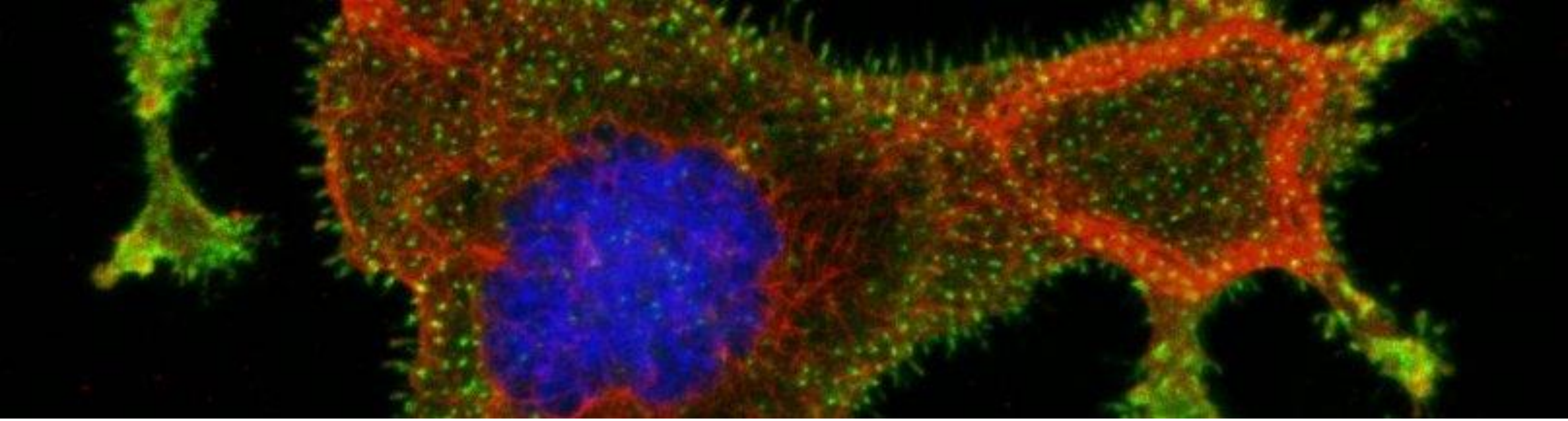

Image Analysis with Fiji

An introductory course for biologists

Jeremy Pike, Image Analyst for COMPARE

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

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Fiji installation and course material

1. Download Fiji:

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

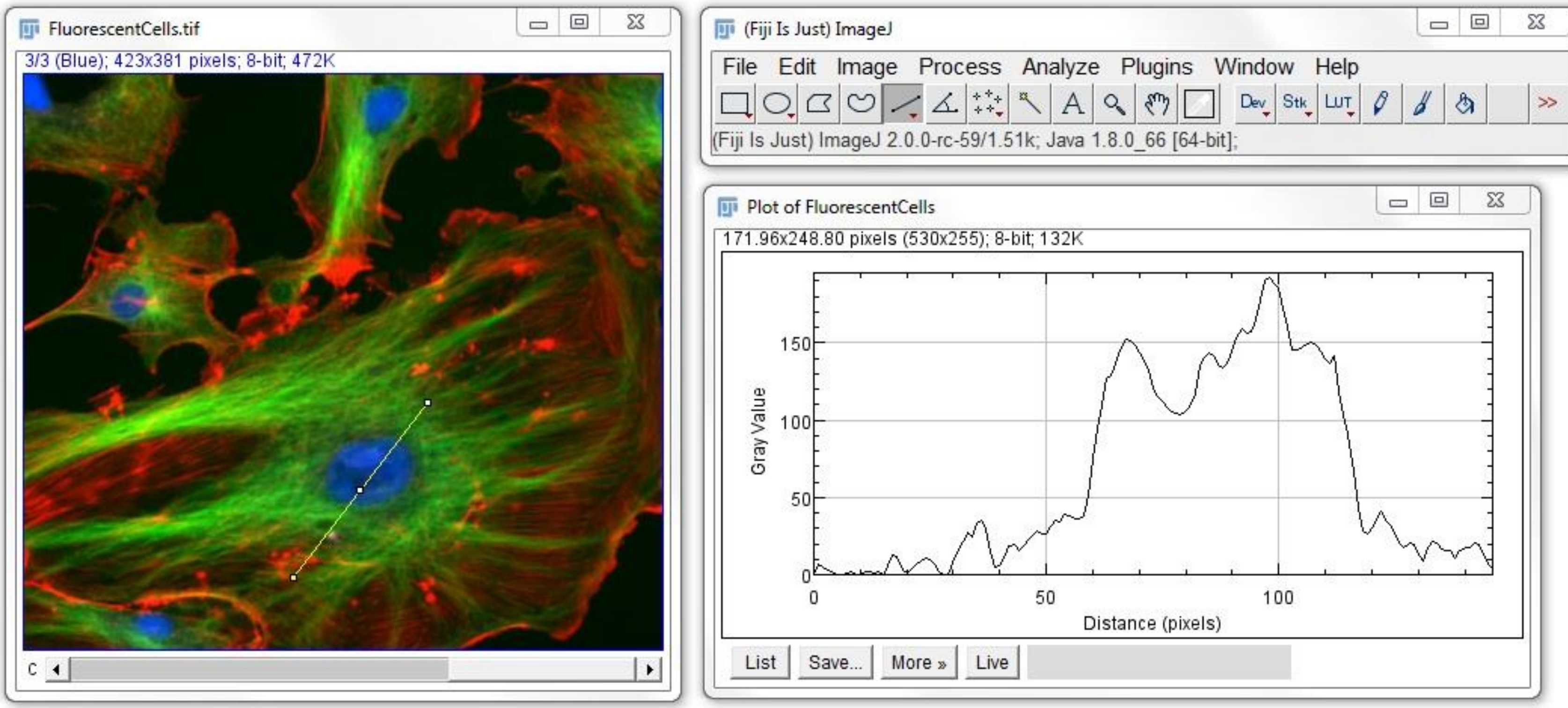
You don't need to install Fiji, just unpack and start ☺

2. Download the course sample data:

https://jeremypike.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.

Part 1: Basics



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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.

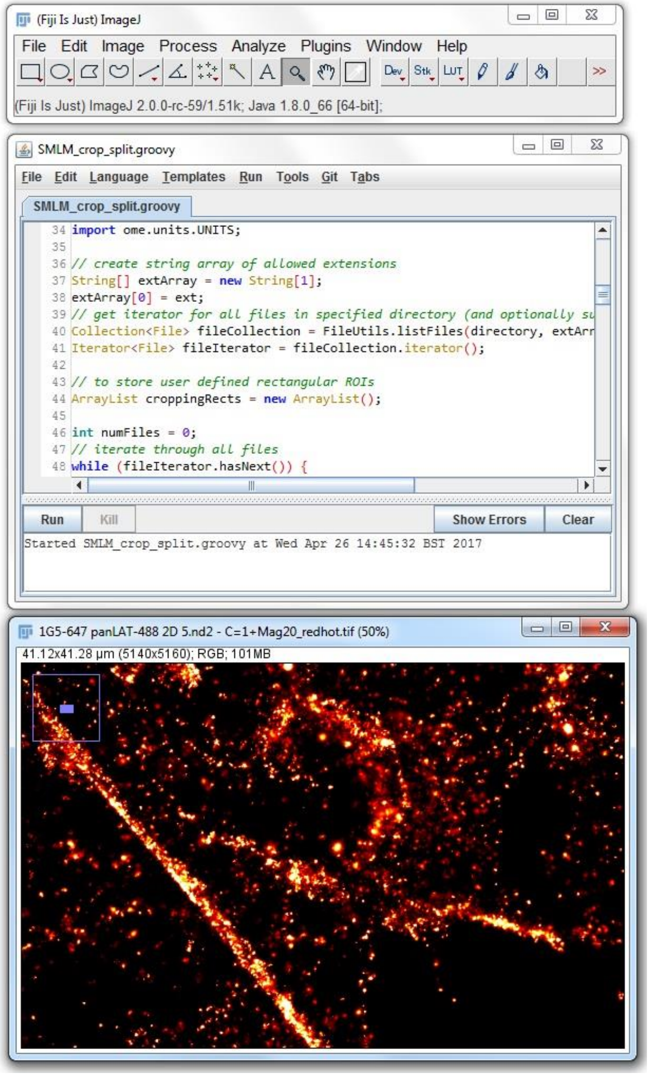
ImageJ2 is a complete rebuild of ImageJ, it is built into Fiji

Rueden, C.T., Schindelin, J., Hiner, M.C. et al. (2017) BMC Bioinformatics 18, 529 (2017)

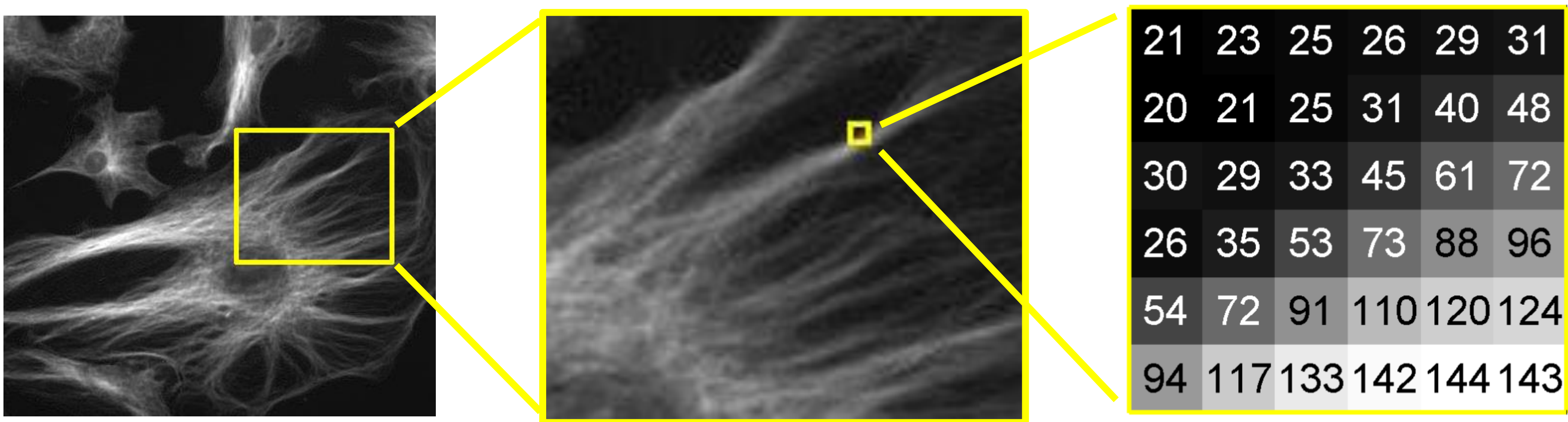
If in doubt get Fiji!

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)



Digital images are simply arrays of numbers



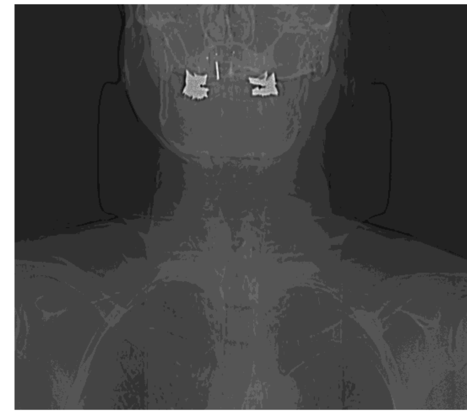
Note pixels are samples of intensity at a spatial point, not little squares!

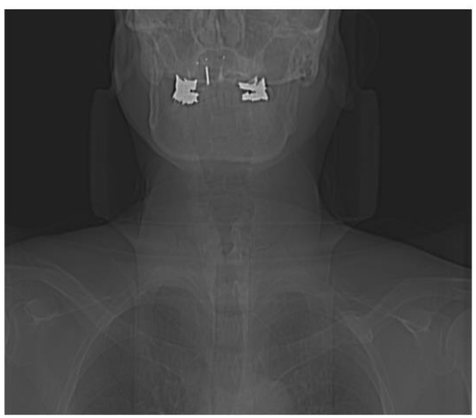
Dynamic range is the number of values each number can take

