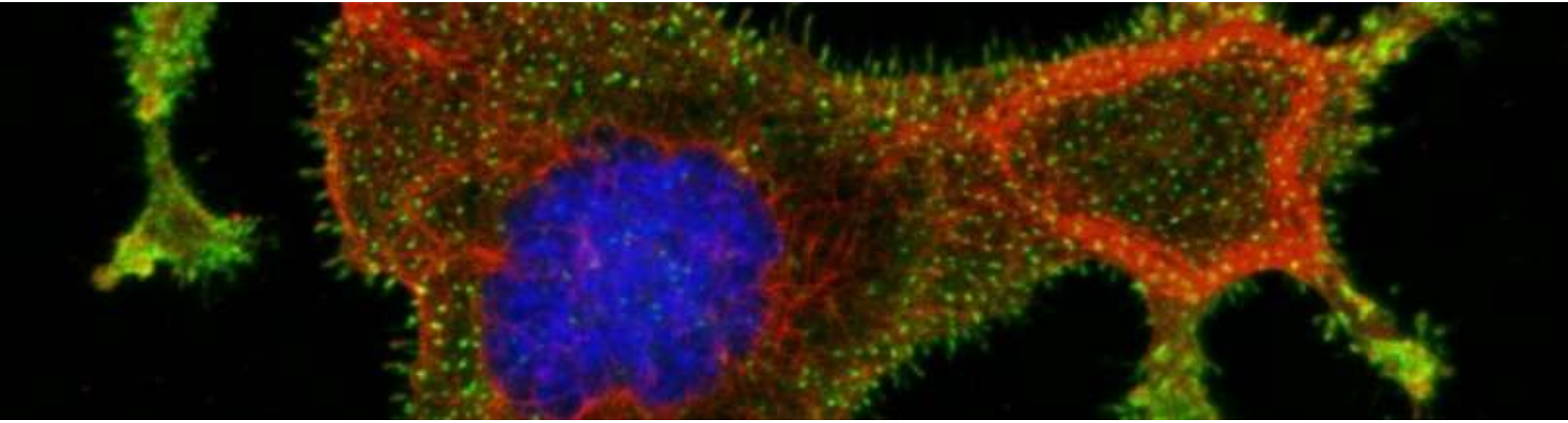

Image Analysis with Fiji

An introductory course for biologists

COMPARE Advanced Imaging

Course website:
https://compareuob.github.io/image-analysis-with-fiji/

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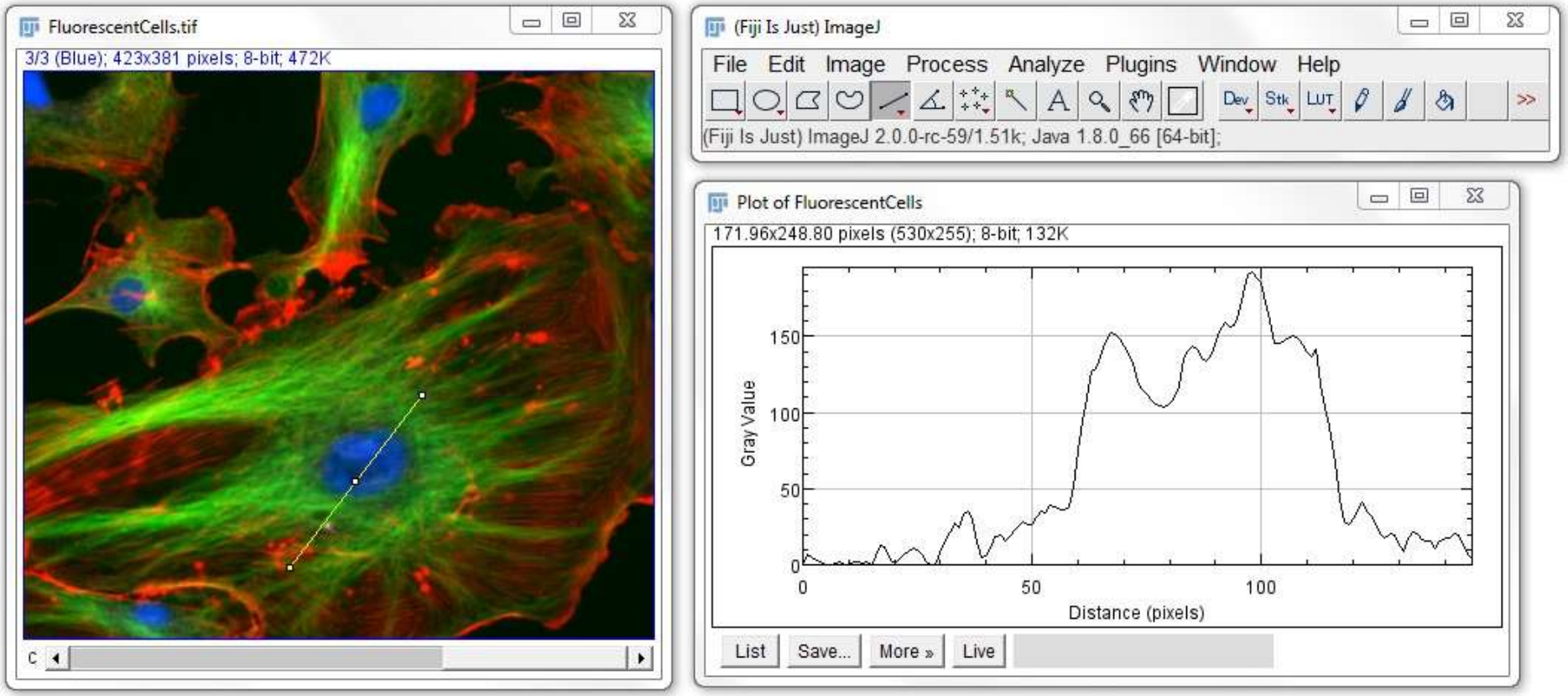
COMPARE
CENTRE OF MEMBRANE PROTEINS AND RECEPTORS

Part 1: Introductrion



ImageJ, Fiji and ImageJ2 ...

ImageJ is an *open source* image processing and analysis software application

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012), Nature methods 9(7): 671-675.

Fiji is a distribution of ImageJ with loads of really useful *plugins* pre-installed

Schindelin, J., Arganda-Carreras, I. & Frise, E. et al. (2012), Nature methods 9(7): 676-682.

ImageJ2 is a complete rebuild of ImageJ, it is built into Fiji

Rueden, C. T., et al. (2017), preprint arXiv:1701.05940.

If in doubt get Fiji!

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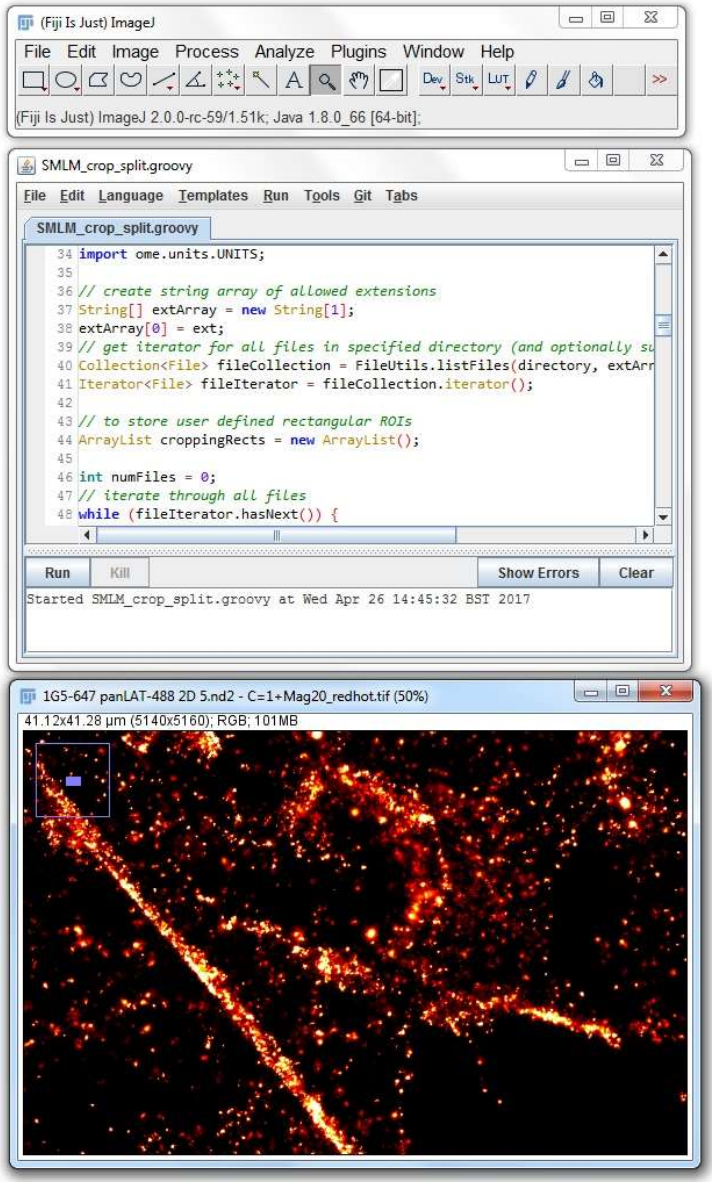
Why use Fiji?

- Open source
- Very popular
- Wide range of sophisticated user-written plugins
- Great for beginners all the way to developers
- Macros and scripting for easy automation
- Interoperability with other software (e.g. KNIME)



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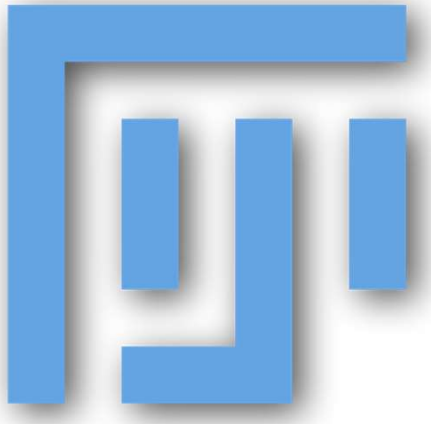
Fiji installation and course material

1. Download Fiji:



http://imagej.net/Fiji/Downloads

You don't need to install Fiji, just unpack and start ☺

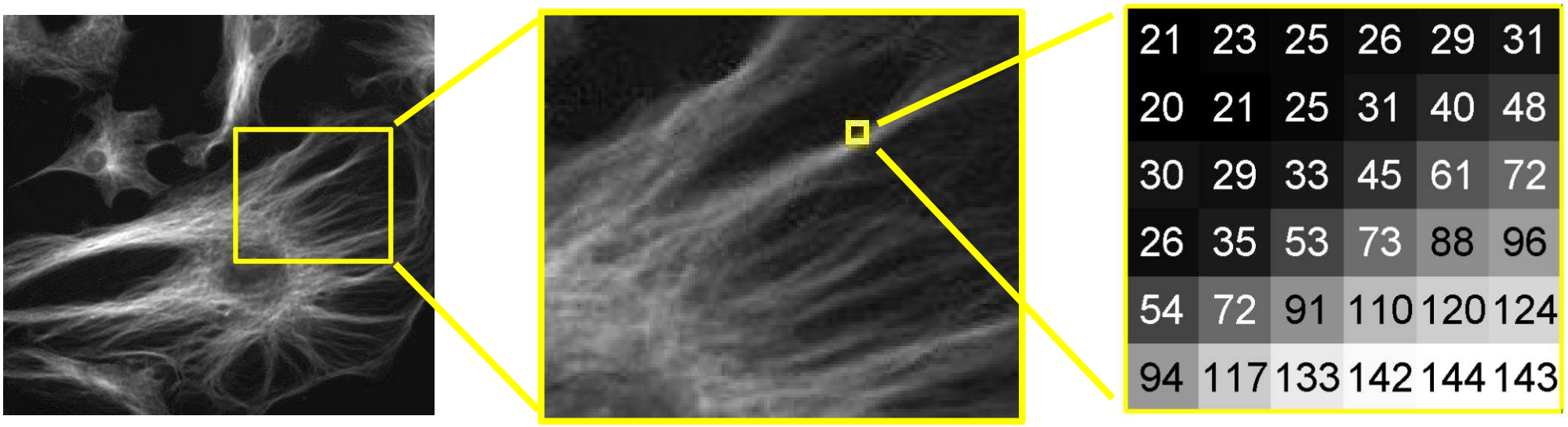
2. Download the course sample data:

https://compareuob.github.io/image-analysis-with-fiji/

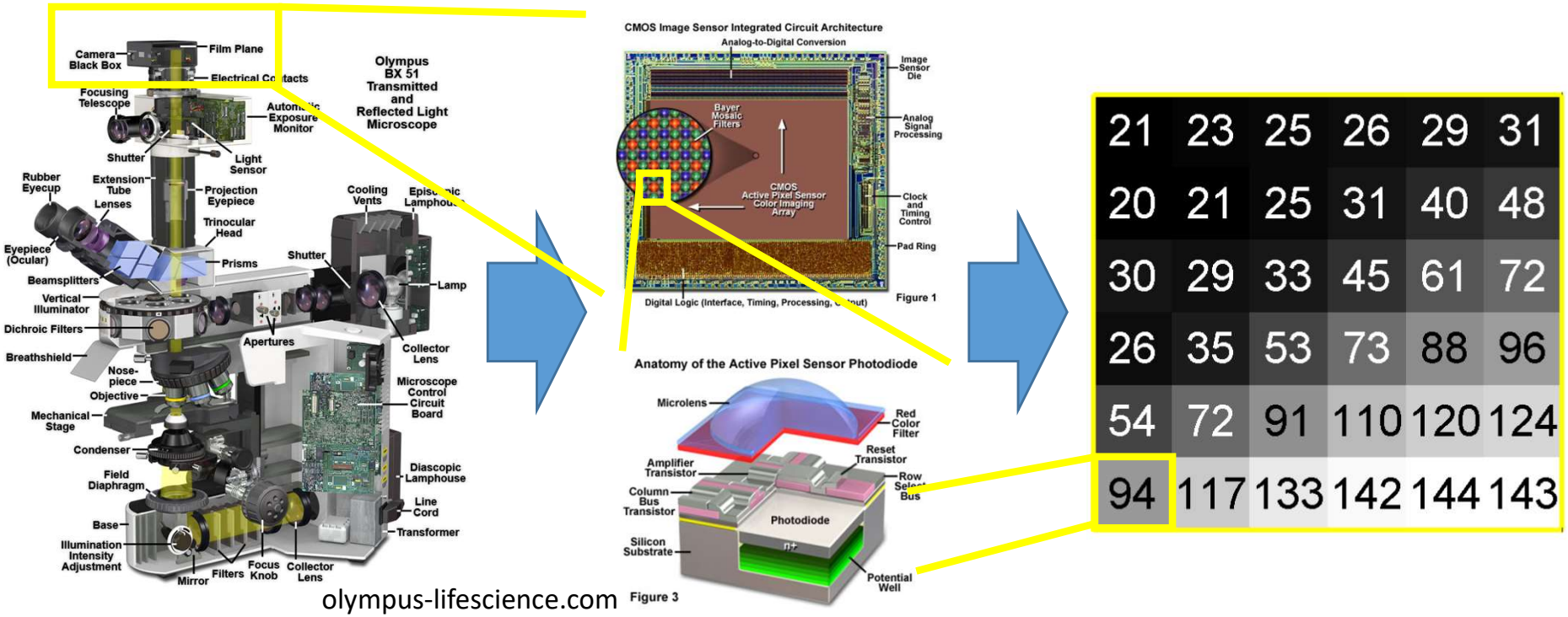
Click sample data zip file and unpack. You can also get copies of the exercises, slides and macros if you want.

