# Image Analysis with Fiji

An introductory course for biologists
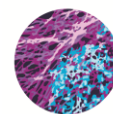
Jeremy Pike, Image Analyst for COMPARE

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

**IN PARTNERSHIP:**
The Universities of Birmingham and Nottingham

**COMPARE**
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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 ☺
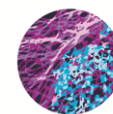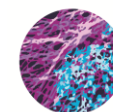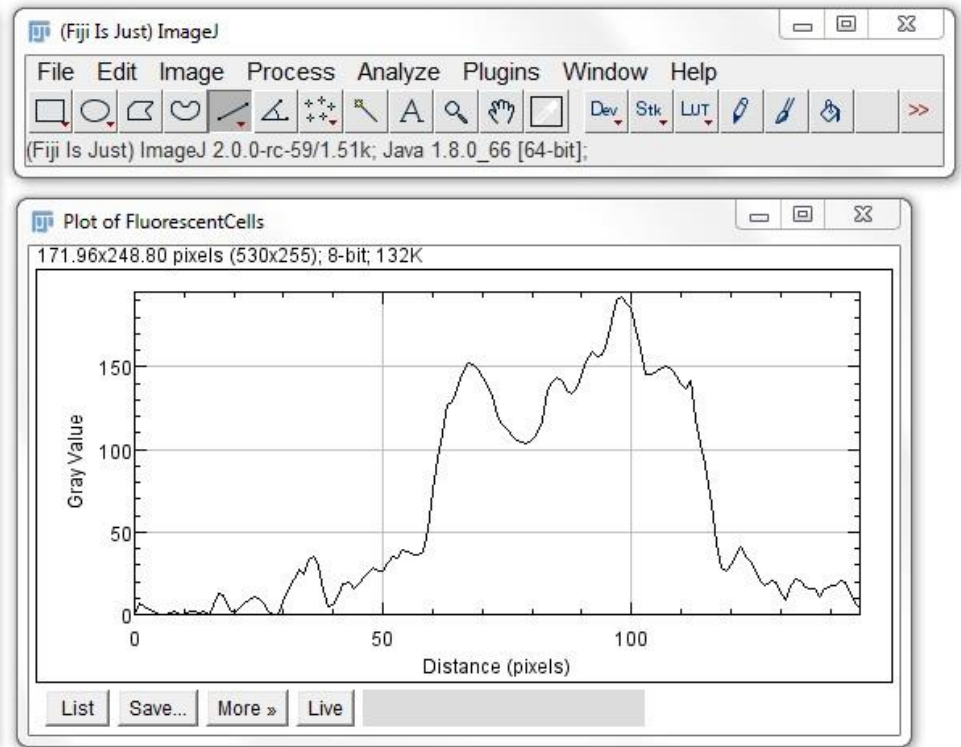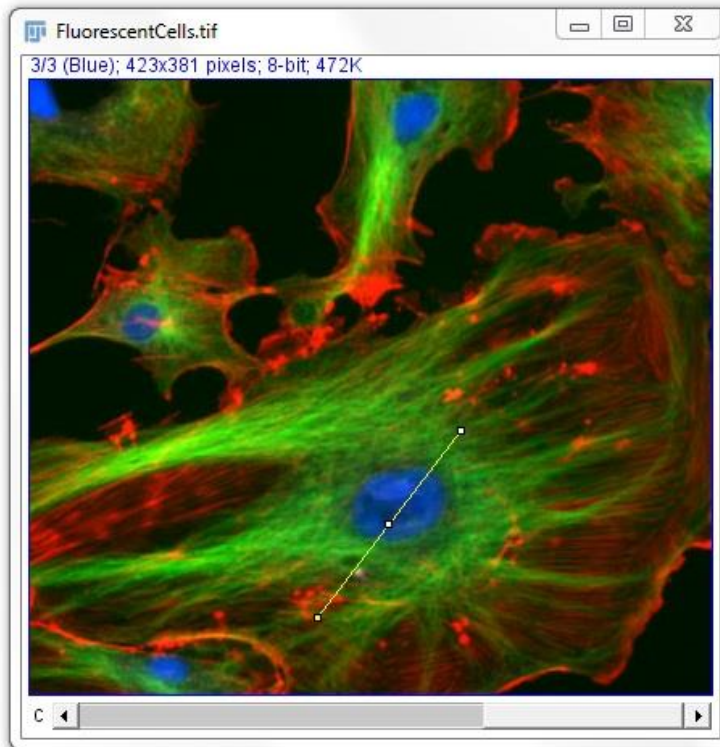
2. Download the course material:

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

Click whole course as a zip archive and unpack

# Part 1: Basics

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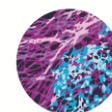
**ImageJ2** is a complete rebuild of ImageJ, it is built into Fiji

*Rueden, C. T., et al. (2017), preprint arXiv:1701.05940.*
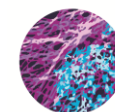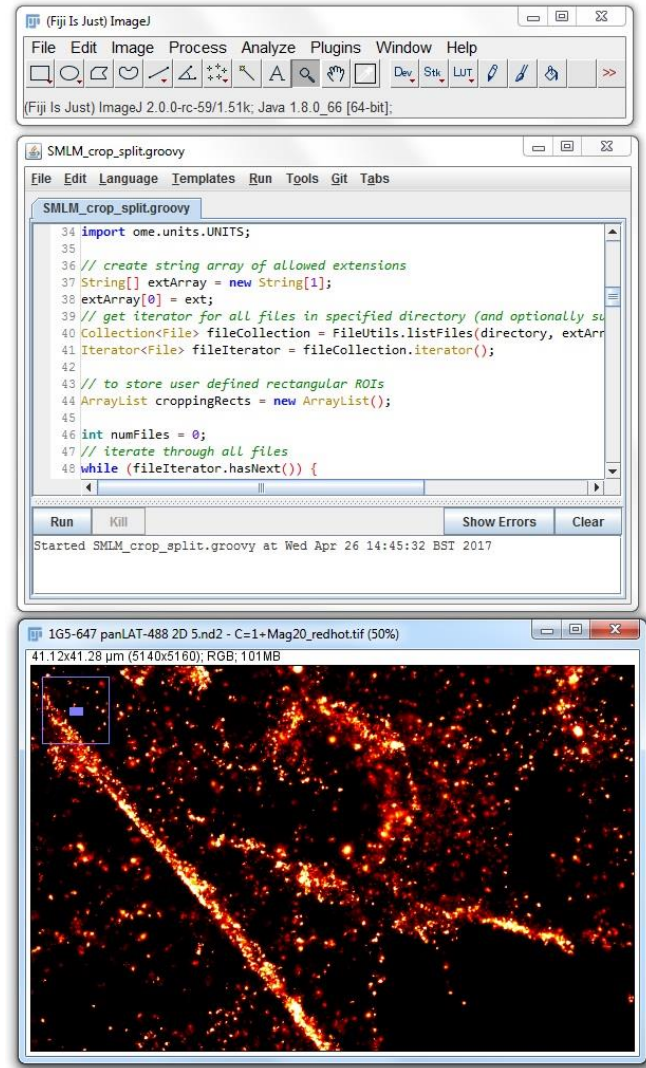
### If in doubt get Fiji!

# 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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# Digital images are simply arrays of numbers



| 21 | 23 | 25 | 26 | 29 | 31 |
| 20 | 21 | 25 | 31 | 40 | 48 |
| 30 | 29 | 33 | 45 | 61 | 72 |
| 26 | 35 | 53 | 73 | 88 | 96 |
| 54 | 72 | 91 | 110 | 120 | 124 |
| 94 | 117 | 133 | 142 | 144 | 143 |
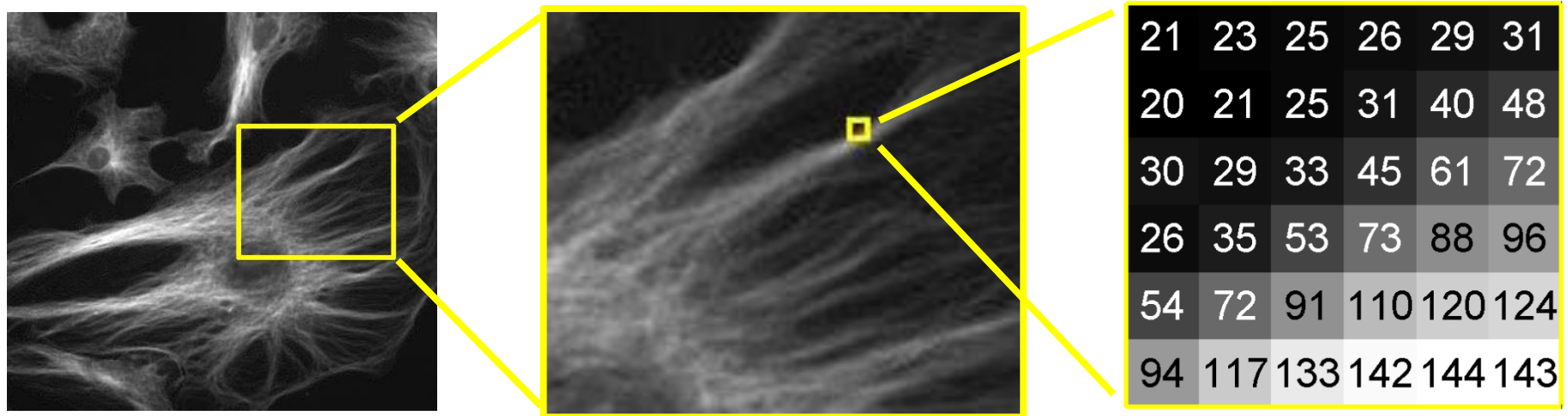
Note pixels are samples of intensity at a spatial point, not little squares!