1 bit = 2

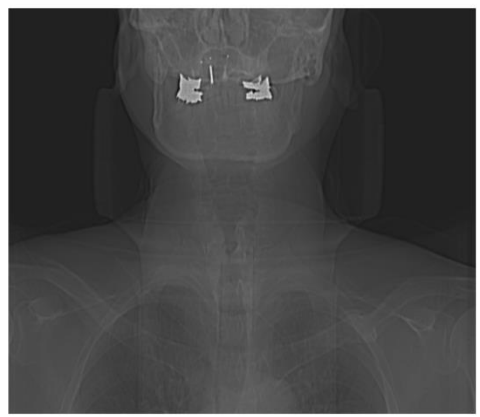
2 bit = 2^2

4 bit = 2^4

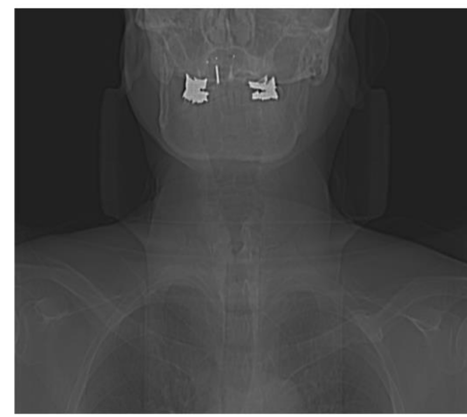




8 bit = 2^8

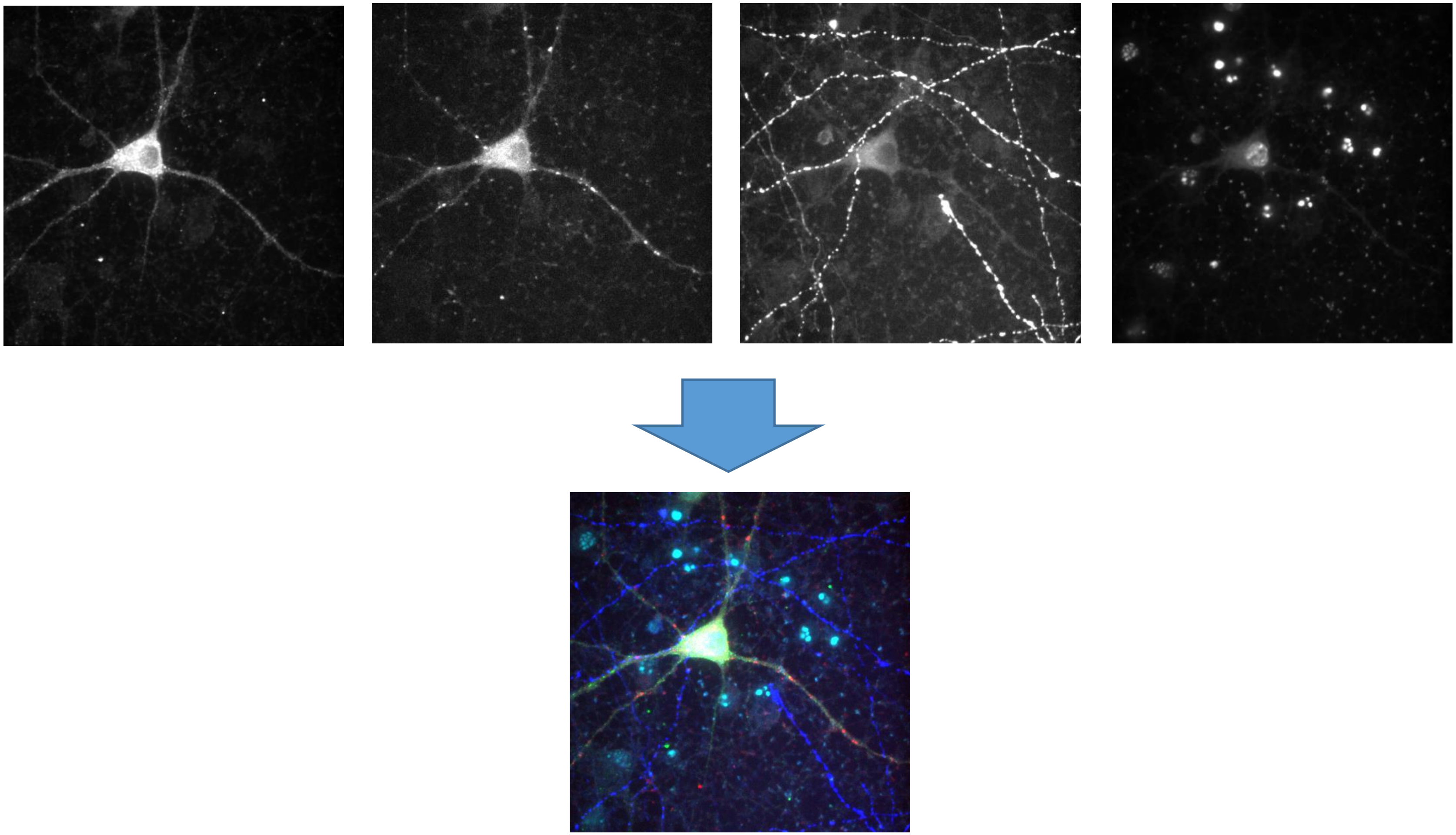


12 bit = 2^{12}



16 bit = 2^{16}

Multi Channel Data



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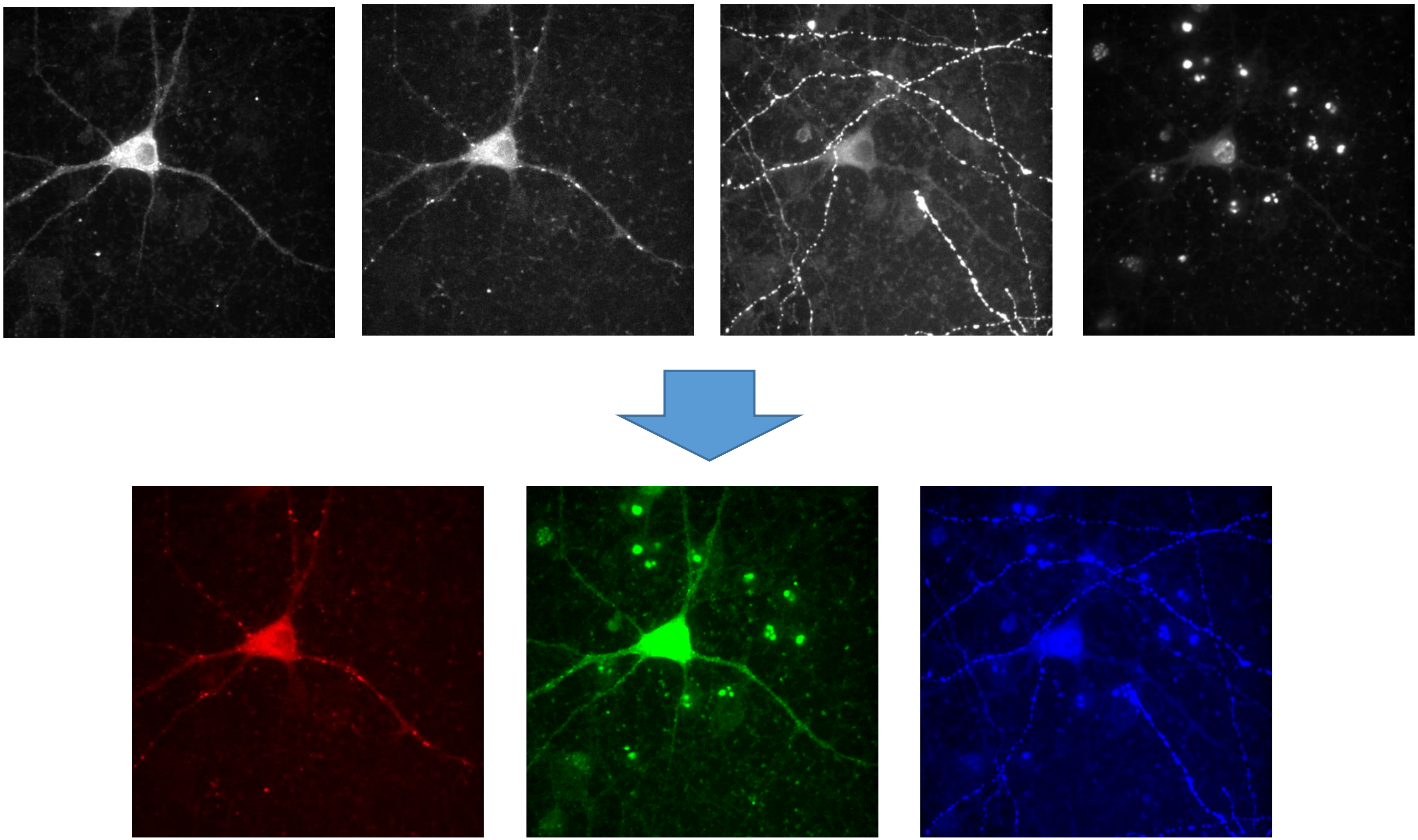
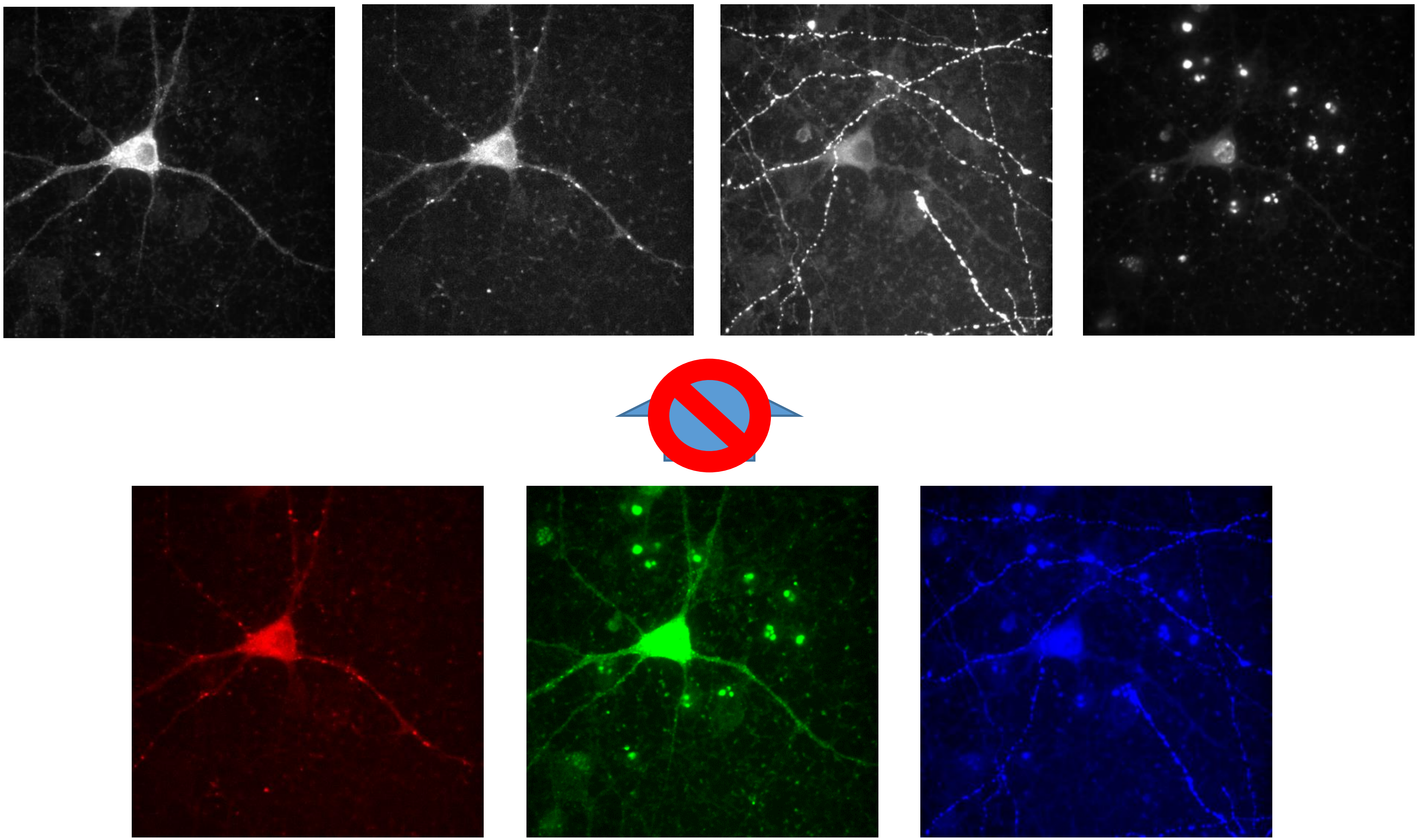
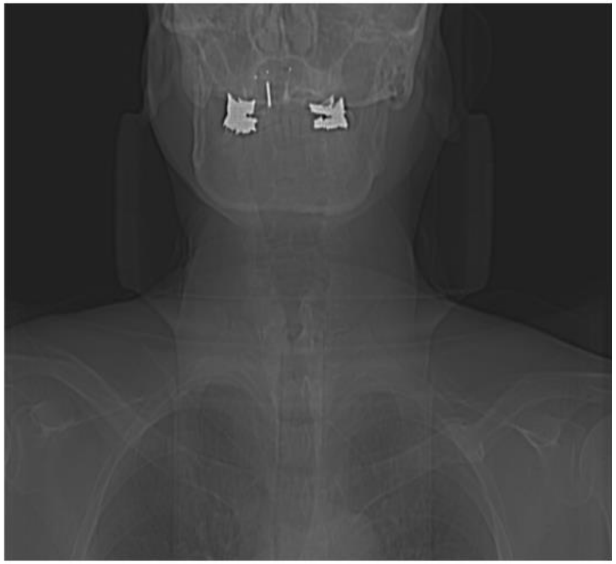
RGB Colour Images



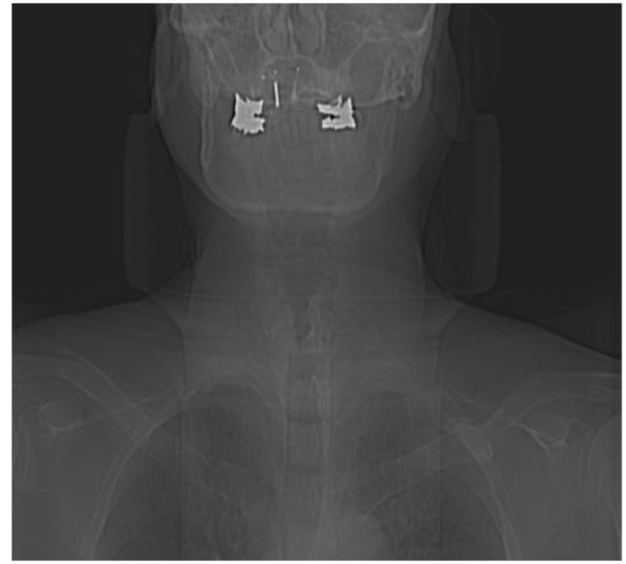
RGB Colour Images



What can we “see” on a monitor?



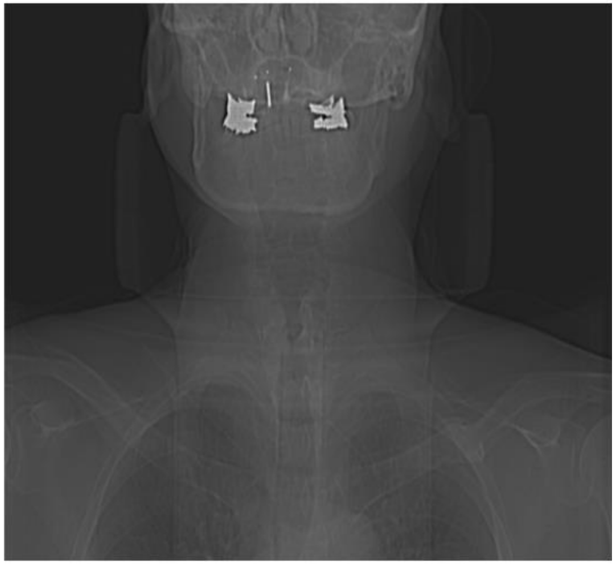
8 bit $= 2^8$



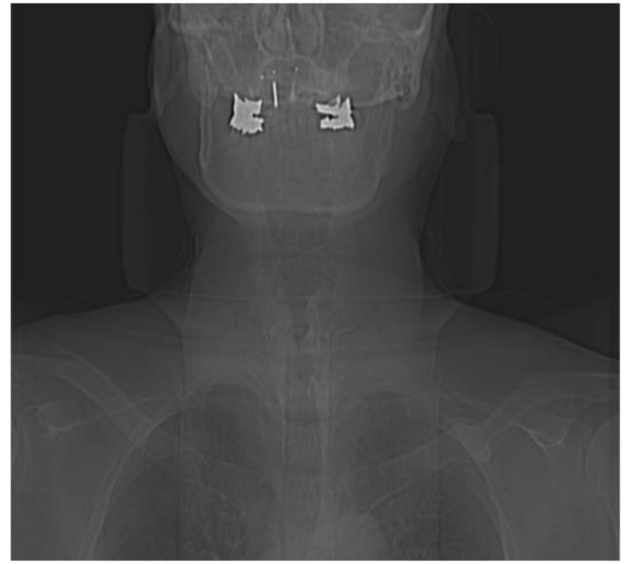
16 bit $= 2^{16}$

Can you tell the difference?

What can we "see" on a monitor?



