Weatherall Institute of Molecular Medicine

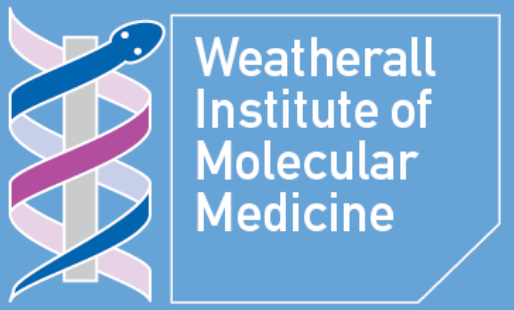
UNIVERSITY OF OXFORD

The MRC Weatherall Institute of Molecular Medicine is a strategic alliance between the Medical Research Council and the University of Oxford

MRC Medical Research Council

Introduction to ImageJ

Dr. Dominic Waithe
CBRG Workshop, April 2016



First lecture

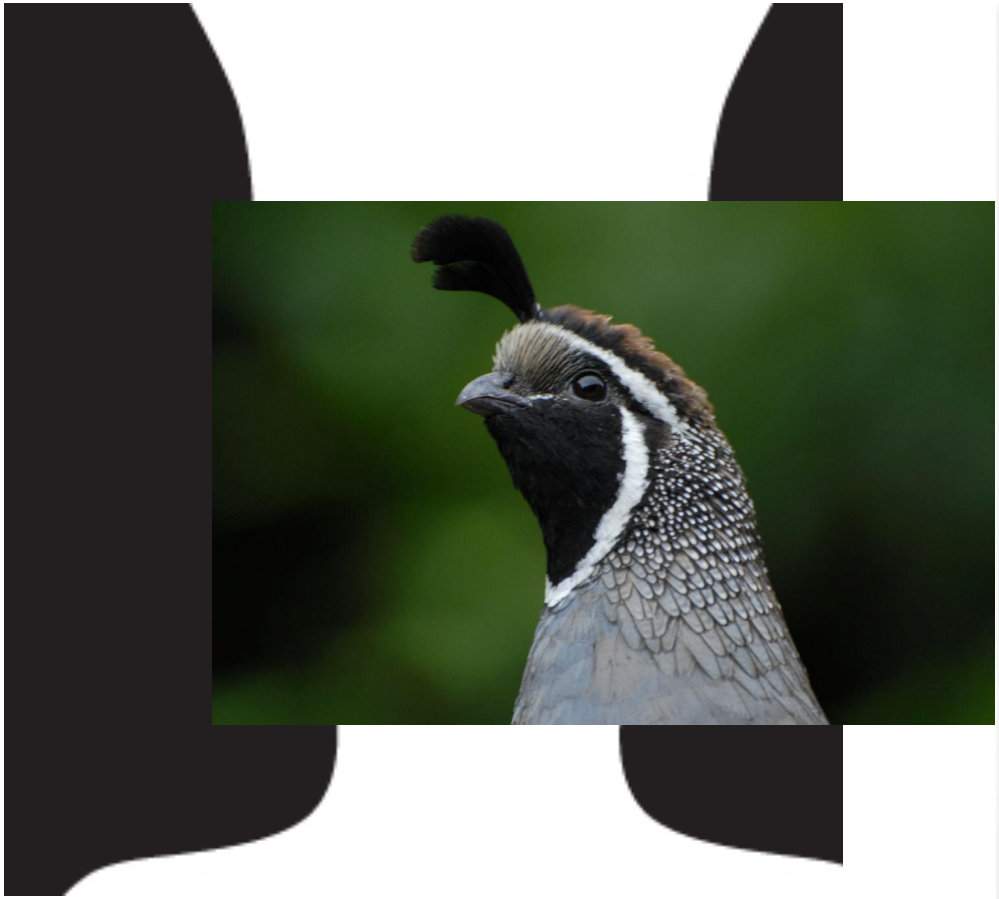
- Image Formation
- ImageJ
- Understanding your Image
- Some analysis
- Practical

Image Formation

‘A picture is worth a thousand + words’ - pearls of wisdom.

Weatherall Institute of Molecular Medicine

Image formation and content

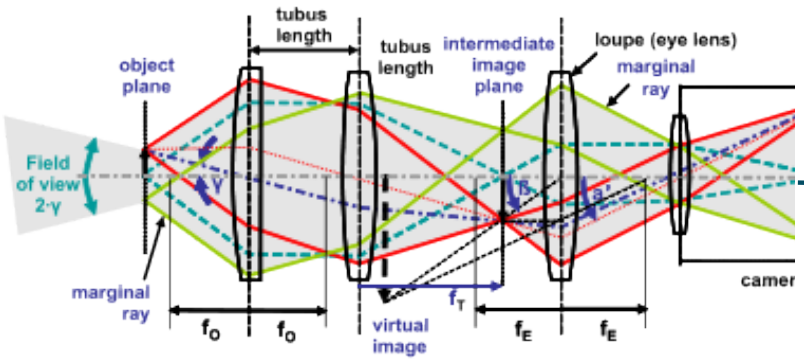


- We as humans naturally try and put things into categories based on our conditioning.

Source: http://blog.deannaknippling.com/?p=2424

Detection of light through microscopy

Optics



Filter Wheel

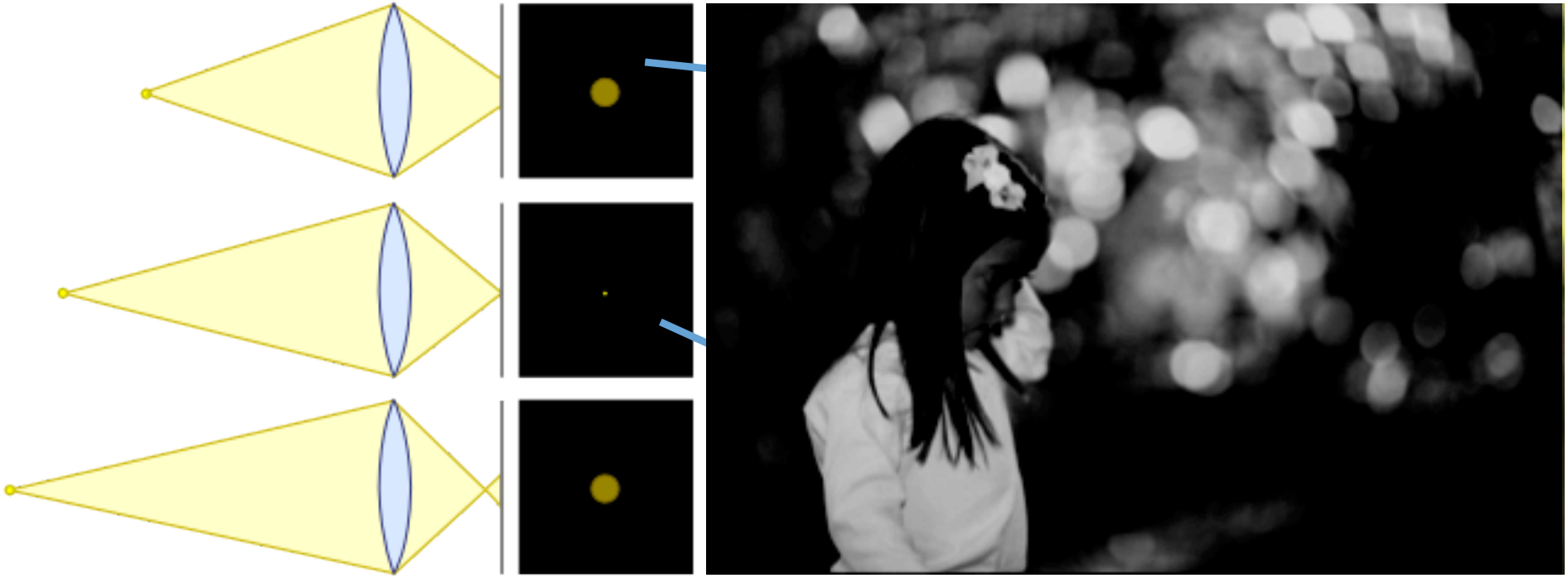
CCD

PMT

- The detection equipment determines much about how light is detected, the wavelengths and the quantities.

Source: http://en.wikipedia.org/wiki/File:CCD.jpg, http://www.hamamatsu.com/us/en/R11540.html, Tim Weyrich

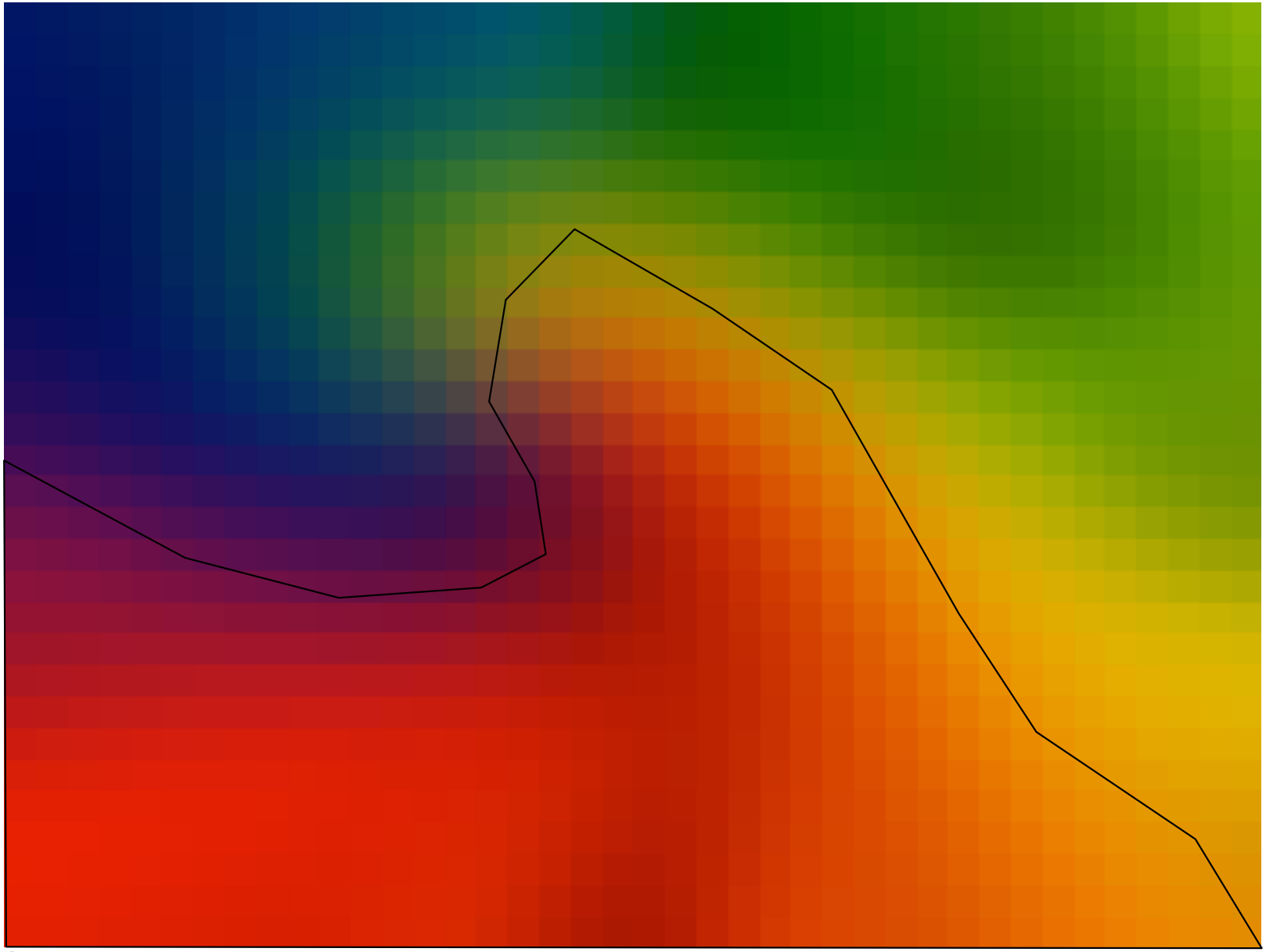
What is an image?



•A typical photo or wide-field microscopy image contains light from the focal plane (girl) as well as from behind and in front of the focal plane.