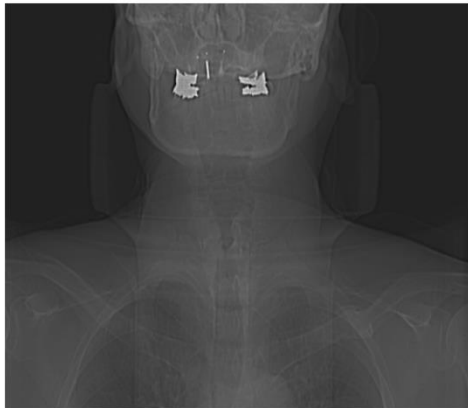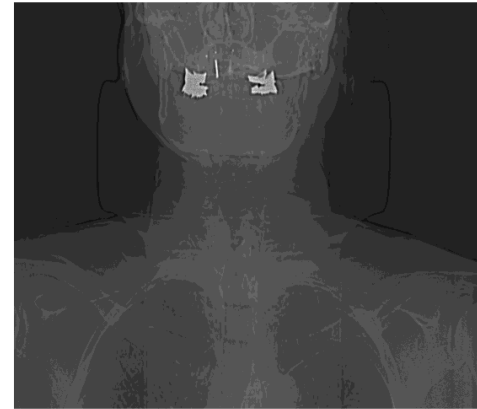# Dynamic range is the number of values each number can take
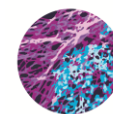
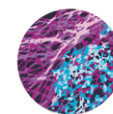1 bit = 2
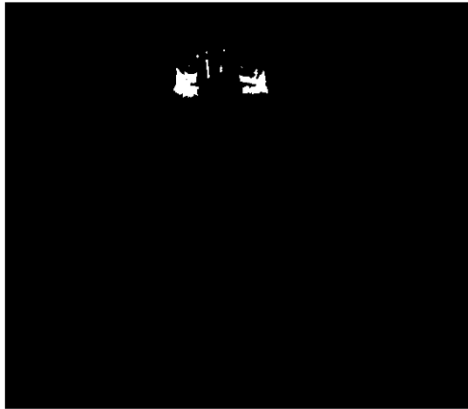
2 bit = $2^2$

4 bit = $2^4$



8 bit = $2^8$

12 bit = $2^{12}$

16 bit = $2^{16}$

# Multi Channel Data

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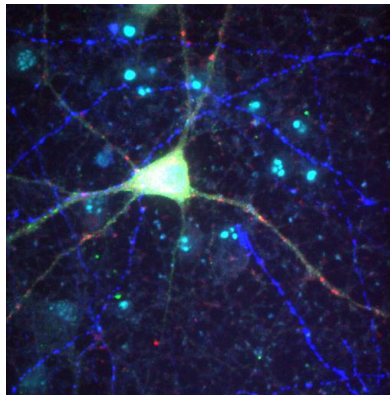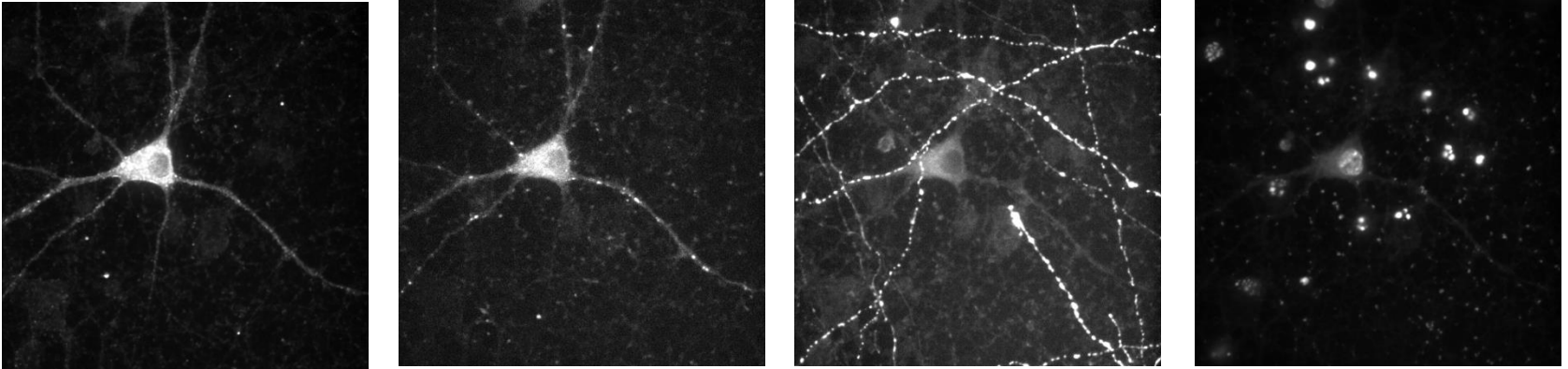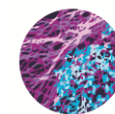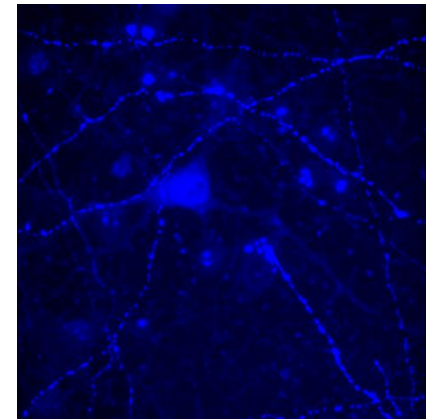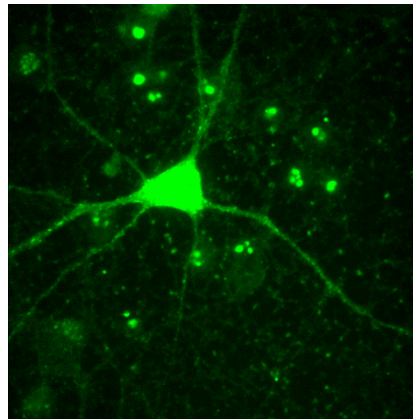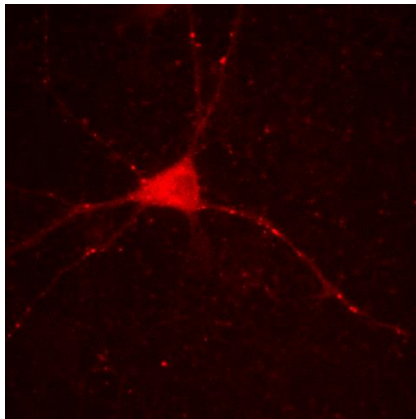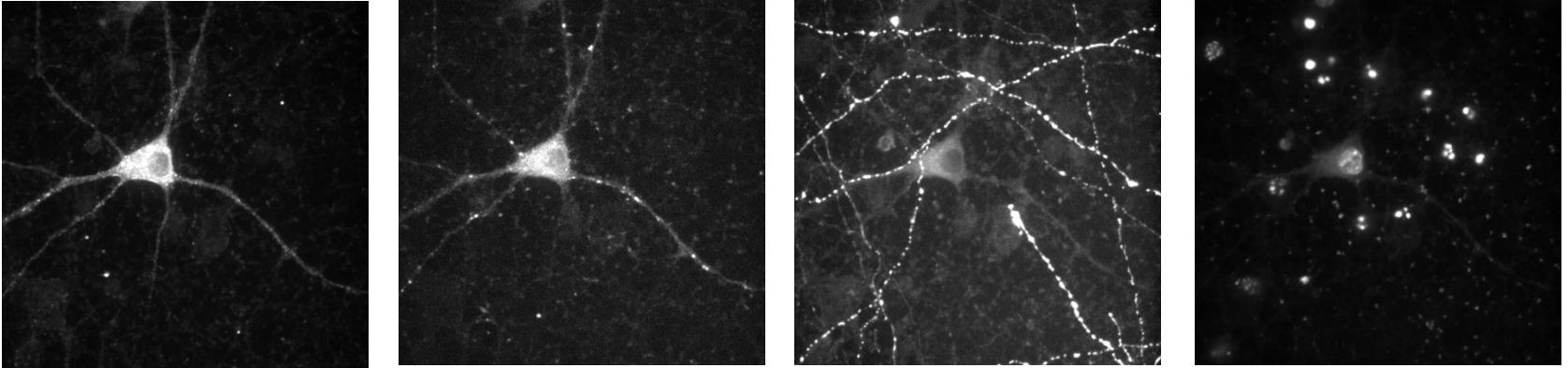
# RGB Colour Images

# RGB Colour Images
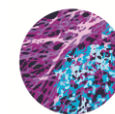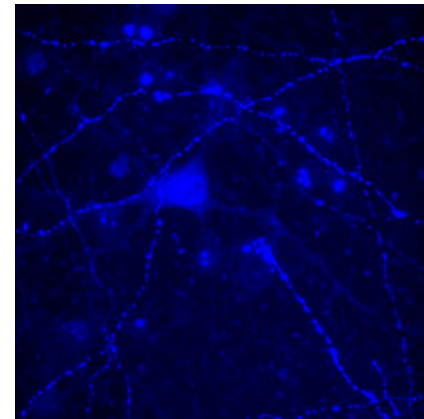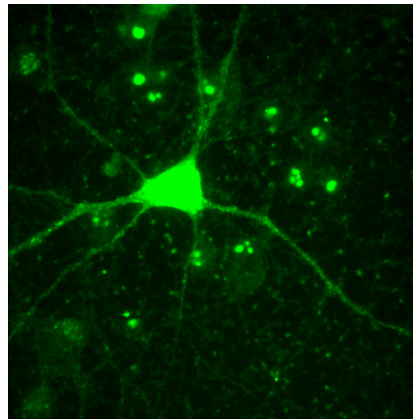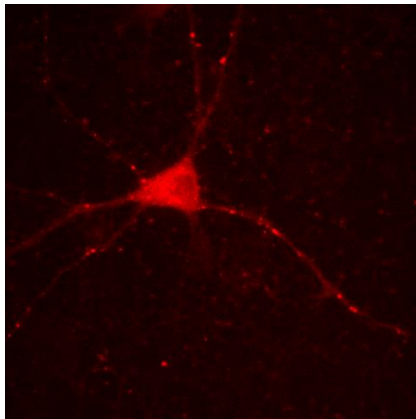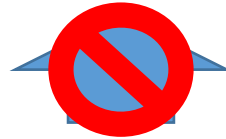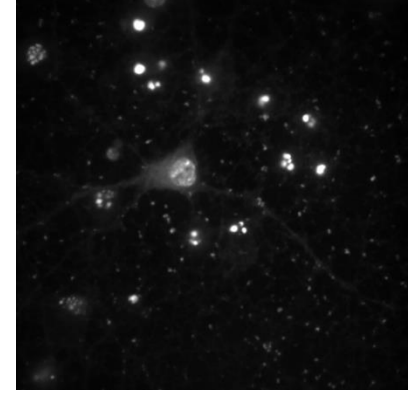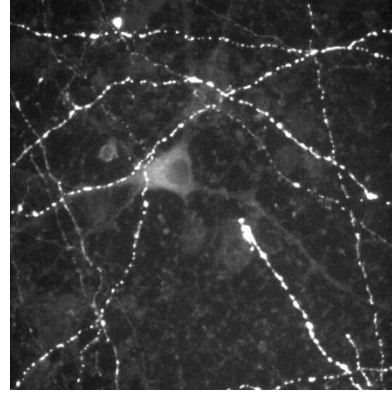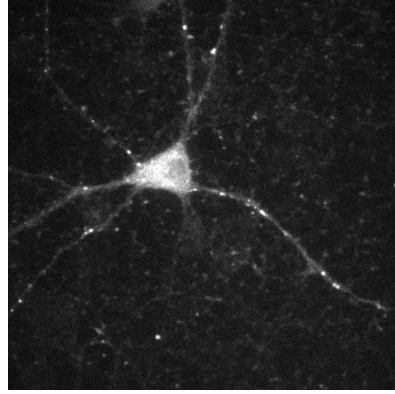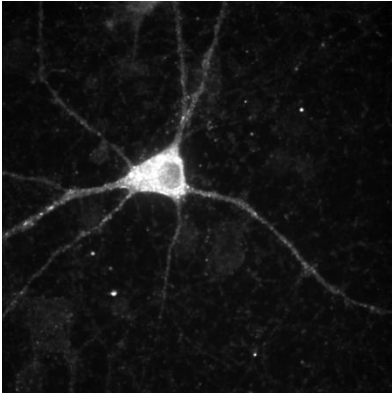
# What can we "see" on a monitor?
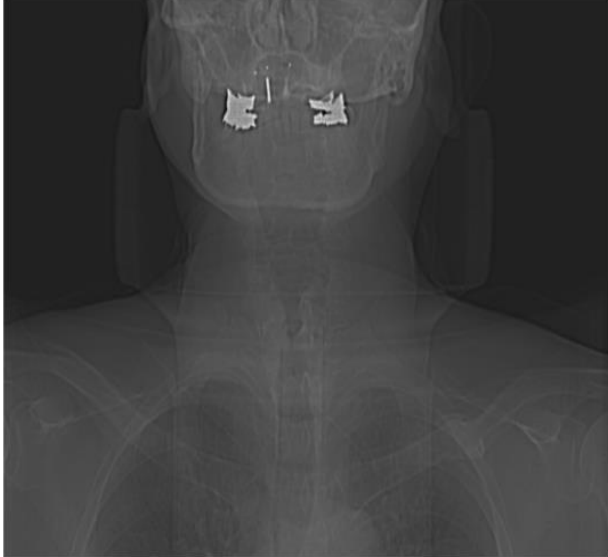


