

# Introduction to image analysis

Adam Tyson

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April 2018

# Course website



[bit.ly/ICR-image](http://bit.ly/ICR-image)

- Slides
- Sample images
- Software

**ICR** The Institute of Cancer Research

**Introduction to Image Analysis**

**Course Materials**

Course slides (.pdf)  
Course slides (.pptx)

Sample Images (.zip)

FLU download (OSX)  
FLU download (Windows)  
FLU download (Linux)

Deconvolution toolbox

**Details**

April 17<sup>th</sup>, 10am-3pm  
Training Room, Chester Beatty Labs, 237 Fulham Road.

**Summary**

Targeted at those new to image analysis. No experience whatsoever is required.  
Applicable to any microscopy method in use at the ICR.

The course will cover some of the fundamentals of image analysis, using examples in the popular open-source software - ImageJ.

The focus will be on general image analysis principles which can be applied in any software package. Many of the example images will be from microscopy, but the principles are applicable to any imaging modality.

**Aims**

- Allow those new to microscopy to feel comfortable carrying out basic image analysis independently.
- To provide a starting point for the more advanced image analysis methods that are becoming necessary in many areas of biomedical science.

**Contents**

- ImageJ/FLU
- Histograms, thresholding & segmentation
- Smoothing & background subtraction
- Morphological operators (erosion, dilation, opening, closing)
- Watershed
- Gradients & edge detection
- Boolean algebra (AND, NOT, OR, XOR)
- Deconvolution
- Advanced segmentation using machine learning
- Object measurement
- ImageJ automation & macros
- Experiment planning
- Analysis as a method (e.g. super resolution microscopy)

This project is maintained by Adam Tyson  
Advanced Image Analyst and Microscopist, Institute of Cancer Research

Hosted on GitHub Pages — Theme by [orderedlist](#)

# Course website - download



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- Using ImageJ/FIJI (but everything is general)
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## Disclaimer:

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# Questions

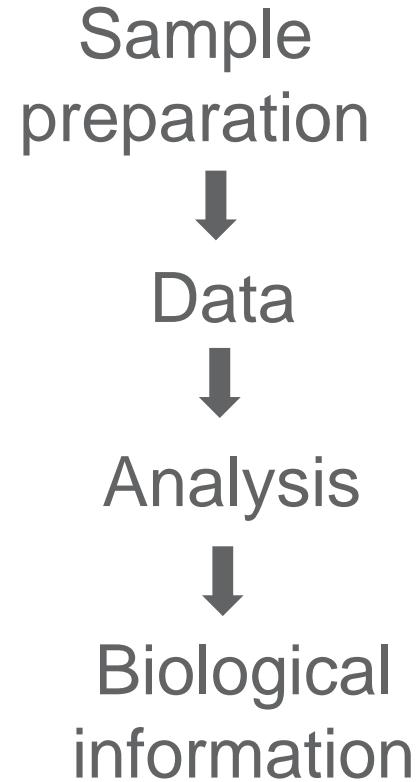
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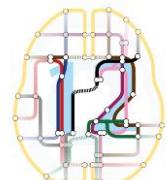
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- Which analysis software?
- A bit / no / lots of experience?
- Any specific aims?

# First (and most important lesson)

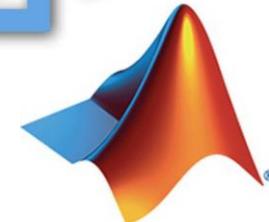
Test your analysis  
**before** you  
acquire data!



