MicroPICell

Introduction to Bioimage Analysis with Fiji/ImageJ

Pasteur NEUBIAS Course on Bioimage Analysis
Early Career Investigators Track
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cnrs BioCore Nantes Université

Who has done image analysis before?
Who has used Fiji/ImageJ before?
What are your backgrounds?

Plan for today

I/ Bioimage Analysis (BIA)

II/ Fiji

III/ Fundamentals

IV/ Image ethics

V/ Quantification

Hands-on 1: Count nuclei and determine which ones are green

Hands-on 2: Quantify the number of red spots per nucleus

VI/ Intro to automation

Plan for today

I/ Bioimage Analysis (BIA)

- What do you mean, you want to « quantify » things in images?
- Piloting experiments all the way
- The typical BIA workflow
- Software: the usual suspects

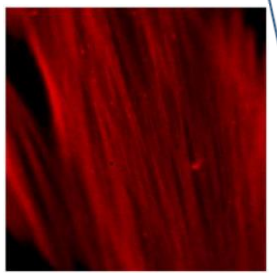
II/ Fiji

- Flavors of ImageJ
- Installing and updating
- Plugins
- Opening images

I/ BioImage Analysis (BIA): What do you mean when you say you want to quantify things in a biological image?

I/ BIA: Typical things you'd want to measure in biological images

Object counts? Density per region? Shape comparison? Surface %? Intensity? Object class? Diameter? Distance to another region?



Fazeli, Elnaz, Robert Haase, Michael Doube, Kota Miura, et David Legland. « From Cells to Pixels: Bridging Biologists and Image Analysts Through a Common Language », 2024.

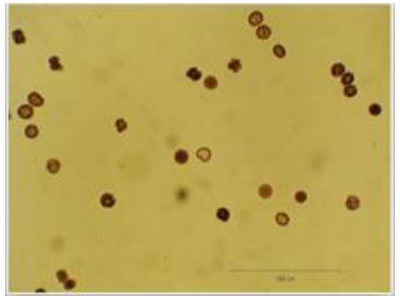
I/ BIA: Pilot your experiments all the way!

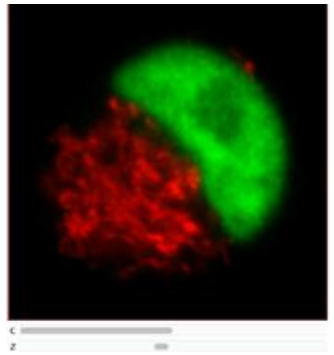
It is a great idea to pilot your experiment all the way to the analysis and visualization!

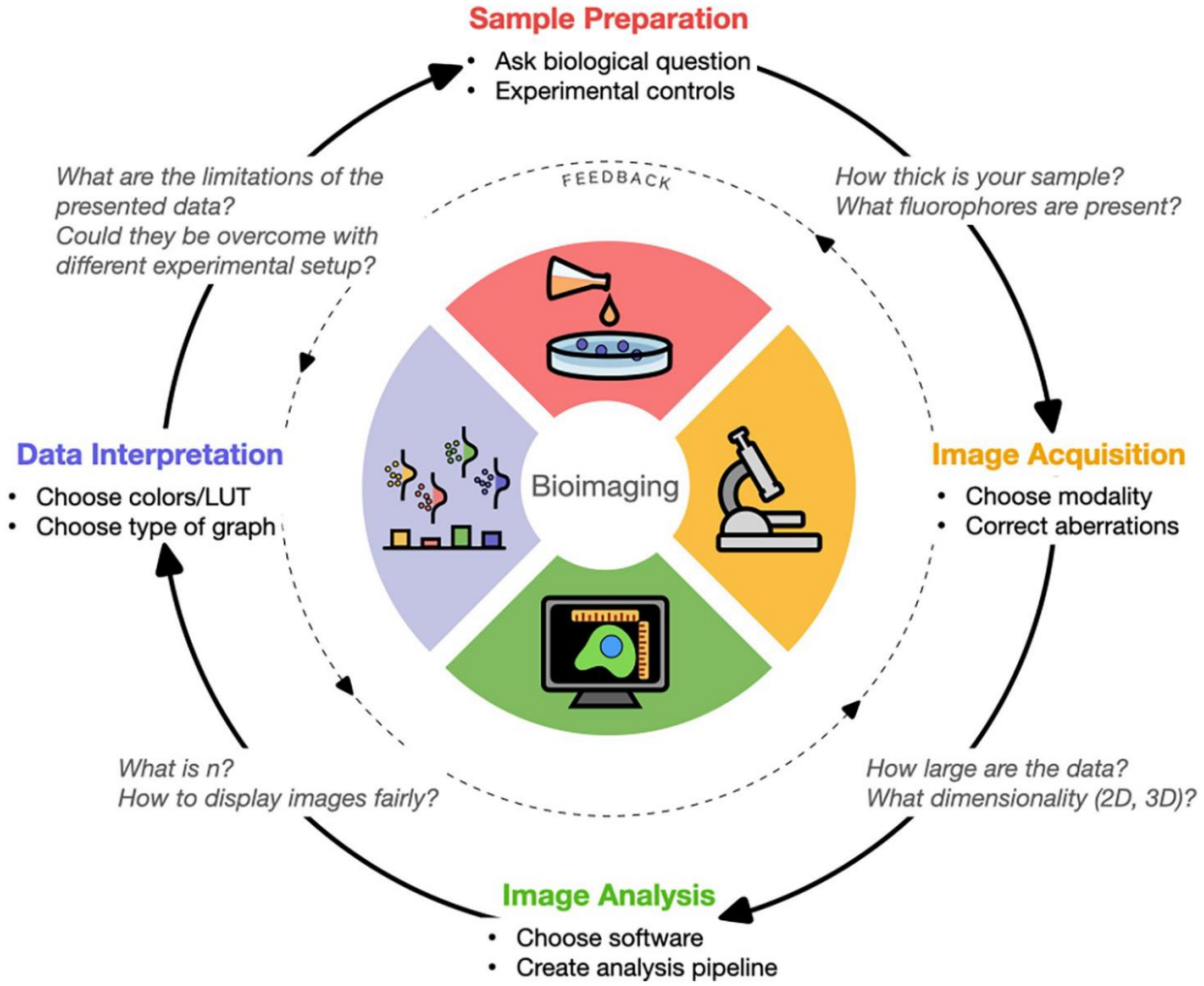
“Do I need to segment surfaces? Individual objects?”

“What type of control do I need?”

“What type of microscopy am I going to use? Is there a marker that could help?”

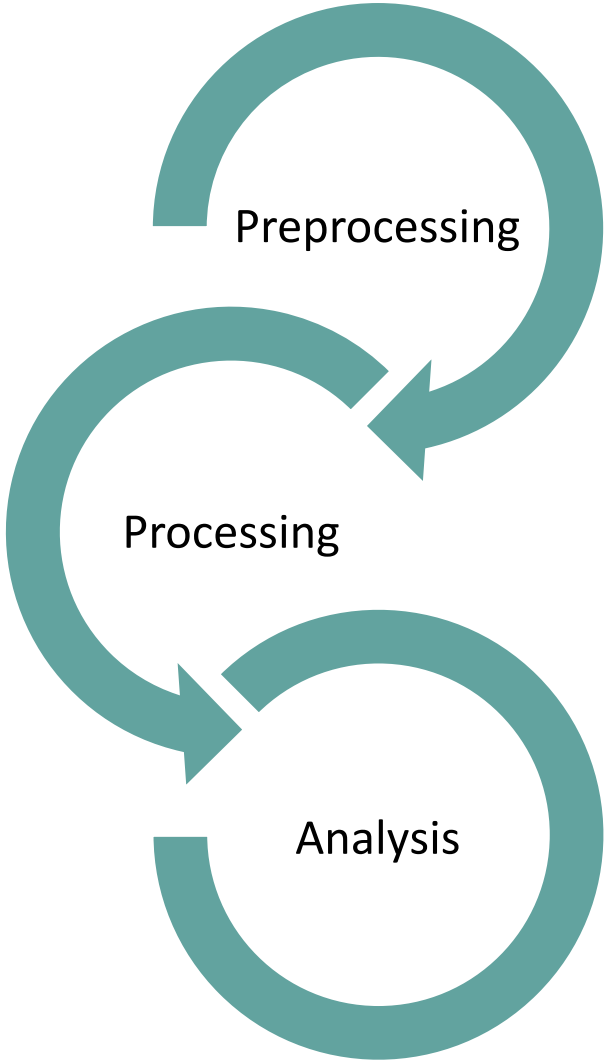






Senft, Rebecca A., Barbara Diaz-Rohrer, Pina Colarusso, Lucy Swift, Nasim Jamali, Helena Jambor, Thomas Pengo, et al. « A Biologist’s Guide to Planning and Performing Quantitative Bioimaging Experiments ». *PLOS Biology* 21, nº 6 (27 juin 2023): e3002167. https://doi.org/10.1371/journal.pbio.3002167.

I/ BIA: Typical bioimage analysis workflow



Prepare image = correct artefacts, enhance quality, potentially reduce the size

Image

Detection, segmentation, tracking, feature extraction…

Numbers

Quantification, clustering, and visualisation

Conclusions

From pixels to numbers to statistics to biological conclusions: “information condensation”

I/ BIA: Software: the usual suspects

Open source

Fiji/ImageJ

This workshop

Ilastik

Icy

CellProfiler
cell image analysis software

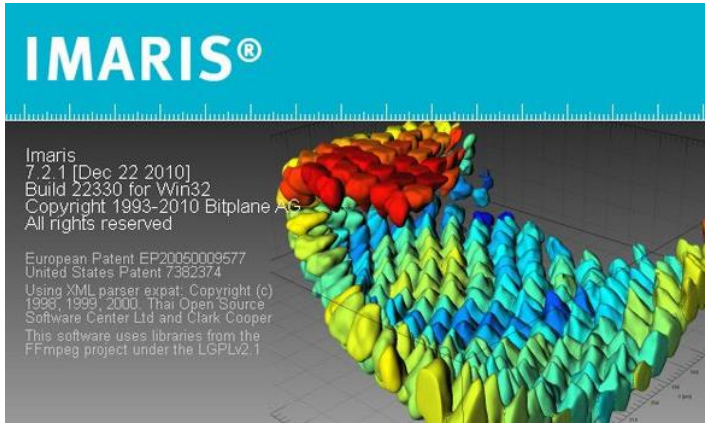
python™

Napari

jupyter

QuPath

Commercial



Haase, Robert, Elnaz Fazeli, David Legland, Michael Doube, Siân Culley, Ilya Belevich, Eija Jokitalo, Martin Schorb, Anna Klemm, et Christian Tischer. « A Hitchhiker's Guide through the Bio-Image Analysis Software Universe ». *FEBS Letters* 596, nº 19 (2022): 2472-85. https://doi.org/10.1002/1873-3468.14451.

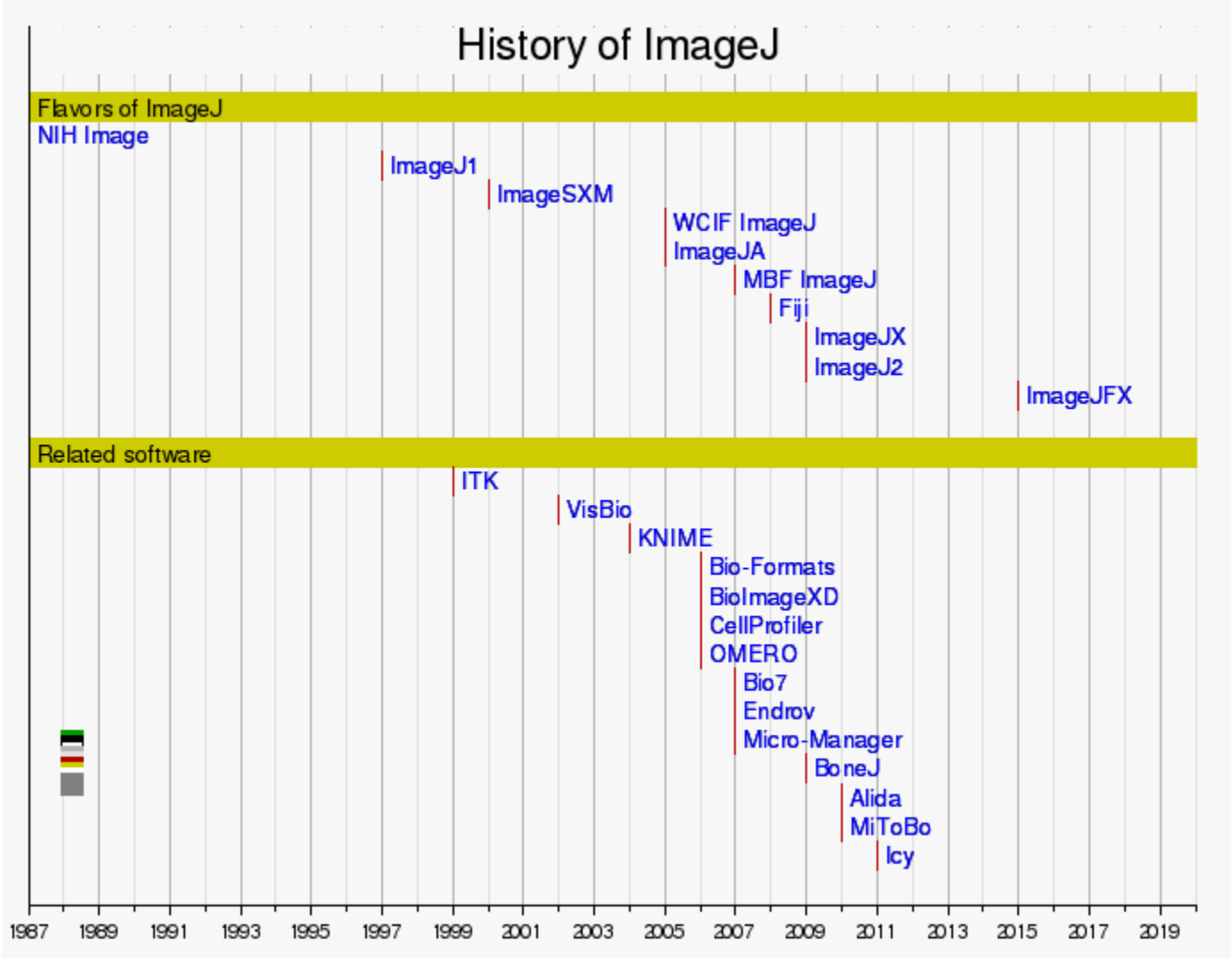
II/ Fiji: installing and updating, installing plugins

II/ Fiji: Flavors of ImageJ

There are a many different derivatives of ImageJ with very similar names, and some confusion is inevitable. Below is a table which should help to clarify the purpose of each. For the historical context of these projects, see History below.