8 bit = $2^8$

16 bit = $2^{16}$

Can you tell the difference?
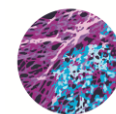
# What can we "see" on a monitor?
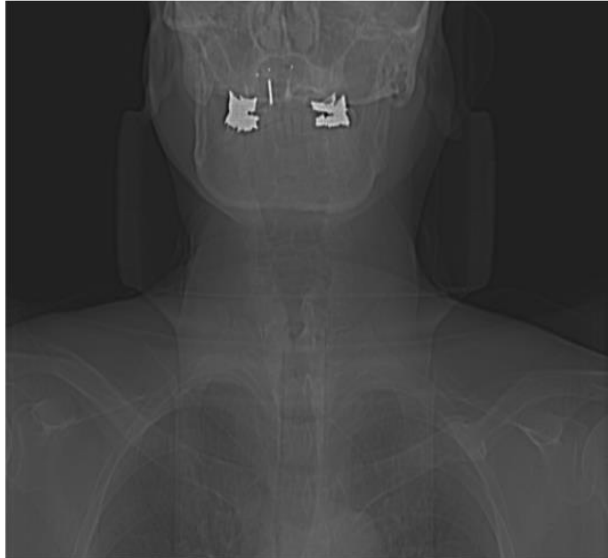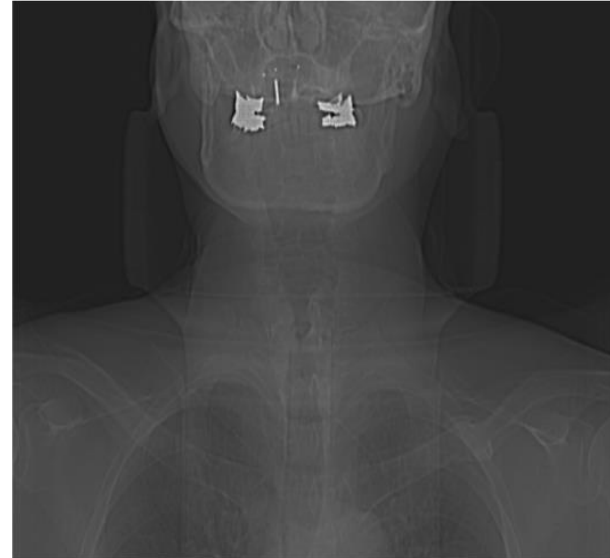


8 bit = $2^8$



16 bit = $2^{16}$

- 8 bit display range

- 3 x 8bit RGB for colour display

- What bit-depth can our eyes detect?
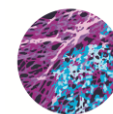
# Microscopy data

- Image: 2D    XY

- Stack: 3D    XYT      XYZ    XYC

- Hyperstack: 4D, 5D

XYZT

XYZC

XYZCT

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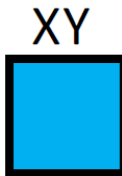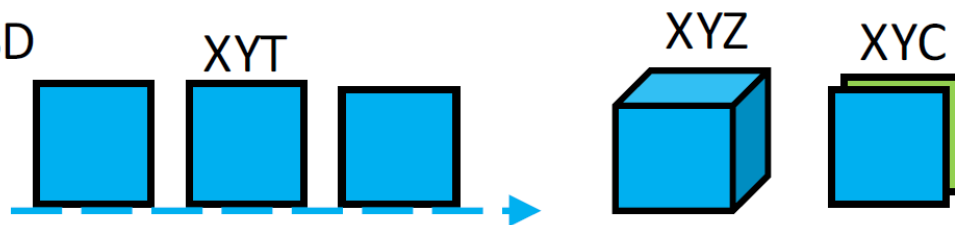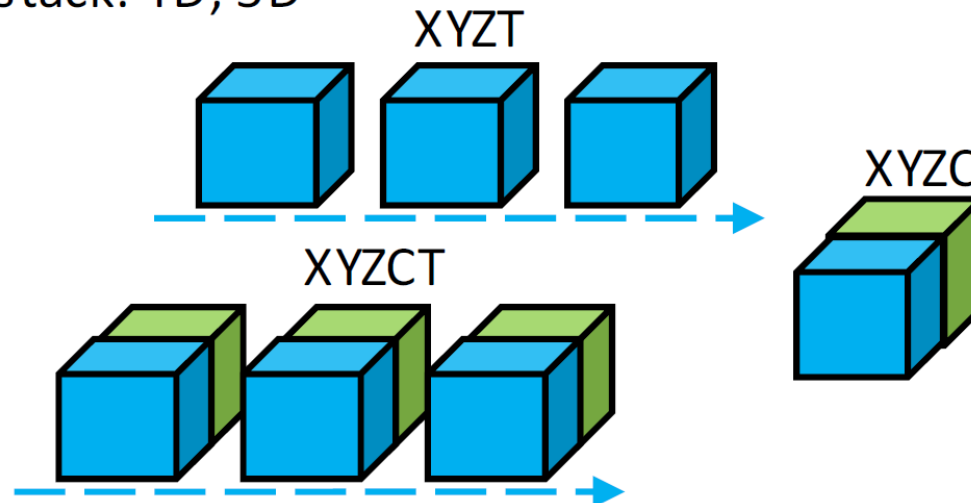
# Volume rendering in Fiji

We recommend using ClearVolume
http://imagej.net/ClearVolume
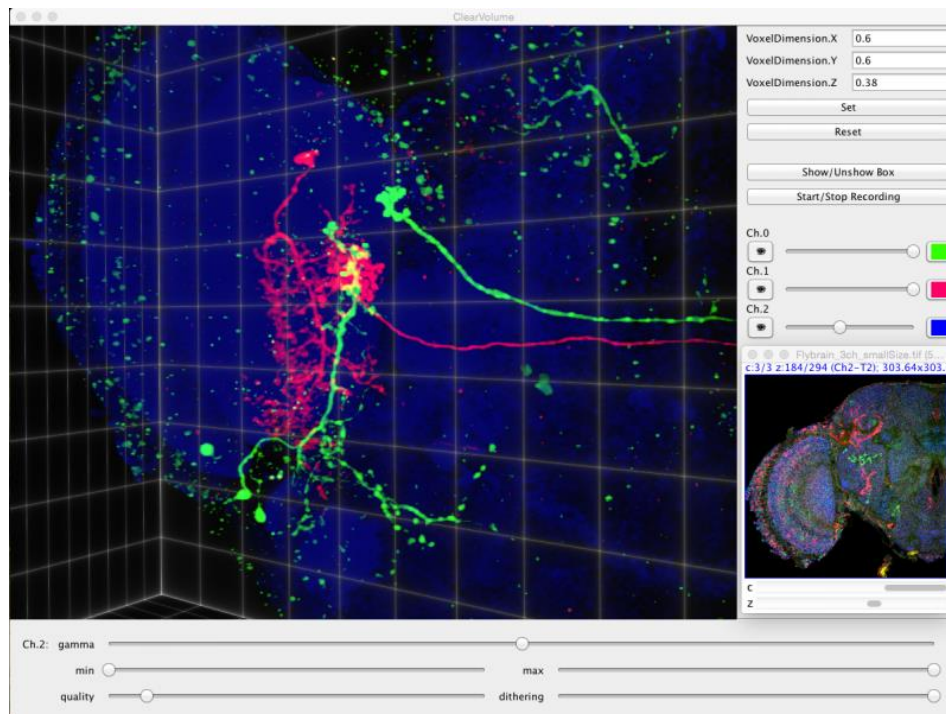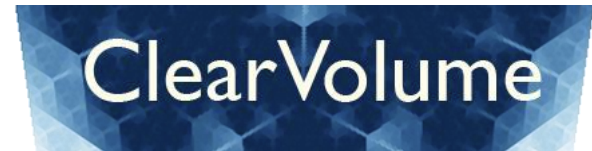


*Royer, L. A., Weigert, M. & Günther, U. et al. (2015), Nature Methods 12(6): 480-481.*
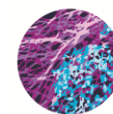
# Common file formats