Note: if installing on your own computer, you may also need to install Java. Plugins may also have their own requirements.

Digital images are just arrays of numbers



But the numbers are complicated



olympus-lifescience.com

Pixel values are not point measurements from the sample, and are not volume measurements from squares/cubes in the sample. They are a complex function of the optics of the microscope and the camera sensor, applied to the sample. It is important to correct for these effects where possible before performing standard analysis.

An image is the sum of many point spread functions

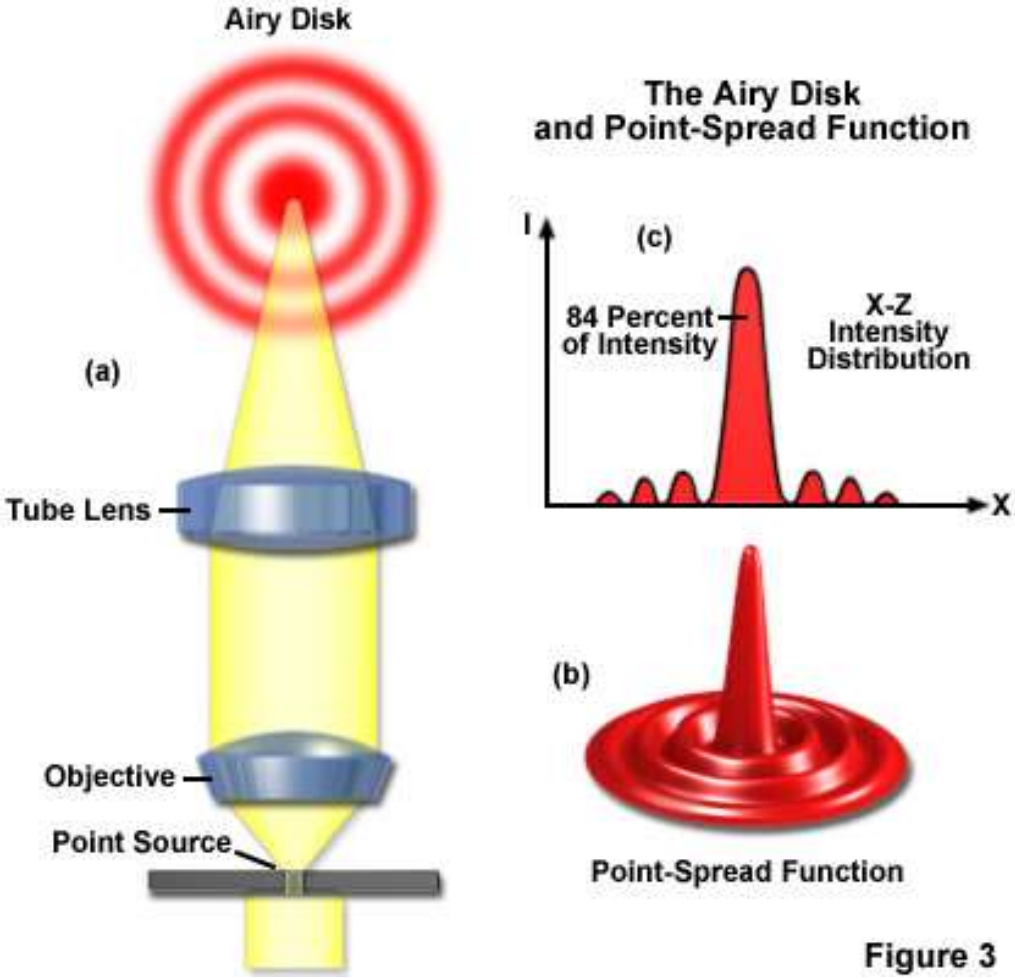


Figure 3

"Each element of the primary image is a small diffraction pattern, and the actual image, as seen by the eyepiece, is only the ensemble of the magnified images of these patterns"

Born and Wolf, Principles of Optics

What determines the resolution limit in light microscopy

- ~~The number of pixels in an image~~
- ~~The magnification of the objective lens~~
- The width of the point spread function
  - Numerical aperture of objective lens
  - Wavelength of light
  - Refractive index of immersion medium
  - Note: This also applies to illumination!

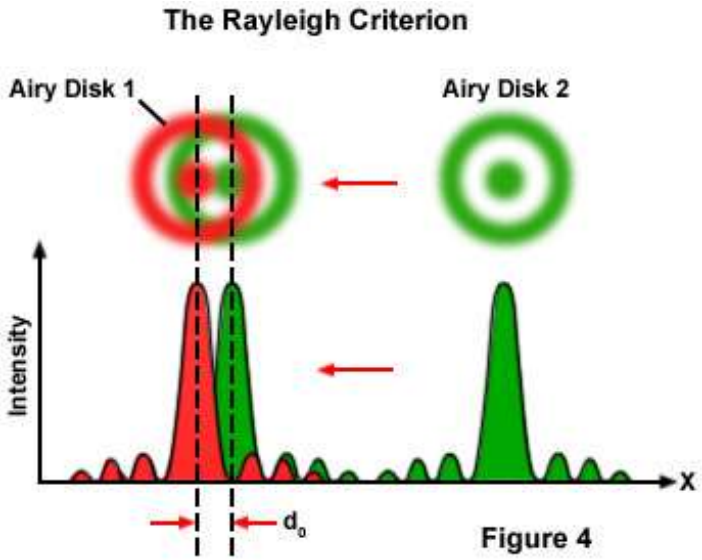


Figure 4

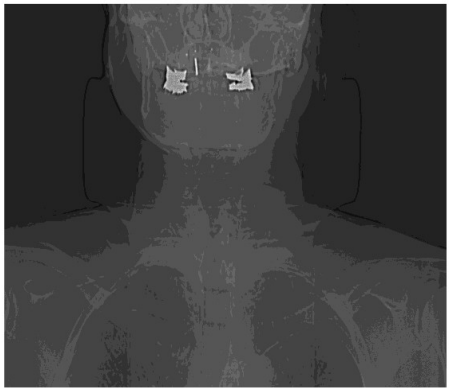
http://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html

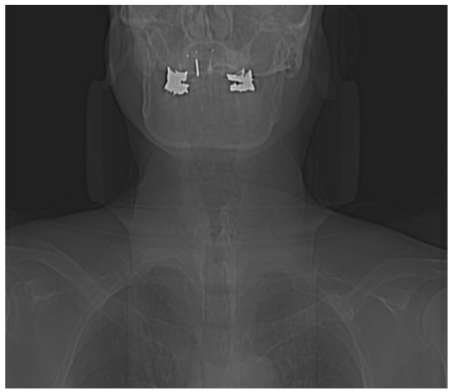
Dynamic range is the number of values each number can take

1 bit = 2

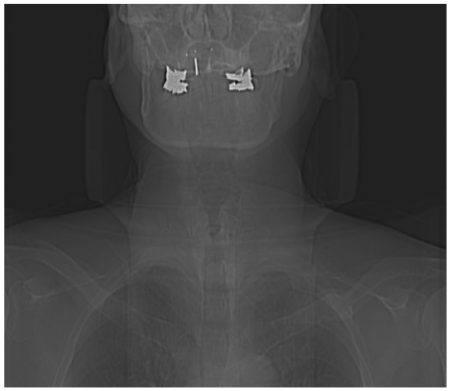
2 bit = 2^2 = 4

4 bit = 2^4= 16

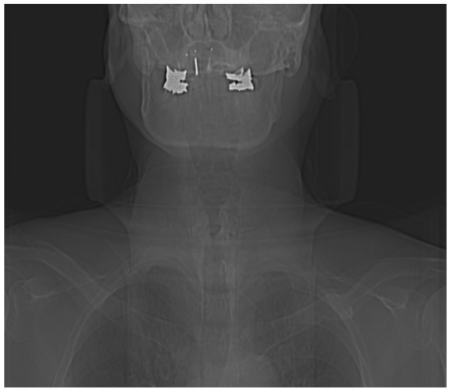




8 bit = 2^8 = 256



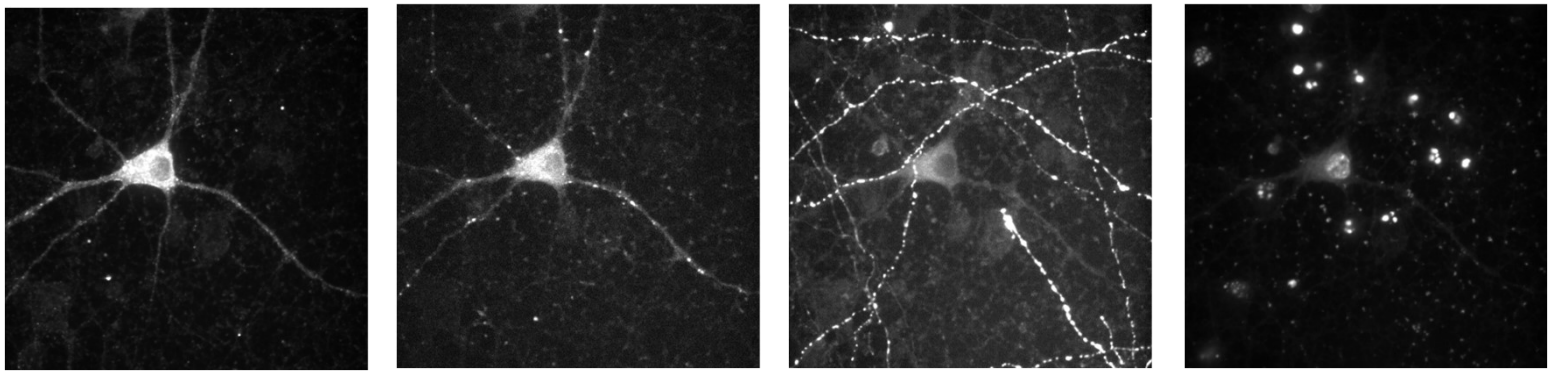
12 bit = 2^{12} = 4 096

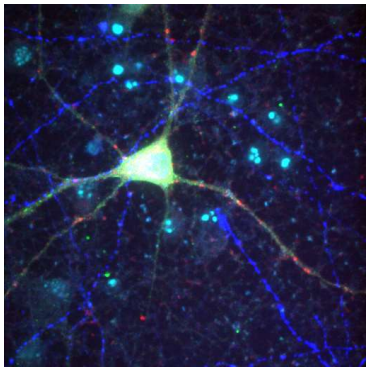


16 bit = 2^{16} = 65 536

Bit depth choice is often a compromise between dynamic range vs imaging speed and file size

