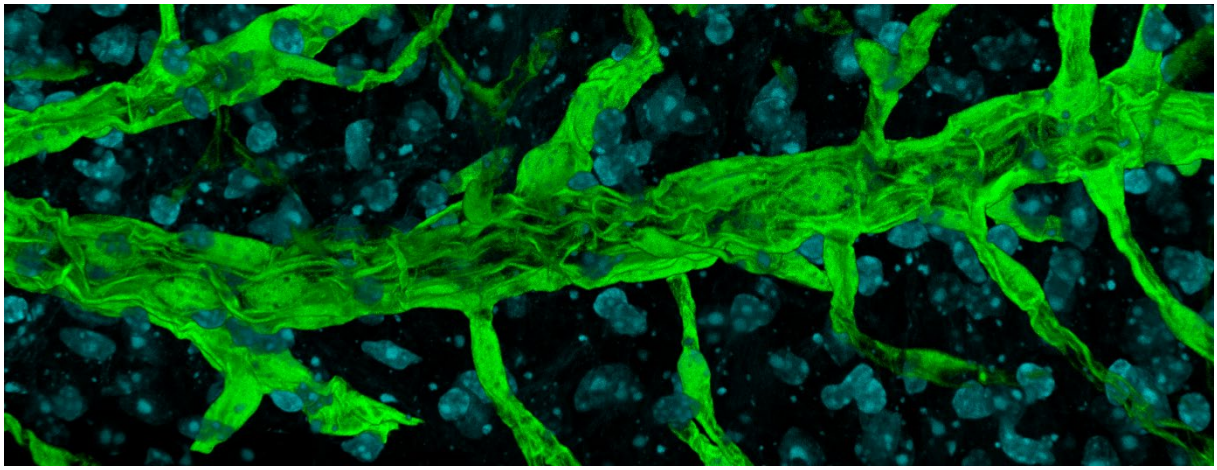
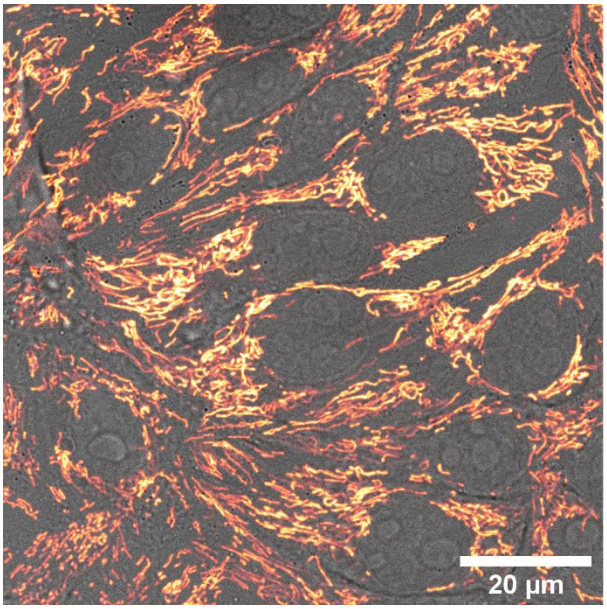
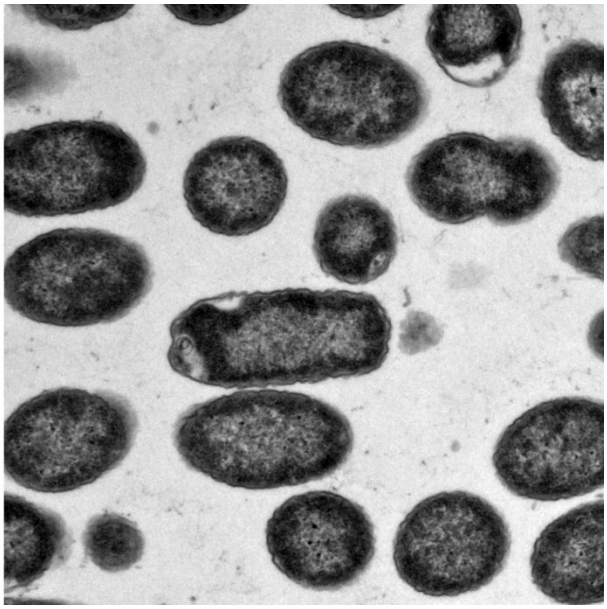
Intro to Image Processing with FiJi

Practical Examples







Center for Microscopy and Image Analysis
University of Zurich

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This script was written as a short summary for the users of Center for Microscopy and Image Analysis, University of Zurich, Switzerland.

