

Image Analysis with Fiji

An introductory course for biologists

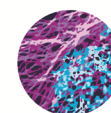
Jeremy Pike, Image Analyst for COMPARE

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:

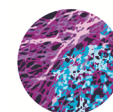
<https://jeremypike.github.io/image-analysis-with-fiji>

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



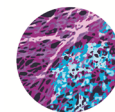
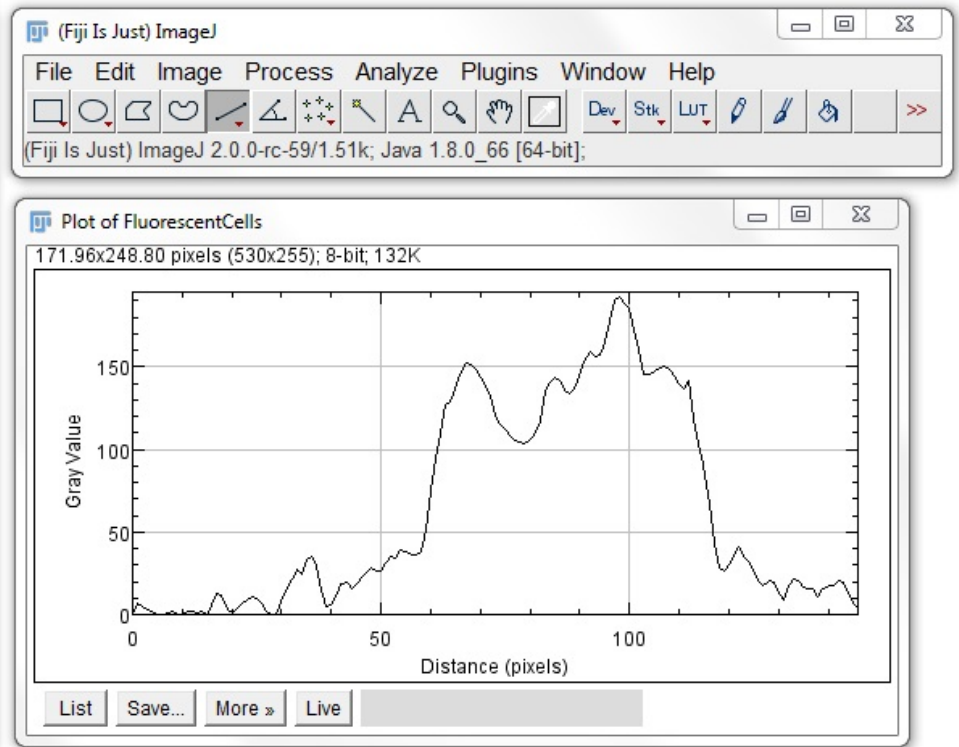
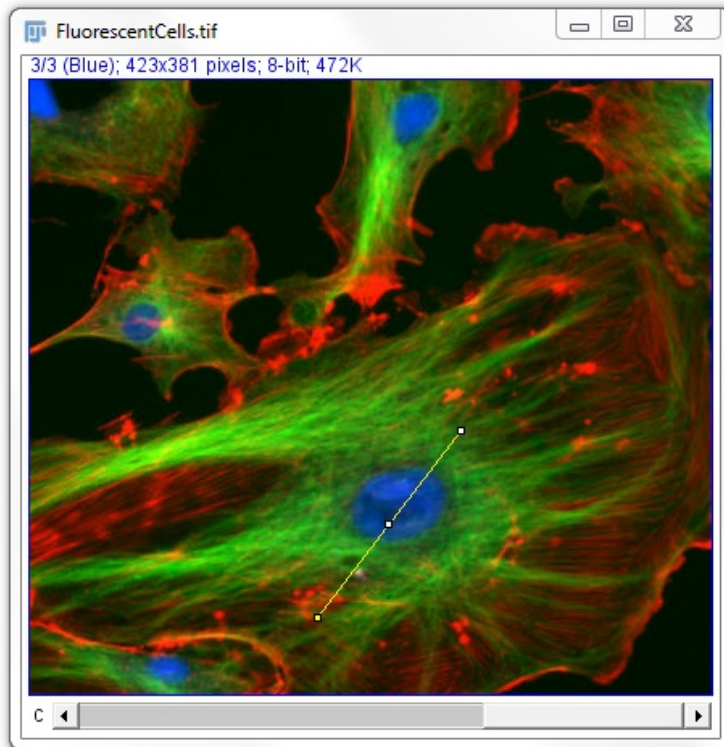
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Part 1: Basics



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.



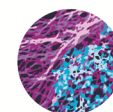
ImageJ2 is a complete rebuild of ImageJ, it is built into Fiji

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

If in doubt get Fiji!

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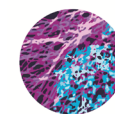
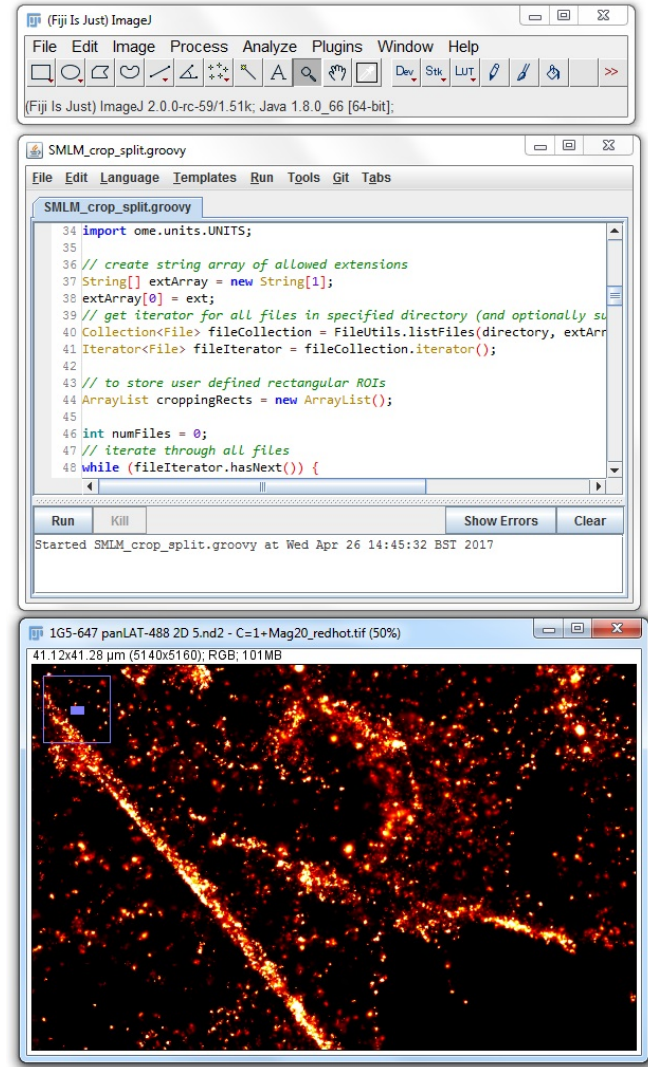
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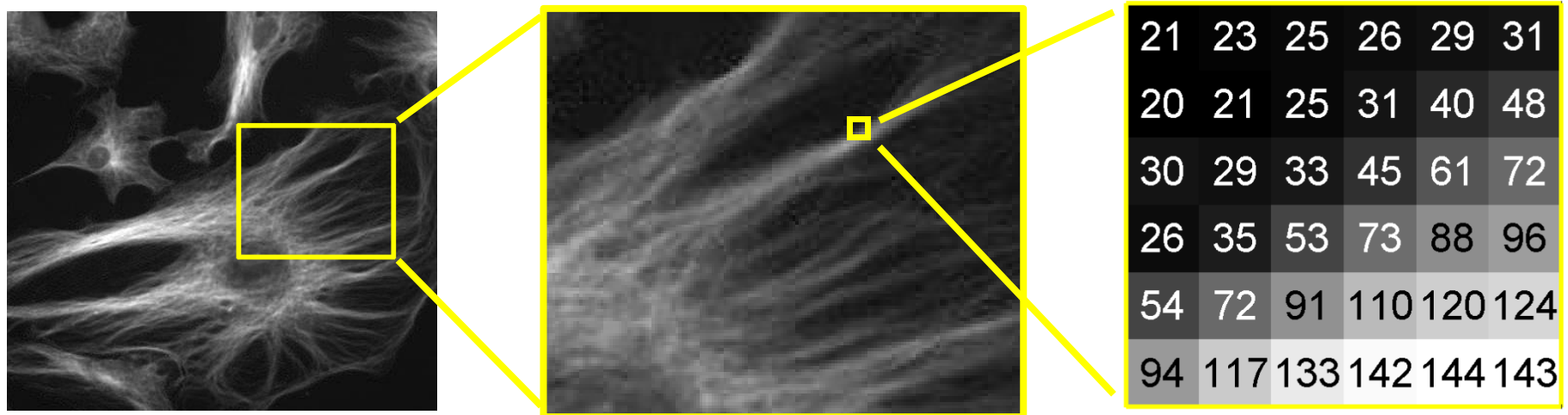
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Why use Fiji?