# Software



SlideBook

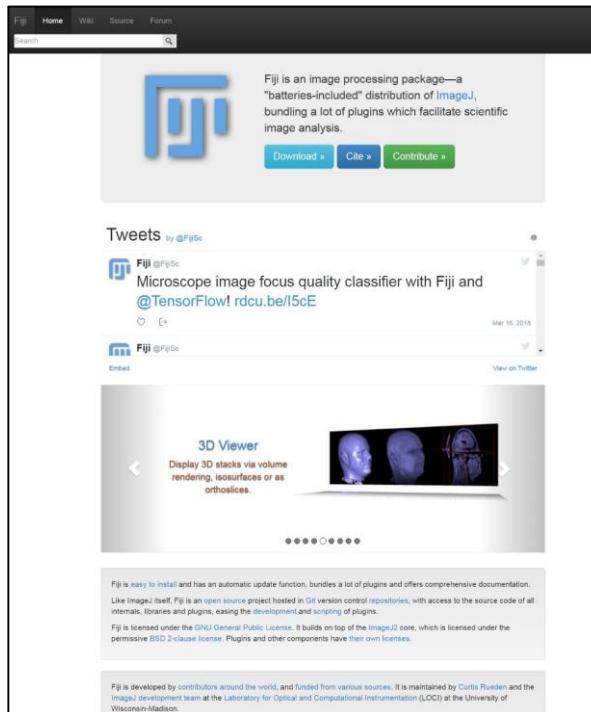


Imaris 9

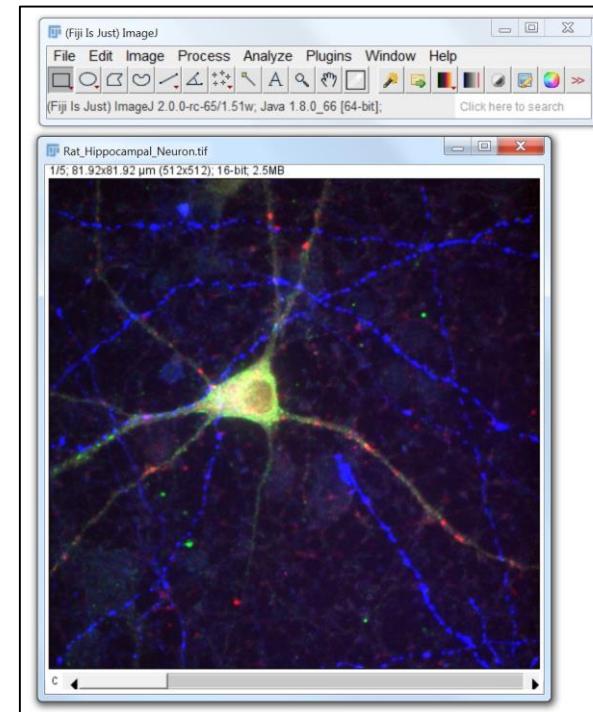
julia



# FIJI (FIJI is just ImageJ)

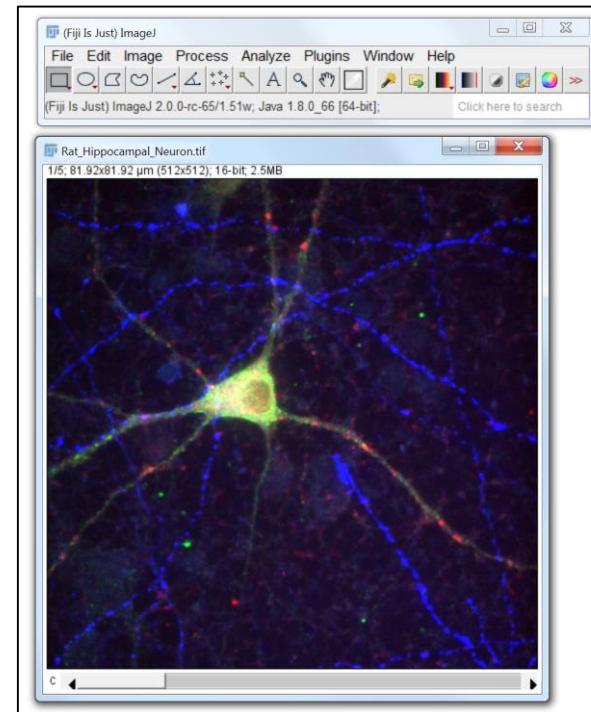
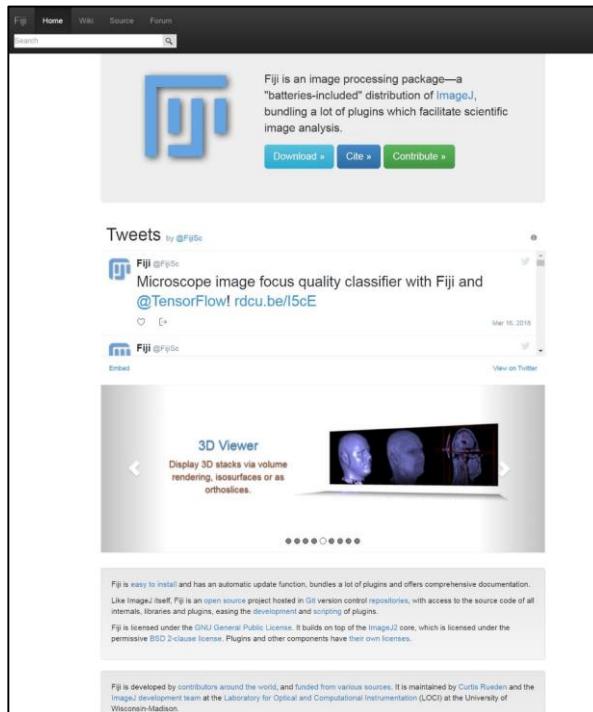