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This script was written using Fiji/ImageJ 1.52i.

Contents

1. Abbreviations

ROI: Region of interest

LUT: Look-up table

Stack: Data structure with a set of related images of the same size and bit depth.

Slices: The images that make up a stack.

Hyperstack: Multidimensional data structure, extending image stacks to four (4D) or five (5D) dimensions: x (width), y (height), z (slices), c (channels or wavelengths) and t (time frames).

Voxel: In stacks, a pixel (which represents the smallest 2D image data) becomes a voxel (volumetric pixel), i.e. an intensity value on a regular grid in a three dimensional space.

2. Remarks

This script was written for practical training purposes by the Center for Microscopy and Image Analysis, University of Zurich. Theory is kept to a minimum.

Images - *Center for Microscopy, ZMB;* Sample courtesy - *Prof. Bruno Weber, Inst. Of Pharmacology, UZH and Prof. Andrew Hall, Inst. Of Anatomy, UZH*

The script should be used as a guideline for training.

Highlighting styles used in this script:

File names are set in green (e.g. Cell_Division_Pinhole_0_5AU_Nyquist_7386.lif)

Step 1.
Practical steps are set in italic. ***Keyboard shortcurts here***

```
Macro code:     run("Enhance Contrast", "saturated=0.35");
```

3. Software: FiJi

This script gives a basic introduction to scientific image processing. All exercises can be done using ImageJ or **Fiji** which is just a version of ImageJ supplemented with many useful plugins. It can be downloaded for free from

https://fiji.sc/

Downloadable distributions are available for Windows, Mac OS X and Linux. It can read many image and microscope formats including TIFF, GIF, JPEG, BMP, DICOM, FITS, IMS, LIF, CZI, 'raw'.

Install your own copy of FiJi and selected plugins in your home folder if you are using the virtual machines available to the users of the Center of Microscopy and Image Analysis. Otherwise save it locally in your computer.

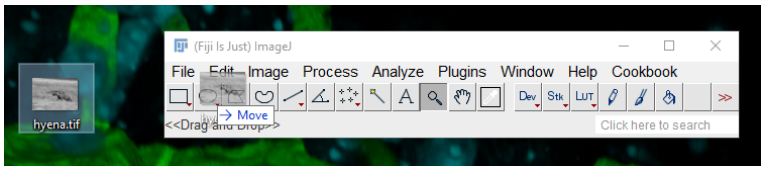
4. Plugins and Updating

When installing FiJi from the Downloads page, the latest *stable* version is available. However FiJi and its various plugins are frequently updated so you should check if you have the latest updates through

Step 1.
Help>Update

Here you can also manage the update sites and install additional useful plugins which are not installed by default.

5. Opening Standard File types



Files can be opened in two ways, **Drag&Drop** the file in the task bar OR through

Step 2. *File>Open*

For some file formats, you may want to explicitly invoke the Bio-Formats Importer to override the default behavior of Fiji. Bio-Formats is largely integrated with the File/Open command of Fiji but might be useful for certain formats and also gives you some advanced options.

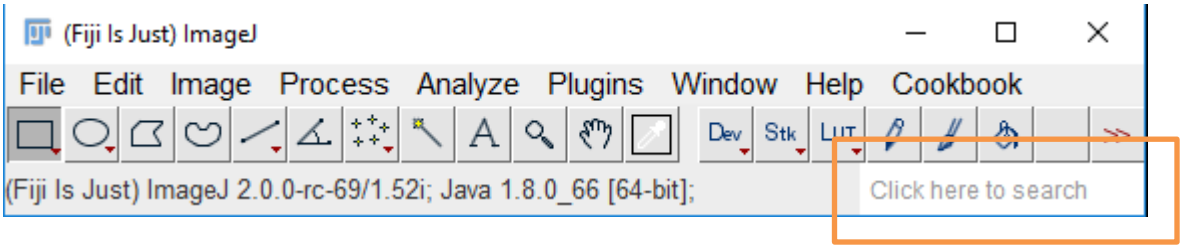
Step 3. *File>Import>Bio-Formats*

You can find an updated list of the supported file formats here:

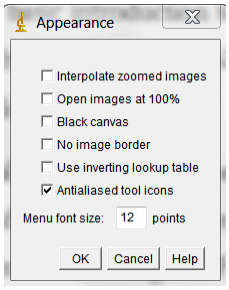
https://www.openmicroscopy.org/bio-formats/