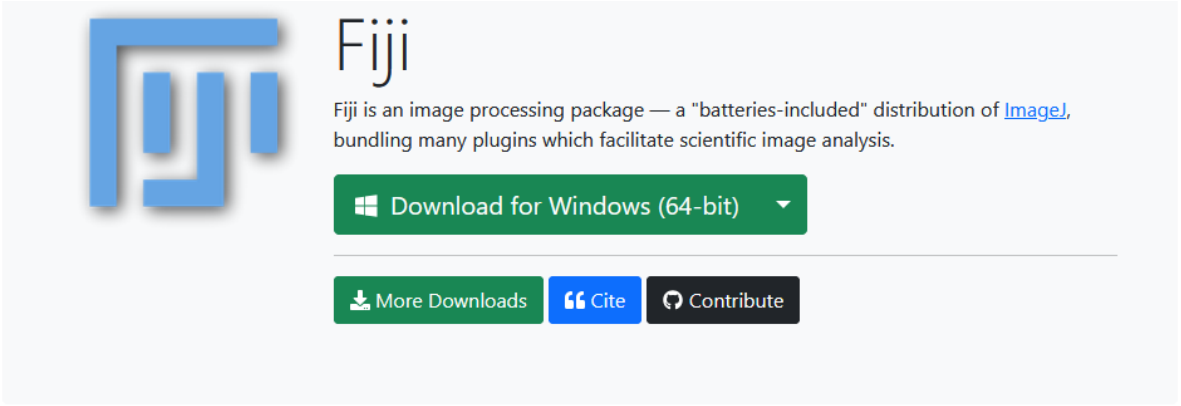
	Name	Author/Maintainer(s)	Description	Initiated	Status
	ImageJ	Wayne Rasband	The original version of ImageJ which has been in development since 1997. It has a strong, established user base, with thousands of plugins and macros for performing a wide variety of tasks. Sometimes referred to as **the original ImageJ** or **ImageJ 1.x** for technical clarity, or informally with the shorthands **ImageJ1** or **IJ1**.	1997	Active
	ImageJ2	ImageJ2 developers	A new version of ImageJ targeting scientific multidimensional image data. It is a complete rewrite of the original ImageJ, but includes the original ImageJ with a compatibility layer, so that old-style plugins and macros can run the same as always. ImageJ2 provides several significant new features, such as an automatic updater, and improved scripting capabilities.	Dec. 2009	Active
	Fiji	Fiji contributors	**F**iji **i**s **J**ust **I**mageJ, with extras. It is a distribution of ImageJ and ImageJ2 with many plugins useful for scientific image analysis in fields such as life sciences. It is actively maintained, with updates released often.	Dec. 2007	Active
	ImageJ.JS	Wei Ouyang	ImageJ.JS is a web version of ImageJ that runs in the browser without installation, compiled from Java to JavaScript using the Cheerpj compiler and integrated with the ImJoy plugin system. It's accessible from https://ij.imjoy.io and also supports mobile devices and tablets.	2020	Active

II/ Fiji: Flavors of ImageJ

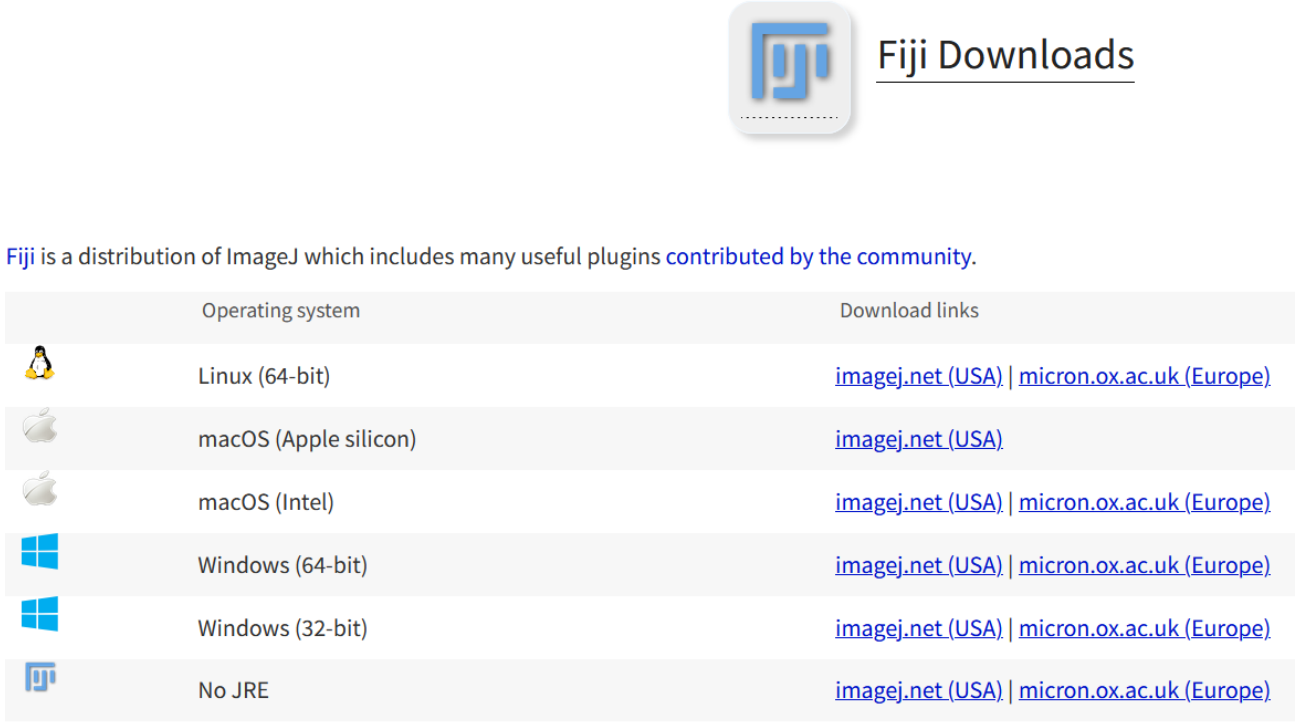


II/ Fiji: Installation

https://fiji.sc/



https://imagej.net/software/fiji/downloads

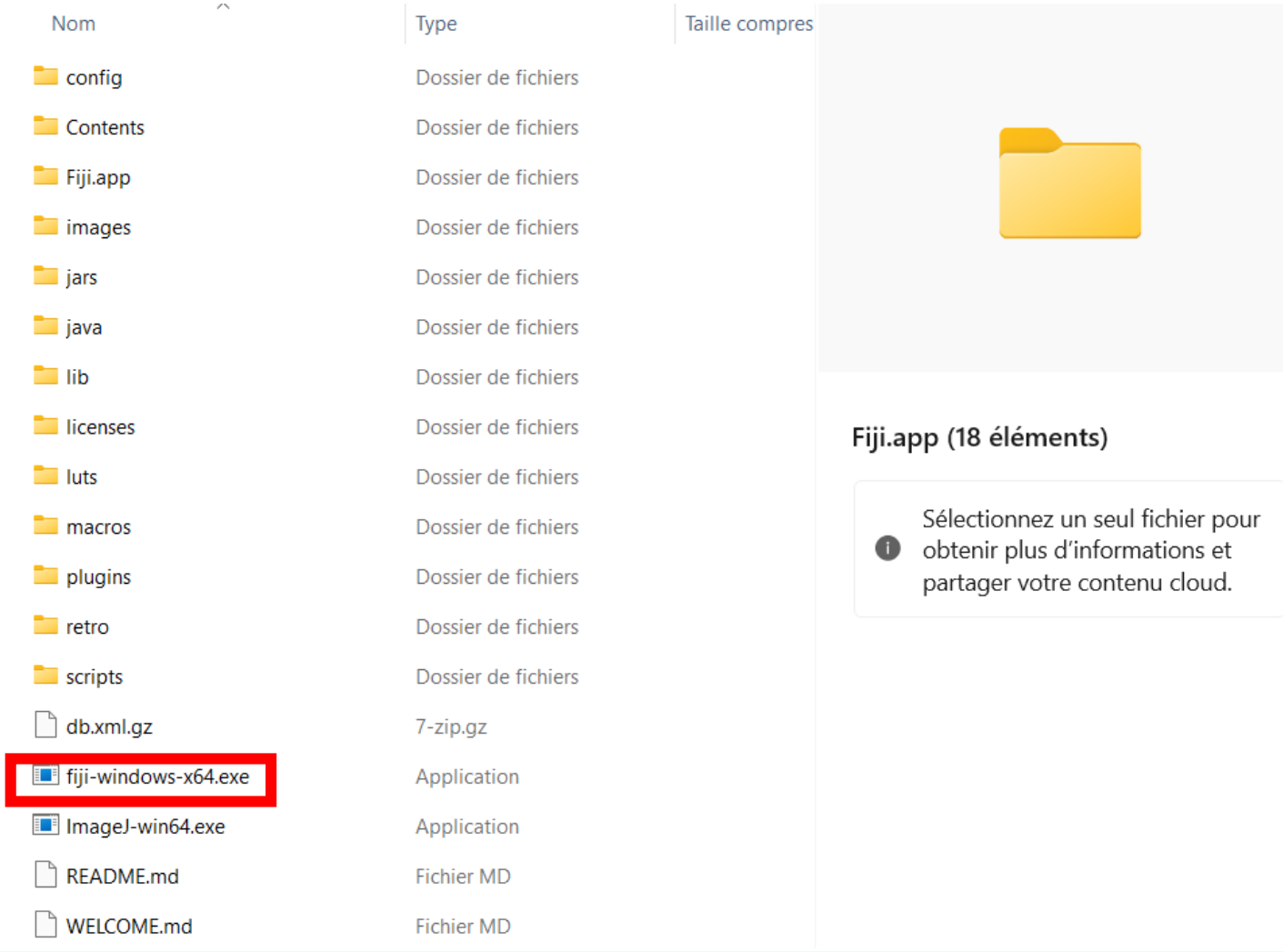


https://imagej.net/contribute/citing

- Download for your OS
- Unzip (/!\ Somewhere you have full permissions!)

II/ Fiji: Installation

Explore your Fiji directory



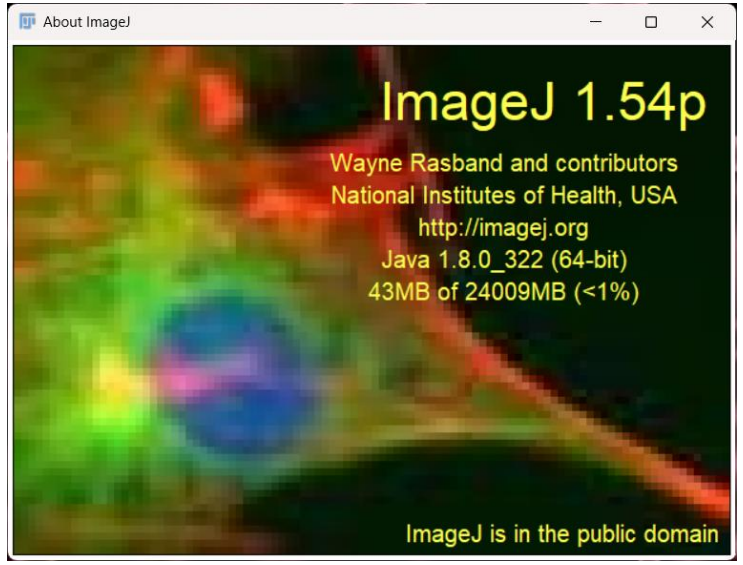
To launch Fiji,

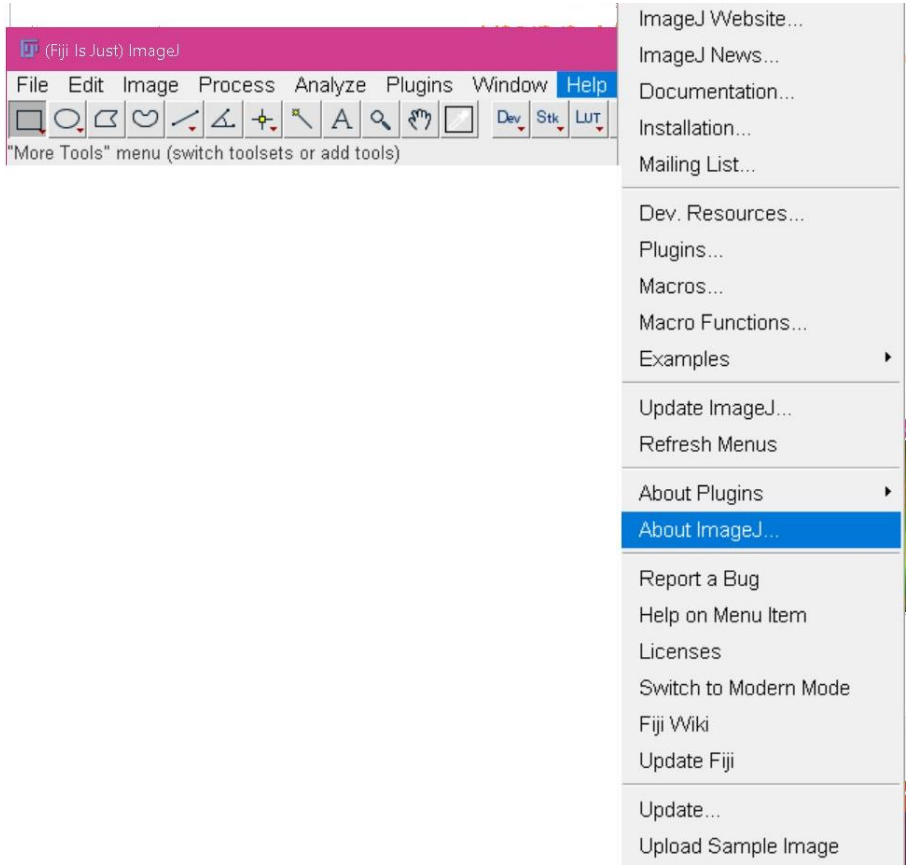
double click on the .exe

You can also create a shortcut on your desktop.