8 bit = 2^8

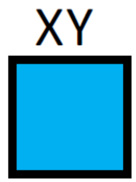


16 bit = 2^{16}

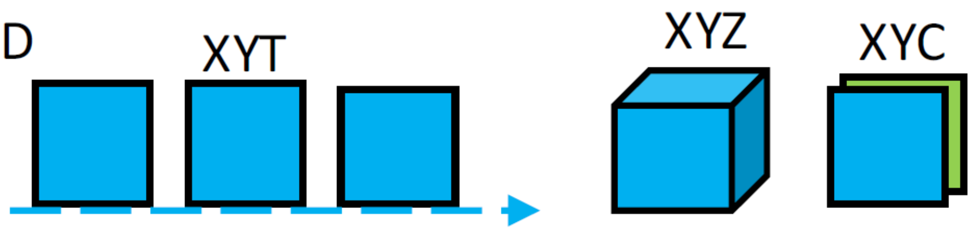
- 8 bit display range
- 3 x 8bit RGB for colour display
- What bit-depth can our eyes detect?

Microscopy data

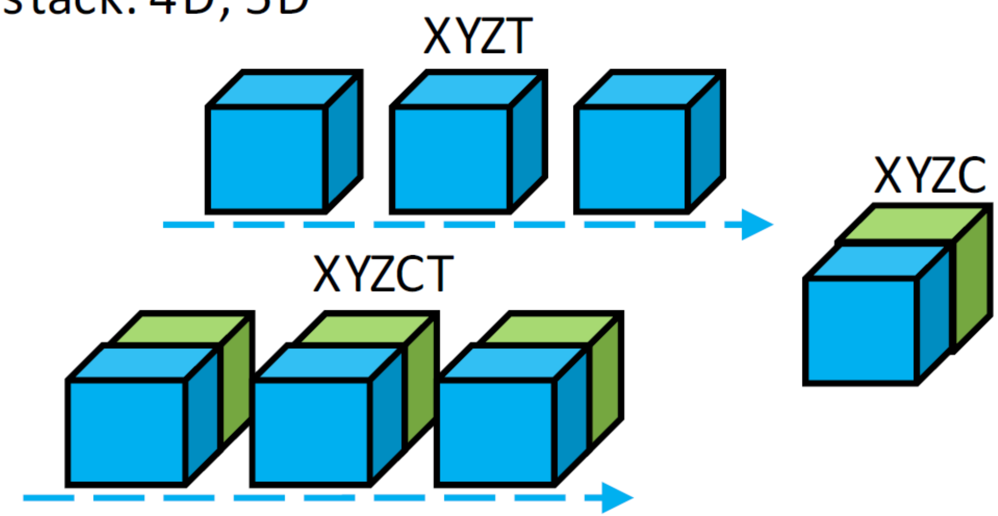
- Image: 2D



- Stack: 3D



- Hyperstack: 4D, 5D



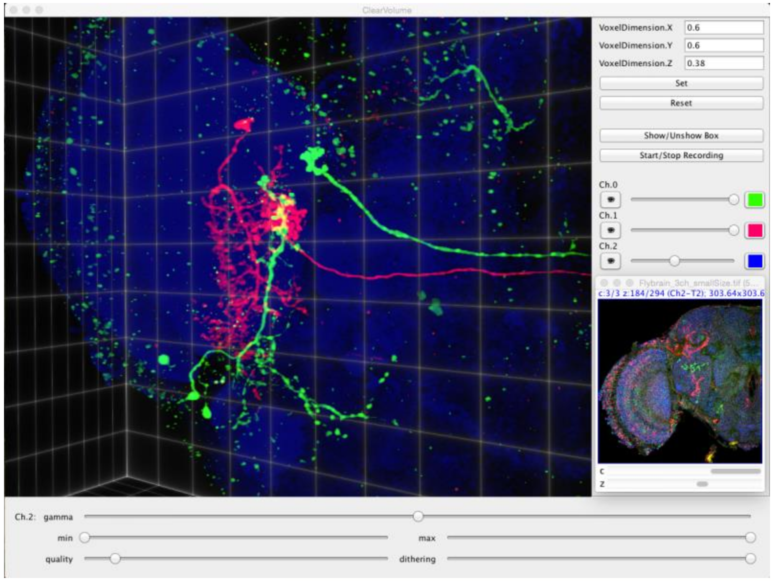
Volume rendering in Fiji

There are plugins available for Fiji, for example

- Clear Volume
http://imagej.net/ClearVolume

- Volume Viewer
https://imagej.nih.gov/ij/plugins/volume-viewer.html

- 3D Viewer
https://imagej.net/plugins/3d-viewer/



Or commercial software:

- Arivis Vision4D, Imaris etc

Common file formats

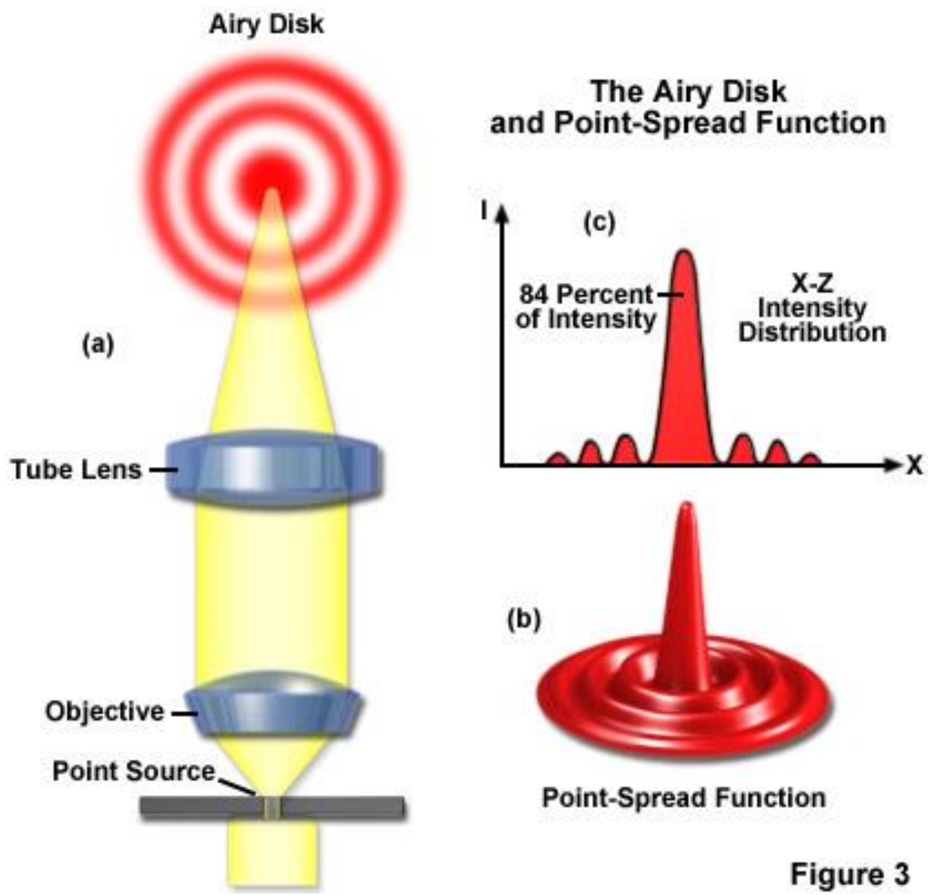
• **TIFF** is a good choice
– Lossless storage of data
– Header tags for metadata

• **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

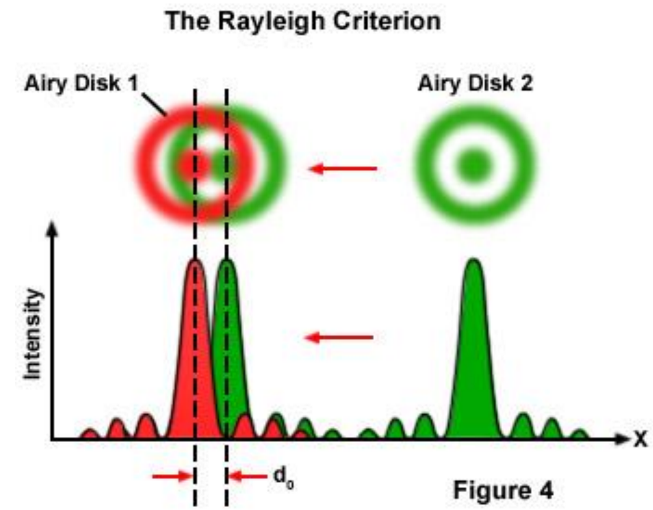


"Each element of the primary image is a small diffraction pattern, and the actual image, as seen by the eyepiece, is only the ensemble of the magnified images of these patterns"

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



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

Part 2: Introduction to Image Processing and Analysis

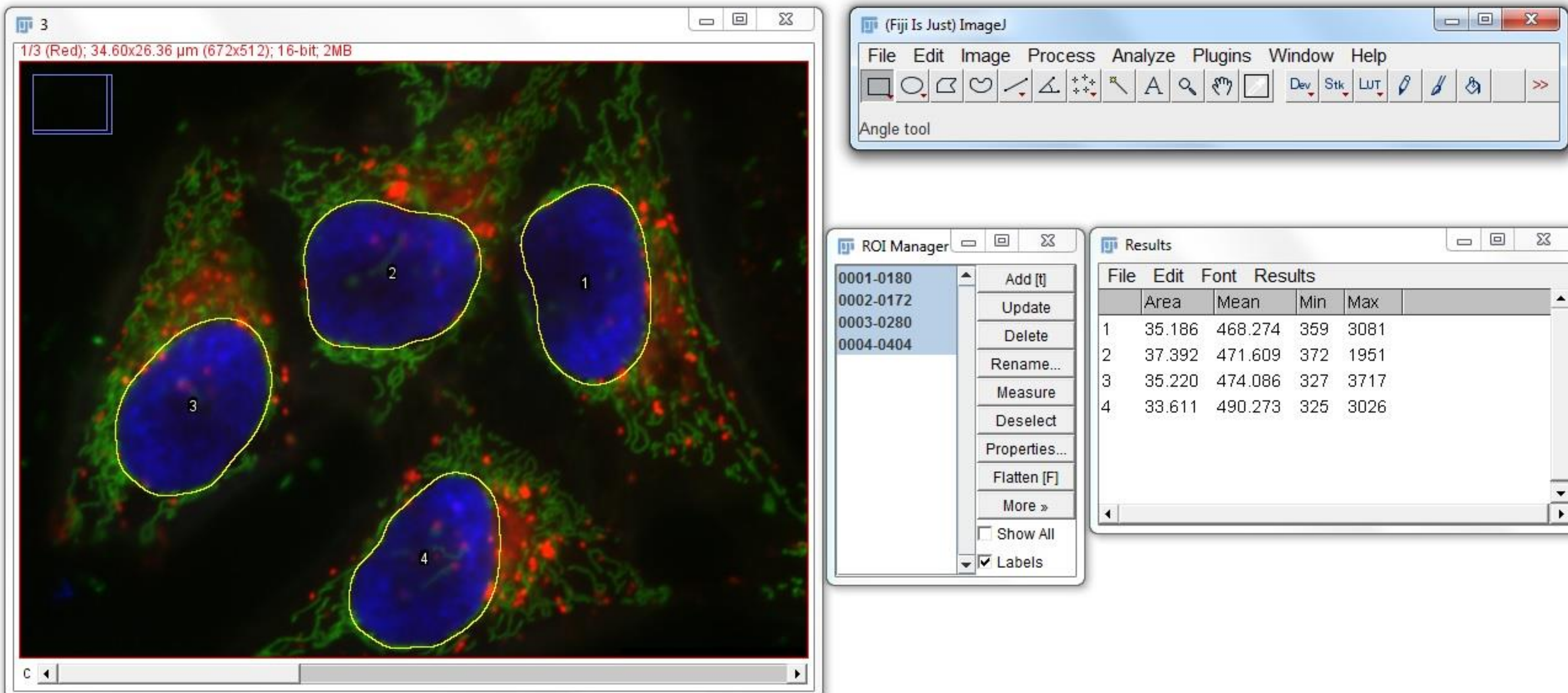


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Image Processing:

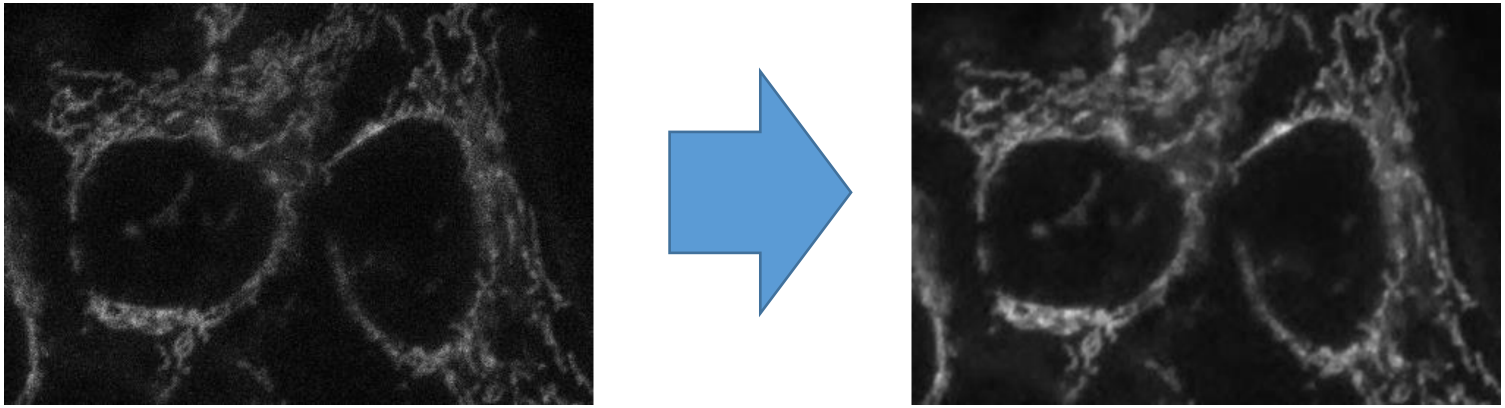
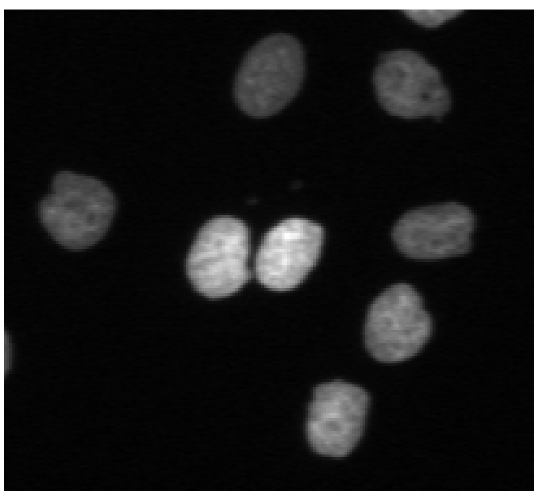
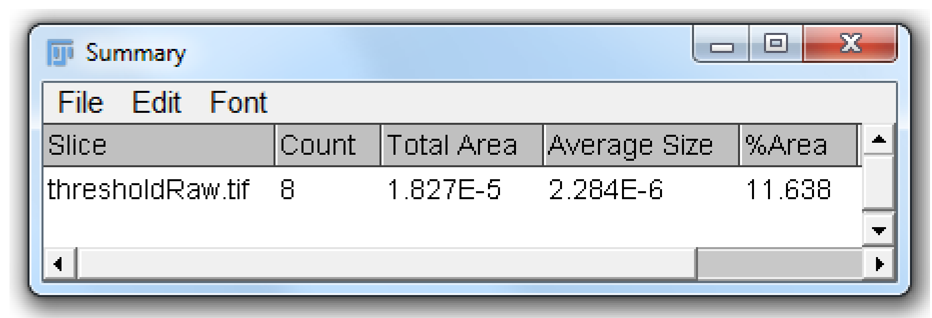