6. The command Finder

ImageJ has many features and sometimes it can be hard to find and keep track of all of them. In Fiji version 1.51 and higher, you can type your search term in to the white box at the end of the taskbar and a new window will pop up with your search results



7. Digital Image – representation



Please check if **Fiji** is configured as follows:

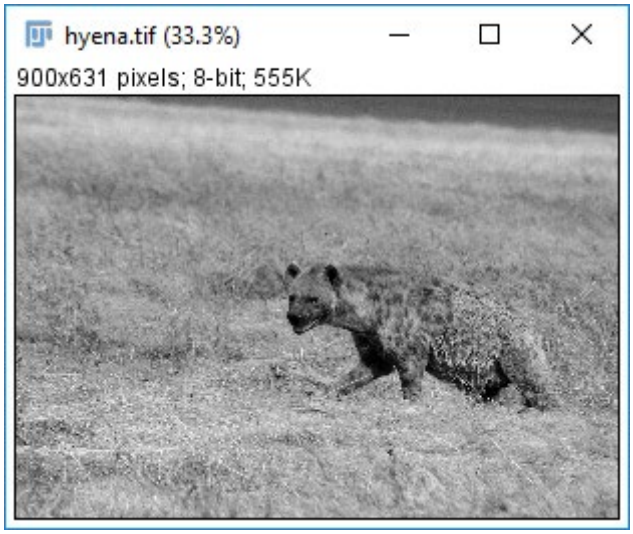
Step 4. *Edit>Options>Appearance:*

Please uncheck the 'Interpolate zoomed images'

This allows you to see the individual pixels on the screen when zooming images.

8. Metadata & Image Info

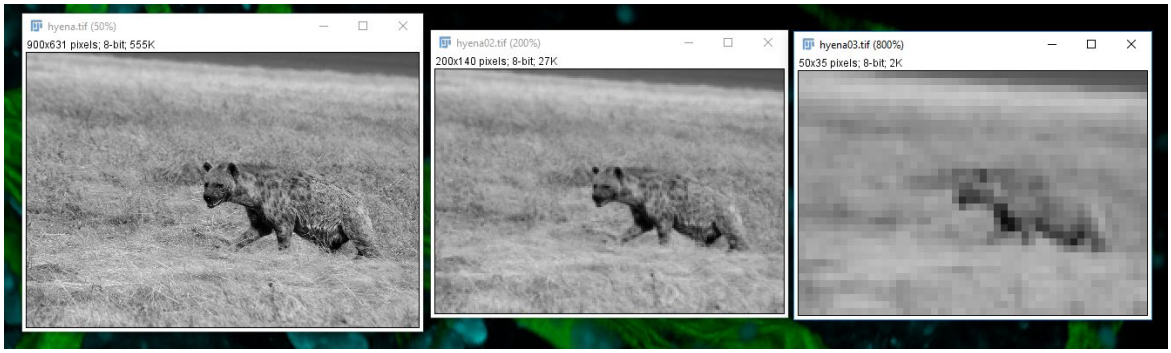
Step 5. *Open the file "Hyena.tif" contained in the folder provided for this training session.*
Step 6. *Magnify the image until pixels are seen (, press Alt to demagnify).*
Step 7. *Individual pixel values can be visualized by placing the cursor over pixels.*



Once you open an image or stack of images, notice the information at the top of the image window.

1. The top line contains the name of the image.
2. "900x631 pixels" is the image size. The units depend on how the image is calibrated. In this case, the units are pixels indicating the image is NOT calibrated.
3. The memory your image takes up on the hard drive.

Step 8. *Open the files "Hyena02.tif" and "Hyena03.tif".*



What is the size of each image? How many pixels are necessary for resolving the animal?

In scientific imaging the pixel size will depend on the microscope you are using and the magnification factor (camera, objective plus any zoom applied during imaging). This information (metadata) can be embedded in the image file or is sometimes found in a text or log file that is generated by the microscope software along with the image and is accessible by Fiji.

Step 9. *Open the file "Cells_DIC.tif".*

Note that in this case the image is calibrated and the units shown are in µm.