- **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

# An image is the sum of many point spread functions



Figure 3
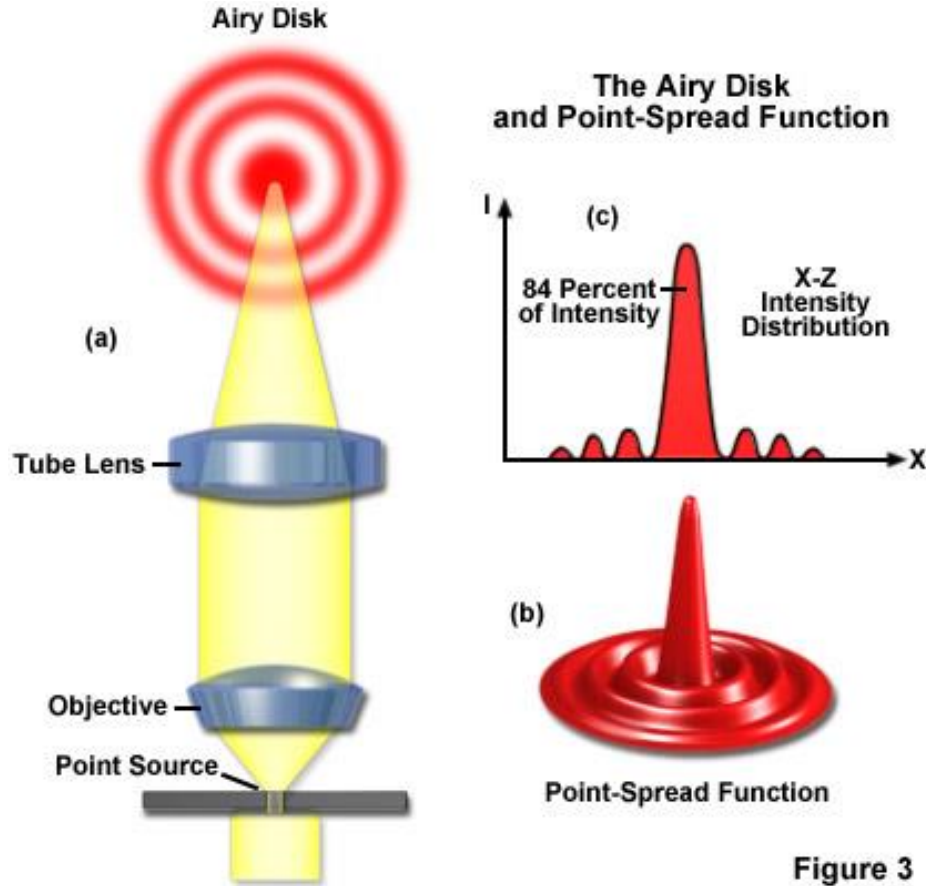
*"Each element of the primary image is a small diffraction pattern, and the actual image, as seen by the eyepiece, is only the ensemble of the magnified images of these patterns"*

**Born and Wolf**, Principles of Optics

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# What determines the resolution limit in light microscopy
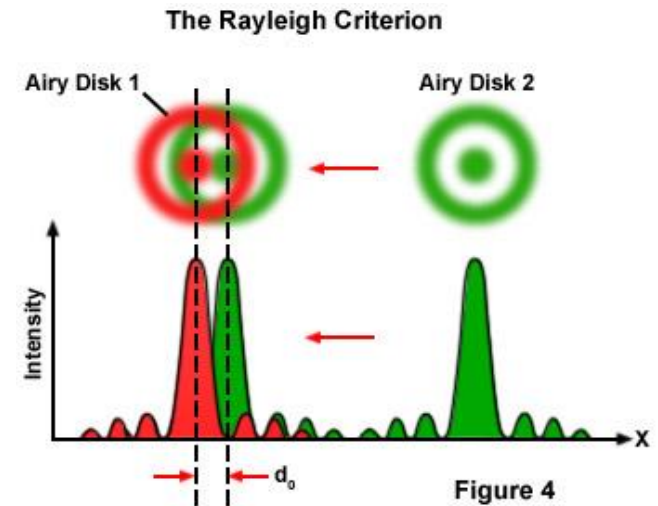
- ~~The number of pixels in an image~~

- ~~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

**The Rayleigh Criterion**

Airy Disk 1

Airy Disk 2

Intensity

$d_0$

X

Figure 4

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

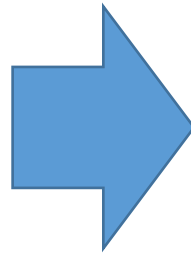# Part 2: Introduction to Image Processing and Analysis
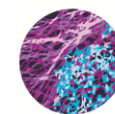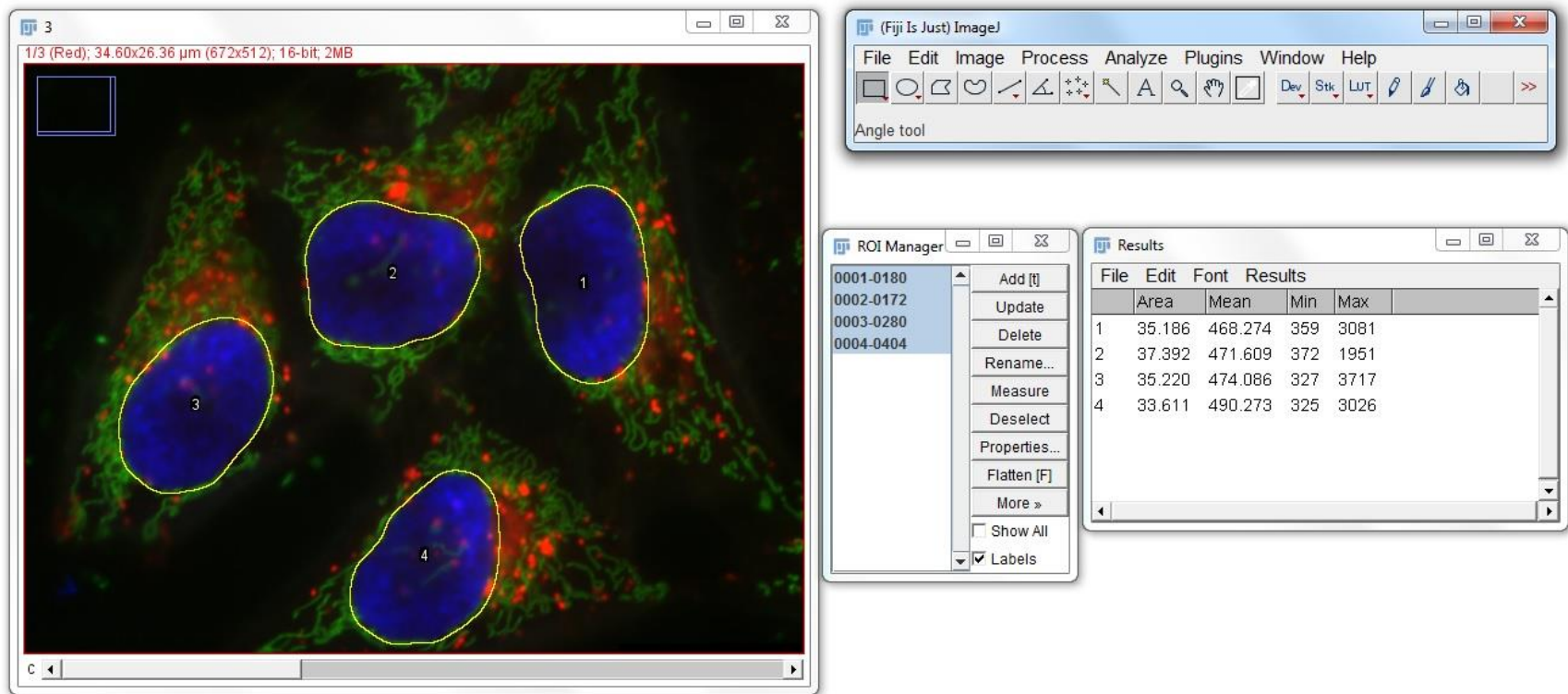
# Image Processing:
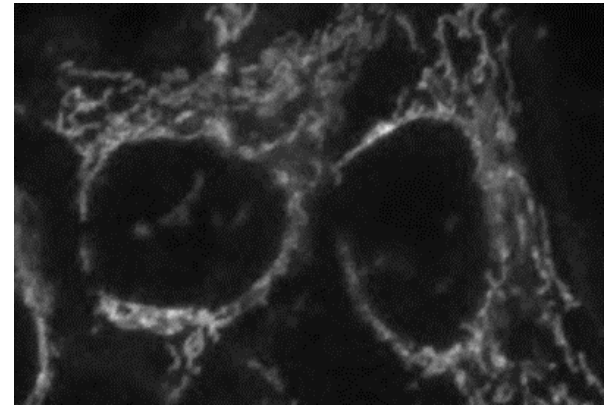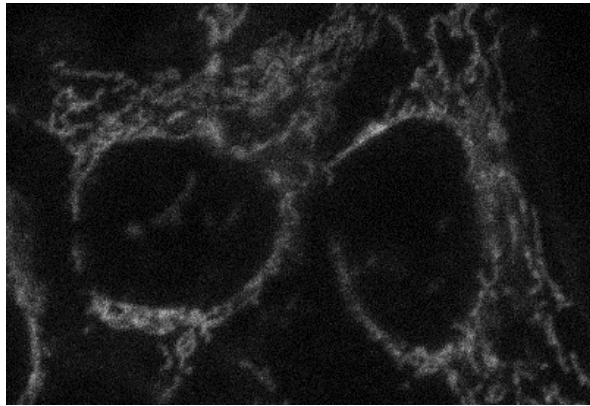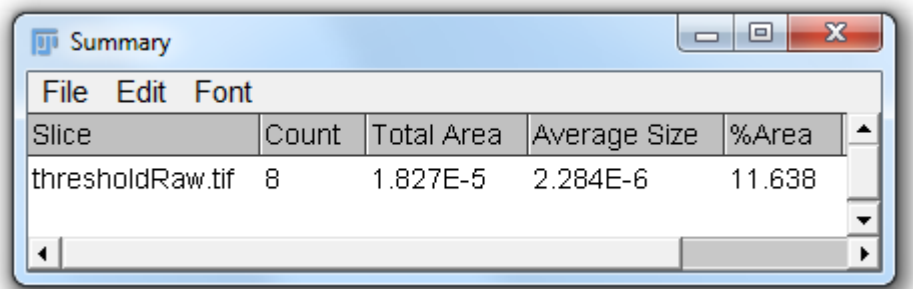


# Image Analysis:
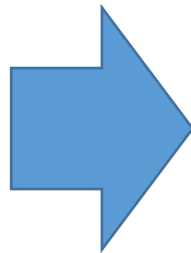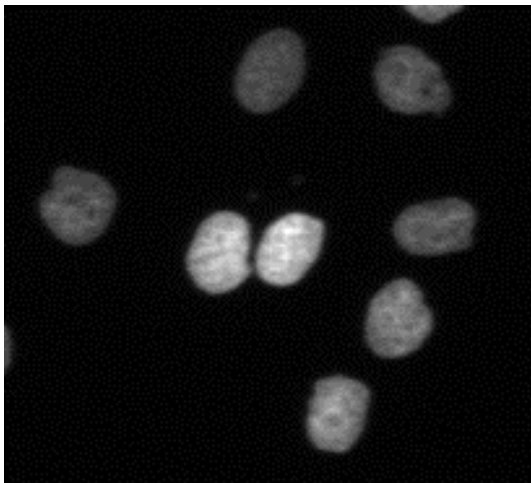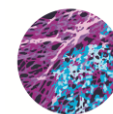
# Why do computational processing and analysis?

