Introduction to Bioimage Analysis

Micron Advanced Microscopy Course

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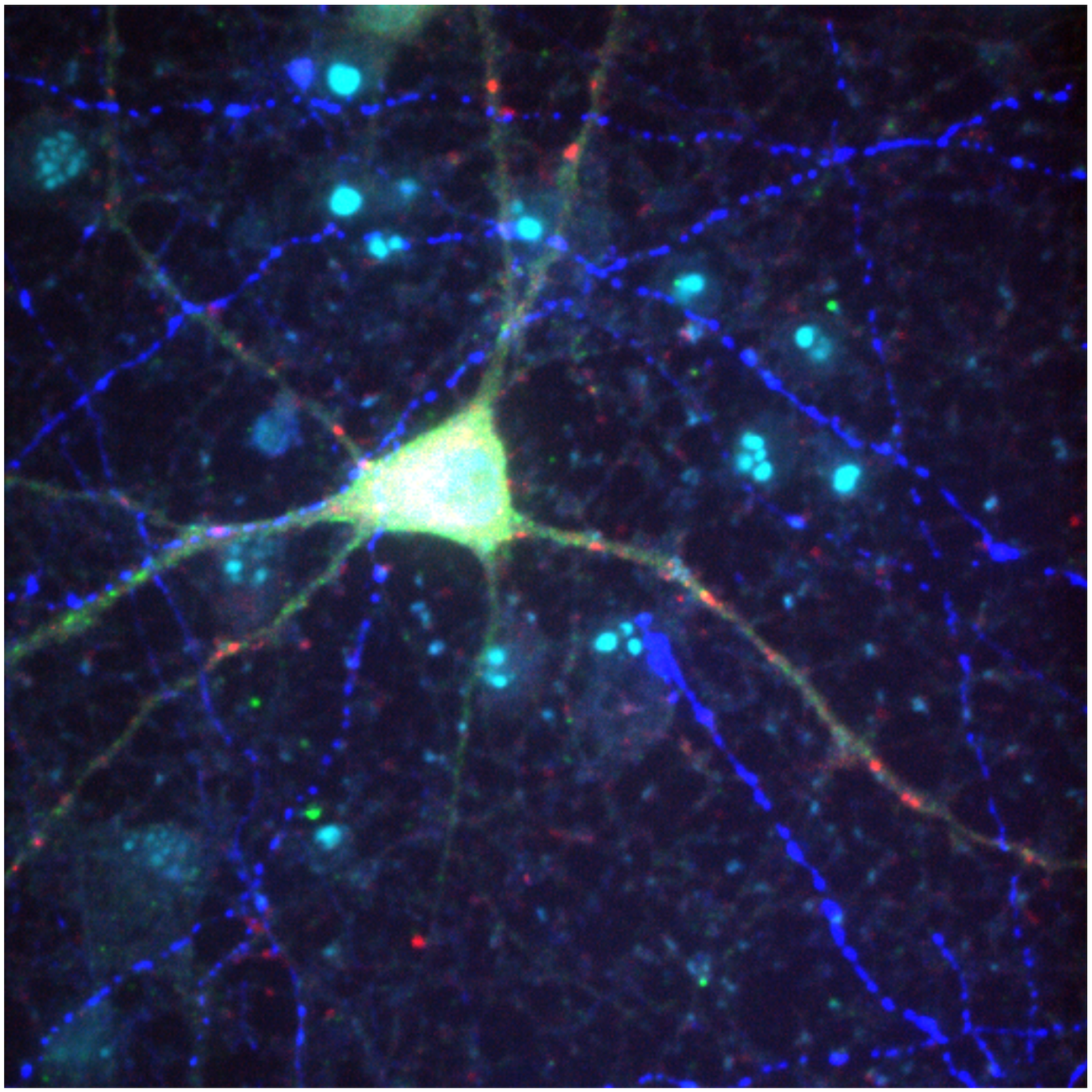
What is Image Analysis?



- precise
- unbiased
- reproducible

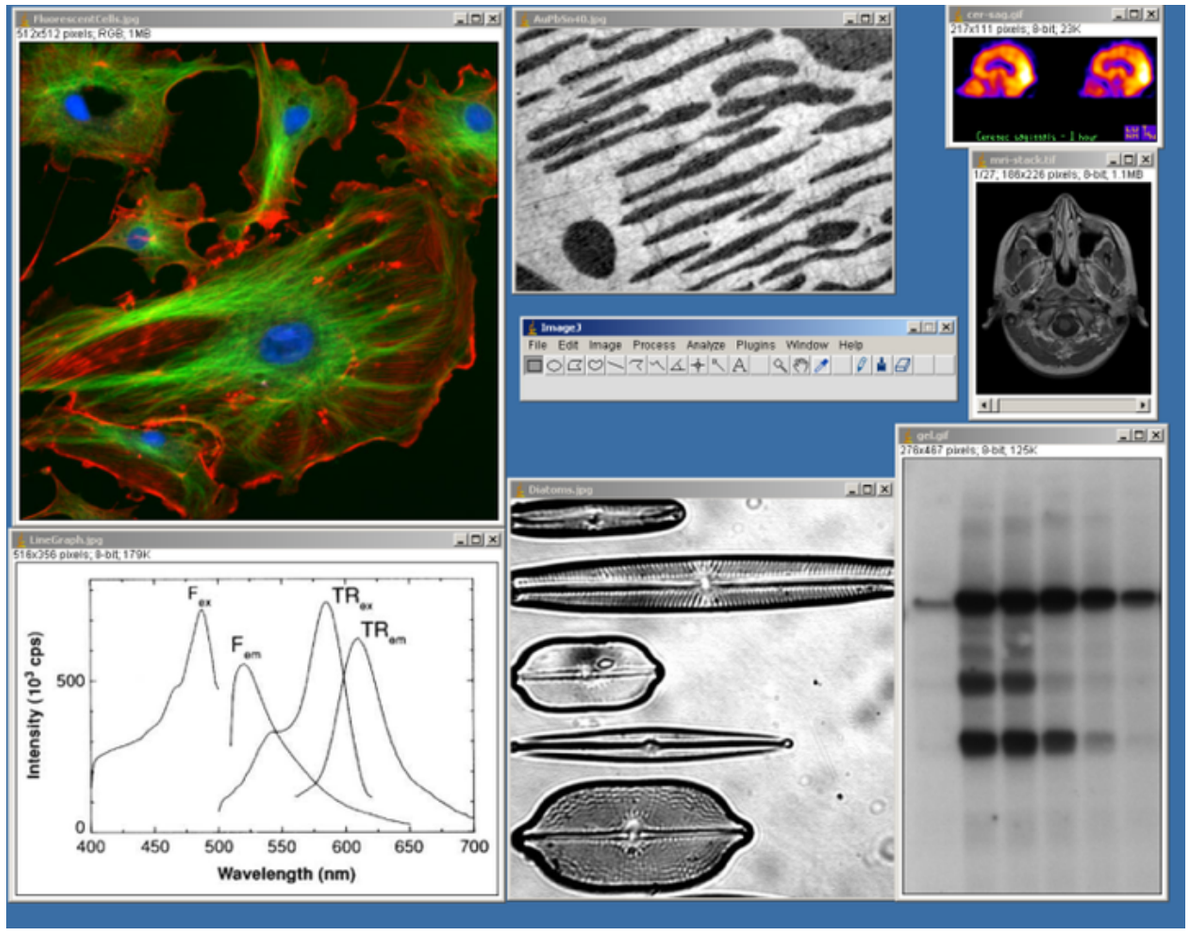
Available Image processing and Analysis Software

Zen LE software
Image-Pro
Drishti
Volocity
ImageJ
Oko-Vision
CellProfiler
Clemex Vision PE
Fiji
IN Cell Investigator
Nikon Z-C1
Omero
Image Surfer
MetaMorph
Imaris
Matlab
ImageTool
Photoshop
Mathematica
MCID
ImageTrak
Image Metrology
MIATool
Icy
SimpleWare
ParaView
Reconstruct
SoftWoRx
FLIMfit
Leica LAS
Sinema
AutoQuant
MeVisLab
VIAS
Reconstruct
Voxx2
BioImage XD

medicine
microscopy
remote sensing
astronomy
materials science
machine vision
security
robotics
geology
optical character recognition
assay micro-plate reading
metallography
defence
filtering

Source: http://en.wikipedia.org/wiki/Image_analysis, http://www.hsr.it/research/organization/services-open-labs/alembic-advanced-light-and-electron-microscopy-bioimaging-center/9/#fs35

General analysis software: Fiji/ImageJ



- Originally created by Wayne Rasband at the NIH in 1997 as ImageJ.
- Free and easy to get running on all systems.

Source: Source: http://fiji.sc/Fiji

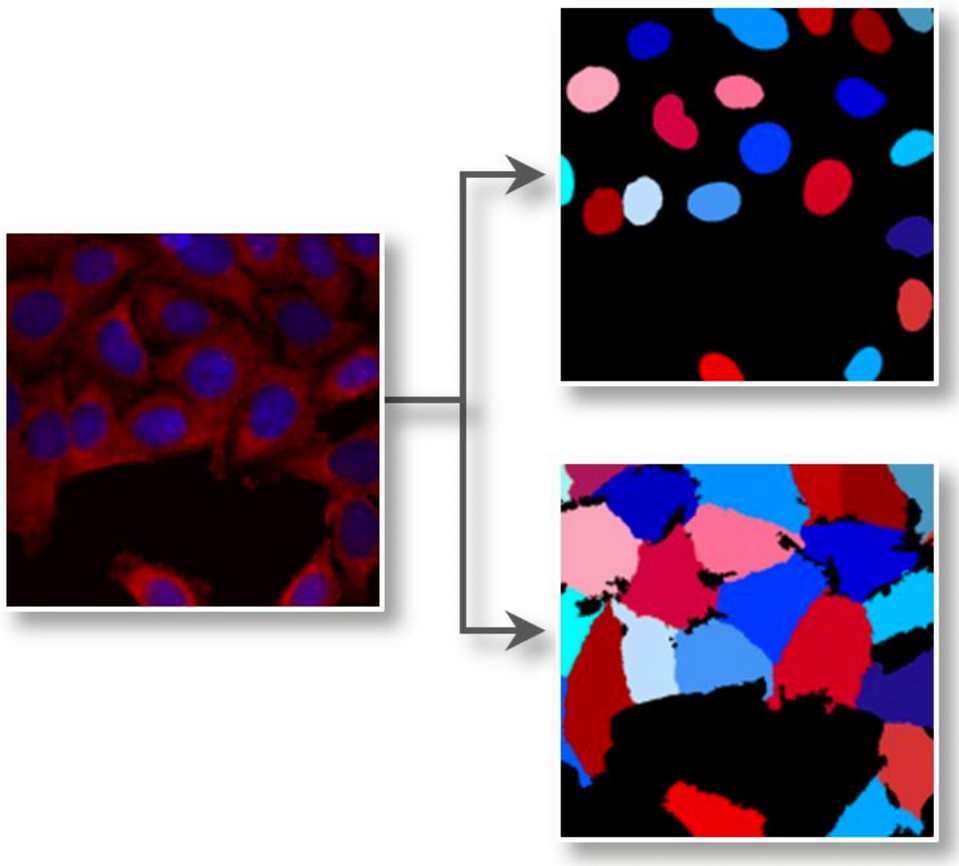
Installation of FIJI

Get it from: https://imagej.net/Fiji/Downloads

- Doesn't need installation - just unzip/uncompress and run.
  - Don't save it in "Program Files"!
- Updated regularly
  - Use lifeline versions if needed to avoid updating issues

Date	Downloads						Description
	64-bit	32-bit	macOS	64-bit	32-bit	no-JRE	
2017 May 30							The final version of Fiji using Java 6, for all platforms.
2015 December 22							Just prior to starting the transition to Java 8.
2014 November 25							Just prior to a big update to facilitate reproducible builds.
2014 June 02							Just prior to some big changes to ImageJ2 under the hood.
2013 July 15							Just prior to extensive changes reconciling Fiji with ImageJ2.

General analysis software: Cell Profiler

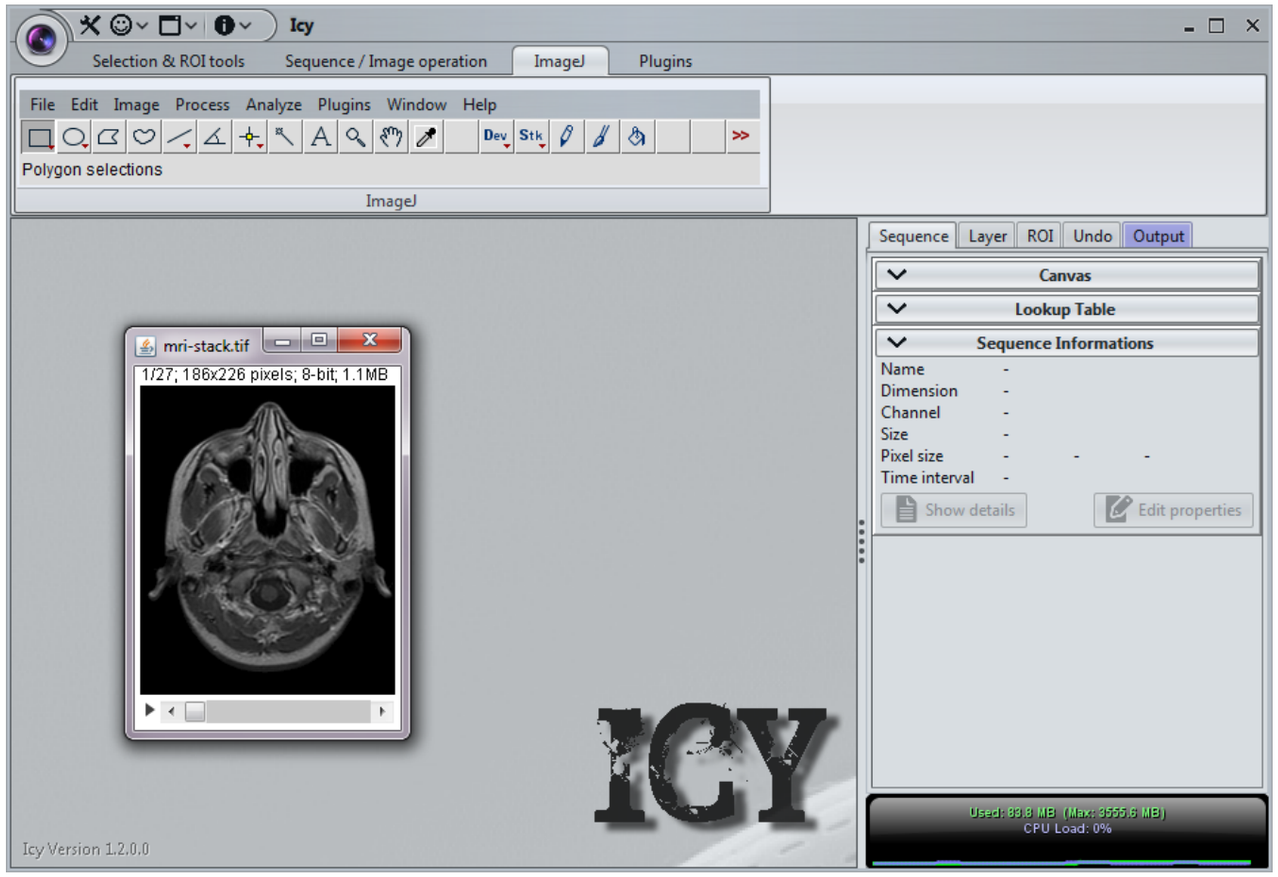


CellProfiler
cell image analysis software

- CellProfiler is free open-source software designed to enable biologists without training in computer vision or programming to quantitatively measure phenotypes from thousands of images automatically.

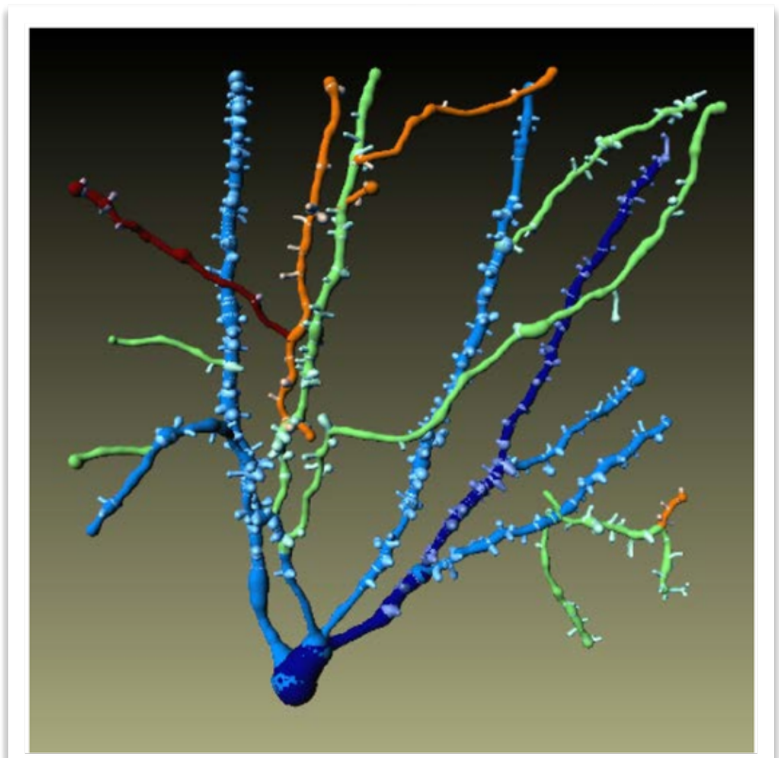
Source: http://www.cellprofiler.org/ started by Anne E. Carpenter and Thouis Jones

General analysis software: Icy



- Icy has been created by the Quantitative Image Analysis Unit at Institut Pasteur. Free and easy to get running on all systems.

Source: http://www.bioimageanalysis.org/



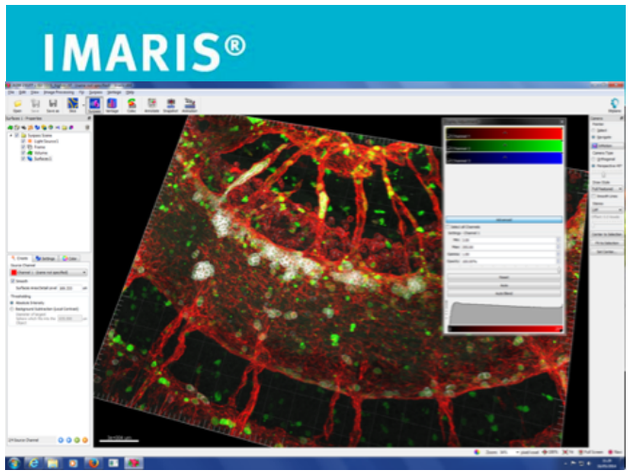
3D
datasets

Source: Imaris Filament tracer

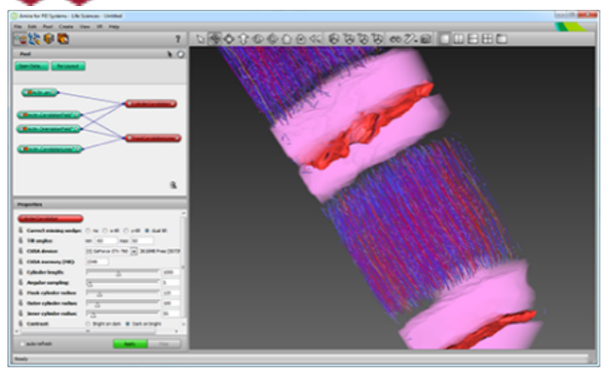
Waithe 2019

3D Software

VAA3D 挖三维



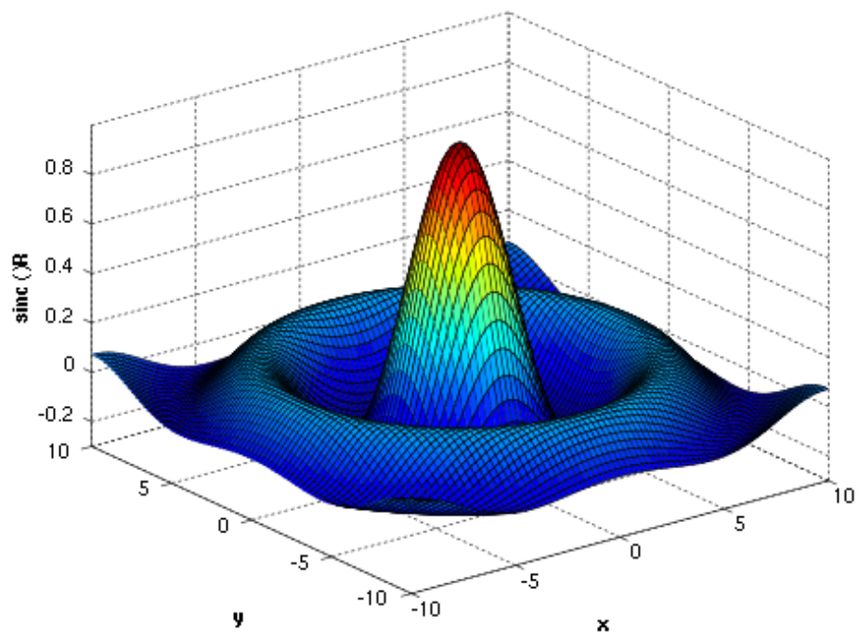
FEI™ AMIRA



- Volume Visualisation
- Automatic and manual identification of objects in 3D
- Tracking and Colocalisation in 3D
- generally expensive

Source: http://www.fei.com/software/amira-3d-for-life-sciences/ http://www.bitplane.com/imaris/imaris, www.vaa3d.org/

Matlab and Python



Matlab is popular tool for technical computing. Integrated programming environment. Images are imported as arrays of numbers.