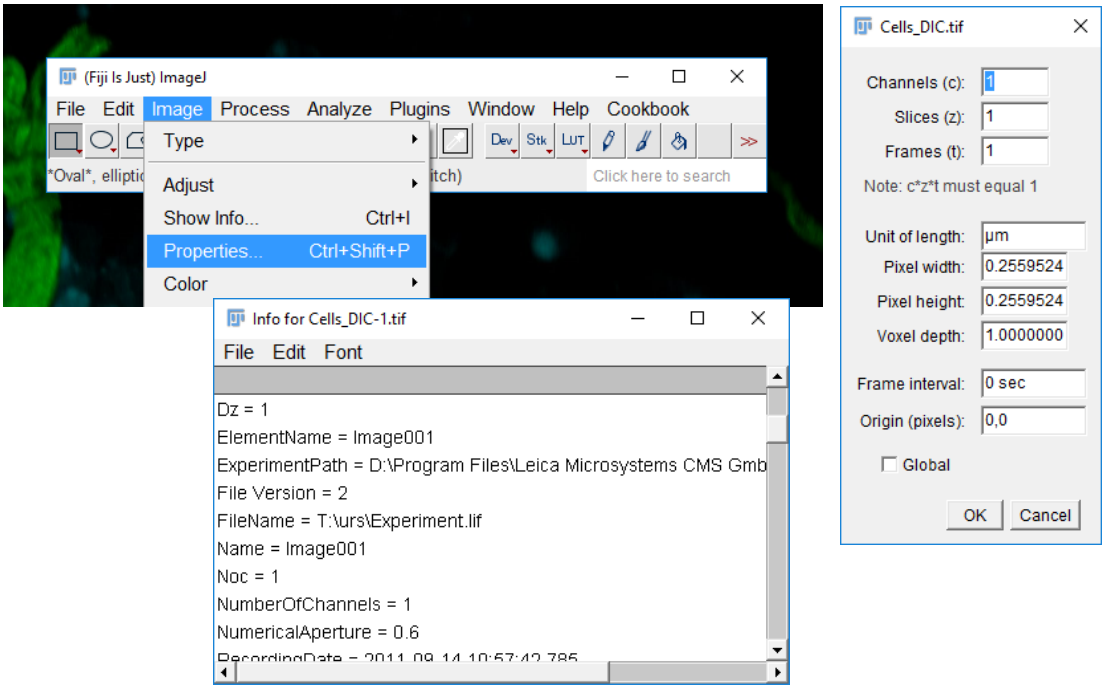
Size, area, distance, etc. measurements made on a calibrated image will be given in units matching the unit of length you entered. Uncalibrated images give measurements in pixels.

Some of the information contained in the image metadata can be accessed through

Step 10.

Image>Properties or ***Ctrl+Shift+ P***

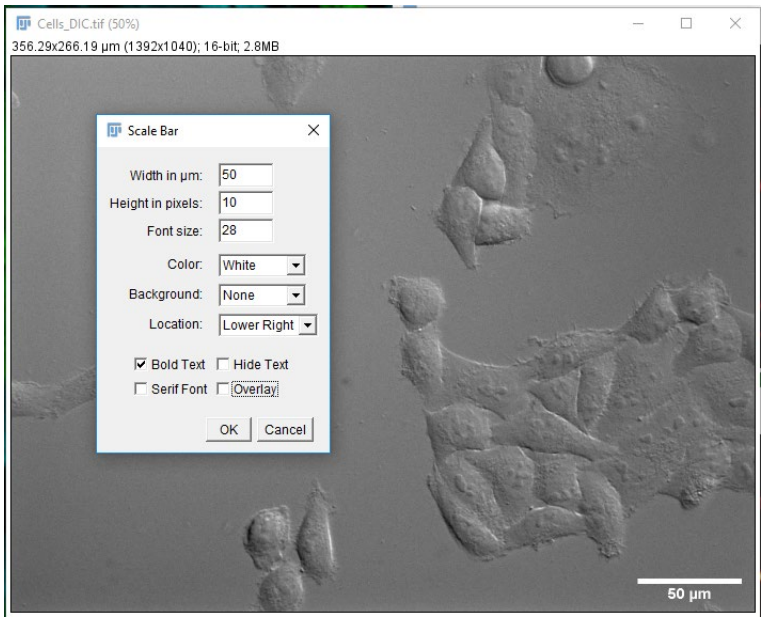
Image>Show Info ***Ctrl+I***



Based on this information you can also include a scale bar in your image through the menu

Step 11.

Analyze> Tools > Scale Bar



Unclick Overlay if you want these change to be permanent and embed the scale bar into the image.