- 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)



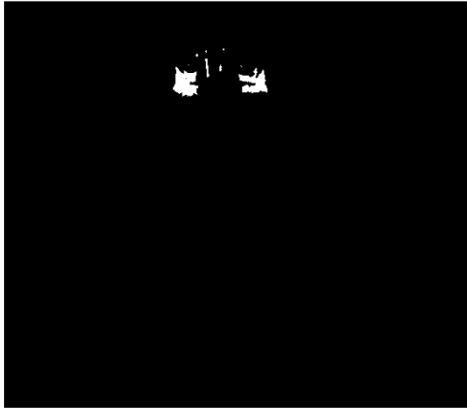
Digital images are simply arrays of numbers



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

Dynamic range is the number of values each number can take

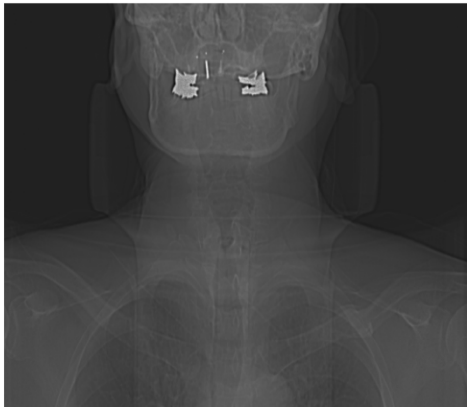
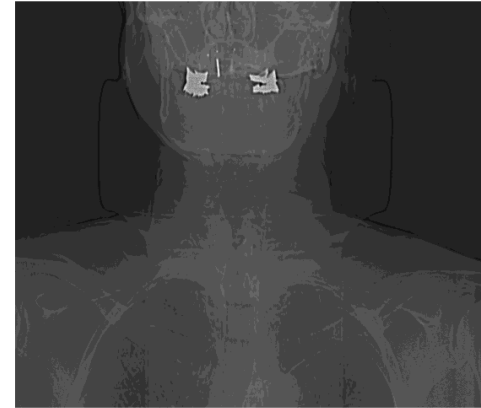
1 bit = 2



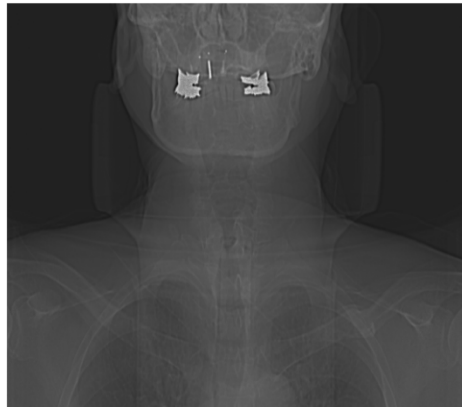
2 bit = 2^2



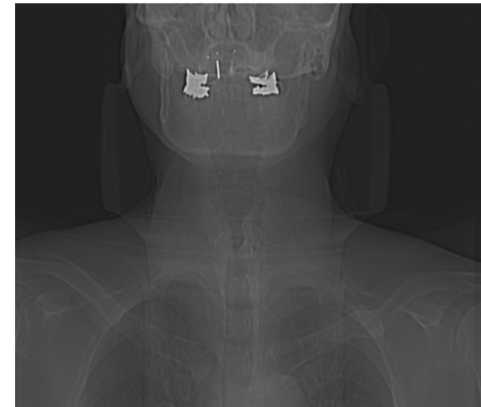
4 bit = 2^4



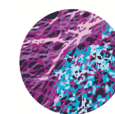
8 bit = 2^8



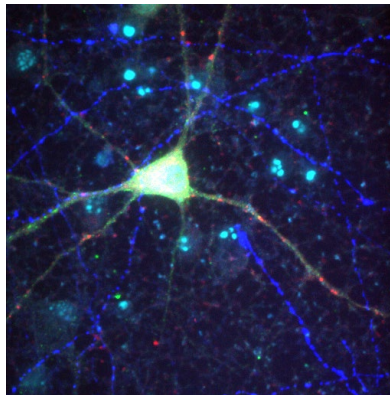
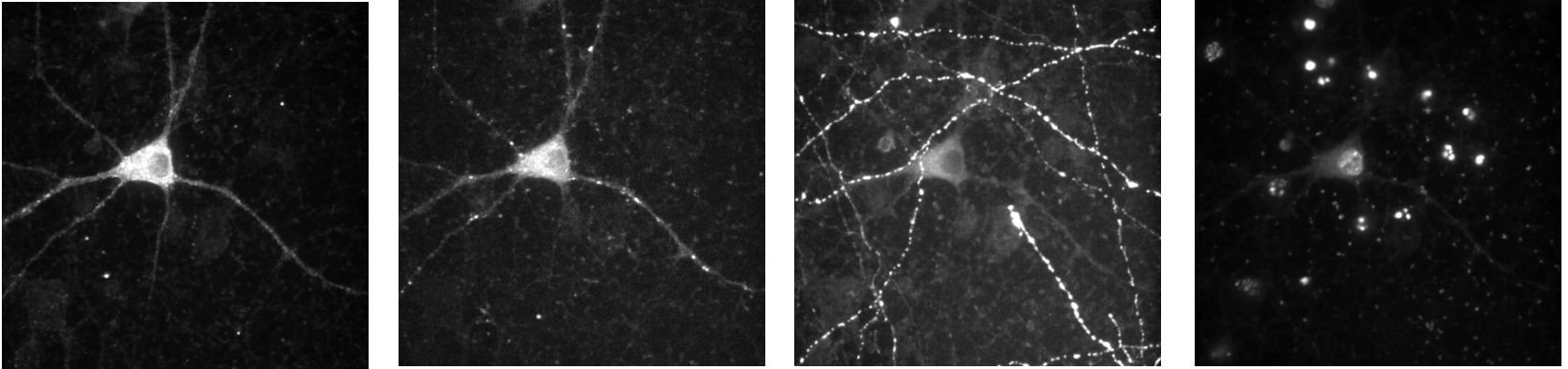
12 bit = 2^{12}



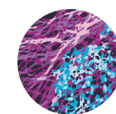
16 bit = 2^{16}



Multi Channel Data

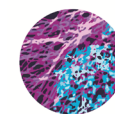
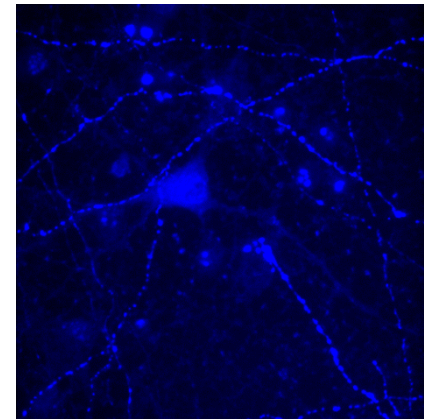
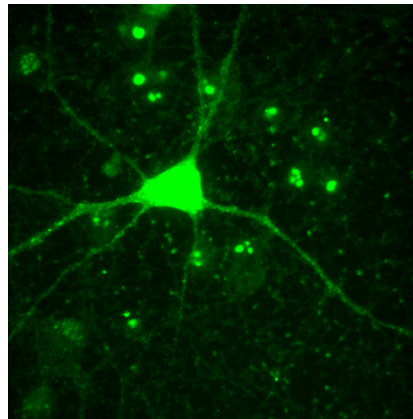
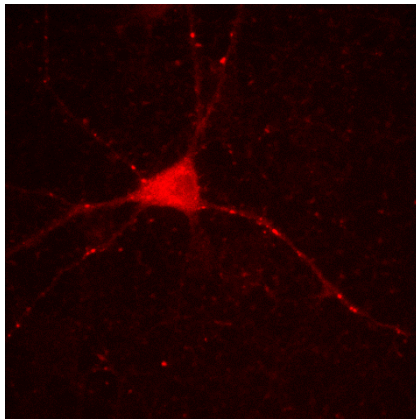
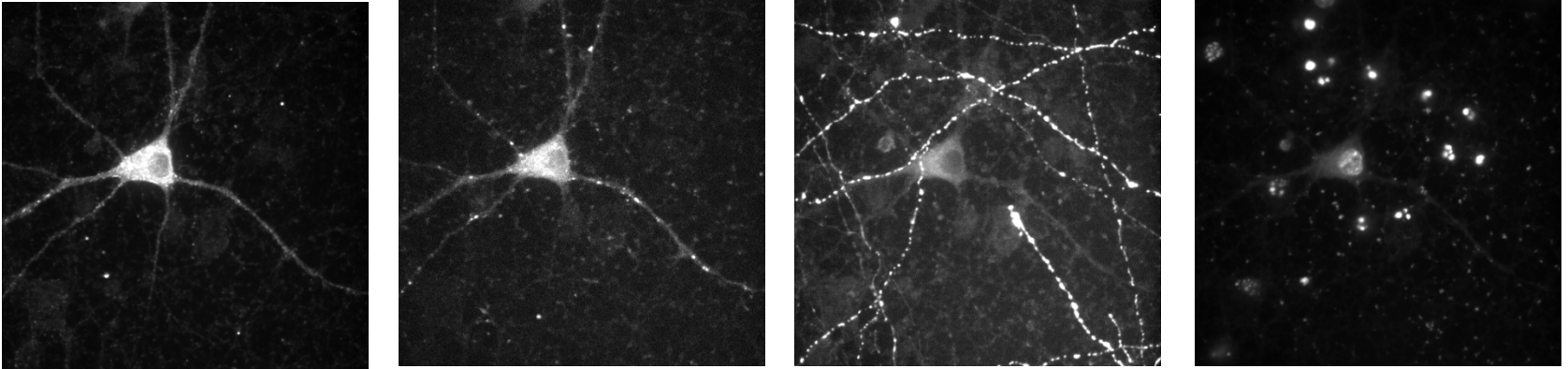