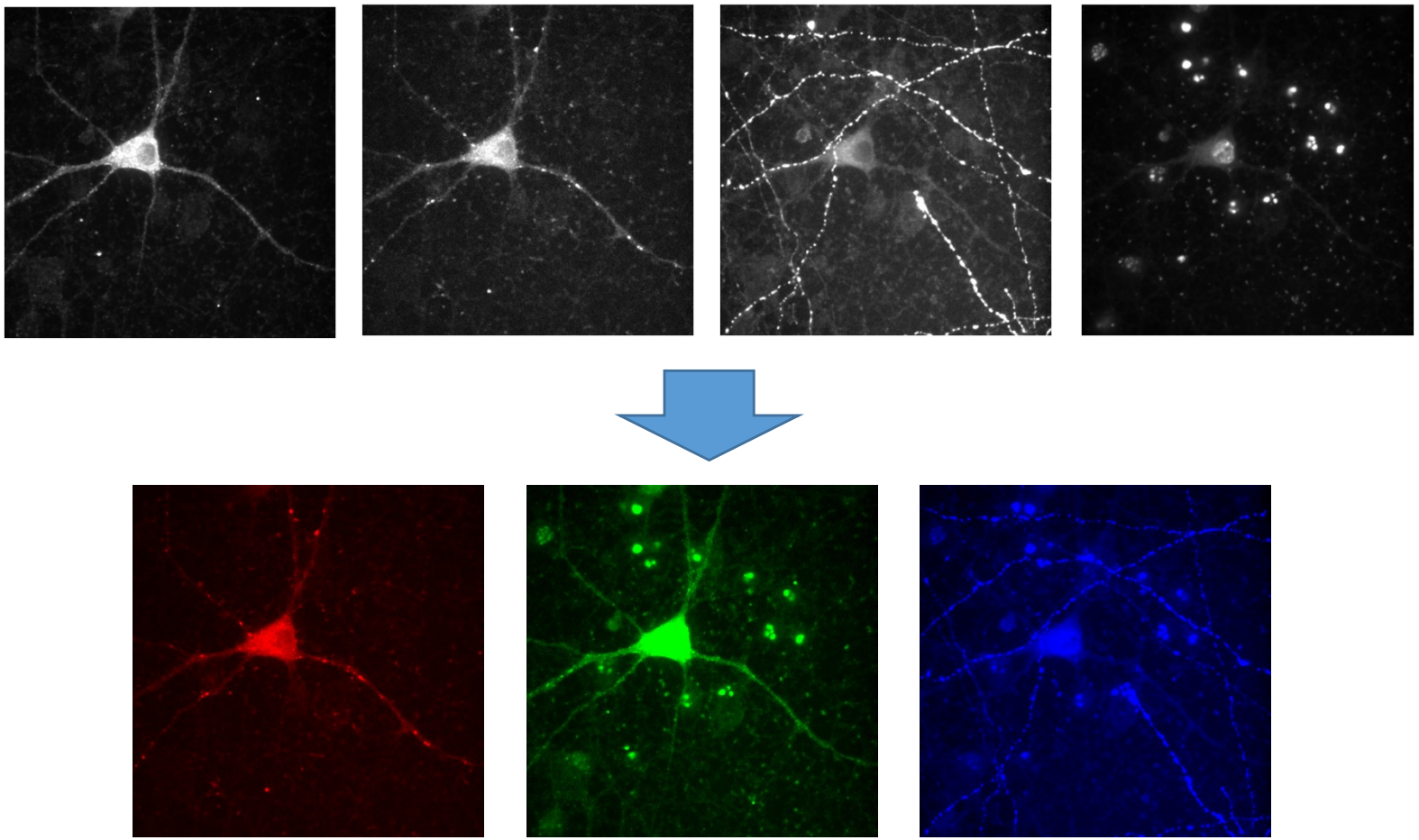
Multi Channel Data





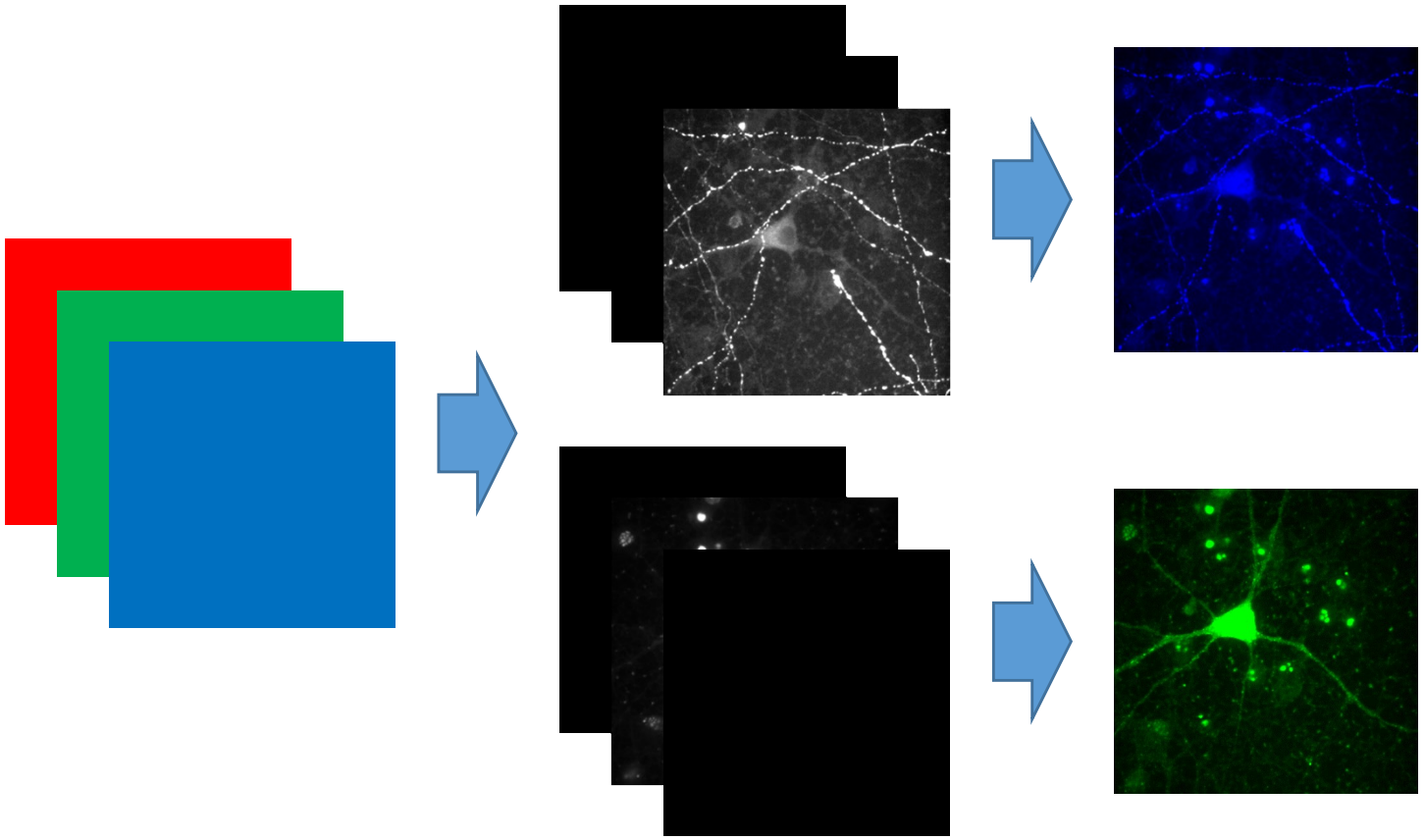
Channels usually come from different cameras attached to different parts of the optical path, so do not necessarily show exactly the same parts of the sample. Alignment/registration should be done before further analysis.

Colouring channels



Can assign colours to channels for better visualisation. This is an artificial process.

Colouring channels

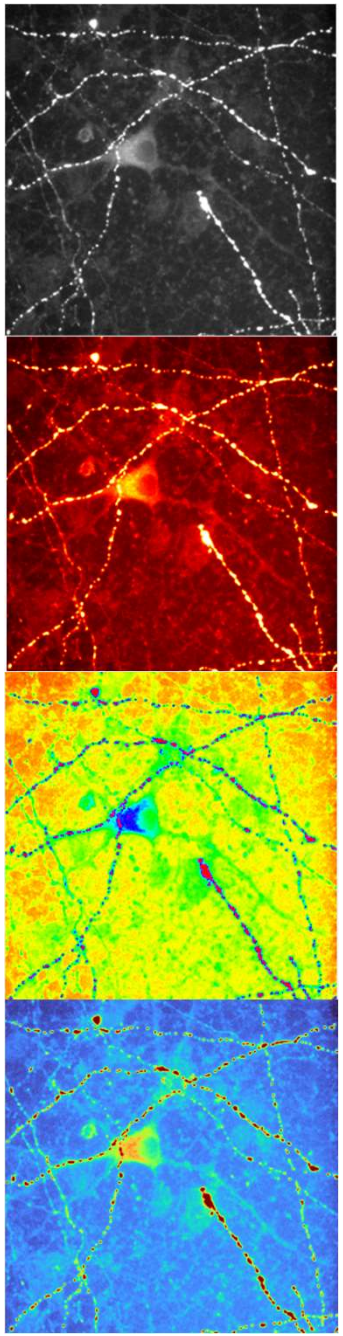


Can create arbitrary colours by placing monochrome images in RGB channels with different weights. Can then create composite images by adding images together.

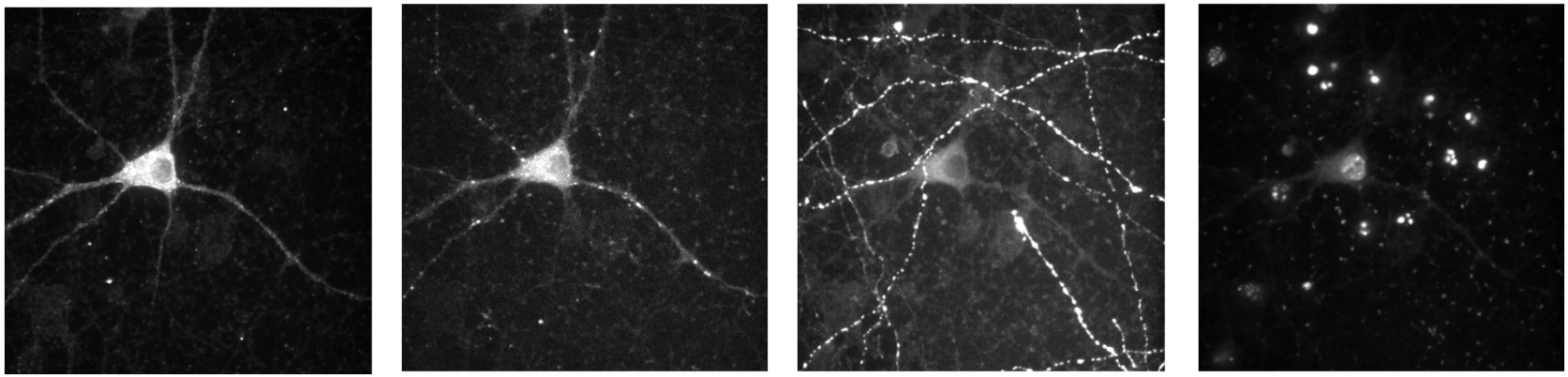
Look up tables (LUTs)

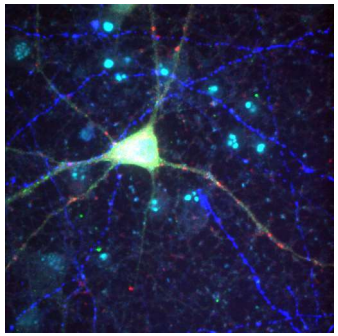
- Can use colour maps to aid visualisation of single channel data
- Colour maps use look up tables to assign colour vectors to scalar intensities
- Colour maps are *arbitrary choices* and *perception is subjective*
- Some colour maps are more accessible (e.g. colourblindness)

Intensity	Red	Green	Blue
0	0	0	0
1	2	1	0
2	4	2	1
…	…	…	…
254	1	254	126
255	0	255	127



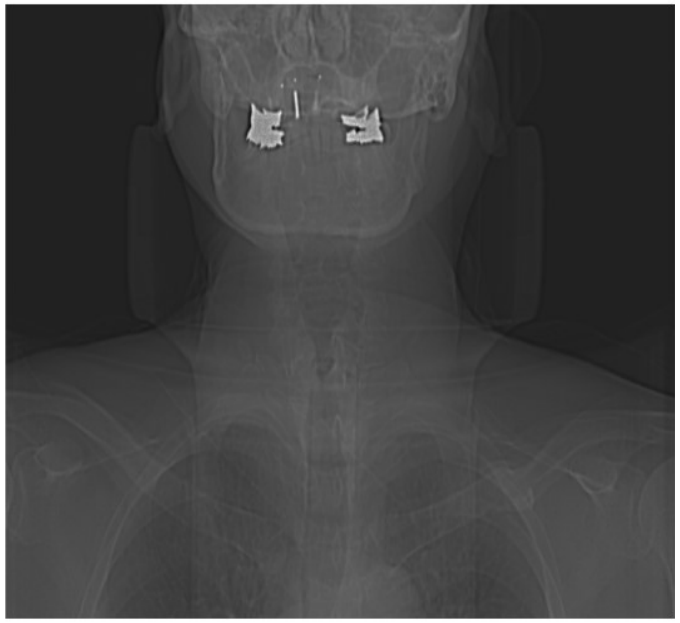
Combining channels



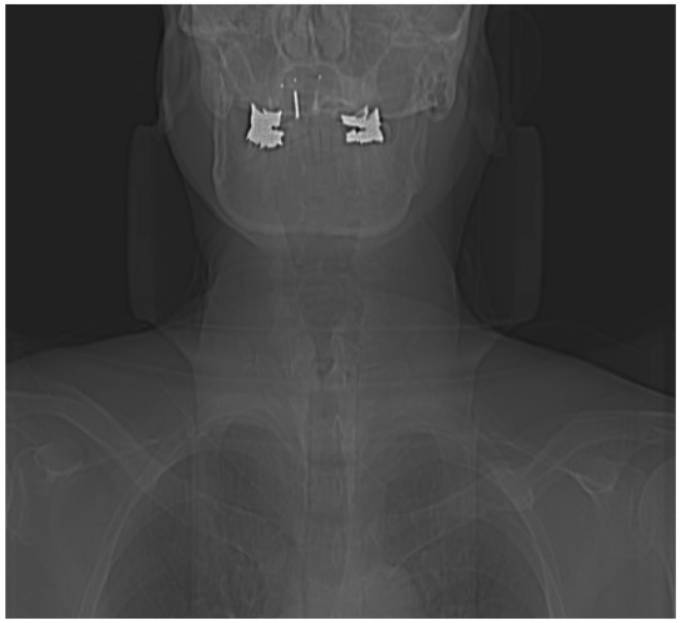


Creating RGB visualisations is a one-way process. Cannot in general disentangle into original channels. Always keep the raw data where possible

What can we “see” on a monitor?



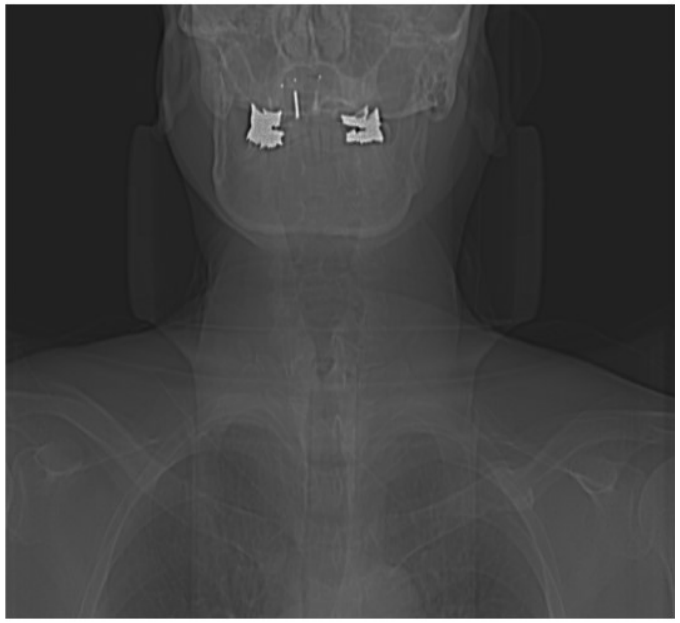
8 bit = 2^8 = 256



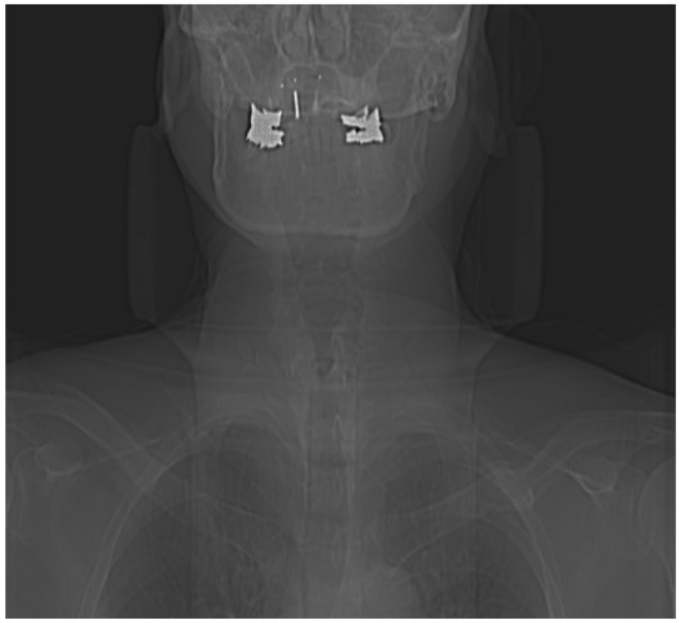
16 bit = 2^{16} = 65 536

Can you tell the difference?

What can we “see” on a monitor?