II/ Fiji: Installation

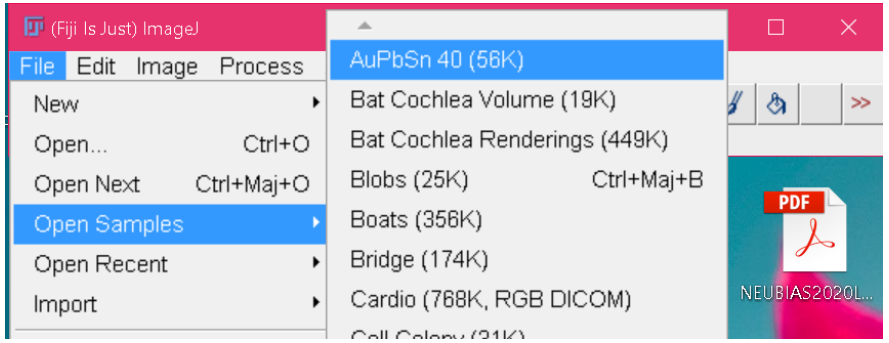
Check the version: *Help > About ImageJ*





II/ Fiji: Installation

Try to do *File > Open Samples > AuPnSn 40 (56k)*

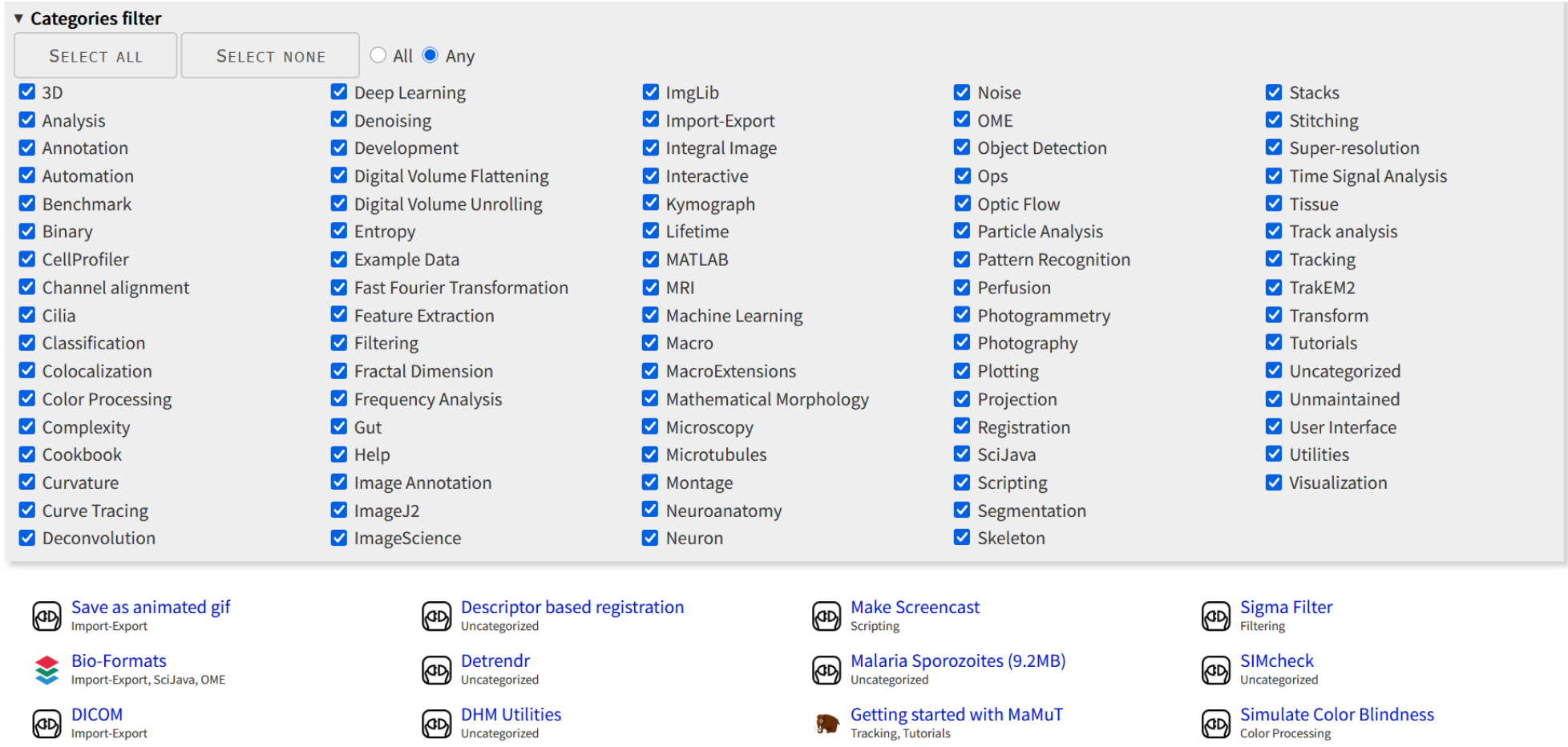


Do you get

?

II/ Fiji: Installation: Plugins

Extending Fiji: plugins: https://imagej.net/list-of-extensions, https://imagej.net/list-of-update-sites/



II/ Fiji: Installation: Plugins: *Hands-on!*

Example of plugin installation: Stardist

Step 1 — **Open Fiji Updater on Fiji** — *Help > Update...*

Step 2 — **Click on Manage update sites and check the following sites** — *Update sites : **CSBDeep**, **StarDist** and **TensorFlow***
Tip: use the Search bar!

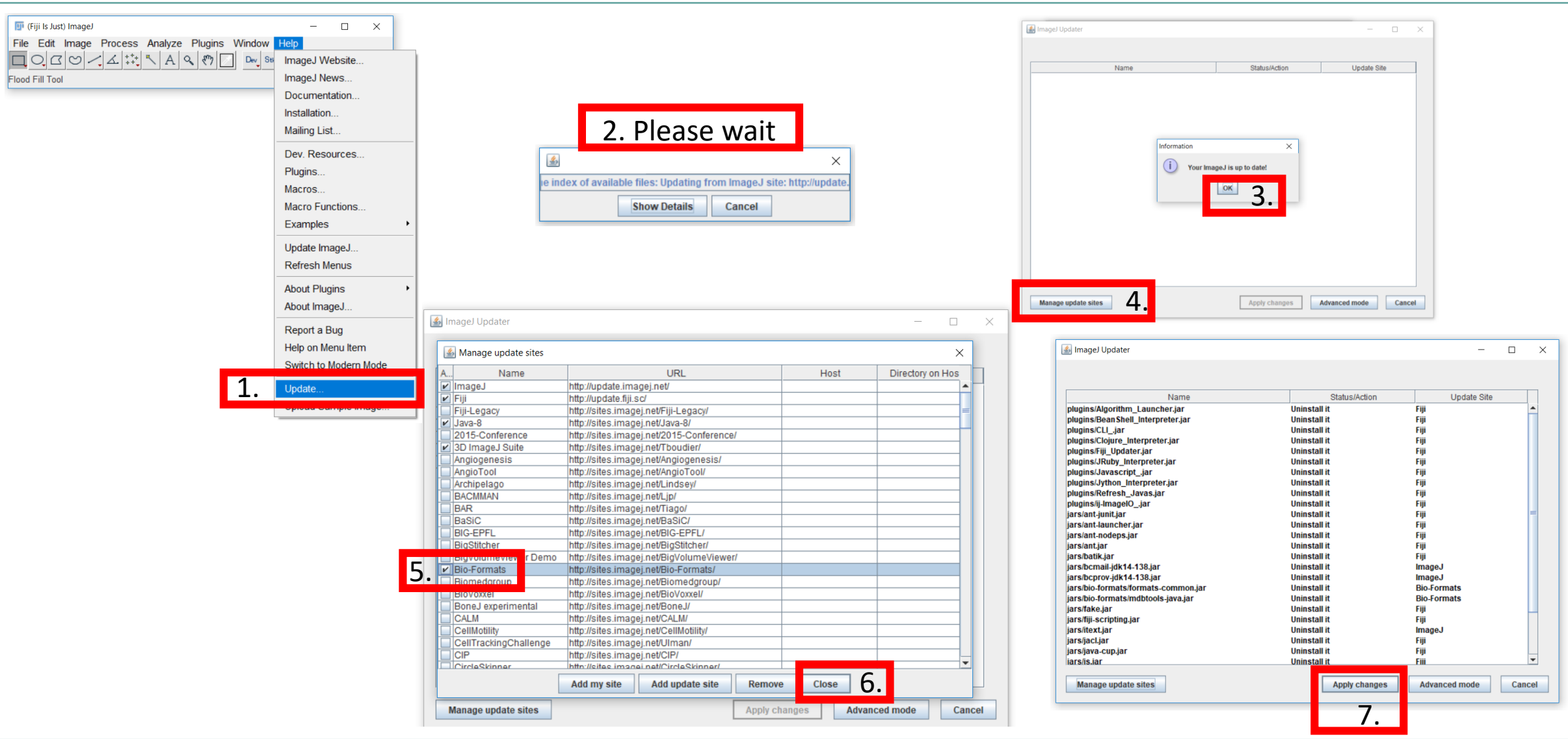
Step 3 — **Close Manage update sites and Click on Apply changes**

Step 4 — **Restart Fiji/ImageJ** — *Sometimes a simple Help> Refresh can do the job*

Step 5 — **The plugin is ready to use** — *It should appear under Plugins>StarDist*

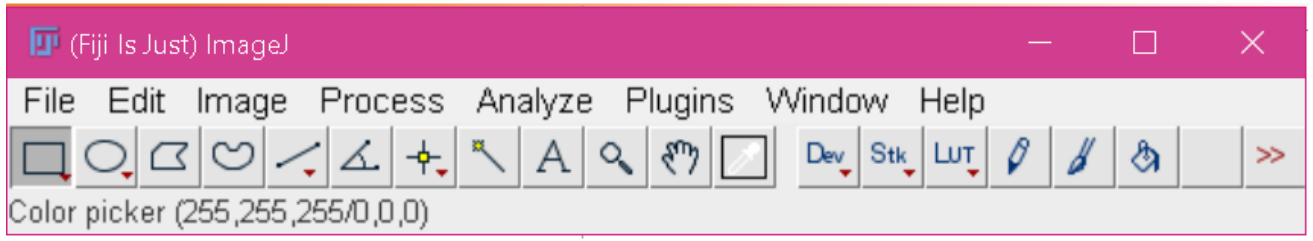
https://imagej.net/plugins/stardist

II/ Fiji: Installation: Plugins: *Hands-on!*



II/ Fiji: Exploring the menu

Going through the menu



II/ Fiji: Opening an image: *Hands-on!*

Two methods:

- File > open
- Drag and drop onto the Fiji bar

Step 1	Open boats.tif
Step 2	Move your cursor around on the image
Question	**What do you notice?**

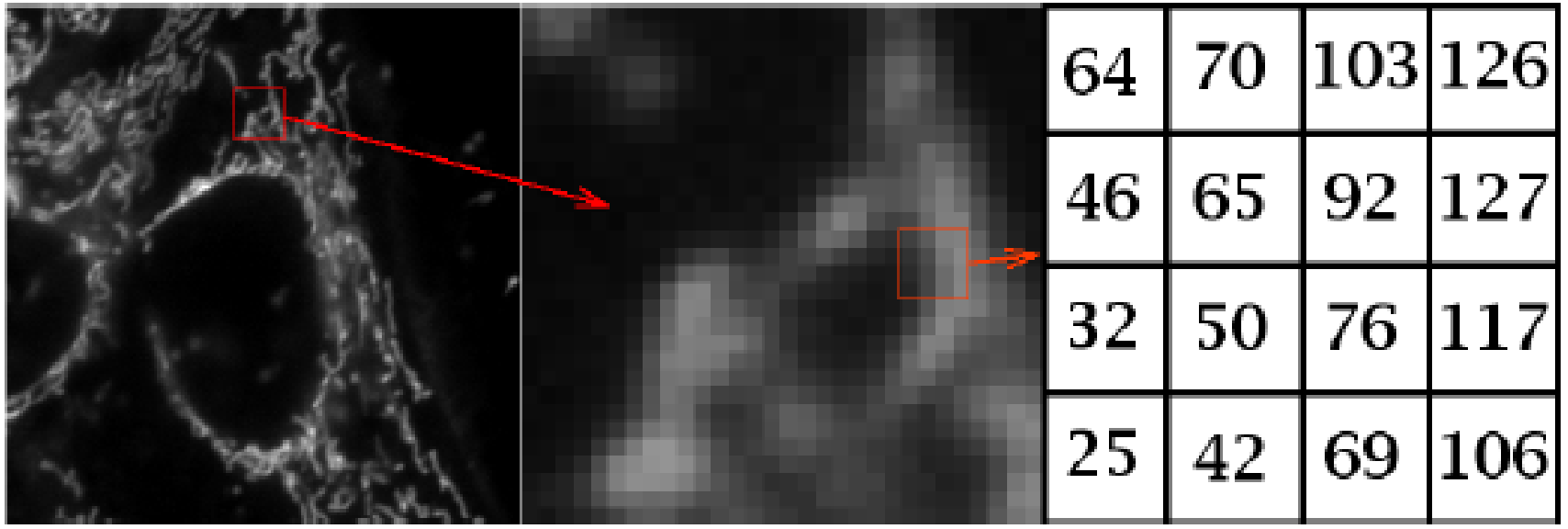
Rule number 1 in Fiji:

Anything you will do on an image will apply on the **active image**,
i.e. the last image…

- which you have opened,
- or clicked on,
- or which was created by a process!

III/ Fundamentals: What are digital images?

III/ Fundamentals: What is a digital image?



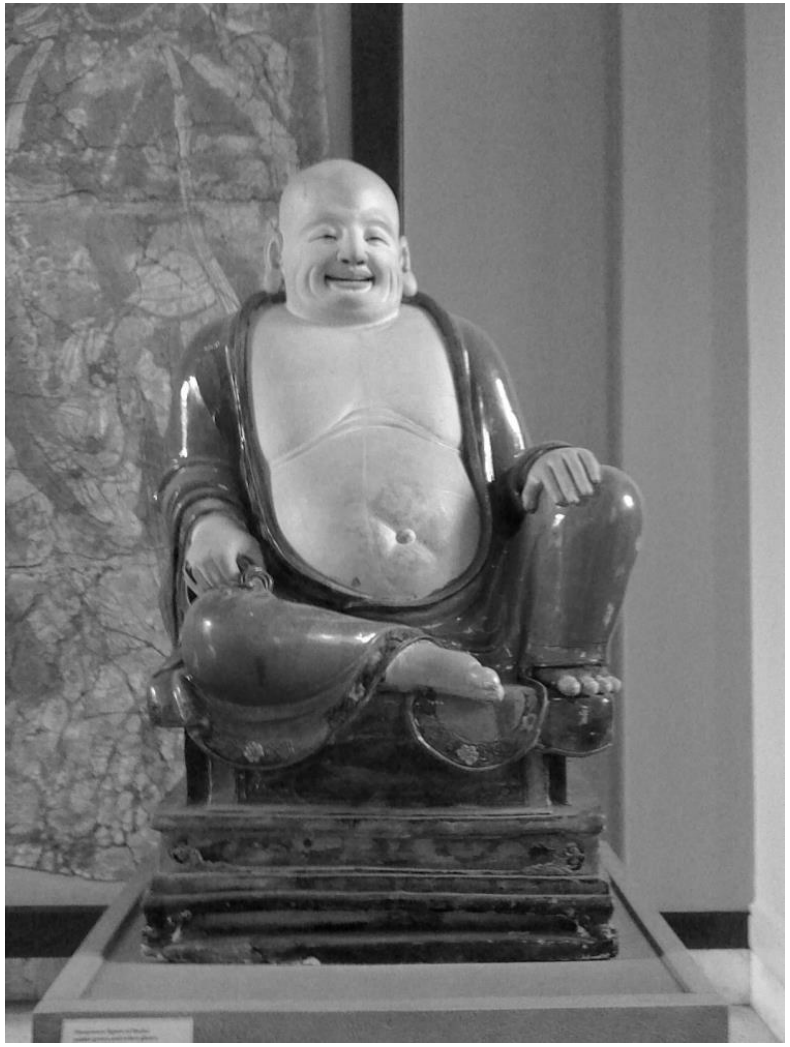
64	70	103	126
46	65	92	127
32	50	76	117
25	42	69	106