Image Analysis:





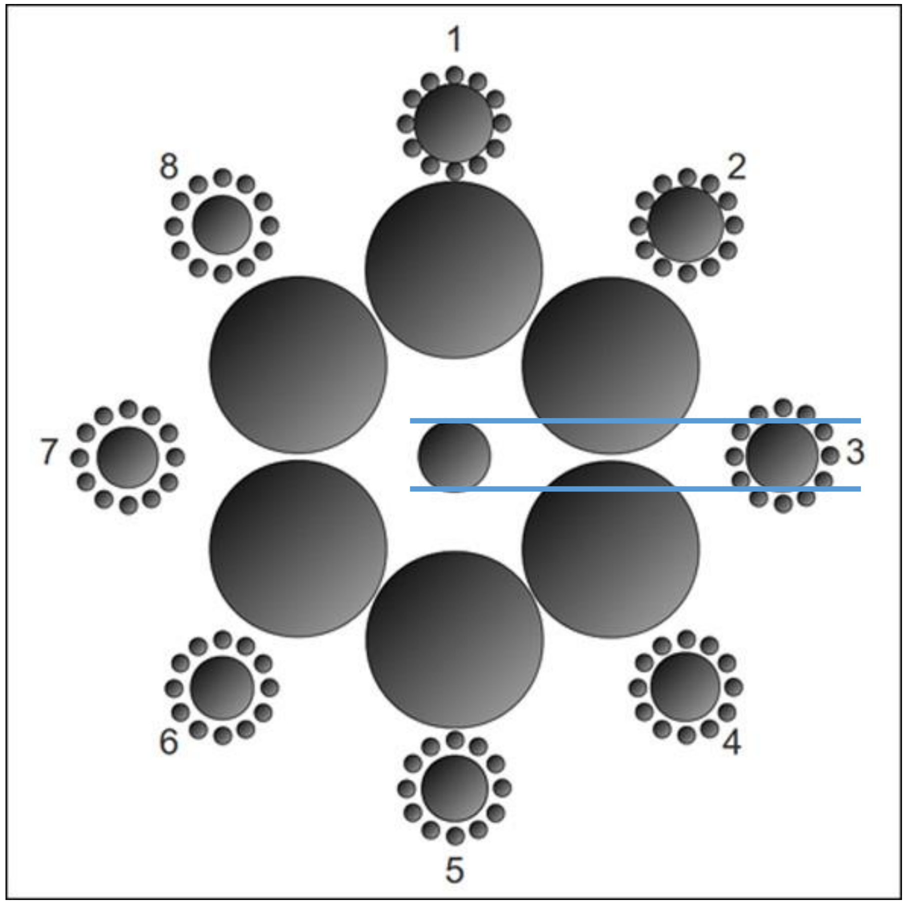
Slice	Count	Total Area	Average Size	%Area
thresholdRaw.tif	8	1.827E-5	2.284E-6	11.638

Why do computational processing and analysis?

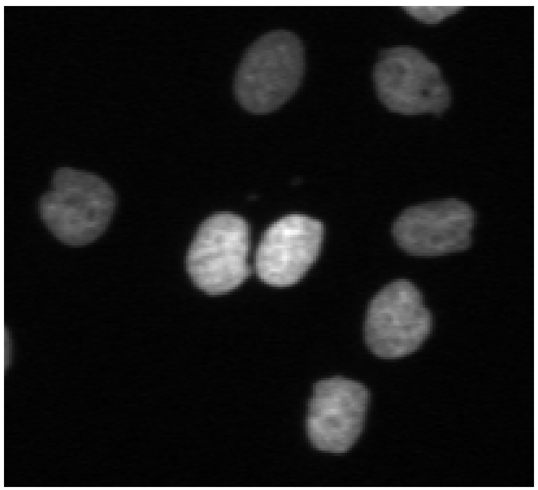
- Its quantitative
- Its unbiased
- Can enhance understanding of the data
- Can be automated for processing of large datasets

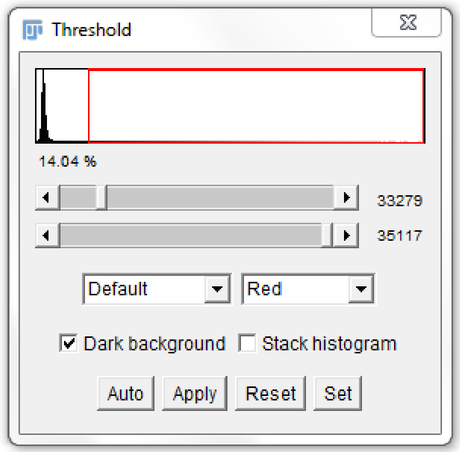
"The first principle is that you must not fool yourself - and you are the easiest person to fool. So you have to be very careful about that. After you've not fooled yourself, it's easy not to fool other scientists. You just have to be honest in a conventional way after that."
Richard Feynman

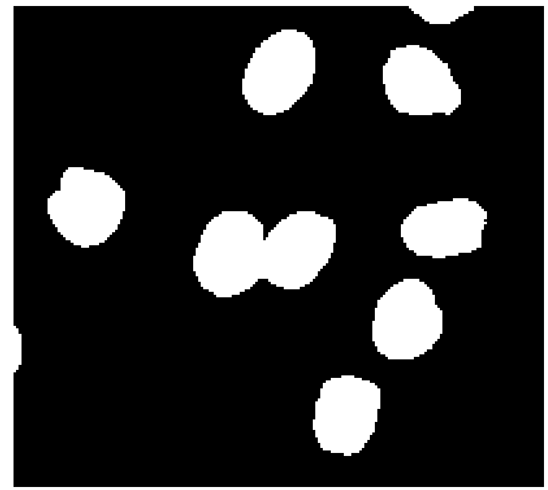
What outer circle is the same size and the central circle?



Intensity based thresholding to segment objects

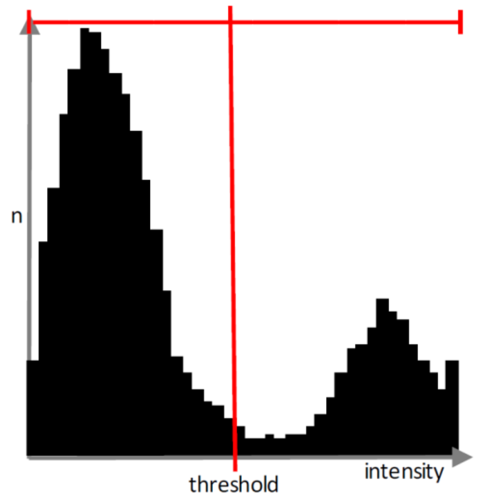






Automated threshold values are preferable to manual selection

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

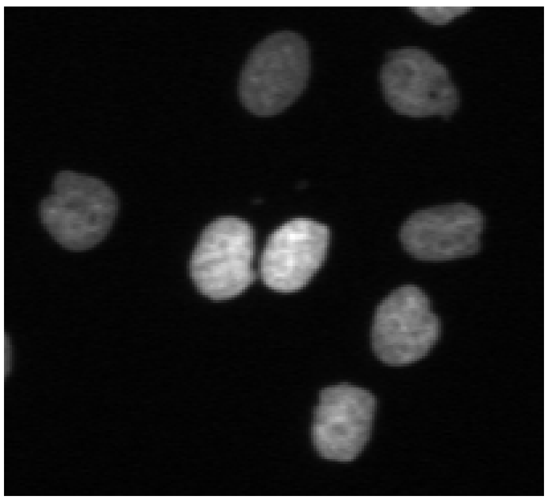


Otsu, N (1979), IEEE Trans. Sys., Man., Cyber. 9: 62-66.

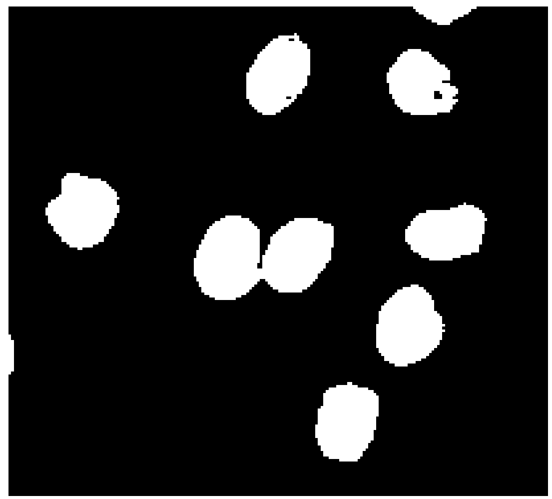
- Li thresholding minimises the cross entropy between the original and segmented images.

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

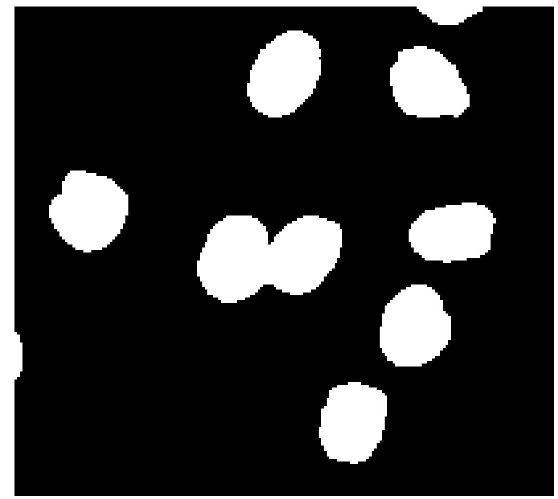
Automated threshold values are preferable to manual selection



Raw Data

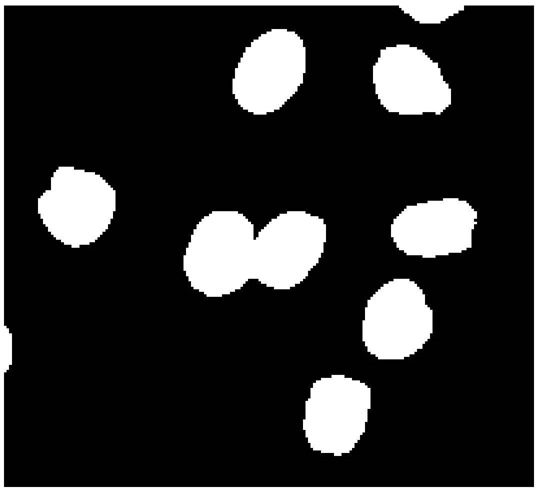
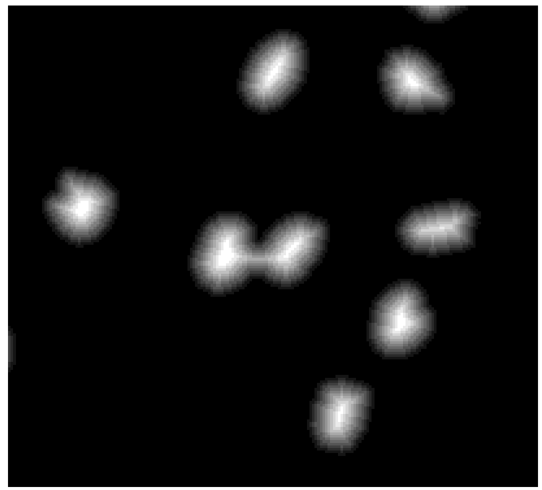
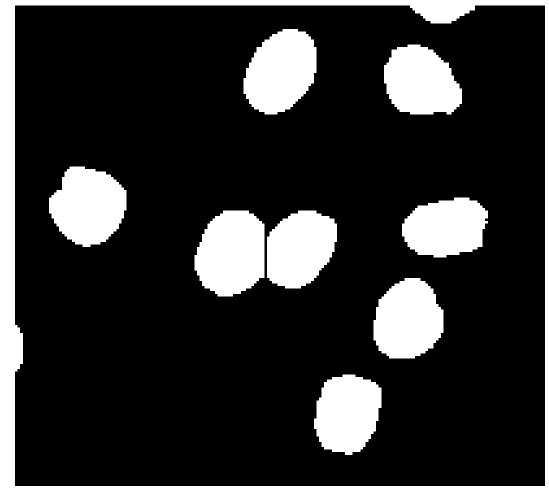


Otsu Threshold



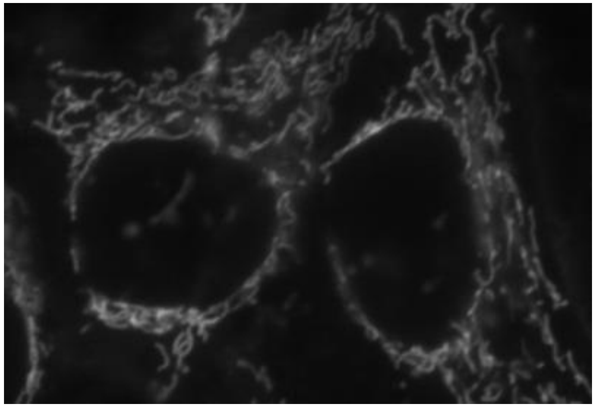
Li Threshold

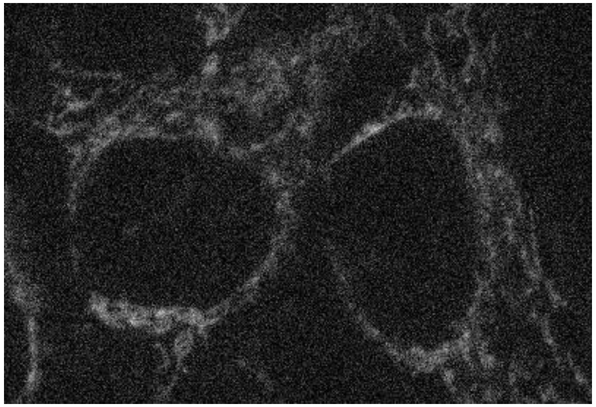
Watershed transformation to separate touching objects