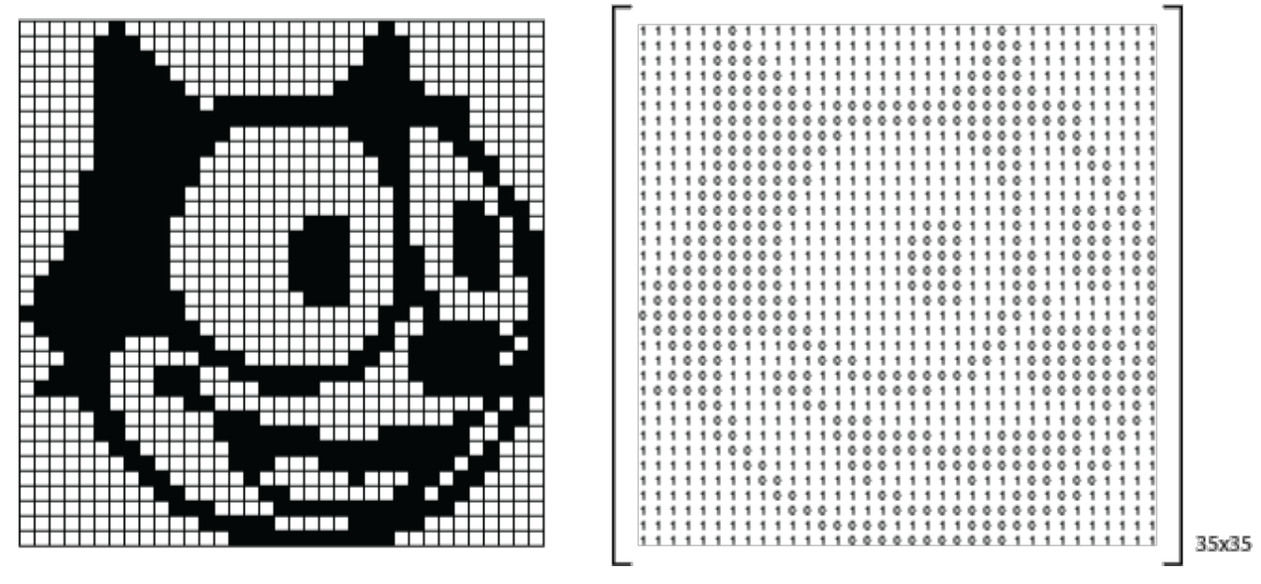
Python is free and very versatile scripting language growing in popularity.

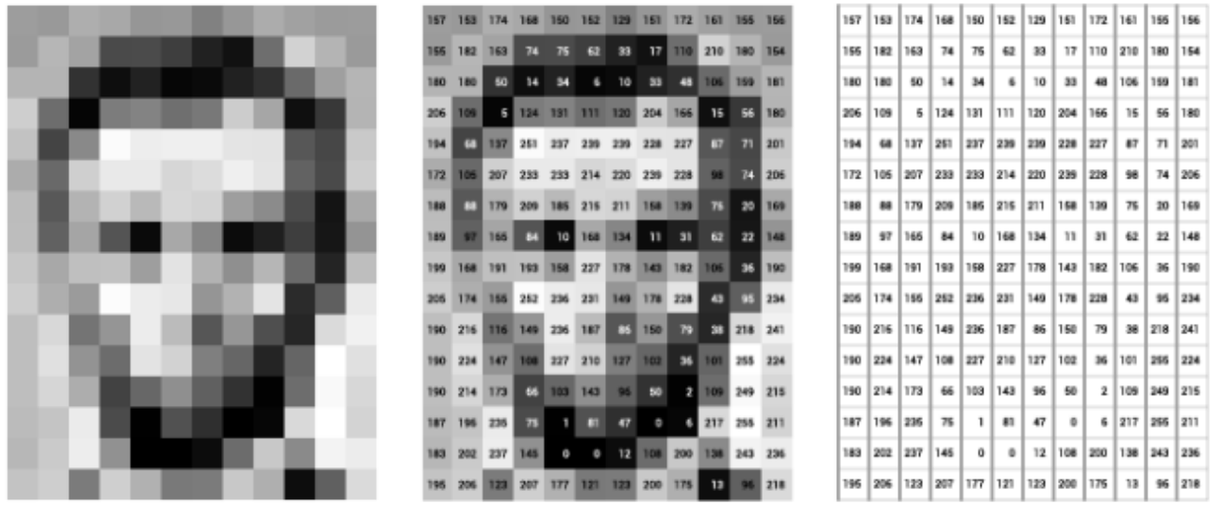
Matlab and python has many tools used for segmentation and analysis of data.

Both include visualisation tools for end-2-end analysis.

Source: en.wikipedia.org/wiki/MATLAB

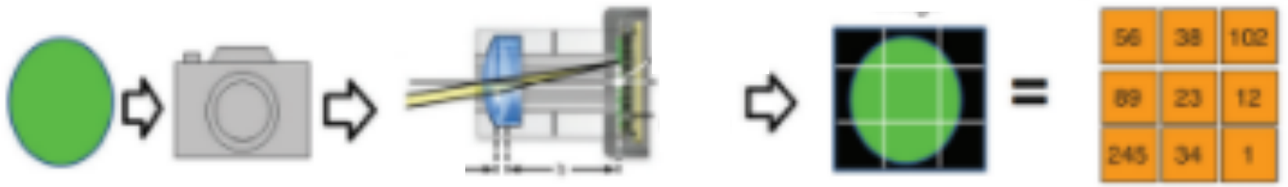
An Image is a matrix of numbers

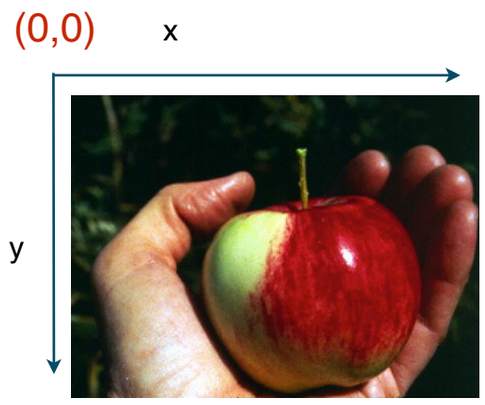




Source: http://blog.kleinproject.org/, https://towardsdatascience.com/understanding-images-with-skimage-python-b94d210afd23

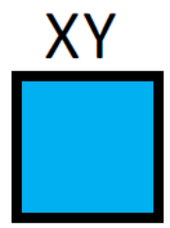
Pixel size





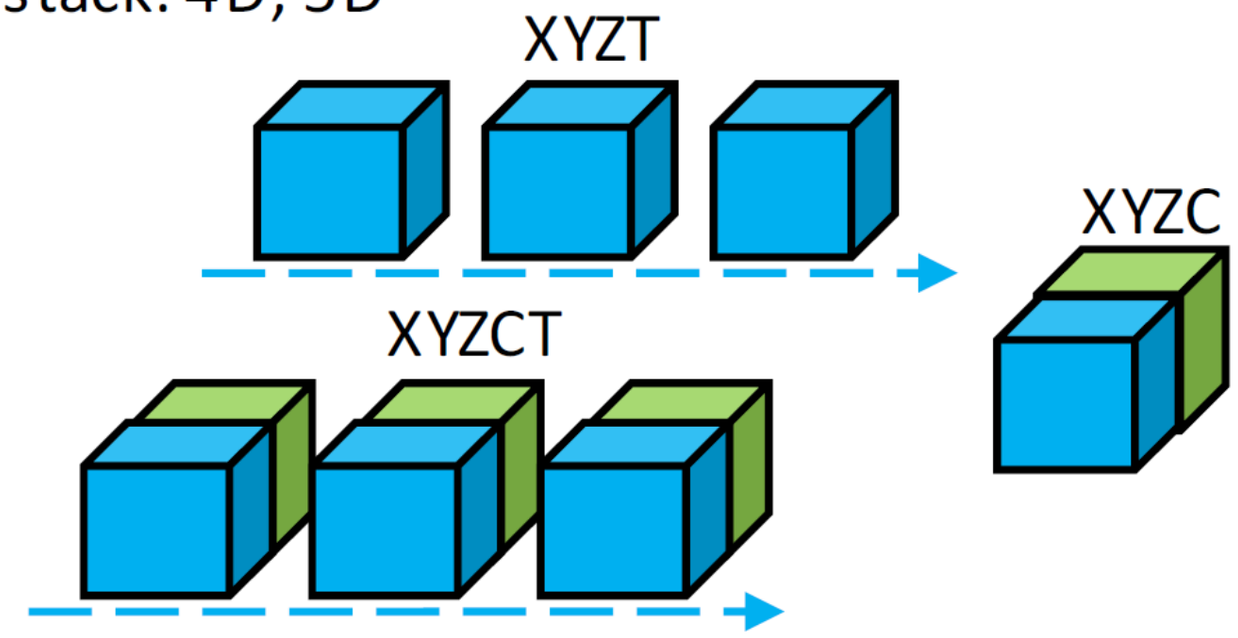
Multiple Dimensions

- Image: 2D



- Stack: 3D

- Hyperstack: 4D, 5D

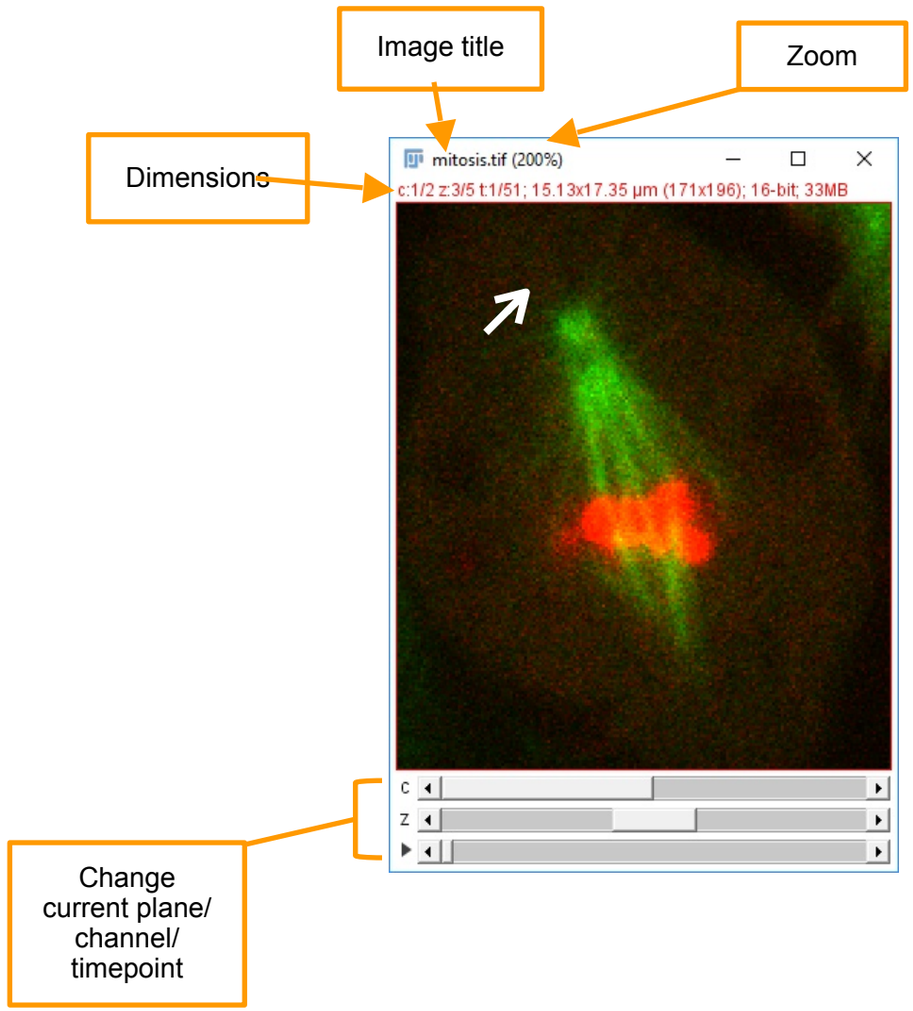


neubias
network of european bioimage analysts

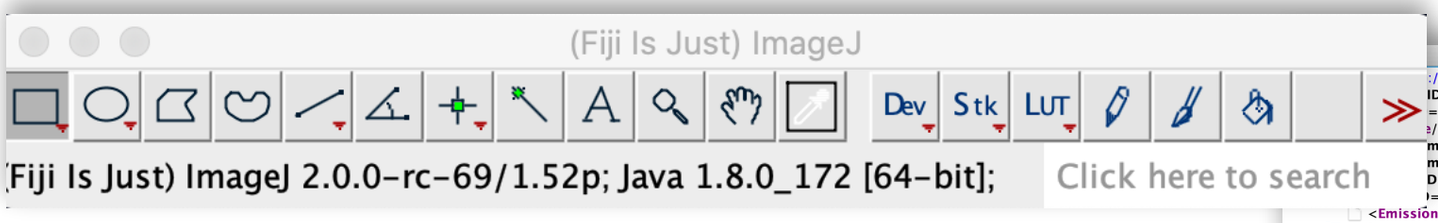
Opening an Image with FIJI

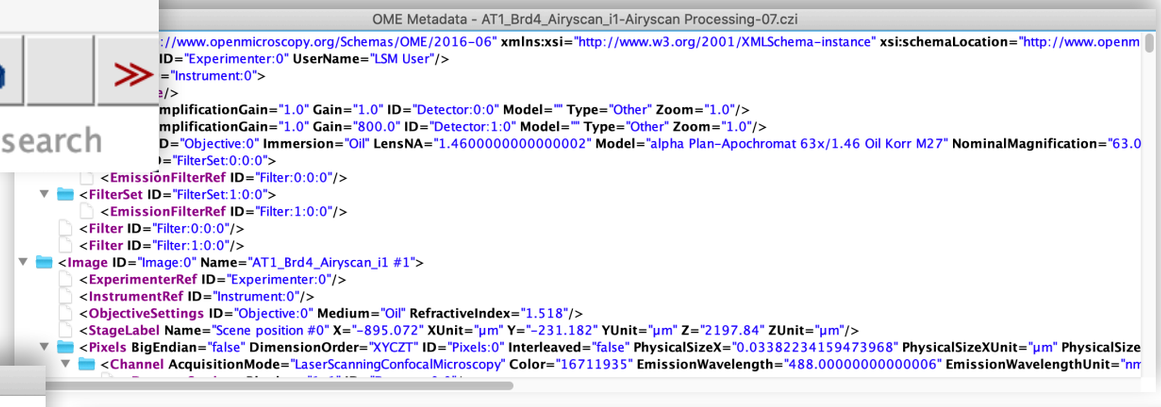
How to interpret an image in ImageJ

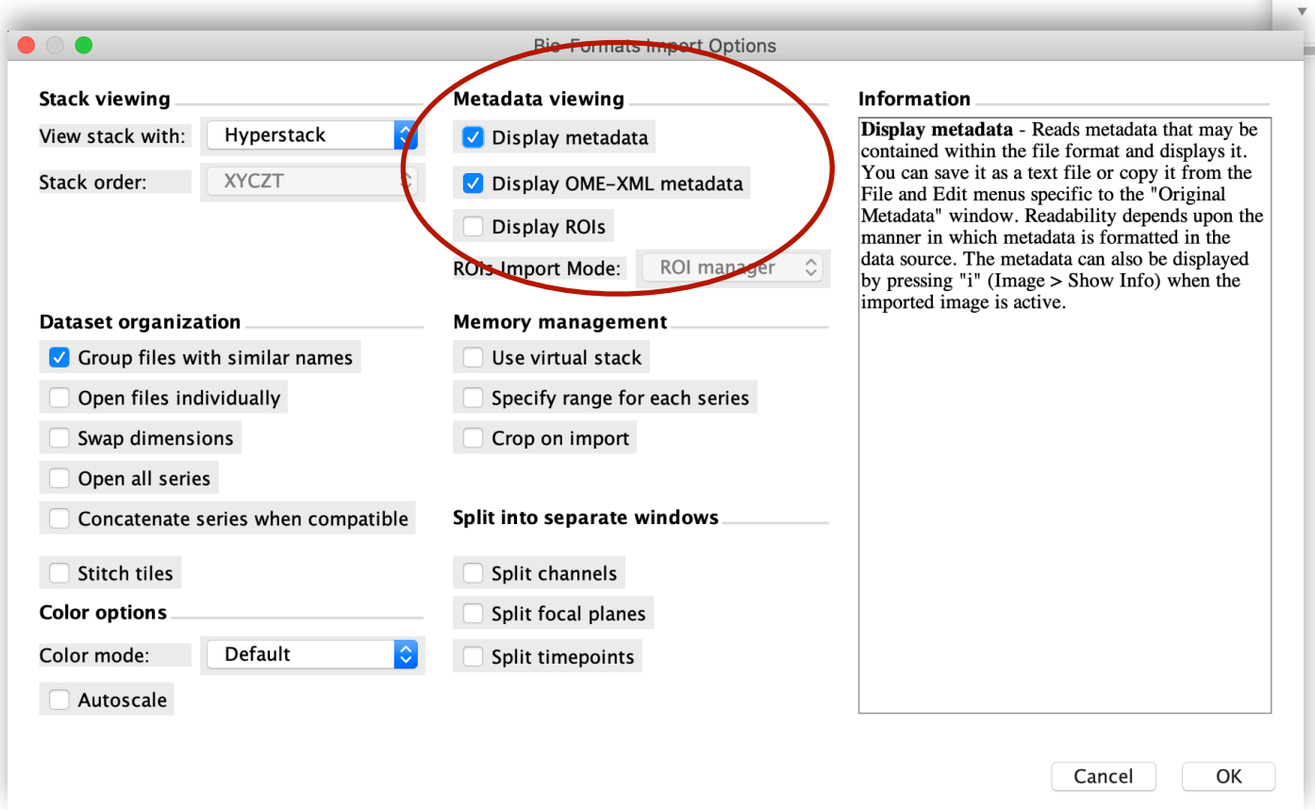
- Image Title
  - Includes extension of the file
- Image zoom
- Dimensions and other info
  - Number of channels (c); number of Z planes (z); number of Timepoints (t); size (scaled);
  - size (pixels, x & y); Bit depth; image size
- Dimension sliders

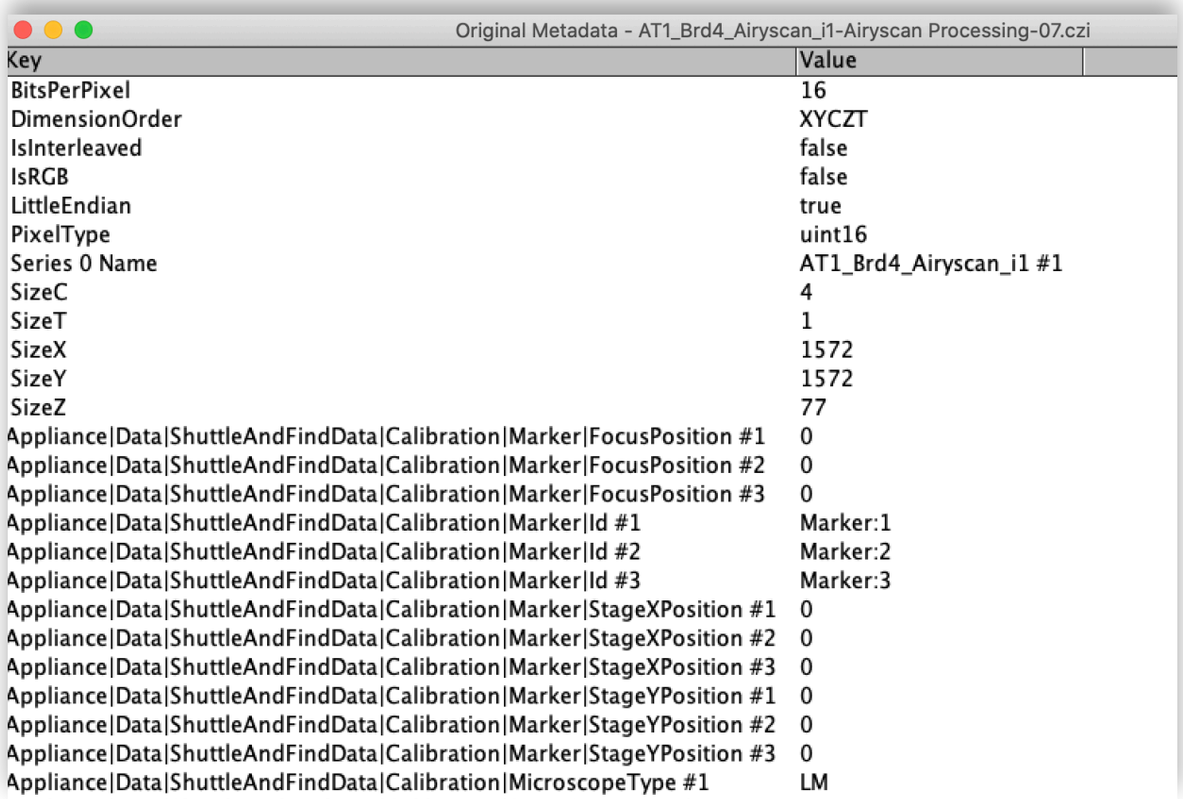


Accessing metadata with FIJI









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IsRGB	false
LittleEndian	true
PixelType	uint16
Series 0 Name	AT1_Brd4_Airyscan_i1 #1
SizeC	4
SizeT	1
SizeX	1572
SizeY	1572
SizeZ	77
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #1	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #2	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #1	Marker:1
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #2	Marker:2
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #3	Marker:3
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageXPosition #1	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageXPosition #2	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageXPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #1	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #2	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|MicroscopeType #1	LM

Metadata can be found when you import images to Fiji.

Metadata can be found through the image-> show info option also.