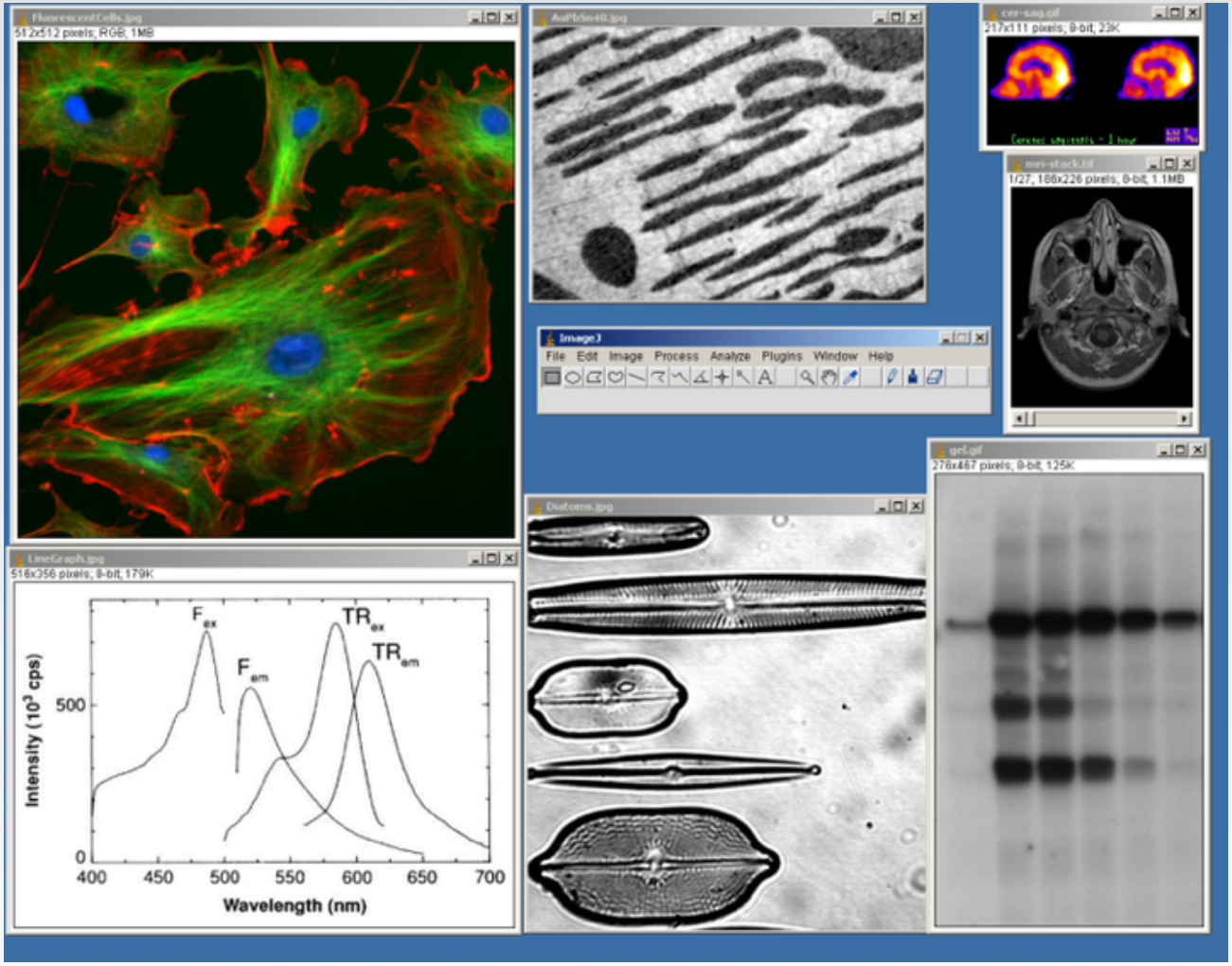
Source: http://upload.wikimedia.org/wikipedia/commons/8/8a/Josefina_with_Bokeh.jpg

continuous world to discrete computer



Source: www.colourvision.co.za

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.

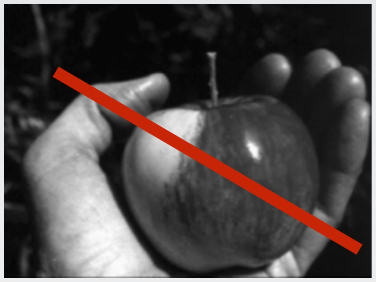
Source: Source: http://fiji.sc/Fiji

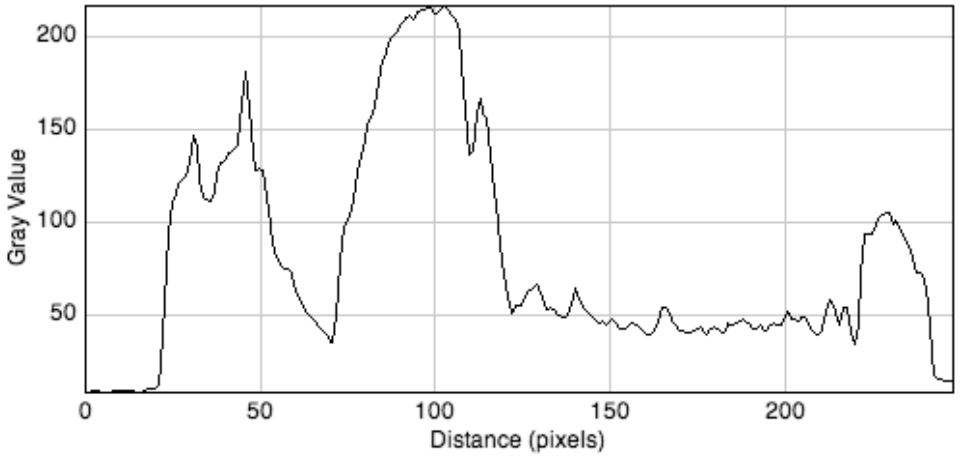
Image representation

Grayscale Computer images are 2D arrays of numbers:

[93],	[23],	[23],	[155],	[155],	[155]
[107],	[**198**],	[**198**],	[140],	[140],	[140]
[121],	[11],	[11],	[7],	[7],	[7]
[135],	[**235**],	[**235**],	[198],	[198],	[198]
[149],	[114],	[114],	[**213**],	[**213**],	[**213**]
[163],	[**187**],	[**187**],	[9],	[9],	[9]
[8],	[80],	[80],	[150],	[150],	[150]
[22],	[**187**],	[**187**],	[20],	[20],	[20]
[16],	[165],	[165],	[111],	[111],	[111]
[158],	[15],	[15],	[34],	[34],	[34]
[**200**],	[120],	[120],	[69],	[69],	[69]

Global stats: min, max, mean, median values.





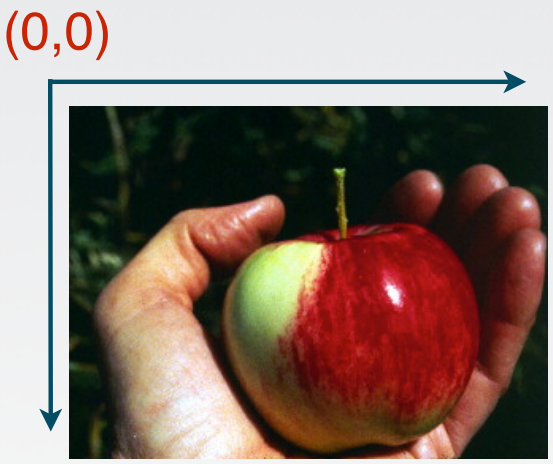
Calculate intensity values along a line or within a region.

Image spatial quantisation

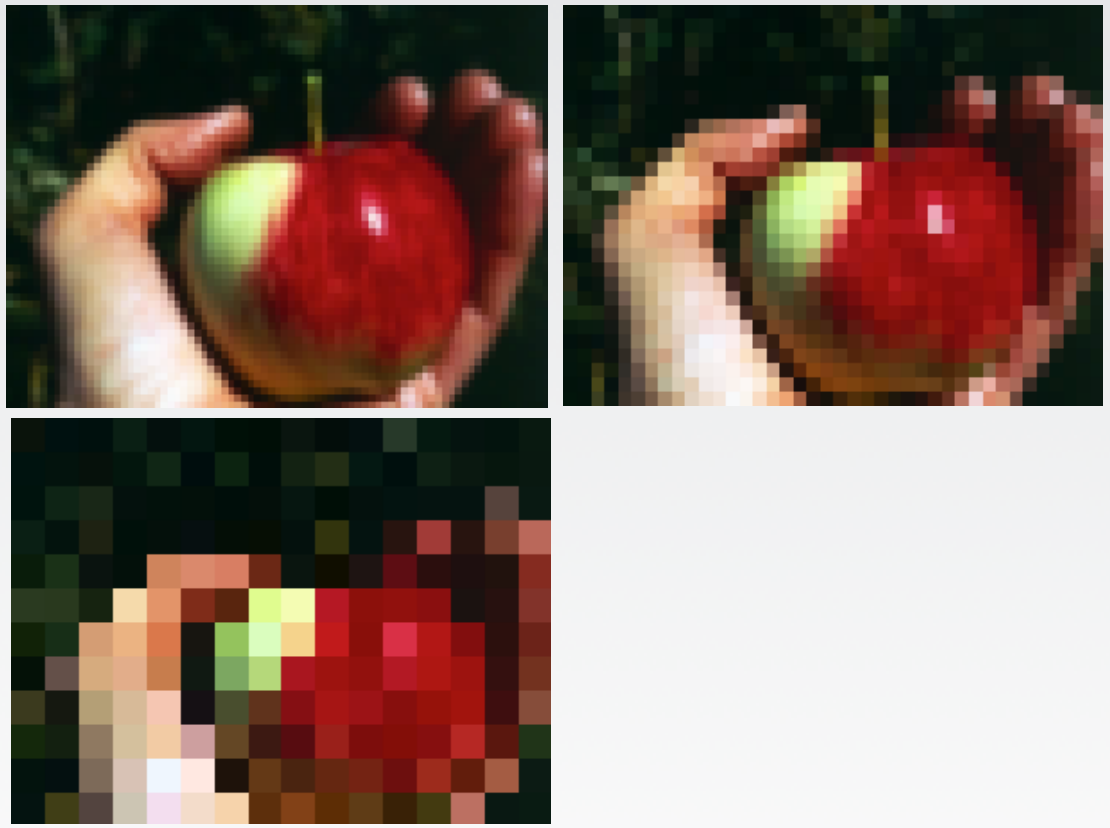
Digital representation.

Co-ordinate system

of image.



Resolution (no. pixels x,y).
Spatial quantisation



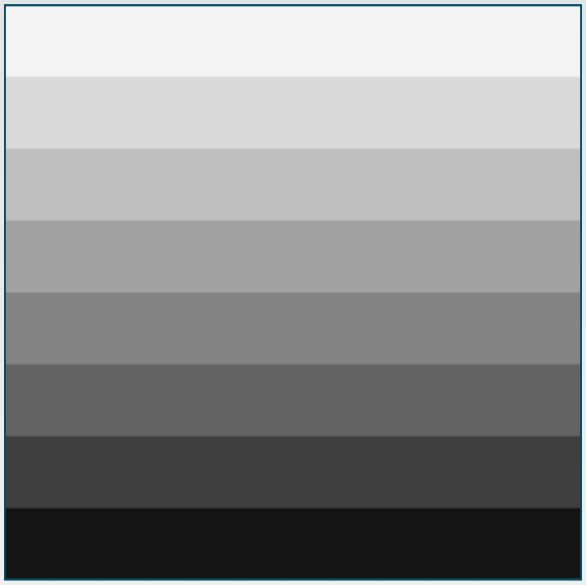
Source: http://en.wikipedia.org/wiki/File:95apple.jpeg