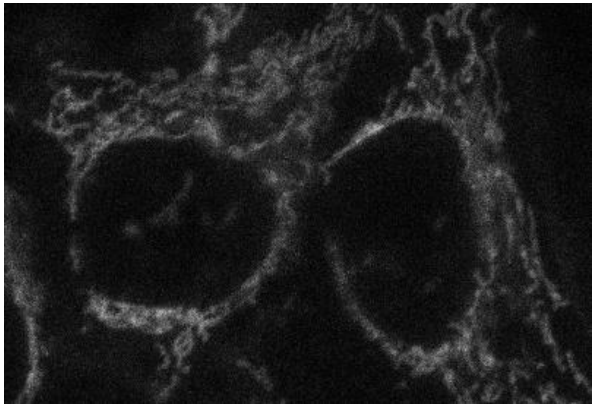
- Seeds placed at local minima of distance map and dilated
- This can be visualised as flooding the distance map

Noise is image corruption from the acquisition process





Gaussian

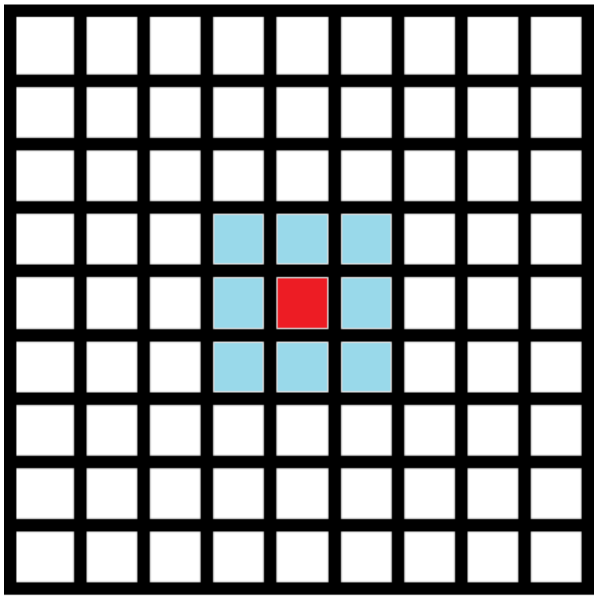


Poisson

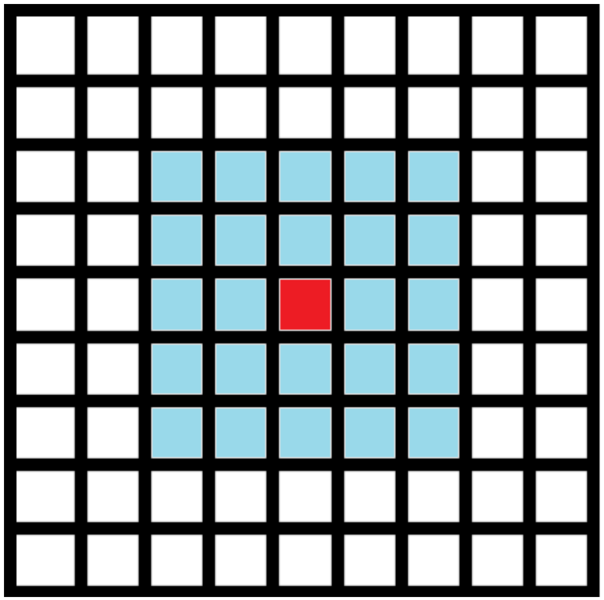
Impulse

Image filters and convolution

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



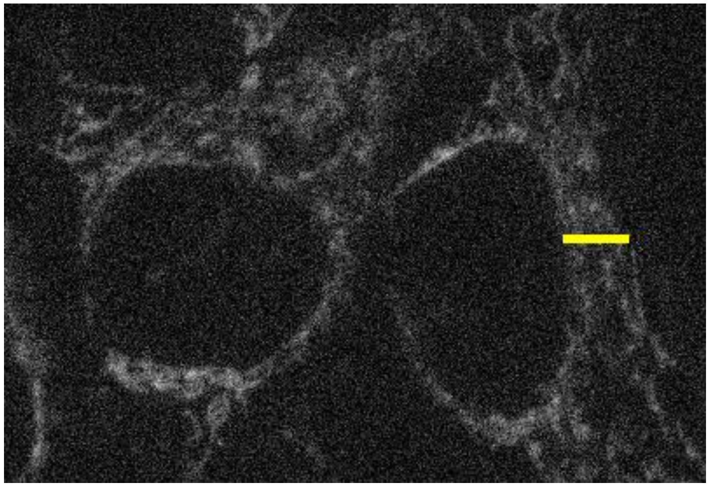
3x3 Neighbourhood

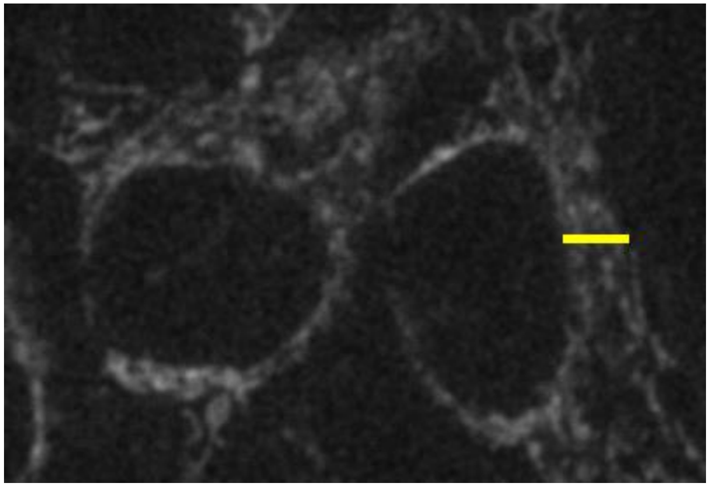


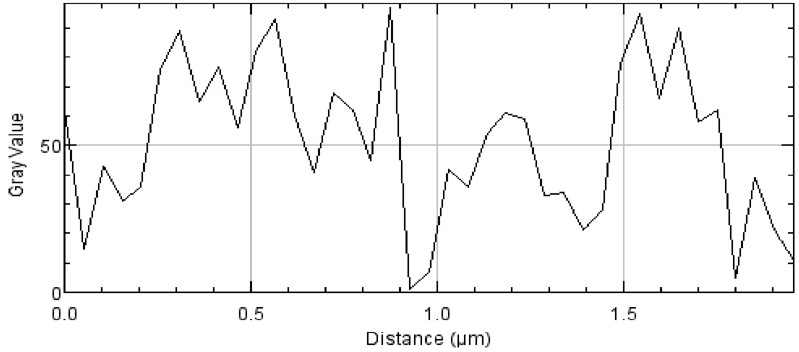
5x5 Neighbourhood

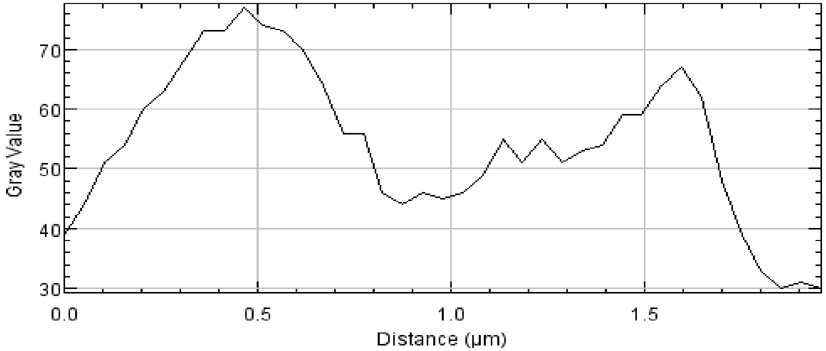
Mean filter

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



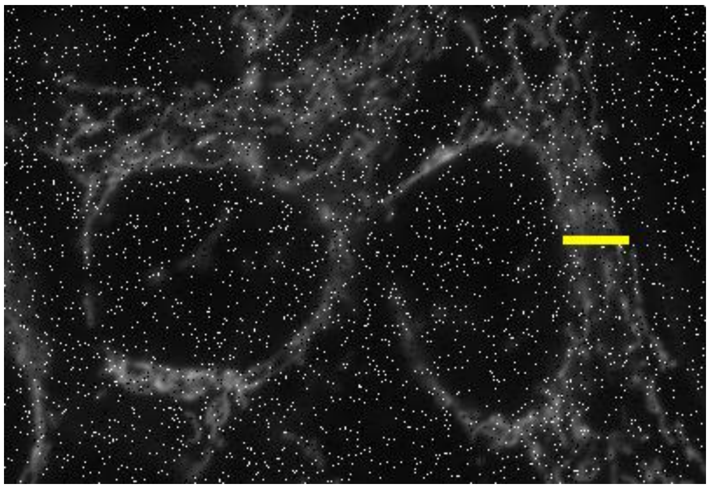


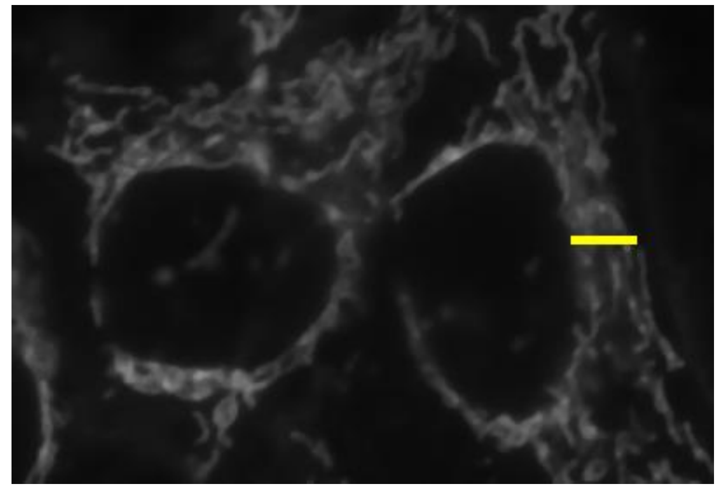


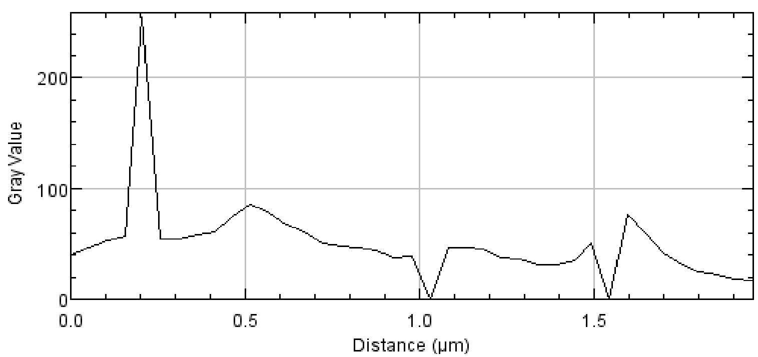


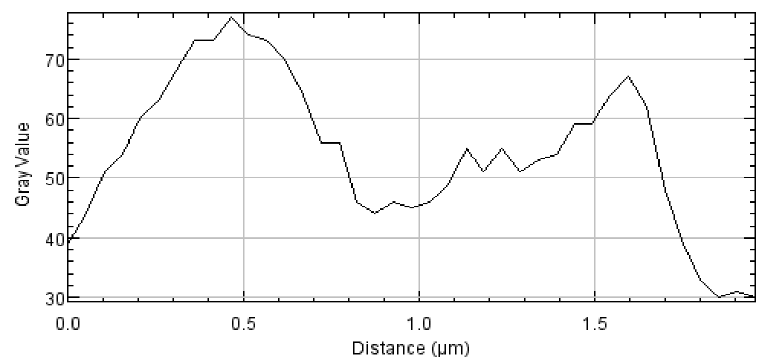
Median filter

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





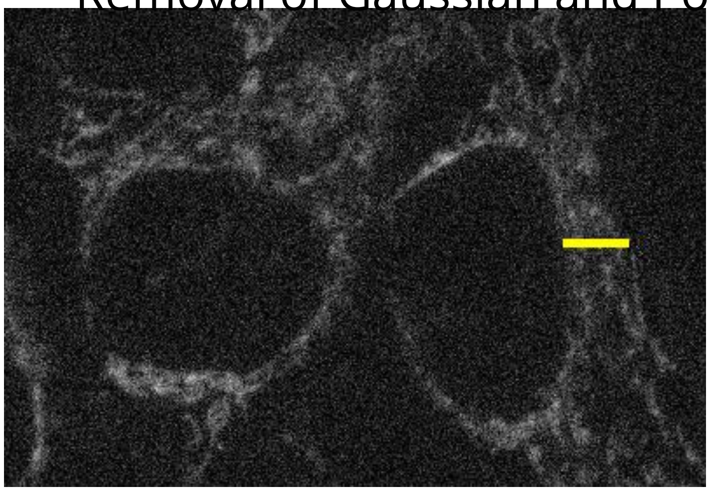


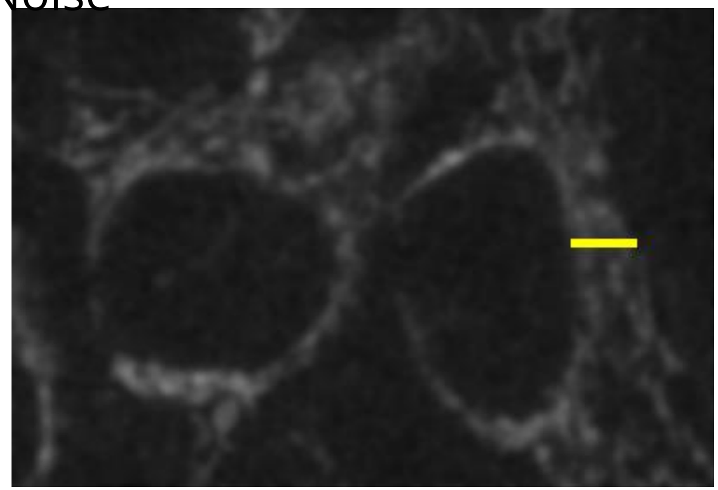


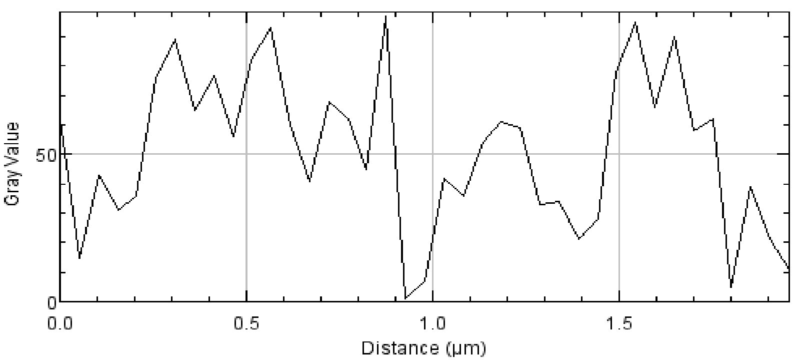
Gaussian filter

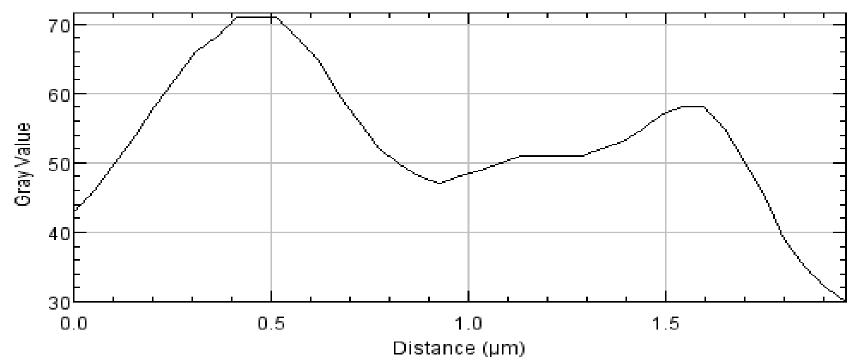
- Contribution of neighbourhood pixels weighted by Gaussian profile