Image File Formats

Lossless compression:

TIFF,
Raw

Lossy - compression:

JPEG, JPEG 2000,
TIFF, BMP, PNG,
Movie formats

Proprietory microscopy files:

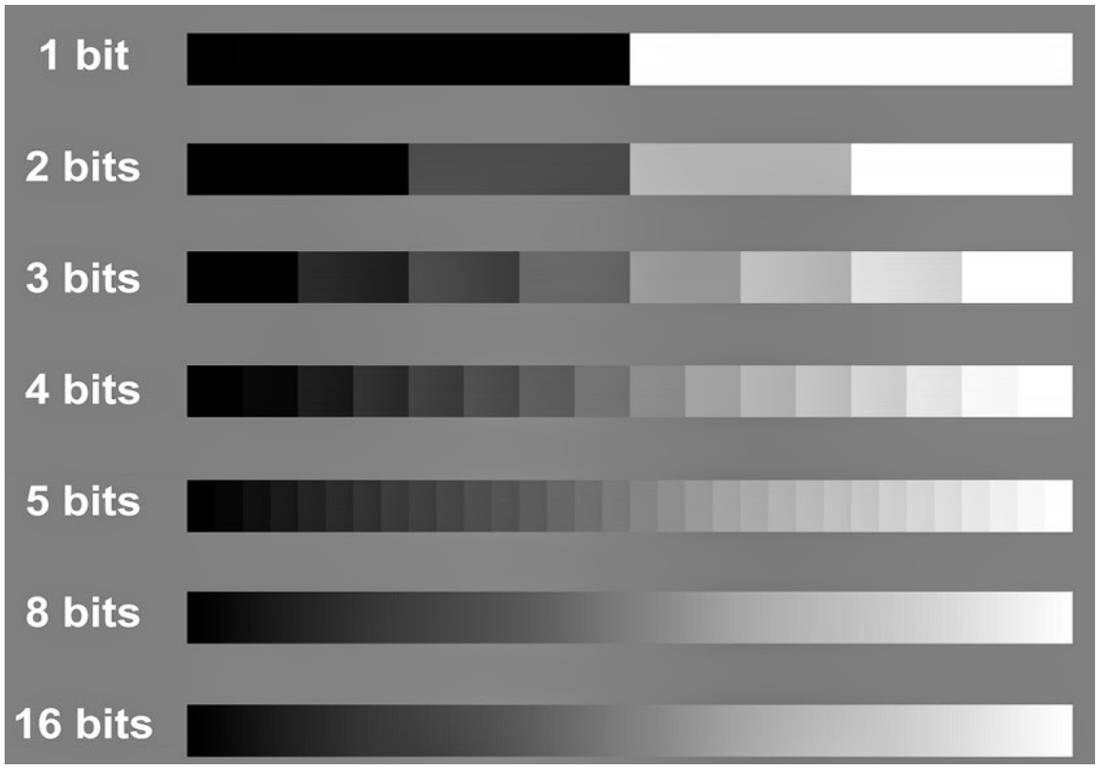
Format	Extensions
3i SlideBook	.sld
Andor Bio-Imaging Division (ABD) TIFF	.tif
AIM	.aim
Alicona 3D	.al3d
Amersham Biosciences Gel	.gel
Amira Mesh	.am, .amiramesh, .grey, .hx, .labels
Amnis FlowSight	.cif
Analyze 7.5	.img, .hdr
Andor SIF	.sif
Animated PNG	.png
Aperio AFI	.afi, .svs
Aperio SVS TIFF	.svs
Applied Precision CellWorX	.htd, .pnl
AVI (Audio Video Interleave)	.avi
Axon Raw Format	.arf
BD Pathway	.exp, .tif
Becker & Hickl SPC FIFO	.spc
Becker & Hickl SPCImage	.sdt
Bio-Rad Gel	.1sc
Bio-Rad PIC	.pic, .raw, .xml
Bio-Rad SCN	.scn
Bitplane Imaris	.ims
Bruker MRI	
Burleigh	.img
Canon DNG	.cr2, .crw
CellH5	.ch5
Cellomics	.c01, .dib
cellSens VSI	.vsi
CellVoyager	.xml, .tif
DeltaVision	.dv, .r3d
DICOM	.dcm, .dicom
ECAT7	.v

Source: https://docs.openmicroscopy.org/bio-formats/5.8.2/supported-formats.html

Grayscale

8-bit, 16-bit, 32 bit

Attention when converting between different bit-depth!



2 levels

4 levels

8 levels

16 levels

32 levels

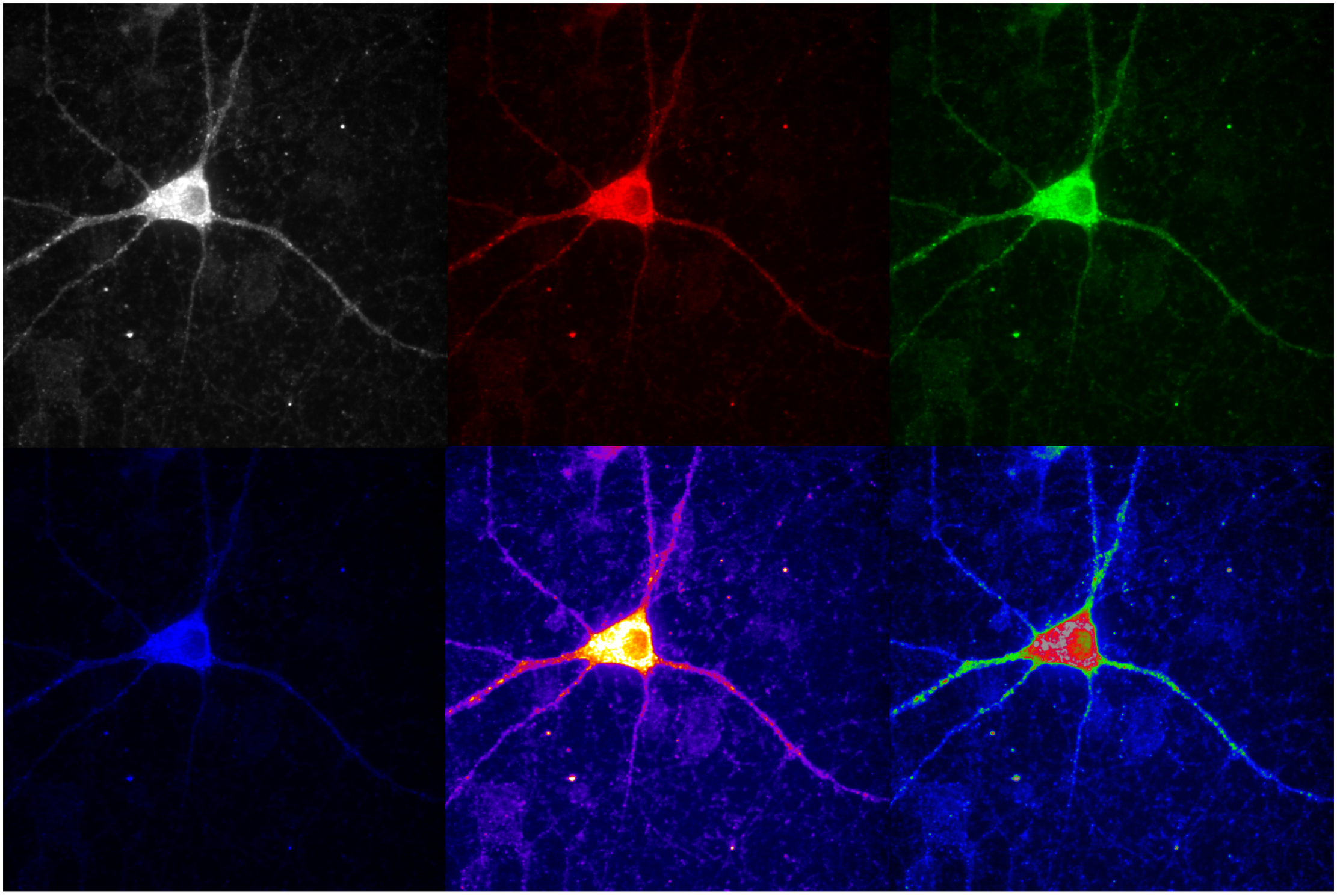
256 levels

65536 levels

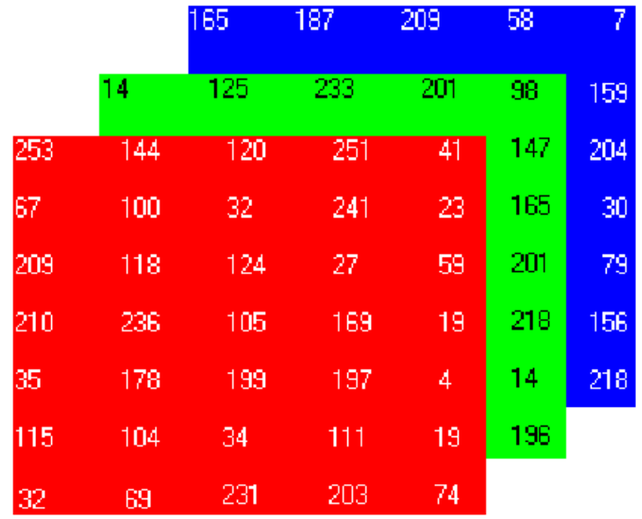
32-bit images:

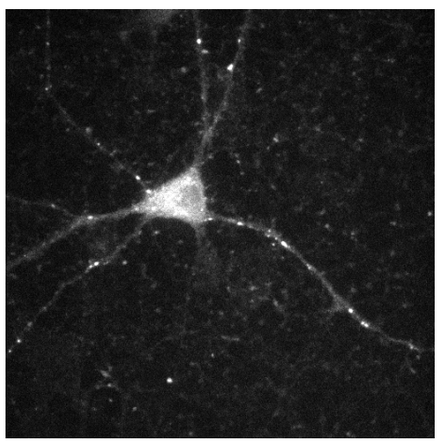
- Can be positive/negative
- represent floating point numbers
- Useful for image maths

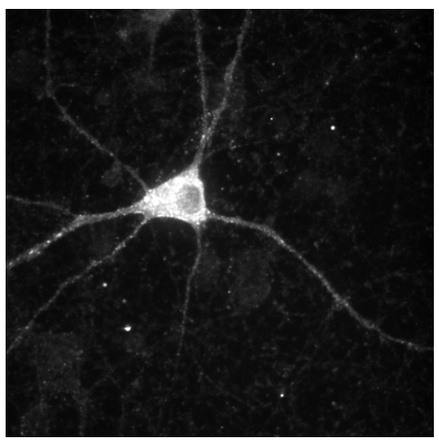
Look up tables

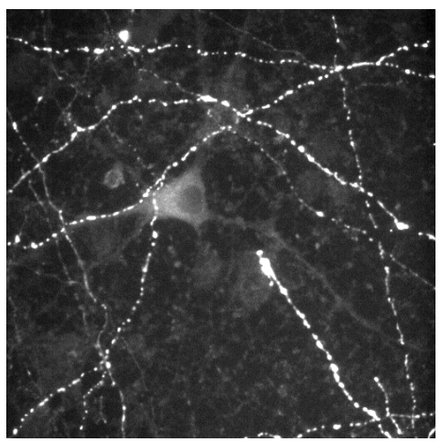


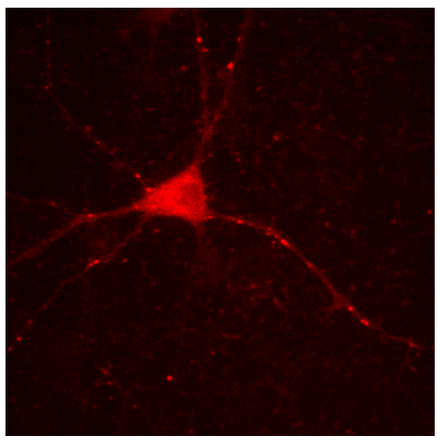
RGB Images

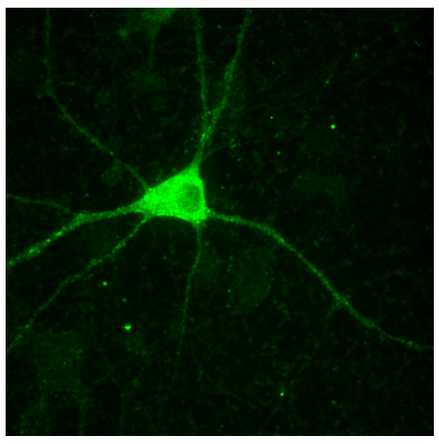

165 187 209 58 7
14 125 233 201 98 159
253 144 120 251 41 147 204
67 100 32 241 23 165 30
209 118 124 27 59 201 79
210 236 105 169 19 218 156
35 178 199 197 4 14 218
115 104 34 111 19 196
32 69 231 203 74

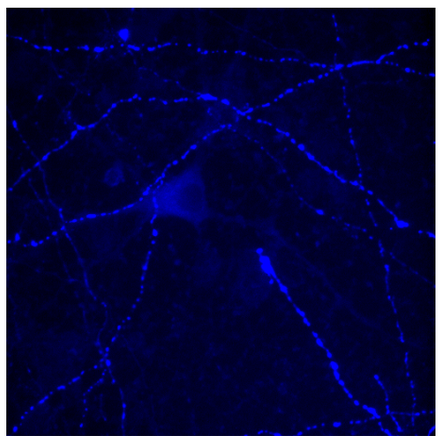


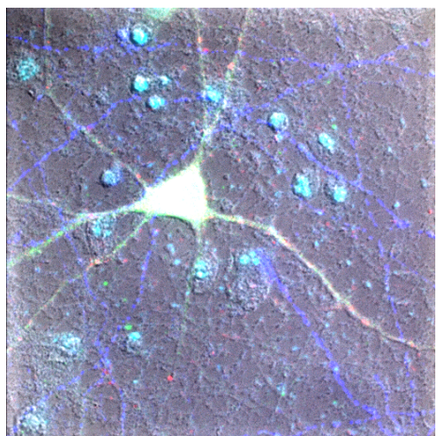




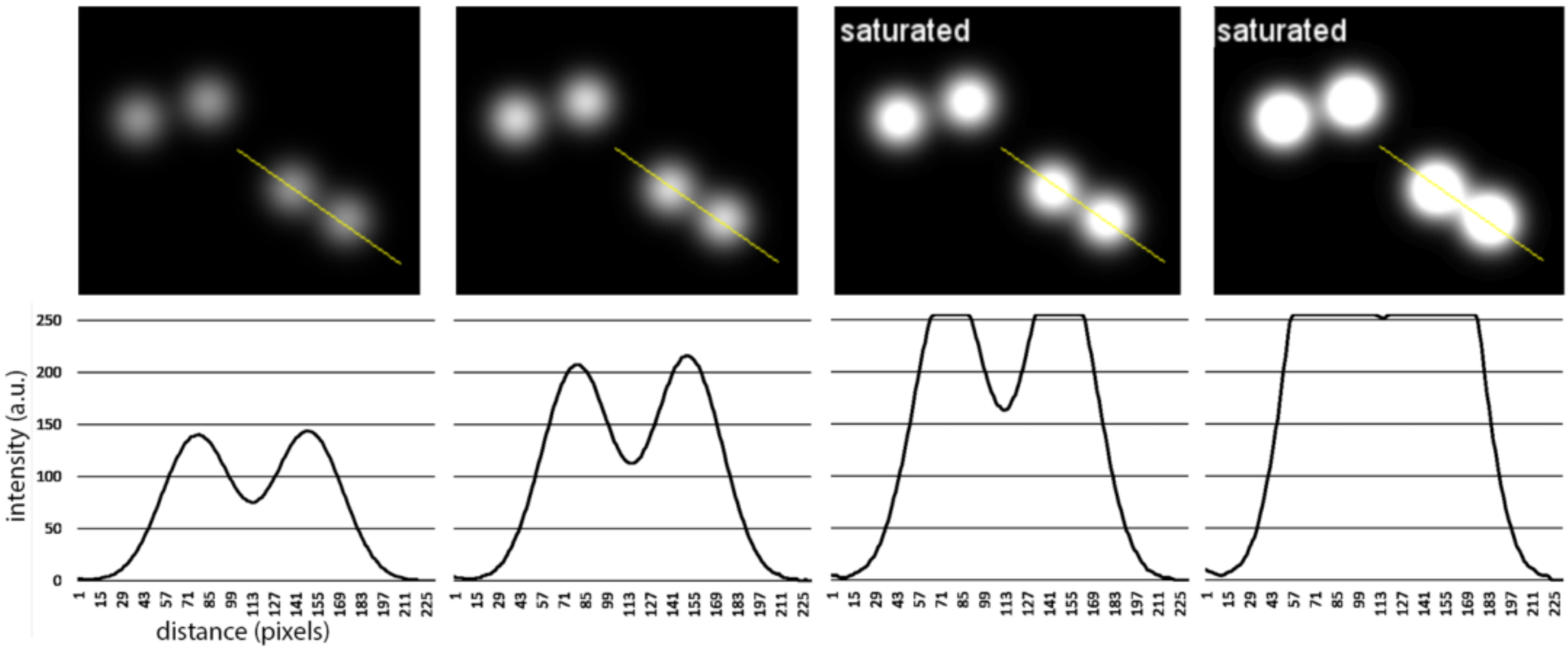






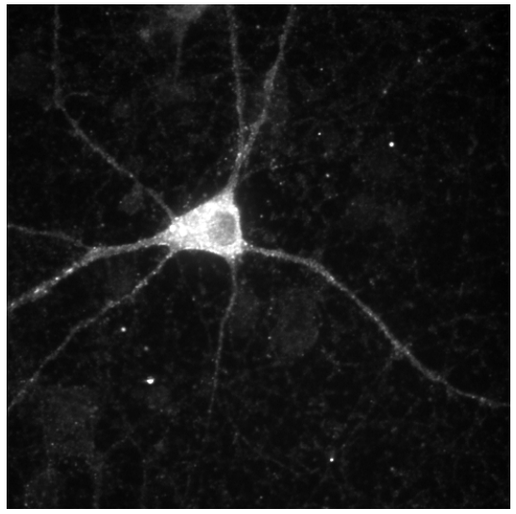


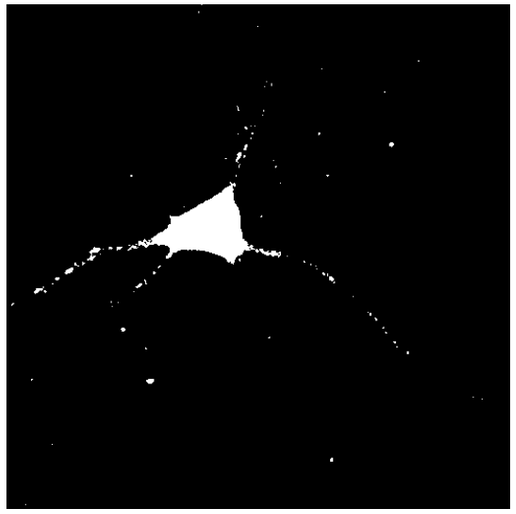
Dead Pixels and Saturated Pixels

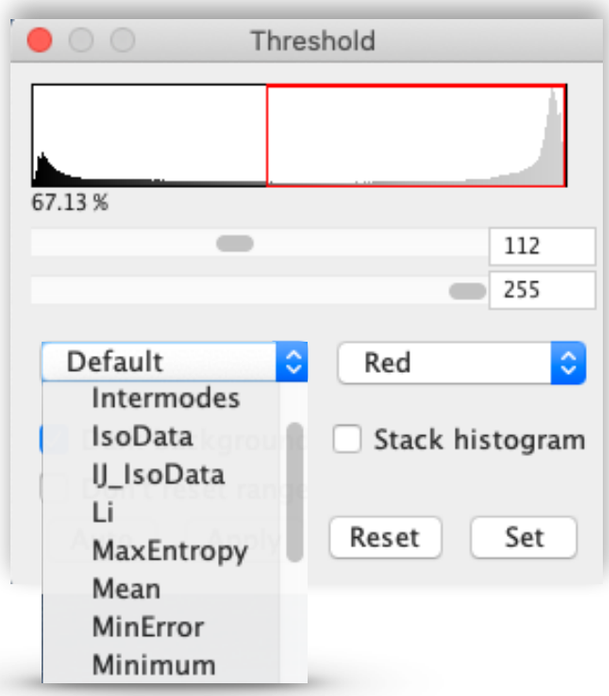


Source: http://microscopynotes.com/imagej/saturation/index.html

Segmentation - thresholding







Source: https://en.wikipedia.org/wiki/Thresholding_(image_processing)

Make Binary
Convert to Mask

Erode
Dilate
Open
Close-

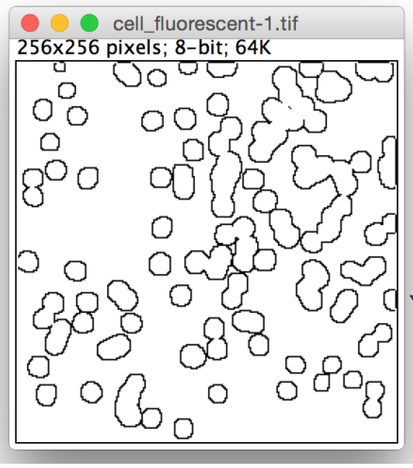
Outline
Fill Holes
Skeletonize

Distance Map
Ultimate Points
Watershed
Voronoi

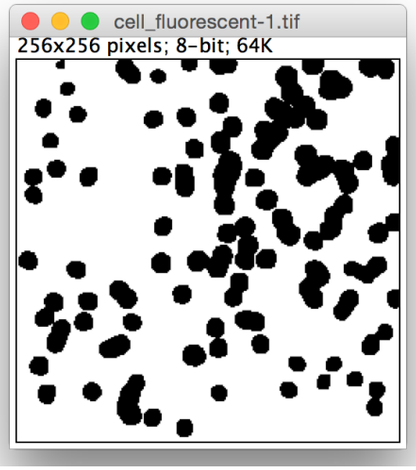
Options...