8 bit = 2^8

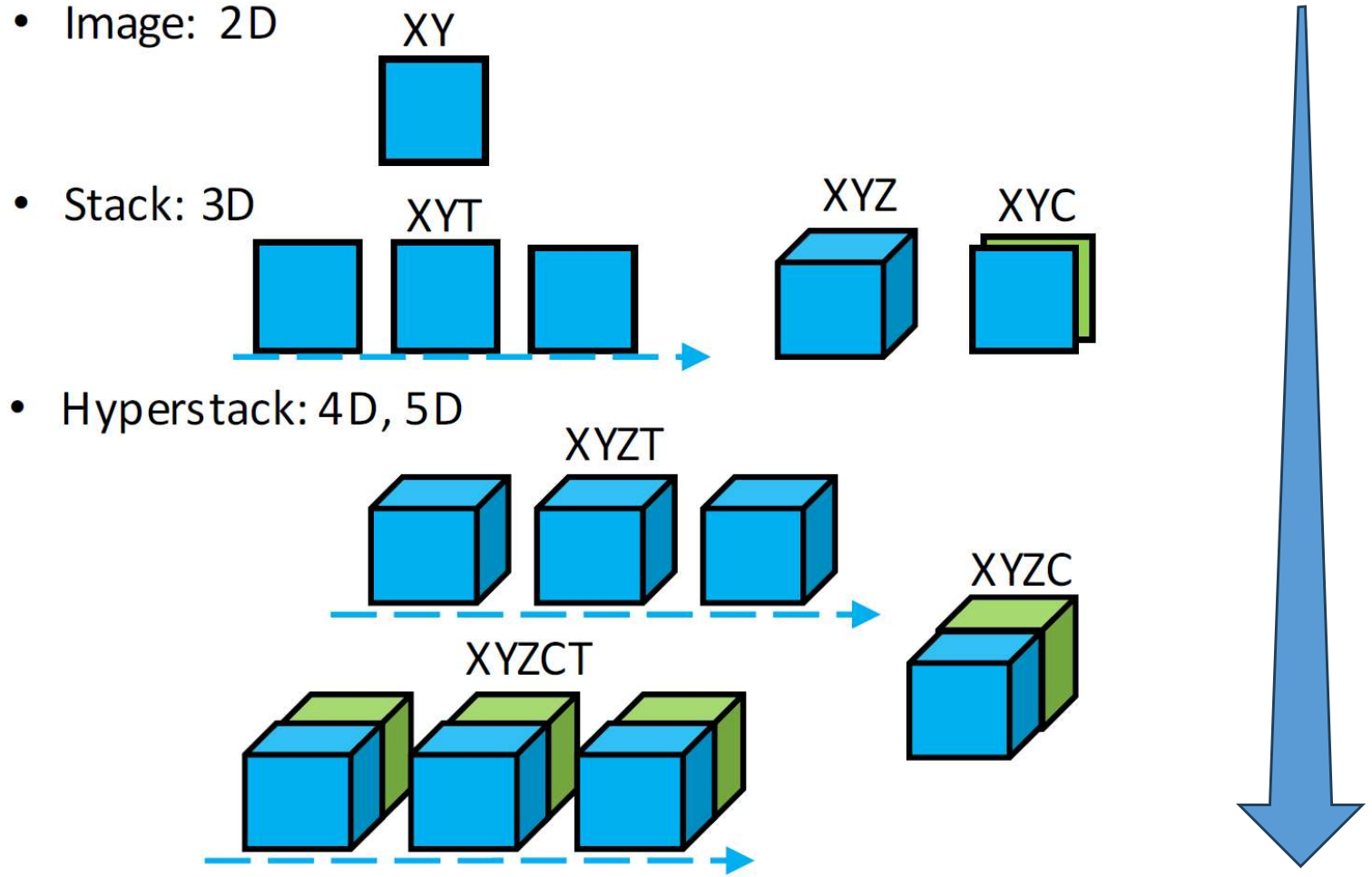


16 bit = 2^{16}

- 6, 8, 10, or 12 bit display range
- 3 x *n* bit RGB for colour display
- What bit-depth can our eyes detect?
- What bit-depth can image analysis algorithms leverage?
- Does this depend on the algorithm?

Microscopy data

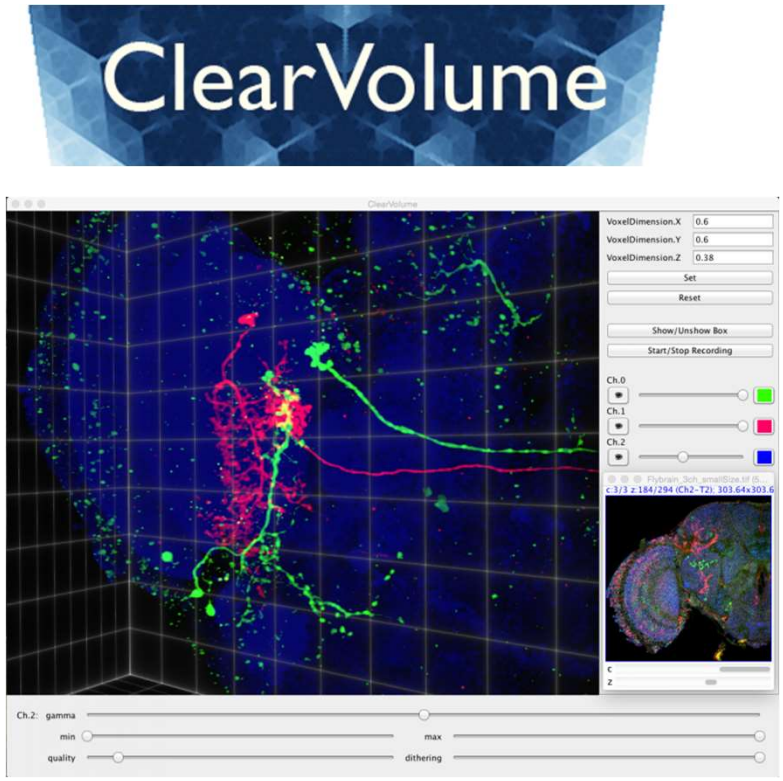
File size
Processing time
Hardware requirements



Everything is a multidimensional array at the end of the day.
Remember your dimensions!

Volume rendering in Fiji

- ClearVolume is often a good option (https://imagej.net/plugins/clearvolume)
- Note: may be slow if your computer does not have a suitable GPU
- May not work at all if the dataset is larger than your computer's memory
- If so, can:
  - Downsample dataset before viewing it
  - Try a different viewer such as sciview (https://imagej.net/plugins/sciview)



Royer, L. A., Weigert, M. & Günther, U. et al. (2015), Nature Methods 12(6): 480-481.

Common file formats

• **TIFF** is a good choice
– Lossless storage of data
– Header tags for metadata

• **Proprietary formats from microscope vendors** (e.g. lif, nd2, czi)
– Often just a TIFF wrapper
– Easy handling or 5D data, and lots of metadata added automatically
– The Bio-Formats plugin will load most formats

• **PNG** should only be used for transfer and display
– Lossless compression
– No metadata
– RGB only

• **JPEG** should not be used for scientific images
– Lossy compression discards information and causes artefacts

- Exercises

Part 2: Introduction to Image Processing and Analysis

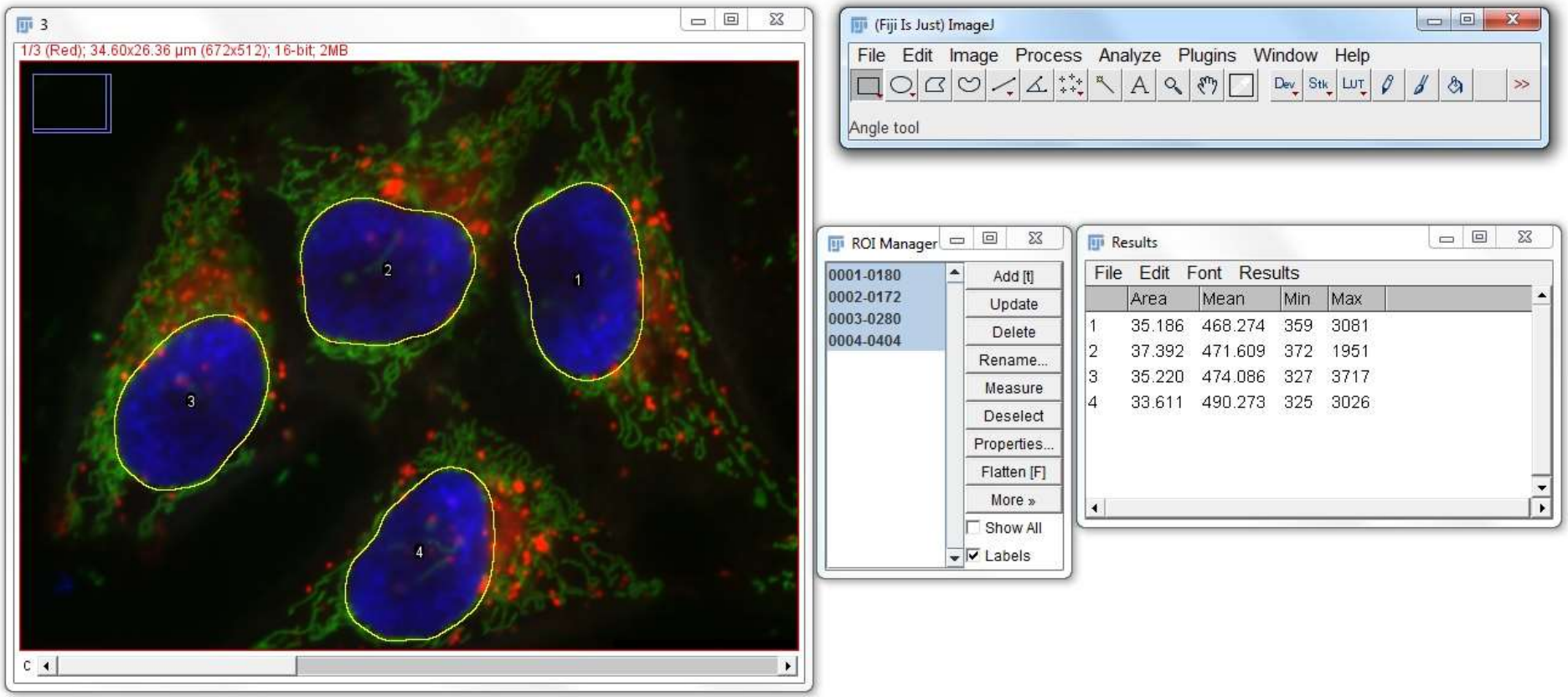


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Image Processing:

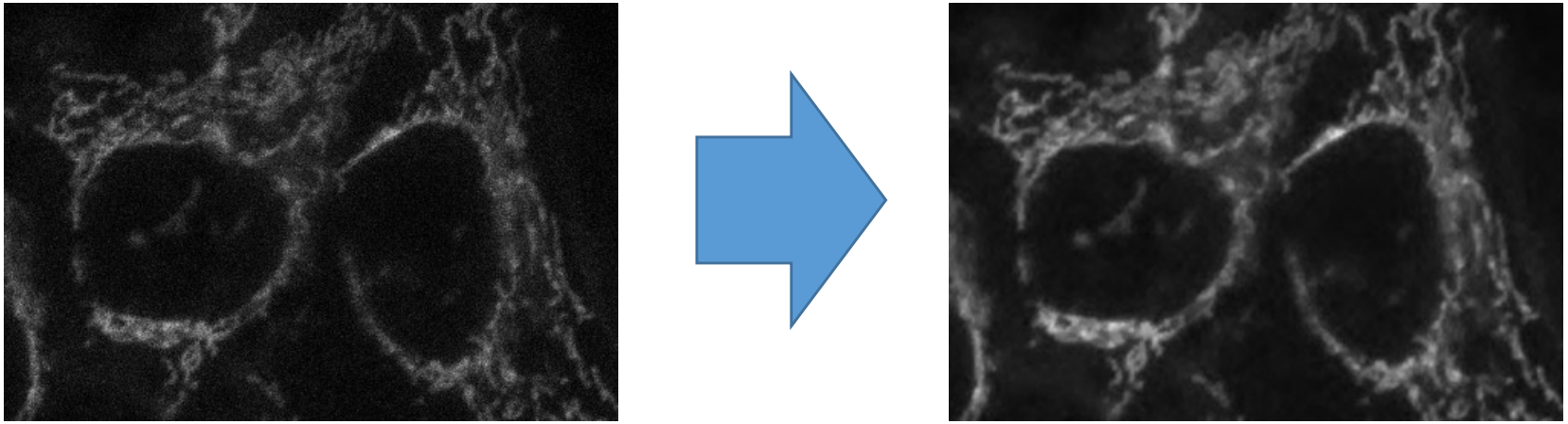
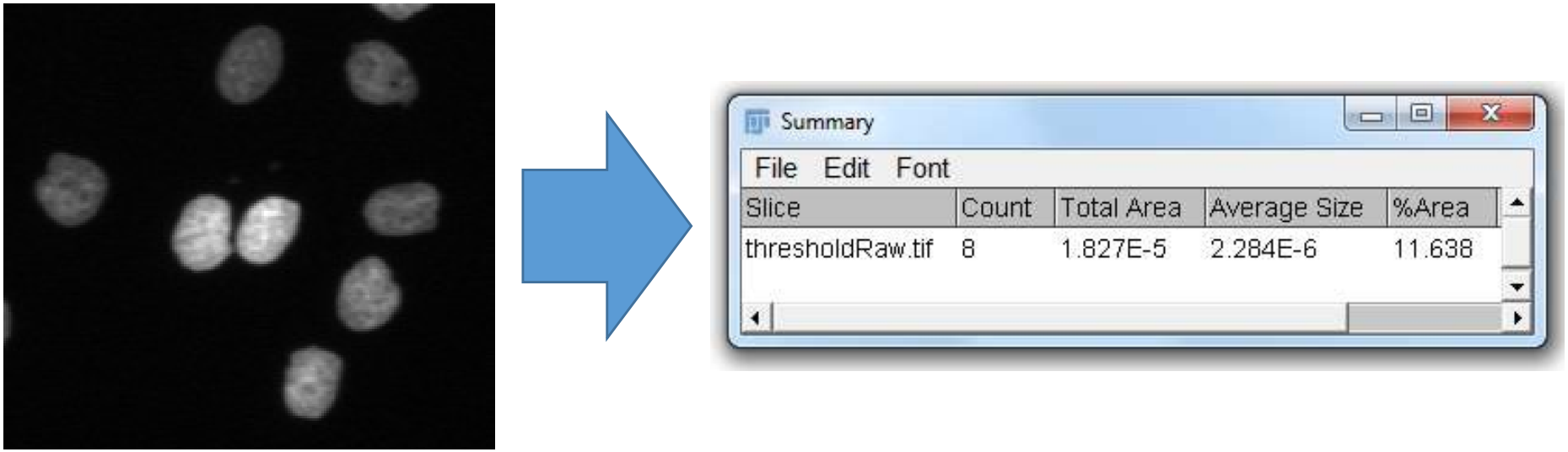


Image Analysis:



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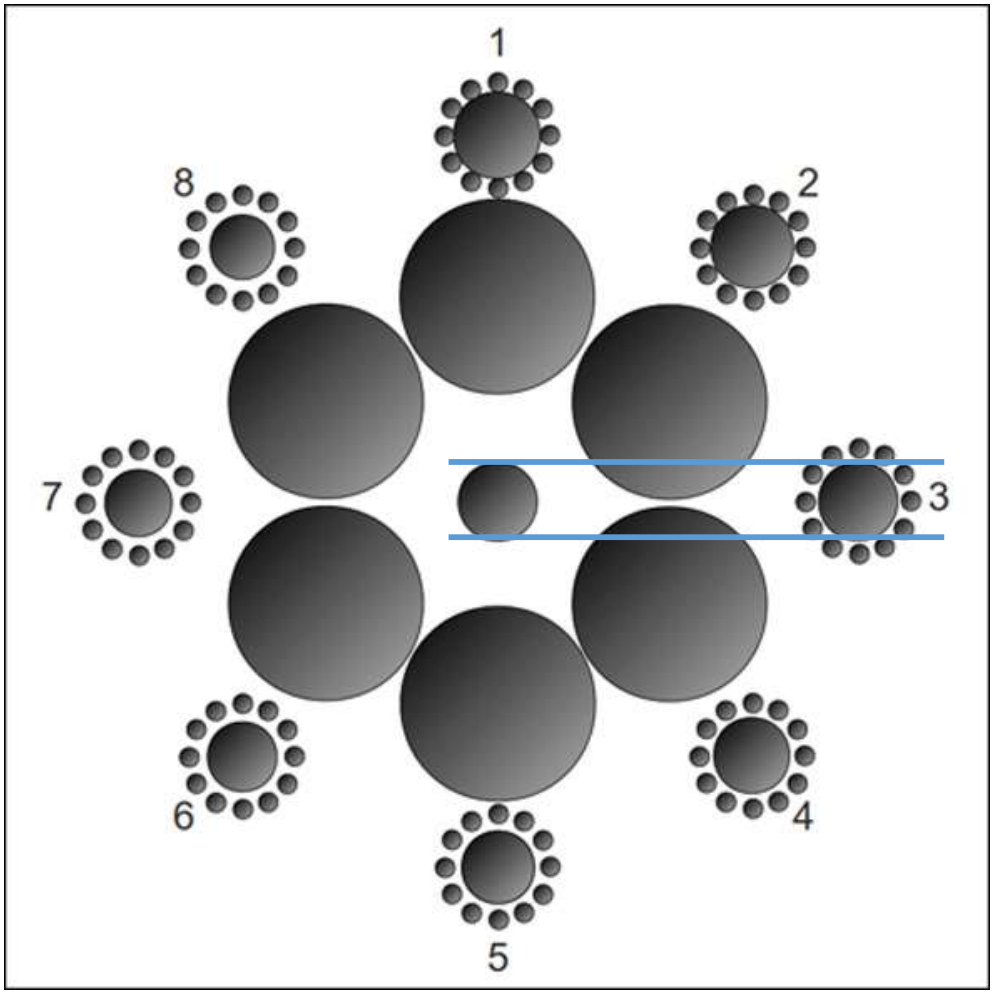
Why do computational processing and analysis?