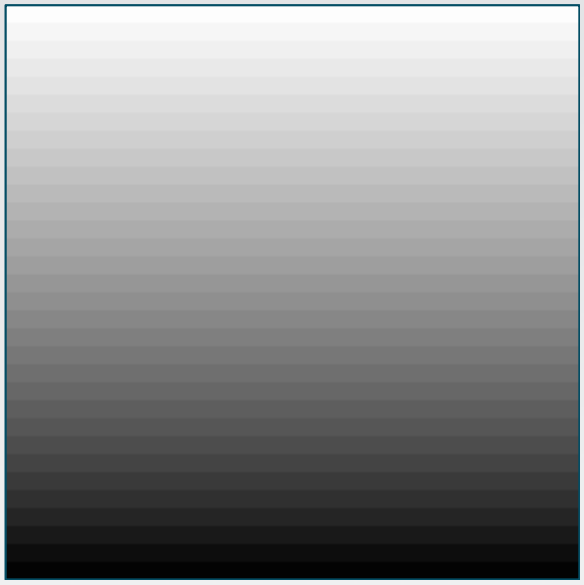
Image intensity quantisation

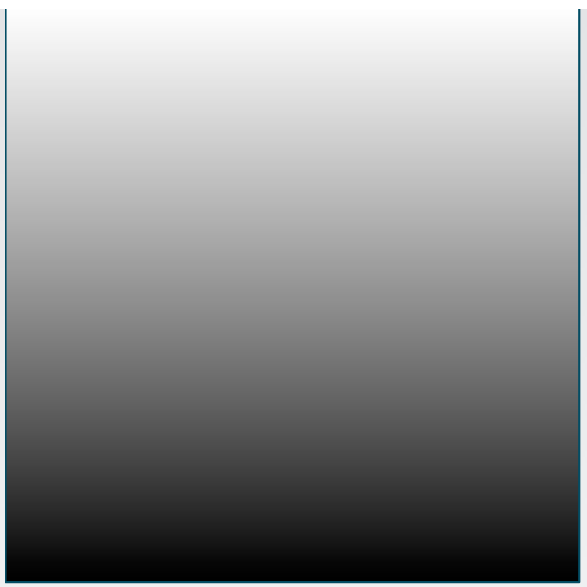
3-bit (2^3) 8 levels

5-bit (2^5) 32 levels

8-bit (2^8) 256 levels
16-bit (2^16) 65536 levels

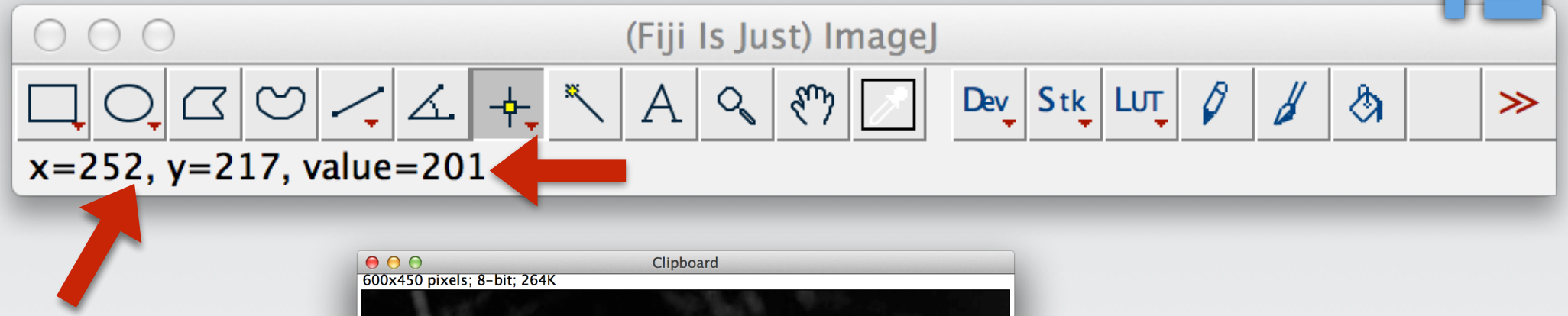






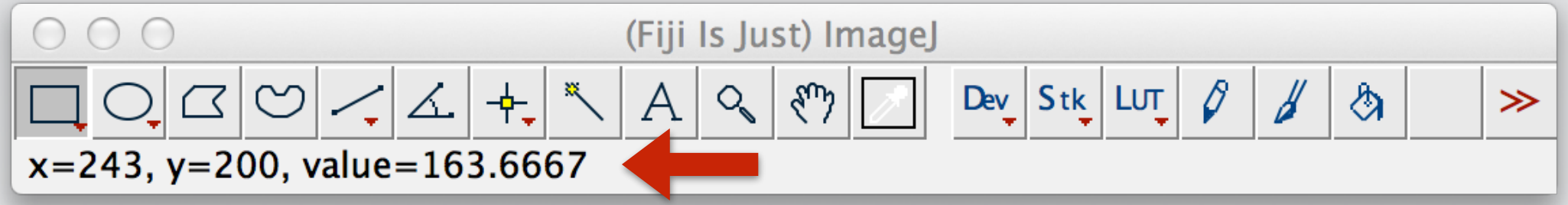
In reality most digital monitors can only show up to 8-bit.
This is why 8-bit and 16-bit images appear the same on a monitor.

Image representation



Inspecting your images is quick and easy using ImageJ/Fiji

Floating point numbers

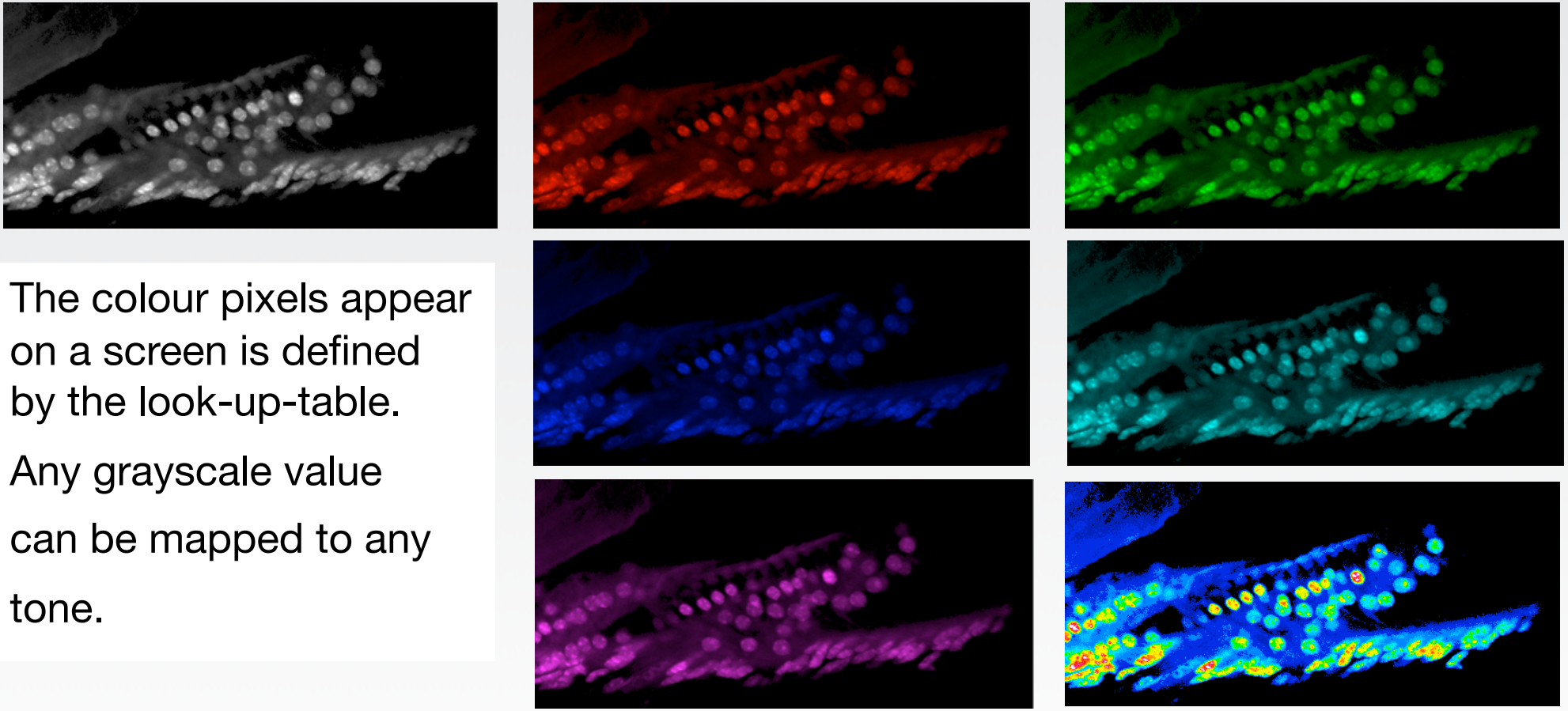


32-bit images represent floating point numbers. Useful for image maths.

Sour

Image intensity

Pixel values when visualised on a screen can be coloured in a number of different ways, independent of the actual pixels values.



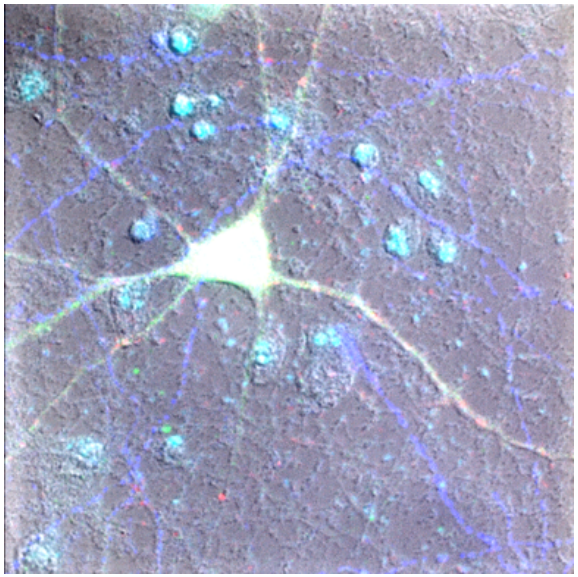
The colour pixels appear on a screen is defined by the look-up-table.

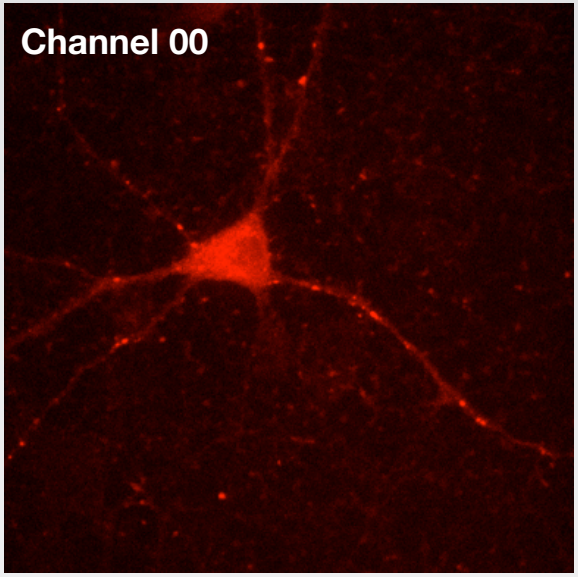
Any grayscale value can be mapped to any tone.