Morphological operators

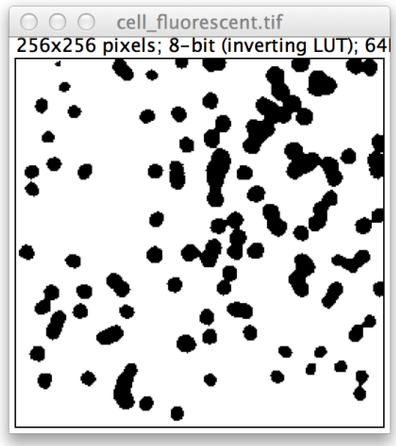
Discrete morphological operators

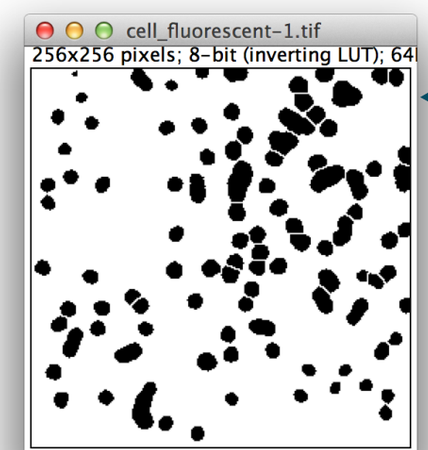


Outline: Find outline

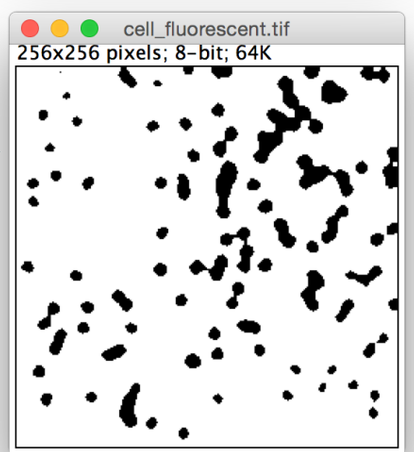


Dilation: Enlarge blobs



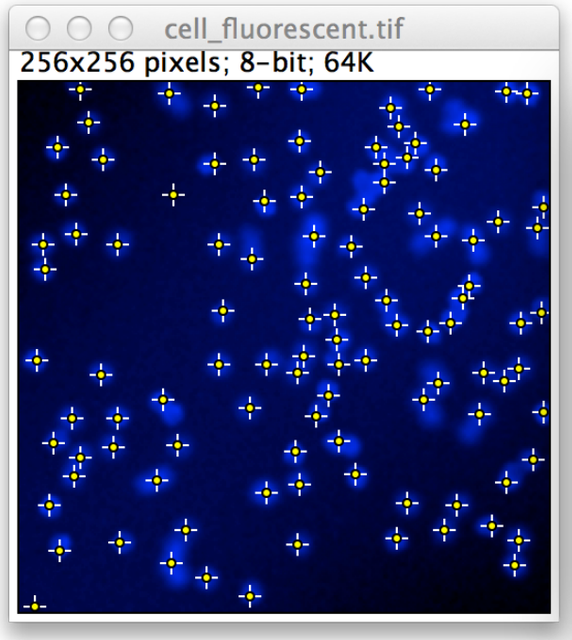


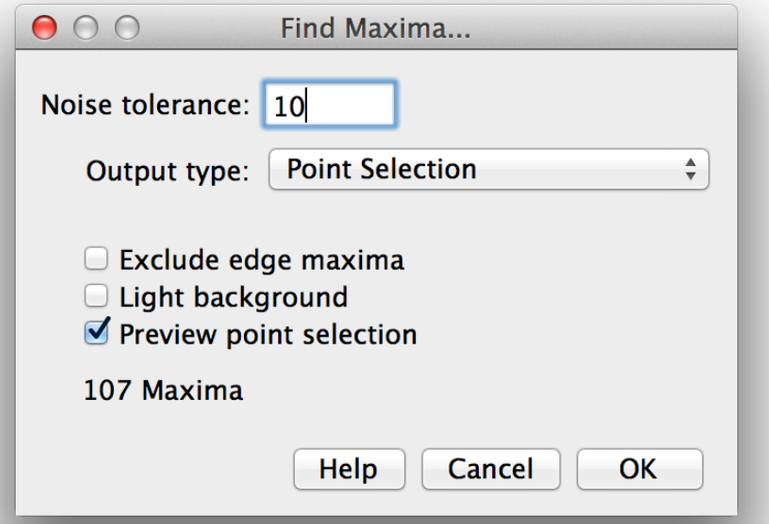
Watershed: Splits blobs

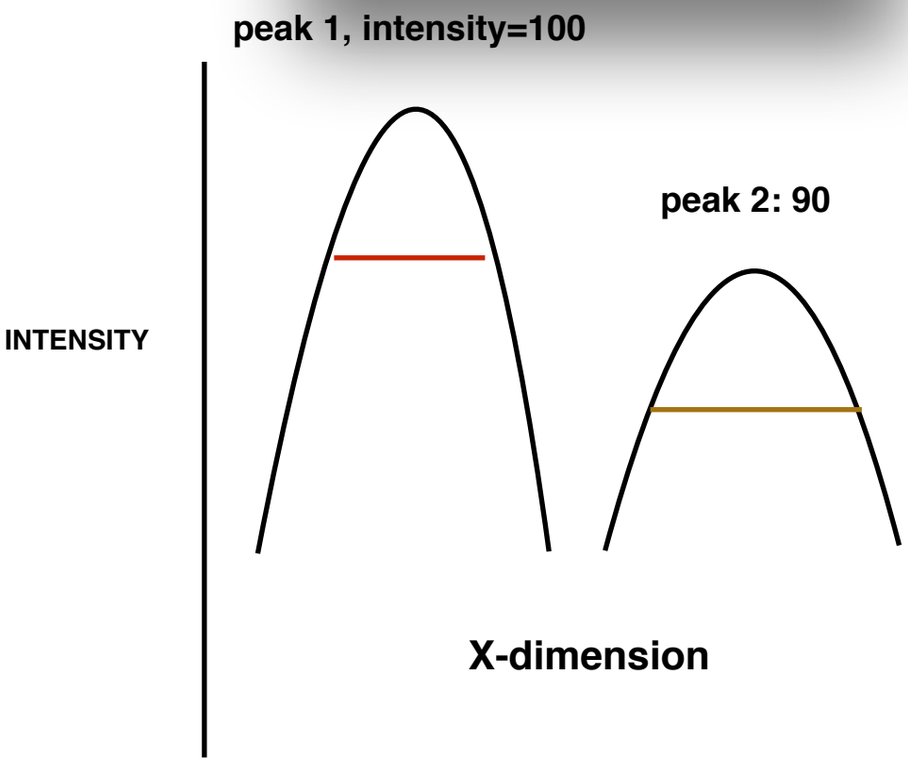


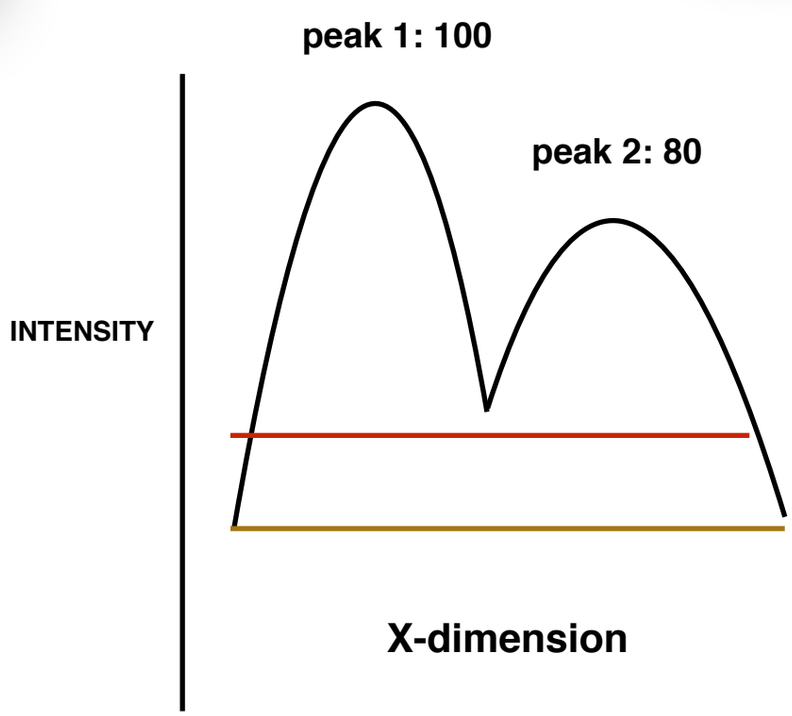
Erosion: Shrink blobs

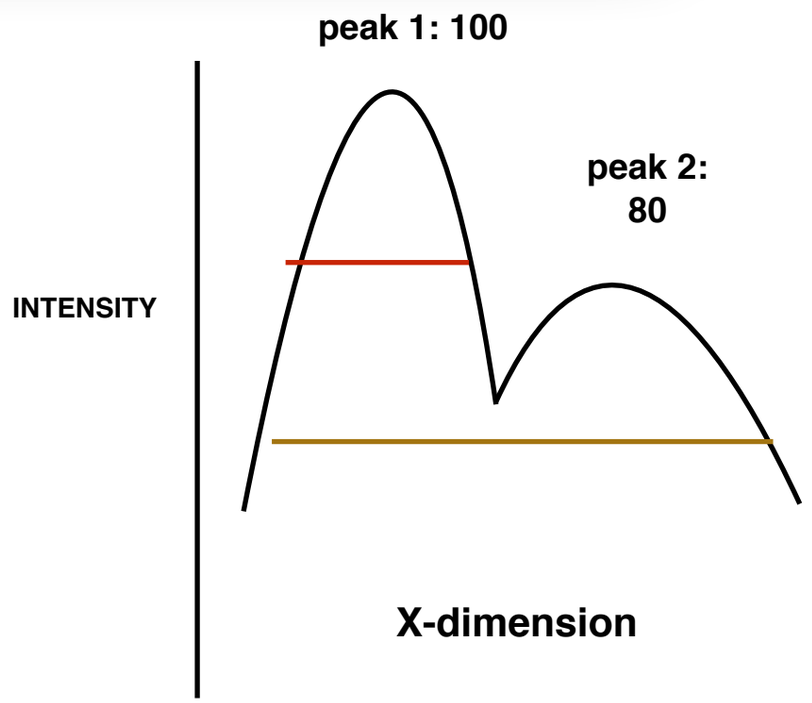
2d peak finding: Process -> Find Maxima



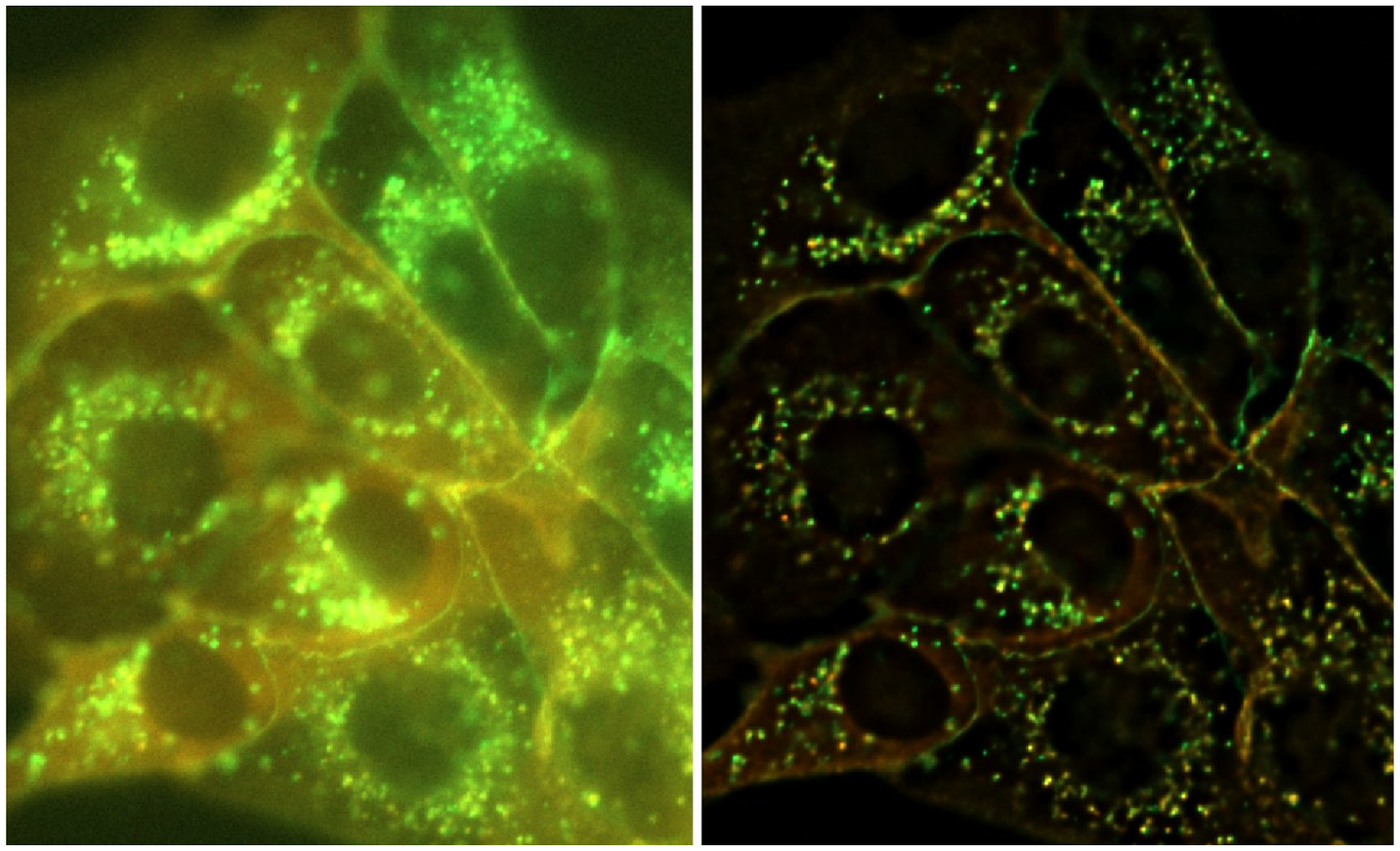




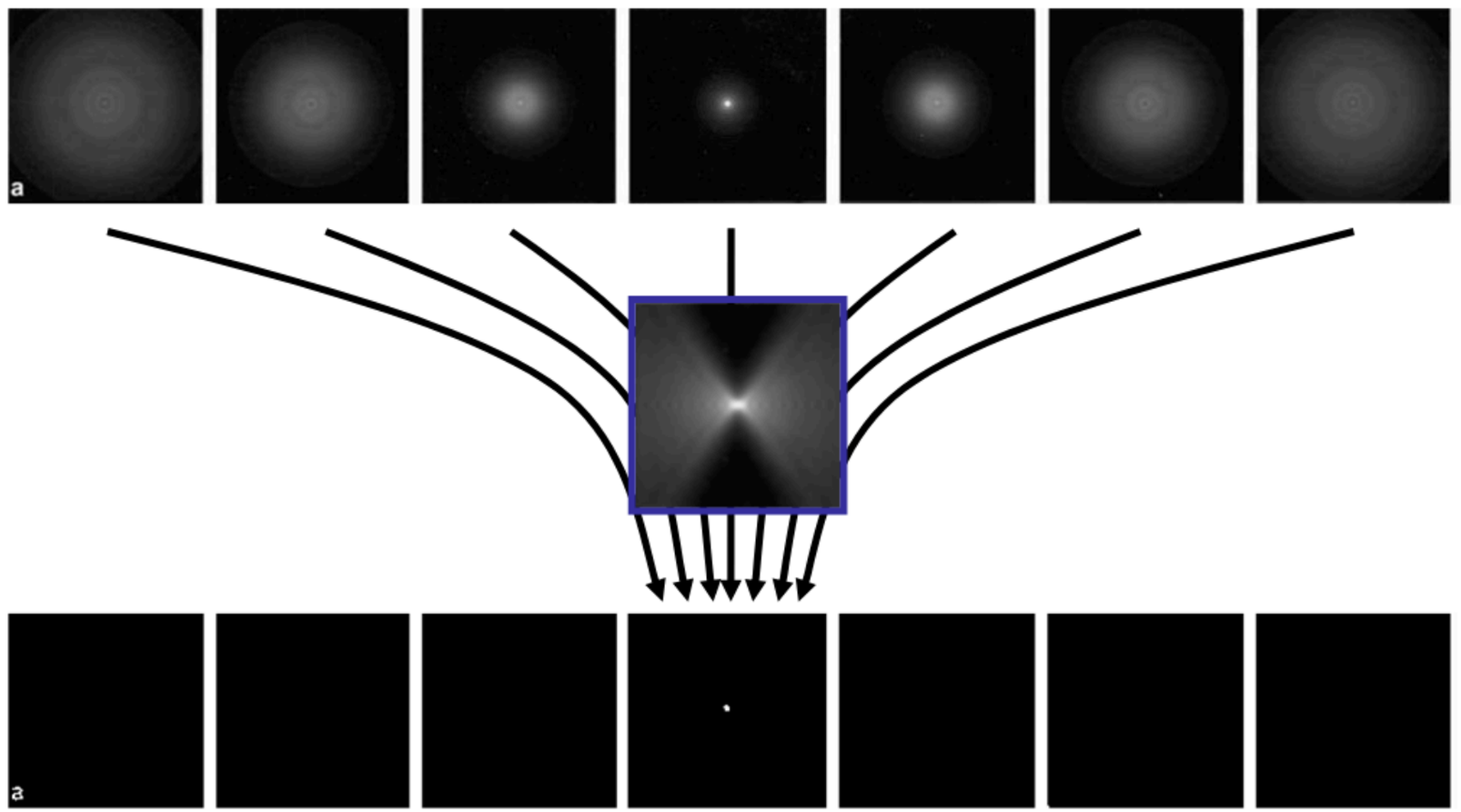




Deconvolution



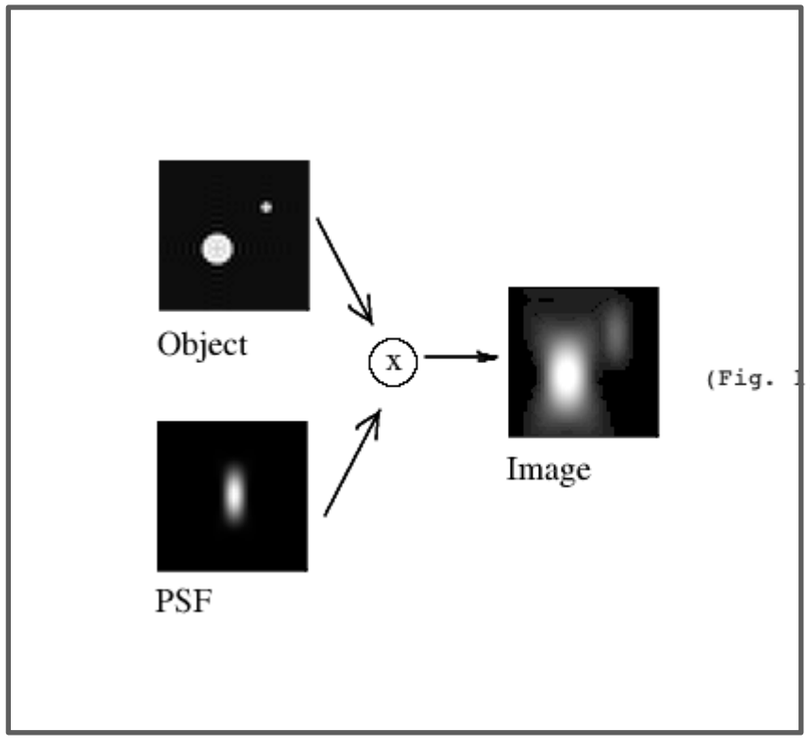
https://svi.nl/ImageGallery



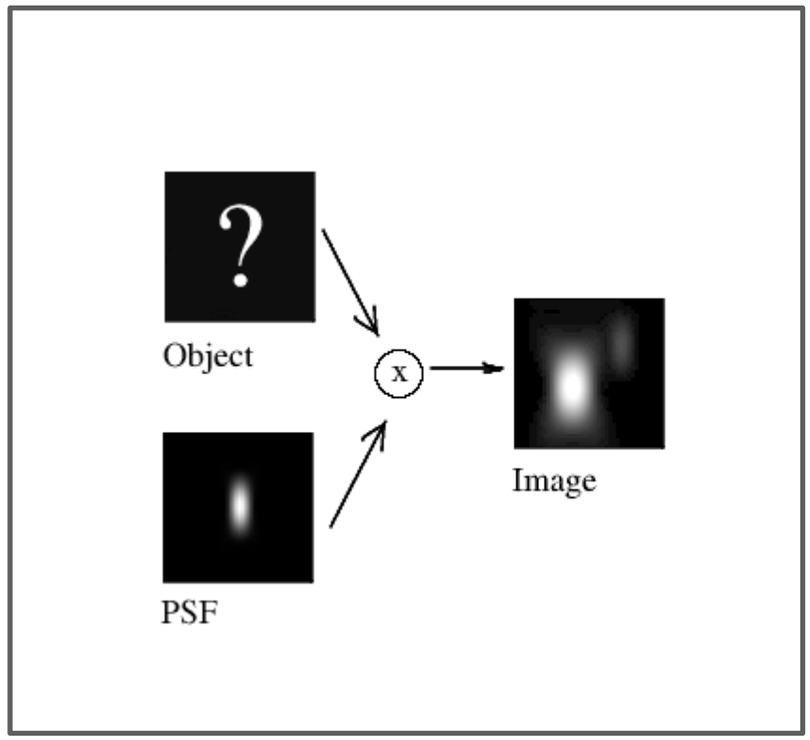
Out-of-focus information is moved back to its estimated origin

Source: https://www.imperial.ac.uk/media/imperial-college/medicine/facilities/film/Deconvolution-training-140219.pdf

Deconvolution



(Fig. 1)



Source: https://svi.nl

Deconvolution software

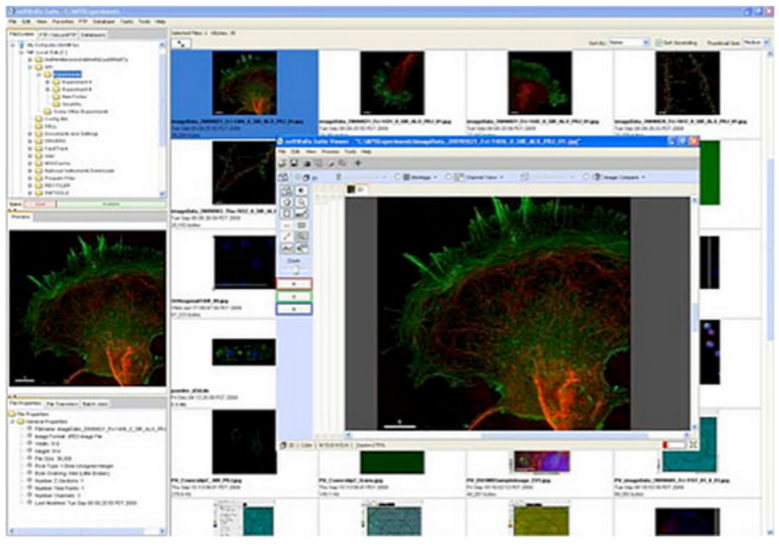
Deconvolution

- Image restoration
- Volume Visualisation
- Some analysis

Software is Expensive.