- Its quantitative

- Its unbiased

- 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***
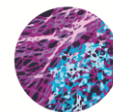
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# What outer circle is the same size and the central circle?

# Intensity based thresholding to segment objects

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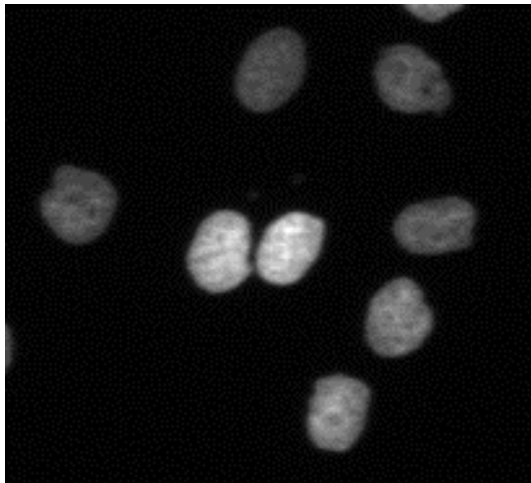
# Automated threshold values are preferable to manual selection

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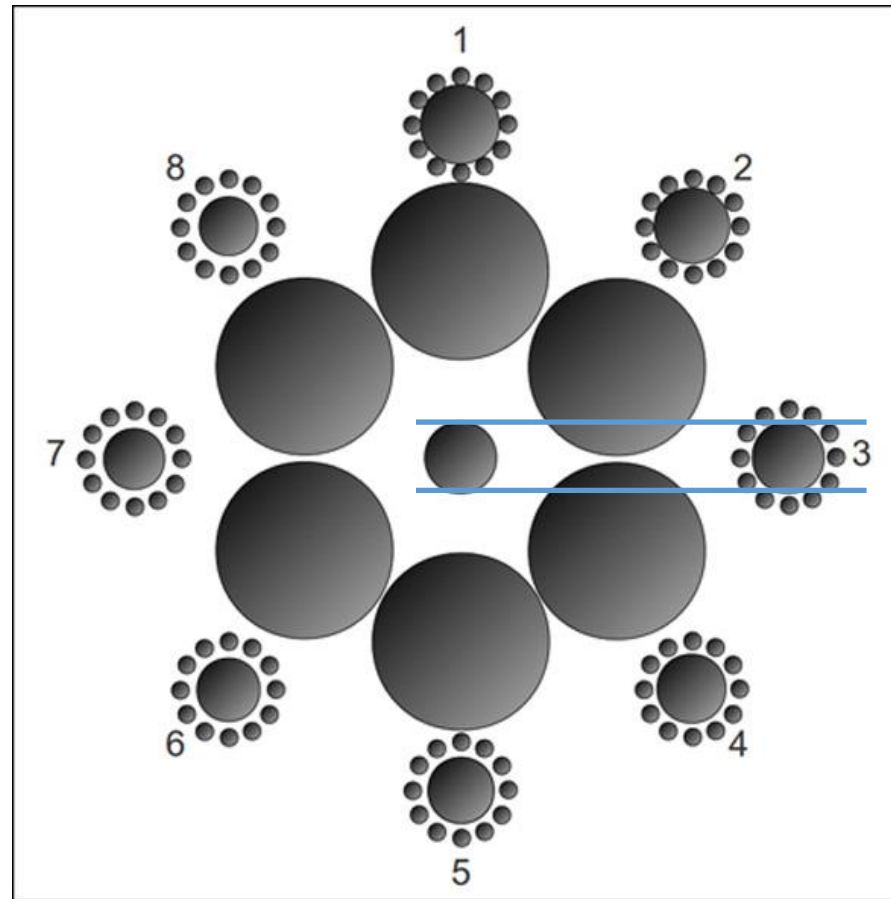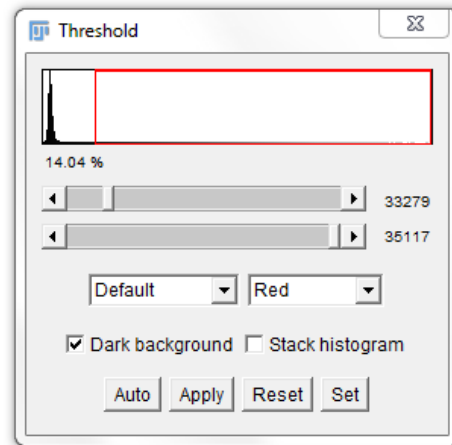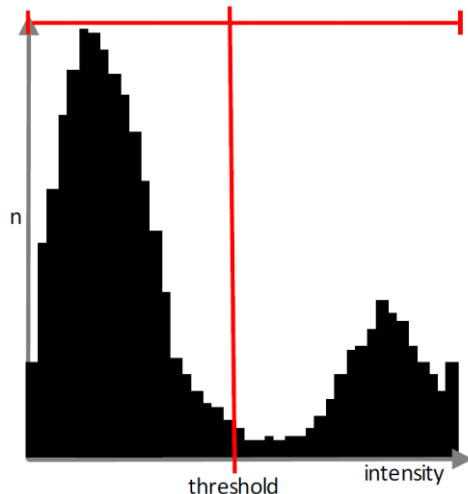


*Otsu, N (1979), IEEE Trans. Sys., Man., Cyber. 9: 62-66.*

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

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

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# Automated threshold values are preferable to manual selection
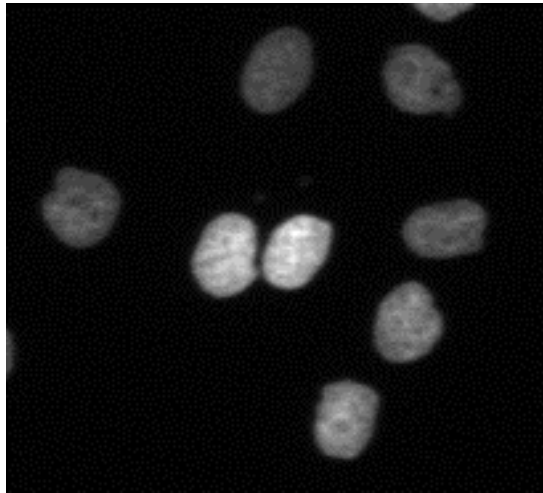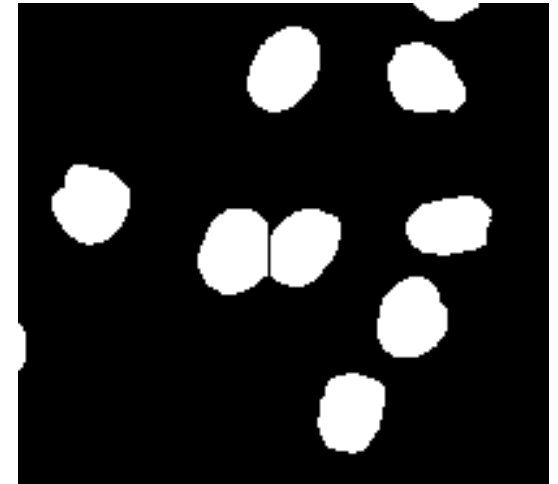


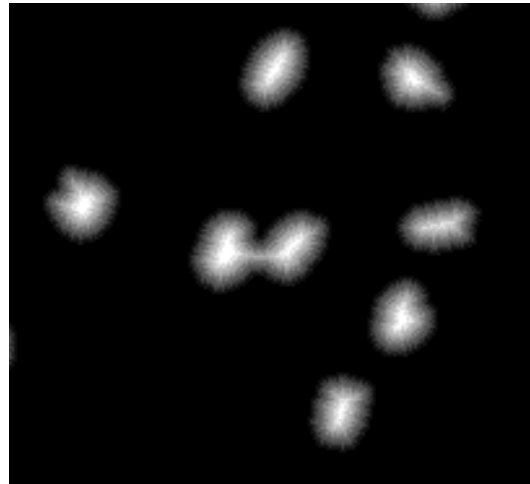Raw Data             Otsu Threshold             Li Threshold

# 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
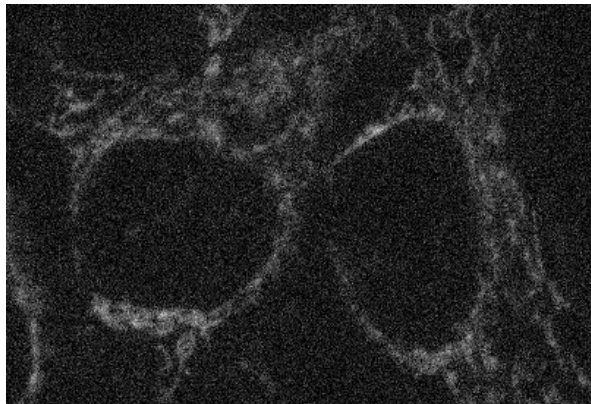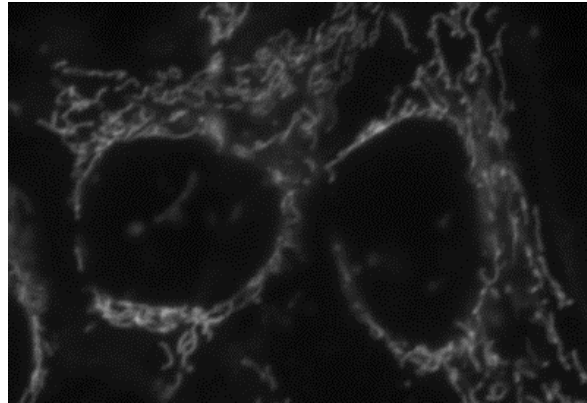


