Scientific Imaging with ImageJ

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June 23rd, 2022

Introduction

The aim of this session is to

- improve handling of scientific images for quantification and illustation,
- understand core concepts about images,
- discover ImageJ.

Online ressources are available here:

- `http://imagej.nih.gov`
- `https://imagej.net`
- `https://forum.image.sc`

Context

Quantification

- Measure objects properties (intensity, area, shape, ...)
- Count objects
- Relationship (co-occurrence, co-localisation), hierarchical.
- Speed, motion, ...

Illustration

- Use ImageJ to prepare the elements of a figure and import them in Illustrator.
- Relationship between markers
- Localisation of organelles
- Scale of objects

Image integrity

When preparing the figure:

- Apply adjustments to the entire image
- Track the sequence of manipulations (scripts/macro)

Include in the legend or methods section the following information:

- Equipment (microscopes/objective lenses, cameras, detectors, filters) and acquisition software used. microscope controlled by Zen black and equiped with a 63x/1.4 oil objective using a 488nm laser and GaAsp detectors.
- Time and space sampling (pixel size), image bit depth, temperature, imaging medium, fluorochromes
- Mention the look up table applied.
- Processing software and manipulations (deconvolution, 3D reconstructions, volume rendering, filtering, nonlinear operation, thresholding and projection).

Undocumented image manipulations can lead to accusations of research misconduct

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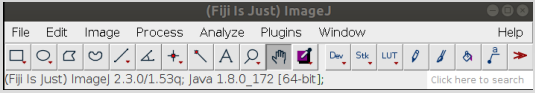
Nature, ed. 2022. URL: https://www.nature.com/nature-portfolio/editorial-policies/image-integrity#microscopy.

Office of Research Integrity, ed. 2022. URL: https://ori.hhs.gov/education/products/RIandImages/default.html.

The ImageJ software



- ImageJ is a Java based image processing software.
- Java programs run on the java virtual machine (JVM)
- Can run on many operating system (Microsoft Windows, Mac OS, Linux, ...)
- Developed by Wayne Rasband since 1997 at the National Institute of Health
- ImageJ eco-system
  - ImageJ2: rewrite of ImageJ for multi dimensional data
  - Fiji: image processing package built around ImageJ2

CT Rueden et al. *BMC Bioinformatics* 18.1 (2017).
CA Schneider, WS Rasband, and KW Eliceiri. *Nature Methods* (July 2012).

Installation & Updating

First Installation

- Download Fiji from `https://imagej.net/software/fiji/downloads`.
- Unzip and save somewhere on your hard drive where default users have access.
- In Windows open the folder and double ckick on ImageJ-win64 and create a shortcut.

Updating

- To update ImageJ to the latest version Help » Update ImageJ.
- To install/update plugins collections and manage update sites Help » Update

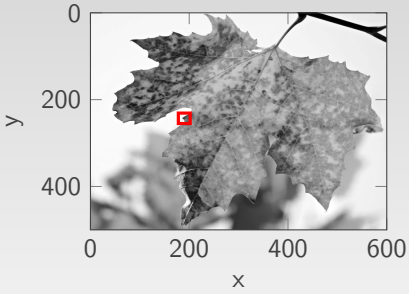
Fiji.app folder content

The Fiji.app folder is organized into several subfolders:

- `jars` : contains the main jar (eg ij-1.53q.jar) and extra java dependencies
- `macros` : contains macros you installed and the `StartupMacros.fiji.ijm`
- `plugins`: contains the jar (java artifacts) of the plugins
- `scripts`: contains a few matlab scripts
- `lut` : look up tables (mapping for intensity to displayed colors)

Image is data

- Sensors converts the number of detected photo-electrons into an electric volatage
- This voltage is then digitized into a number by the A/D converted.
- The image can be seen as an array of values with columns x and rows y starting at the top left corner at $(0, 0)$.



Try it yourself

- Open a sample image : File » Open Sample » Blobs
- Click on the >> icon and tick Pixel Inspector

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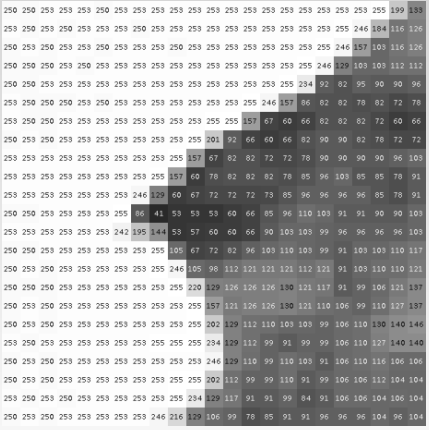

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General image formats

- Digital images can be saved from the volatile memory (RAM) of a computer to its persistent memory (HDD/SSD) as files with various formats.
- Most file formats include some sort of compression:
  - Lossless compression (PNG): original values can be exactly retreived
  - Lossy compression (JPEG): information is lost when storing the image
  - Both (TIF): some formats are containers which can include various type of compression
- ImageJ can read and write natively a few format such as TIFF, PNG, JPG
- Use TIF for saving intermediate results (with LZW compression) and PNG for figures

Microscope vendor image formats

Each microscopy company has developed a format that can store multiple series with each a multi-channel multi-plane image stack.