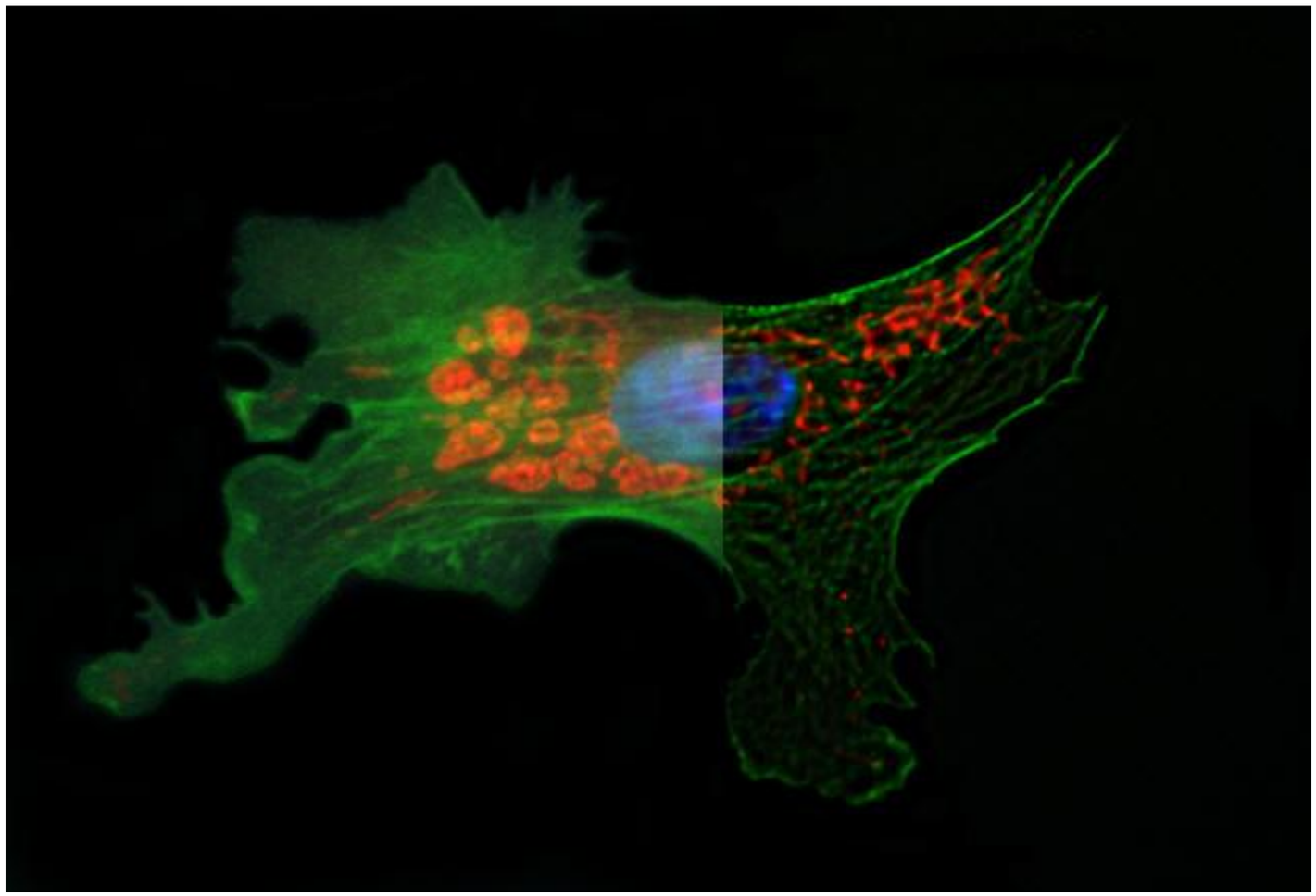
Some new deep learning solutions are becoming available.
e.g. CARE
http://csbdeep.bioimagecomputing.com/

SoftWoRx



Source: http://www.svi.nl/HuygensSoftware, http://www.mediacy.com/index.aspx?page=AutoQuant, http://api.gehealthcare.com/api/softworx-suite.asp

Deconvolution



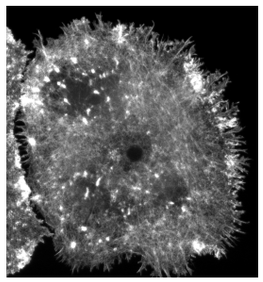
Be aware of artefacts!

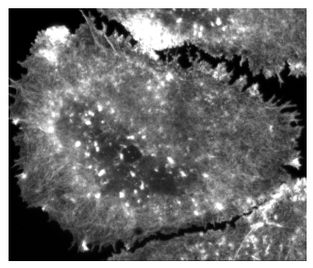
https://svi.nl/ImageGallery

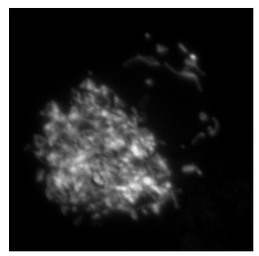
Colocalisation statistics

How similar are these images?

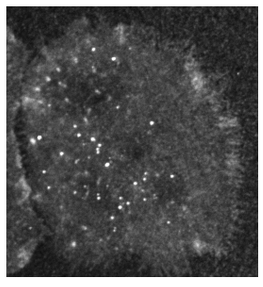
Channel 1

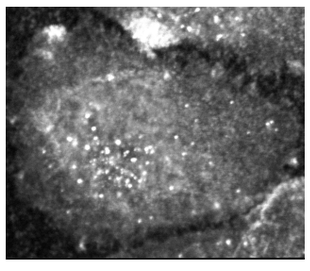




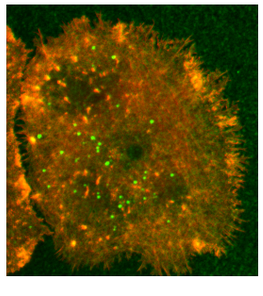


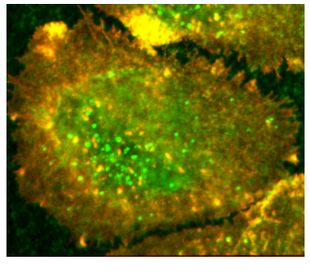
Channel 2

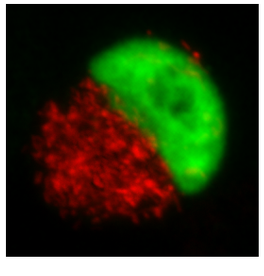




Composite



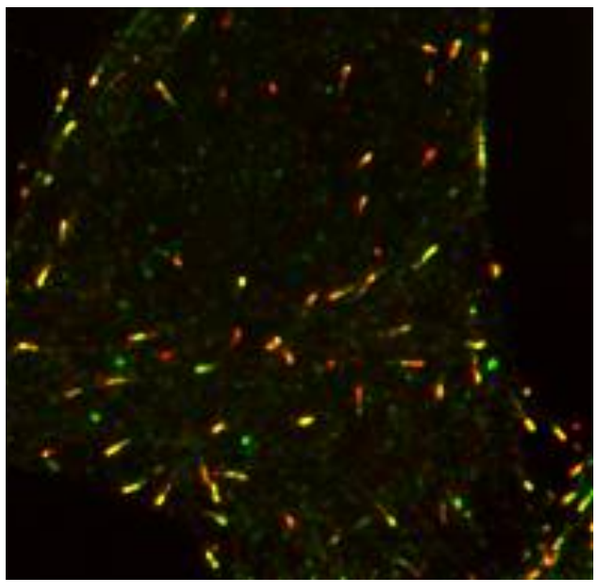




Three ways to evaluate colocalisation

Visualisation

First, apply a LUT to each channel, then overlay them



Quantification

Image considered as a **collection of pixels**

Look for a link between intensities from both channels

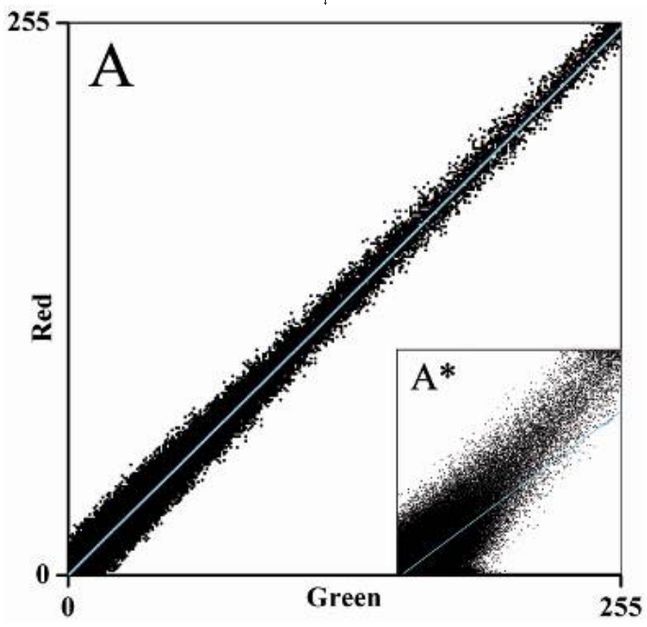
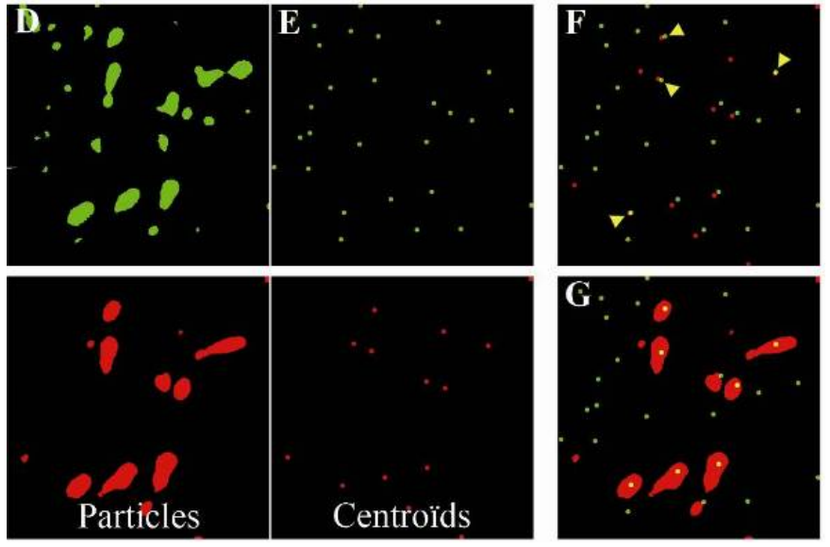
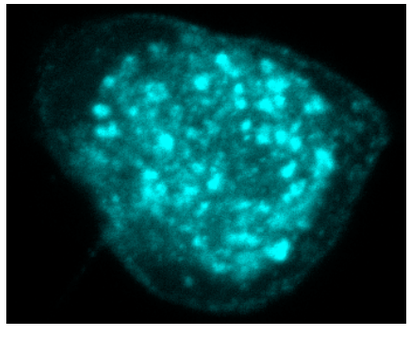


Image considered as a **collection of objects**

First, identify the structures, then look for an overlap, either partial or total between objects



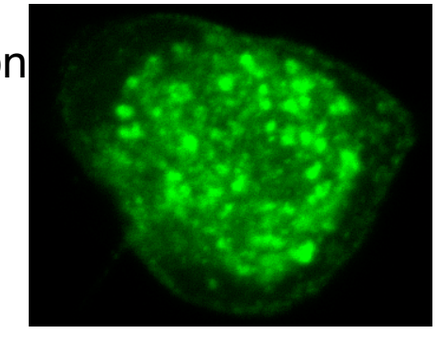
Pearson's Correlation Coefficient

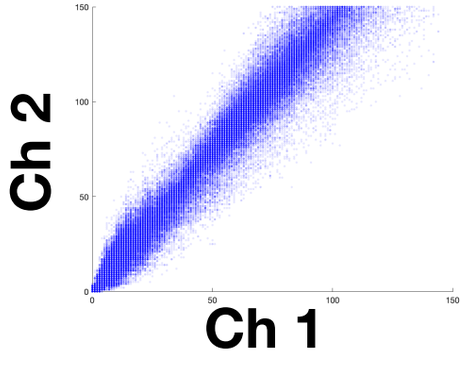


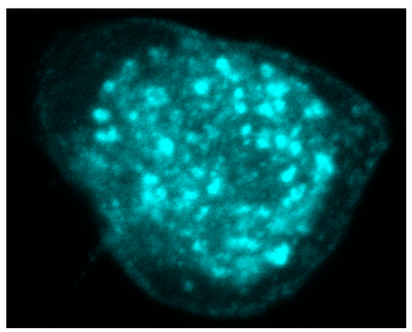
correlation

0.8

VS



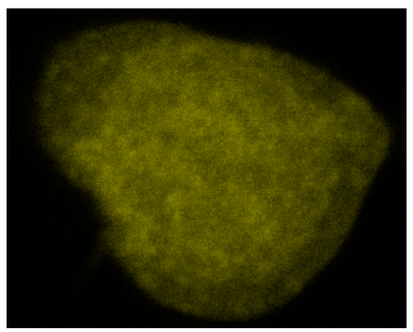


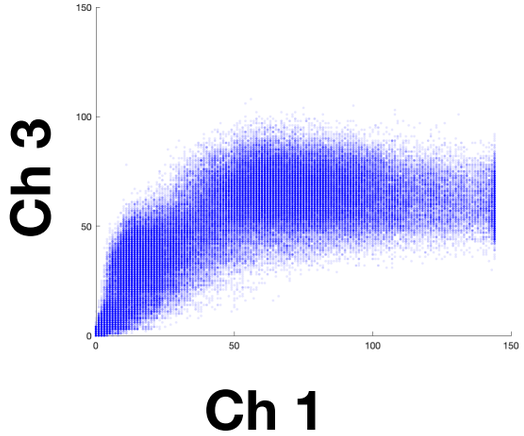


correlation

0.2

VS





Images which have similar 'spatial' distribution of pixel values will be highly-correlated. We can use this to establish colocalisation.

Waithe 2019

Pearson’s product-moment correlation test

Pearson’s equation:

$$r = \frac{\sum \left(R_i - \bar{R}\right) \times \left(G_i - \bar{G}\right)}{\sqrt{\sum \left(R_i - \bar{R}\right)^2 \times \sum \left(G_i - \bar{G}\right)^2}}$$

if r is 1.0 means correlation

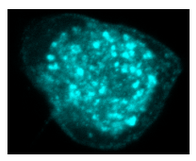
if r is close to ‘0.0’ no correlation.

if r is -1.0 it means anti-correlation.

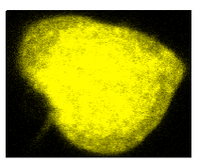
R refers to one channel, G refers to Green channel. G or R with a bar refers to mean intensity in that channel. ‘i’ refers to each pixel in image. Sigma (big E) refers to sum. So sum of all pixels minus their mean.

VS high

r =0.85



VS low

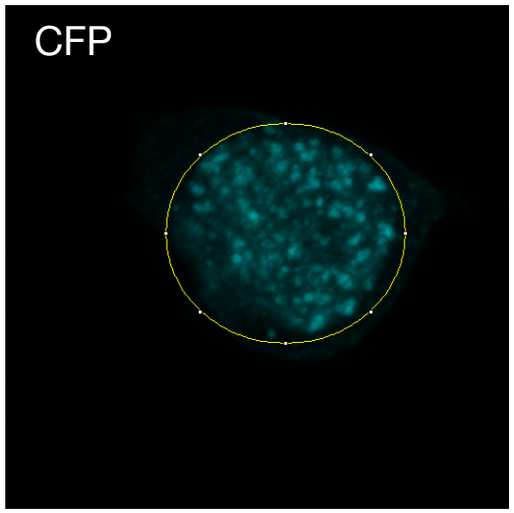


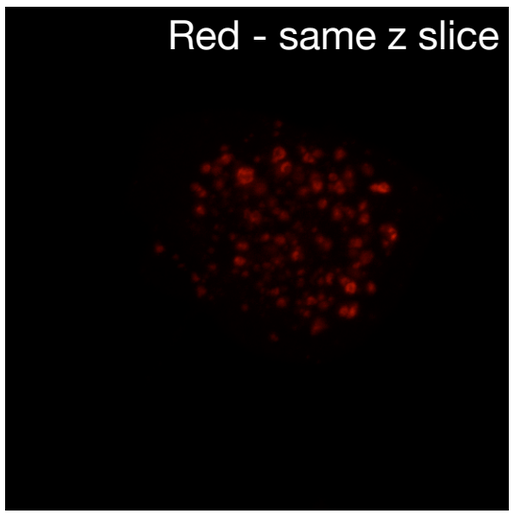
r =0.25

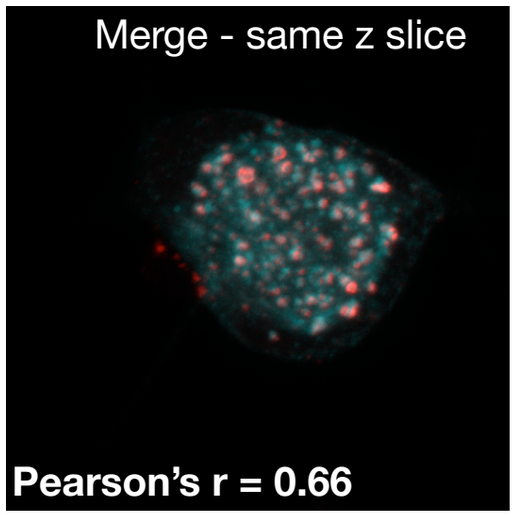
- Dimensionless and normalised comparison. Can be used on any two images as long as they are the same spatial size and don’t have too many black pixels

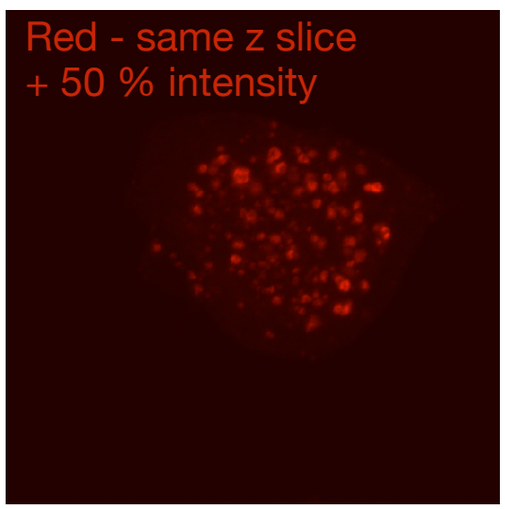
Source: http://en.wikipedia.org/wiki/Correlation_coefficient

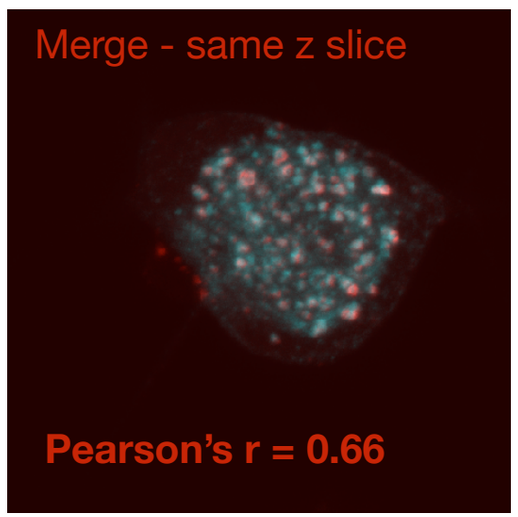
Pearson’s test is insensitive to global intensity











- Pearson’s test is (within reason) insensitive to linear changes in intensity.
- This is good, it looks at trends rather than absolute values.
- This means expression variation between cells does not ruin experiment

Pearson's test is sensitive to bleed-through and noise

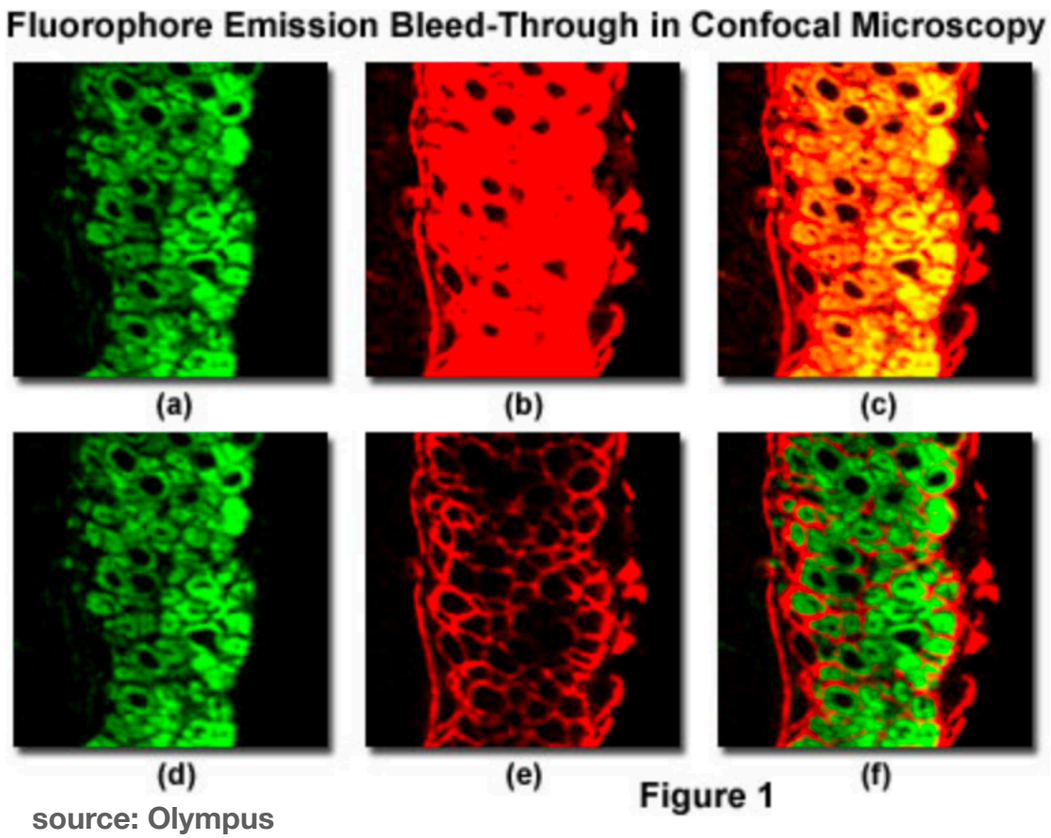
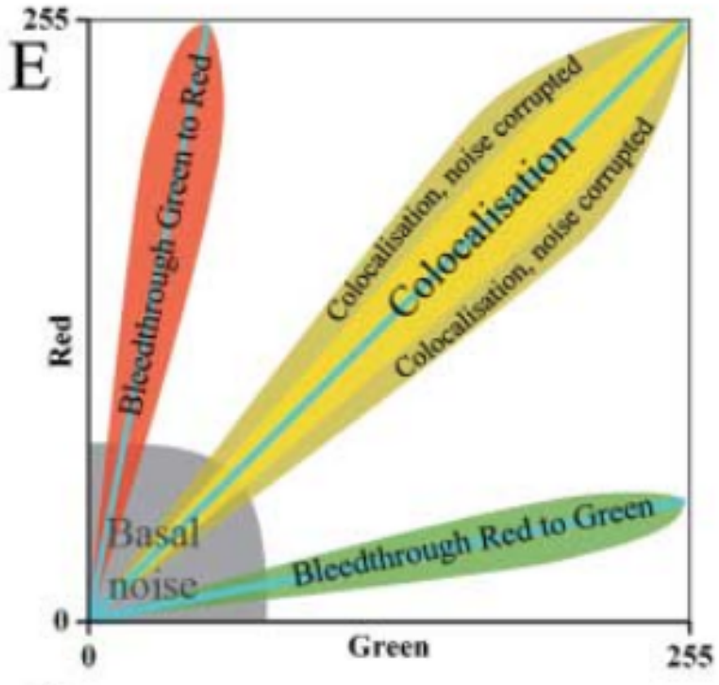


Figure 1

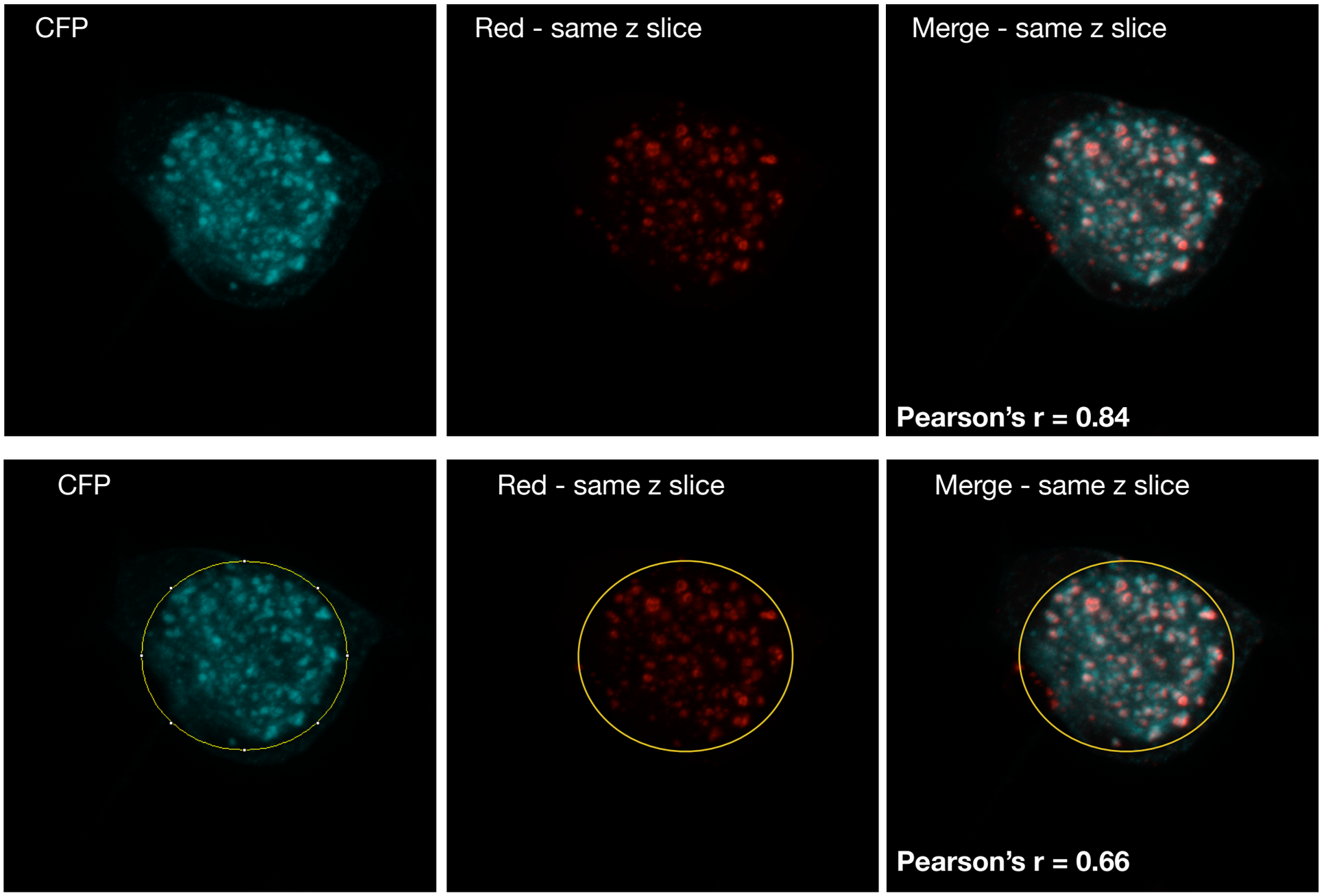
source: Olympus



222 S. BOLTE AND F. P. CORDELIÈRES

- Spectral bleed through will artificially increase the correlation coefficient.
- Noise will artificially decrease the correlation coefficient.