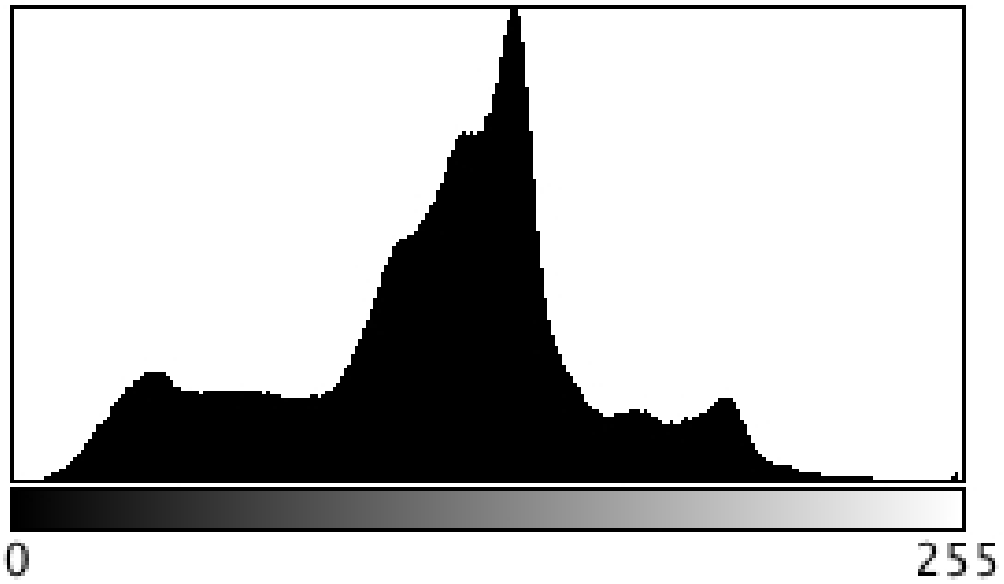
It is a matrix! There are only numbers, that we're going to name **intensity**

The cells are pixels (PICTure ELement)

In 3D = voxels (VOlumoc Element)

III/ Fundamentals: Histogram



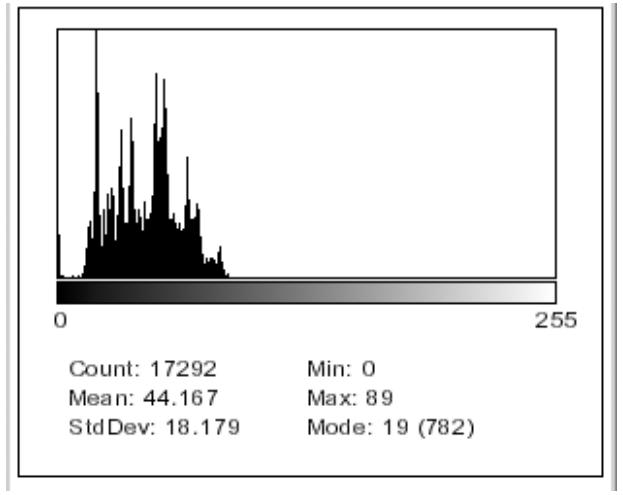


Count: 1920000
Mean: 113.658
StdDev: 42.343
Bins: 256

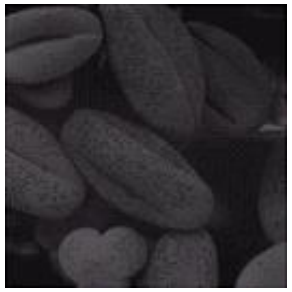
Min: 0
Max: 255
Mode: 135 (33747)
Bin Width: 0.996

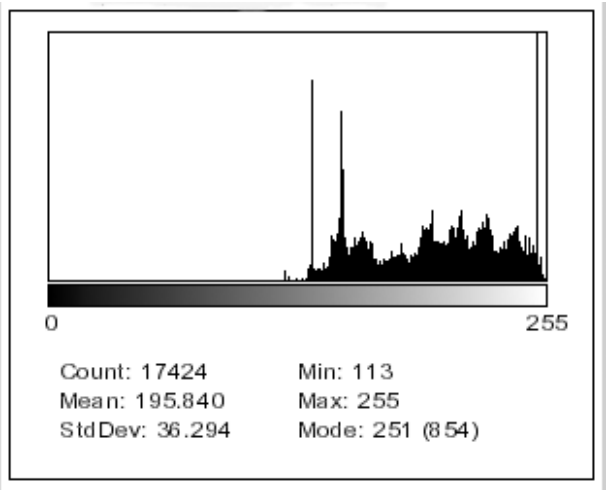
III/ Fundamentals: Using the full dynamic range of the camera

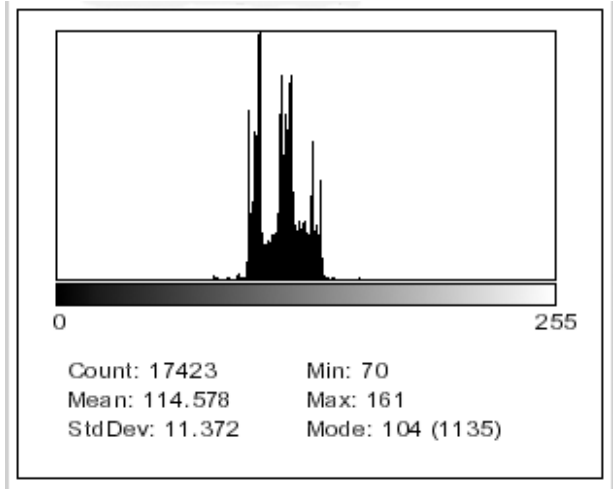
= ability to read very bright and very dark signals together

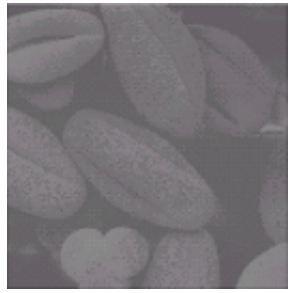


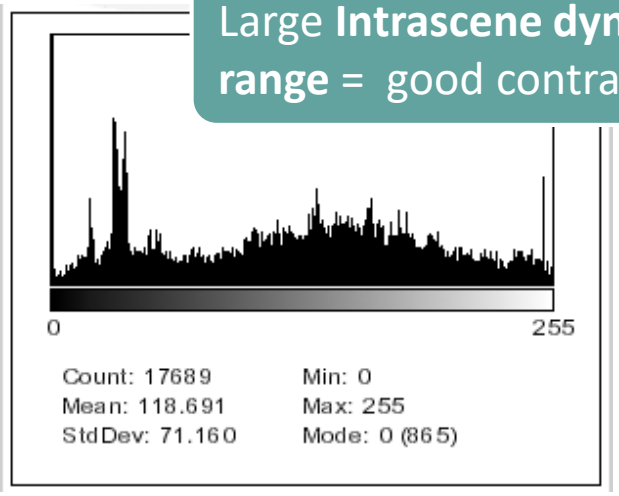
Analyze > Histogram (**h**)





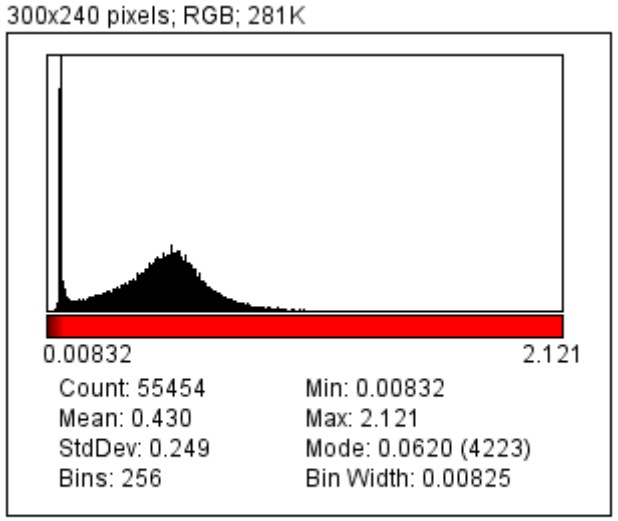


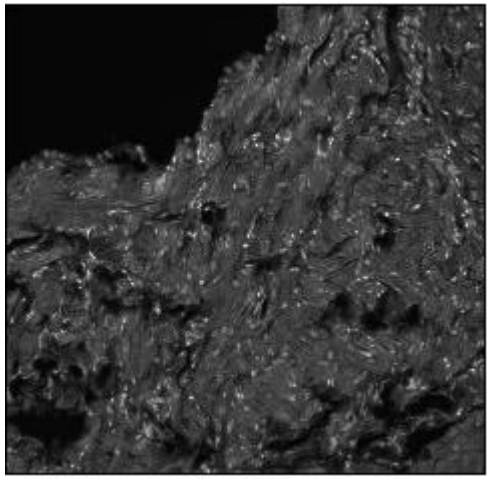




Large **Intrascene dynamic range** = good contrast!

III/ Fundamentals: Saturation

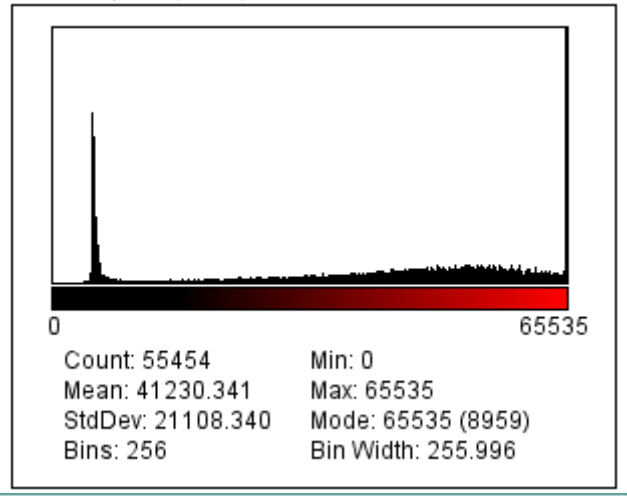


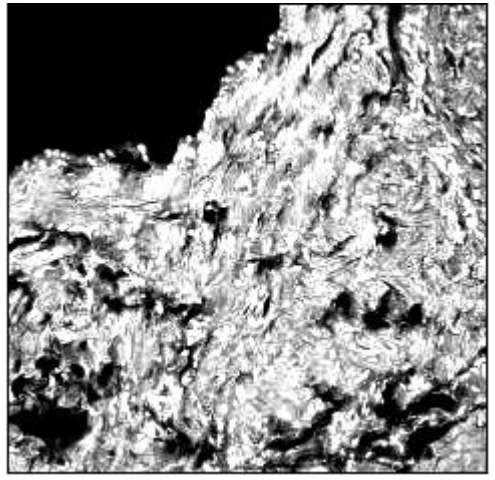


List Copy Log Live

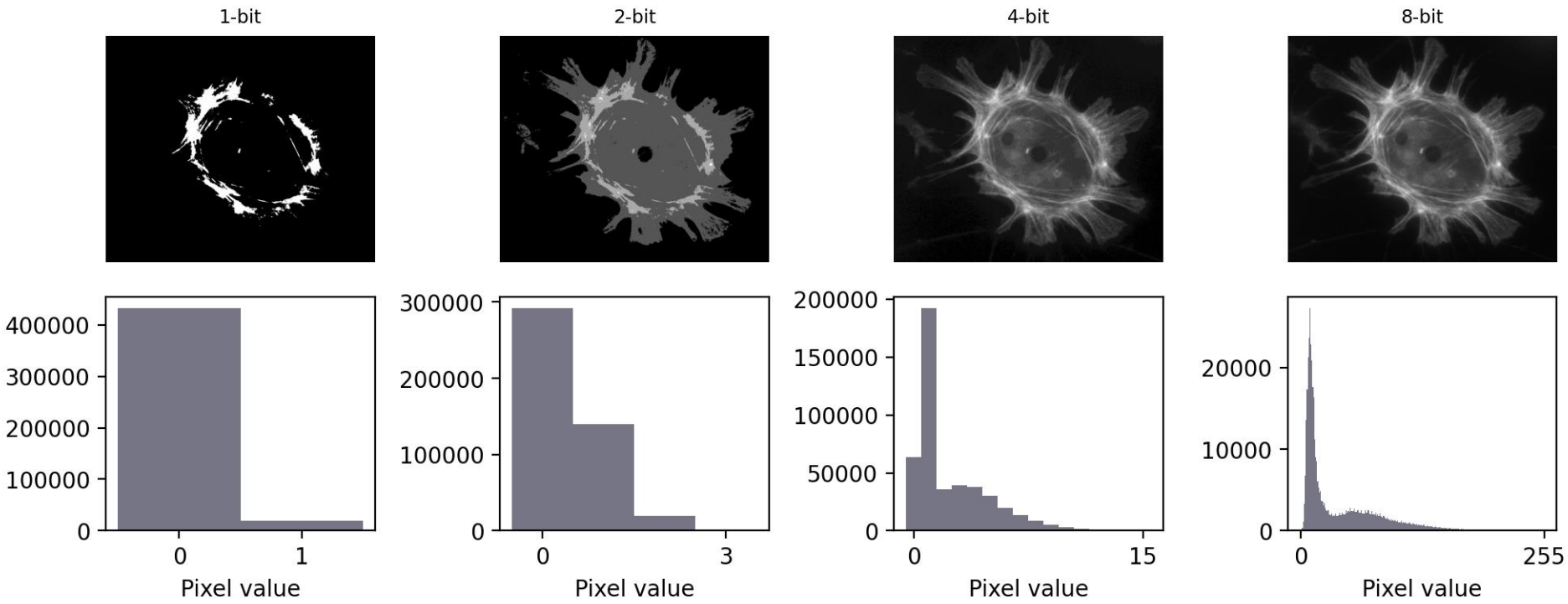
value=0.668

count=410





III/ Fundamentals: Bit depth



1-bit

Decimal	Binary
0	0
1	1

2-bit

Decimal	Binary
0	00
1	01
2	10
3	11

8-bit

Decimal	Binary
0	00000000
1	00000001
2	00000010
3	00000011
4	00000100
5	00000101
6	00000110
7	00000111
...	...
255	11111111

- 8-bit: 0, 1, .., 255 (2^8 - 1)
- 16-bit: 0, 1, .., 65 535 (2^16 - 1)
- 32-bit: 0, 1, .., 1 070 000 000 (2^32 - 1)

https://bioimagebook.github.io/chapters/1-concepts/3-bit_depths/bit_depths.html#fig-bit-depths-demo

III/ Fundamentals: Detector type vs Image type

Detector type	Range	Image type on the computer
8-bit	0 - 255	8-bit
12-bit (most PMTs & cameras)	0 - 4095	16-bit
14-bit (some EMCCD cameras)	0 - 16383	
16-bit (some PMTs)	0 - 65535	
ø	~ -Infinity - +Infinity (real numbers)	32-bit
Special		
Color cameras	RGB (color image)	3 layers of 8-bit each (red, green & blue)

Much better dynamic range, good for intensity comparing

On the computer, everything is a multiple of 1 byte (=8 bits)