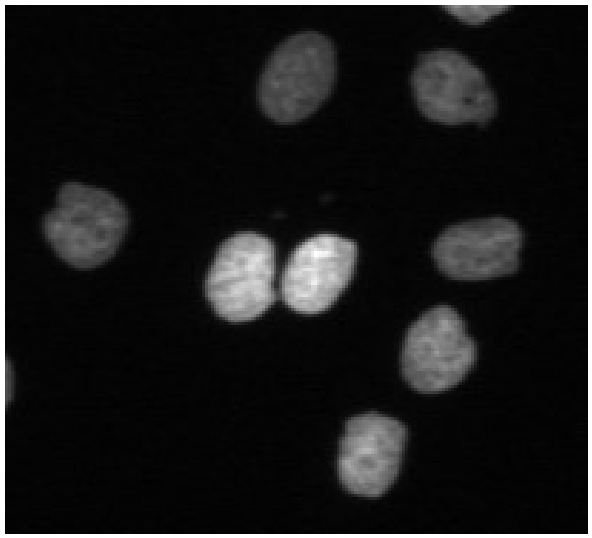
- It's quantitative
- It's unbiased (in principle)
- Can enhance understanding of the data
- Can be automated for processing of large datasets

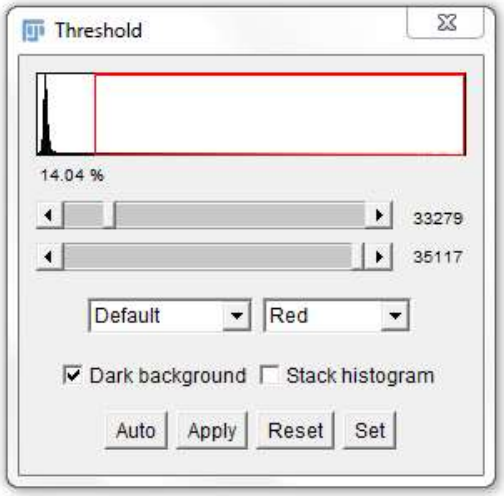
"The first principle is that you must not fool yourself - and you are the easiest person to fool. So you have to be very careful about that. After you've not fooled yourself, it's easy not to fool other scientists. You just have to be honest in a conventional way after that."
Richard Feynman

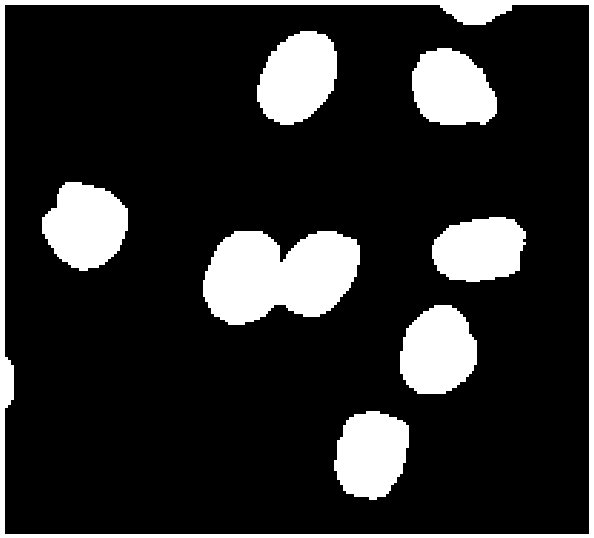
What outer circle is the same size and the central circle?



Intensity based thresholding to segment objects

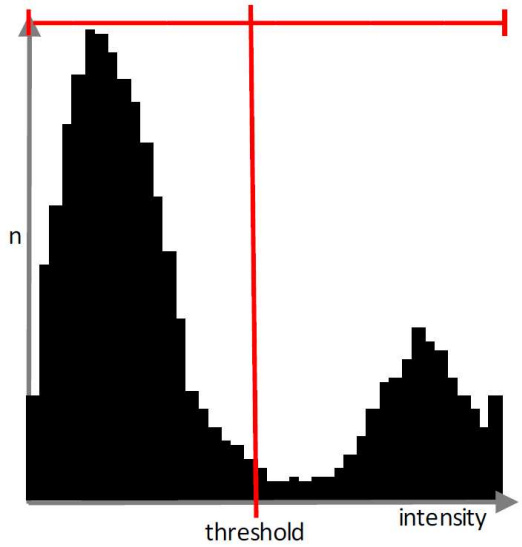






Automated threshold values are preferable to manual selection

- Otsu thresholding assumes there are two classes (signal and background) and maximises the intra-class variance.

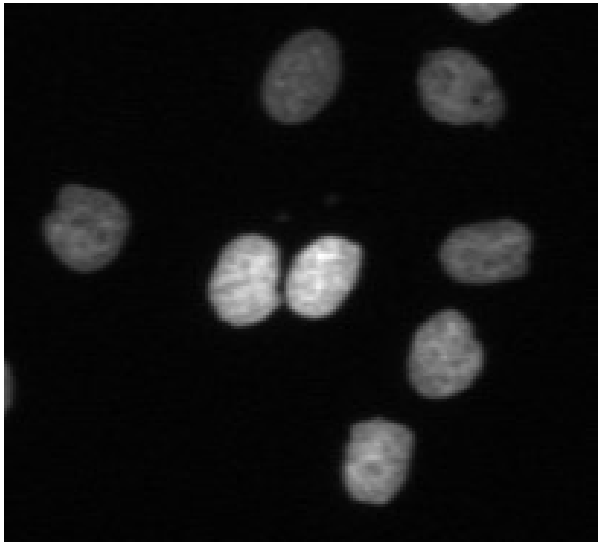


Otsu, N (1979), IEEE Trans. Sys., Man., Cyber. 9: 62-66.

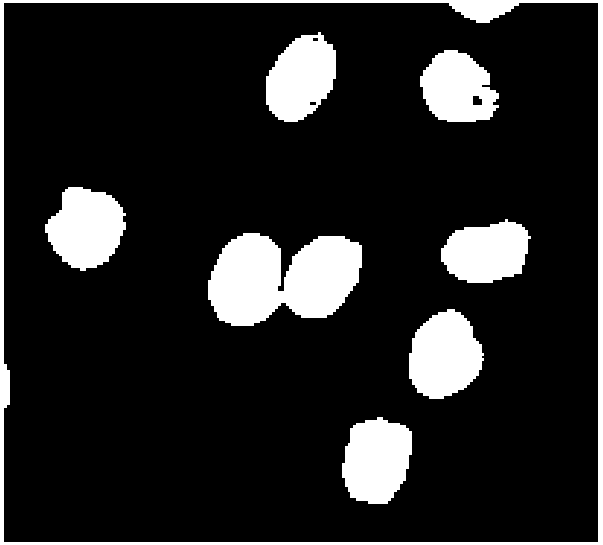
- Li thresholding minimises the cross entropy between the original and segmented images.

Li, CH & Tam, PKS (1998), Pattern Recognition Letters18(8): 771-776

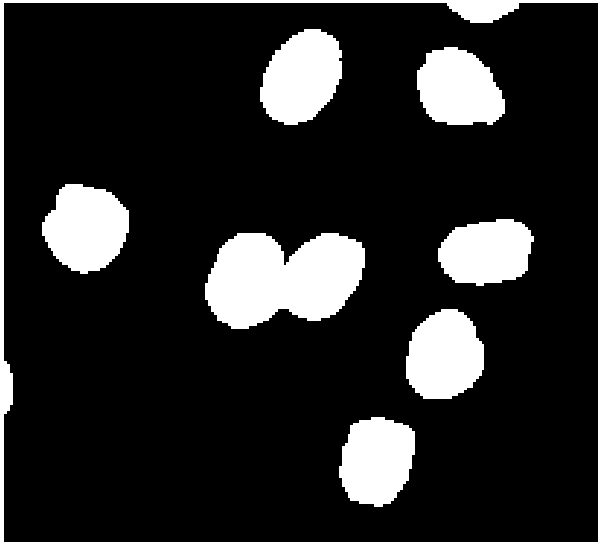
Automated threshold values are preferable to manual selection



Raw Data

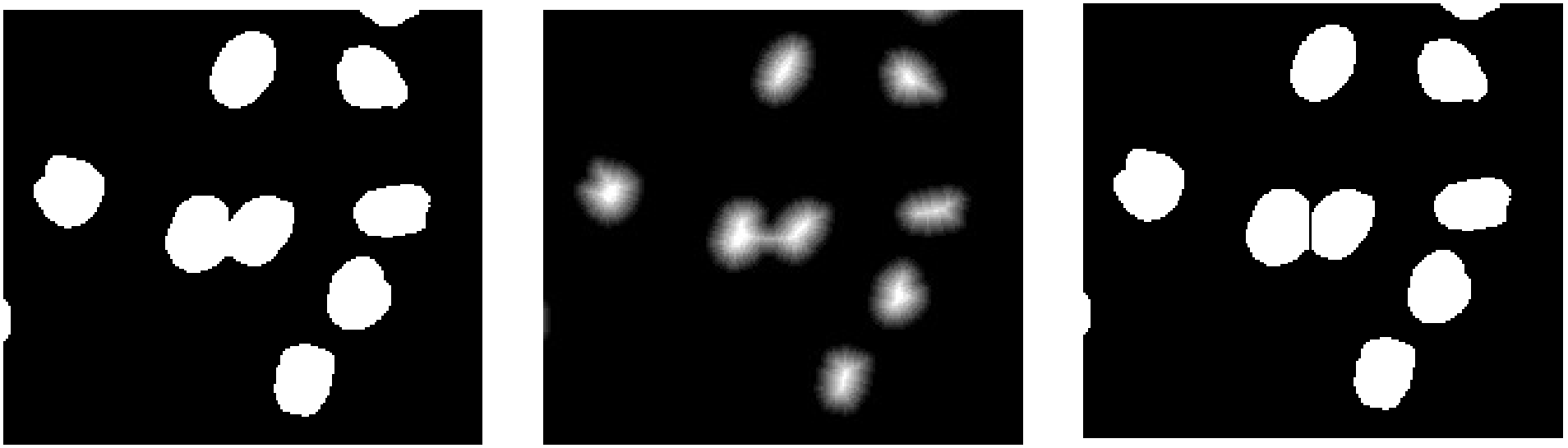


Otsu Threshold



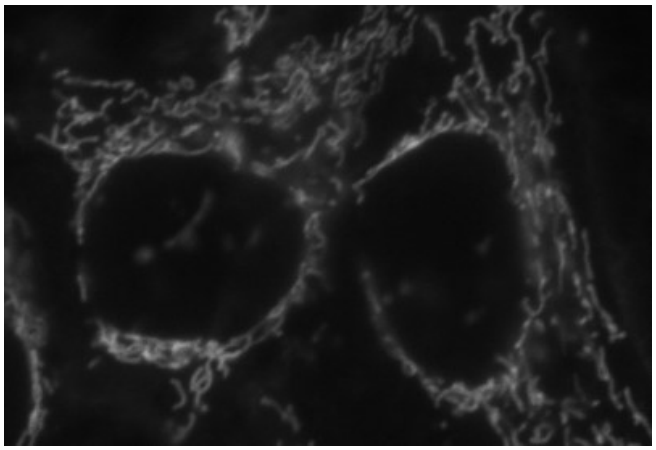
Li Threshold

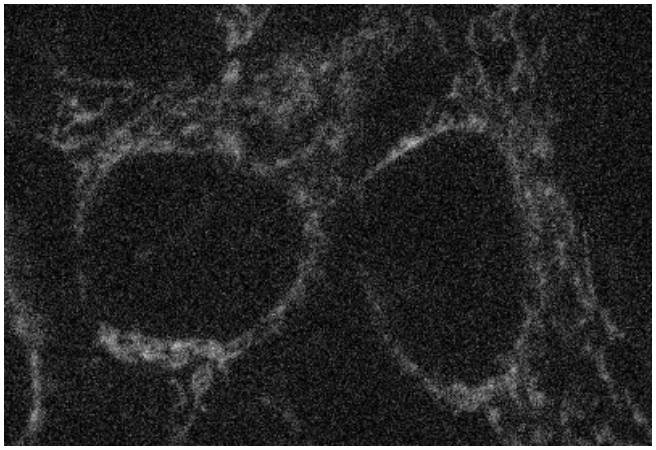
Watershed transformation to separate touching objects



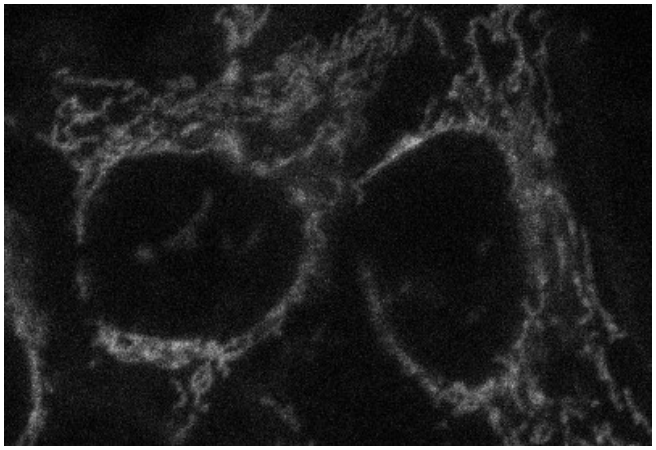
- Seeds placed at local minima of distance map and dilated
- This can be visualised as flooding the distance map