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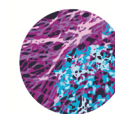
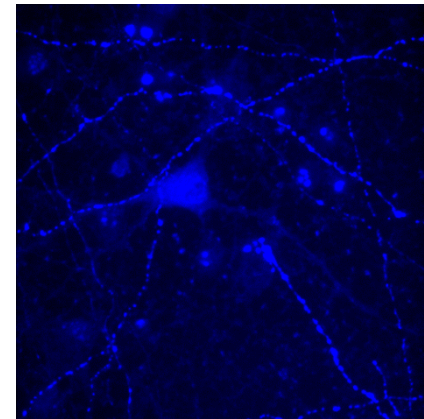
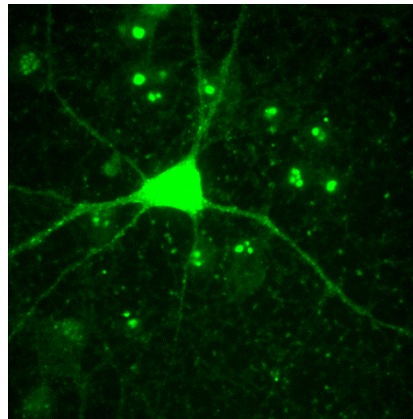
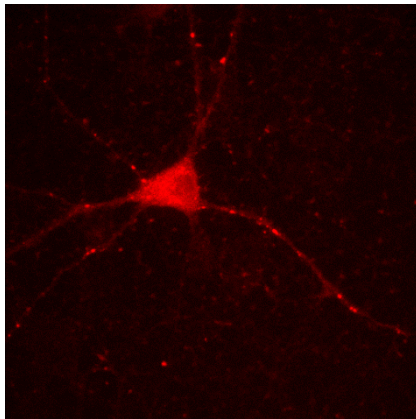
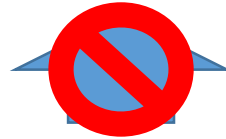
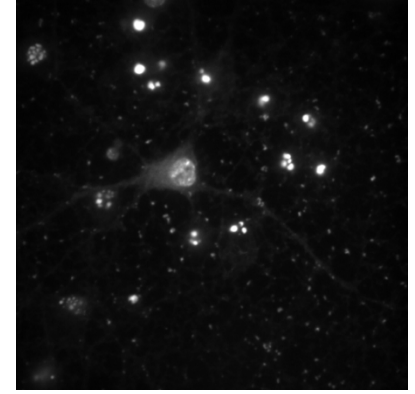
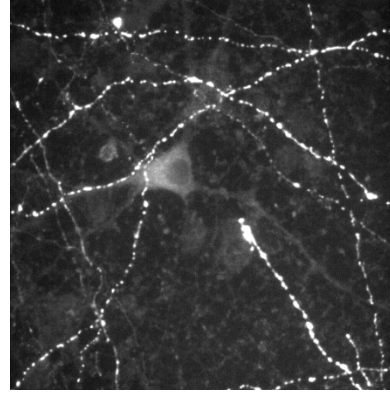
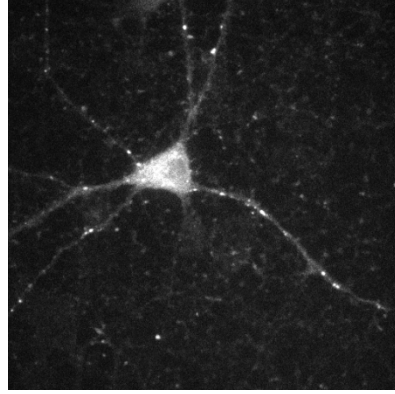
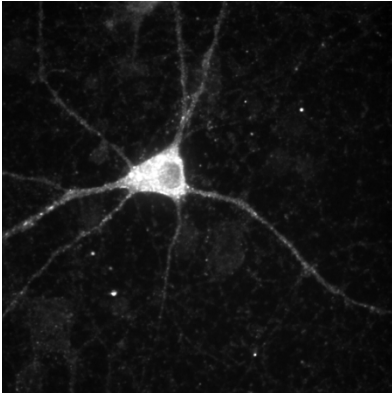


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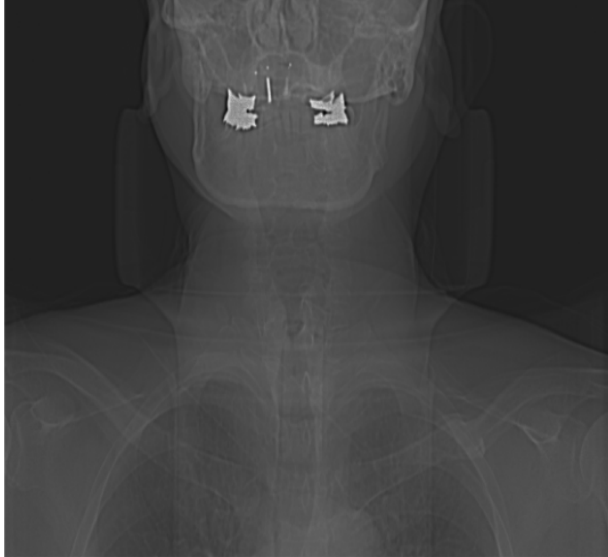
RGB Colour Images



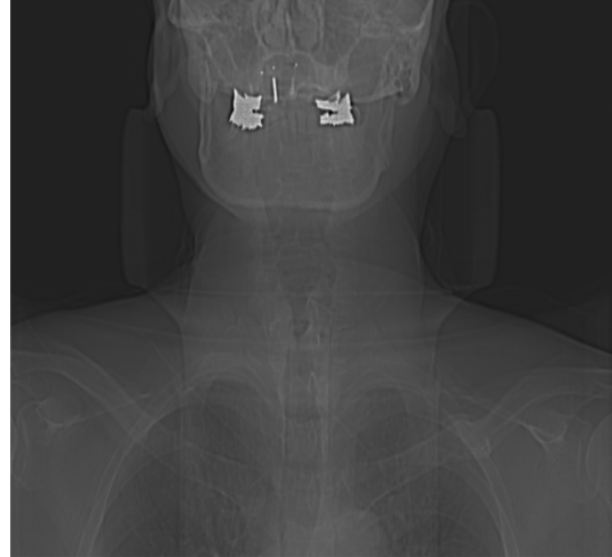
RGB Colour Images



What can we “see” on a monitor?

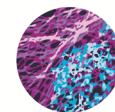


8 bit = 2^8

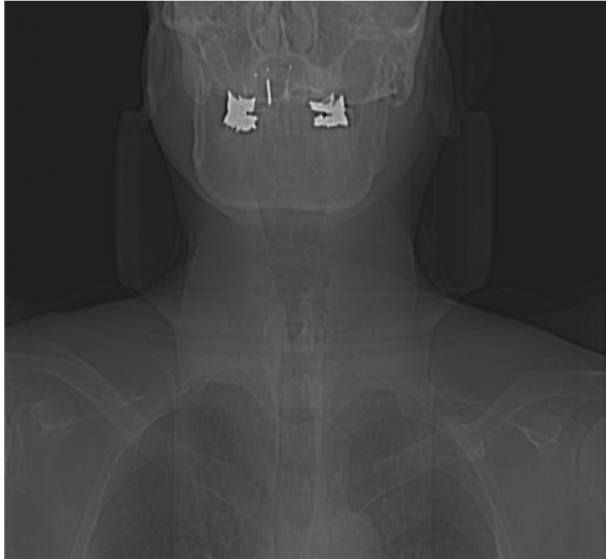


16 bit = 2^{16}

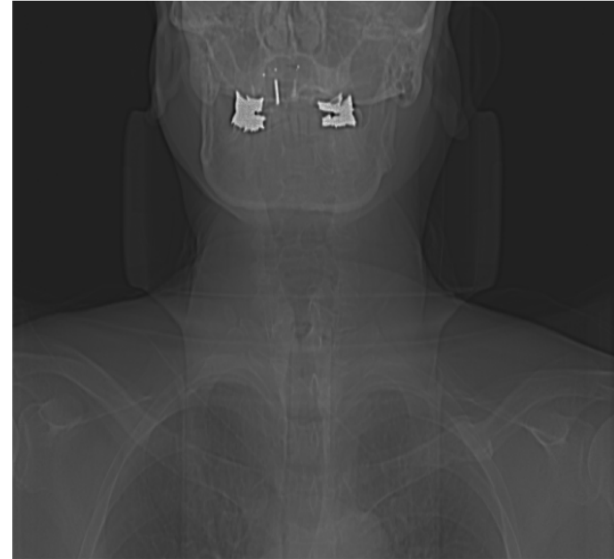
Can you tell the difference?



What can we “see” on a monitor?

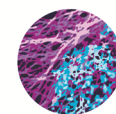


8 bit = 2^8



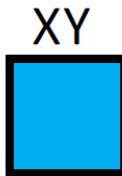
16 bit = 2^{16}

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

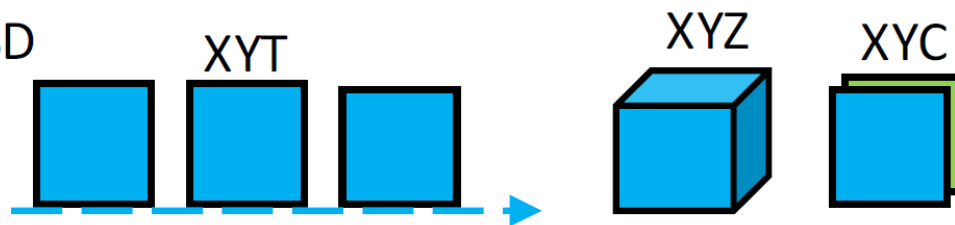


Microscopy data

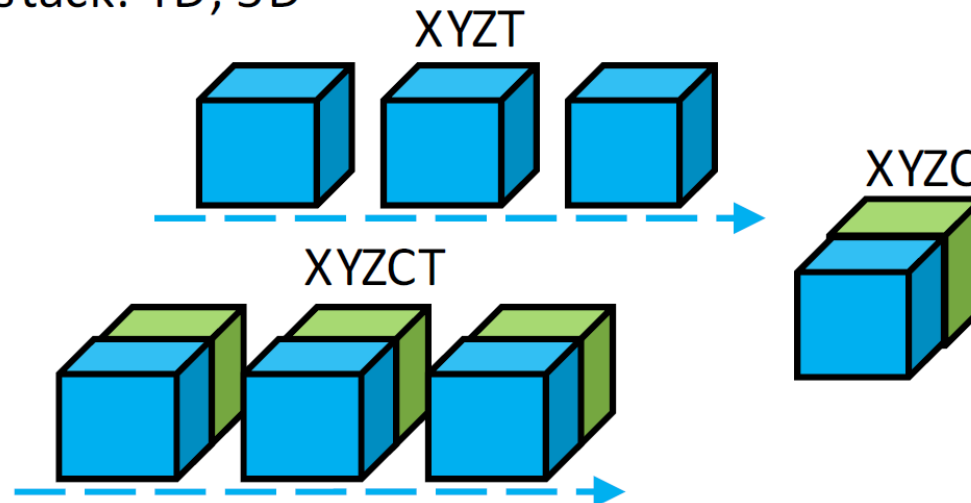
- Image: 2D



- 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

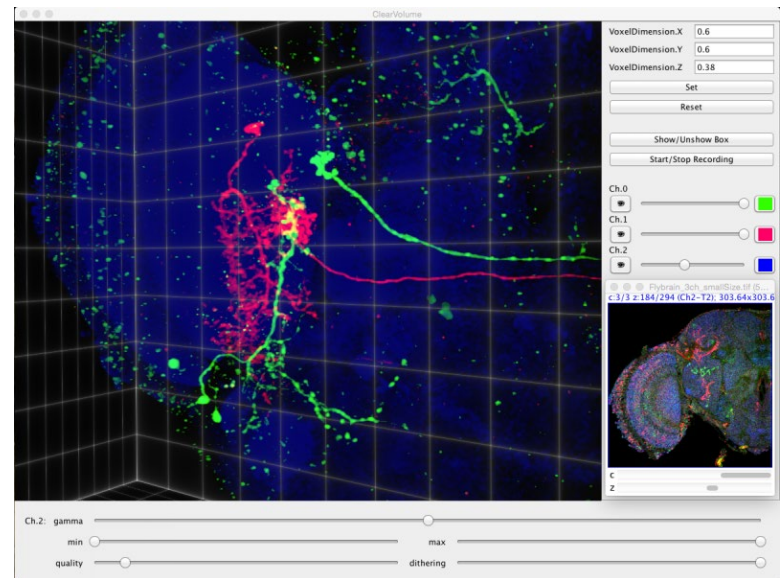
<https://imagej.nih.gov/ij/plugins/volume-viewer.html>