The only representation that permits negative numbers and floating point numbers

Slide: J-Y Tinevez

III/ Fundamentals: Display: Brightness Contrast

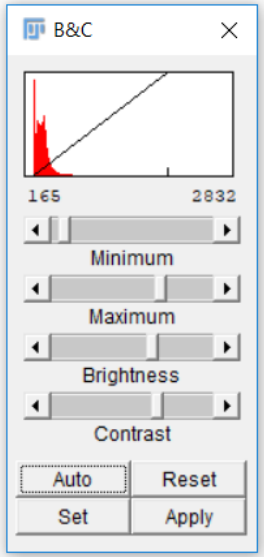


Image > Adjust > Brightness/Contrast…
Shift + C

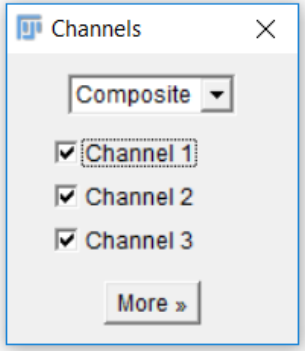
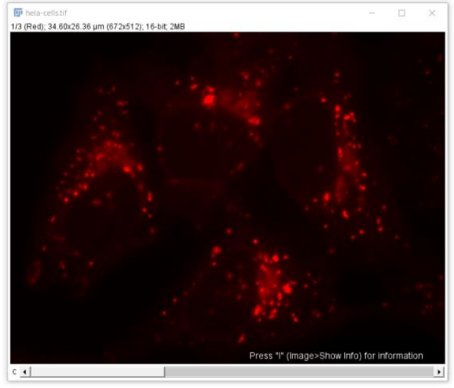
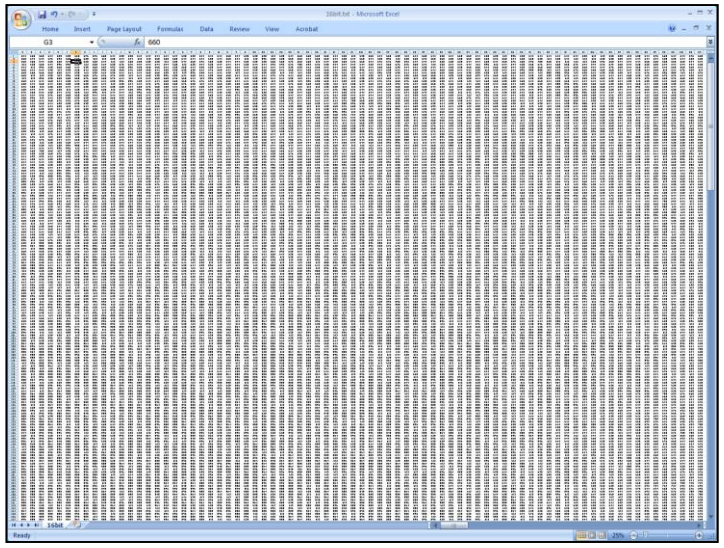
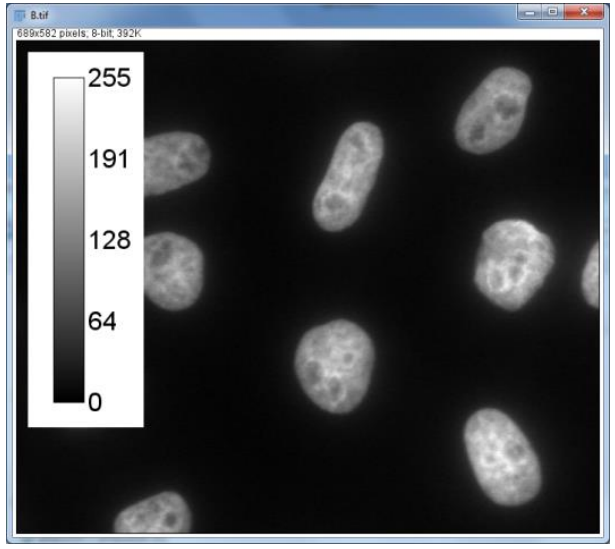


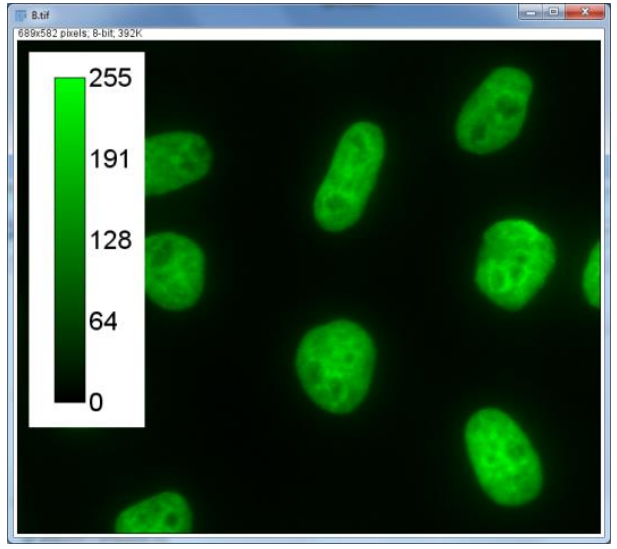
Image > Color > Channels Tool…
Shift + Z

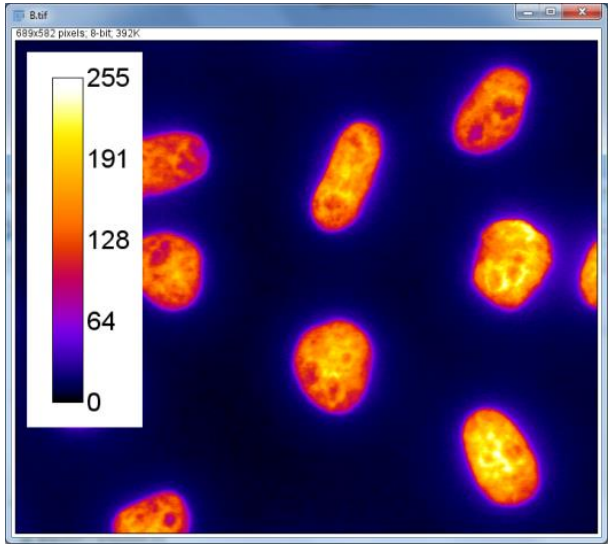


III/ Fundamentals: Display: Lookup Table (LUT) or colormap









Viridis LUT

Red	Green	Blue	Color
68	1	84	
72	25	107	
70	47	124	
64	67	135	
56	86	139	
48	103	141	
41	120	142	
35	136	141	
30	152	138	
34	167	132	
53	183	120	
83	197	103	
121	209	81	
165	218	53	
210	225	27	
253	231	36	

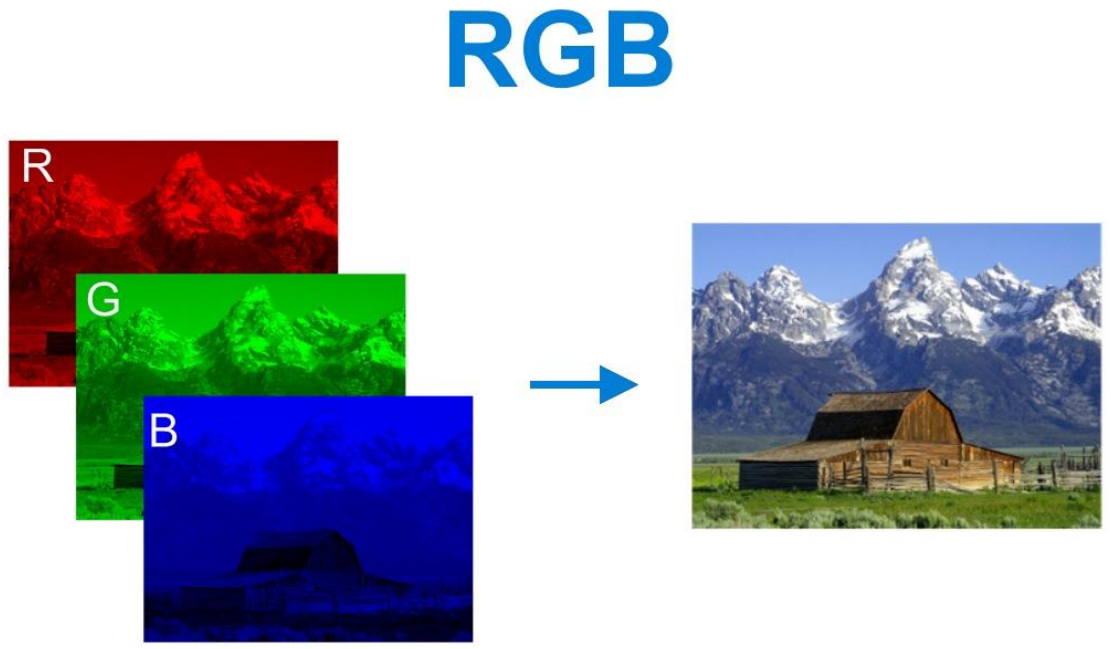
LUT in RGB on Greyscale images: the image is displayed thanks to the mixing of 3 RED, GREEN and BLUE values for one given greyscale value.

III/ Fundamentals: RGB images

Step 1 Open FluorescentCells.tif

Step 2 Open petanque.tif

Question **What are the differences?**



RGB images : each pixel has **three** values for RED, GREEN and BLUE

III/ Fundamentals: *Read the information about an image*

File>Open Samples> Mitosis

Aim: Get the dimensions, the sampling or pixel size (voxel in 3D), the bit depth and the dynamic range of mitosis

Step 1	**Get the dimensions (in X? Y? Z? C? T?)**	*Observe the Information above the image. Alternatively Image> Show Info*
Step 2	**Get the bit depth**	*Observe the Information above the image. Alternatively Image> Show Info*
Step 3	**Get the pixel size/ voxel size**	*Image>Properties*
Step 4	**Get the dynamic range**	*Process>Histogram (min, max), Alternatively Image>Show Info*

III/ Fundamentals: *Read the information about an image*

File>Open Samples> Mitosis

Aim: Get the dimensions, the sampling or pixel size (voxel in 3D), the bit depth and the dynamic range of mitosis

Step 1	**Get the dimensions (in X? Y? Z? C? T?)**	X=171, Y=196, Z=5, C=2, T=51, 0,14286 seconds between frames
Step 2	**Get the bit depth**	Bit-depth = 16 bits
Step 3	**Get the pixel size/ voxel size**	Pixel(Voxel in 3D) Size XYZ= 0.0885x0.0885x1 µm3
Step 4	**Get the dynamic range**	c1 1582-6440; c2 1614-15787 Bonus: C1 and C2 content? Drosophila S2 cell expressing GFP-Aurora B and mCherry-tubulin

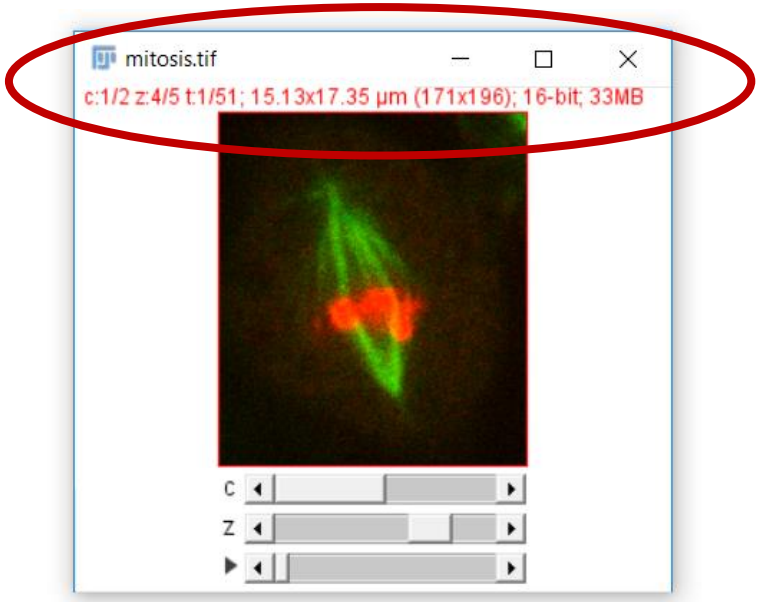
III/ Fundamentals: Show Info

Drosophila S2 cell expressing GFP-Aurora B and mCherry-tubulin
fusion protein undergoing mitosis. Courtesy of Eric Griffis.

(Fiji Is Just) ImageJ 2.0.0-rc-69/1.52i; Java 1.8.0_172 [64-bit]; Windows 10 10.0; 3145MB of 12141MB (25%)

Title: mitosis.tif
Width: 15.1335 µm (171)
Height: 17.3460 µm (196)
Depth: 5 µm (5)
Size: 33MB
Resolution: 11.2994 pixels per µm
Voxel size: 0.0885x0.0885x1 µm^3
ID: -26
Bits per pixel: 16 (unsigned)
Display ranges
1: 1582-6440
2: 1614-15787
Frame: 7/510
Frame interval: 0.14286 sec
Channel: 1/2
Slice: 4/5
Frame: 1/51
Composite mode: "composite"
No threshold
ScaleToFit: false
Uncalibrated
URL: http://imagej.nih.gov/ij/images/Spindly-GFP.zip
Screen location: 1171,328 (2048x1152)
Coordinate origin: 0,0,0
No overlay
No selection

Image > Show Info (**i**)



III/ Fundamentals: Hyperstacks

Image 2D

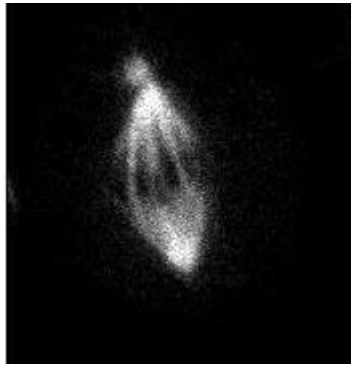


Image 3D:

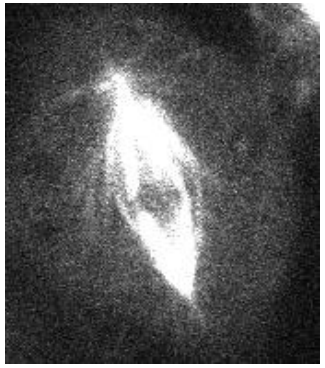
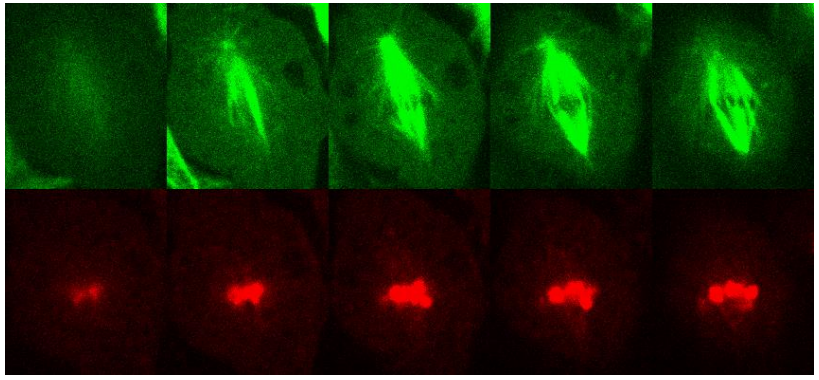
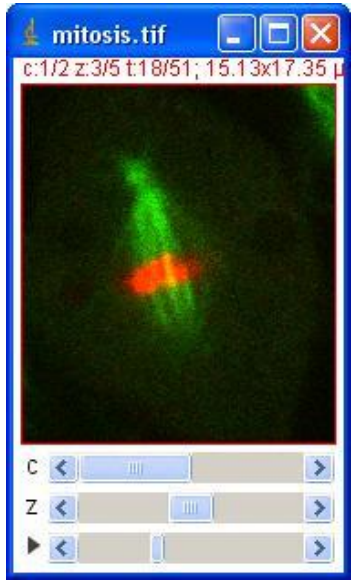