Noise is image corruption from the acquisition process

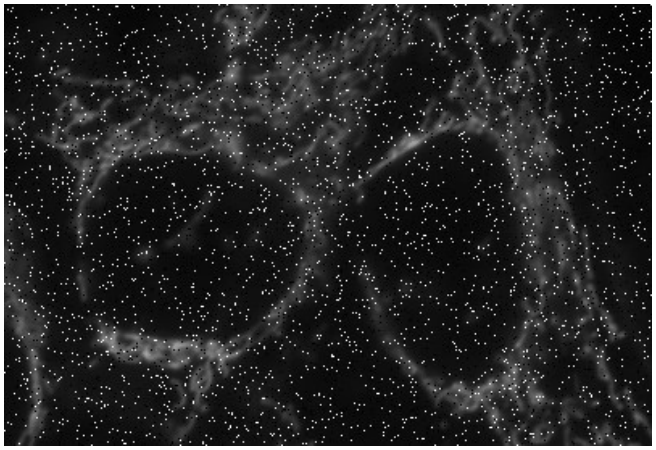




Gaussian



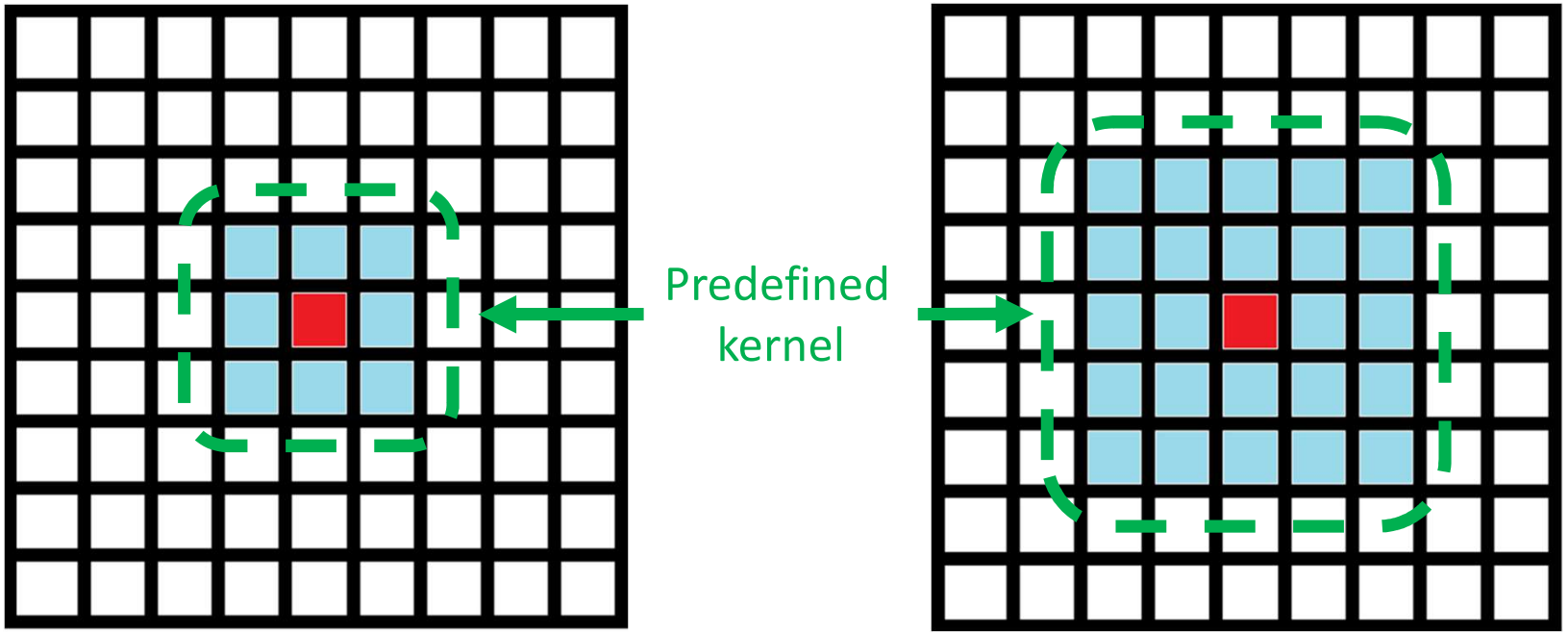
Poisson



Impulse

Image filters and convolution

- The value for a pixel in the filtered image is dependent on pixels in the local neighbourhood
- Pre-define a filter kernel and apply it to the neighbourhood of each pixel

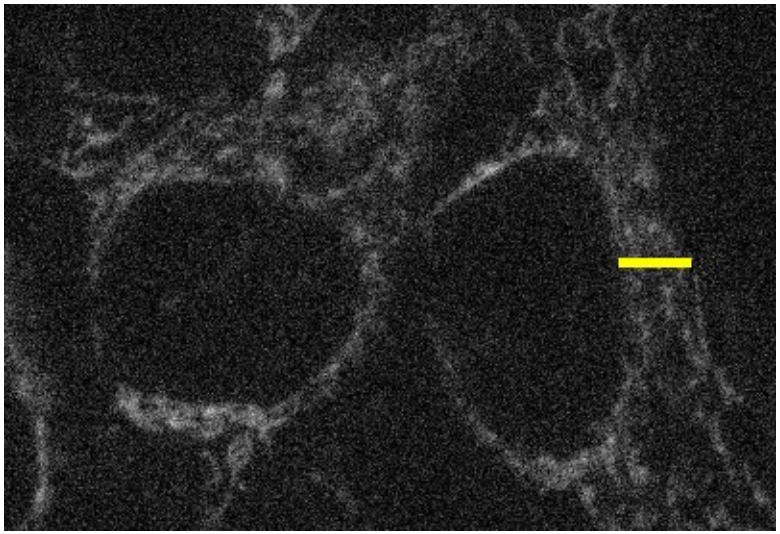


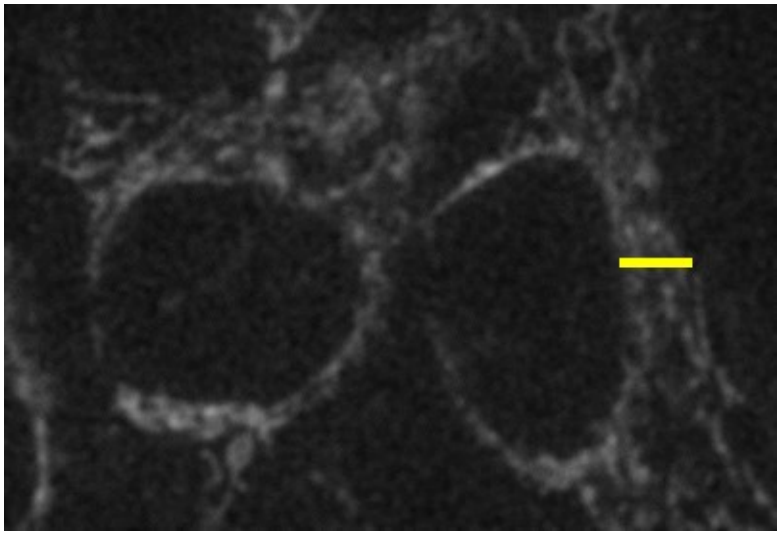
3x3 Neighbourhood

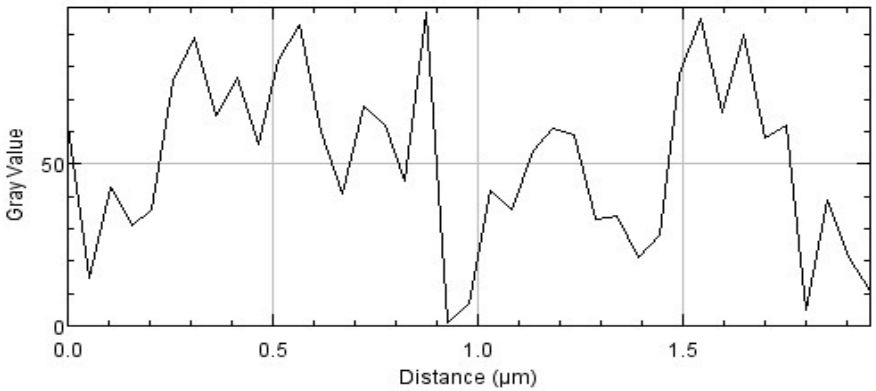
5x5 Neighbourhood

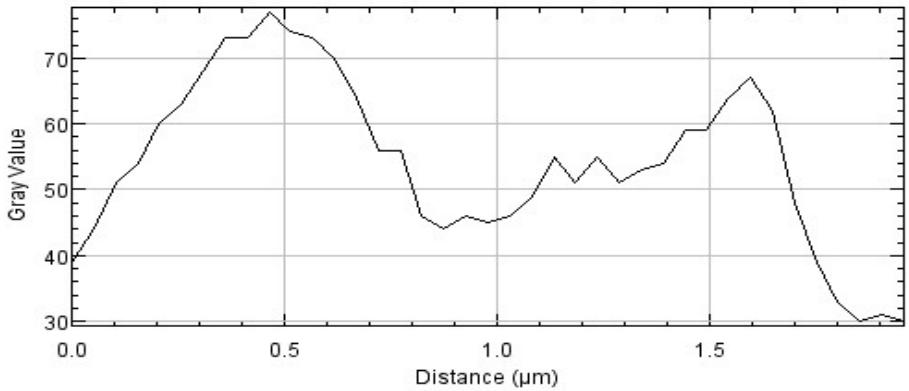
Mean filter

- Pixel values given by mean over neighbourhood
- Removal of Gaussian and Poisson Noise









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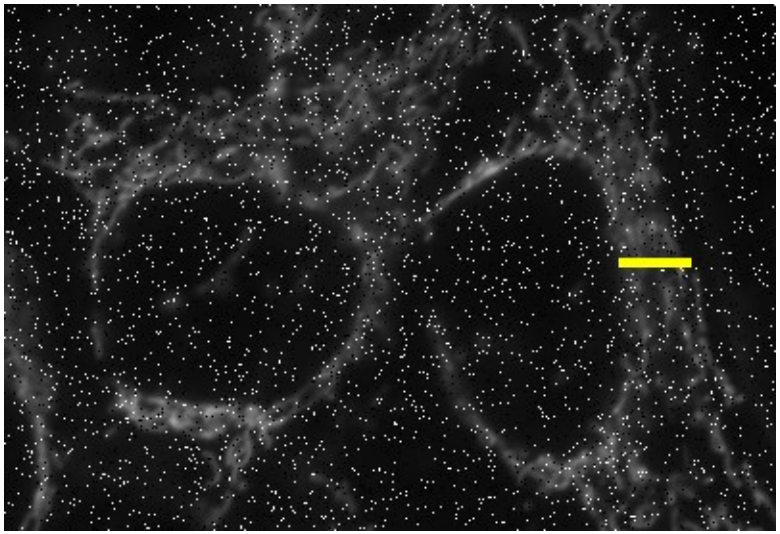
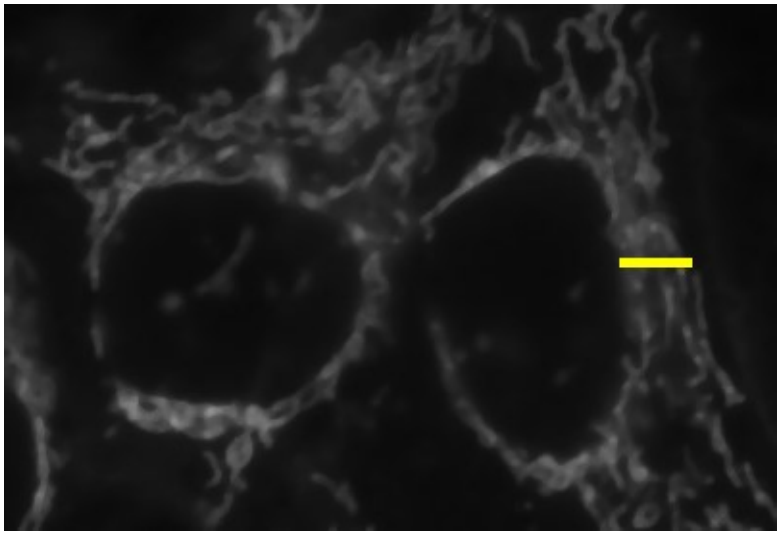
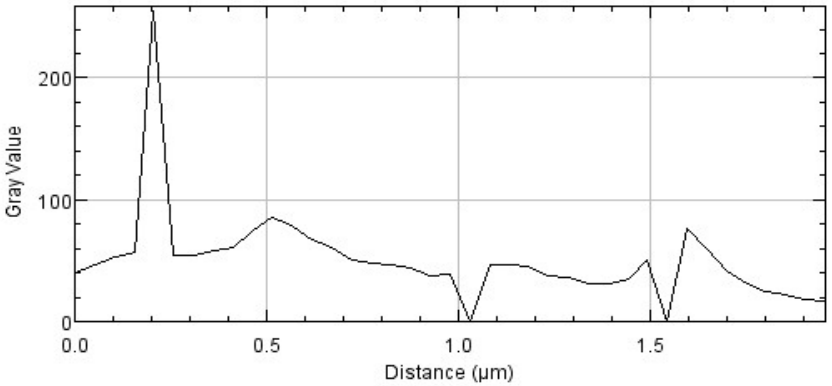
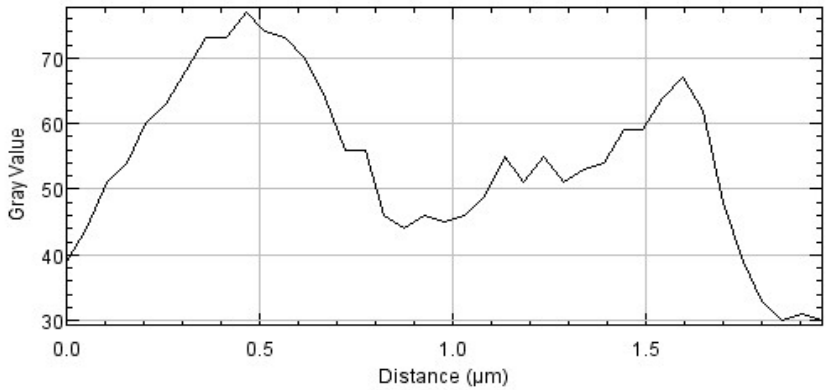
Median filter

- Pixel values given by median over neighbourhood
- Removal of salt and pepper (impulse) noise