- 3D Viewer

<https://imagej.net/plugins/3d-viewer/>

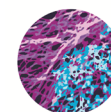
Or commercial software:

- Arivis Vision4D, Imaris etc



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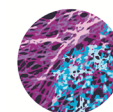
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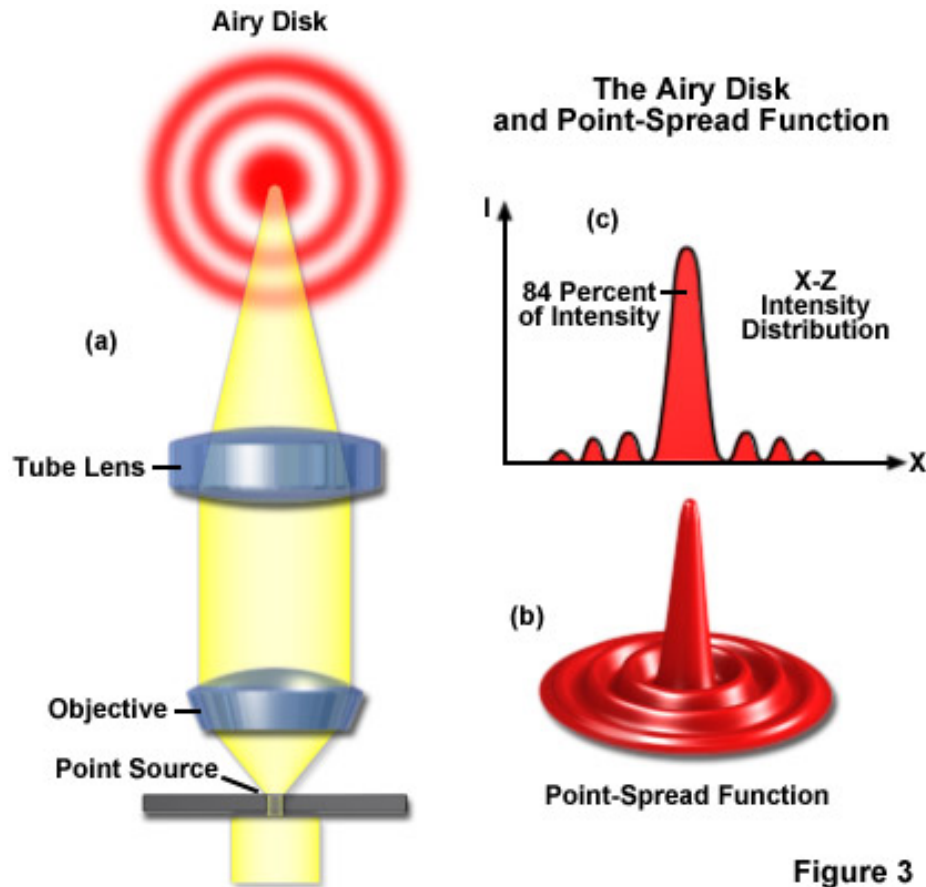
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Common file formats

- **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 of 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



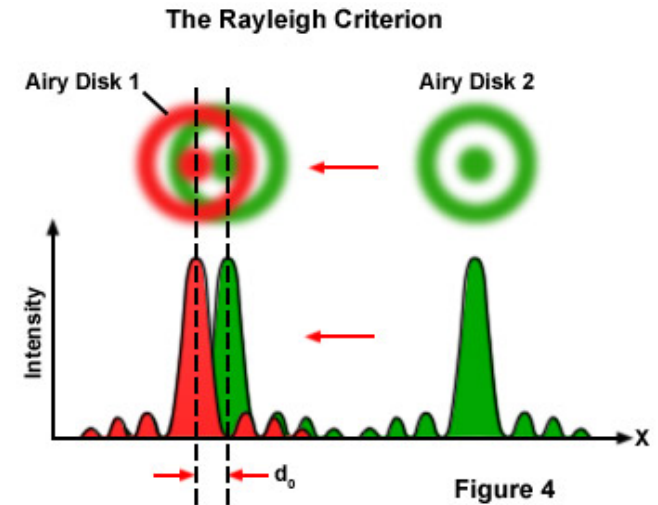
"Each element of the primary image is a small diffraction pattern, and the actual image, as seen by the eyepiece, is only the ensemble of the magnified images of these patterns"

Born and Wolf, Principles of Optics

Figure 3

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

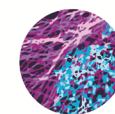
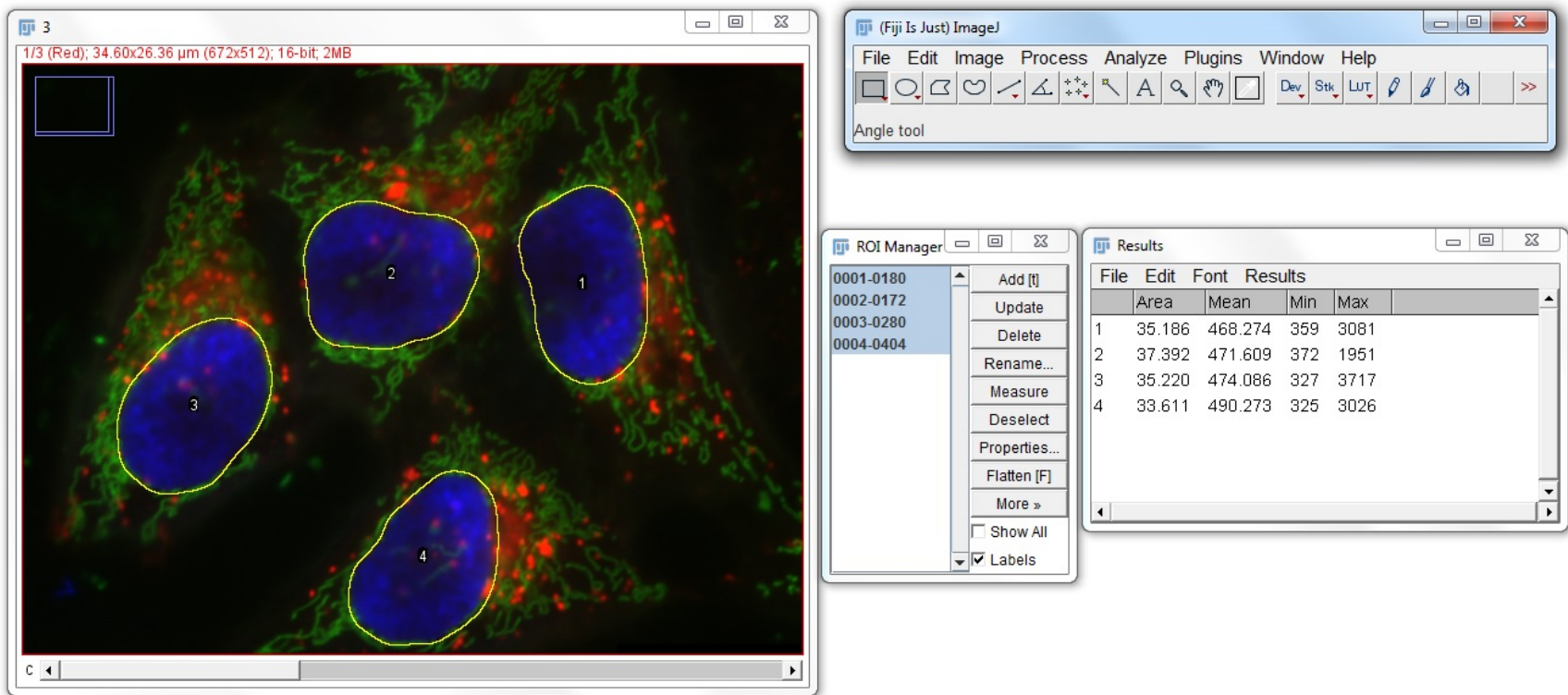


Image Processing:

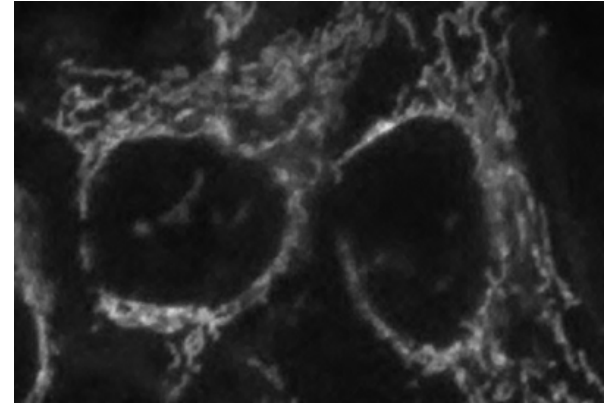
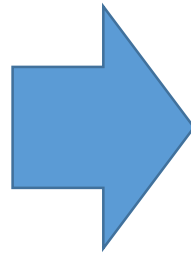
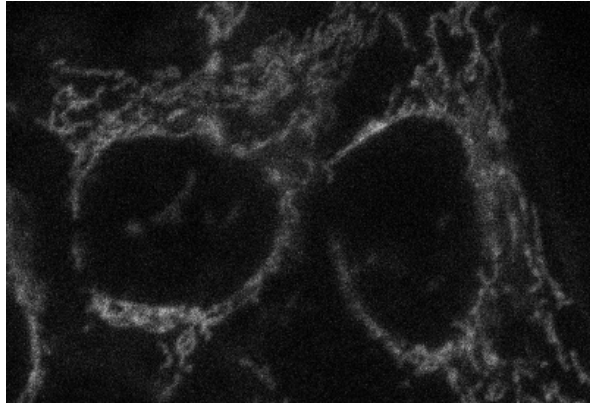
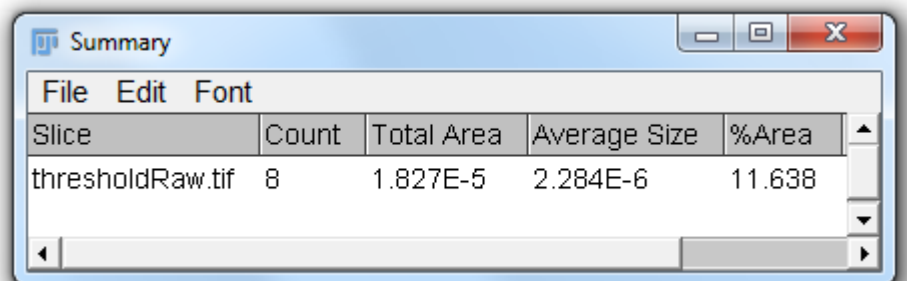
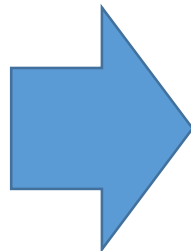
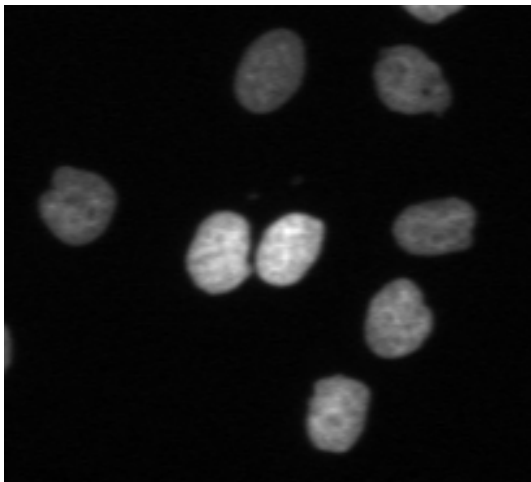


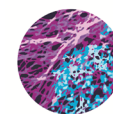
Image Analysis:

A screenshot of a software window titled "Summary". It contains a table with 5 columns: Slice, Count, Total Area, Average Size, and %Area. The table has one data row for "thresholdRaw.tif".

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

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Why do computational processing and analysis?

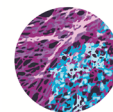
- Its quantitative
- Its unbiased
- Can enhance understanding of the data
- Can be automated for processing of large datasets

"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

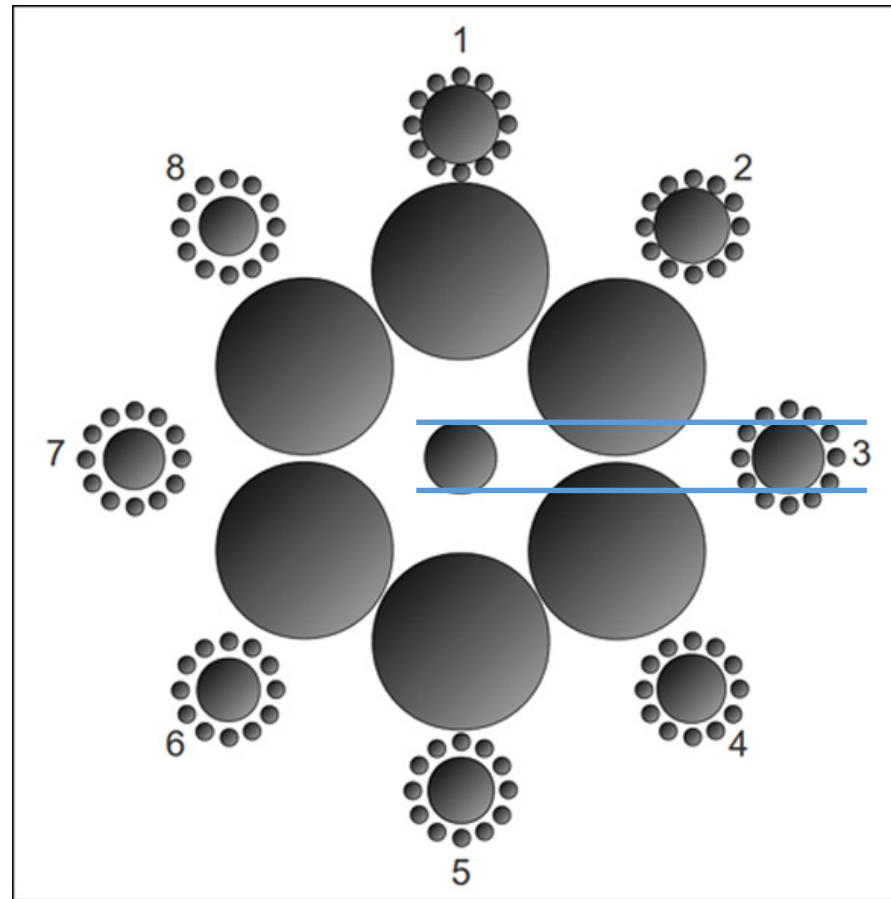
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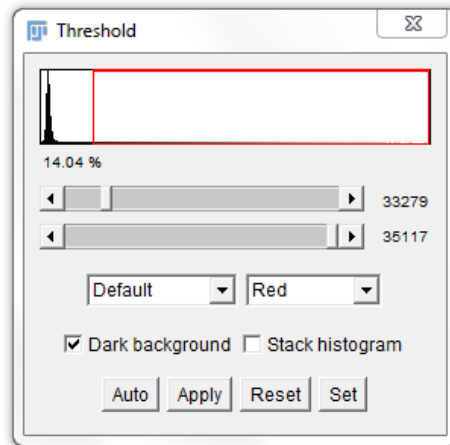
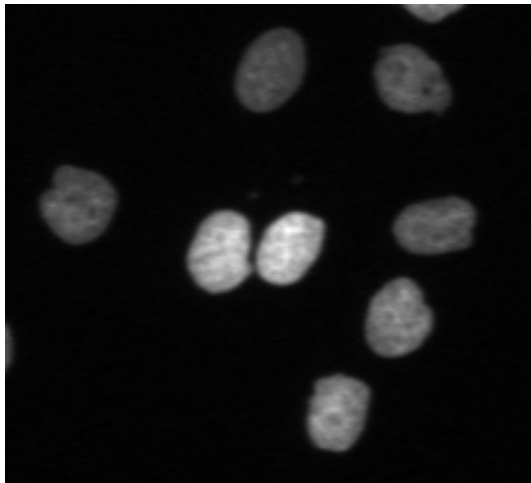


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What outer circle is the same size and the central circle?

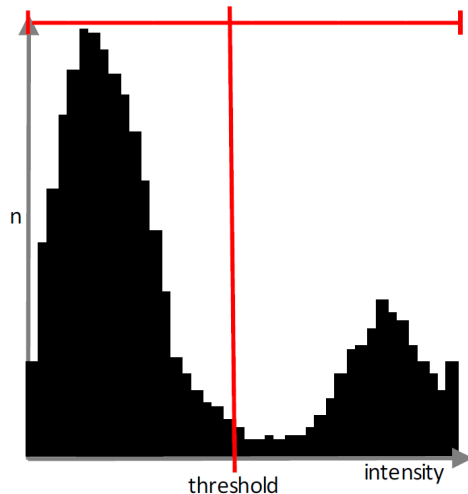


Intensity based thresholding to segment objects



Automated threshold values are preferable to manual selection

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

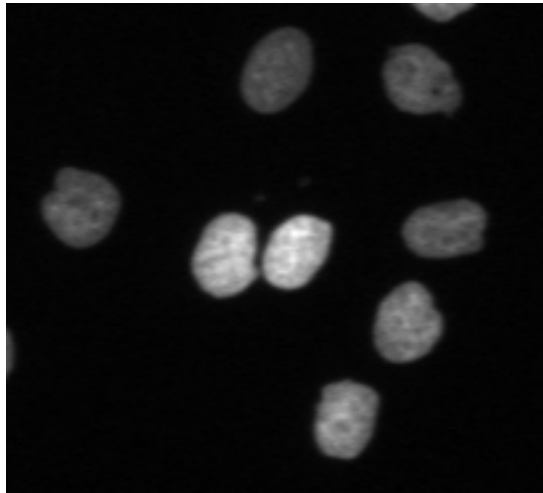


Otsu, N (1979), IEEE Trans. Sys., Man., Cyber. 9: 62-66.

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

Li, CH & Tam, PKS (1998), Pattern Recognition Letters 18(8): 771-776

Automated threshold values are preferable to manual selection



Raw Data



Otsu Threshold



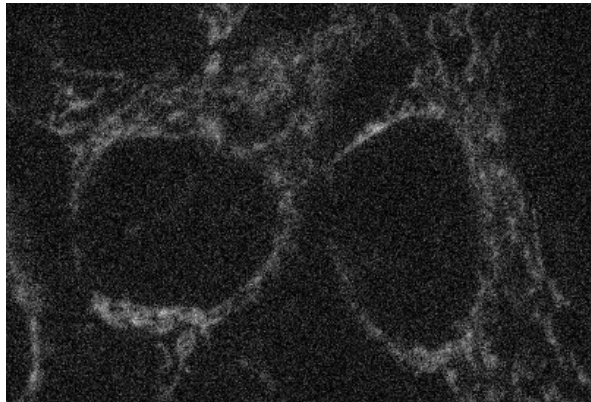
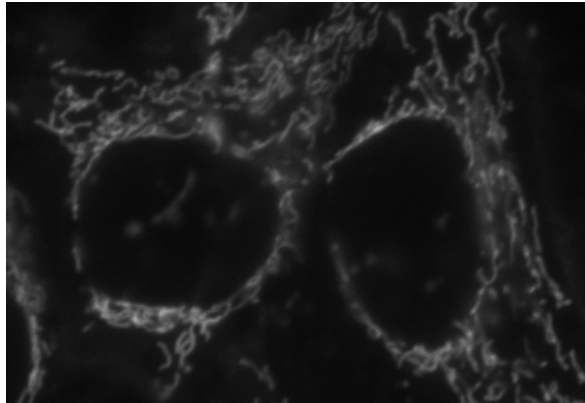
Li Threshold