Image 4D+: Hyperstack



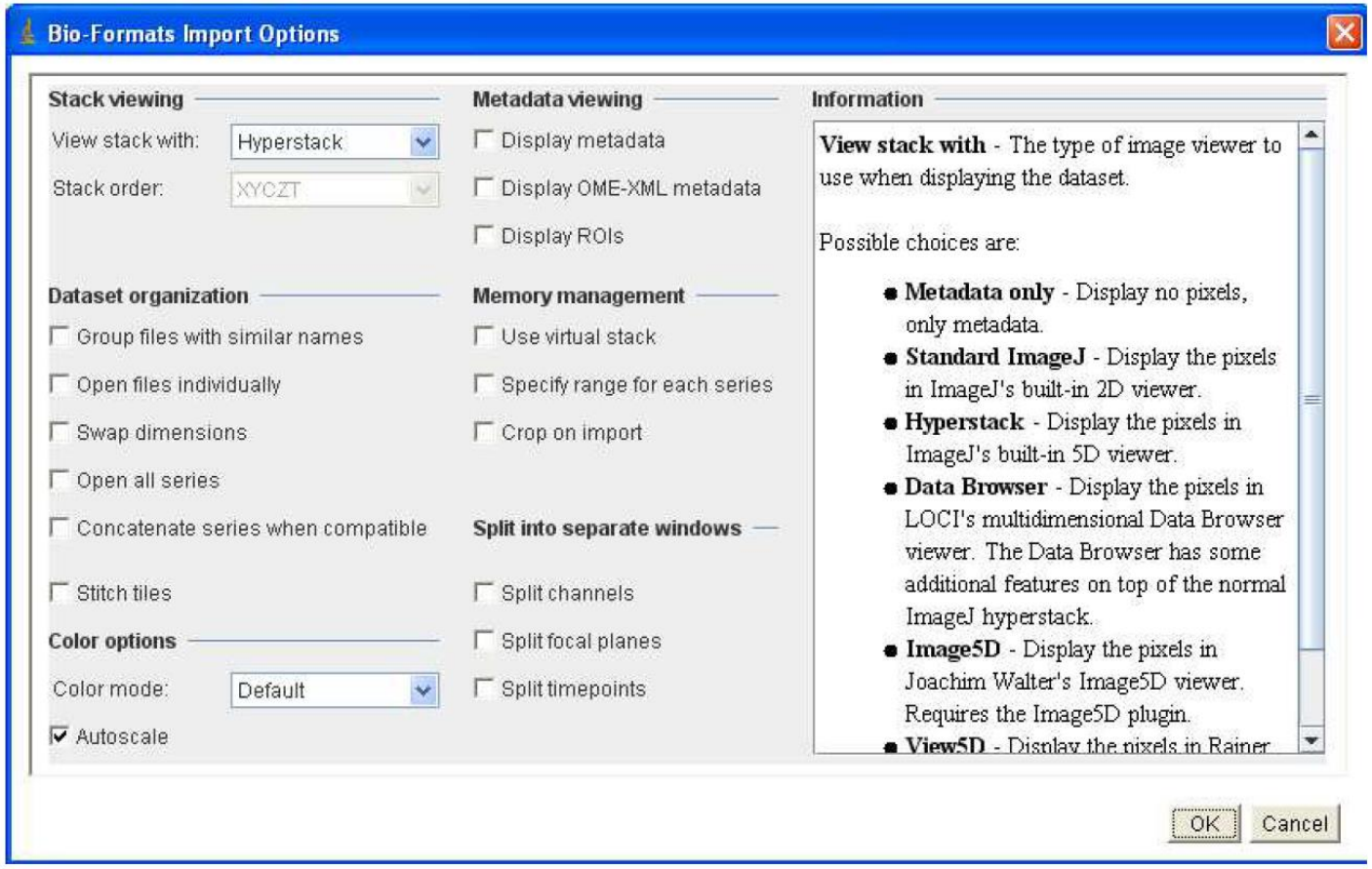


Hyperstack = 5 dimensions

- x
- y
- z (slice)
- channel
- time (Frame)

III/ Fundamentals: Hyperstacks

Can be opened with Bio-Formats



III/ Fundamentals: Calibration: *Hands-on!*

Step 1	**Open petanque.tif**	*Can you measure the distance between the ball and the jack? In cm?*
Step 2	**Draw a line on something you know the size of**	*Hint: he's holding a 20 cm ruler ;)*
Step 3	**Calibrate the image!**	*Analyze > Set scale*
Step 4	**Alternative method**	*If you know the pixel size, input it directly in Image>Properties*
Step 5	***Measure the distance in cm between the ball and the jack!***	

Analyze > Set scale

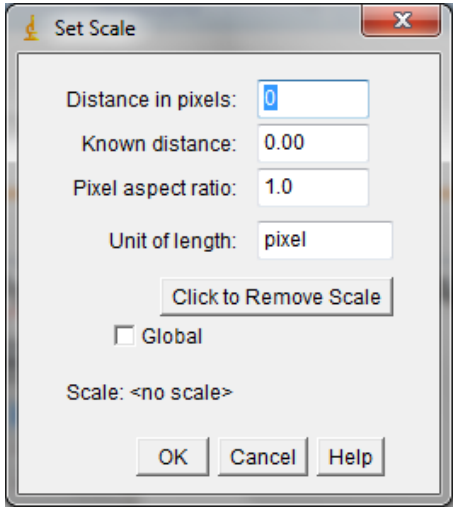
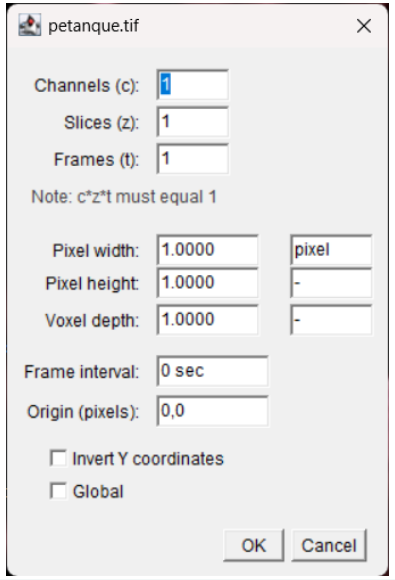


Image > Properties…



III/ Fundamentals: Image format and image storage: *Hands-on!*

Step 1	Try to drag and drop the MysteryFile.txt	Parse it. Comment.
Step 2	Try to open using File>Import>Text Image	Comment, hover the mouse over pixels
Step 3	Modify the pixel size	Image>Properties Changes unit values to um (=microns) and Pixel width and height to 0.5
Step 4	Duplicate the image twice using Image> Duplicate…, do it twice	Give it a new title. You should have 3 times the same image
Step 5	Save the first image as .tif File> Save As > Tiff	Then close first image
Step 6	Save the second image as .txt File> Save As > Text Image	Then close second image
Step 7	Save the third image as .jpg File> Save As > Jpeg	Then close third image

III/ Fundamentals: Image format and image storage: *Hands-on!*

Now look at the directory content. What do you notice?

MysteryFile.tif	16/01/2019 18:39	Image TIFF	3 559 Ko
MysteryFile.jpg	16/01/2019 18:39	Fichier JPG	141 Ko
MysteryFile.txt	16/01/2019 18:39	Document texte	7 119 Ko

Open the 3 images again (Do not forget to use Import>Text Image for the .txt)

Different?

III/ Fundamentals: Image format and image storage: *Hands-on!*

Conclusions:

Some formats store the metadata (here pixel size) in addition to the pixel data (ex: .tif)

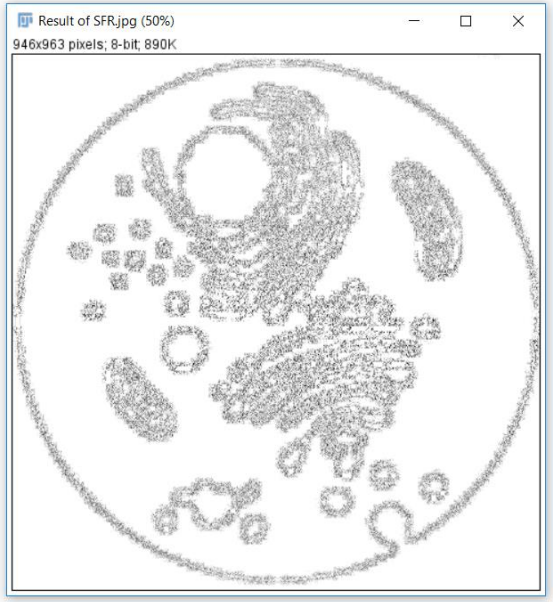
The way the data is stored is different -> compression

Jpeg image we created

-

Tiff image we created

=



Jpeg – tiff (pixel by pixel difference)

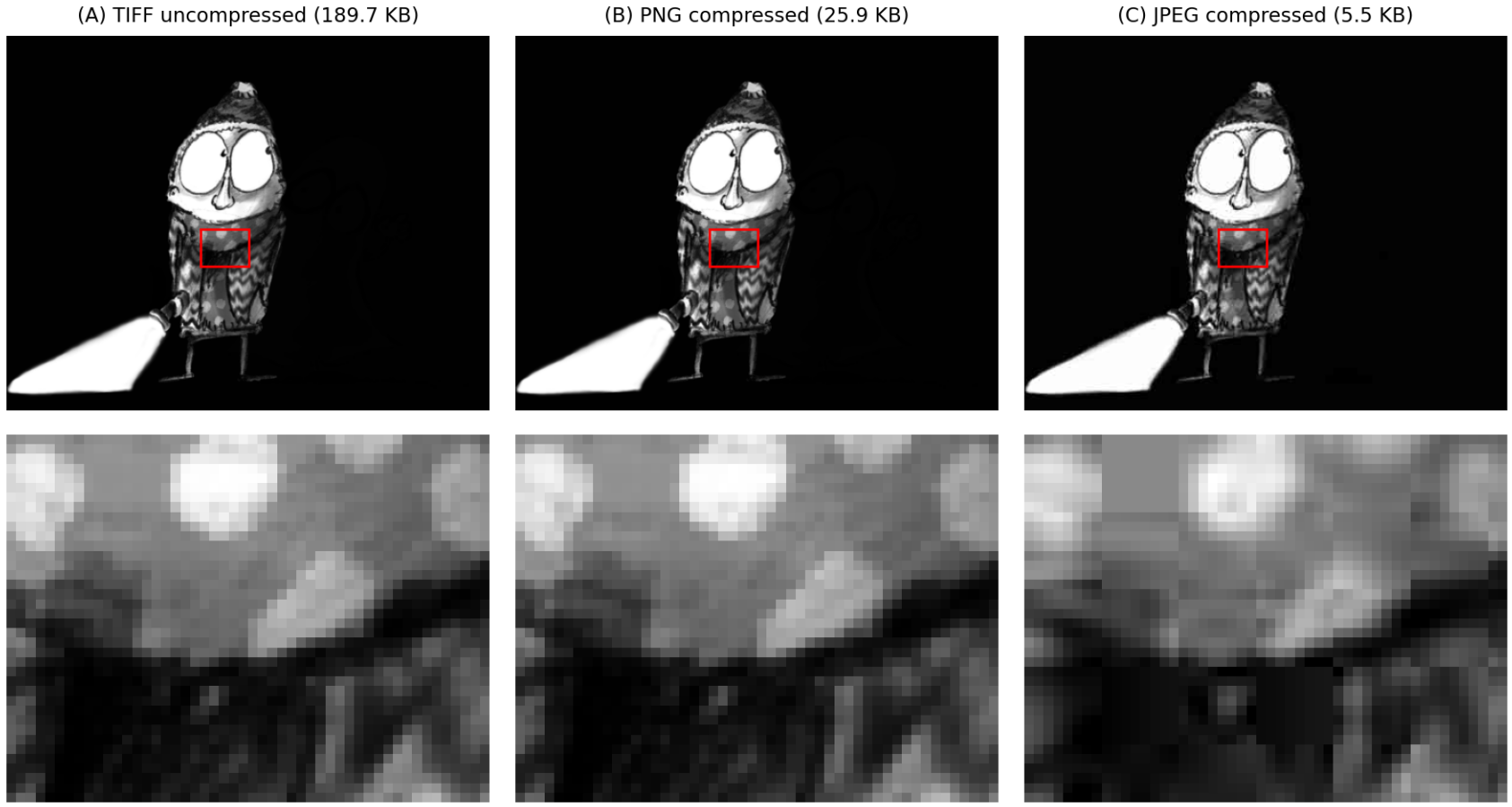
III/ Fundamentals: Image format and image storage

Images files= pixel values and metadata (dimension, image type, bit-depth, pixel size, microscope parameters…)

Format	Extensions	Main use	Compression	Comment
TIFF	.tif, .tiff	Analysis, display (print)	None, lossless, lossy	Very general image format
OME-TIFF	.ome.tif, .ome.tiff	Analysis, Display (print)	None, lossless, lossy	TIFF, with standardized metadata for microscopy
Zarr	.zarr	Analysis	None, lossless, lossy	Emerging format, great for big datasets – but limited support currently
PNG	.png	Display (web, print)	Lossless	Small(ish) file sizes without compression artefacts
JPEG	.jpg, .jpeg	Display (web)	Lossy (usually)	Small file sizes, but visible artefact

III/ Fundamentals: Image format and image storage

https://bioimagebook.github.io/chapters/1-concepts/6-files/files.html



III/ Fundamentals: Recap: concepts

Digital Images
They are matrices!

Histograms
An eagle-eye view of your data. Great to see what is wrong with it (e.g. saturation)

Dynamic range
The ability of the sensor to image bright and dark signal simultaneously: if low, bright signals can saturate the sensor while weak become lost in the noise. Adjust your settings so you make good use of it!

Bit depth
How finely chopped your signal is: the bigger it is, the more shades of grey, the more details can be replicated in an image. The greater the bit depth, the better the full dynamic range can be resolved. Make sure you have the adequate one when sitting at the microscope

Display adjustments
- **Brightness & contrast:** they do not change your data, just the way it is represented on the screen.
- **LUT:** Same. Pick the best for a snapshot of your data.

Hyperstacks
3D = stack. 4D+=Hyperstack

Calibration
Demonstrated that the *metadata* is crucial in microscopy! Beware of lossy formats.