P’s test is sensitive zero pixels and saturation



- Pearson’s test doesn’t ignore ‘0’ pixels and noise within calculation.
- Coloc 2 plugin does warn you however: The ratio between zero-zero pixels and other pixels is larger 0.37. Maybe you should use a ROI.

Sensitivity to resolution

The « diagnostic » placed for co-localisation should always be stated relative to a particular <u>resolution</u> and <u>sampling rate</u>.

- **In cell biology:**

the two proteins are at the same location

- **The statistical point-of-view:**

Considering the current resolution, it might not be excluded that the two proteins are indeed at the same location

neubias
network of european bioimage analysts

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neubias
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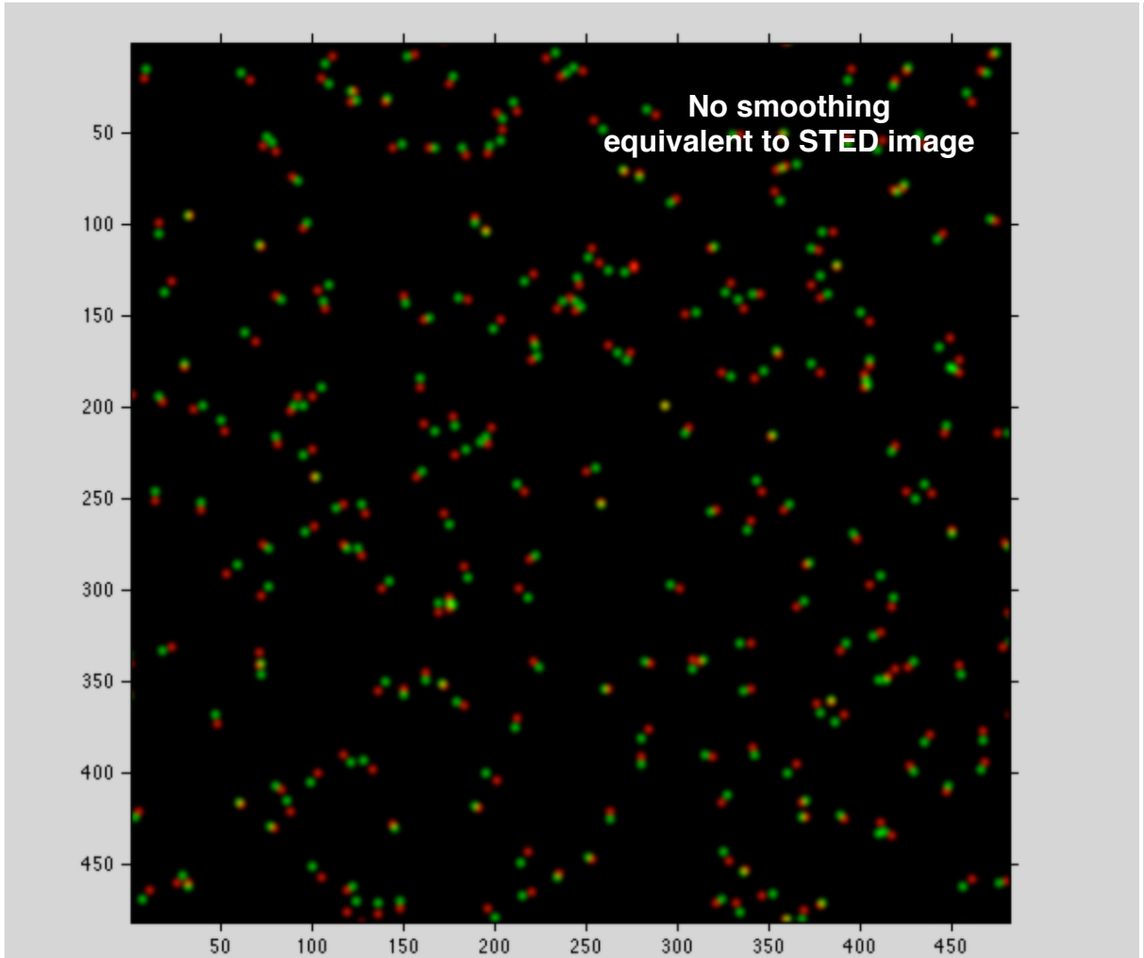
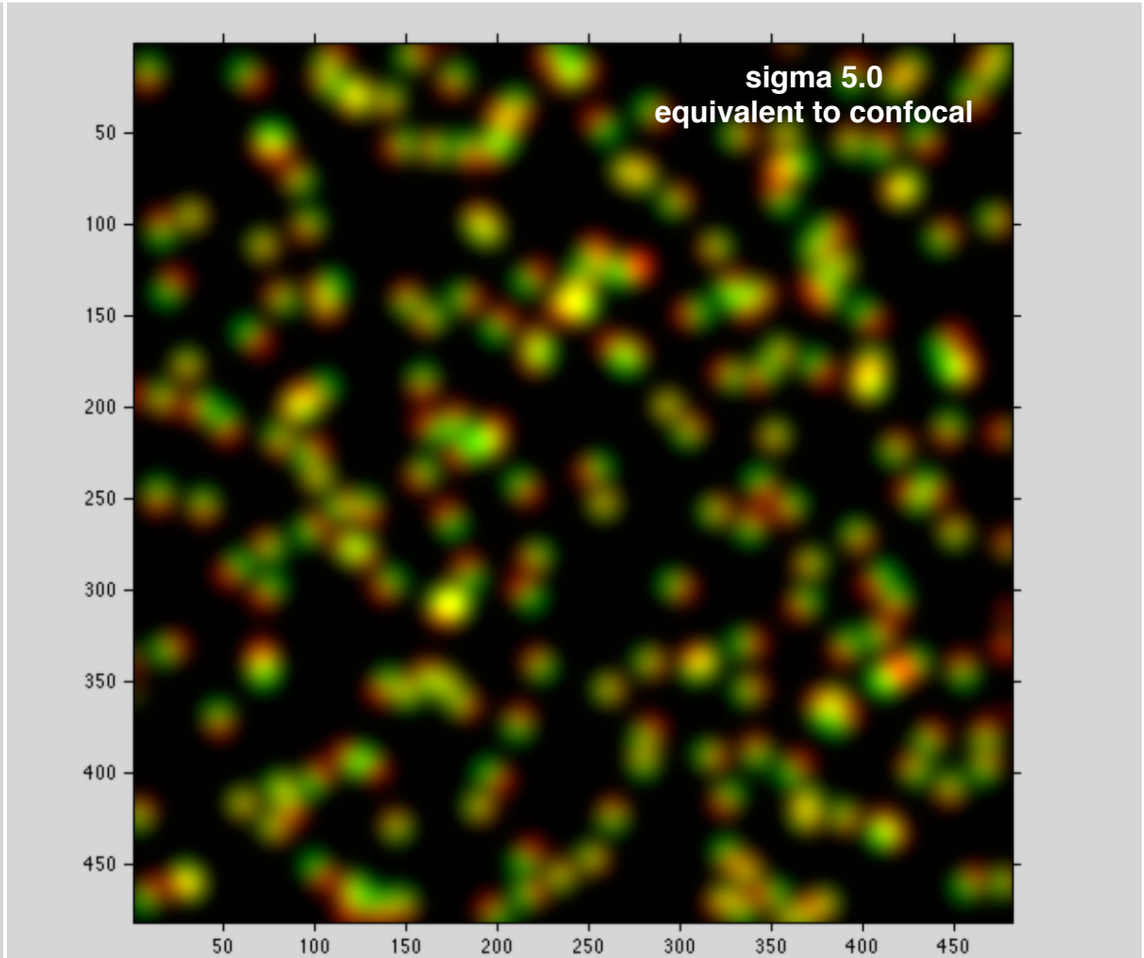
Sensitivity to resolution



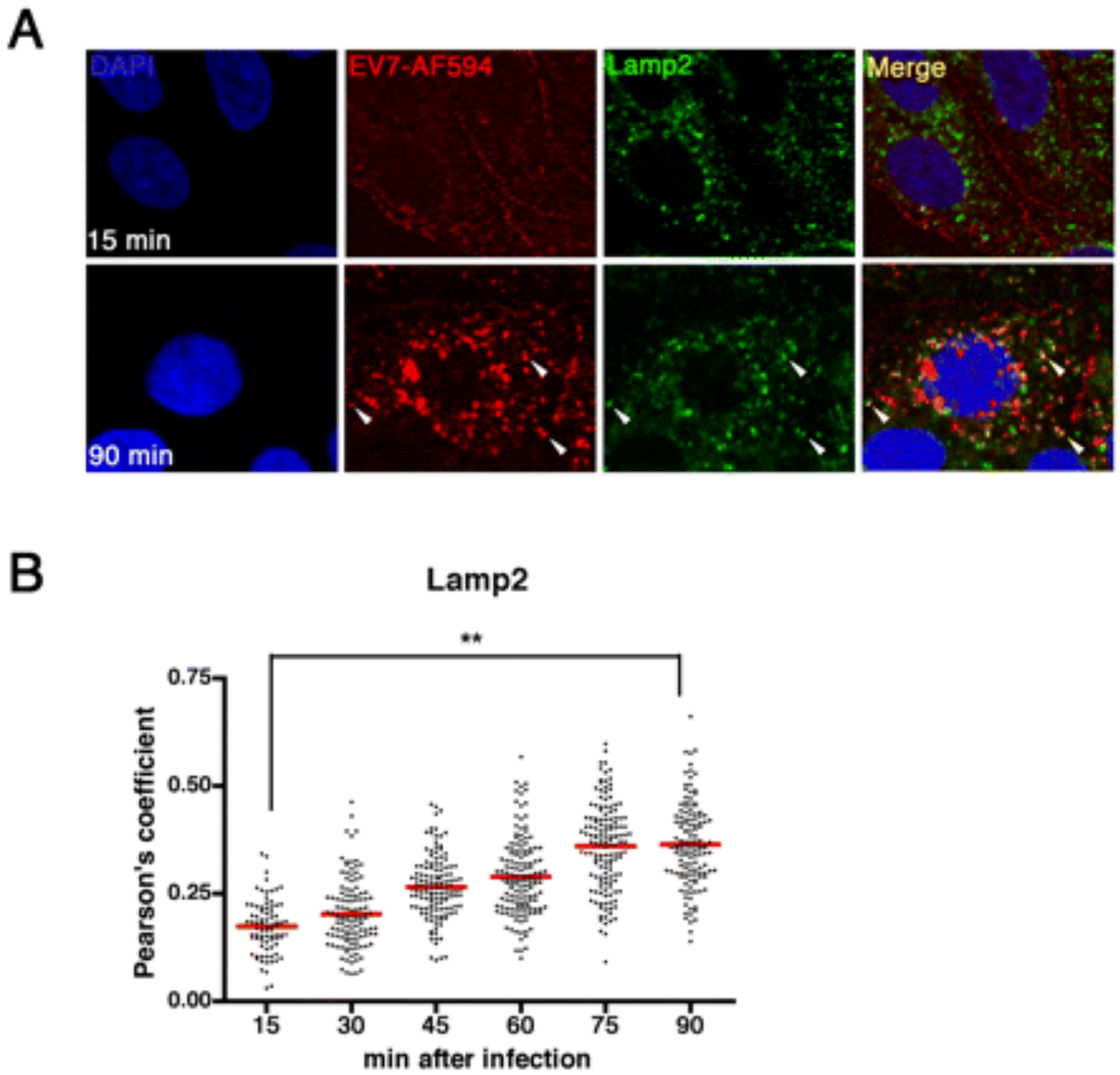
r = 0.23



r = 0.84

A good example of pearson's test for colocalisation

Comparing EV7 staining with Lamp2 over time.

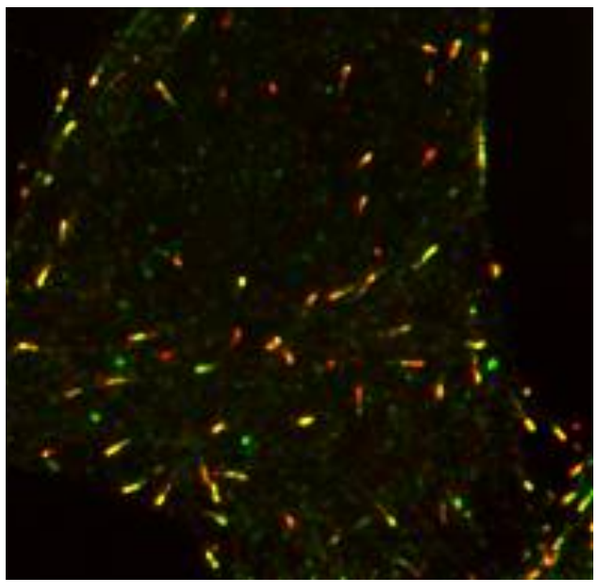


Source: http://mbio.asm.org/content/3/2/e00304-11/F5.expansion.html

Always consider the limitations of the method and chose your Experimental settings and controls carefully.

Visualisation

First, apply a LUT to each channel, then overlay them



Quantification

Image considered as a **collection of pixels**

Look for a link between intensities from both channels

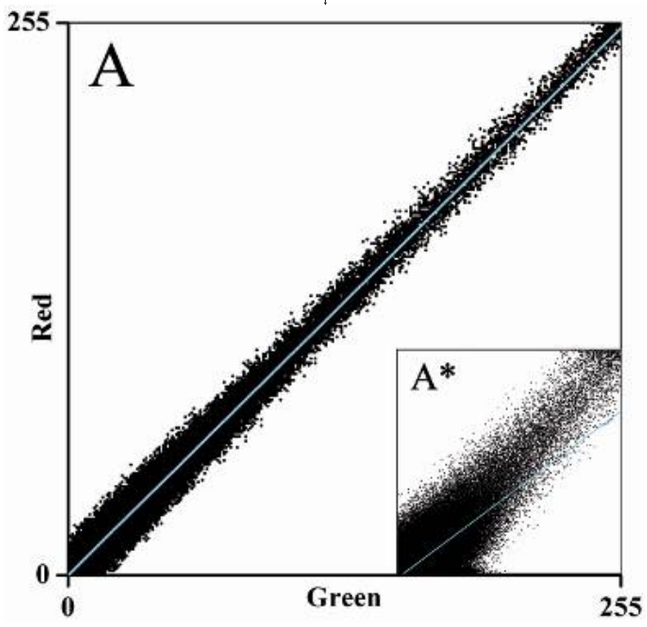
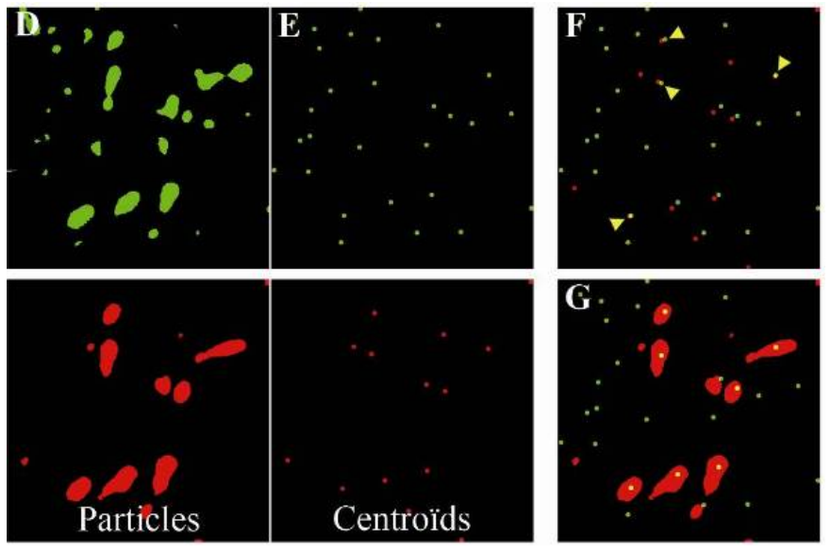
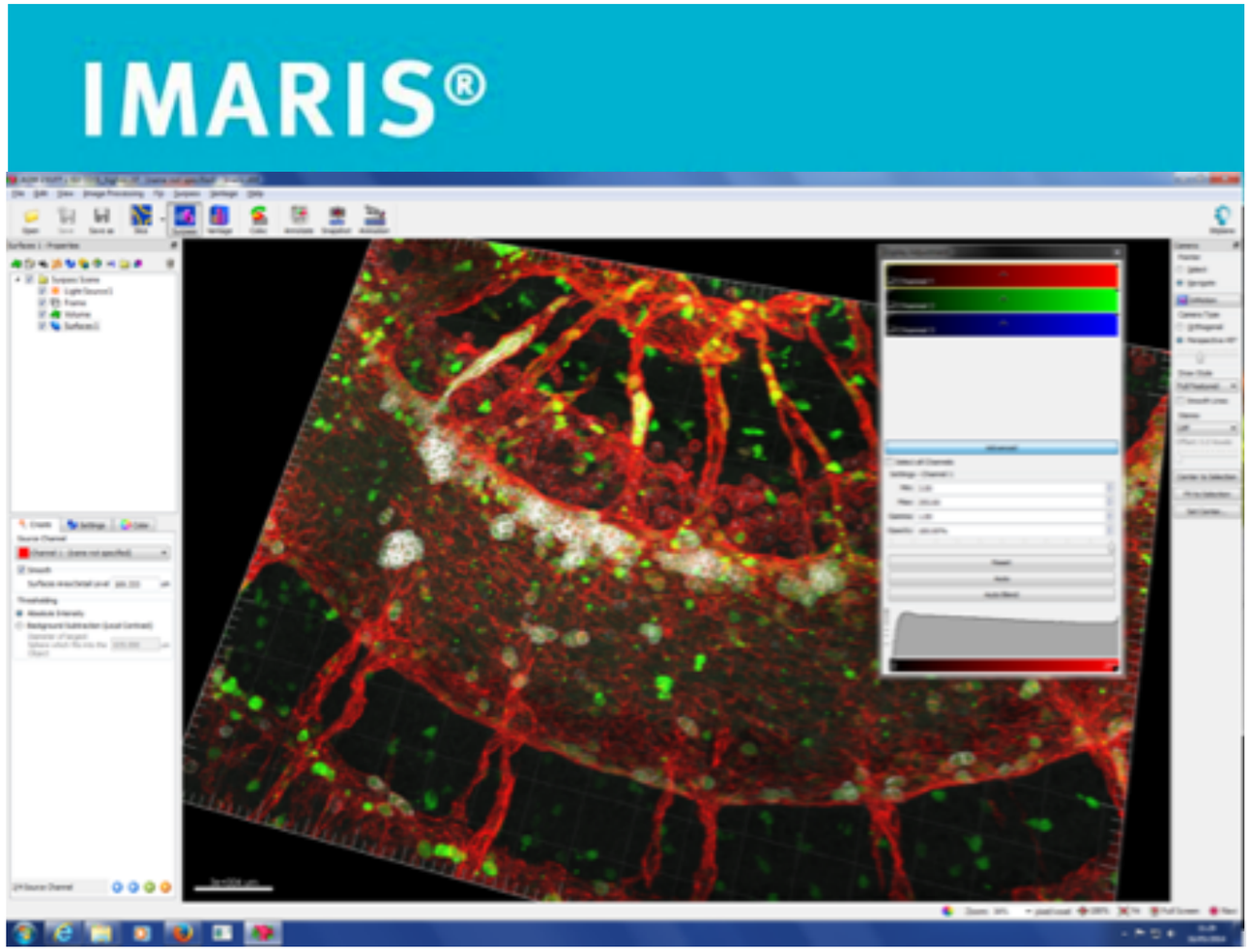


Image considered as a **collection of objects**

First, identify the structures, then look for an overlap, either partial or total between objects