<https://fiji.sc/>



<http://forum.imagej.net/>

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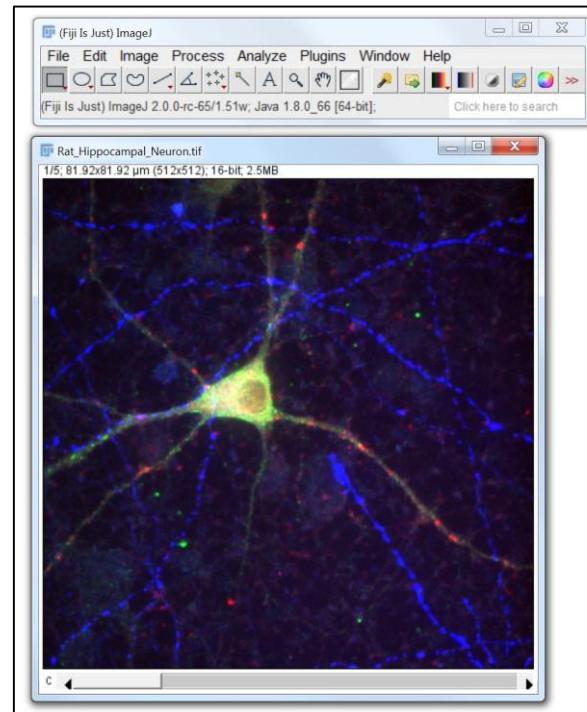
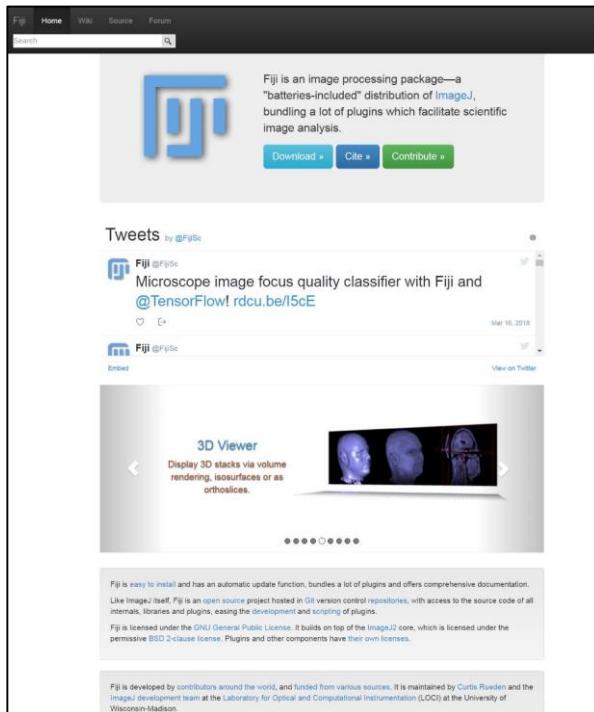


- ImageJ with lots preinstalled

<https://fiji.sc/>

<http://forum.imagej.net/>

# FIJI (FIJI is just ImageJ)



- Free
- Open source
- Cross platform
- Flexible
- Huge community

<https://fiji.sc/>

<http://forum.imagej.net/>

# FIJI - installation

- Download
- Windows
  - Open fiji-win64.zip in “windows explorer” & click “extract all files” to e.g. Desktop
- Linux (e.g. Ubuntu)
  - Open fiji-linux64.zip with “archive manager” & extract to e.g. Desktop
- Mac (OSX)
  - Open fiji-macosx.dmg, drag and drop Fiji.app to applications (or anywhere else).
  - May need to go to “System Preferences > Security & Privacy” and allow unidentified developers.