III/ Fundamentals: Recap: commands

Image > Adjust > Brigthness/Contrast (**Shift + c**)
Image > Show info (**i**)
Image > Properties (**Shift + p**)
Image > Color > Channels Tool (**Shift + z**)
Analyze > Histogram (**h**)
Image > Lookup Tables

List of shortcuts: https://imagej.net/ij/docs/shortcuts.html

III/ Fundamentals: Other basic commands

Edit > Invert (**Shift + i**)
Image > Duplicate (**Shift + d**)
Image > Crop (**Shift + x**)
Image > Rename
Image > Transform > Rotate/Flip …
Image > Adjust > Size
Image > Color > Split Channels/Merge Channels
Selection > Select All (**a**)
Selection > Select None (**Shift + a**)
Selection > Restore (**Shift + e**)
Selection > Add to Manager (**t**)

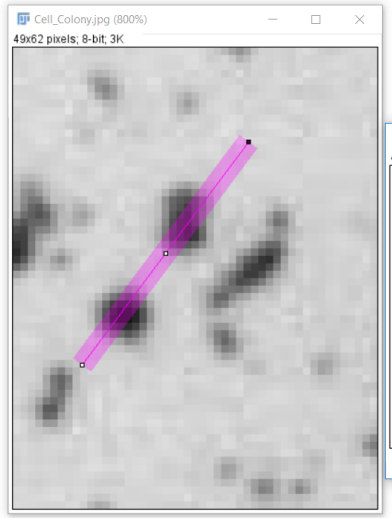
III/ Fundamentals: still a lot left to learn!

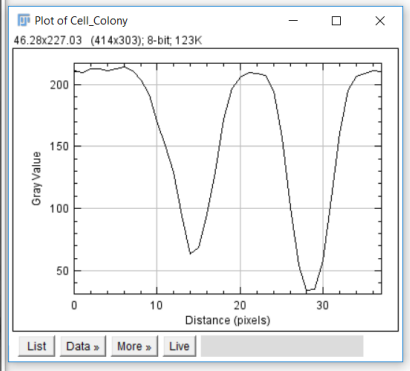
Image > Stacks > Z Project

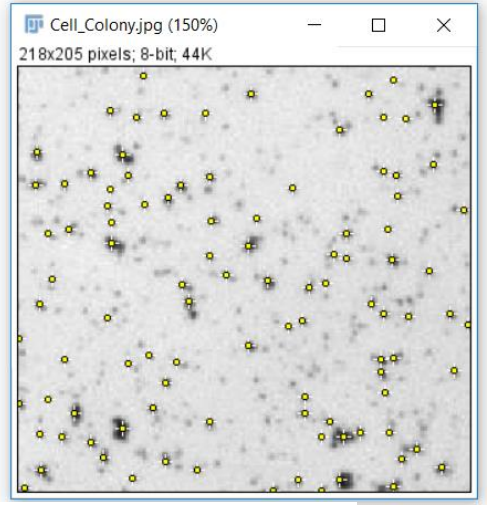
Process > Find maxima

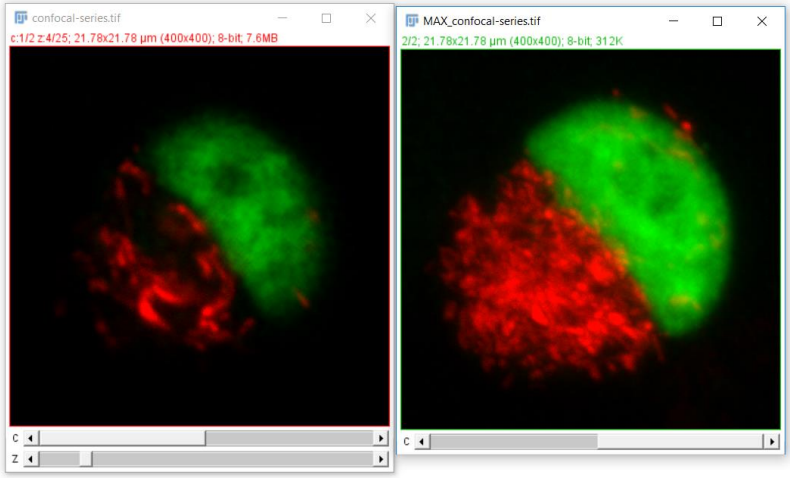
Analyze > Plot profile

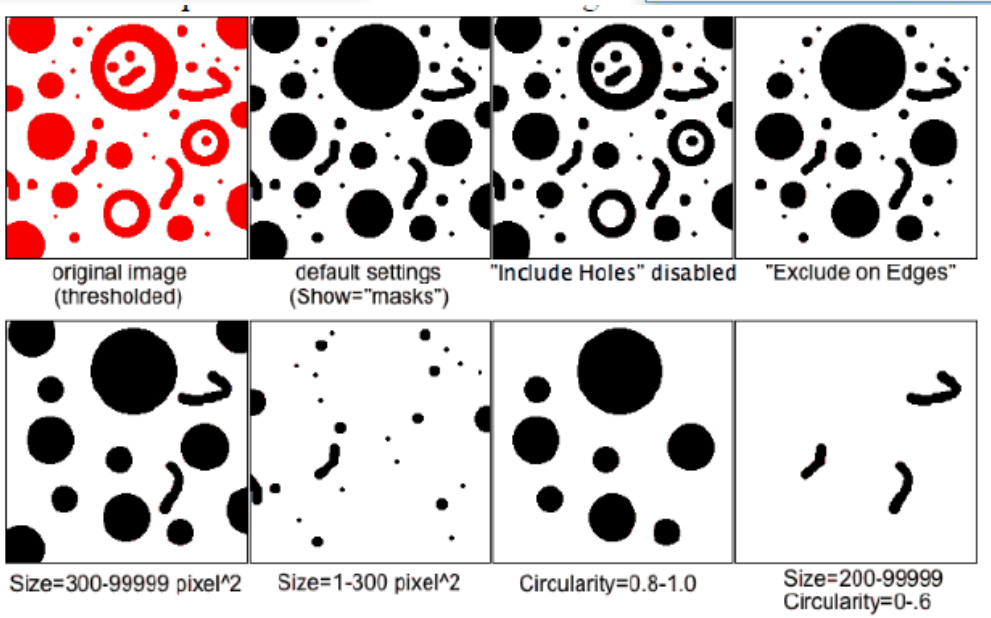
Analyze > Analyze Particles











IV/ Image ethics

IV/ Image ethics: Ethical guidelines for the appropriate use and manipulation of scientific digital images (Cromey, 2010):

1/ Scientific digital images are **data that can be compromised** by inappropriate manipulations.

2/ Manipulation of digital images should only be performed on a **copy** of the unprocessed image data file (Always keep the original data file safe and unchanged!).

3/ **Simple adjustments** to the **entire** image are **usually acceptable**.

4/ **Cropping** an image is **usually acceptable**.

5/ Digital images that will be compared to one another should be **acquired under identical conditions**, and any **post-acquisition image processing** should also be **identical**.

6/ **["Local"] manipulations** that are specific to one area of an image and are not performed on other areas are **questionable**.

Cromey, Douglas W. « Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images ». *Science and Engineering Ethics* 16, nº 4 (2010): 639-67. https://doi.org/10.1007/s11948-010-9201-y.

IV/ Image ethics: Ethical guidelines for the appropriate use and manipulation of scientific digital images (Cromey, 2010):

7/ Use of **software filters to improve image quality** is usually **not recommended** for biological images.

8/ **Cloning or copying objects** into a digital image, from other parts of the same image or from a different image, is **very questionable**.

9/ **Intensity measurements** should be performed on **uniformly processed image data**, and the data should be **calibrated** to a known standard.

10/ **Avoid** the use of **lossy compression**.

11/ **Magnification** and **resolution** are important.

12/ Be **careful when changing the size** (in pixels) of a digital image.

Cromey, Douglas W. « Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images ». *Science and Engineering Ethics* 16, nº 4 (2010): 639-67. https://doi.org/10.1007/s11948-010-9201-y.

IV/ Image ethics: Ressources for good practices

https://focalplane.biologists.com/2021/05/25/preparing-your-manuscript-guidelines-for-writing-microscopy-methods-and-figures/

https://www.nature.com/documents/nprot-guide-to-preparing-final-artwork.pdf

1. Heddleston JM, Aaron JS, Khuon S, Chew TL. 'A guide to accurate reporting in digital image acquisition – can anyone replicate your microscopy data?' J Cell Sci. 2021 Mar 30;134(6):jcs254144. doi: 10.1242/jcs.254144.

2. Aaron J, Chew TL. 'A guide to accurate reporting in digital image processing – can anyone reproduce your quantitative analysis?' J Cell Sci. 2021 Mar 30;134(6):jcs254151. doi: 10.1242/jcs.254151.

3. Jambor H, Antonietti A, Alicea B, Audisio TL, Auer S, Bhardwaj V, Burgess SJ, Ferling I, Gazda MA, Hoeppner LH, Ilangovan V, Lo H, Olson M, Mohamed SY, Sarabipour S, Varma A, Walavalkar K, Wissink EM, Weissgerber TL. 'Creating clear and informative image-based figures for scientific publications' PLoS Biol. 2021 Mar 31;19(3):e3001161. doi: 10.1371/journal.pbio.3001161.

4. Schmied C and Jambor HK. Effective image visualization for publications – a workflow using open access tools and concepts [version 2; peer review: 2 approved]. F1000Research 2021, 9:1373 (https://doi.org/10.12688/f1000research.27140.2).

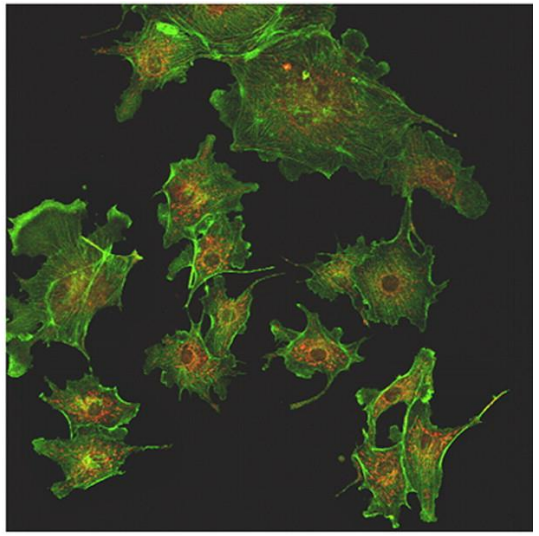
5. Schmied, Christopher, Michael S. Nelson, Sergiy Avilov, Gert-Jan Bakker, Cristina Bertocchi, Johanna Bischof, Ulrike Boehm, et al. « Community-Developed Checklists for Publishing Images and Image Analyses ». *Nature Methods*, 14 septembre 2023, 1-12. https://doi.org/10.1038/s41592-023-01987-9.

6. Miura, Kota, et Simon F Nørrelykke. « Reproducible image handling and analysis ». *The EMBO Journal* 40, nº 3 (février 2021). https://doi.org/10.15252/embj.2020105889.

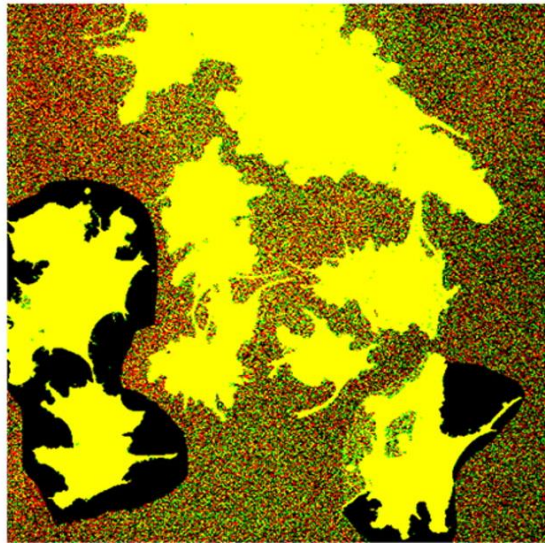
IV/ Image ethics: Mishandling can be intentional

Rossner & Yamada, JCB 2004

Manipulated image



Manipulation revealed by contrast adjustment

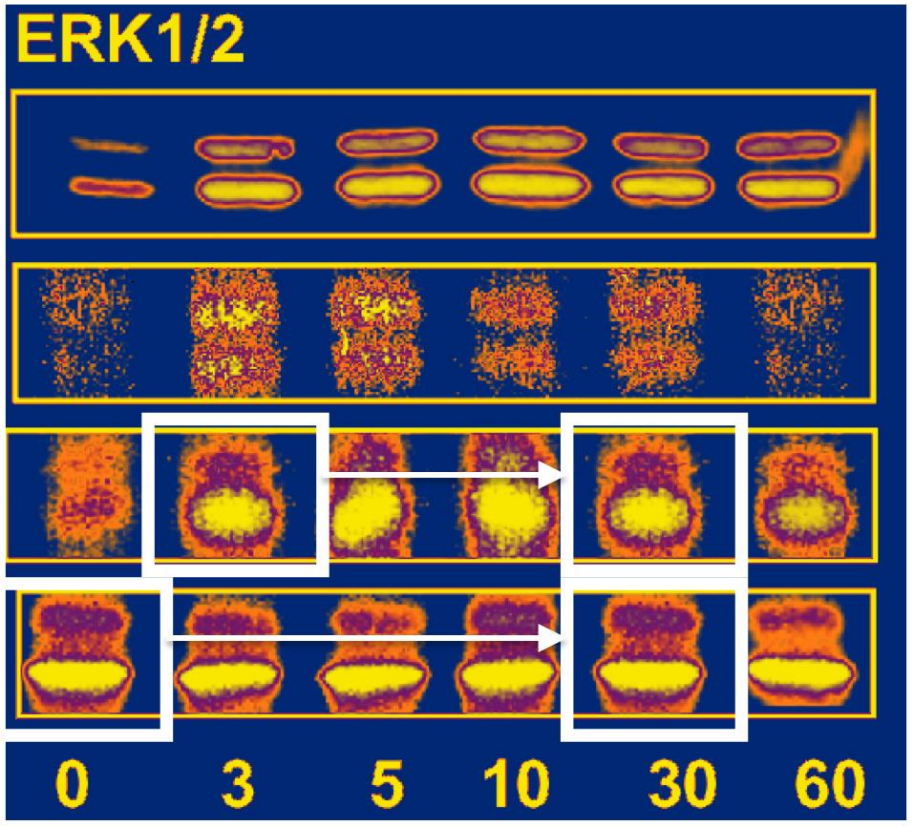


Copy / pasting cells into an image.

Slide: J-Y Tinevez

IV/ Image ethics: Mishandling can be intentional

Simple tools to detect image manipulation.



Copy/paste within images.
But you can detect them easily with false color LUT.

Solution to avoid mishandling? Yes, REPORTING!