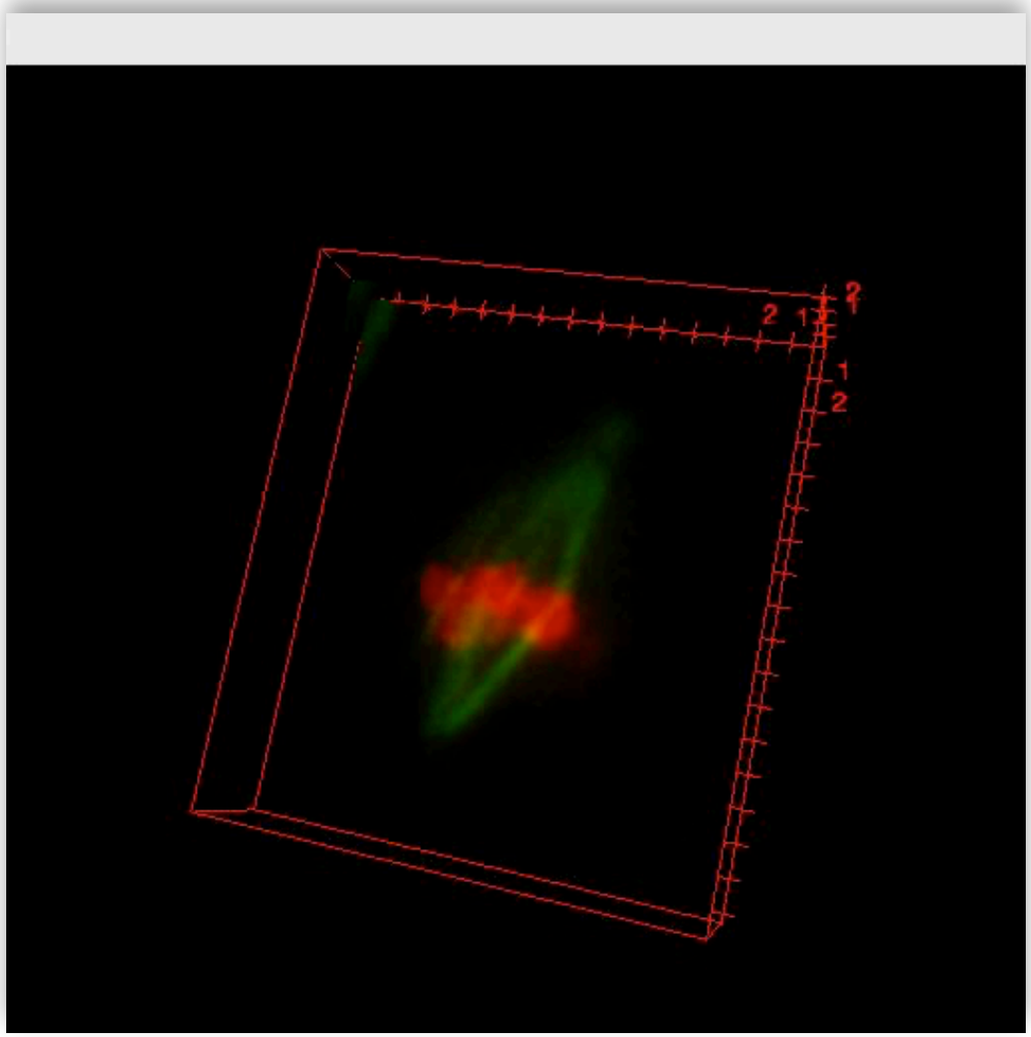


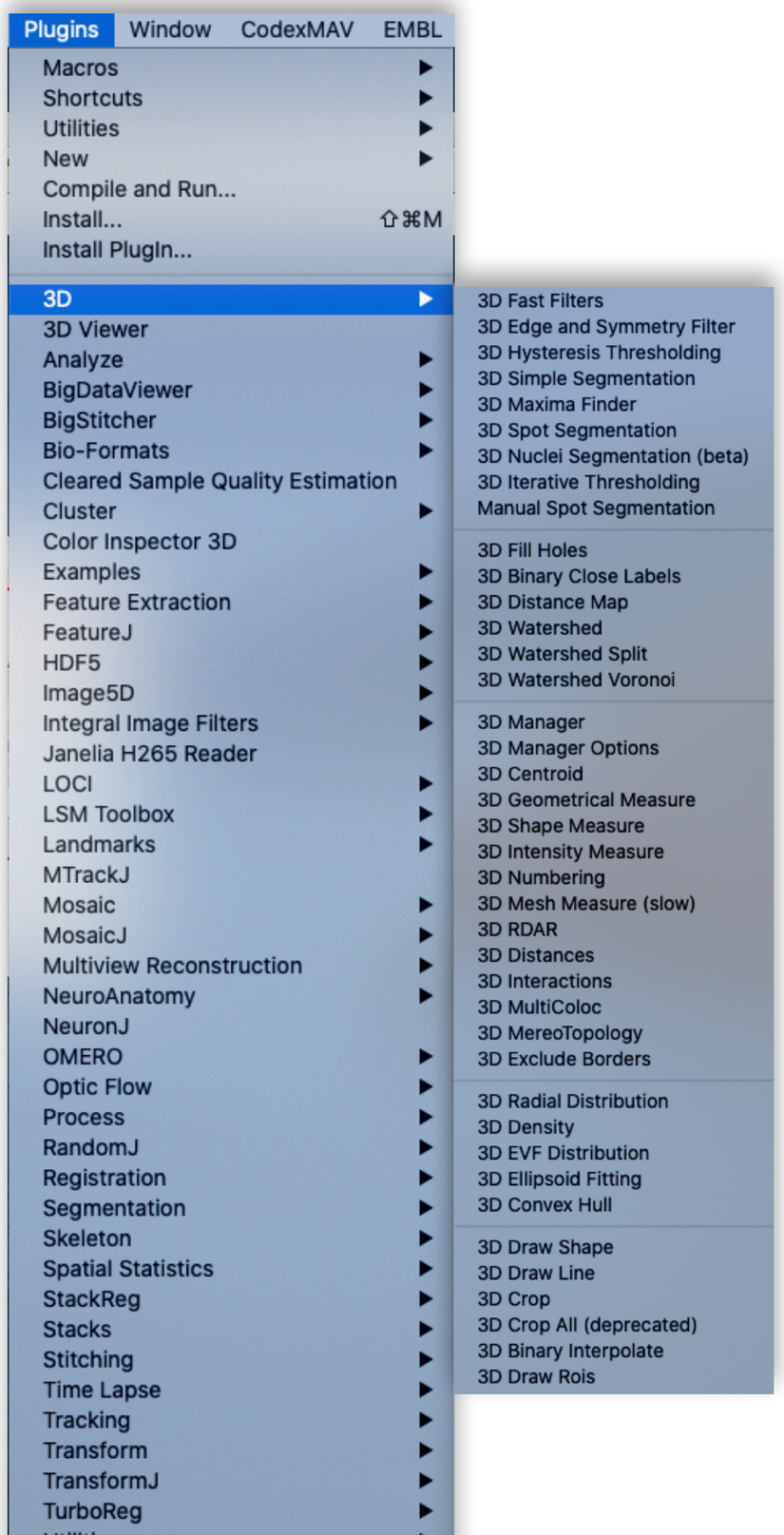
3D visualisation & Analysis



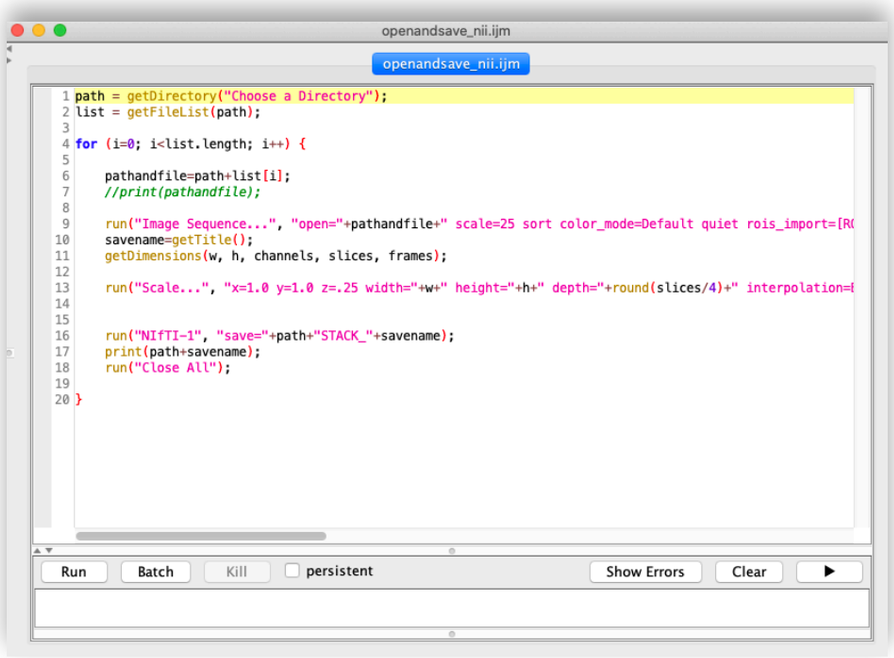
- Volume Visualisation
- Automatic and manual identification of objects in 3D
- Tracking and Colocalisation in 3D

3D visualisation & Analysis





Macro Programming



Take nice images!

- **No saturated pixels**
- **Good signal to noise**
- **Low background**

- **Keep imaging conditions the same: laser power, gain, resolution, etc.**

- **Microscopes have to be aligned**
- **Be mindful of chromatic aberrations**

- **Think about image Analysis before acquiring hundreds of images!**

Don’t!

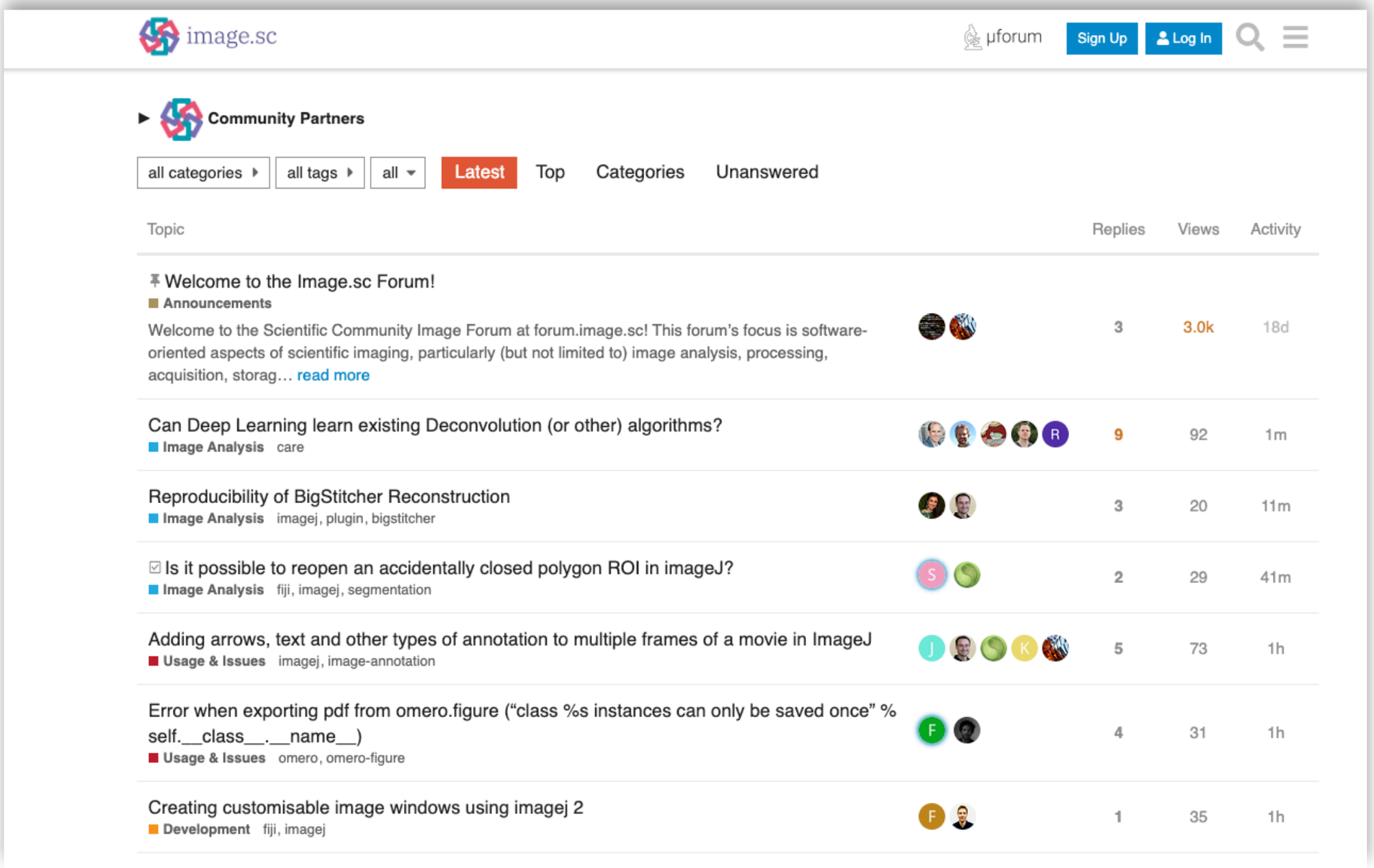
- **Adjust the brightness and contrast equally in each image or else include colour scale.**

- **Do not disguise faint structures in your image by adjusting brightness to hide it.**

- **Do not remove or change pixels in background.**
- **Don’t apply non-linear transforms to image (e.g. change gamma).**

- **Don’t do anything you cannot justify in your methods.**

Image Analysis questions for later



Source: image.sc

Thank you!

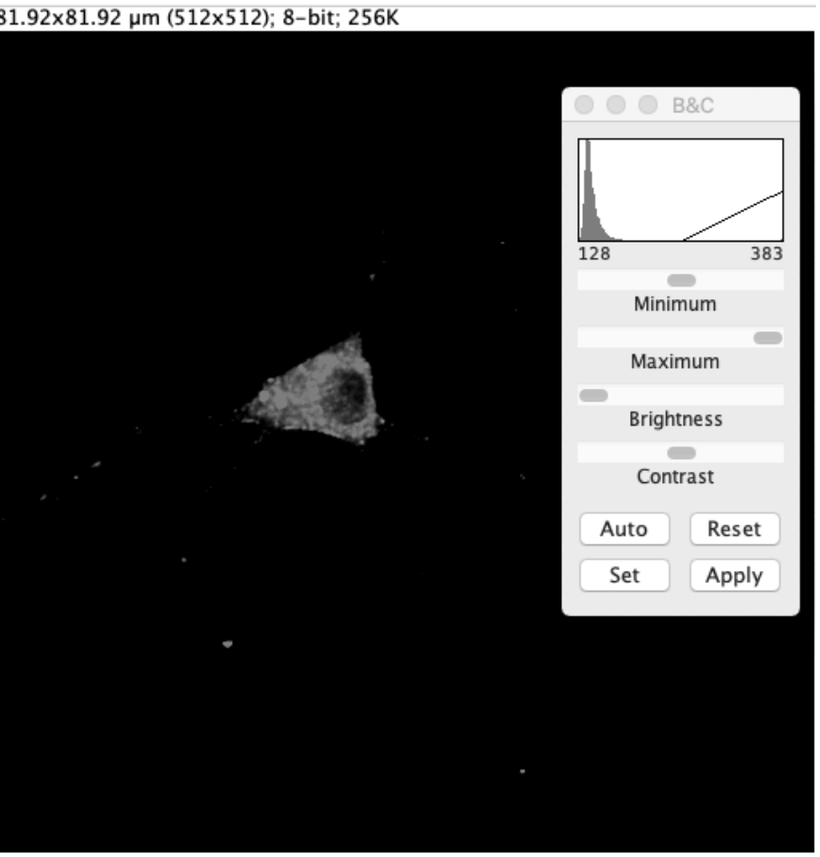
Ulrike.Schulze@rdm.ox.ac.uk

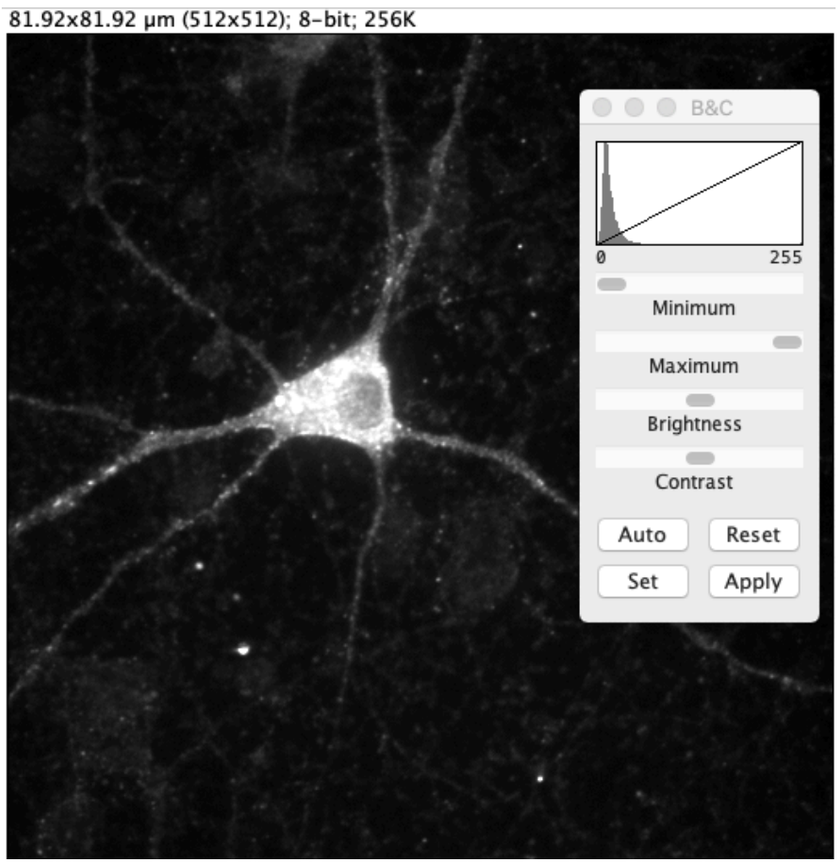
Thank you!