Watershed transformation to separate touching objects

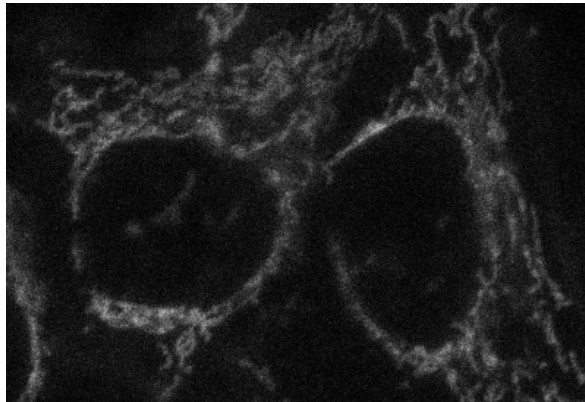


- Seeds placed at local minima of distance map and dilated
- This can be visualised as flooding the distance map

Noise is image corruption from the acquisition process



Gaussian



Poisson



Impulse

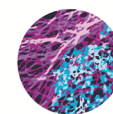
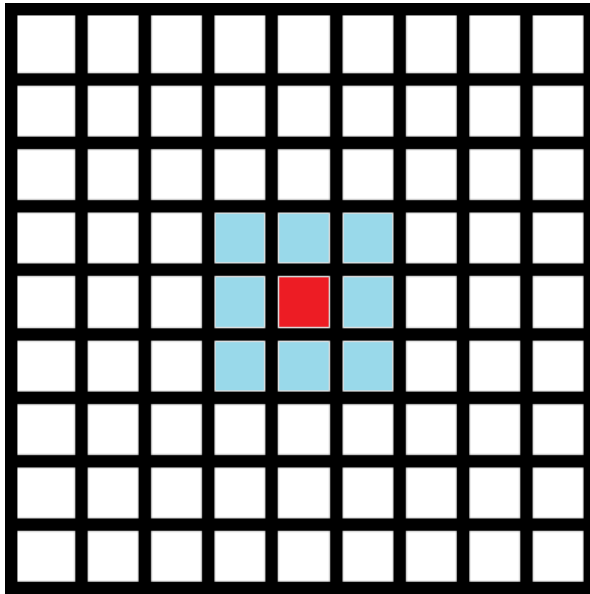
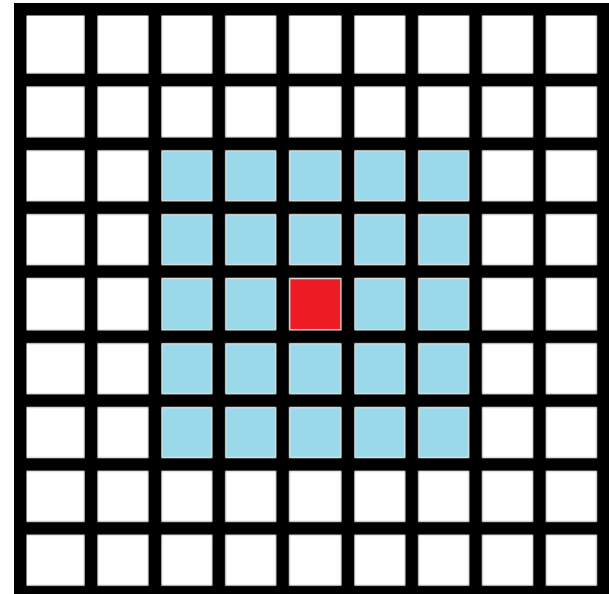


Image filters and convolution

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



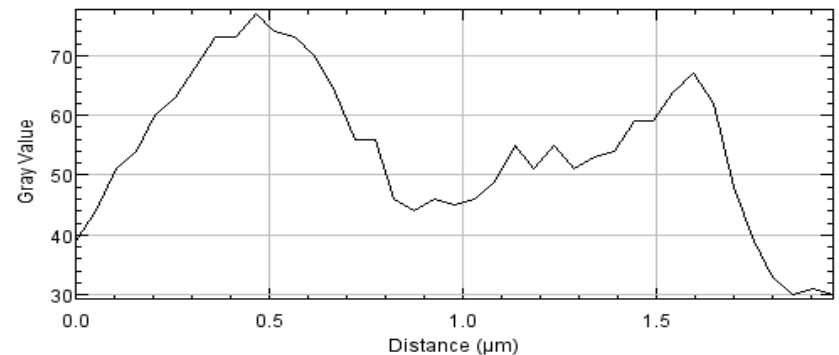
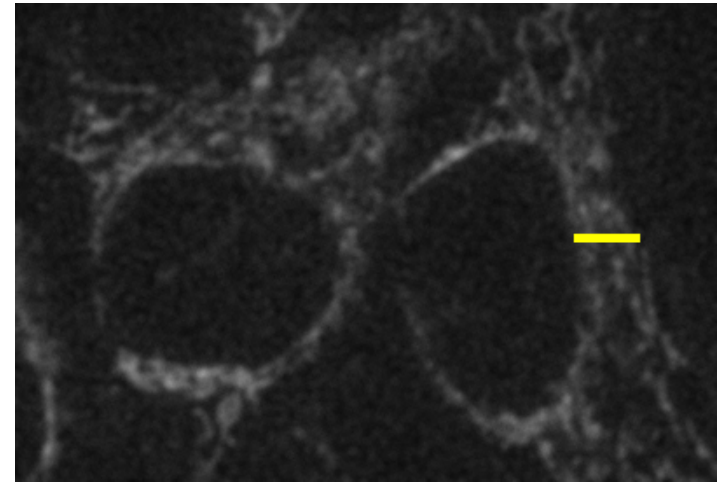
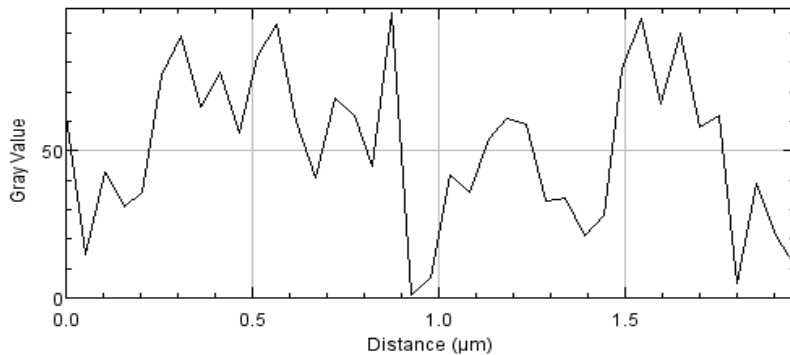
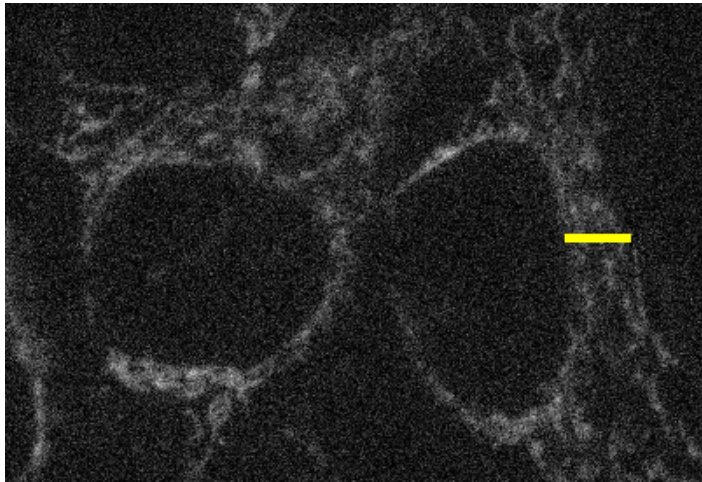
3x3 Neighbourhood



5x5 Neighbourhood

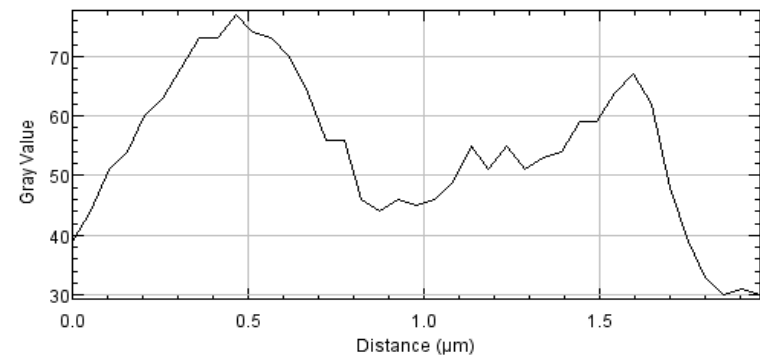
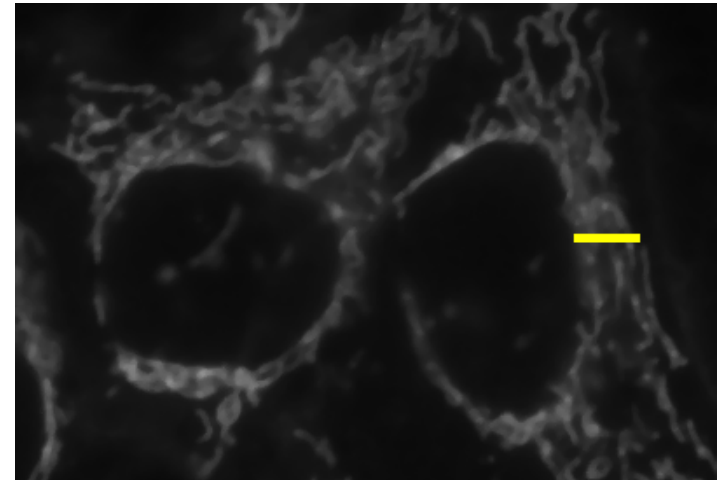
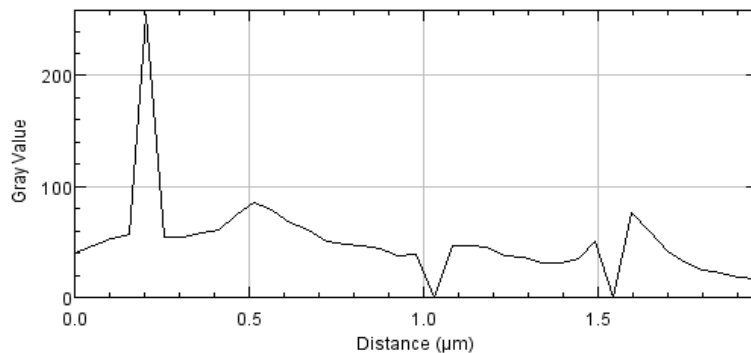
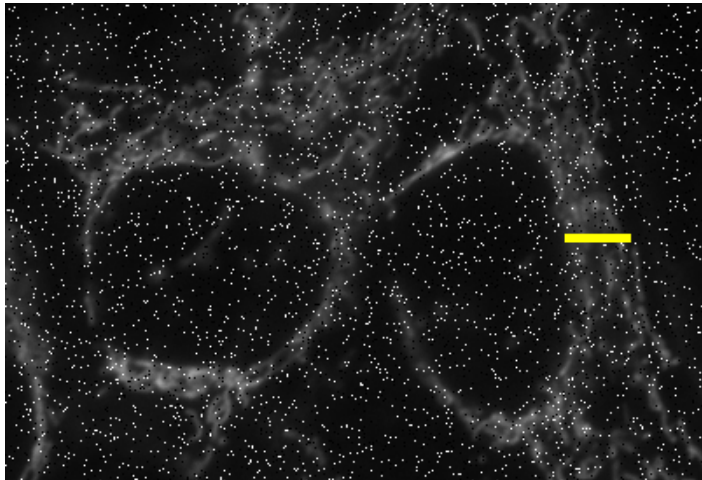
Mean filter