9. Duplicate

You can create a copy of your image by

Step 12.
Image>Duplicate ***Ctrl+Shift+ D***

This is a standard procedure, especially if you wish to compare your processed image to the original. The Undo option is not applicable to some of the processing options and this ensures that you can easily revert your steps without opening the source file again.

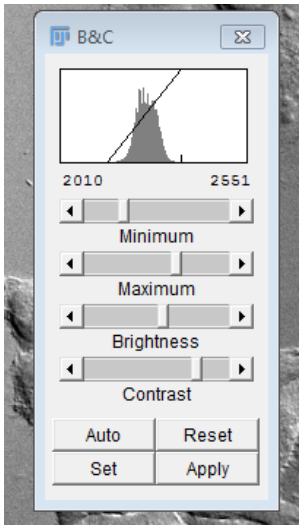
It can also be used to create a new image from a selection.

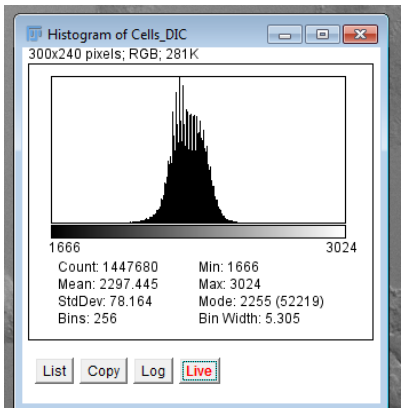
If you wish to revert to your originally saved image you can also use the command

File>Revert ***Ctrl+ R***

10. Brightness and Contrast - Dynamic Range

Step 13.
Select Image>Adjust>Brightness/Contrast ***Ctrl+ Shift+C***





You can adjust the slider 'minimum, maximum, brightness, contrast'.

"Minimum" or maximum set either the bottom or the top of the display range.

"Brightness" adjusts both the top and bottom brightness values displayed without changing the size of the range displayed while the "Contrast" increases or decreases the size of the displayed range.

"Auto" button chooses the best display range, by saturating 0.35% of the pixels in your image.

The "Reset" button resets the image to its original (full) display range.

You can also use "Set" to type in values for the top and bottom of the display range.

Make sure you avoid saturated pixels and clipping.

None of these changes affect pixel intensity values UNLESS you press the “Apply” button. This will compress the intensity distribution of your image. Usually there is no need to use this option and it should not be used when you want to quantify or compare images.

Step 14. *S*
elect Analyze>Histogram

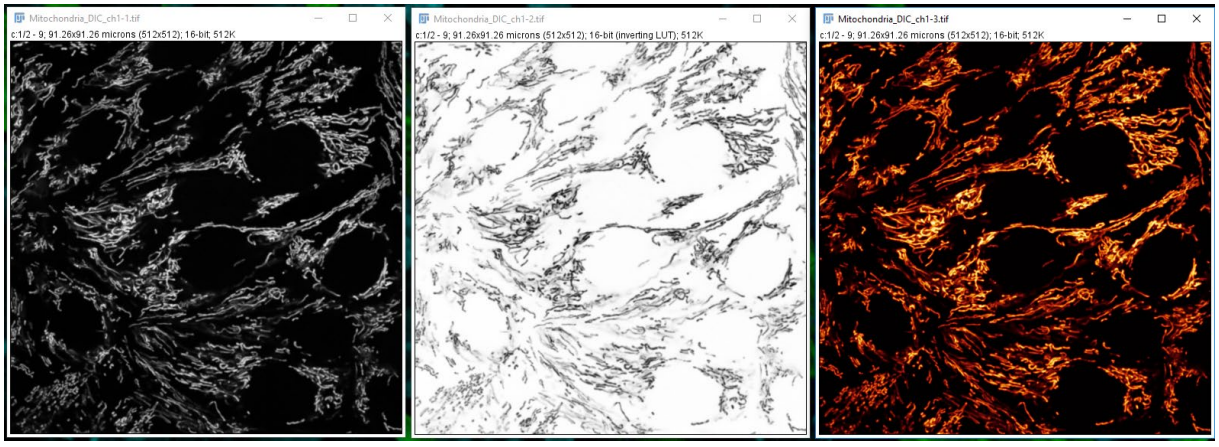
Activate the Live button and change your brightness and contrast settings. When you hit “Apply” this changes will alter the histogram of your image.

Step 15. *S*
elect Process>Math>Gamma and adjust the image with different gamma values.