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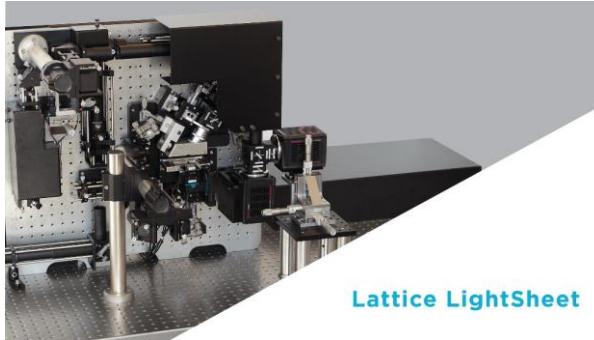
[FIJI download \(Linux\)](#)

[Deconvolution toolbox](#)

# Why computational image analysis?

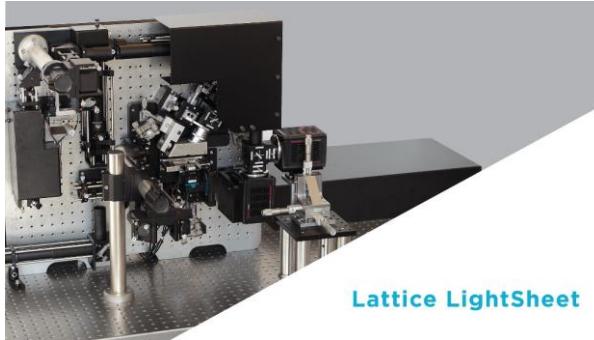
# Why computational image analysis?

- Quantity of data (TB's per day)



# Why computational image analysis?

- Quantity of data (TB's per day)
- Reproducibility, comparison



**Functional Specialization of the Axon Initial Segment by Isoform-Specific Sodium Channel Targeting**  
Johannes J. Letzkus,<sup>1,2</sup> Maarten H.P. Kole,<sup>1,2</sup> and Greg J. Stuart<sup>1,\*</sup>  
Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria; <sup>2</sup>Center for Neural Science, New York University, New York, NY 10003, USA

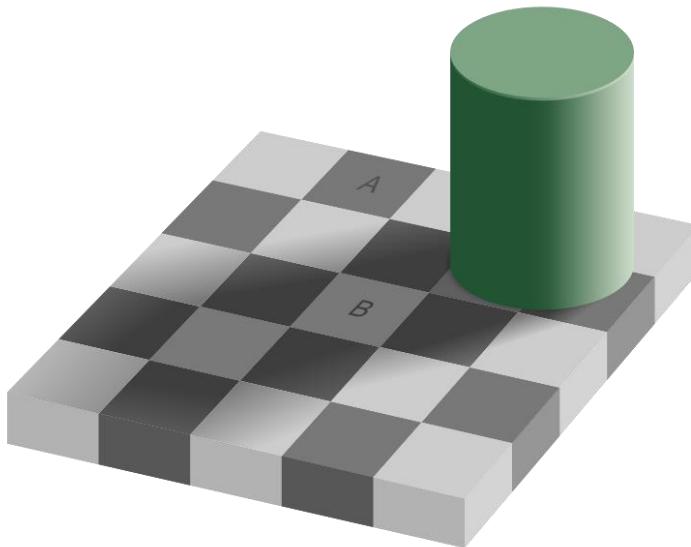
**Axon Initial Segment Kv1 Channels Control Axonal Action Potential Waveform and Synaptic Efficacy**  
Maarten H.P. Kole,<sup>1,2</sup> Johannes J. Letzkus,<sup>1,2</sup> and Greg J. Stuart<sup>1,\*</sup>  
A Selective Filter for Cytoplasmic Transport at the Axon Initial Segment  
Ai-hong Song,<sup>1</sup> Dong Wang,<sup>1</sup> Gang Cai,<sup>1</sup> and Kristian L. Hedstrom<sup>1</sup>  
AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity  
Kristian L. Hedstrom,<sup>1</sup> Yasuhiro Ogawa,<sup>1,2</sup> and Matthew N. Rasband<sup>1,2</sup>  
<sup>1</sup>Department of Neuroscience, University of Connecticut Health Center, Farmington, CT 06032  
<sup>2</sup>Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030