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



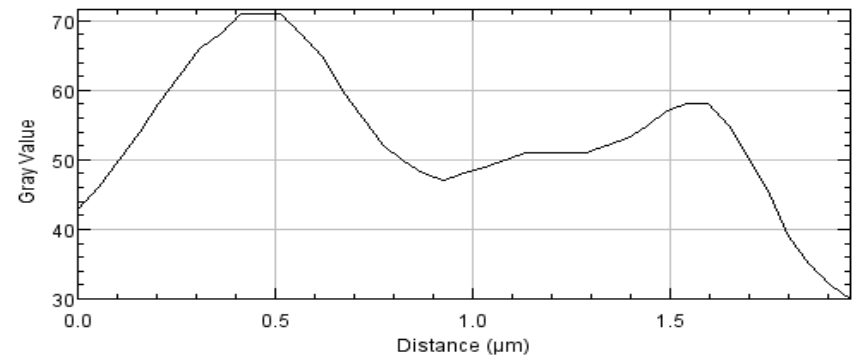
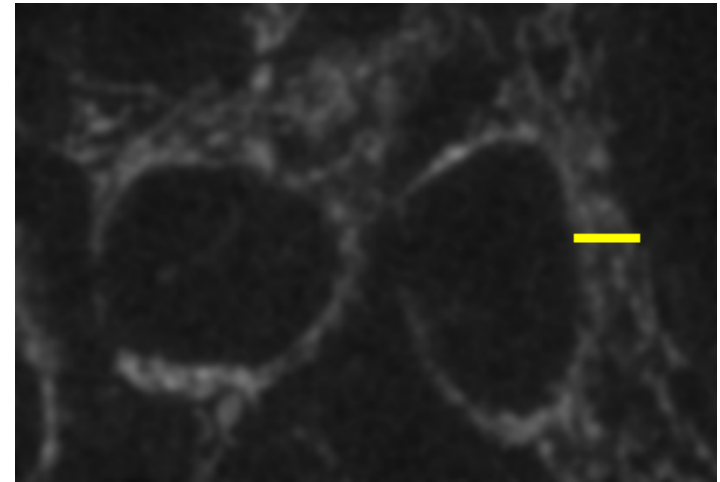
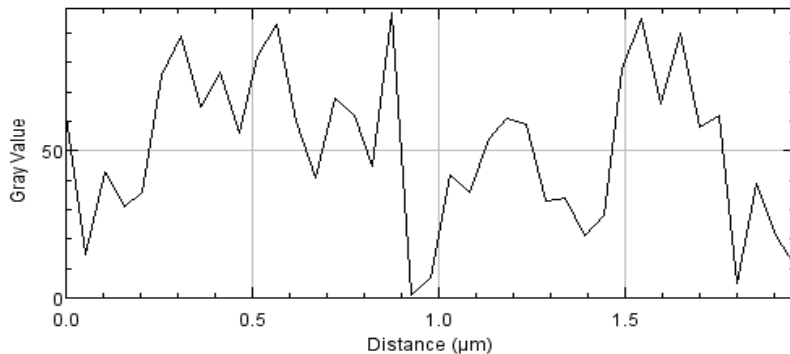
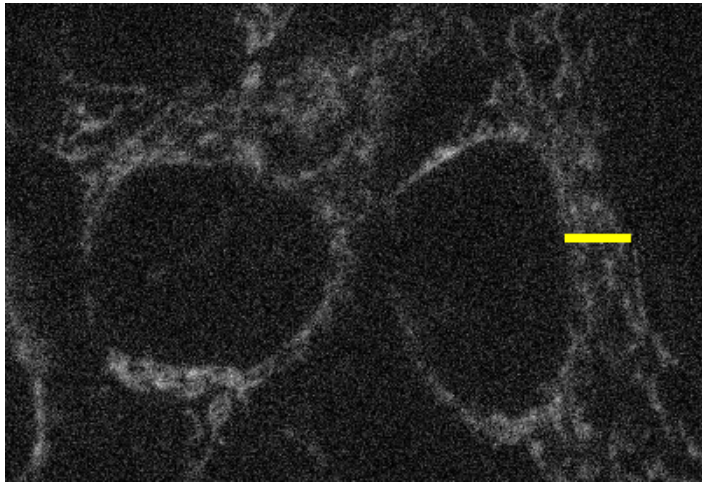
Median filter

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



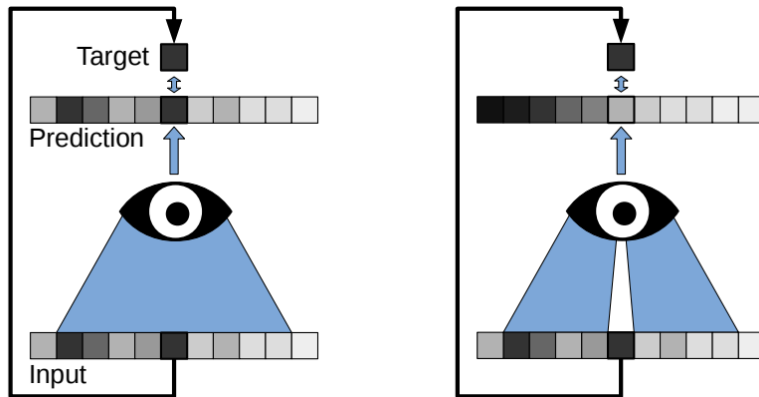
Gaussian filter

- Contribution of neighbourhood pixels weighted by Gaussian profile
- Removal of Gaussian and Poisson Noise

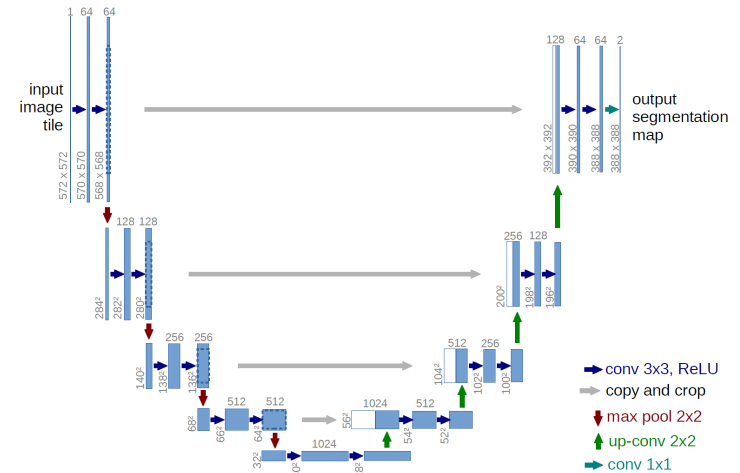


Deep learning based denoising

Self-supervised denoising: Noise2Void [1]



UNET architecture [2]



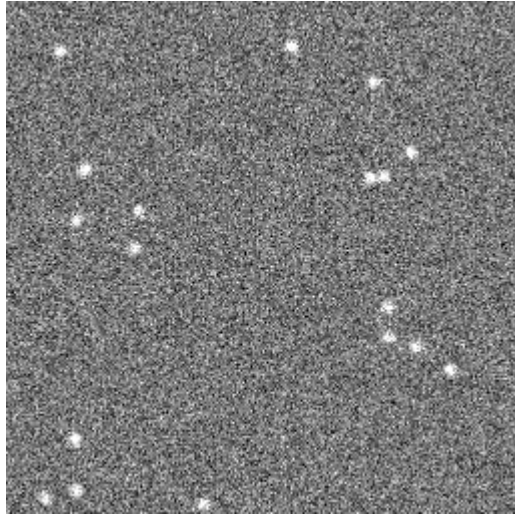
Fiji plugin available: <https://imagej.net/plugins/n2v>

[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.