11. Pseudocoloring Images

Look up tables (LUTs) are fundamental in highlighting the desired features of an image. The human eye can only perceive relatively few different shades in one scene. Therefore pseudo-colouring images can make the data more insightful.

Step 16. *S*
elect Image>Lookup tables and use different LUTs. Any image can be used (e.g. ***Mitochondria_DIC_ch1.tif or kidney.tif*** *for a time series.)*



If you wish to export the image so the color information is available in other programs, choose

Step 17.
Image >Type> RGB Color.

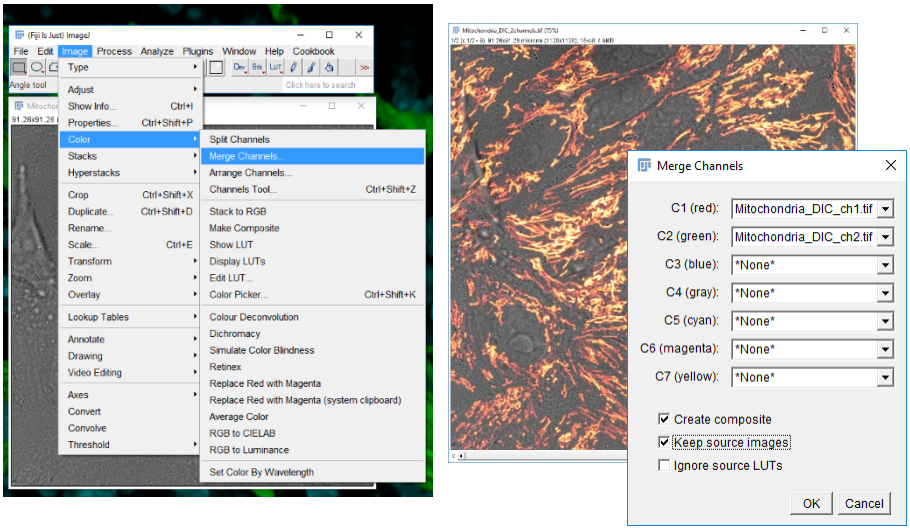
You may also include a scale bar, which can be added to the RGB image, as illustrated in 9. If you want to visually relate your LUT to the image intensity values, you can also include a calibration bar

Step 18.
Analyze>Tools>Calibration Bar

12. Stacks

Step 19.

Open the files Mitochondria_DIC_ch1.tif and Mitochondria_DIC_ch2.tif



Often we acquire images with multiple spectral channels (multi colors). These images might be saved individually as grayscale files but you can easily merge them into stacks and visualize them as multi-color images.

Step 20.

Image>Color>Merge Channels

These files need to have the same physical size, the same number of slices per stack and have the same image type (in this example 1128x1128 pixels, 1 time point and 16-bits). You can also use this command if you have additional dimensions such as time series or z stacks.

"Create Composite" creates a stack while maintaining the image type. If you unclick this option an RGB file will be created instead. "Ignore source LUTs" can be used if you want to apply a different color in the merge than the original LUTs. Alternatively you can also explore

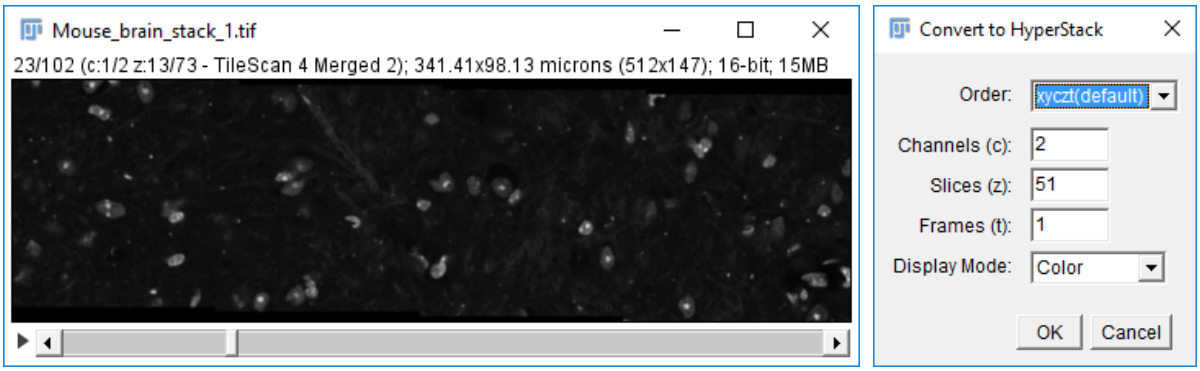
Step 21.