**Activity-dependent regulation of the axon initial segment fine-tunes neuronal excitability**  
Matthew S. Grubb<sup>1</sup> & Juan Burrone<sup>1</sup>  
Costa M. Colbert and Daniel Johnston  
Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030

**Ankyrin-Based Ion Channel Densities in the Axon Initial Segment**  
Action potential generation requires a high sodium channel density in the axon initial segment  
Directs GABAergic transmission in the axon initial segment  
Maarten H P Kole<sup>1</sup>, Susanne U Ilshner<sup>1</sup>, Björn M Kampa<sup>1,4</sup>, Stephen R Williams<sup>1,2</sup>, Peter C Ruben<sup>1,3</sup> & Greg J Stuart<sup>1</sup>

# Why computational image analysis?

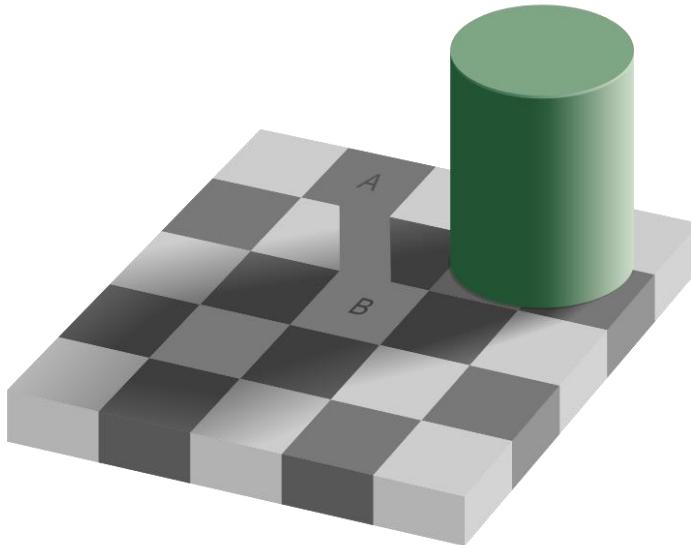
- Visual system relies heavily on context and relative changes.



Checker shadow illusion, Edward Adelson 1995.

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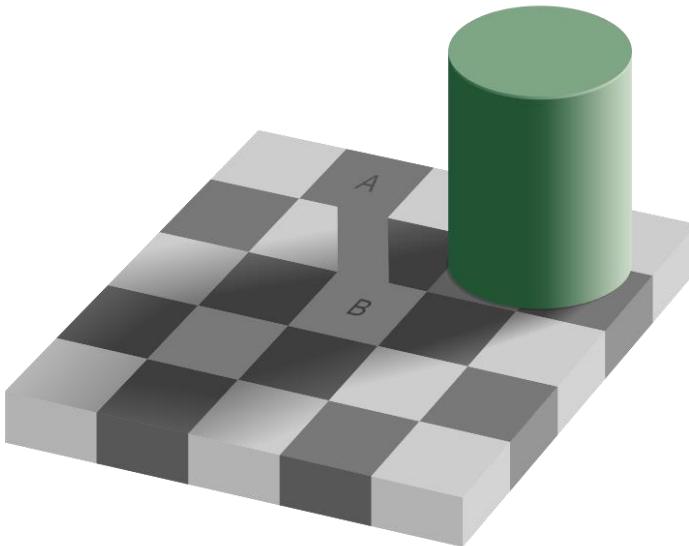
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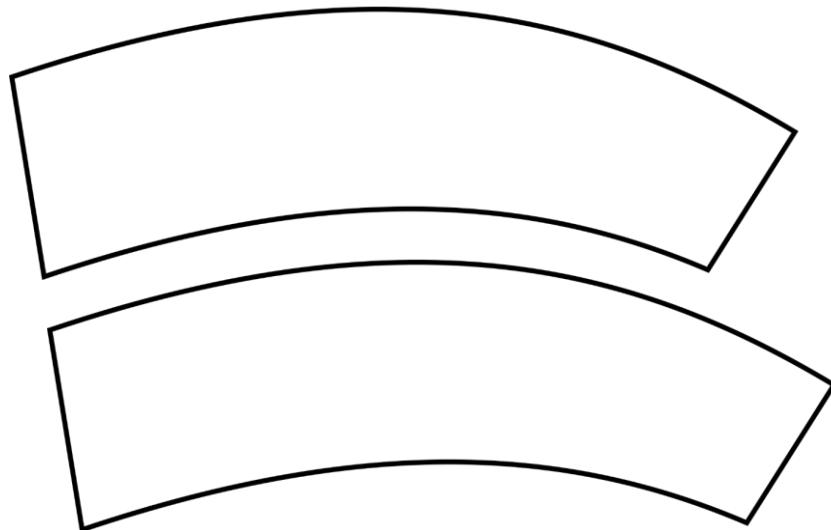
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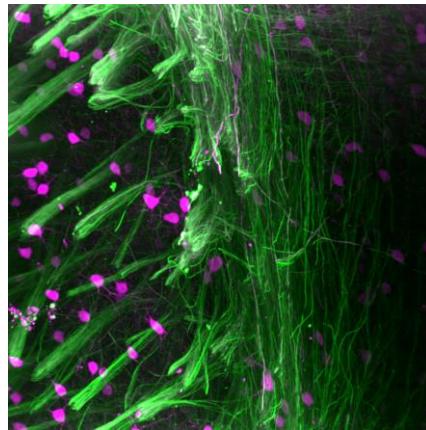