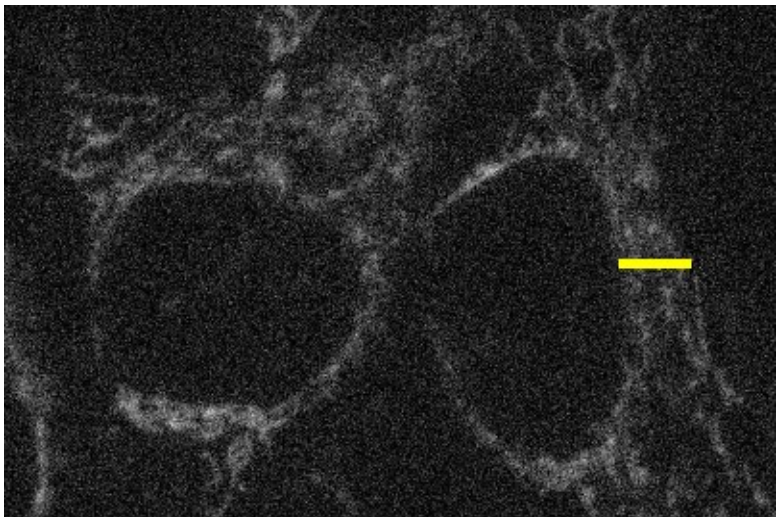


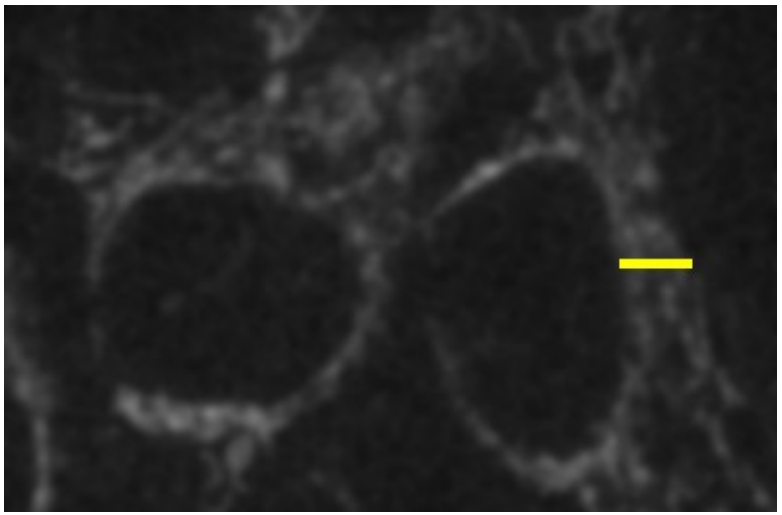


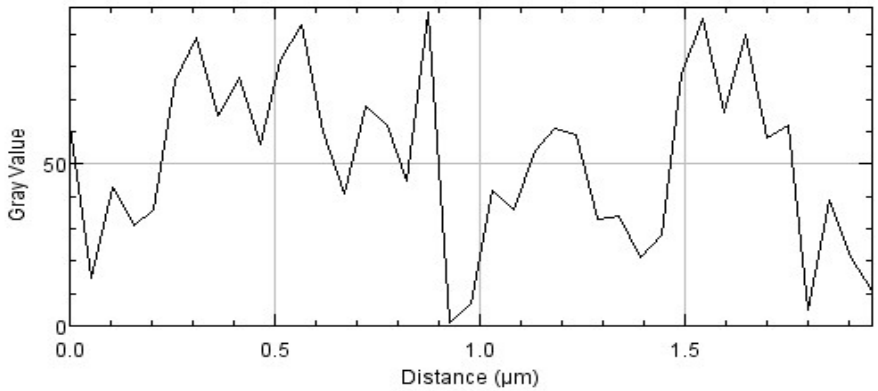
Gaussian filter

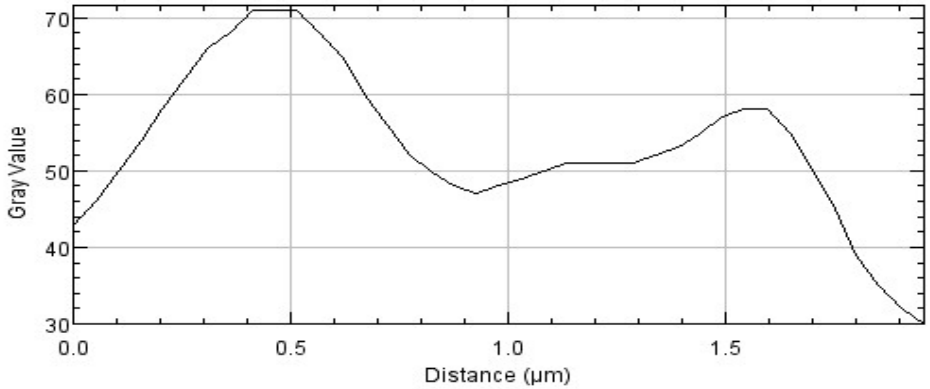
- Contribution of neighbourhood pixels weighted by Gaussian profile

- Removal of Gaussian and Poisson Noise





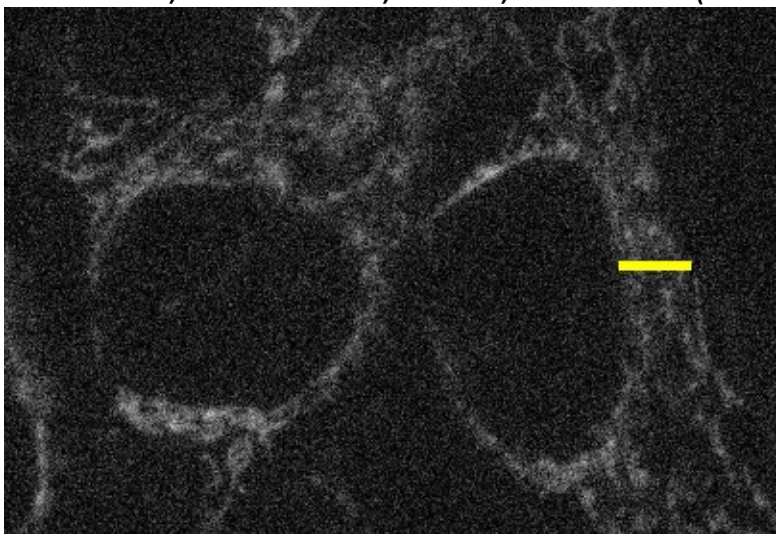


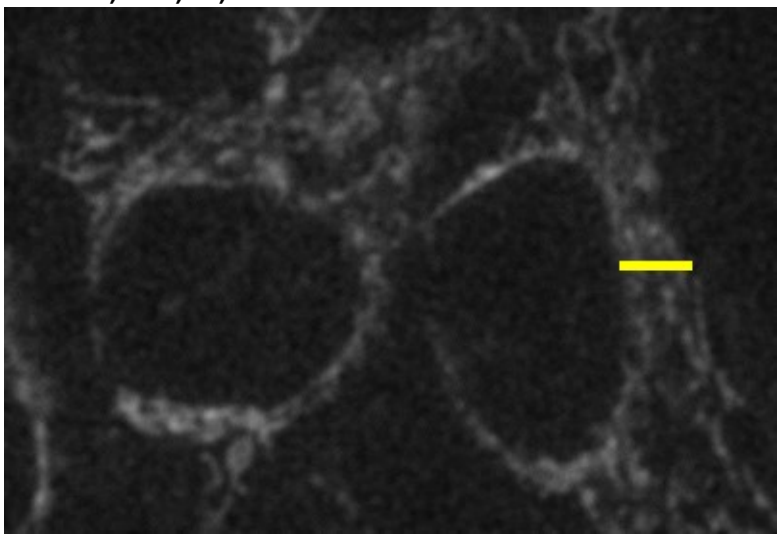


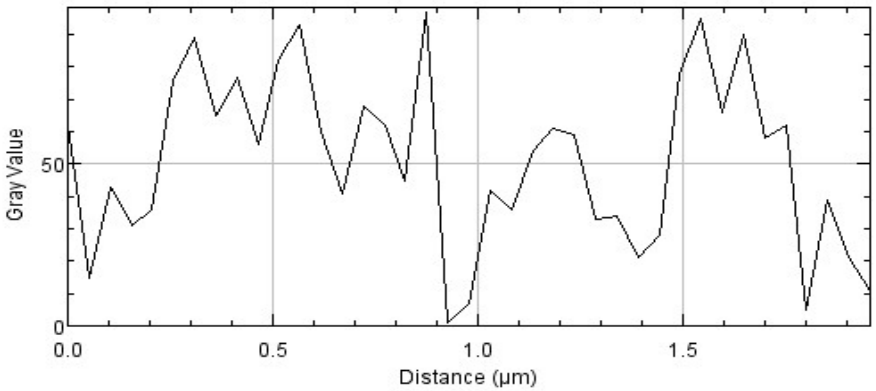
The PureDenoise plugin

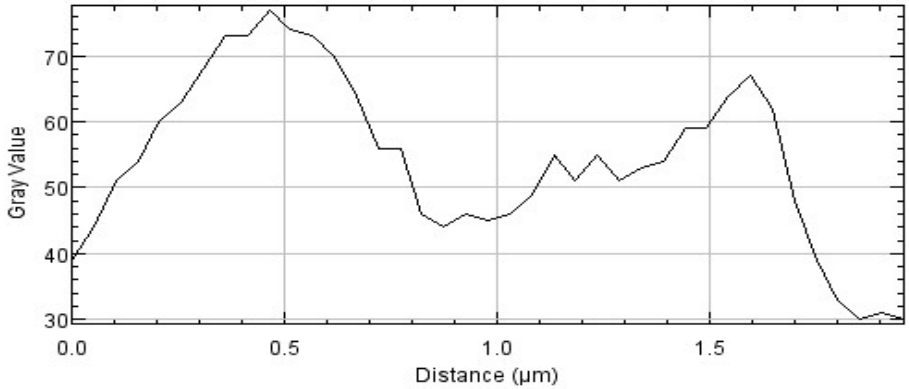
- Sophisticated plugin for removal of Poisson noise
- Also handles noise removal from multiple image samples

F. Luisier, C. Vonesch, T. Blu, M. Unser (2010). Sig. Process., 90, 2, 415-427.









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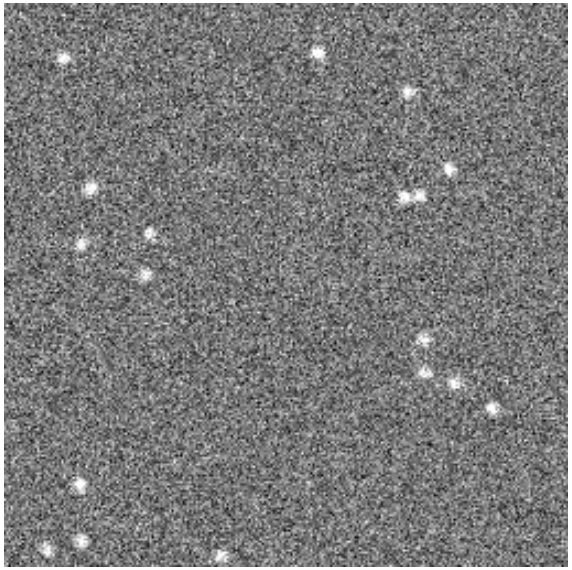
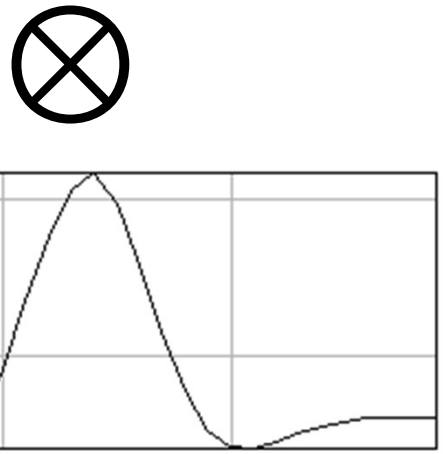
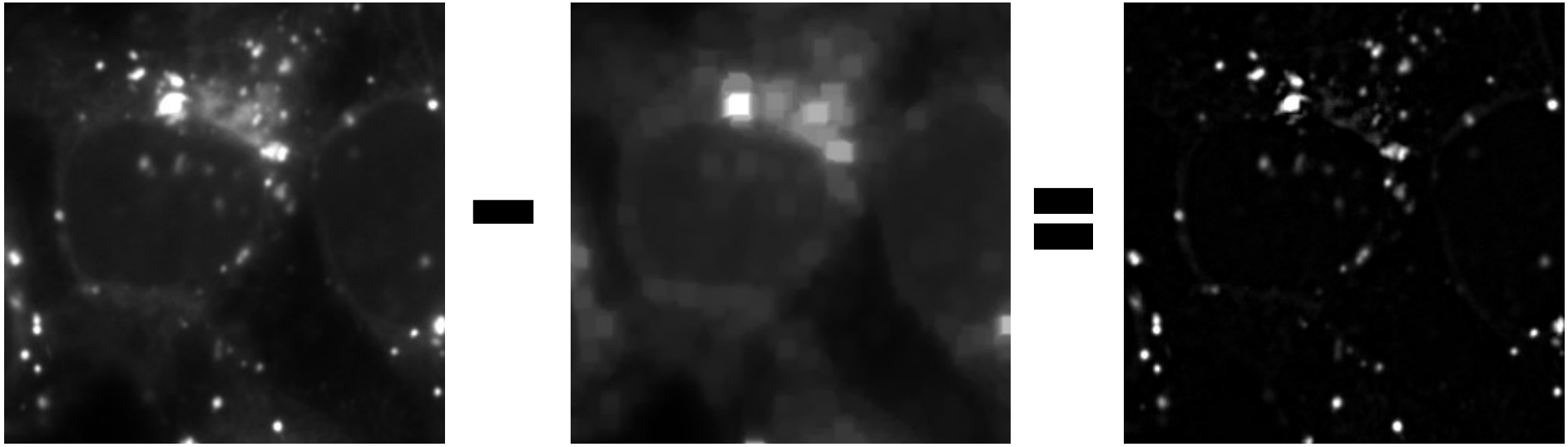
Laplacian of Gaussian filter for spot detection





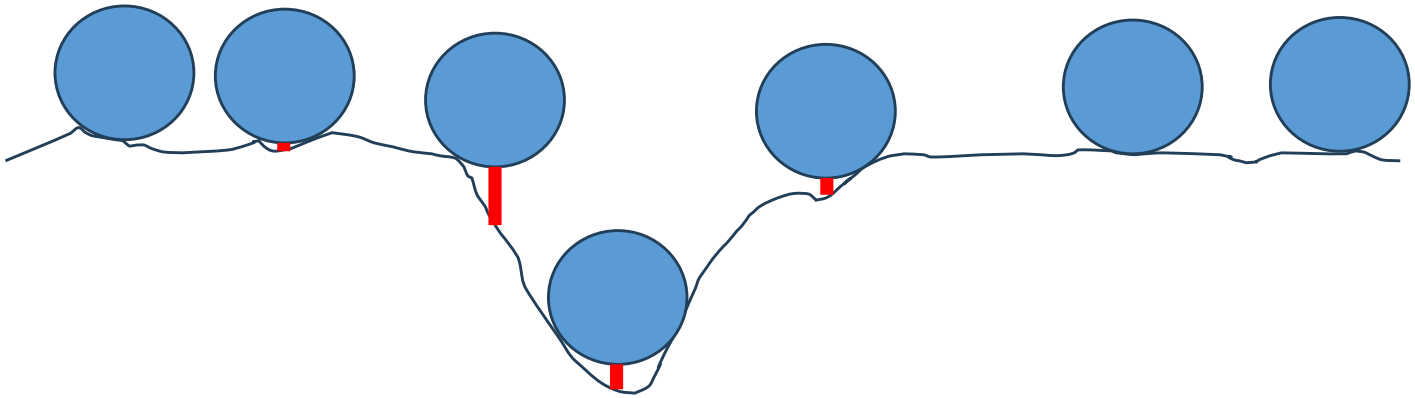
Rolling ball background subtraction

- Background calculated using the mean of a circular local neighbourhood
- The radius of the “ball” should be at least as large as the radius of the largest target