Image>Color>Channel Tool ***Ctrl+ Shift+C***

13. Hyperstacks

Additionally you might encounter multidimensional files such as time series or z stacks with different channels. These can be saved in various ways and can sometimes be challenging to visualize in a single stack. By creating a Hyperstack you will be able to better handle and visualize such data.

Step 22.
Open the file "Mouse_Brain_stack.tif



This file contains a 2 color set with multiple planes, but is displayed as a single stack with 102 slices. To create a Hyperstack go to

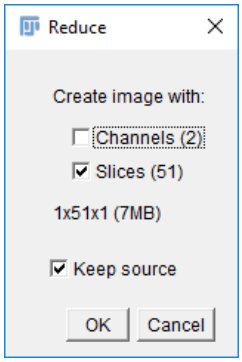
Step 23.
Image> Hyperstacks>Stack to Hyperstacks

You can determine the order of your stack by running through it and looking at what changes first. For example: if your images flip between colors, then the z-plane changes, you have a stack with the default xyczt dimensions. Fill in the number of channels, slices and frames accordingly.

Check that the numbers for channels, slices and frames fit your dataset. If your numbers are not correct ImageJ will not render and will give you an error message.

14. Reduce dimensionality

Conversely you might only need to work on a subset of your hyperstack. You can do this through



Step 24.
Image> Hyperstacks>Reduce Dimensionality

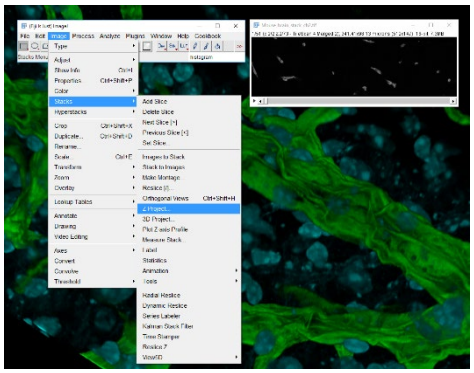
Or

Image> Stacks>Tools>Make Substack

Create a stack containing only the second channel for the following section.

This approach can be also very useful when you want to easily extract a specific channel or time point from a 4D or 5D dataset and resave it with a new filename.

15. Projections and 3D



Z projections compress multiple slices/planes (3D volume) into one plane (2D image) to "flatten" a stack and therefore allowing better representation on a 2D medium such as a screen or paper. One of the most commonly used is the maximum intensity projection, which finds the brightest pixel in the stack for each XY position and projects those into a single XY plane. You can achieve this and explore the multiple projection options in

Step 25.
Image>Stacks>Z Project

Step 26.
Try to make a 3D projection along different viewing axes to simulate a rotation of the image for presentation purposes: Image>Stack>3D Project (-30 to +30° works normally ok). Select different settings.

16. Particle counting and measuring

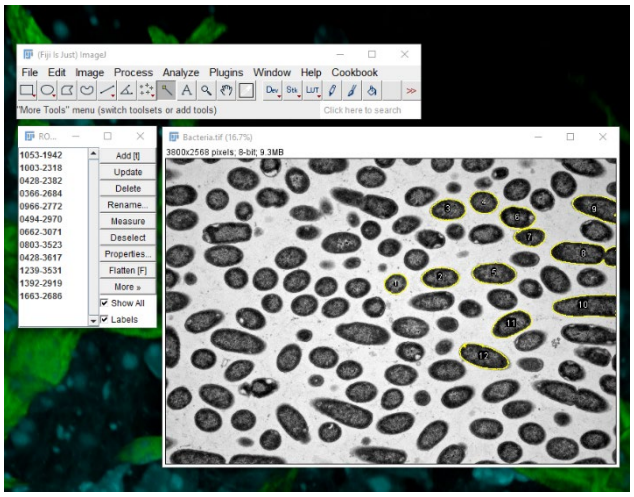


Image segmentation and quantification is the most important and also the most often performed image processing task. Automatic counting and quantification plays a crucial role in many applications including high content imaging and screening.

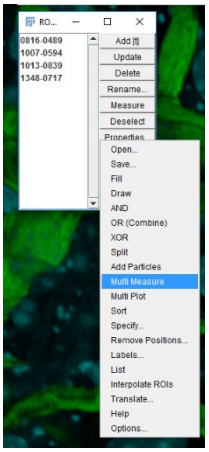
Manually selecting a region of interest (ROI) and measuring variables such as size, shape and intensity is one possible way.