- Removal of Gaussian and Poisson Noise



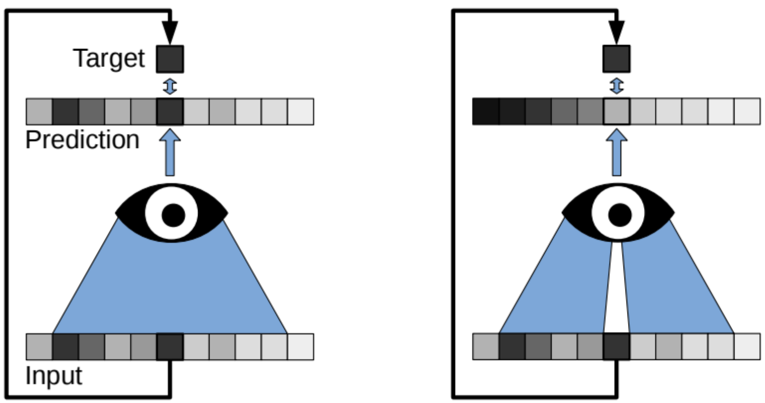




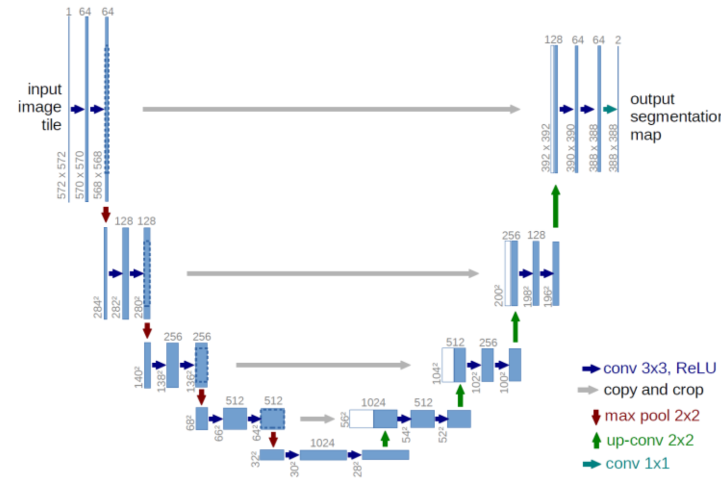


Deep learning based denoising

Self-supervised denoising: Noise2Void [1]



UNET architecture [2]

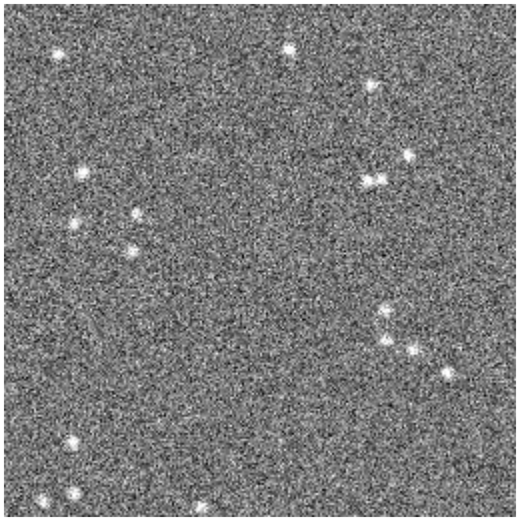


Fiji plugin available: https://imagej.net/plugins/n2v

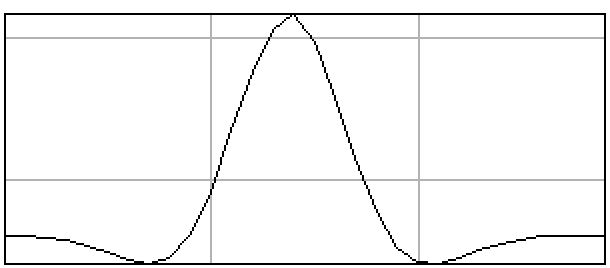
[1] Krull, Alexander, Tim-Oliver Buchholz, and Florian Jug. "Noise2void-learning denoising from single noisy images." Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition. 2019.
https://github.com/juglab/n2v

[2] Ronneberger, Olaf, Philipp Fischer, and Thomas Brox. "U-net: Convolutional networks for biomedical image segmentation." International Conference on Medical image computing and computer-assisted intervention. Springer, Cham, 2015.

Laplacian of Gaussian filter for spot detection



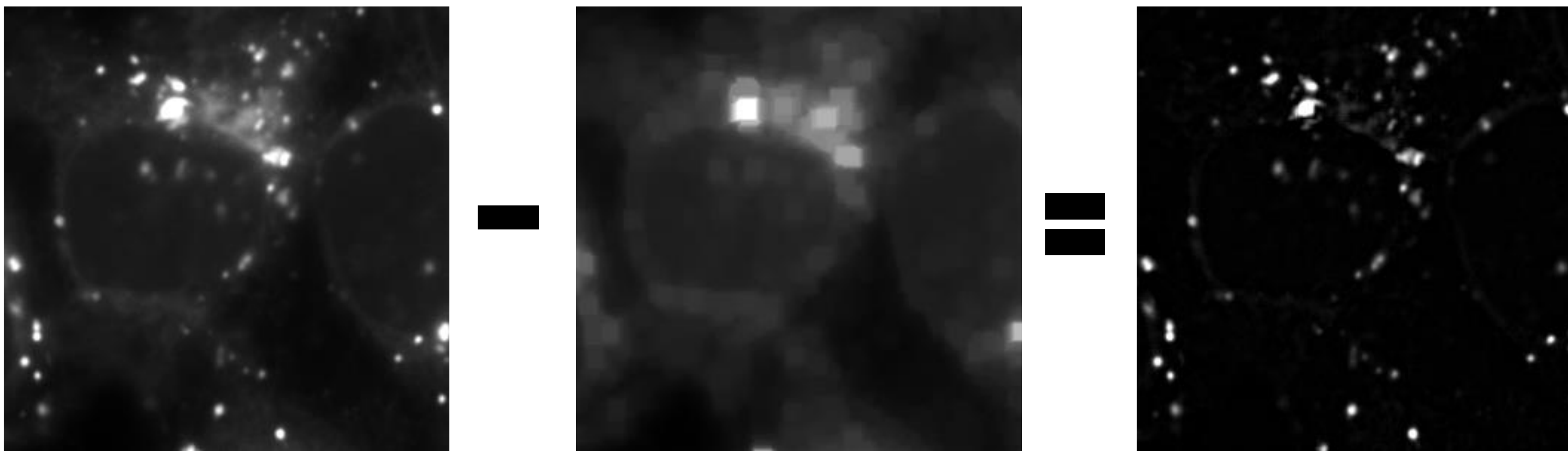
$\otimes$



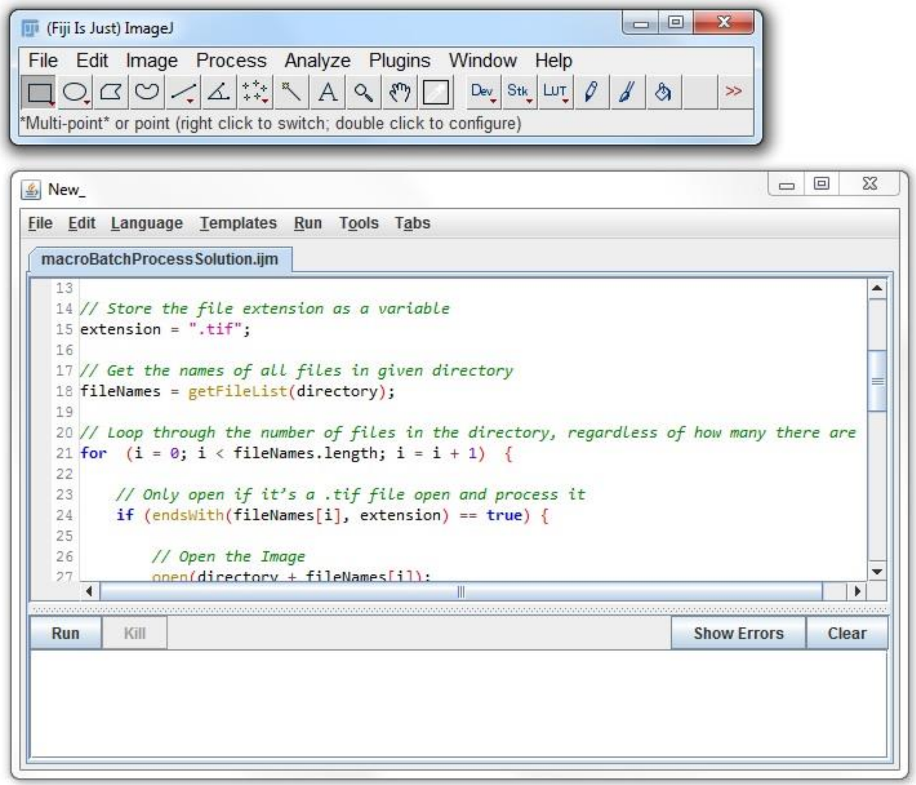
$=$

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



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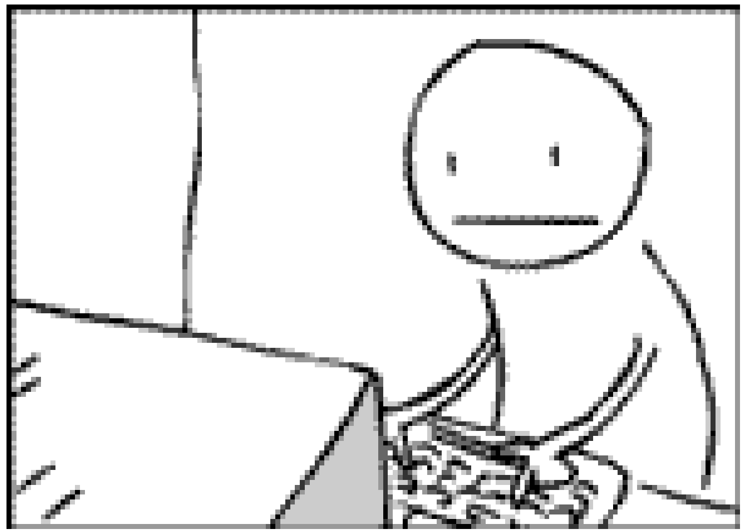
Why automate a workflow?



- Save time and eliminate user mistakes
- Unbiased and consistent approach
- Have a record off what you have done

A macro is just a sequence of ImageJ commands

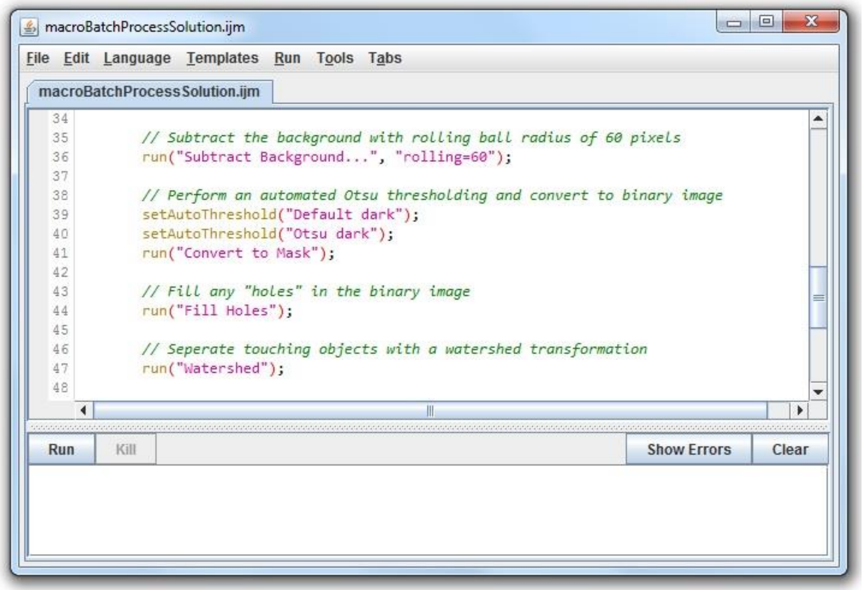
- Simple, easy to learn language
- Calls predefined ImageJ and Java functions
- Any ImageJ menu item can be called with a macro command
- Lots of online tutorials examples and resources including:

http://fiji.sc/Introduction_into_Macro_Programming

The Fiji script editor

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

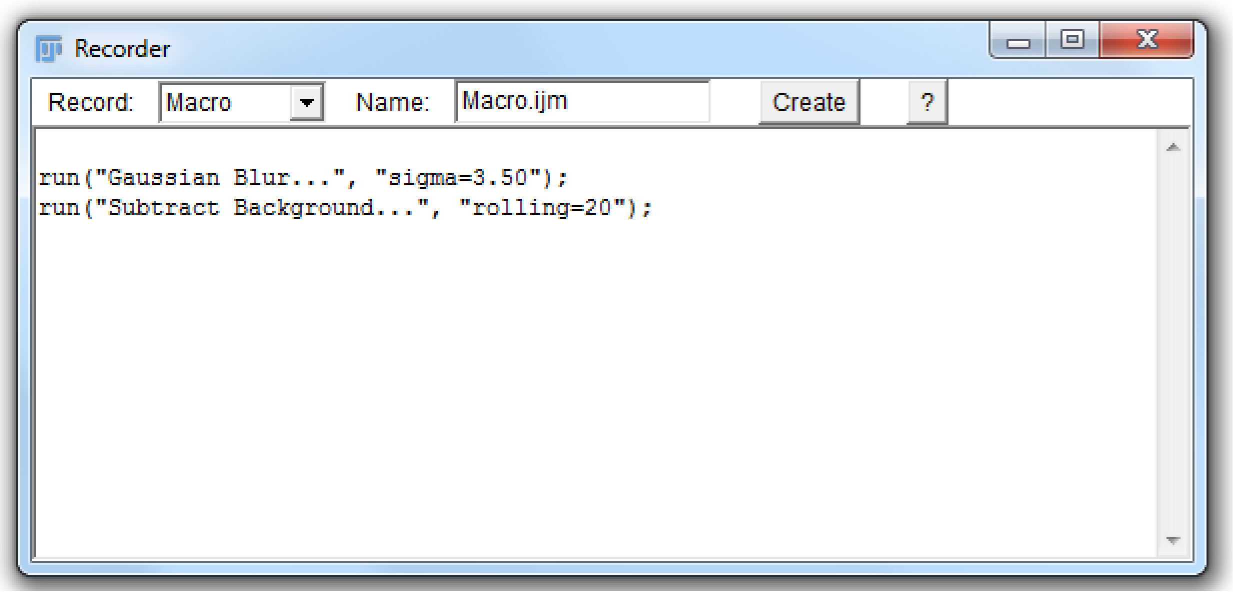
- Syntax highlighting
- Templates menu contains some useful examples