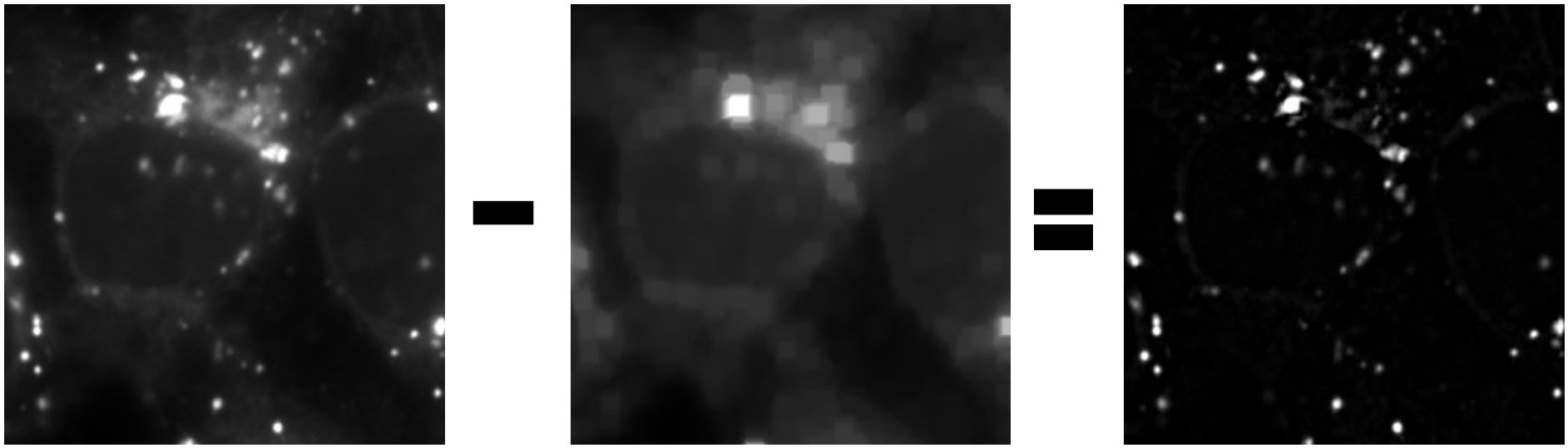
Rolling ball background subtraction

- There are different rolling ball background subtraction algorithms
- The more general algorithm uses a 3-dimensional “ball” where one dimension is the image intensity!
- The apex of the ball is the background value



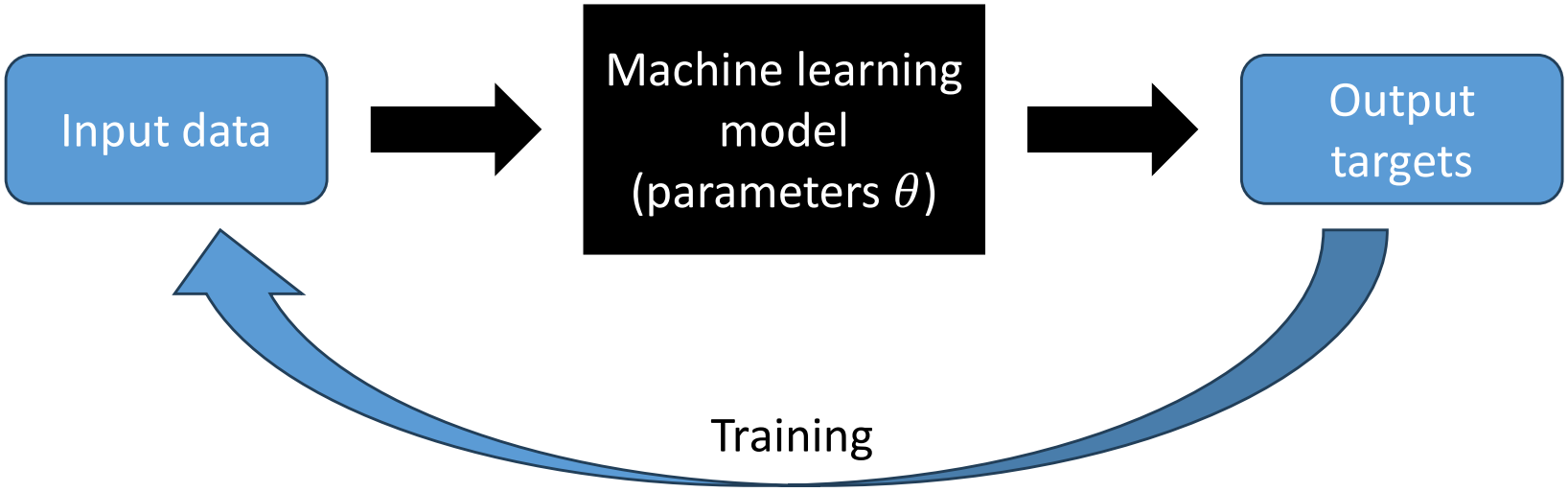
Rolling ball background subtraction

- Background calculated using the mean of a circular local neighbourhood
- The radius of the “ball” should be at least as large as the radius of the largest target
- Note: the “ball” is 3-dimensional and one dimension is the image intensity!



Machine learning/Deep learning

- Current SoTA for many processing and analysis tasks
- Algorithms are defined by millions or billions of parameters
- Algorithms *learn* by minimising the error between their outputs and target outputs *provided by the user*, given a set of input data
- Standard machine learning requires user-provided input and target data, as well as a computer with a powerful GPU
- Once a model has been trained, it can be shared and reused



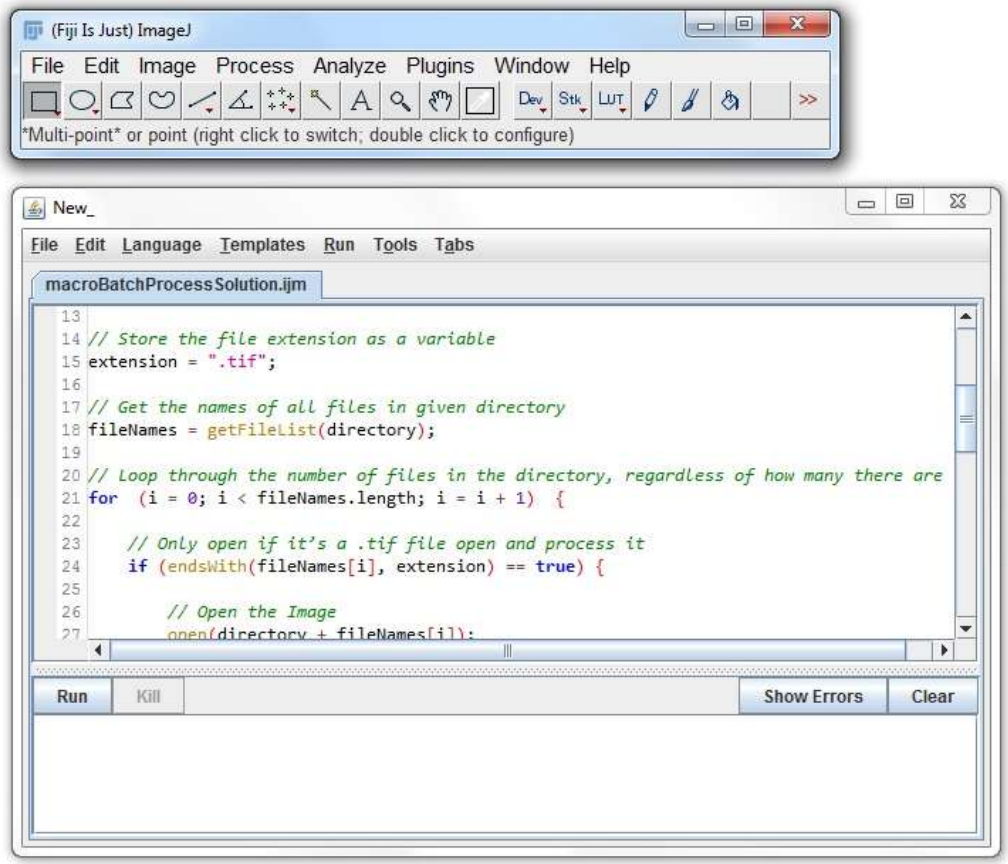
Machine learning/Deep learning

- Once a model has been trained, it can be shared and reused without additional training
- This assumes that the data for the application for which it is used *resembles that on which it was trained*
- Note: the model will usually not warn if new data is not like its training data. It will simply give its best guess at an answer, *which may be bad*
- Can start with DeepImageJ plugin: https://deepimagej.github.io/
- There is a small library of pre-trained models here: https://bioimage.io

deepImageJ

- Exercises

Part 3: Introduction to Macros and Workflow Automation



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Why automate a workflow?

- Save time and eliminate user mistakes
- Less biased and more consistent approach
- Have a record of what you have done

A macro is just a sequence of ImageJ commands

- Simple, easy to learn language
- Calls predefined ImageJ and Java functions
- Any ImageJ menu item can be called with a macro command
- Lots of online tutorials examples and resources including:

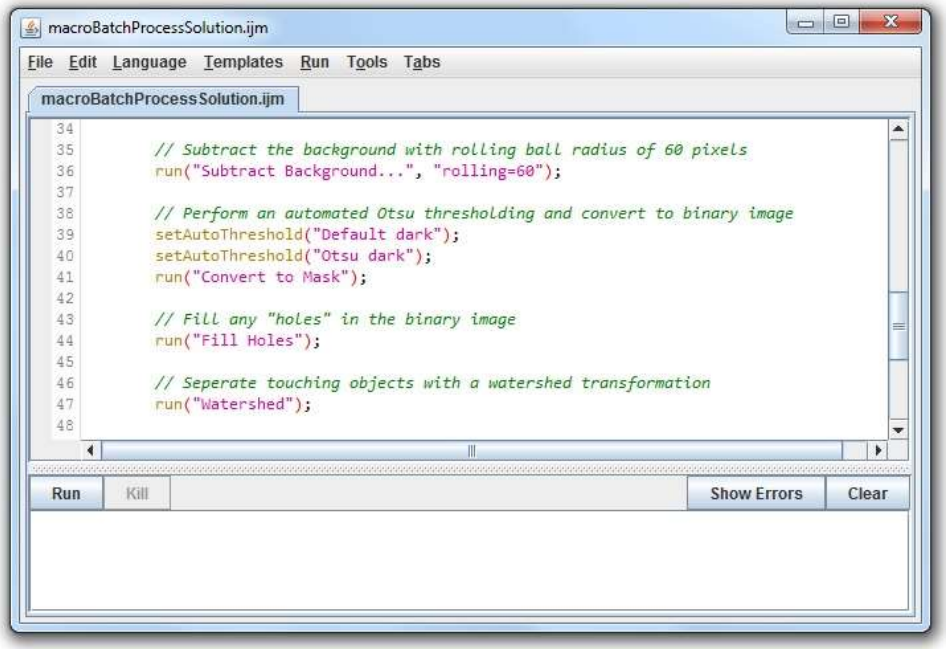
  http://fiji.sc/Introduction_into_Macro_Programming

- ImageJ has dedicated macro language but can write more complex scripts in other languages (e.g. Python)

The Fiji script editor

Plugins -> New -> Macro (or press {)

- Syntax highlighting
- Templates menu contains some useful examples



Macro language

```
var = 4;
```

variable name assignment value line end

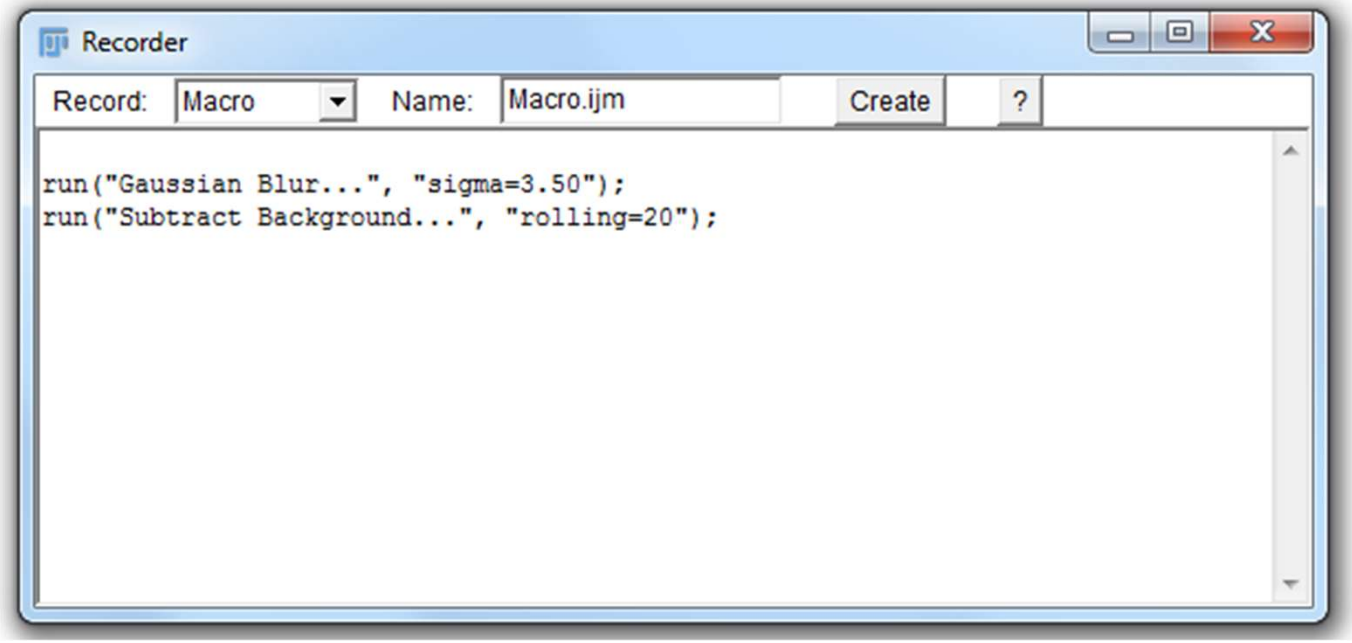
```
out = sum(var_1, var_2);
```

variable name assignment method arguments line end

The Command Recorder

Plugins -> Macros -> Record...

- A really simple way to automate a workflow and make a Macro
- Simply perform the analysis on one image and click create!



For loops

An iterative statement that executes a block of code a specified number of times.

```
for (initialisation; stop condition; increment) {
        do something
    }
```

```
for (i = 1; i <= 10; i = i + 1) {
        run("Add...", "value=" + i);
}
```

Conditional statements

if (condition) {do something}
else if (other condition) {do this instead}
else {do the alternative}

```
if(nImages() == 1) {
      print(getTitle() + " is open.");
}
else if(nImages() > 1) {
      print(nImages() + " images are open.");
}
else {
      print("No images are open.");
}
```

- Exercises

Whats next?

- COMPARE will be organising further courses covering:
  - Segmentation, deconvolution, tracking, colocalization etc
  - Analysis and visualisation of light sheet and SMLM datasets
  - Any suggestions?

- Loads of online resources for further study:
  - ImageJ website
  - ImageJ forum
  - Open source image analysis textbook

- We can work with you on collaborative projects.
  Email (compare@contacts.bham.ac.uk) for enquires.

Acknowledgments

This course was organised and run by the COMPARE Advanced Imaging Facility (COMPARE Advanced Imaging Facility - University of Birmingham).

The materials were adapted from a course originally run by Jeremy Pike (https://github.com/JeremyPike), formerly COMPARE Advanced Image Analysis Specialist.