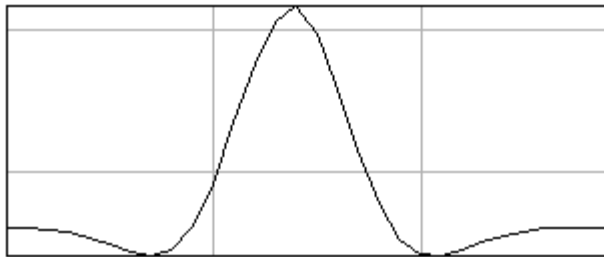
<https://github.com/juglab/n2v>

[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

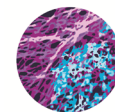


=



IN PARTNERSHIP:

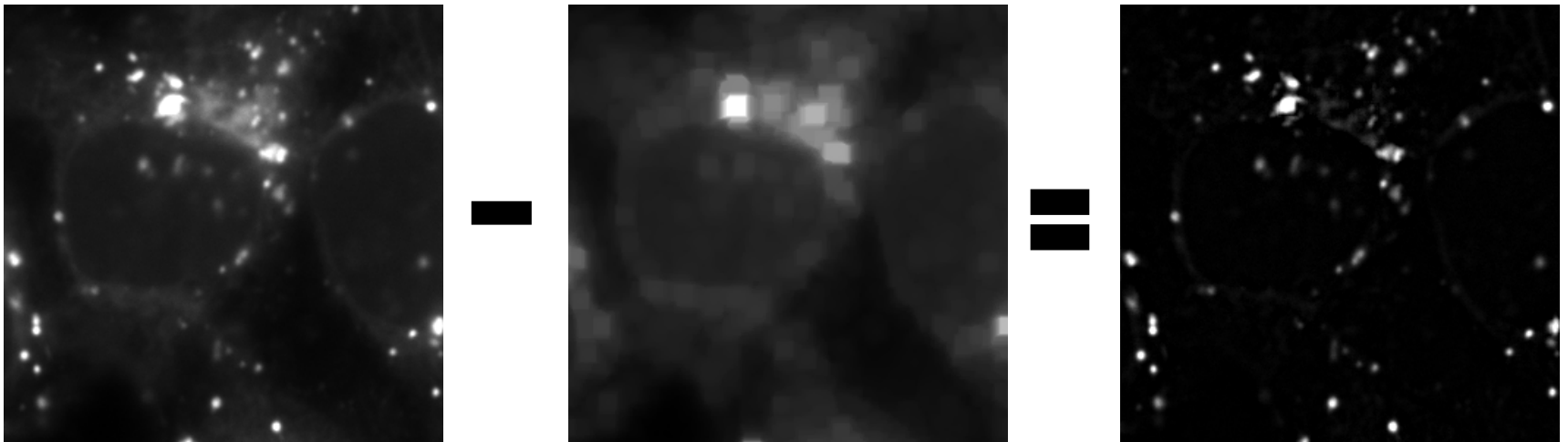
The Universities of Birmingham and Nottingham



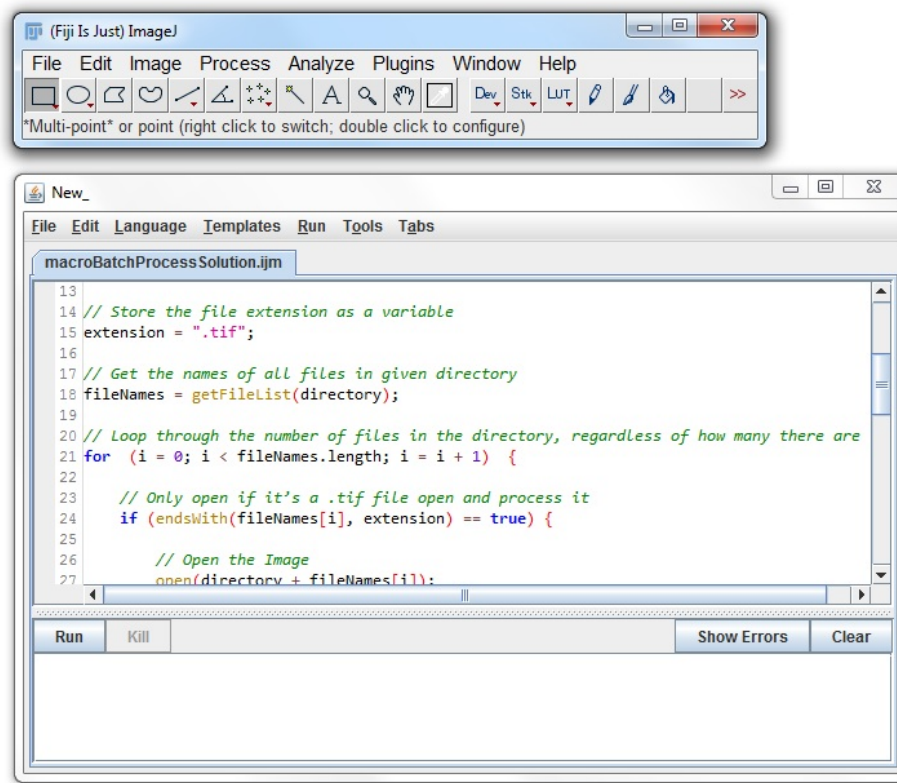
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Rolling ball background subtraction

- 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

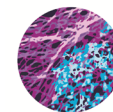


Part 3: Introduction Macros and Workflow Automation



IN PARTNERSHIP:

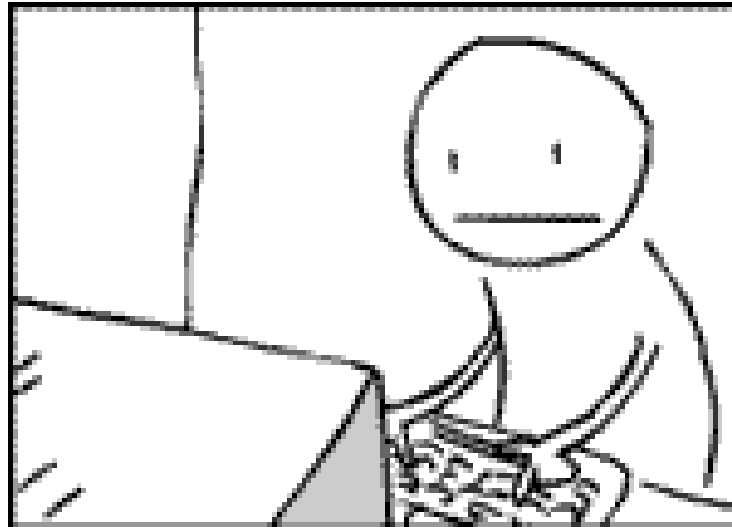
The Universities of Birmingham and Nottingham



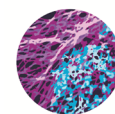
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Why automate a workflow?



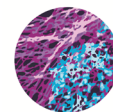
- Save time and eliminate user mistakes
- Unbiased and consistent approach
- Have a record off what you have done



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:

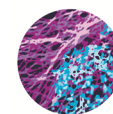
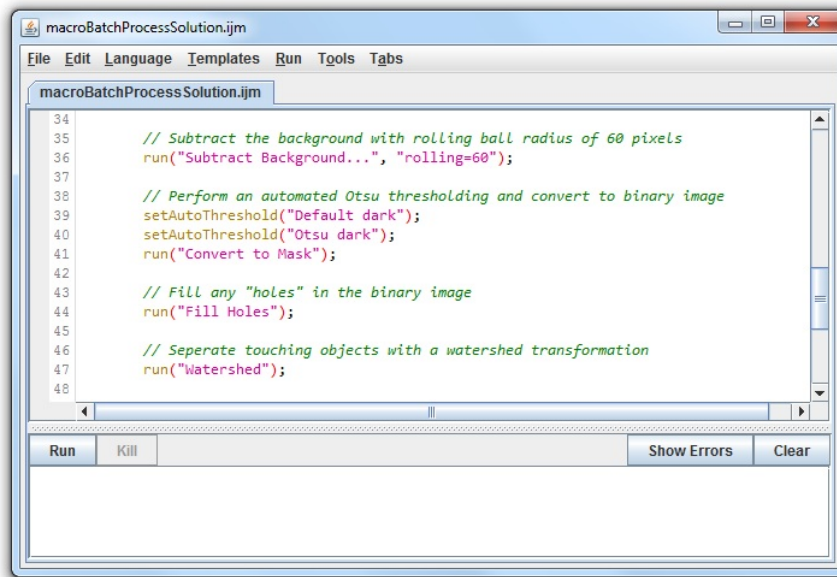
[http://fiji.sc/Introduction into Macro Programming](http://fiji.sc/Introduction%20into%20Macro%20Programming)



The Fiji script editor

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

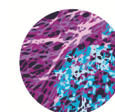
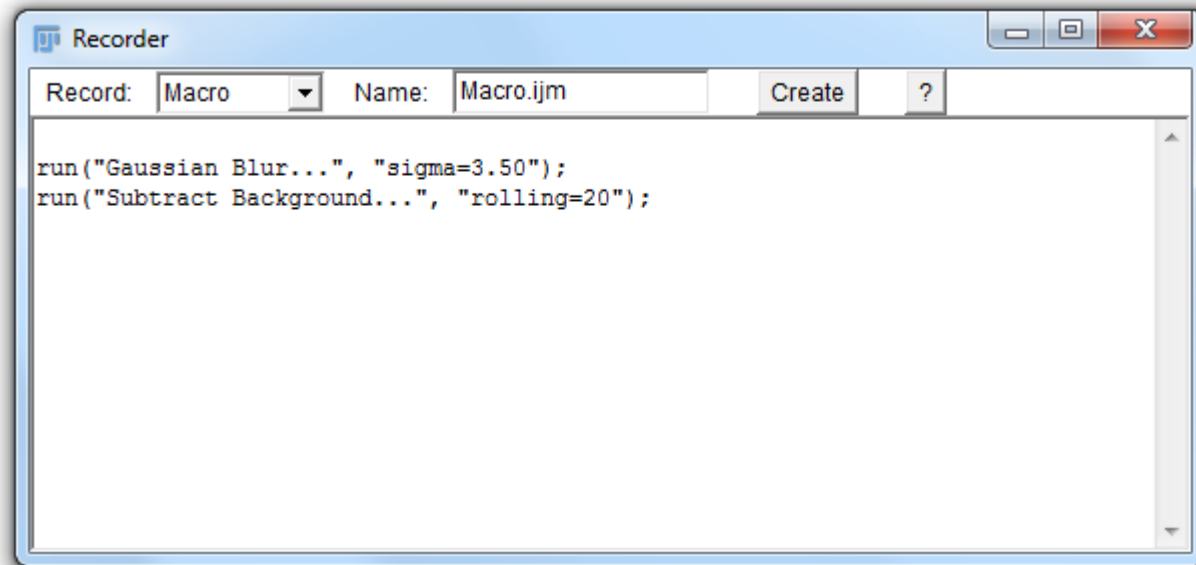
- Syntax highlighting
- Templates menu contains some useful examples



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!

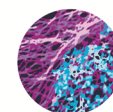


For loops

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);  
}
```



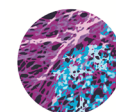
Conditional statements

if (condition) {do something}

else if (other condition) {do this instead}

else {do the alternative}

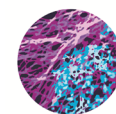
```
if(nImages() == 1) {  
    print(getTitle() + " is open.");  
}  
else if(nImages() > 1) {  
    print(nImages() + " images are open.");  
}  
else {  
    print("No images are open.");  
}
```



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

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

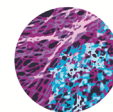
Cancer Research UK Cambridge Institute:

Mark Dunning

Stefanie Reichelt

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