Gaussian
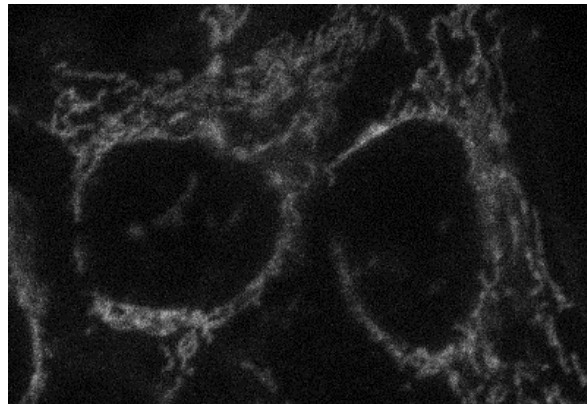
Poisson

Impulse
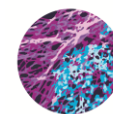
# Image filters and convolution

The value for a pixel in the filtered image is dependent on pixels in the local neighbourhood
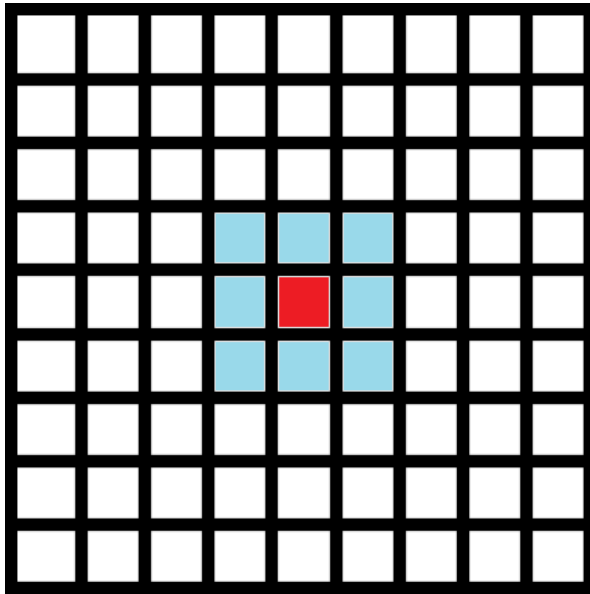


3x3 Neighbourhood
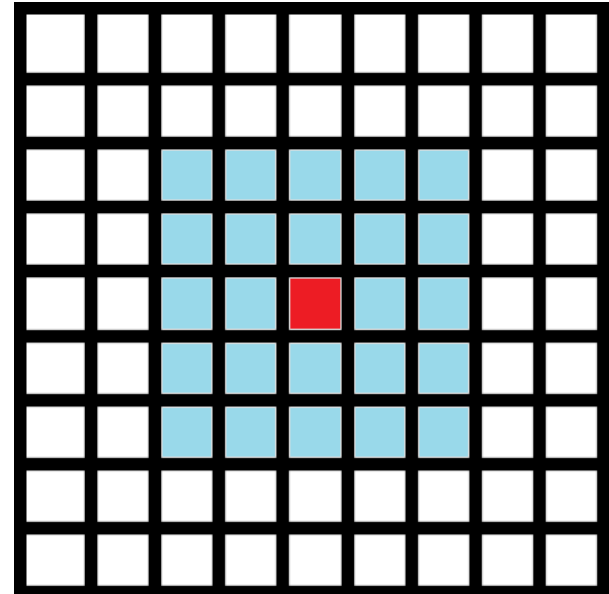


5x5 Neighbourhood

# Mean filter

- Pixel values given by mean over neighbourhood

- Removal of Gaussian and Poisson Noise
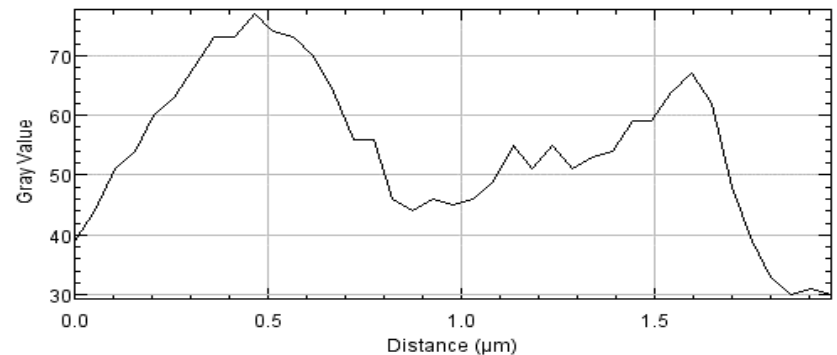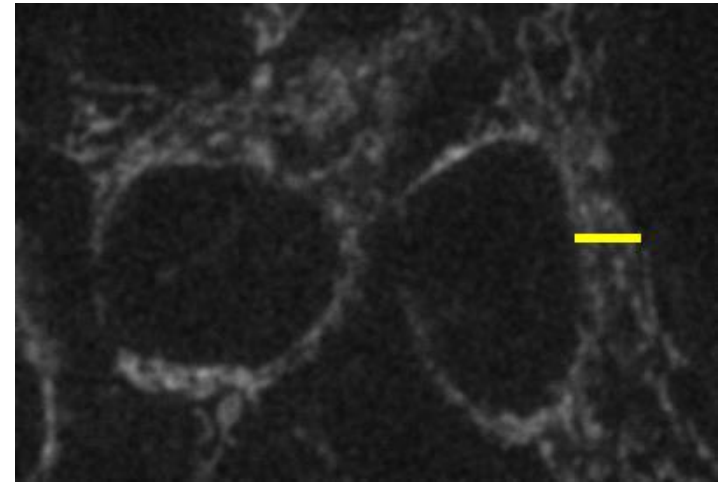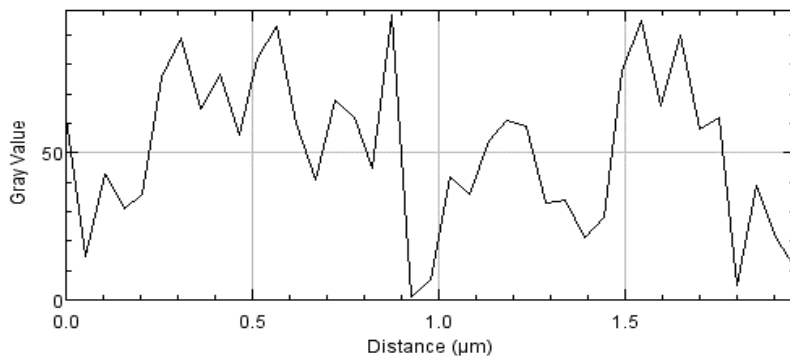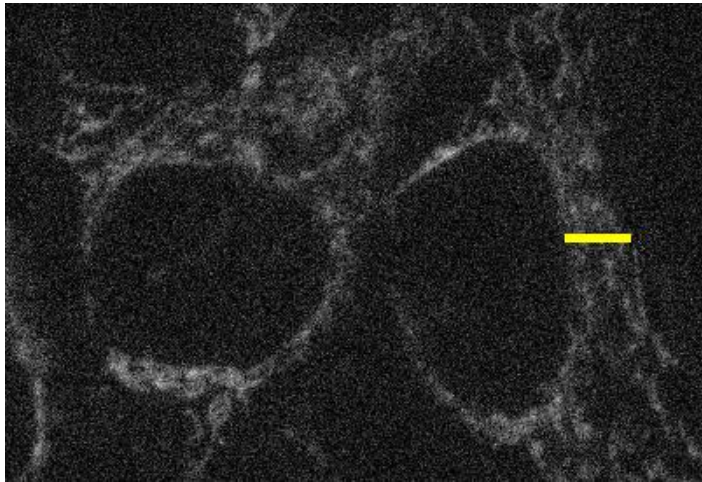
# Median filter

- Pixel values given by median over neighbourhood

- Removal of salt and pepper (impulse) noise

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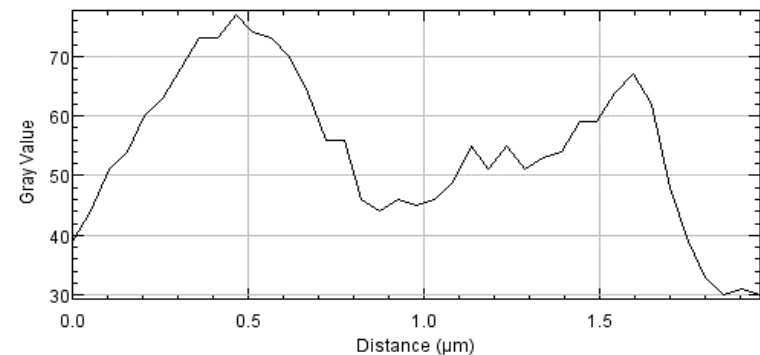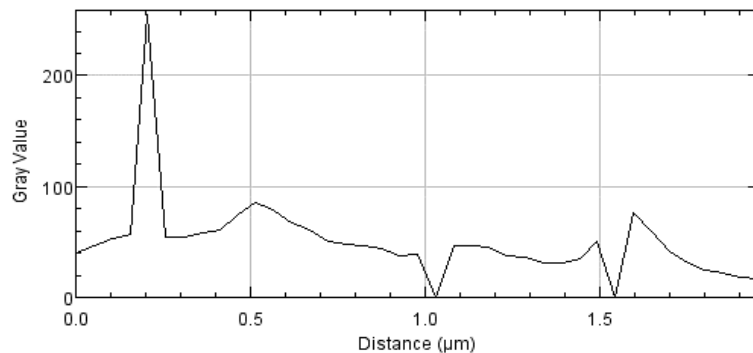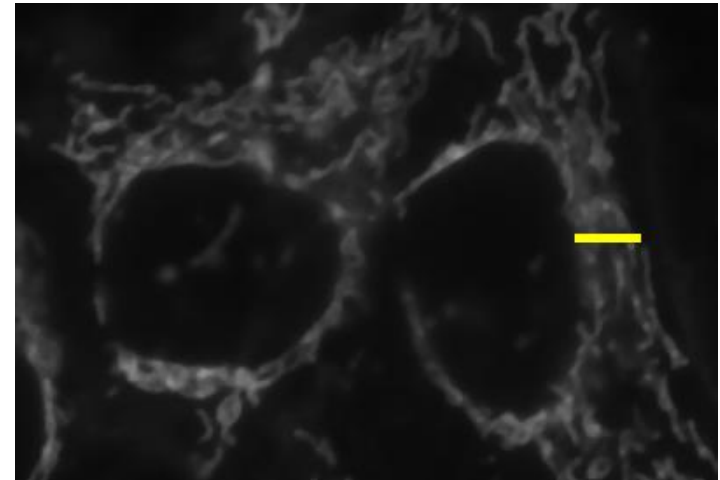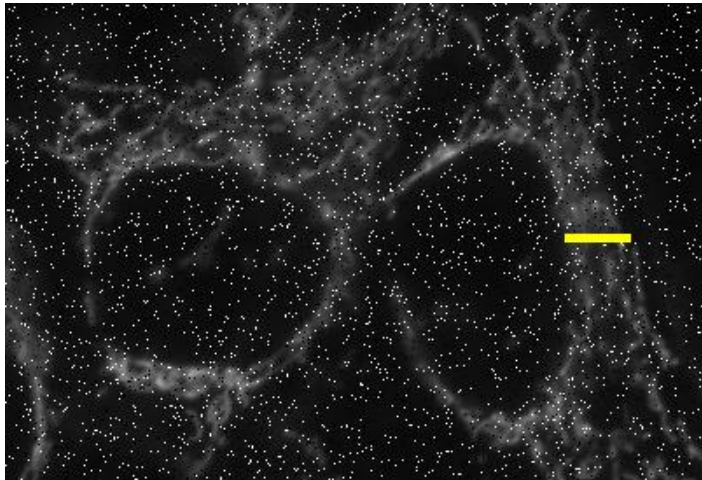
# Gaussian filter