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
  }
```

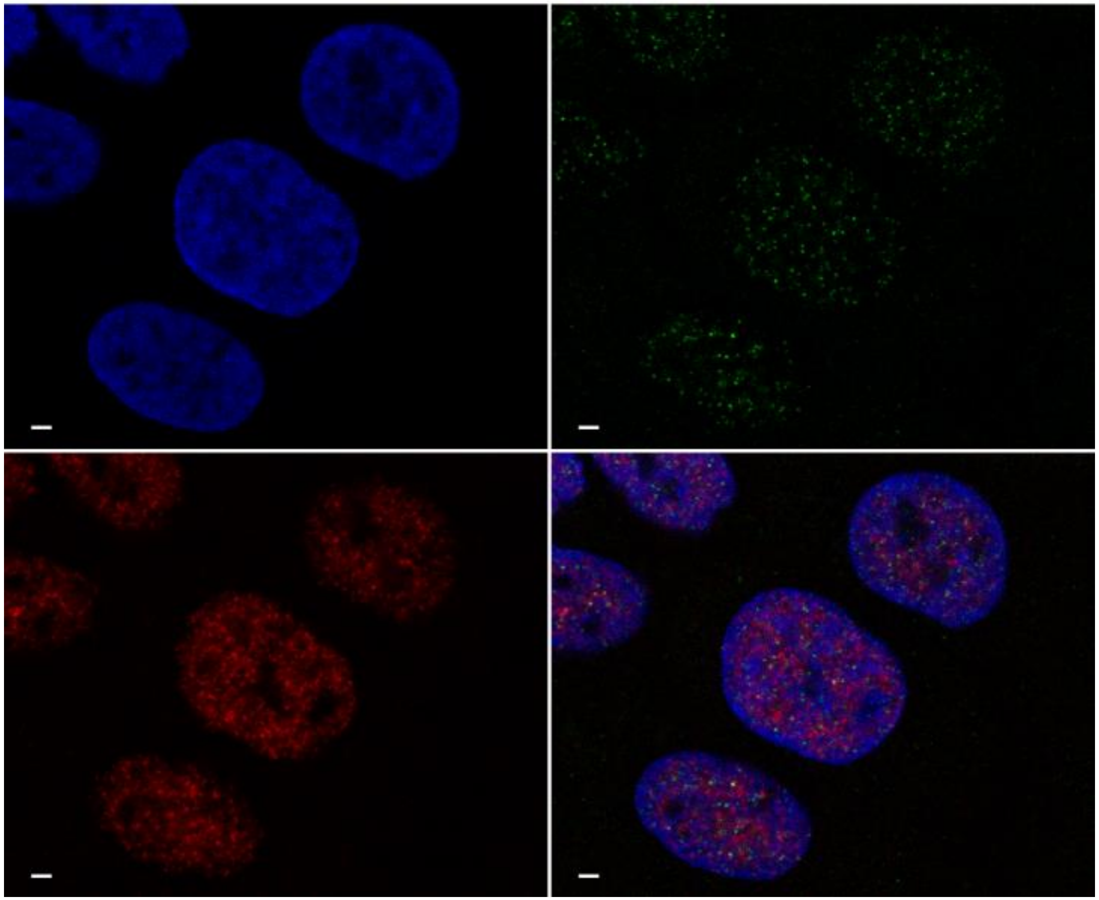
```
for (i = 1; i <= 10; i = i + 1) {
        run("Add...", "value=" + i);
}
```

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.");
}
```

Part 4: Pixel Based Colocalization Analysis



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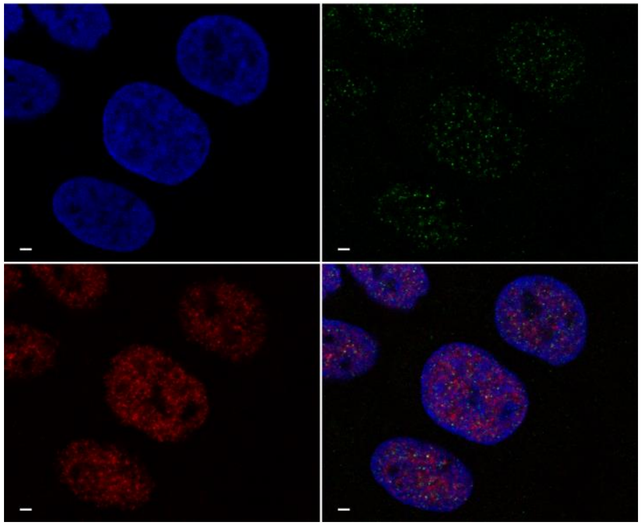
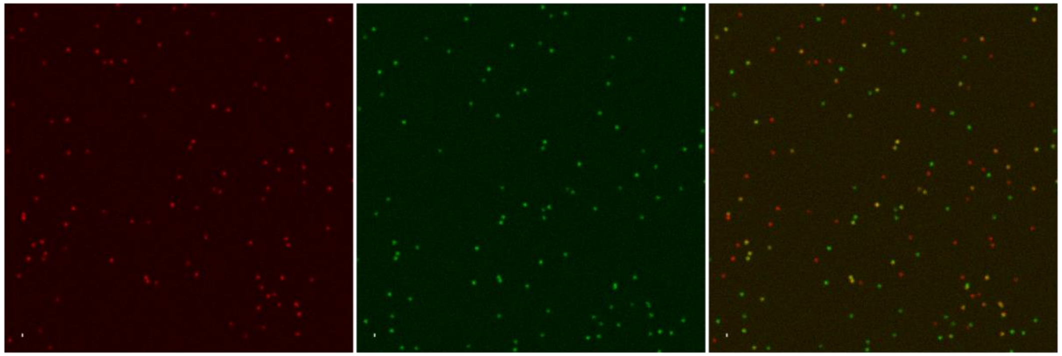
Visualising Colocalization with Colour Overlays

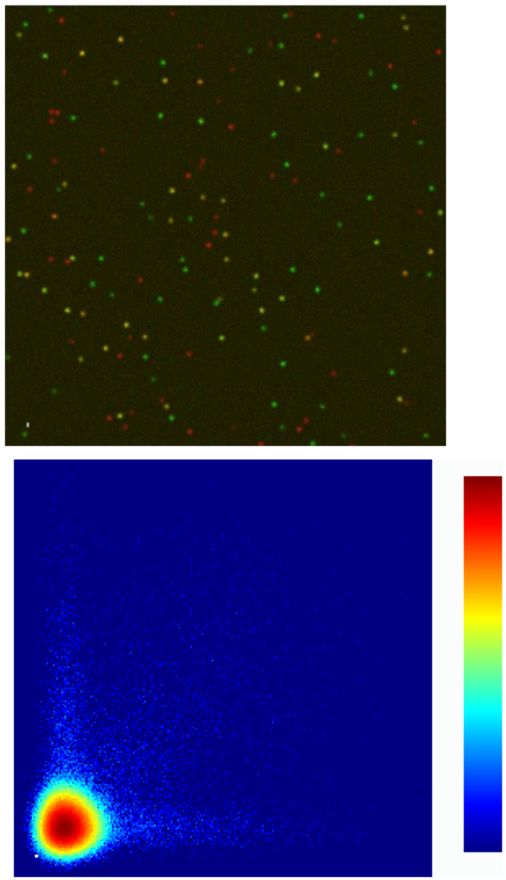




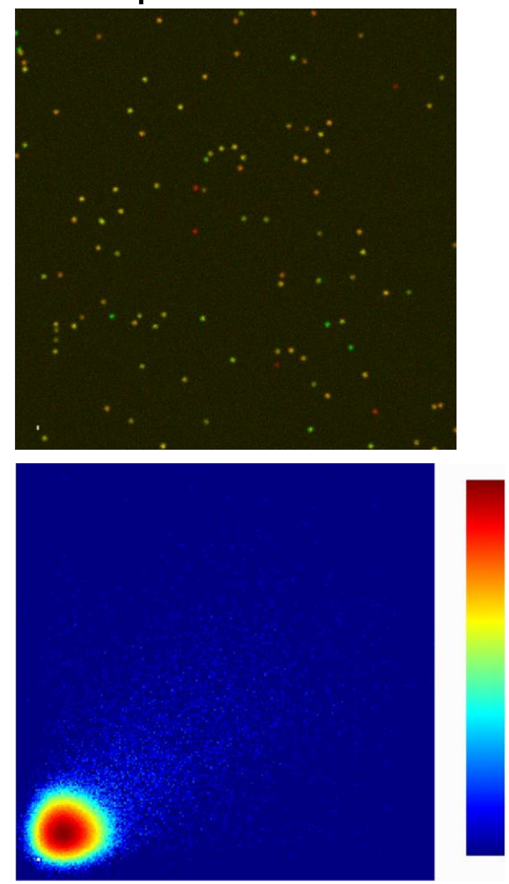
- Not very useful and cannot make any convincing conclusions…
- Visual interpretation is very sensitive to changes in display settings
- Some LUTs are better than others

Visualising Colocalization with Joint-Histograms

50% Spots Colocalized

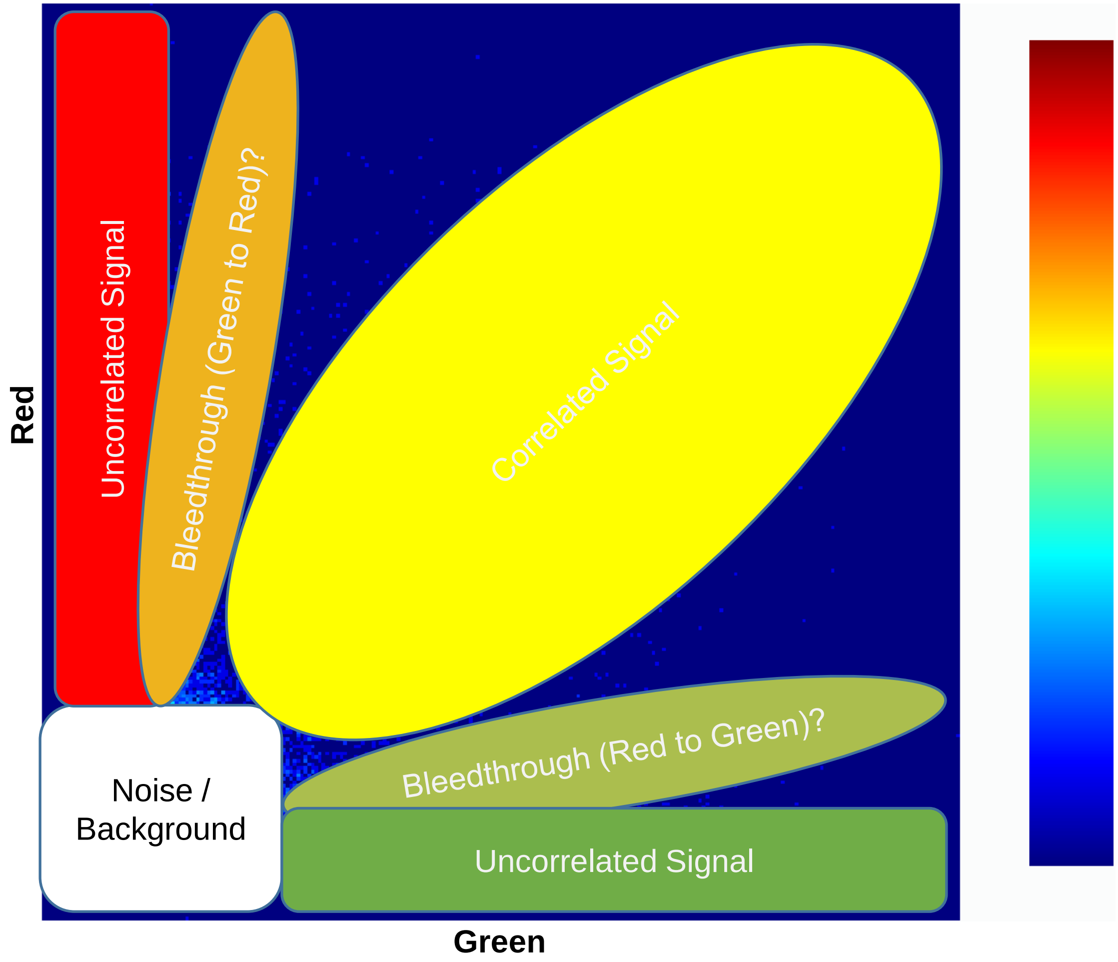


95% Spots Colocalized



- Allows for visual assessment of correlation
- Better than colour overlays but not a replacement for robust quantitative analysis

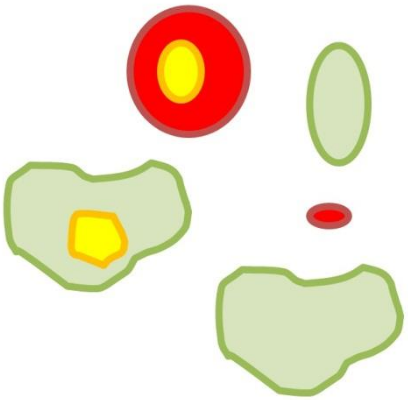
Visualising Colocalization with Joint-Histograms



Quantifying Colocalization

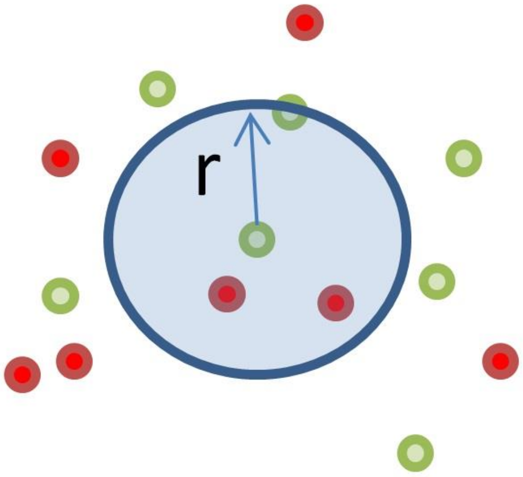
There are two approaches:

Pixel Based



Measures overlap and correlation of signal across individual pixels

Object Based



Spatial analysis using the canter of mass (COM) of each detected object

Lagache, Thibault, et al. "Statistical analysis of molecule colocalization in bioimaging." Cytometry Part A 87.6 (2015): 568-579.