- Zeiss
  - LSM : TIF based file format with additional metadata and LZW compression
  - CZI : JPEG-XR and Zstd lossless compressed image
- Nikon
  - ND2 : JPEG-2000 lossless compression
- Leica
  - LIF : Customized TIF file format
- Olympus
  - OIF: multi file formats with associated images in a folder
  - OIB: store multiple OIF and dependent image in one file

Some standardized image format have also been developed to increase inter-operability:

- OME-TIFF, OME-ZARR, ICS, HDF5

Metadata

- Metadata are essential to the interpretation and processing of the image
- Acquisition software collect additional information that are stored in the files
  - Pixel size
  - Spacing between z planes
  - Detectors
  - Objective
  - Emission wavelength
  - ...
- The Open Microscopy Environment (OME) defines a specification for storing data on biological imaging.
- Findable Accessibility, Interoperability, and Reusability (FAIR) principle.

URL: https://docs.openmicroscopy.org/ome-model/5.6.1/developers/model-overview.htm.
Mark D. Wilkinson et al. *Scientific Data* 3 (Mar. 15, 2016). DOI: 10.1038/sdata.2016.18.
linkert˙metadata˙2010.

Loading an image

- Fiji will detect supported file format when selecting File » Open and use bioformat if needed to load an image from disk to memory.
- You can also use Plugins » Bio-Formats » Bio-Formats Importer to use Bio-formats directly
- The Bio-Formats plugin remember previous settings, so use the tool directly to define the way the drag and drop or File » Open behaves on non-native formats.

Pixel size calibration & scale bar

- Use Image » Properties... to check and set the pixel size in each dimensions
- Use Analyze » Set Scale... to use an known distance to set the scale
- Use Analyze » Tools » Scale Bar to display the scale on a calibrated image

Quantization

- The intensity values are quantized into grey levels
- Images are saved as 8-bit or 16-bit images by acquisition software.
  - For camera sensor (CCD, CMOS), photo-electron are accumulated in wells and converted into analogue voltages that are then digitized. Well depth is in the order of 40000 electrons.
  - For photo-multiplier tubes (PMT) photo-electrons are amplified using a chain of dynodes enabling to count individual photons.

Bit depth	Dynamic range
8	0-255
12	0-4095
14	0-16383
16	0-65535

- Use Image » Type » 8-bit to check & convert an image to 8-bit, using the current dynamic range.
- 32-bit mode will allow to preserve the information if the intensities get out of the initial range.

Brightness & Contrast adjustment

- Image » Adjust » Brightness & Contrast... or ⌥ + ⇧ + C launches the B&C tool
- Pixels intensities are mapped linearly to the displayed intensity.
- Enable to discard unused part of the dynamic range
- Adjust the Minimum and Maximum of the dynamic range which will be displayed.
- Adjust the Brightness and Contrast to change how they are display.
- Press Auto to stretch the intensities between a predefined percentage of saturated pixels. Equivalent to Process » Enhance Contrast... with the normalized option left unticked.
- Press Apply to change the pixel values.
- When changing the image mode (16-bit to 8-bit or 32-bit to 8-bit) the B&C settings are applied to the image.

Brightness & Contrast adjustment

Ty it yourself

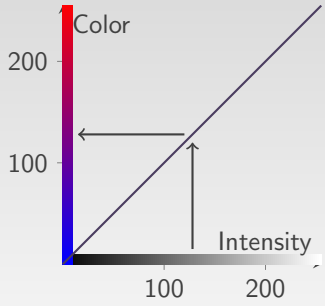
- Open the boat example image File » Open Samples » Boats
- Press the short cut ⌥ + ⇧ + C
- Change the minimum and maximum sliders and observe the image
- Press Set and set the Minimum displayed value to 50 and the maxium displayed value to 200, and press the OK button.
- Finally press Apply and obsere the change of the histogram in the B&C window.