- Contribution of neighbourhood pixels weighted by Gaussian profile

- Removal of Gaussian and Poisson Noise

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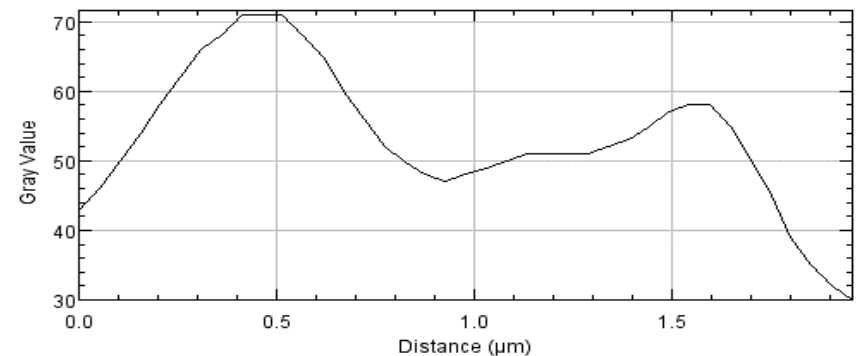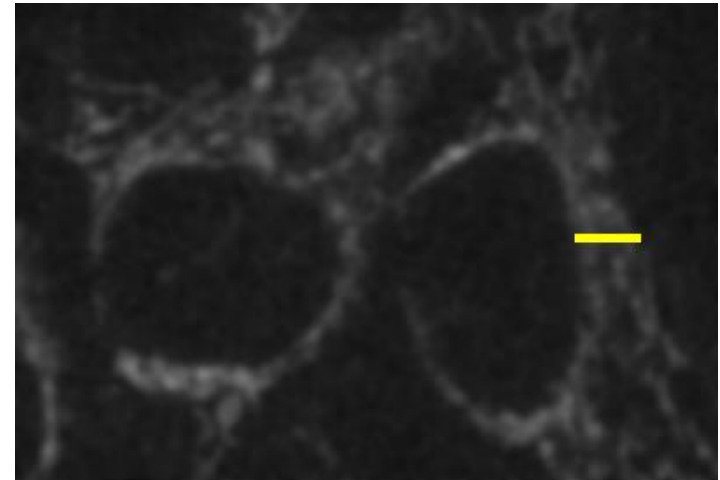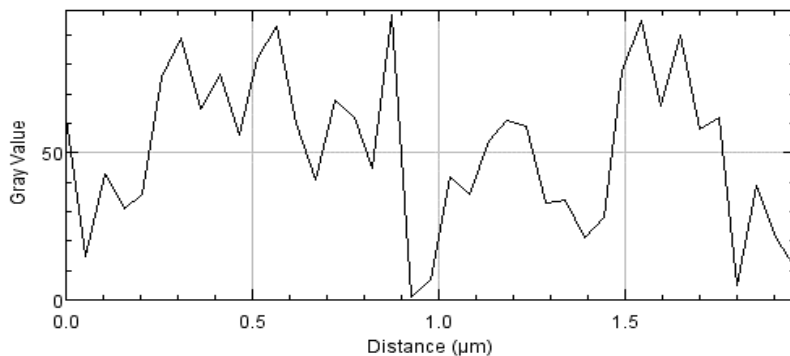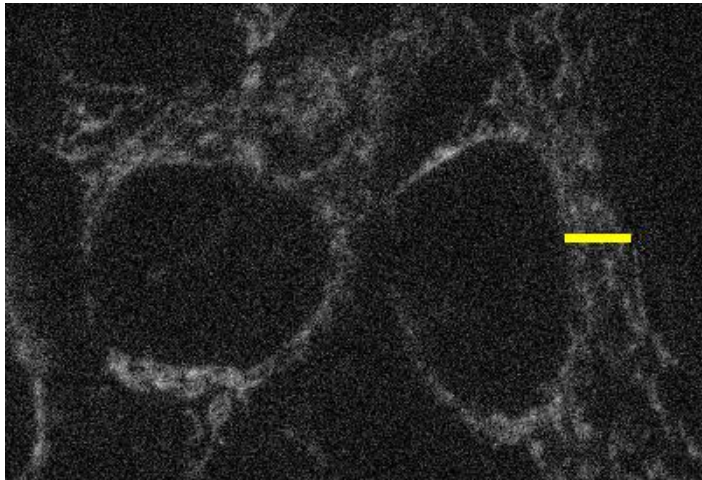
# The PureDenoise plugin

- Sophisticated plugin for removal of Poisson noise

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

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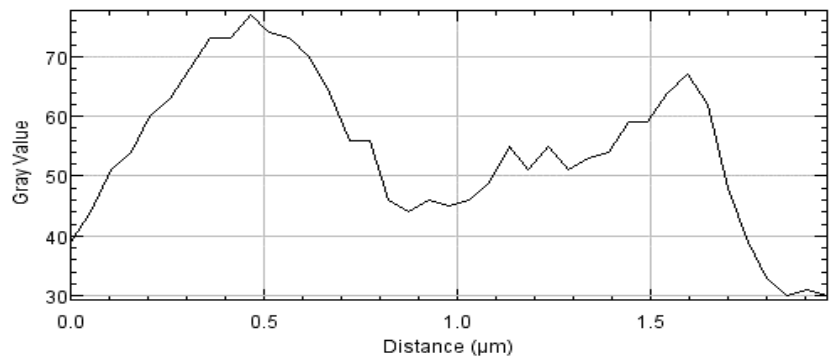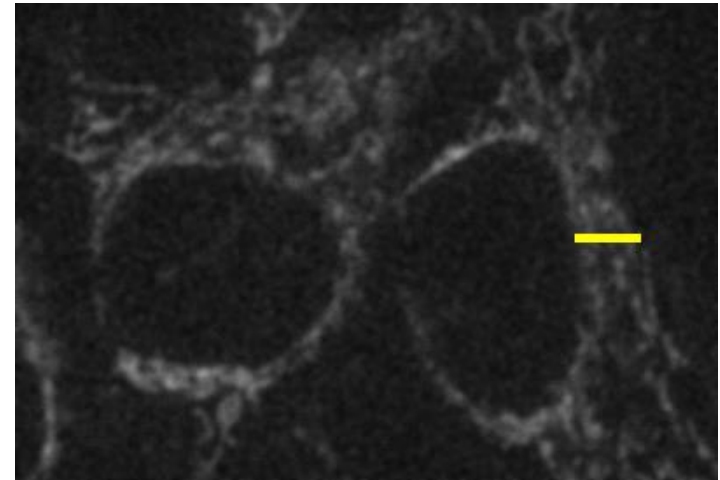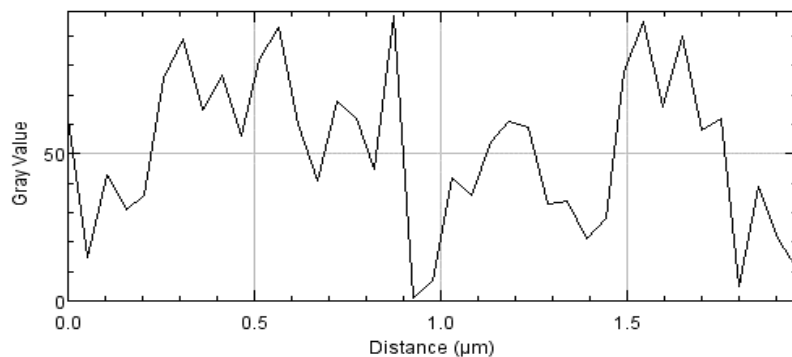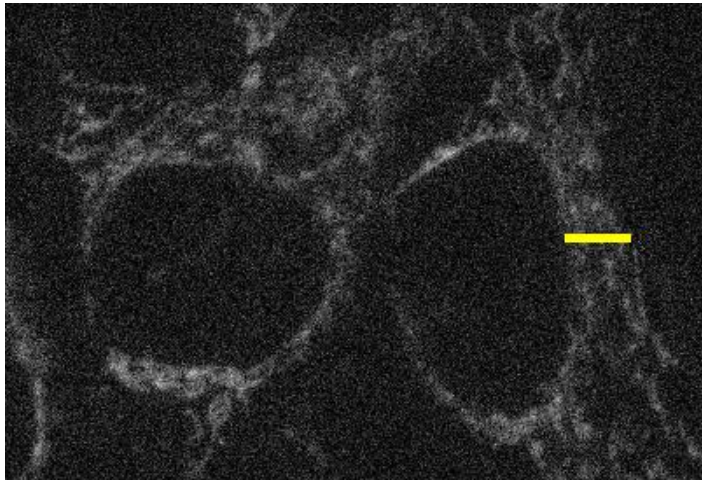
# Laplacian of Gaussian filter for spot detection



=

⊗
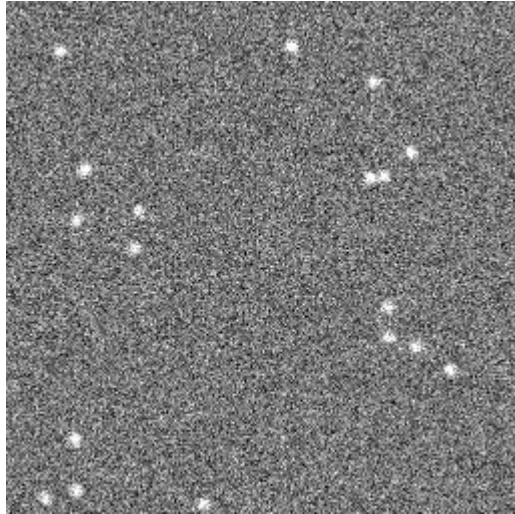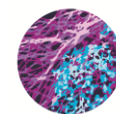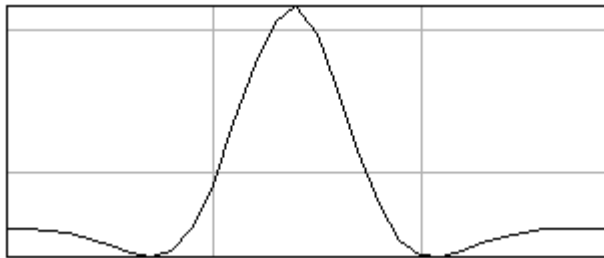
# Rolling ball background subtraction