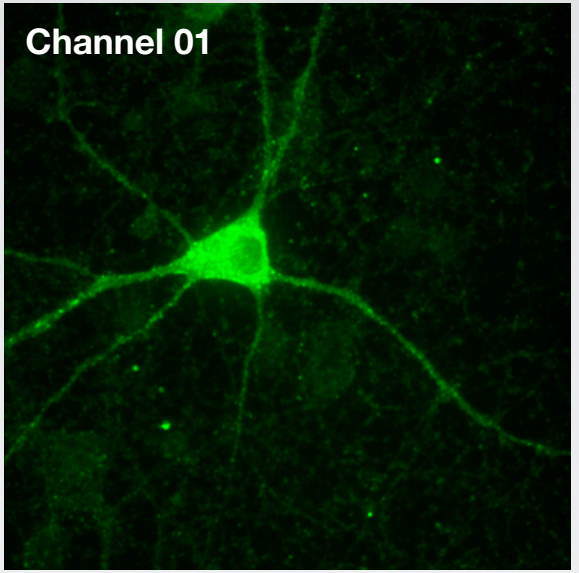
Source: Fiji, organ-of-corti.tif

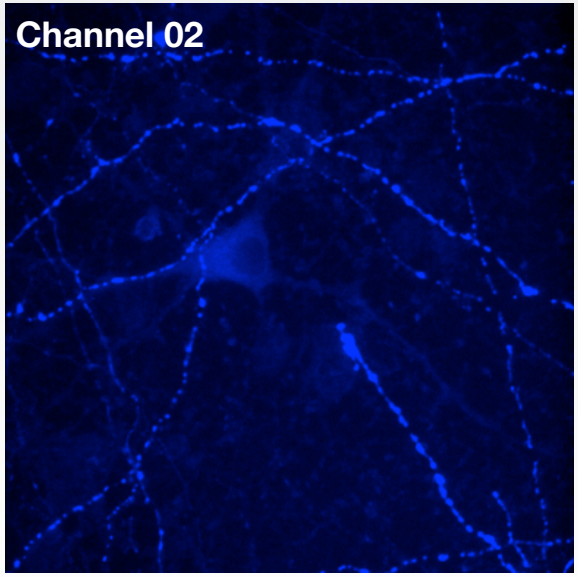
Image representation

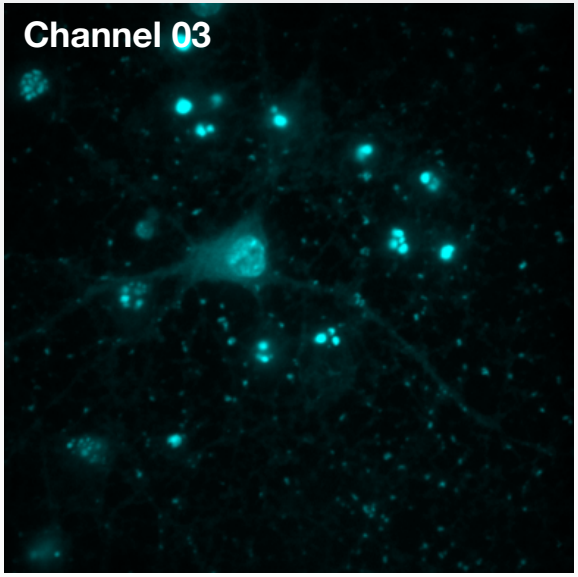
Composite representation

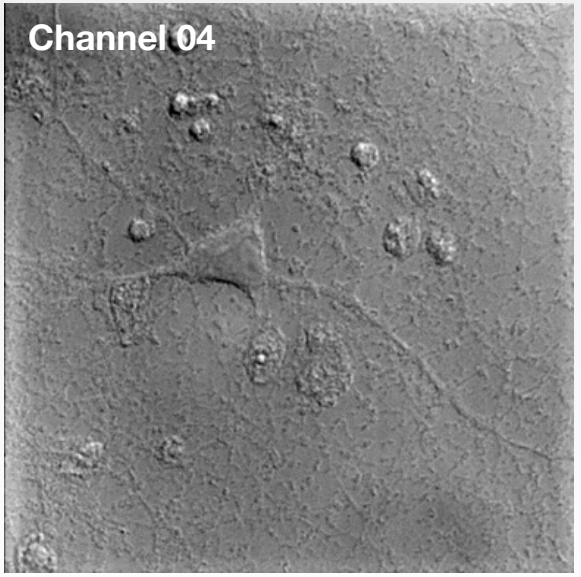








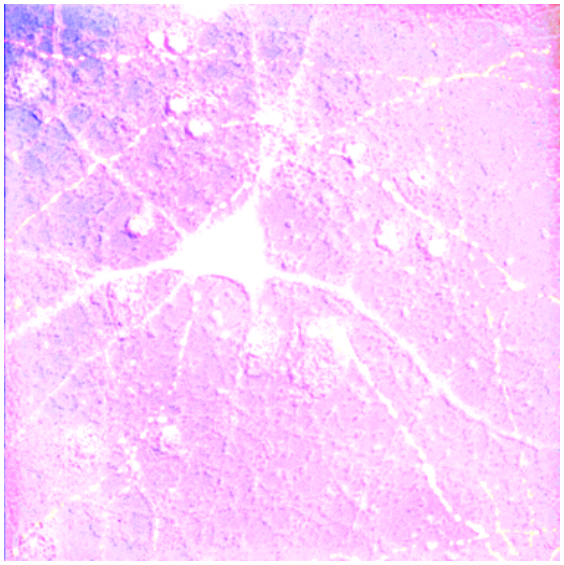


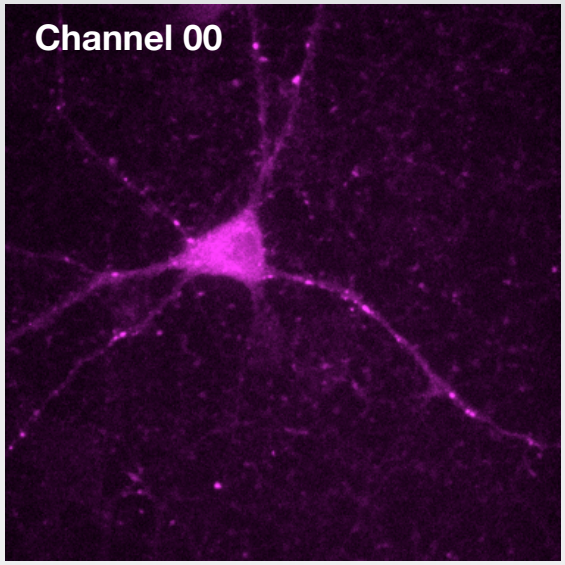


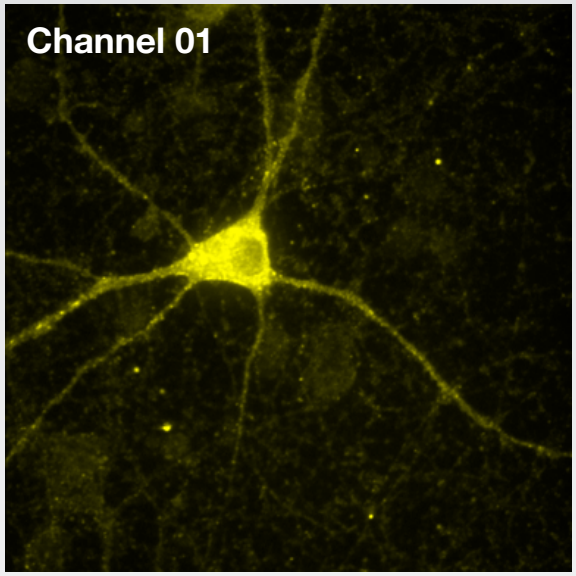
Source: ImageJ Rat_Hippocampal_Neuron.tif.

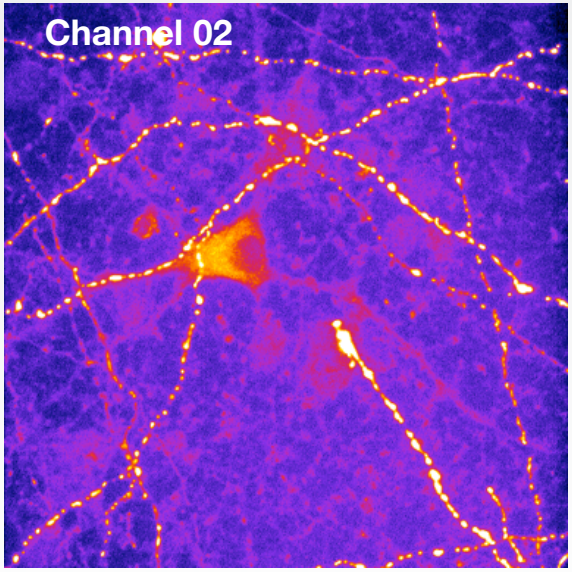
Image representation

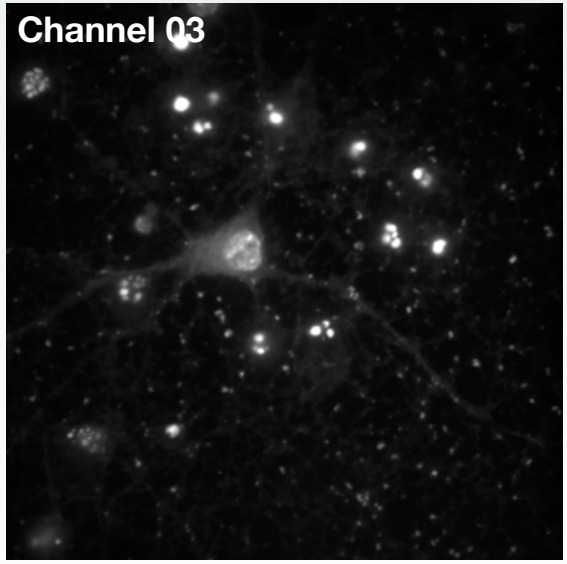
Composite representation

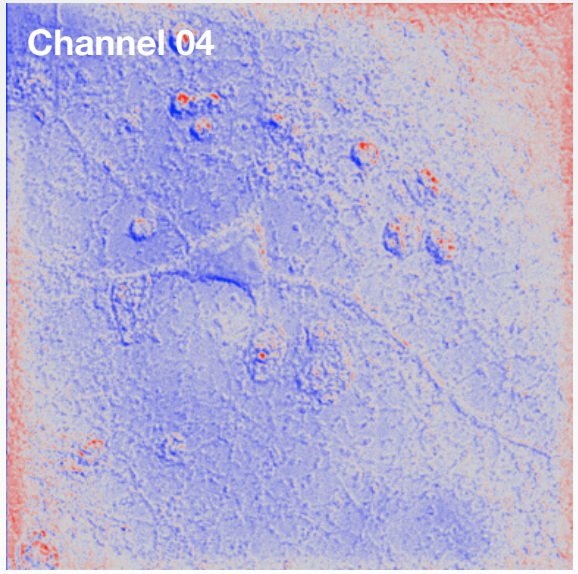










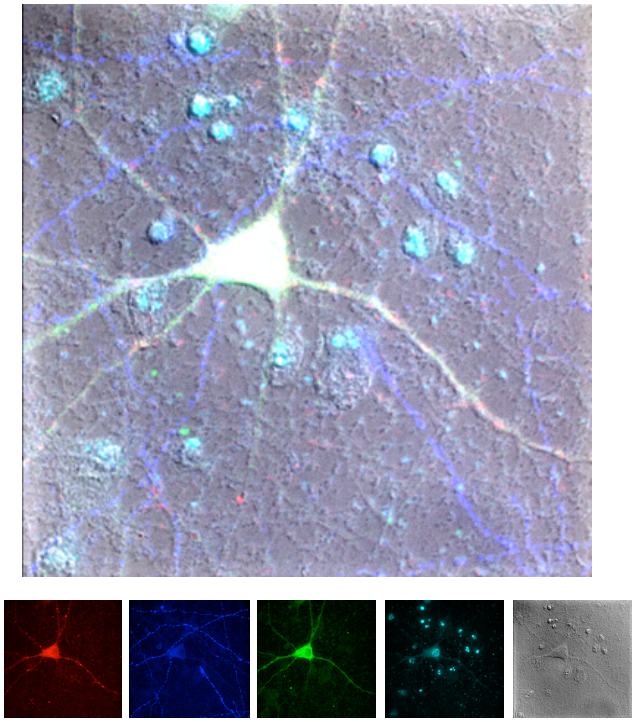


Source: ImageJ Rat_Hippocampal_Neuron.tif.

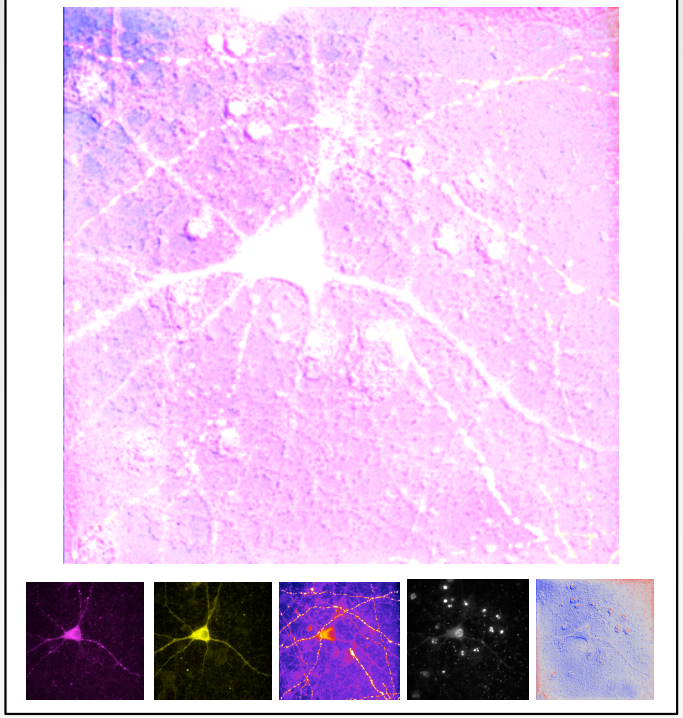
Image inspection

Same information just represented with different colours

Composite representation



Composite representation



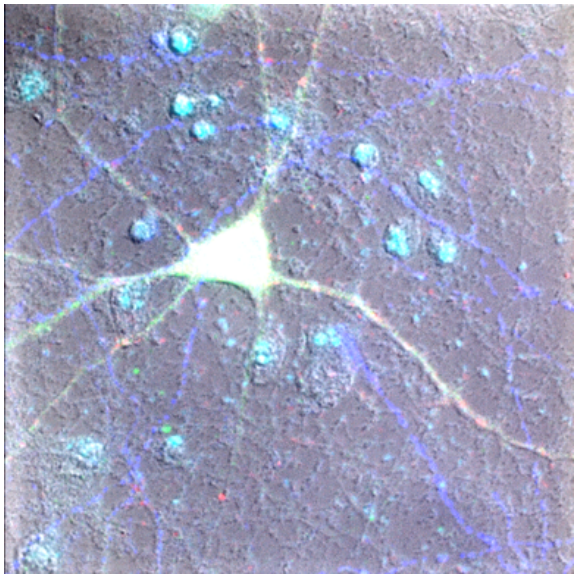
Remember to save in TIFF format if you have many channels (multi-page format).
Don't be tricked by the colour of something. Use Fiji to inspect.

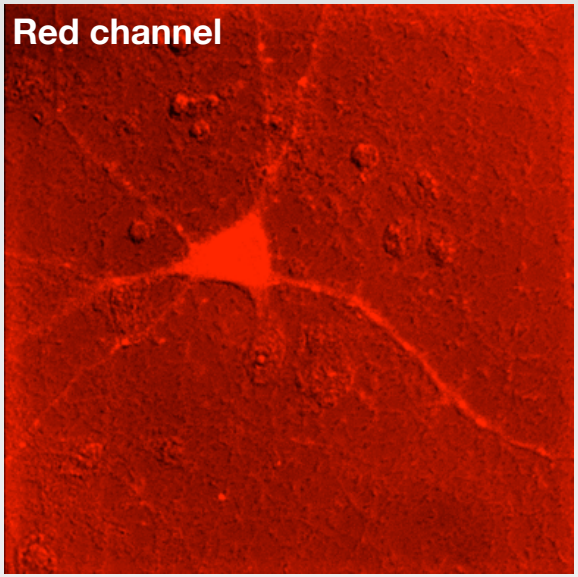
Source: http://fiji.sc/Fiji

Image -> color -> Channels Tool

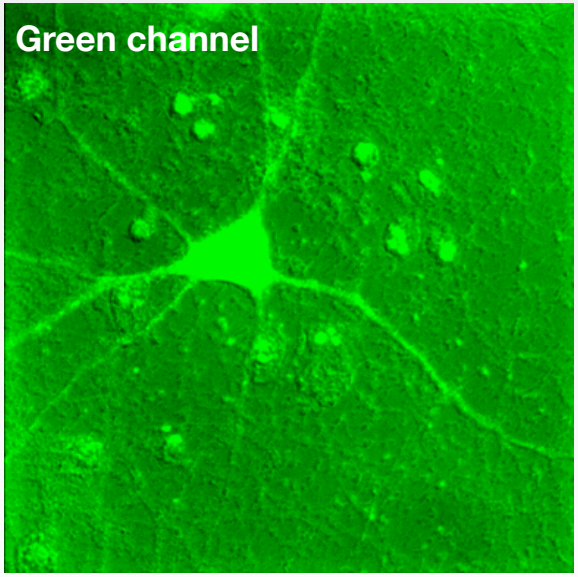
Image representation

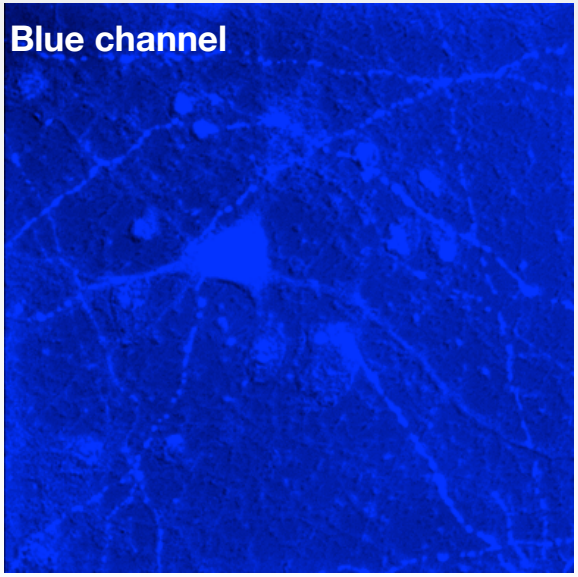
RGB representation





If saved as a conventional image file (e.g. png or jpeg). The image intensity values are distributed amongst the red green and blue channels.