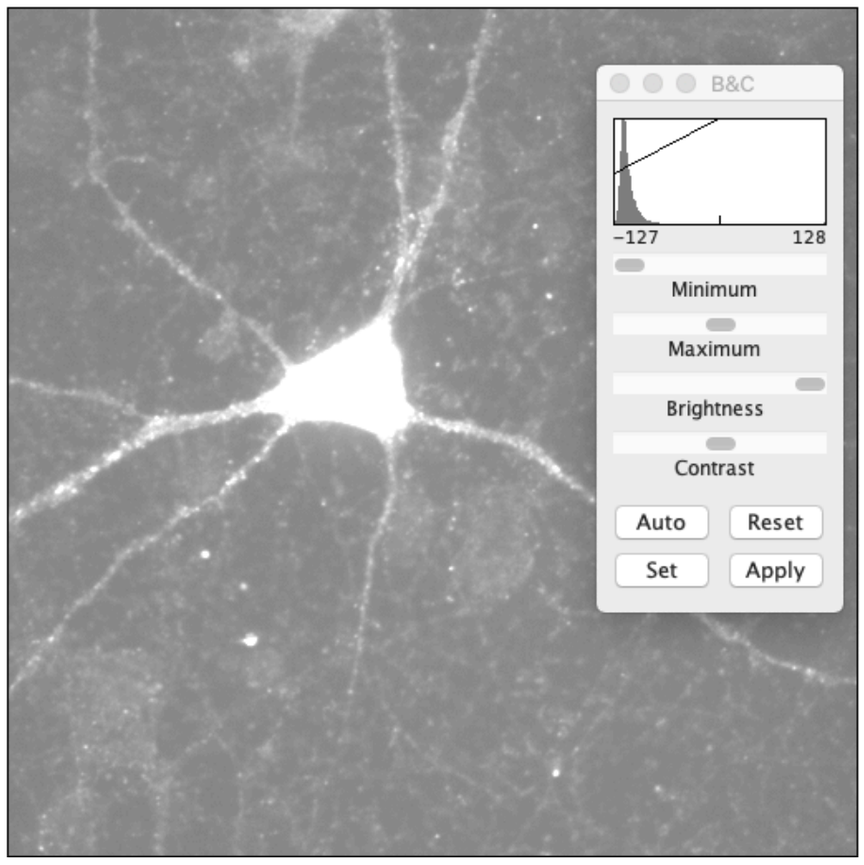
Ulrike.Schulze@rdm.ox.ac.uk

Brightness

Conversion of image intensity to display intensity

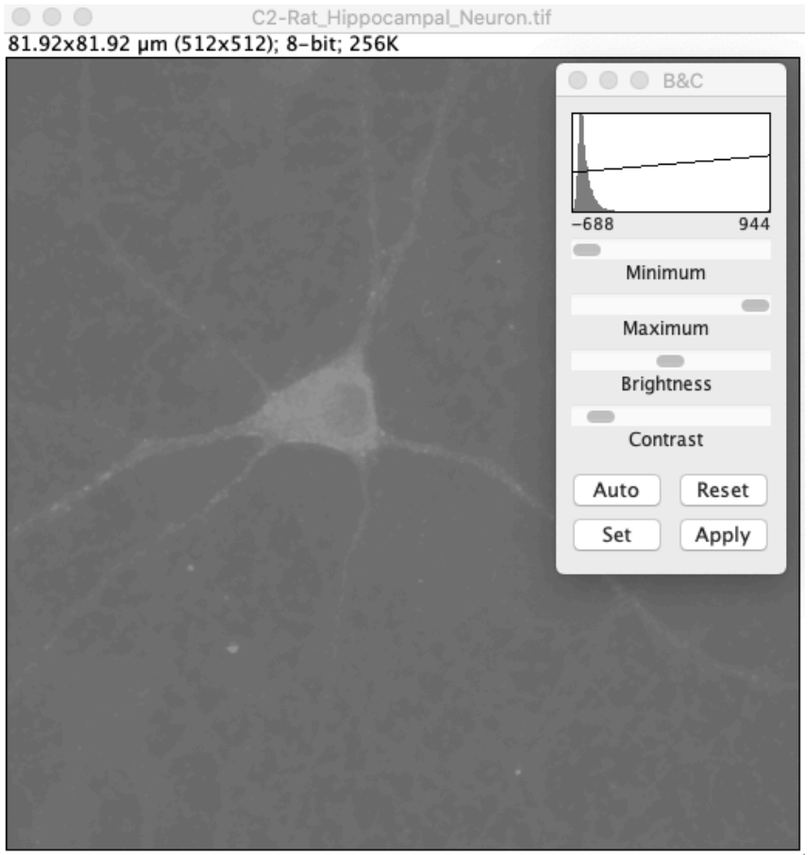


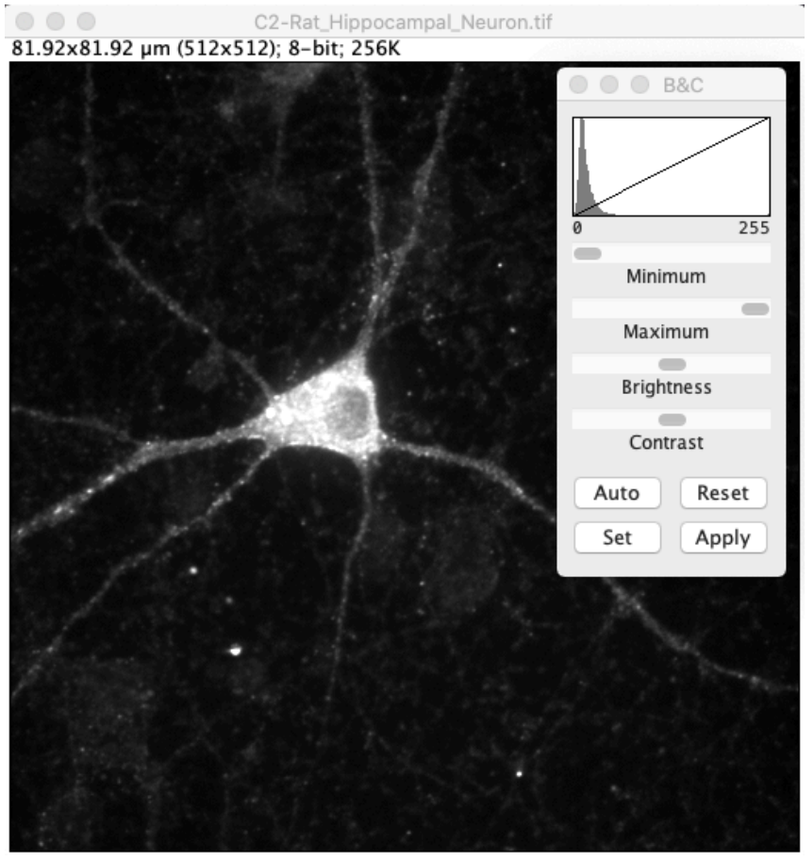


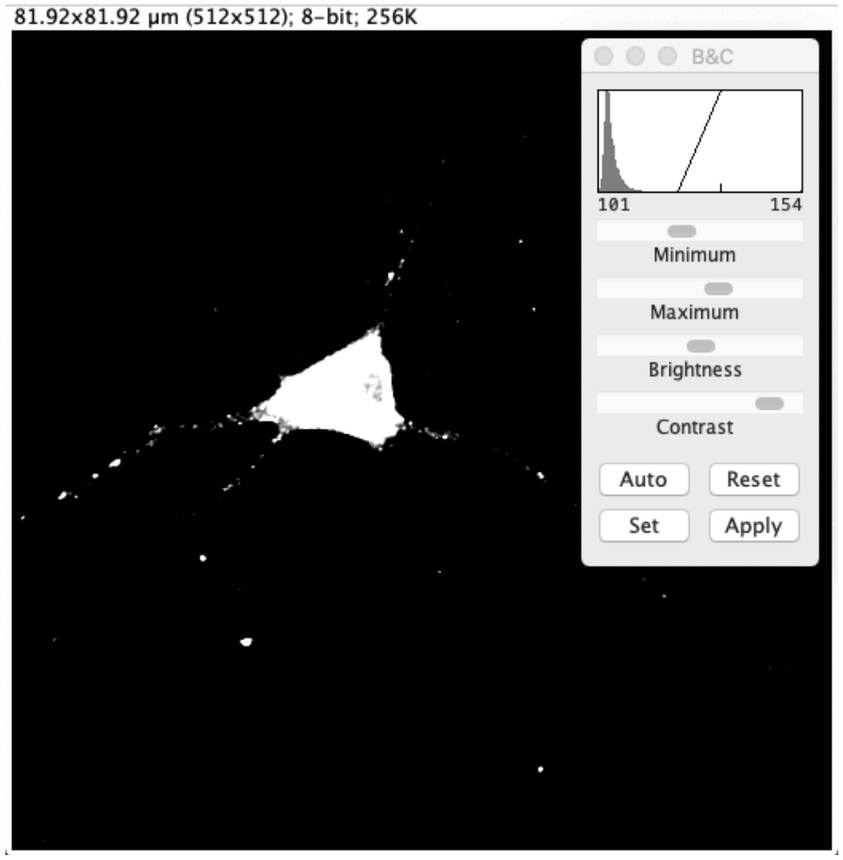


Contrast

ratio of the brightest spot to the darkest spot in the image.
It affects the range of the displayed image

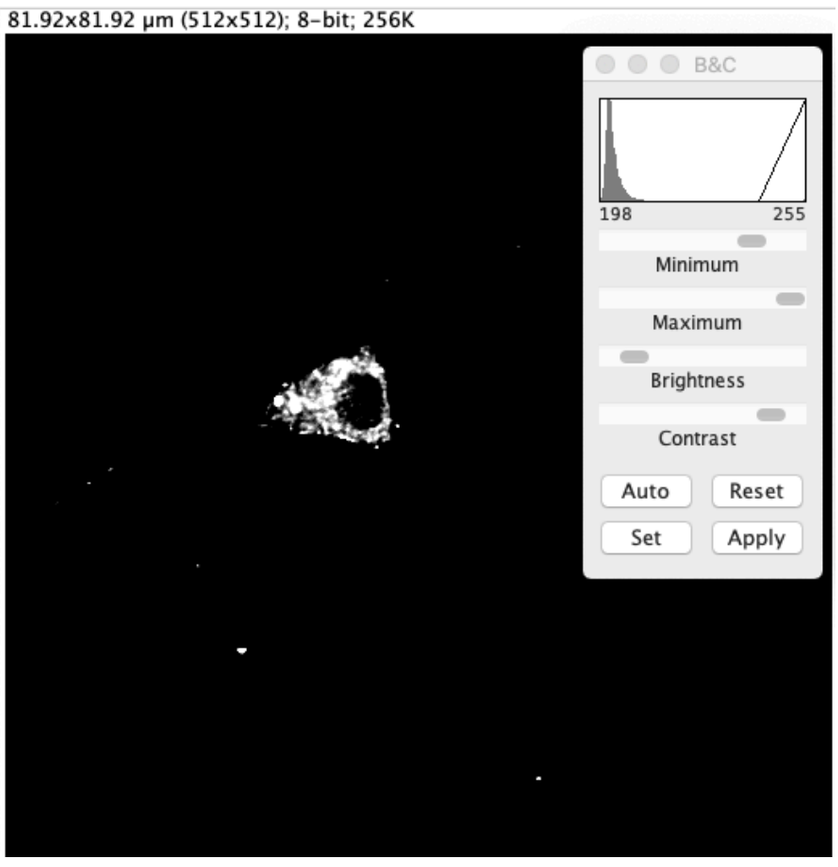


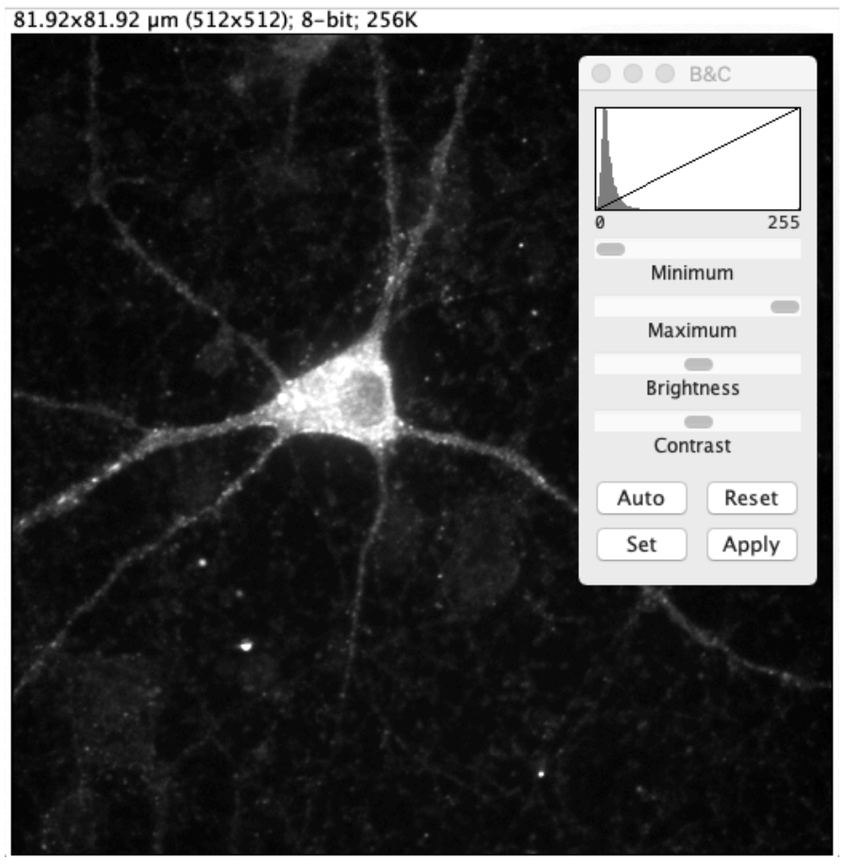


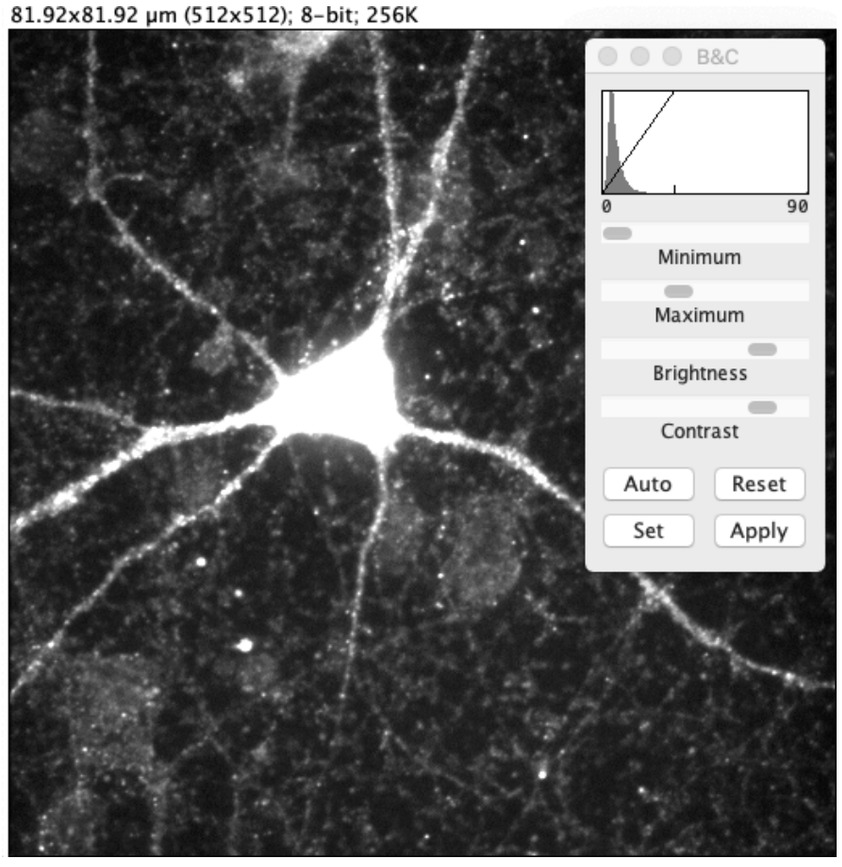


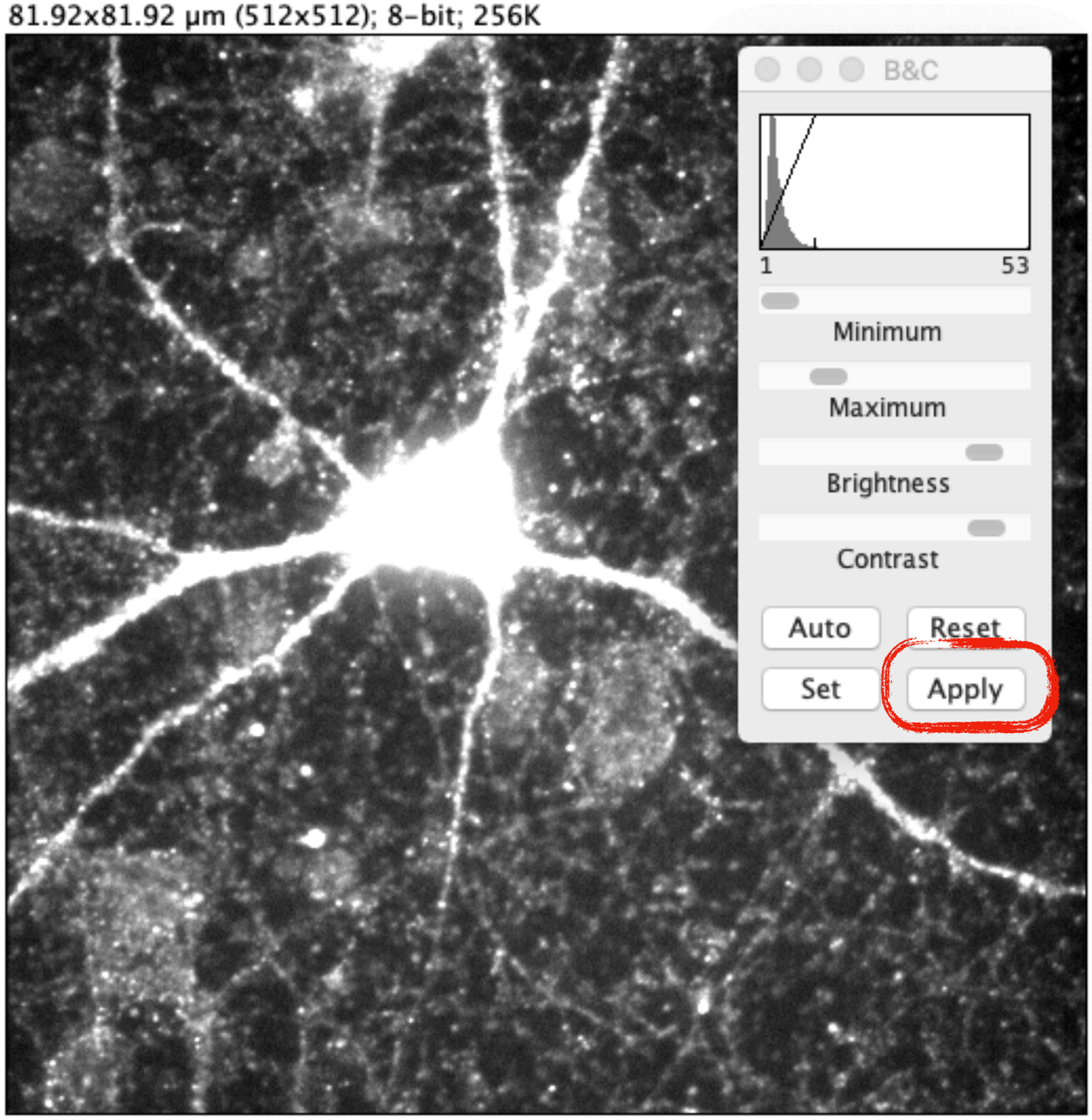
MinMax Slider

Change Brightness and Contrast simultaneously





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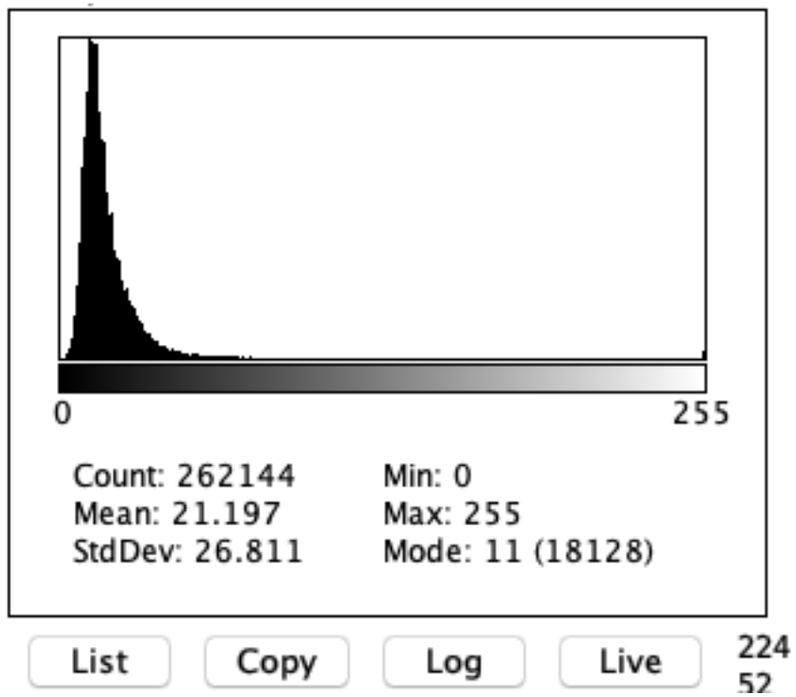
B&C
1
53
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply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List
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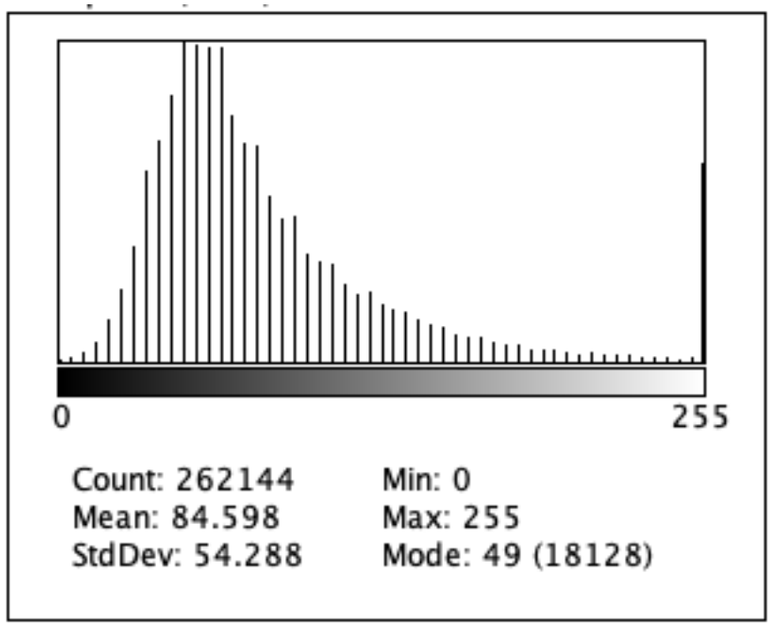
52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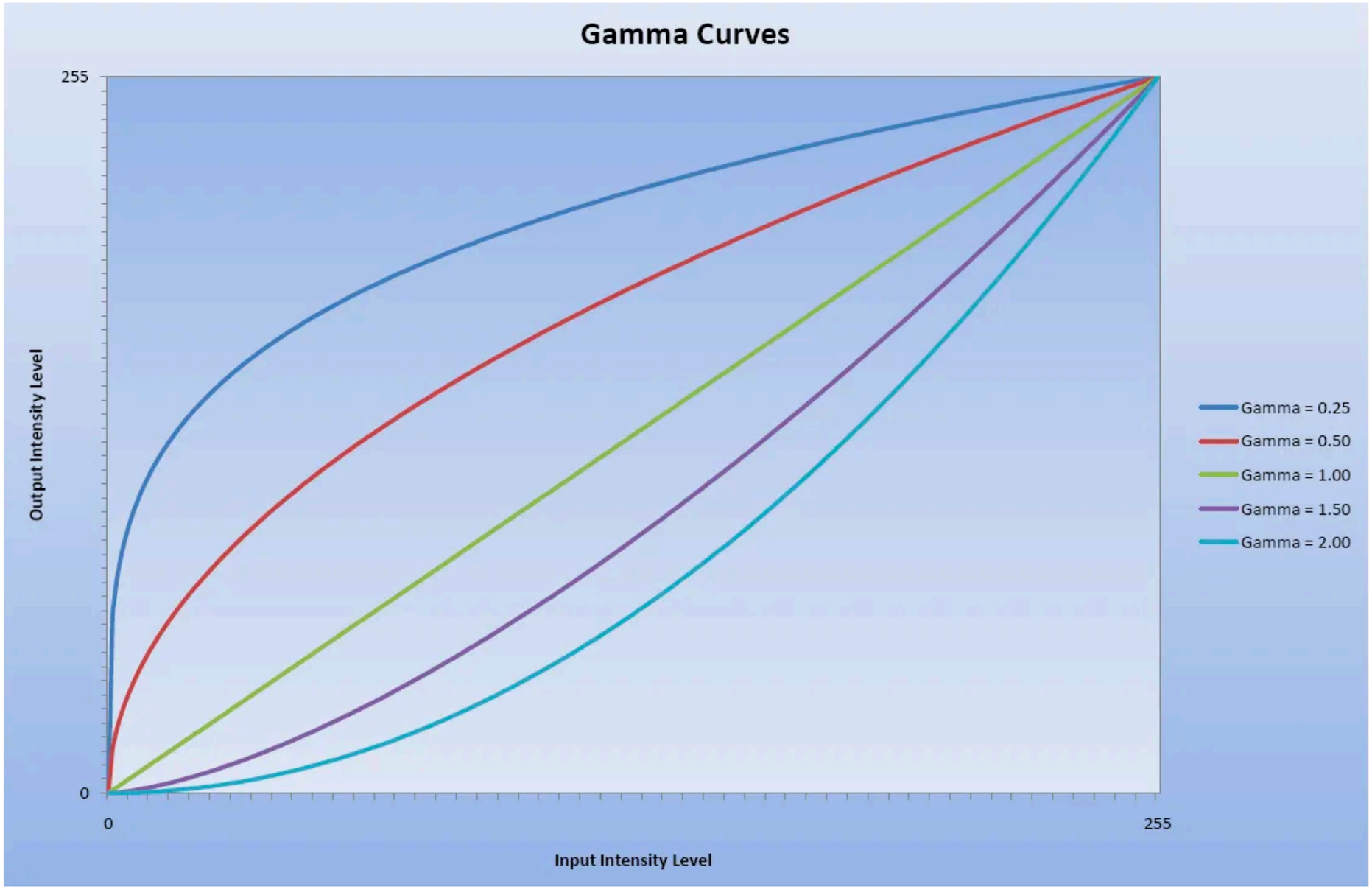
Gamma correction



https://www.dfstudios.co.uk/articles/programming/image-programming-algorithms/image-processing-algorithms-part-6-gamma-correction/

Gamma correction