In ImageJ you can manually define ROIs using the different tools from the main software interface such as: "Rectangle", "Oval", "Polygon selection", etc..

To handle multiple selections in parallel you can add them to the ROI Manager through

Step 27.

Edit>Selection>Add to Manager T



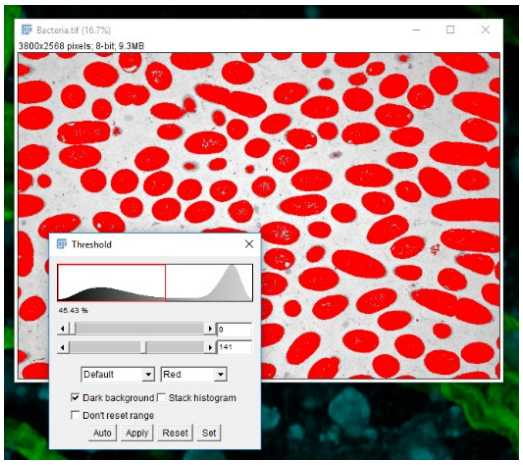
You can then extract the information contained in each individual ROI using the option **Multi-Measure** in the ROI Manager.

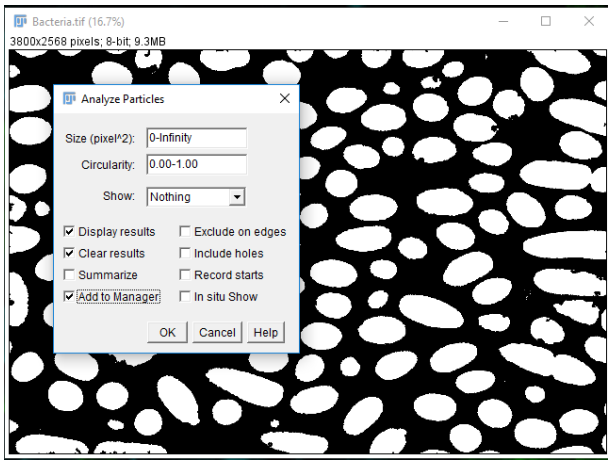
To measure a single selected ROI use

Step 28.

Analyze>Measure M

Automatic segmentation of images has the advantage of allowing for quantification a larger numbers of objects while reducing bias. Here we count bacteria on an electron micrograph using intensity thresholding and an eroding/dilating algorithm on the binary image.





Step 29.

1. *. Open the image **Bacteria.tif**.*
2. *Duplicated the image using Image>Duplicate*
3. *Select Image>Adjust>Threshold to select the object (move the slide bars so that the objects are be in red).*
4. *Apply the selection.*
5. *Using Process>Binary>Fill Holes, none selected areas inside an object are filled.*
6. *Using Process>Binary>Open (or Erode/Dilate) touching structures can be separated until an acceptable selection of the objects is achieved.*
7. *Using Analyze>Analyze Particles all objects are measured (e.g. area, size,…) automatically.*

If you want to select what aspects of your segmentation should be measured and exported, you can define this under

Step 30.

Analyze>Set Measurements

This menu also allows you to “redirect” your measurement and therefore as an example to measure the intensity values in your source and not in the binary segmented file.

17. Saving and Image file formats

Step 31.

1. *Open the image "baboon.tif"*
2. *Save the image as a jpg file through File> Save as.*
3. *Open the saved jpg file and compare the original tif file with the jpg copy (zoom to see the pixels in both images)!*

The JPEG image format uses a lossy compression that leads to image artifacts that are not compatible with quantitative analysis. As such, it should only be used for presentation purposes (where file size can be an issue). Even then a lossless format such as PNG is probably more suitable.

To visualize the loss of information in such a compression you can subtract both images through

Step 32.

Process>Image Calculator

Adjust Brightness and Contrast accordingly.

TIFF is usually the format of choice that supports all ImageJ data types (8-bit, 16-bit, 32-bit float and RGB) and allowing for spatial and density calibration.

18. Literature and further information

- The ImageJ online documentation
    - can be accessed via Help>Documentation...
- The ImageJ Information and Documentation Portal (ImageJ wiki):
    - http://imagejdocu.tudor.lu/
- Several online documents, most of them listed at:
    - http://rsb.info.nih.gov/ij/links.html
- Forum for multiple image processing softwares
    - https://forum.image.sc/