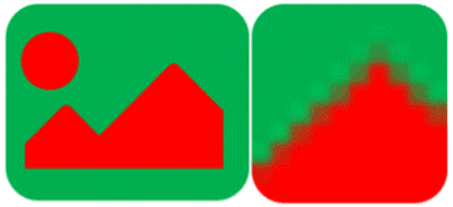
Document all you did to prepare the figures

- Materials and Methods (supp info).
- Jupyter-like Notebooks or scripts to generate the figures.

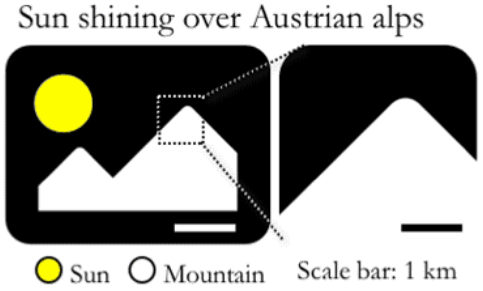
Slide: J-Y Tinevez

IV/ Image ethics: Common mistakes

Schmied C and Jambor HK. Effective image visualization for publications – a workflow using open access tools and concepts [version 2; peer review: 2 approved]. F1000Research 2021, 9:1373 (https://doi.org/10.12688/f1000research.27140.2).

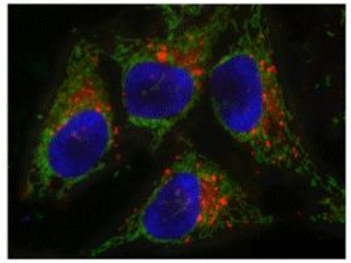


- Compression artifacts
- Inset not indicated
- Not color-blind safe
- Lack of scale information
- Lack of color annotation

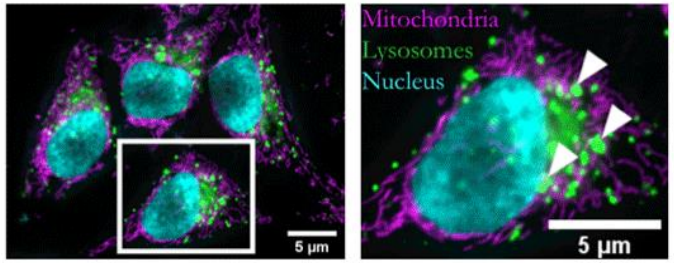


- Image quality good
- Inset origin clear
- Colors accessible
- Scale bar
- Color annotation

Poorly visualized image



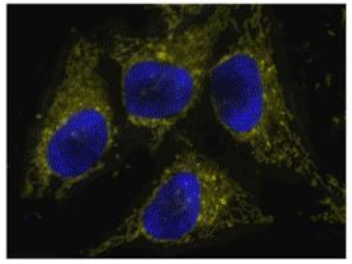
Workflow-based image processing for publication



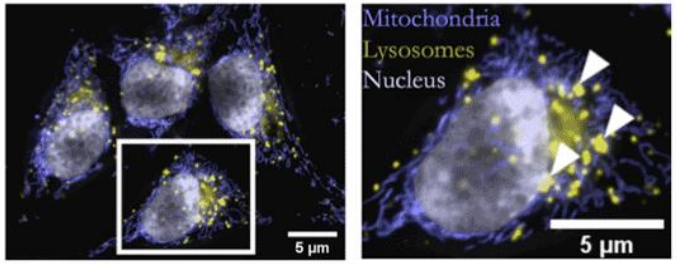
HeLa cells

Test of Image quality:
Color blind (Deuteranopia)
rendering of images

- not color blind safe
- no annotation
- no scale



- colors accessible to color blind
- annotation, annotations color blind safe
- scale bar for image and inset



HeLa cells

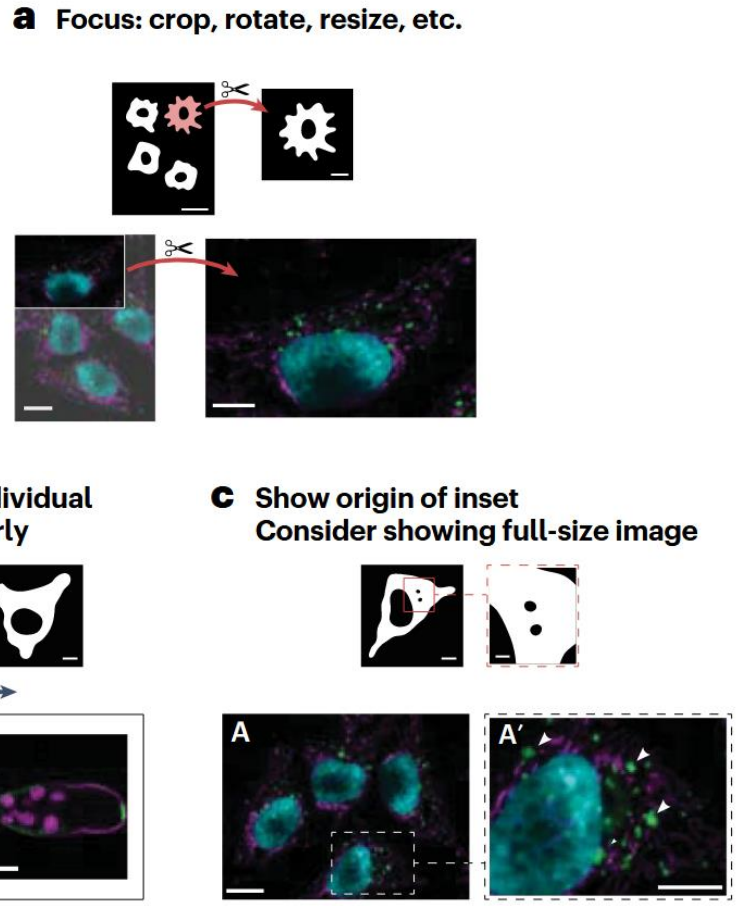
IV/ Image ethics: Good practices

Schmied, Christopher, Michael S. Nelson, Sergiy Avilov, Gert-Jan Bakker, Cristina Bertocchi, Johanna Bischof, Ulrike Boehm, et al. “Community-Developed Checklists for Publishing Images and Image Analyses.” *Nature Methods*, September 14, 2023, 1–12. https://doi.org/10.1038/s41592-023-01987-9.

Checklist for image publishing

Image format

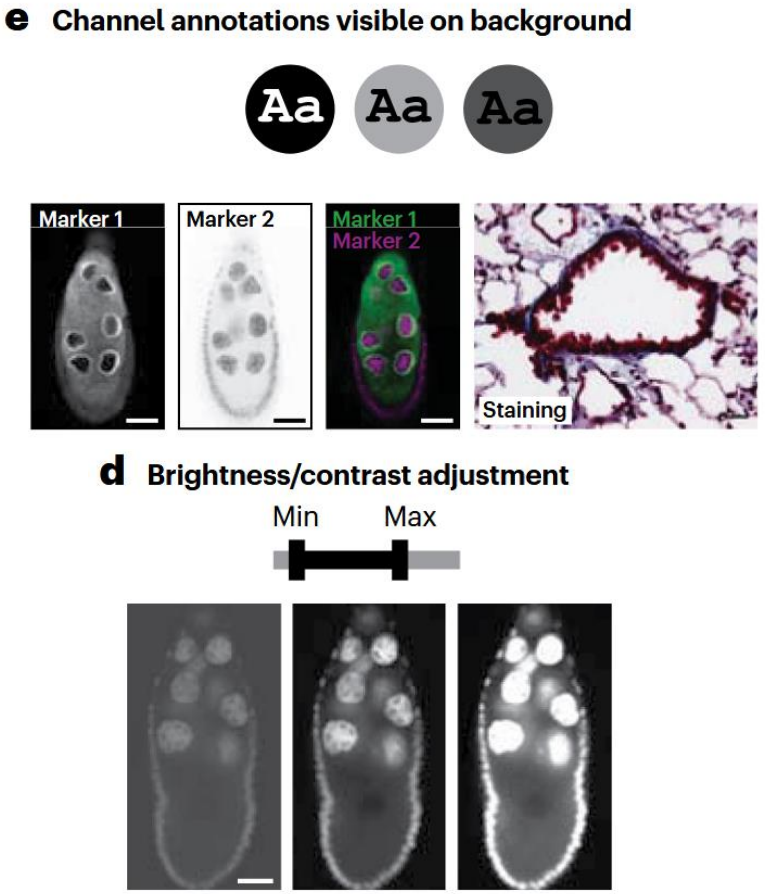
Focus on relevant image content (e.g., crop, rotate, resize)	☐ Minimal
Separate individual images	☐
Show example image used for quantifications	☐
Indicate position of zoom view/inset in full-view/original image	☐
Show images of the range of the described phenotype	☐



IV/ Image ethics: Good practices

Image colors and channels

Annotation of channels (staining, marker, etc.) visible	Minimal
Adjust brightness/contrast, report adjustments, use uniform color scales	
Image comparison: use the same adjustments	
Channel colors: high visibility on the background Best visibility: grayscale	
Multicolors: provide grayscale for each color channel	
Multicolor: if channels are merged, make accessible to color-blind individuals	
Provide intensity scales (calibration bar) for grayscale, color, pseudocolor etc.	Recommended
Pseudocolored images: additionally provide grayscale version for comparison	Ideal
Gamma adjustments: additionally provide linear-adjusted image for comparison	



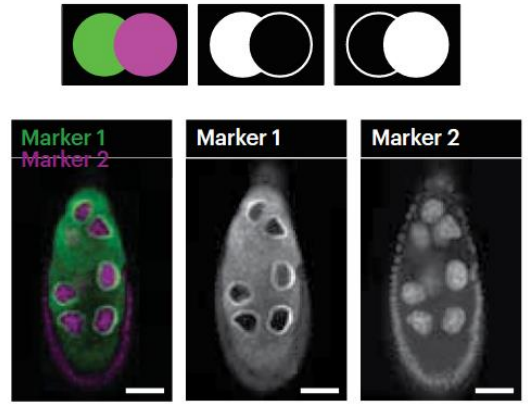
"This is also, why it is important to tell young researchers, that using the same method on all images does not mean that the same things happen on all of them and that they will stay comparable."

IV/ Image ethics: Good practices

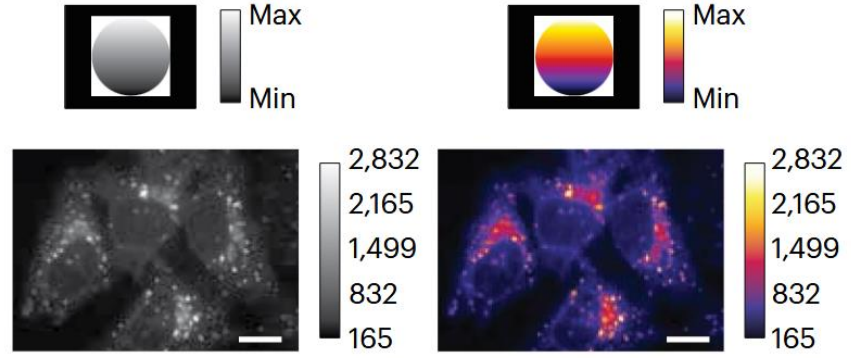
Image colors and channels

Aa Aa	Annotation of channels (staining, marker, etc.) visible	Minimal
Min Max	Adjust brightness/contrast, report adjustments, use uniform color scales	
	Image comparison: use the same adjustments	
	Channel colors: high visibility on the background Best visibility: grayscale	
	Multicolors: provide grayscale for each color channel	
	Multicolor: if channels are merged, make accessible to color-blind individuals	
Max Min	Provide intensity scales (calibration bar) for grayscale, color, pseudocolor etc.	Recommended
	Pseudocolored images: additionally provide grayscale version for comparison	Ideal
	Gamma adjustments: additionally provide linear-adjusted image for comparison	

f Grayscale channels improve visibility of image features



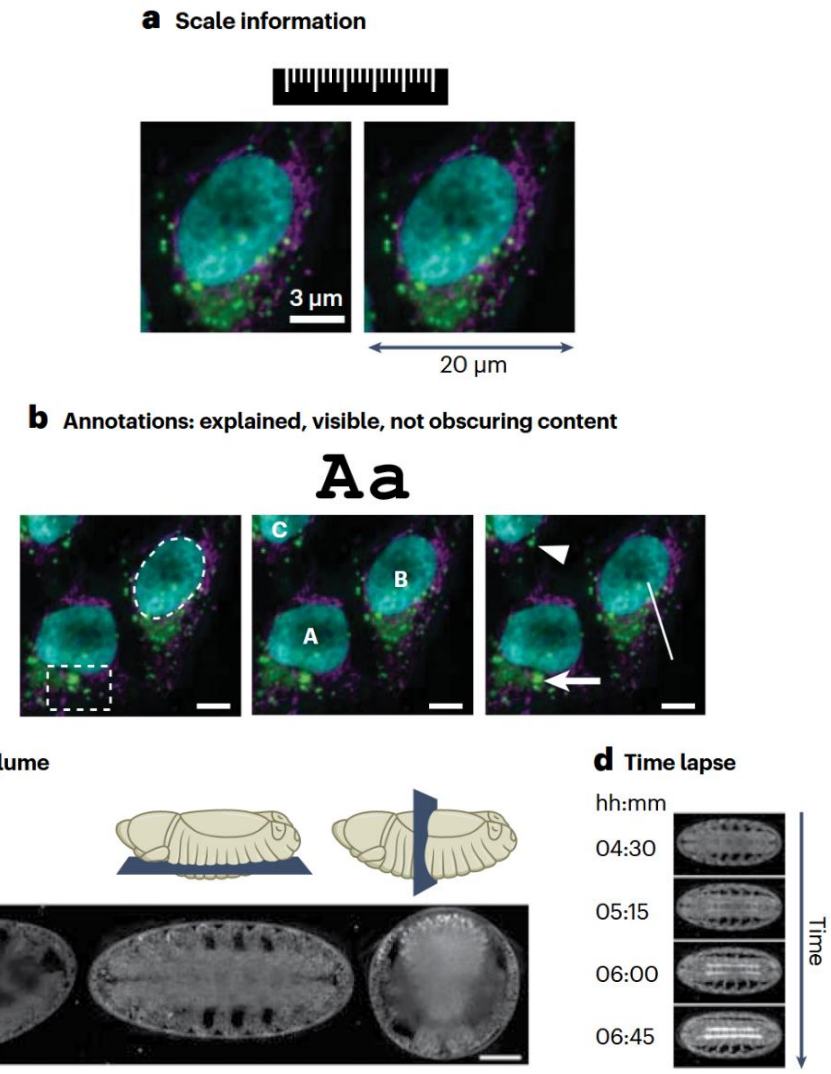
g Explain channel values with calibration scales/indicators



IV/ Image ethics: Good practices

Image annotation

Add scale information (scale bar, image length in figure/figure legend)	☐	Minimal
Explain all annotations (in figure/figure legend)	☐	
Annotations should be legible (line width, size/point size, color)	☐	
Annotations should not obscure key data	☐	
Annotate imaging details important for interpreting the figure (depending on the main message and imaging technique, this may be, e.g., image pixel size, imaging intervals (time-lapse in movies), exposure time or anatomical section)	☐	Recommended



IV/ Image ethics: Good practices