- Background calculated using the mean of a circular local neighbourhood

- The radius of the "ball" should be at least as large the radius of the largest target
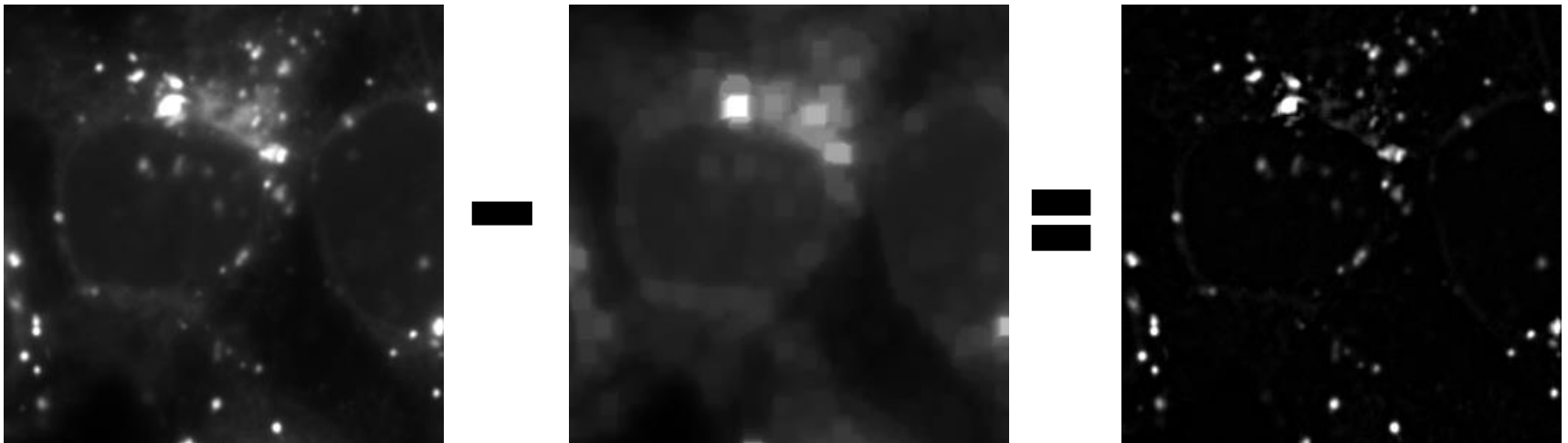
# Part 3: Introduction Macros and Workflow Automation
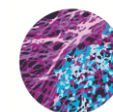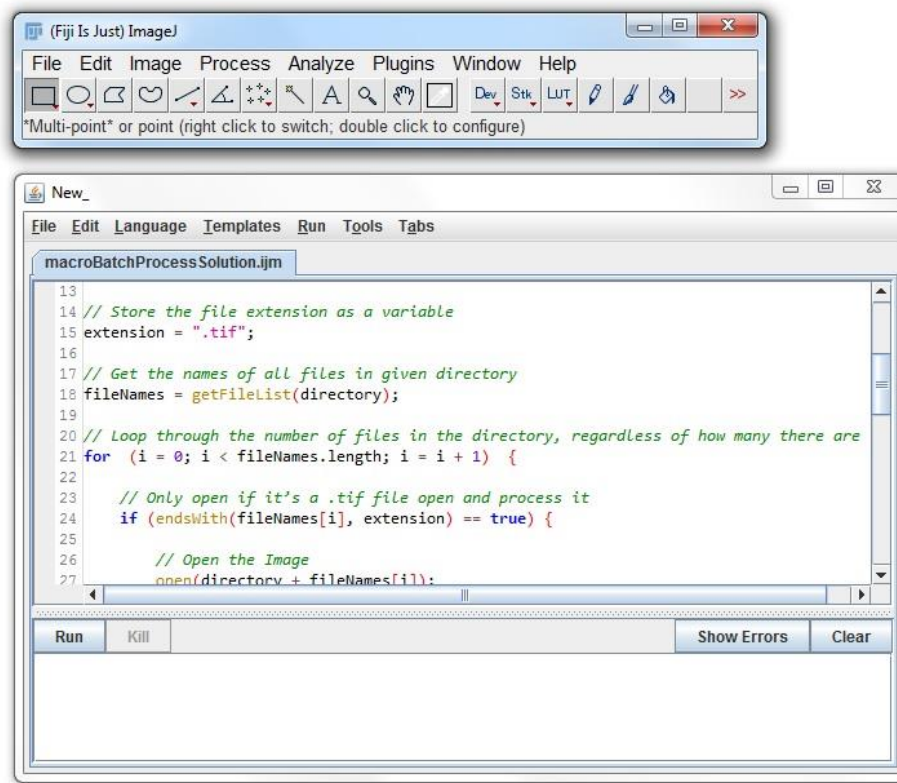
# Common file formats

- **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
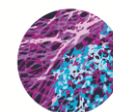
# Why automate a workflow?



- Save time and eliminate user mistakes

- Unbiased and consistent approach

- Have a record off what you have done

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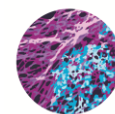
# A macro is just a sequence of ImageJ commands

- 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
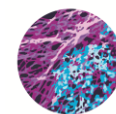
# The Fiji script editor

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

- Syntax highlighting

- Templates menu contains some useful examples

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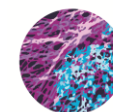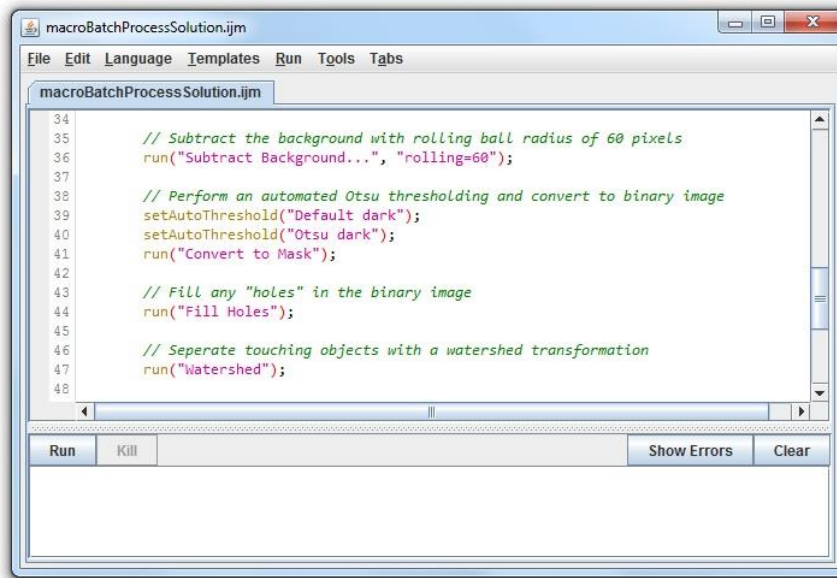
# The Command Recorder

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!

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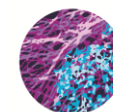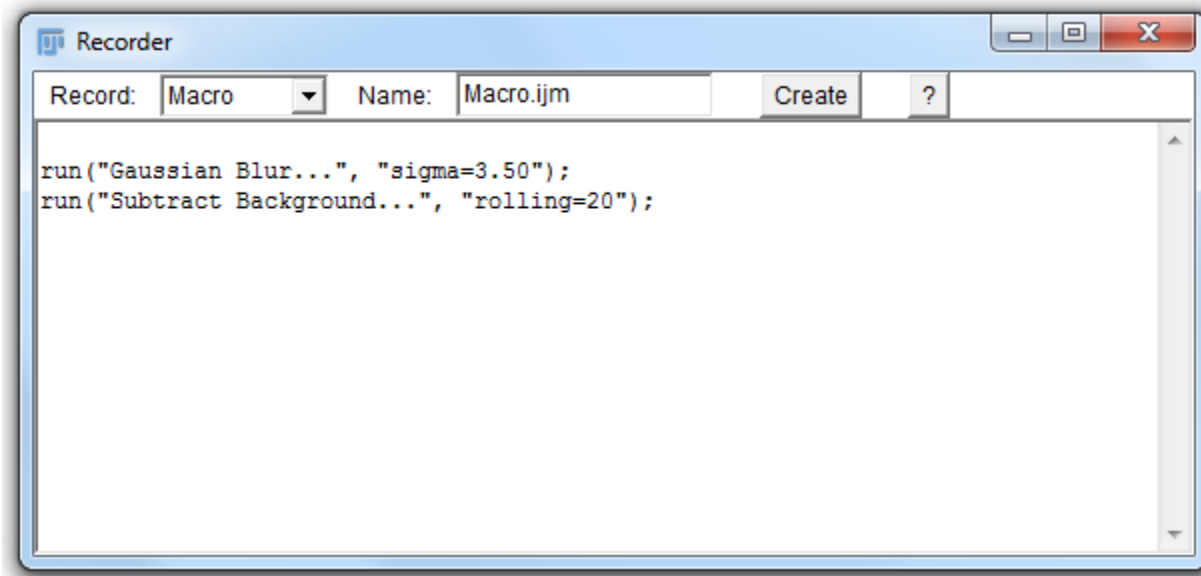
# For loops

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);
}
```

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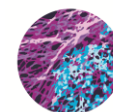
# Conditional statements

**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.");
}
```

# Whats next?

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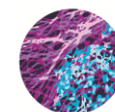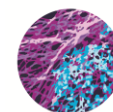    - Analysis and visualisation of light sheet and SMLM datasets
    - Any suggestions?

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

- I can work with you on collaborative projects.
  Email (j.a.pike@bham.ac.uk) for enquires.

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# Acknowledgments

This course was organised and run by the Centre of Membrane Proteins and Receptors (COMPARE), a partnership between the Universities of Birmingham and Nottingham.

http://www.birmingham-nottingham.ac.uk/compare/

The material was adapted from a course original run at the University of Cambridge:
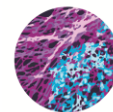
**Gurdon Institute:**
Richard Butler

**Cancer Research UK Cambridge Institute:**
Mark Dunning
Stefanie Reichelt

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