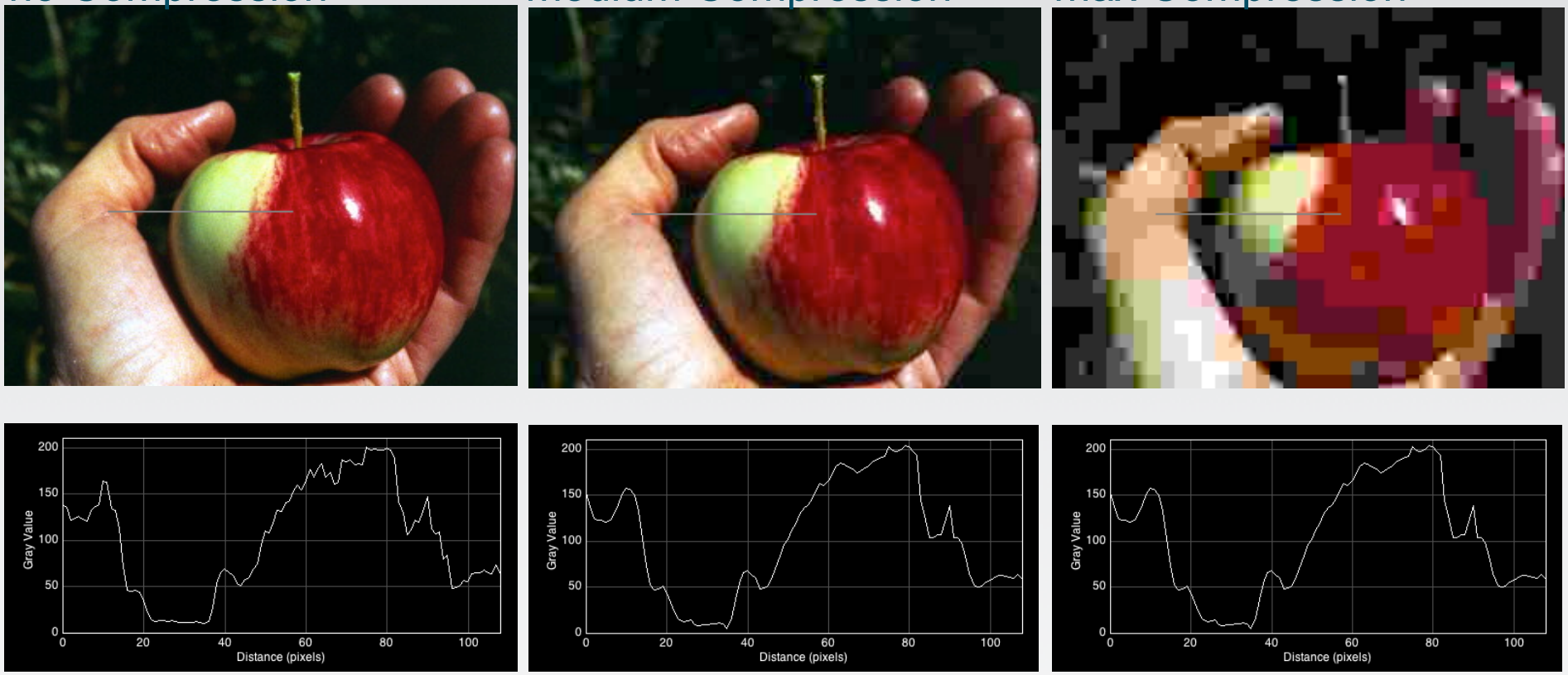


The intensity attributed to each channel is defined by the look-up-table. This is fine for making a picture or figure but bad for analysis as information is mixed and lost.

Source: ImageJ Rat_Hippocampal_Neuron.tif.

Imaging pitfalls: Compression

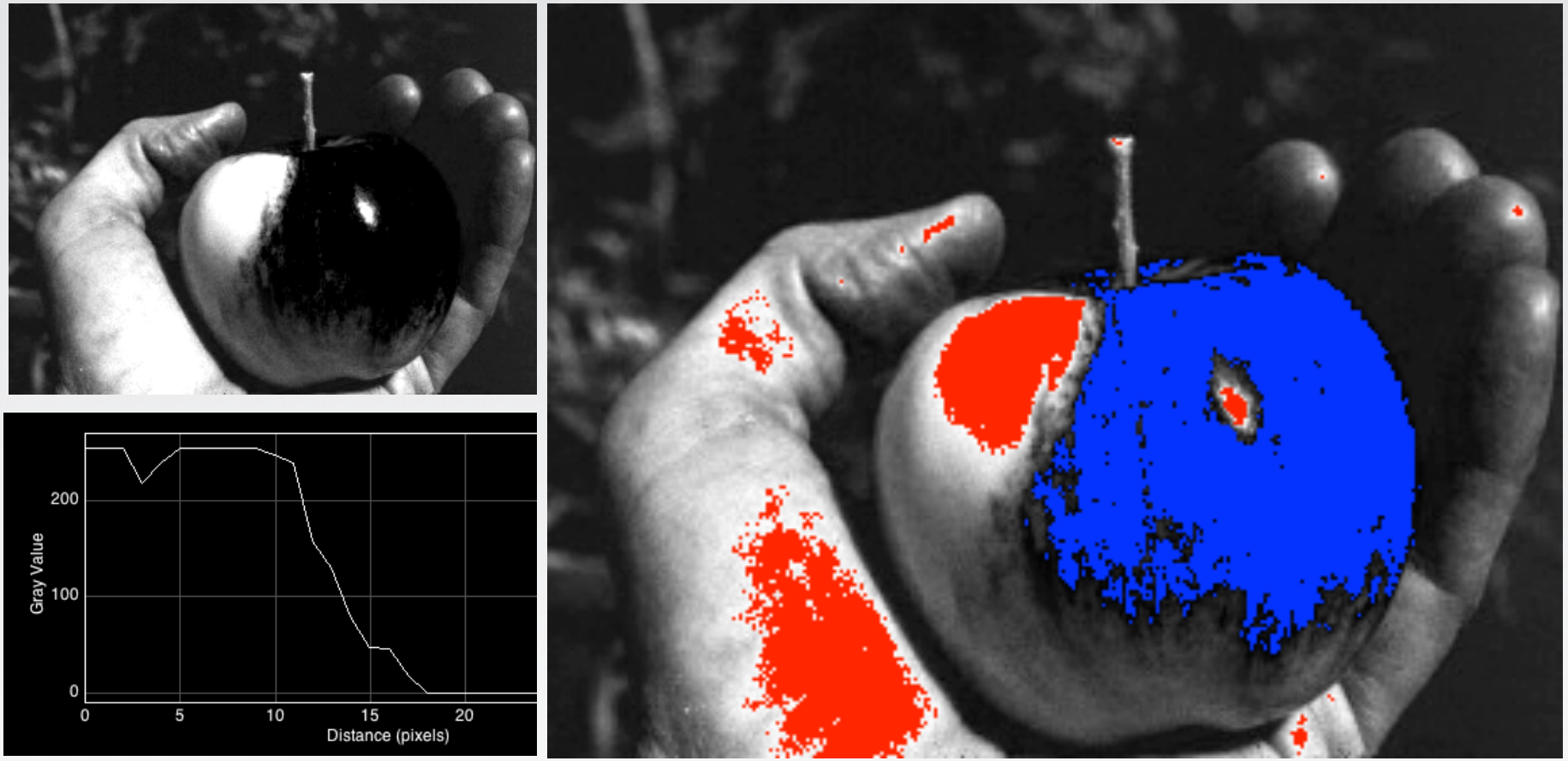
no Compression | medium Compression | max Compression



JPEG, movie formats = LOSSY & BAD (BMP, GIF, PNG also not great)
Compression also reduces certain colours more than others (e.g. blue tones).
TIFF is good, especially OME-TIFF

Source: http://upload.wikimedia.org/wikipedia/commons/c/ce/Quality_comparison_jpg_vs_saveforweb.jpg

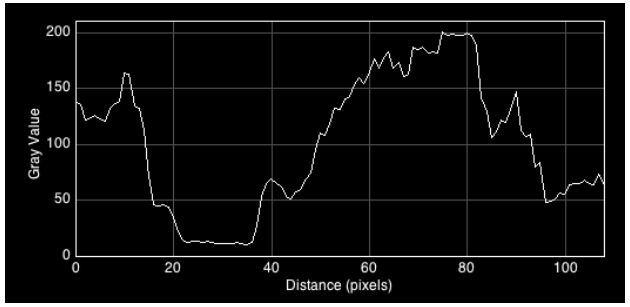
Saturation and zero-pixels



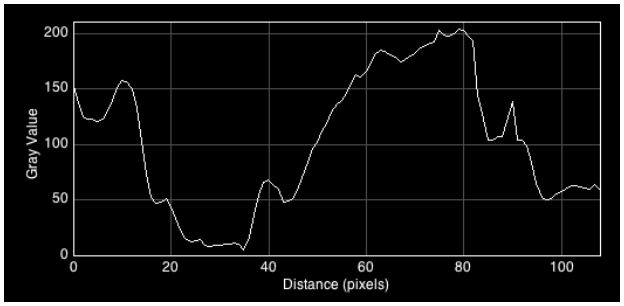
Saturated image pixels in red. Clipped ‘0‘ values in blue.