Histogram

- Shows the frequency of each intensity values
- Use Analyze » Histogram or ctrl + h to display the histogram
- Helps identify clipped values (under/over exposed, saturated)
- Visualise classes of pixels

Look up table (LUT)

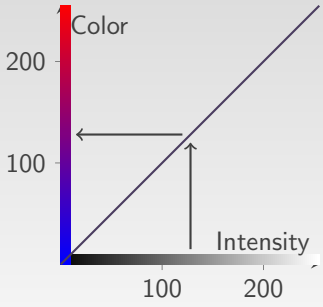
- Intensities are mapped to a color defined by a red, green and blue triplet value for display.
- Converting an image to RGB applies the current LUT (Apply LUT, apply the B&C settings)
- LUT can be inverted (black becomes white, . . .)
- Use LUT to help people with colour blindness



Look up table (LUT)

Try it yourself

- Open a grey scale image File » Open Samples » Boats
- Apply a LUT to the image Image » Look Up Tables » 5 Ramps
- Convert the image to RGB Image » Type » RGB Color
- Observe the values at each pixel
- Open the blobs example File » Open Samples » Blobs and comment on the LUT
- Explore other commands Image » Color » Display LUTs, Image » Color » Edit LUT
- Open the Fluorescent Cells sample and change the red channel to magenta.



Multi-channel images

- Multi-channel images can be easily acquired by microcopes (multiple wavelength/fluorophores, brightfield/DIC, ...)
- There are two types of color images:
  - RGB images store color information into a single (8×3) 24 bit image
  - Composite image are image whose channels are stored as individual (8,16-32-bit) image planes
- On a composite image use Image » Color » Channel Tool or ctrl + ⇧ + z to select the channel to display
- Create a multi channel image using Image » Color » Merge Channels...
- Split channels of an image using Image » Color » Split Channels

Try it yourself

- Open a composite image File » Open Samples » Fluorescent Cells
- Use the channel tool to display each colour individually

Multi-dimensional images

Stack & Hyperstacks

- Images can have many dimensions (axis) associated to them channel c, time t, depth.
- Image stacks represent up to 4 dimensions (xyzc or xytc) with a maximum of 3 channels.
- Hyperstacks can have up to 5 dimensions xyzct with no limits on the number of channels.
- Virtual stacks enable to load only the images planes which are visualised.

To quickly visualize multi-dimensional data, use :

- Orthogonal views (Image » Stack » Orthogonal Views) or ctrl + ⇧ + H can help visualize 3D data
- Maximum intensity projection Image » Stack » Z Project...
- Reslice a stack with Image » Stack » Reslice... or press / each pixel. Activate the BIG-EPFL update site and search for "Extended Depth Of Field"

To synchronize two stacks use Analyze » Tools » Synchronize Windows

Basic manipulation

- Duplicate ctrl + ⇧ + D allows to crop, select channels and slices
- Use + and - or the magnifying glass tool to zoom in and out the displayed image.
- Use the hand tool to pan within a zoomed image.
- Changing the actual pixel size:
  - Image » Scale scale the image, if "create a new image" is not ticked, the image keep the same number of pixels and is cropped or padded as necessary.
  - Image » Adjust » Size... scale the image in place without cropping or creating a new image.
- Use the search bar to look for tools.

Selection / Region Of Interest (ROI)

- Use the rectangle, circle, polygon, freehand, line tools to create selections.
- Use Edit » Selection » Specify to enter manually the coordinate of a shape
- Transfer selection across images using Edit » Selection » Restore Selection or ctrl + ⇧ + E
- Store selection into the ROI manager Edit » Selection » Add to manager or t
- Use Edit » Selection » Select None ctrl + ⇧ + A to be sure no selection is active
- ROI from the ROI Manager can be stored as individual proprietary .roi file or as a collection into a .zip file.
- ROIs can be combined together using AND, OR, XOR logical operation
- Use Edit » Draw and Edit » Fill to set the pixel values on the contours resp. the inside of the shape to the value define by the colour picker tool.

Threshold

- Thresholding an image gives a binary image: 8-bit image with values either 0 or 255 whether the pixel intensity lies within two values (upper & lower).
- Simplest form of image segmentation.
- Use the sort cut ctrl + ⇧ + T to display the threshold tool.
  - Use the sliders to set the upper and lower values
  - Auto thresholds: Fiji includes a set of automatic threshold [1]
  - Stack histogram: the statistics will be computed on the all stack
- Use Apply or Process » Binary » Convert to mask to apply the threshold and make the image binary.
- The menu Process » Binary » Make binary will show a dialog if a threshold has been set.
- The LUT of binary images is inverted