Quantifying Colocalization: Pixel Based

Two types of measures:

Correlation

The Pearson coefficient:

$$R = \frac{\sum_i (C1_i - C1_{av}) \times (C2_i - C2_{av})}{\sqrt{\sum_i (C1_i - C1_{av})^2 \times \sum_i (C2_i - C2_{av})^2}}$$

Co-occurence

The Manders coefficients:

$$M1 = \sum_i \frac{C1_{i,coloc}}{C1_i} \qquad M2 = \sum_i \frac{C2_{i,coloc}}{C2_i}$$

- Together the Pearson and Manders coefficients measure and distinguish between correlation and co-occurence
- For example signal can have a high level of co-occurrence but be weakly correlated
- There are many other pixel based colocalization measures but why?

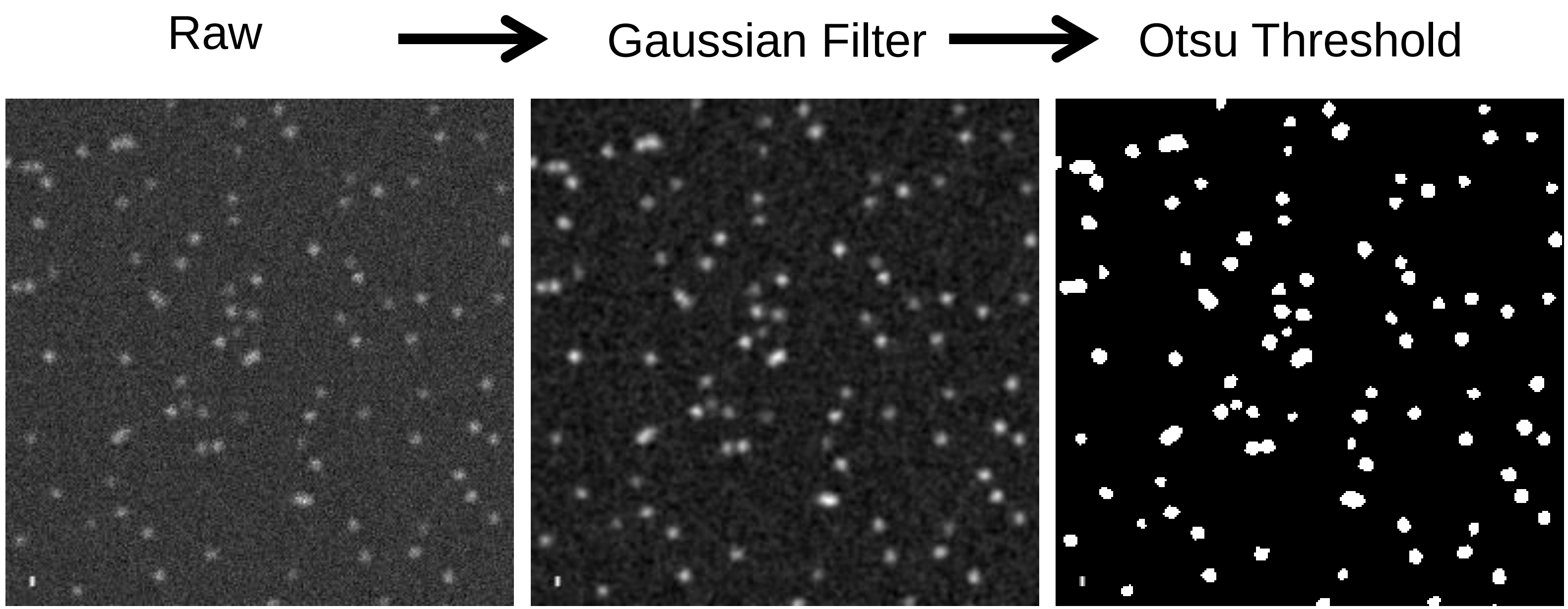
Adler, Jeremy, and Ingela Parmryd. "Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient." Cytometry Part A 77.8 (2010): 733-742.

Image Acquisition and Pre-processing

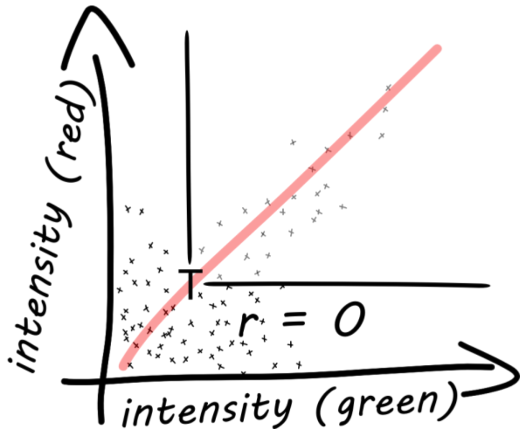
- Care should be taken to avoid cross-talk and bleed-through. Use of single labelled controls is a good idea!
- Watch out for chromatic aberrations
- Pre-processing is important and should not be ignored in colocalization analysis
- Application specific deconvolution, denoising and/or background subtraction steps should be used

Signal Isolation

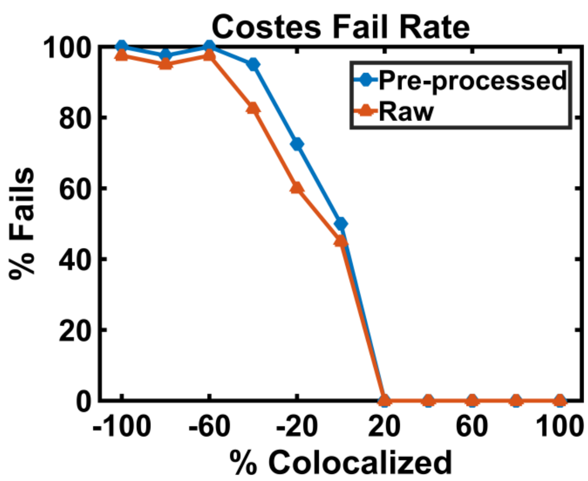
- Essential for calculation of the Manders coefficents and best practice for the Pearson coefficient.
- Aim is to segment the regions in both channels containing biologically relevant signal.
- Needs to be automated!
- There is no “one size fits all” strategy. Need to develop an approach that works reliably for your data.



Costes' Thresholding



- Finds the point on the line of best bit bellow which the Person coefficent ≤ 0
- Sets threshold values for signal isolation at this point

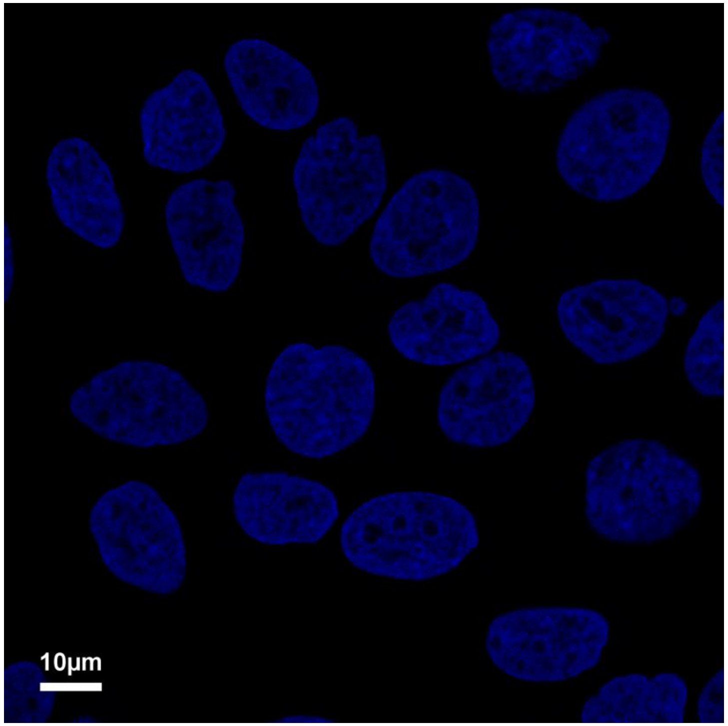


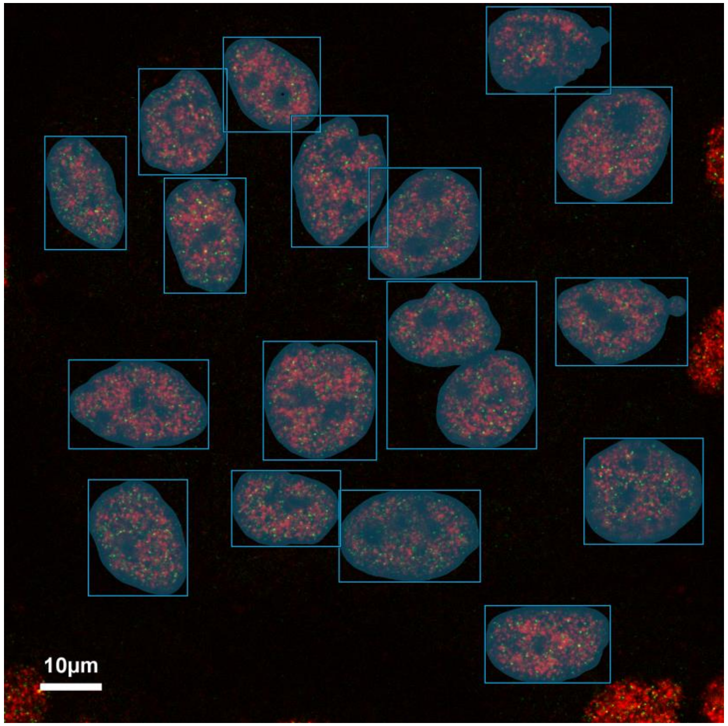
- Be careful, Costes' thresholding assumes a single linear correlation!
- Why use a test that assumes colocalization to test for colocalization?

Pike J., Styles I., Rappoport J.Z., Heath J."Quantifying Receptor Trafficking and Colocalization with Confocal Microscopy." Submitted to Methods 2016.

Regions of Interest (ROIs)

- Often appropriate to restrict (or perform separate) colocalization analysis using ROIs
- This is typically individual cells or nuclei





Statistical Testing

- Formulate a null hypotheses. Typically either:
   1. The signal from both channels is randomly distributed within the ROI
   2. There is no difference in the level of colocalization between two populations

- Option 1: Perform a statistical analysis for each ROI using pixel scrambling or simulations methods
   - Hard to completely remove auto-correlation effects
   - Individual ROIs are typically not very relevant, populations are!

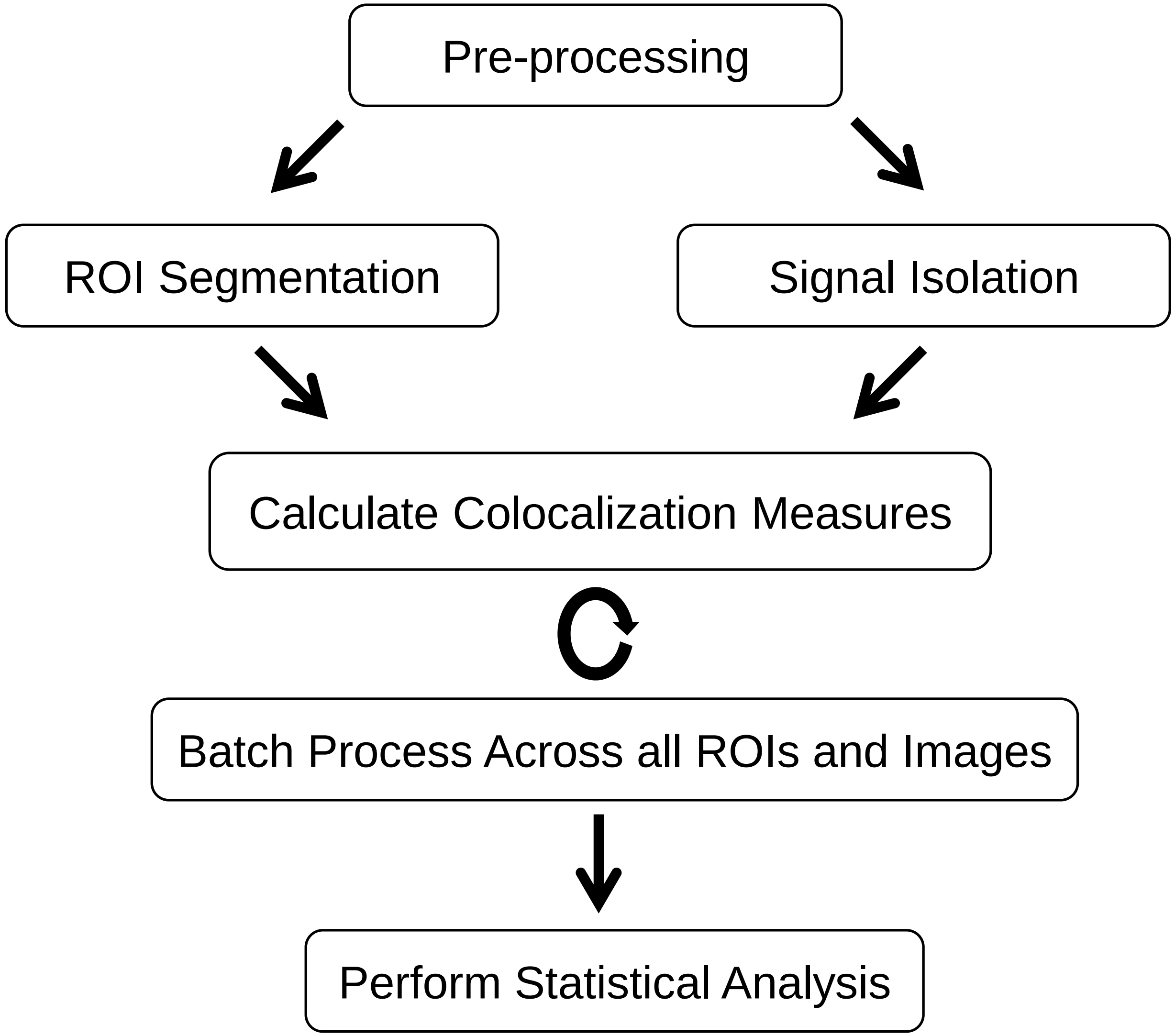
- Option 2: Perform standard statistical tests (eg t-tests) to compare colocalization across populations.

  Hypothesis 1: Subtract the expected value from each ROI measurement and compare the population to zero

  Hypothesis 2: Compare distributions between replicates using two-sample statistical tests

Mcdonald, John H., and Kenneth W. Dunn. "Statistical tests for measures of colocalization in biological microscopy." Journal of microscopy 252.3 (2013): 295-302

Putting It All Together: A Colocalization Workflow



Whats next?

- COMPARE will be organising a more advanced course covering:
  - Segmentation, deconvolution, tracking, colocalization etc
  - Any suggestions?

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

- I can work with you on collaborative projects.

Email (j.a.pike@bham.ac.uk) for enquires.

Acknowledgments

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

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