Imaging pitfalls: Compression

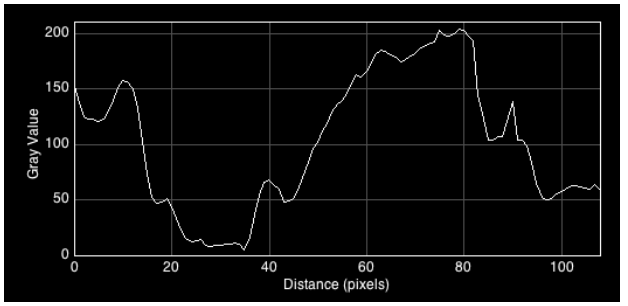
no Compression



medium Compression



max Compression



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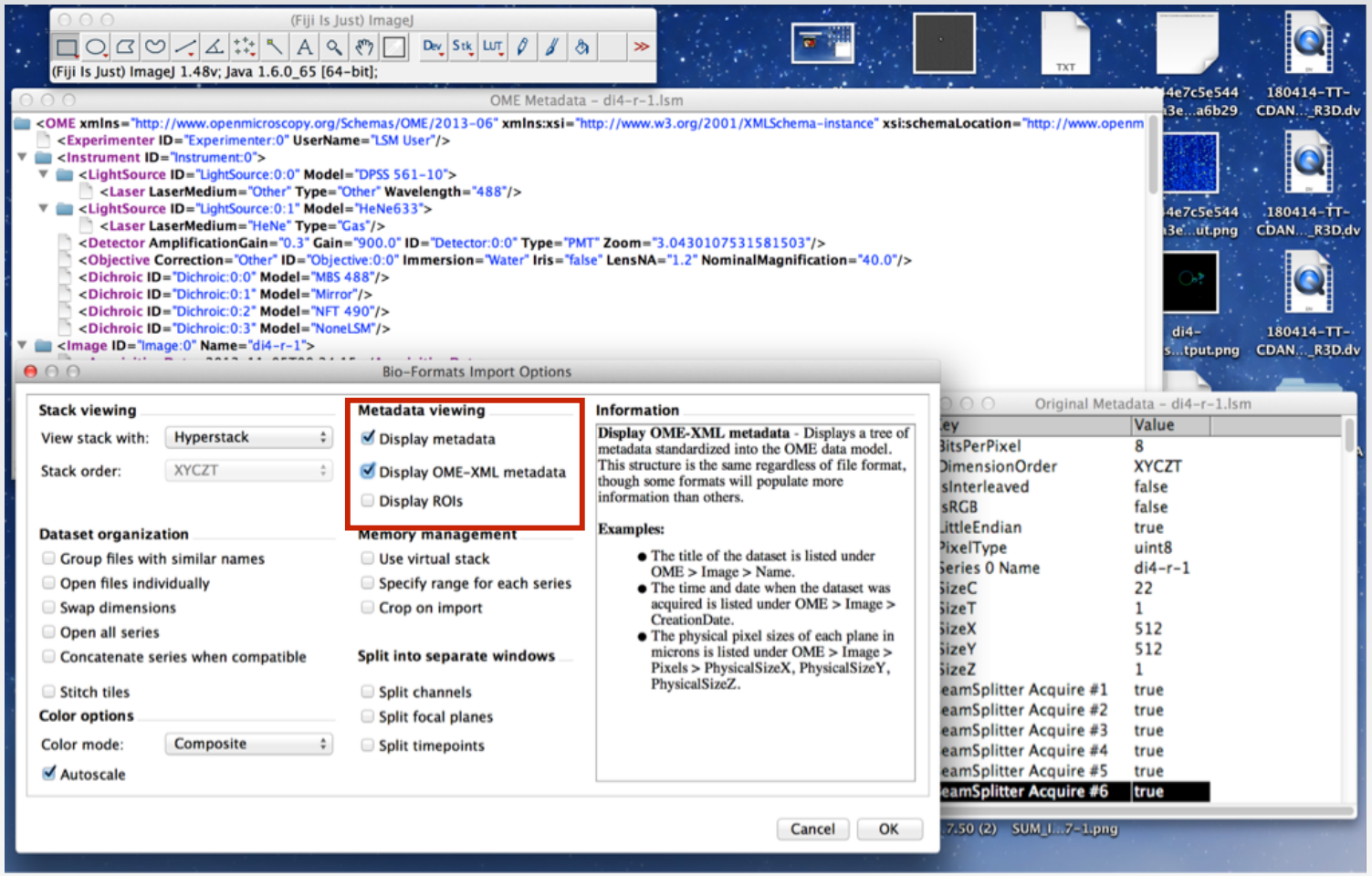
Compression also reduces certain colours more than others (e.g. blue tones).
TIFF is good, especially OME-TIFF

Source: http://upload.wikimedia.org/wikipedia/commons/c/ce/Quality_comparison_jpg_vs_saveforweb.jpg

Accessing metadata



Metadata can be found when you import images to Fiji.

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

Do nots.

- 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.

Ultimately there are no rights and wrongs.

use linear filters

don’t saturate

use background subtr

use non-linear filters

remove no

Use Pearson test

each c

don’t subtract background

don’t use a threshold

Don’t a

sample selectively

Don’t

remove noise

set Threshold manu

sample randomly

each i

don’t remove nois

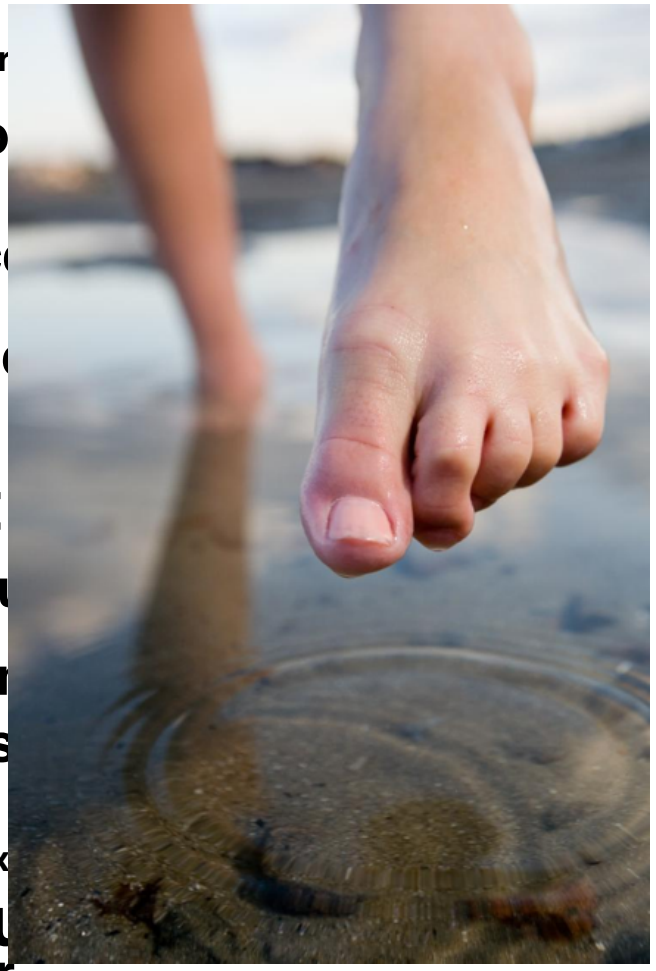
set Threshold automatically

each ex

controls are a mu

use any filter

Adjust intensity range

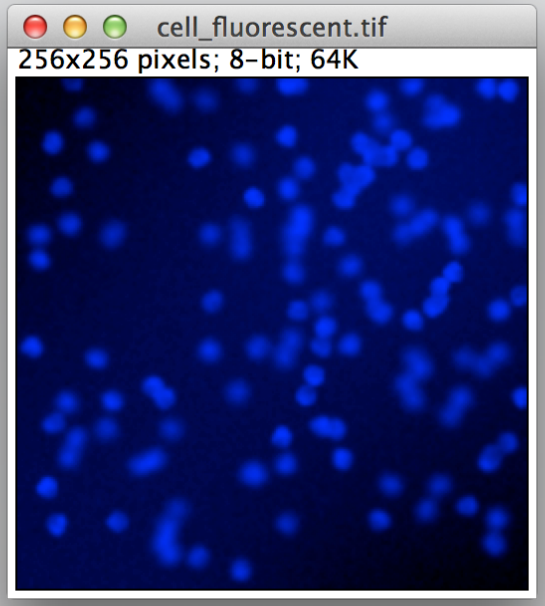


Second Lecture:
Typical analysis approaches using imageJ/FIJI

Source: http://www.wired.com/underwire/2013/10/gravity-future-3d-movies/

Segmentation and morphological operations

Thresholding in ImageJ/Fiji



8-bit original

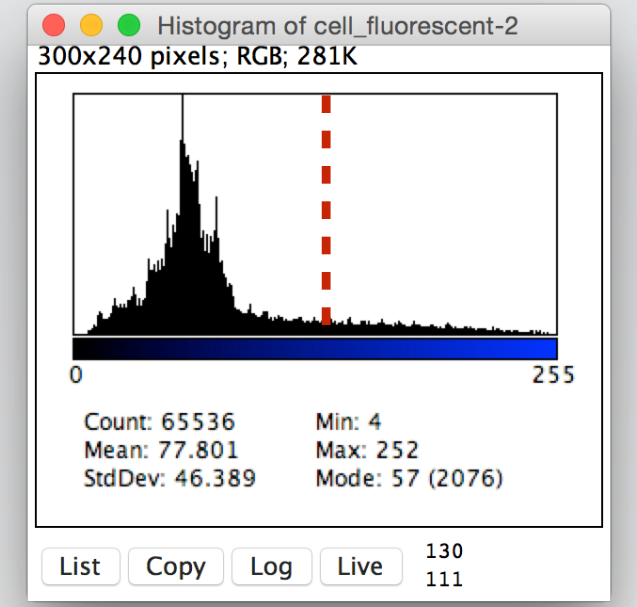
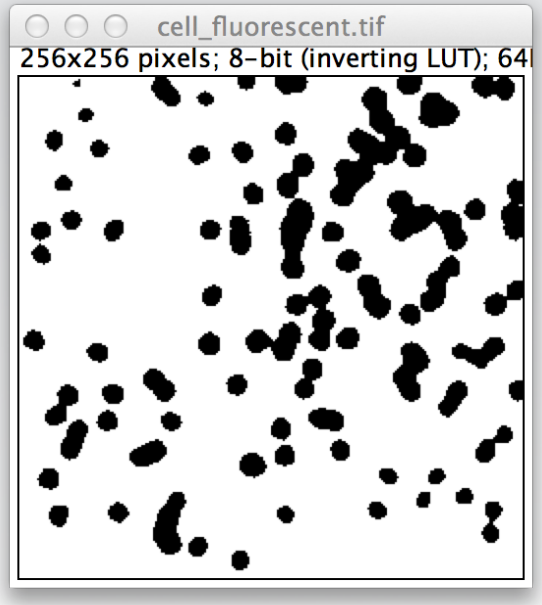


Image histogram

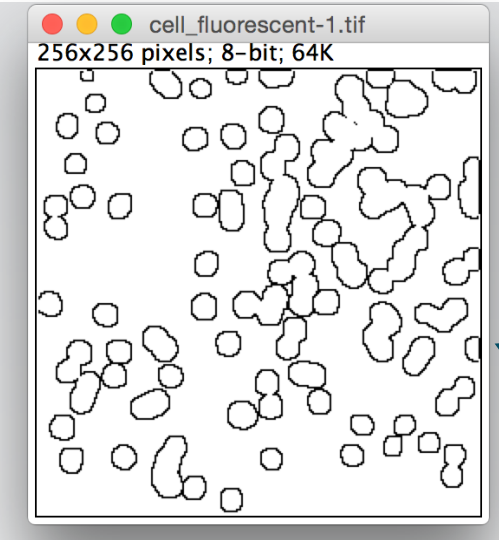


Thresholded (e.g. Otsu)

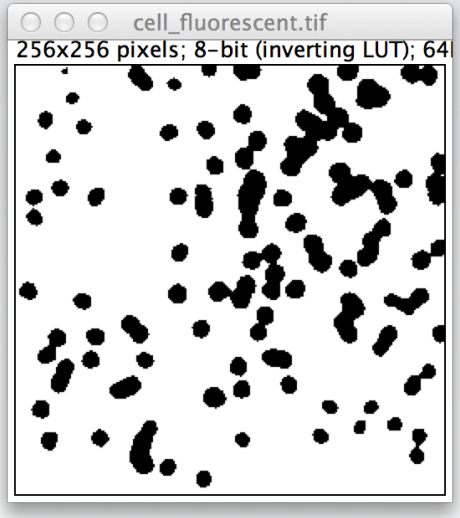
In Fiji you can set a static threshold or choose an algorithm to predict the best partition of the input data. When analysing multiple images in a dataset keep the setting the same, or failing that use the same method (e.g. Otsu).

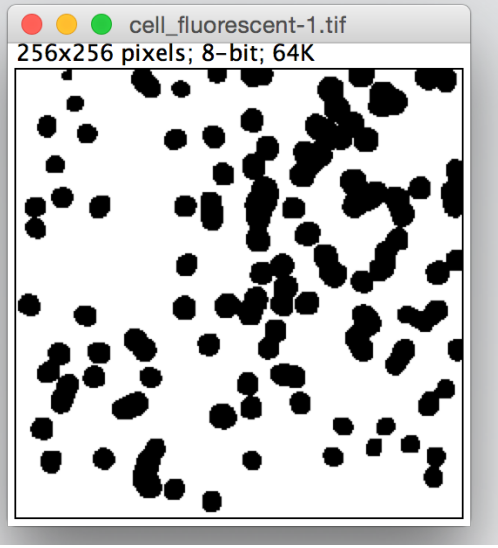
Source:

Discrete morphological operators

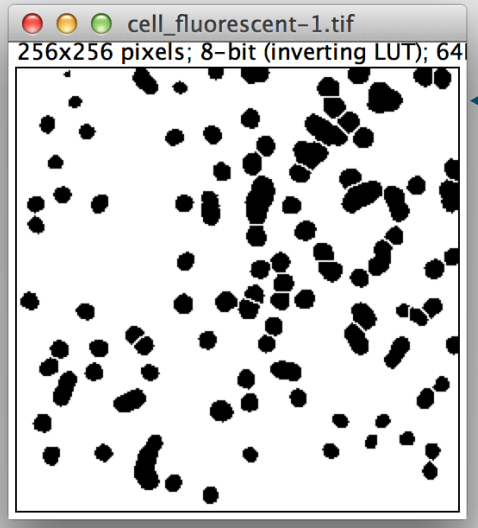


Outline: Find outline

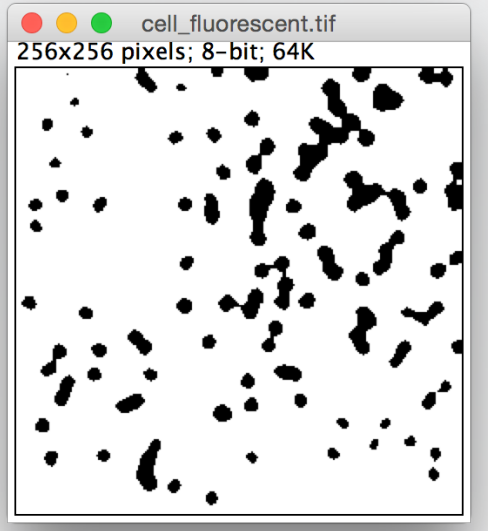




Erosion: Enlarge blobs

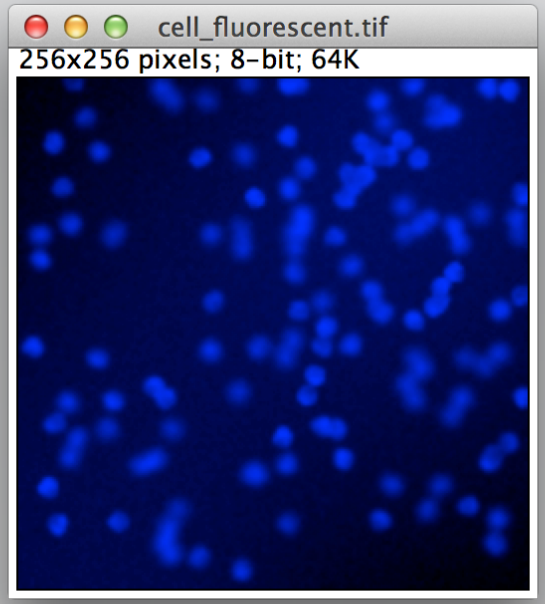


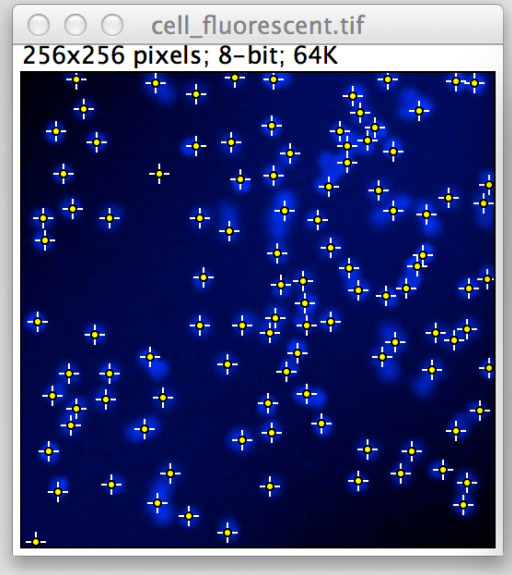
Watershed: Splits blobs

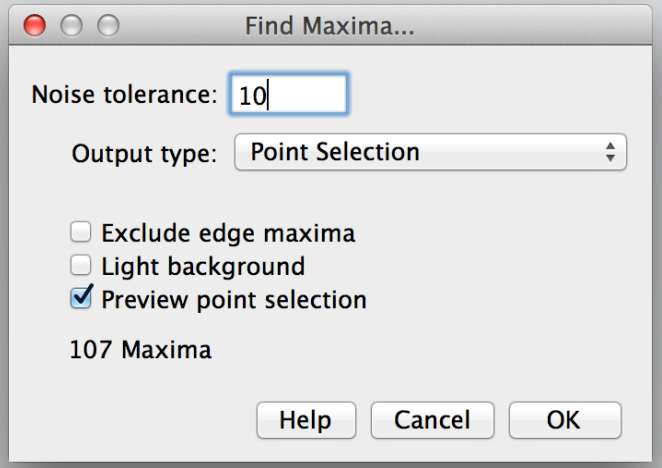


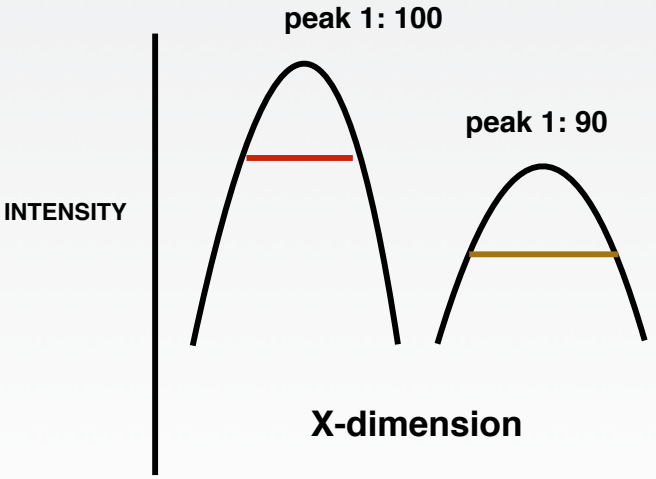
Erosion: Shrink blobs

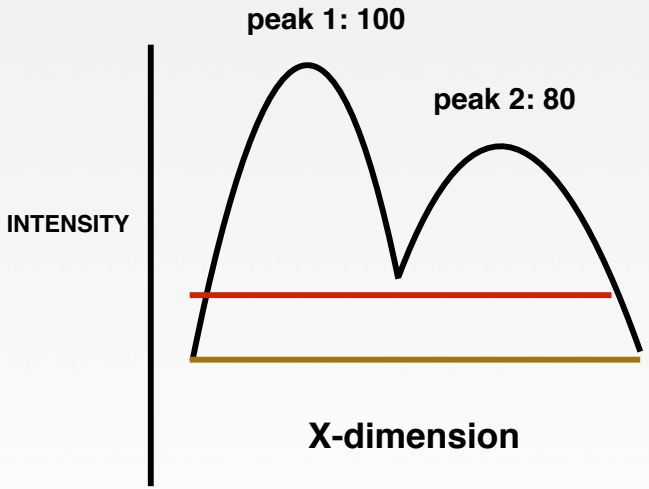
2d peak finding: Process -> Find Maxima

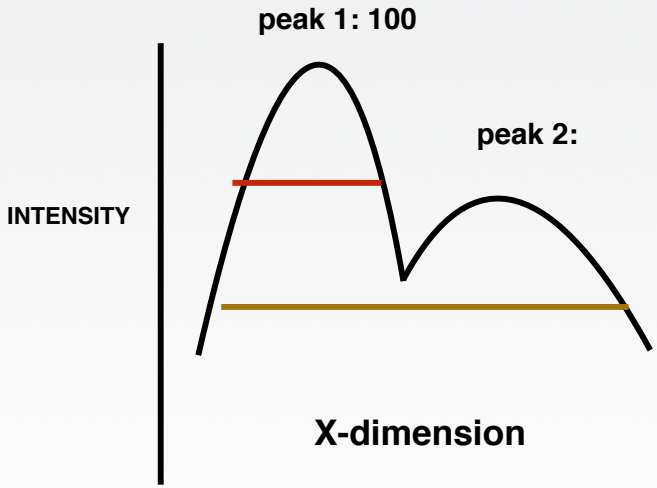










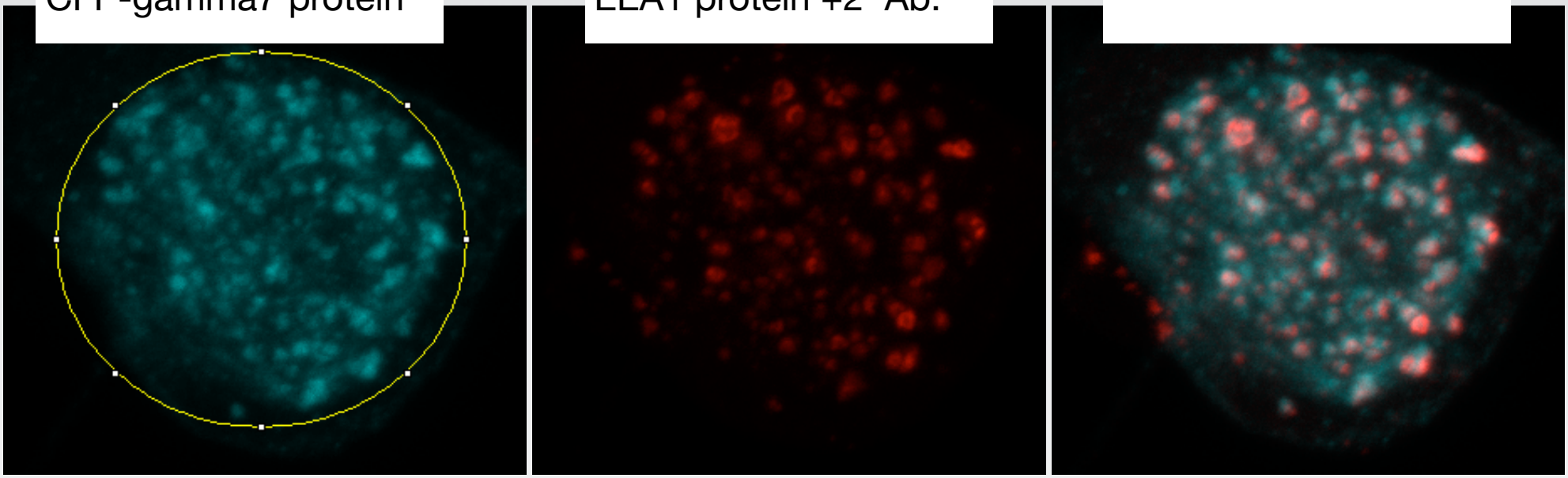


Colocalization

CFP channel
CFP-gamma7 protein

RED channel
EEA1 protein +2^0 Ab.

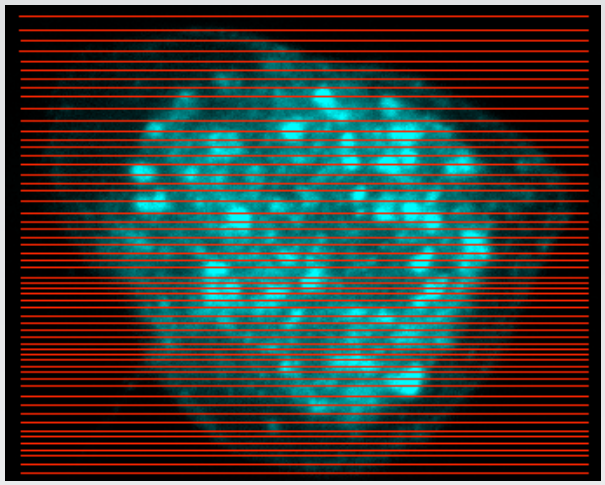
Merge image



Want to find a measure of similarity between the expression patterns of the two proteins within this cell. For this we use correlation (colocalization) to compare the intensity distribution in the different channels and measure the similarity of the two distributions.

Source:

Image (2d array) to list (1d)



=

[93],	[23],	[93],	[155],	[155],	[155]
[107],	[**198**],	[107],	[140],	[140],	[140]
[121],	[11],	[121],	[7],	[7],	[7]
[135],	[**235**],	[135],	[198],	[198],	[198]
[149],	[114],	[149],	[**213**],	[**213**],	[**213**]
[163],	[**187**],	[163],	[9],	[9],	[9]
[8],	[80],	[8],	[150],	[150],	[150]
[22],	[**187**],	[22],	[20],	[20],	[20]
[16],	[165],	[16],	[111],	[111],	[111]
[158],	[15],	[158],	[34],	[34],	[34]
[**200**],	[120],	[**200**],	[69],	[69],	[69]

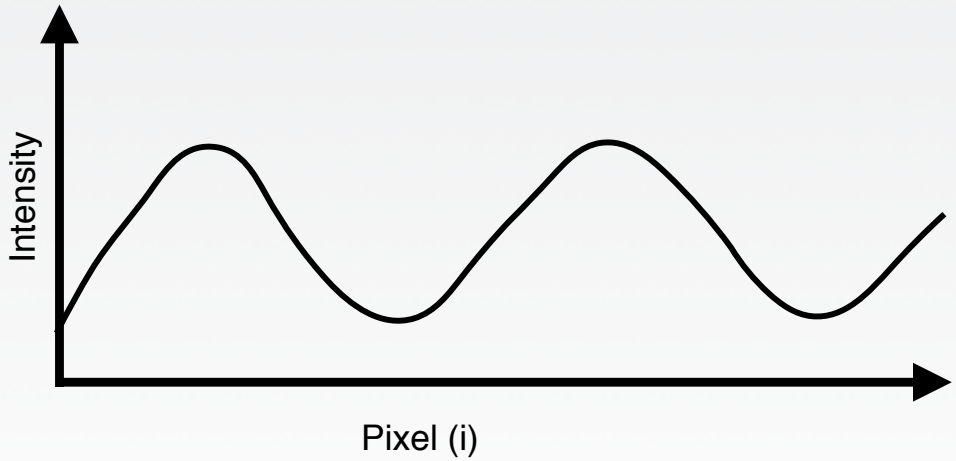
=

155
140
7
198
213
9
150
20
111
34
69

155
140
7
198
213
9
150
20
111
34
69

etc
etc

The software, or with our programming, we take the intensity values and make a long list out of them.