[1] https://imagej.net/plugins/auto-threshold

From thresholds to regions of interests

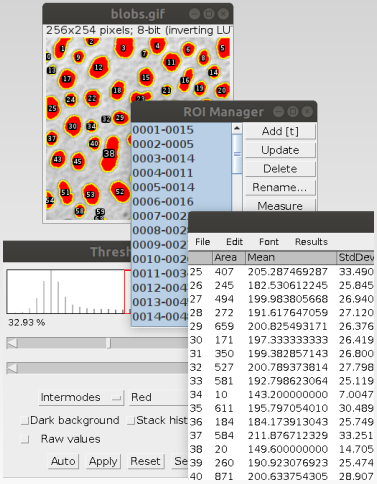
- Use Edit » Selection » Create selection to convert the threshold to a selection (can be a composite selection).
- Use the Analyze » Analyze Particles... to convert connected components into individual ROIs and add them to the ROI Manager.

Try it yourself

1. Open the blob sample image
2. Threshold the image
3. Add connected components to the ROI Manager and get the number of blobs.

Measurements

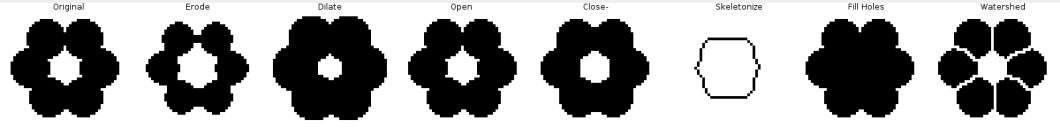
- Use Analyze > Set Measurements to define the quantity to be measured
- Select an individual ROI and press ctrl + M to add measurements to the Result table.
- To measure the properties of all ROI stored in the ROI Manager, select them all with ctrl + A and press Measure



Binary image processing

Binary image processing relies mostly on "morphological image processing" which process image by shapes. It can help refine the mask generated by thresholding:

- Process » Binary » Dilate grow the masks, similar to Process » Filter » Maximum...
- Process » Binary » Erode shrink the masks, similar to Process » Filter » Minimum...
- Process » Binary » Open is equivalent to erode followed by dilate, removes small objects and lines
- Process » Binary » Close- is equivalent to dilate followed by erode, bridge non touching objects etc
- Process » Binary » Watershed separate touching objects
- Process » Binary » Fill Holes will fill holes in the masks



Filtering

Image filtering can help reduce noise

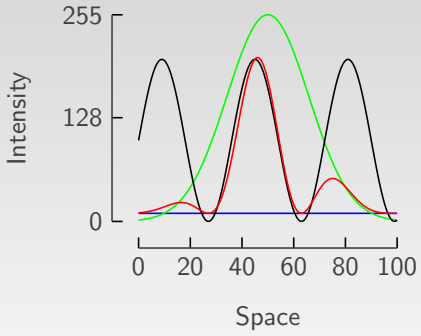
- Box filtering ctrl + ⇧ + S
- Gaussian filtering Process » Filters » Gaussian Blur...
- Median filtering Process » Filters » Gaussian Blur...

Try it yourself

- Create an image with a light grey square on a darker grey square.
- Add noise to the image
- Launch the histogram and press Live
- Apply some filtering to the image
- Apply a threshold

Background correction

- Background level can vary for several reason
  - Uneven illumination
  - Scattering
  - Auto fluorescence
- Simple generic correction
  - Rolling ball from Process › Subtract Background...
  - Top Hat (image - grayscale opening) from Process › Filter › Top Hat...
- Illumination correction
  - BASIC plugin

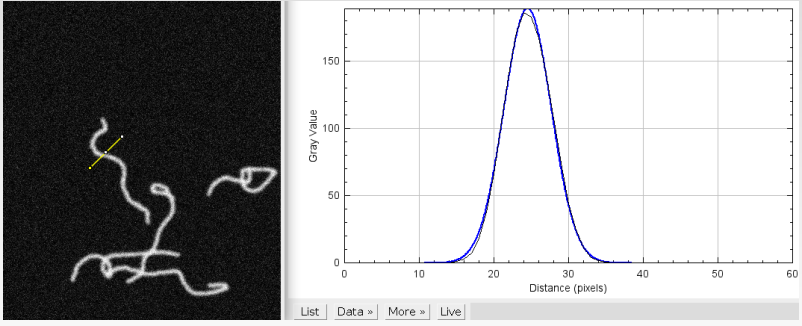


Drift correction

- Install Stackreg from the BIG-EPFL update site
- Plugin » Registration » Stackreg
- The plugin can register drift using several motion models such as
  - Translation
  - Rigid body
  - Rotation
  - Scaled rotation
  - Affine