Source:

Pixel based colocalization, the standard

Pearson's equation:

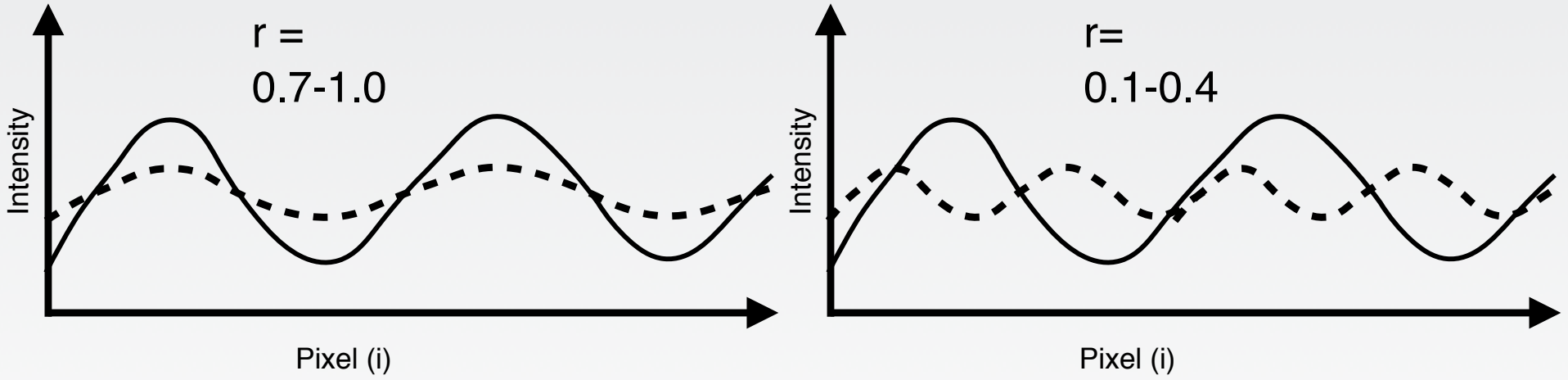
$$r = \frac{\sum \left(R_i - \bar{R}\right) \times \left(G_i - \bar{G}\right)}{\sqrt{\sum \left(R_i - \bar{R}\right)^2 \times \sum \left(G_i - \bar{G}\right)^2}}$$

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.

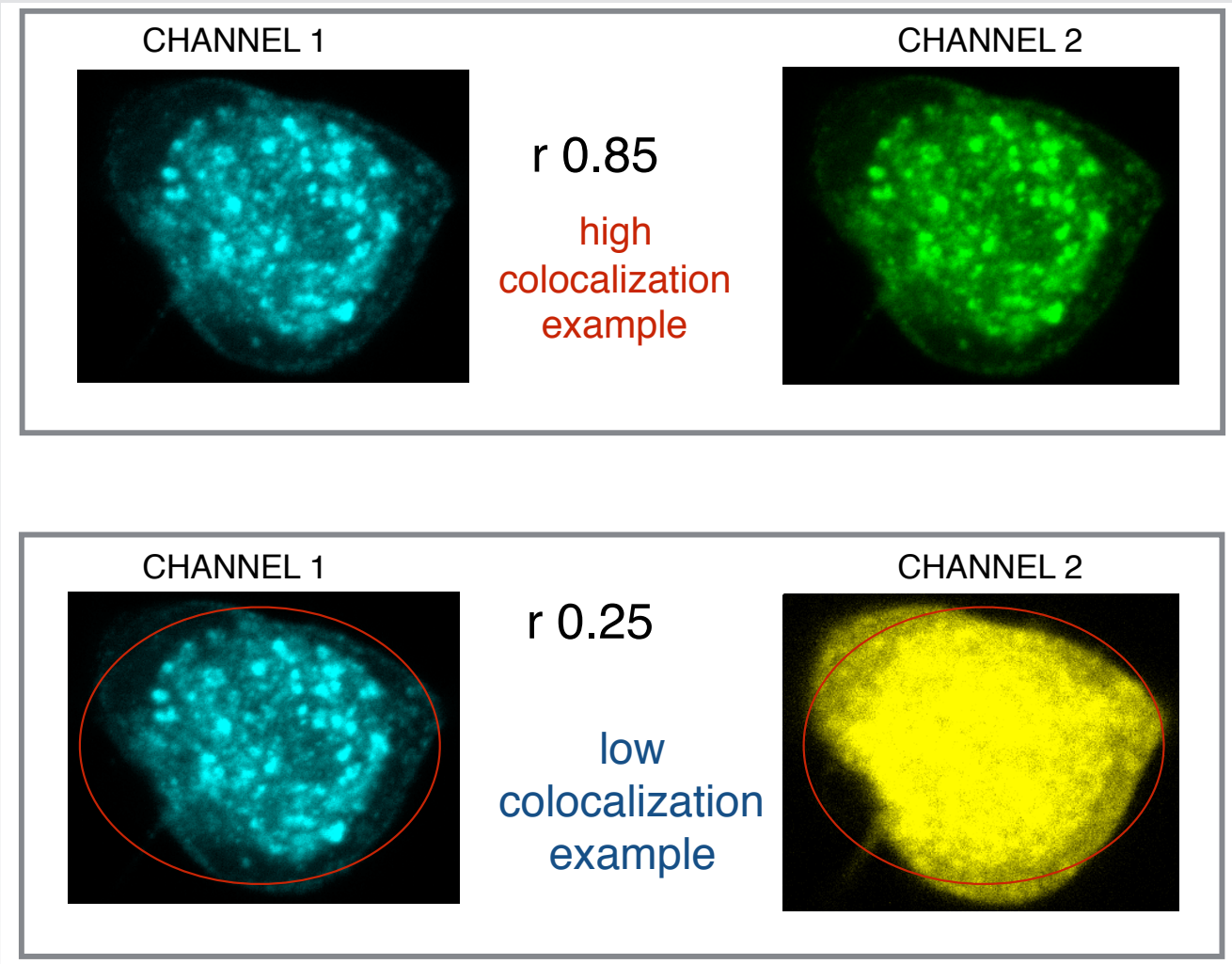
R refers to list of values from one channel, G refers to list from second channel. G or R with a bar refers to mean intensity in that channel. 'i' refers to each pixel in image. Sigma (big E) refers to sum. So sum of all pixels minus their mean.



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

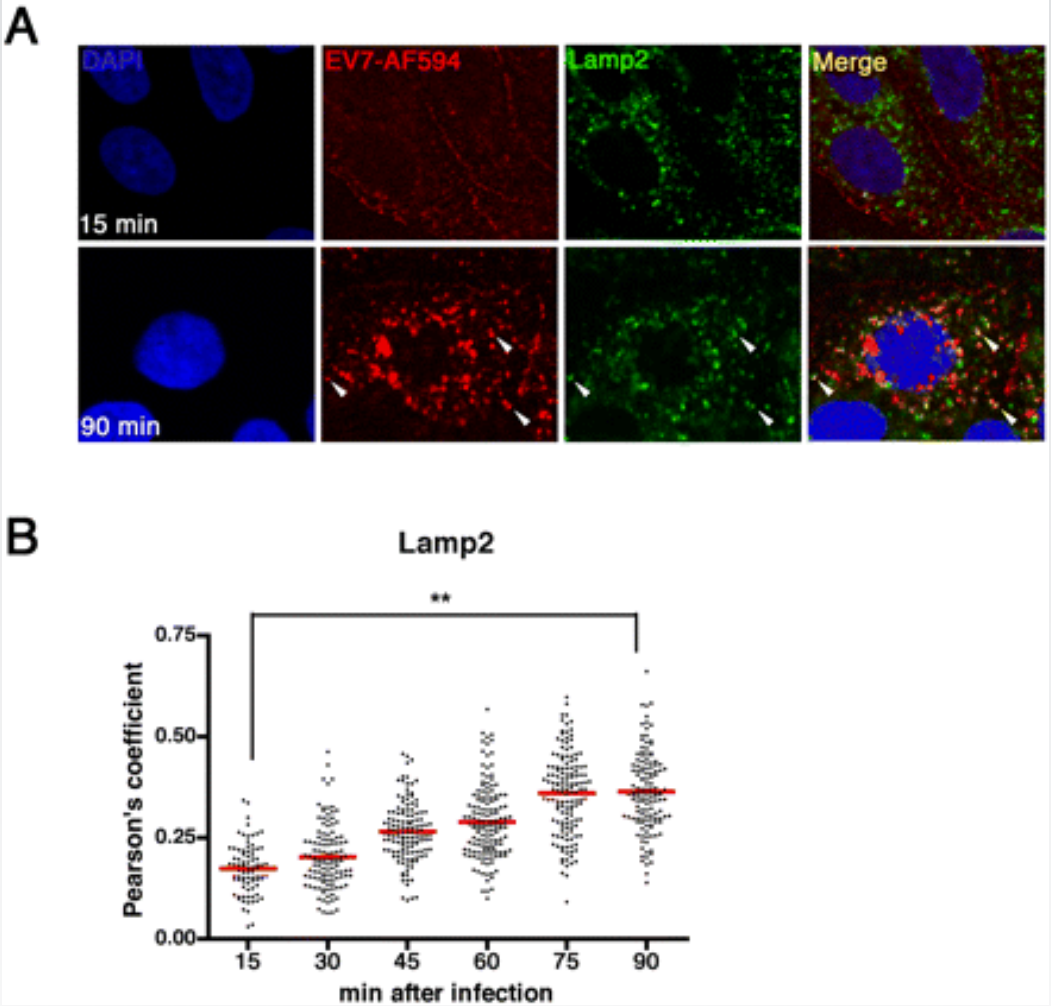
Source: http://en.wikipedia.org/wiki/Correlation_coefficient

Example



Be careful about black/ background pixels, they contribute to the r value and artificially raise the perceived colocalization.

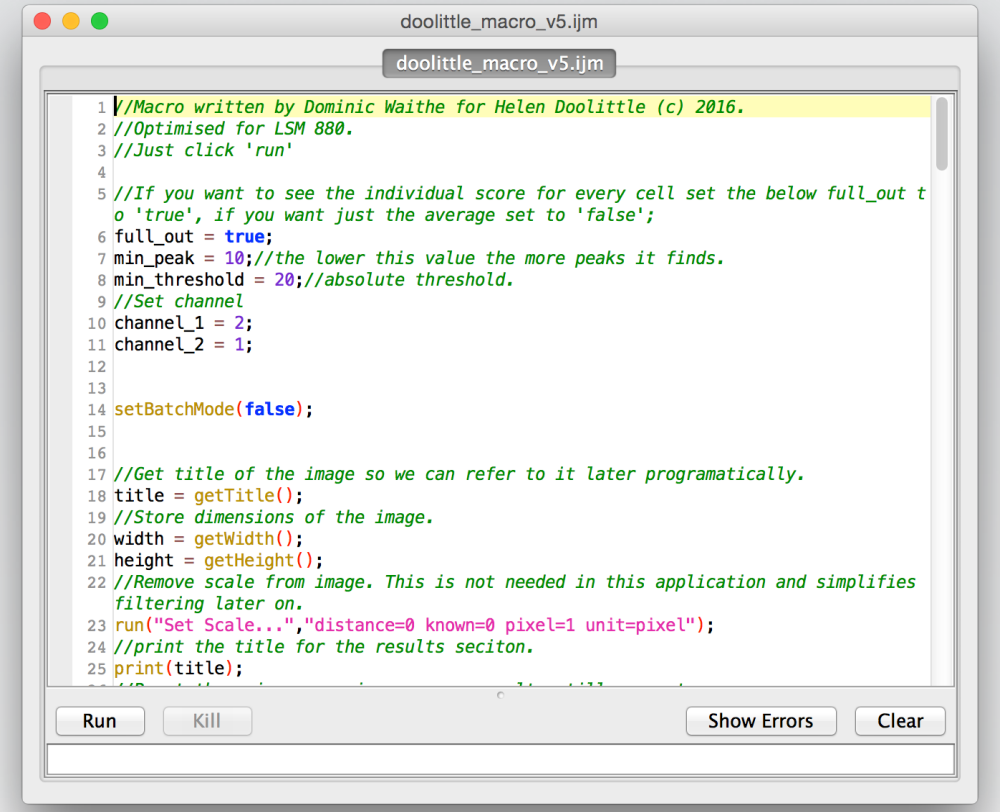
Replication is key: experiments and cells.

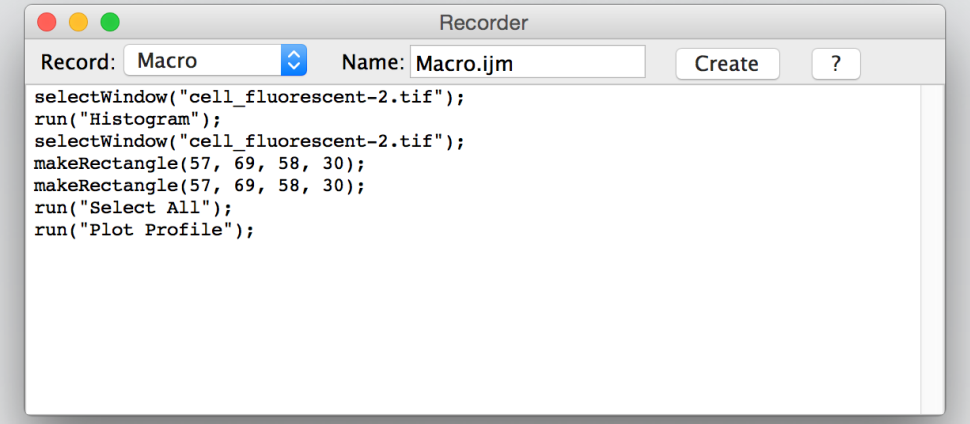


Don’t expect to be able to prove anything without statistical and biological replication.

Source: Comparing EV7 staining with Lamp2 over time. http://mbio.asm.org/content/3/2/e00304-11/F5.expansion.html

Macro writing is very common.





Macros can optimise steps. To learn use the record action function (DEMO), and the internet of course.

Source:

Summary

- Microscopy and image analysis are great ways of testing biological hypothesis.
- But remember methods are based on assumptions.
- Important to know how the different tools work to use them appropriately.

Questions for now or later

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