Line profile

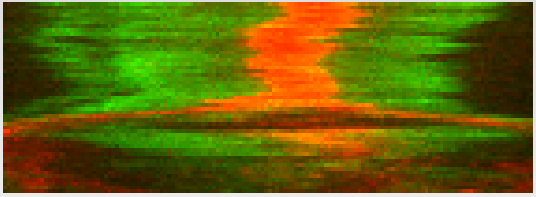
- Line profile enable to measure distances visually.
- Draw a line and select Analyze » Plot Profile or press ctrl + K
- Use Data » Add fit to approximate the data with a model



Kymograph

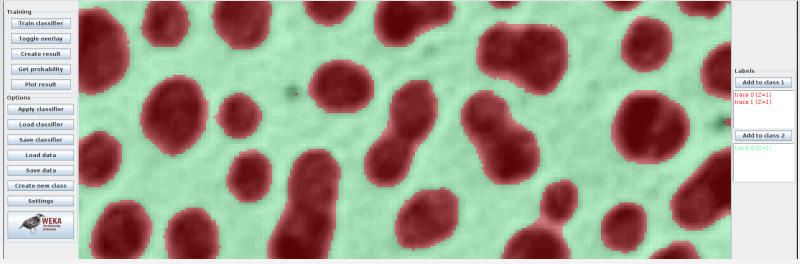
1. Create a maximum intensity projection
2. Select lines using a freehand tool and add them to the ROI Manager
3. Double click on the line tool to access the line width dialog
4. Reslice the original stack

Velocities can be then extracted from the kymographs using a macro for example.



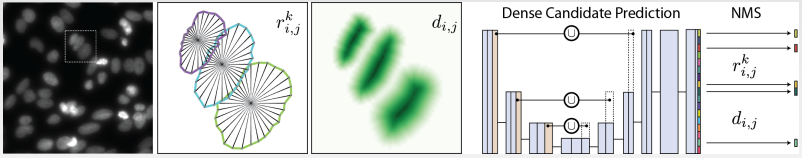
Pixel classifier

- Weka is a machine learning library in Java
- The weka plugin allows to annotate, train, export and apply the classifier



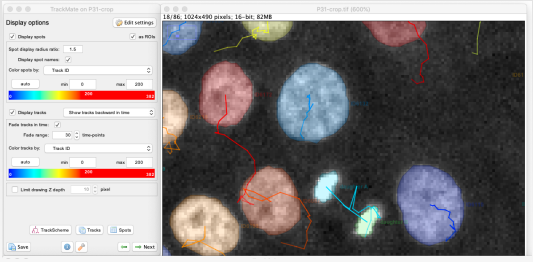
Nuclei segmentation with StarDist

- StartDist is deep learning based approach based on star convex polygons
- It has been trained to identify cell nulcei.
- Install with Help > Update and locate the StarDist and CSBDeep entries
- Launch trackmate from Plugins > StarDist



Tracking with TrackMate

- Detect spots and link them overtime
- Install trackmate using Help » Update and locate the TrackMate entry
- Launch trackmate from Plugins » Tracking » Trackmate
- Trackmate can also use stardist or weka for detecting objects



Basic quantification workflow

1. Open the image
2. Filtering
3. Threshold
4. Refine binary image
5. Extract ROIs
6. Measure

Basic figure preparation workflow

1. Open the image
2. Maximum intensity projection
3. Adjust contrasts
4. Convert to RGB
5. Add a scale bar
6. Export as PNG

Figure preparation tools

Manually

- Convert to RGB, add a scale bar without text, import in Illustrator

figureJ

- Update sites IBMP-CNRS, ImageScience

EZFig

- Update sites: EZF

QuickFigure

- Update sites: QuickFigure

G. Mazo. *PLoS ONE* 16 (Nov. 9, 2021). DOI: 10.1371/journal.pone.0240280.

Extension

ImageJ functionality can be extended and customised using:

- Macros (.txt, .ijm files)
- Scripts (Javascript, Python, Groovy . . .)
- Plugins (in Java)

ImageJ Cheatsheet

Image properties	ctrl + ⇧ + P
Duplicate/crop	ctrl + ⇧ + D
BrightnessContrast	⌥ + ⇧ + C
Channel Tools	ctrl + ⇧ + Z
Add to ROI Manager	t
Restore Selection	ctrl + ⇧ + E
Select All	ctrl + A

Select None	ctrl + ⇧ + A
Histogram	ctrl + H
Orthoslices	ctrl + ⇧ + H
Reslice	/
Threshold	ctrl + ⇧ + T
Measure	ctrl + M
Smooth	ctrl + ⇧ + S
Plot profile	ctrl + K