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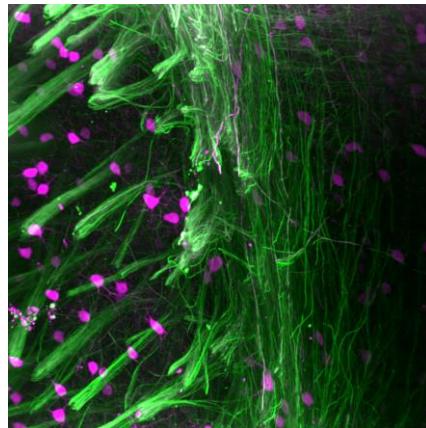
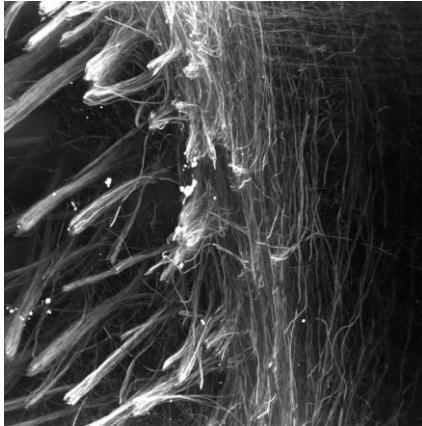


Jastrow illusion, Joseph Jastrow (and others), 1892.

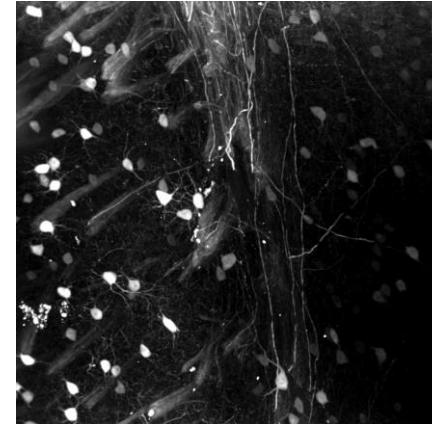
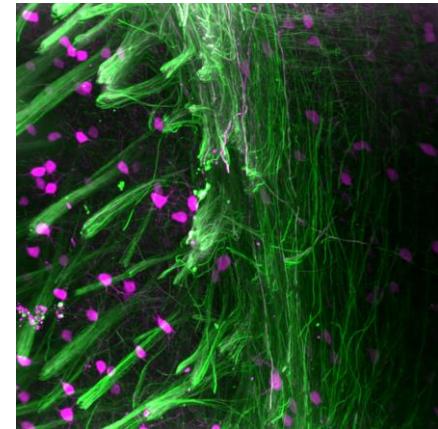
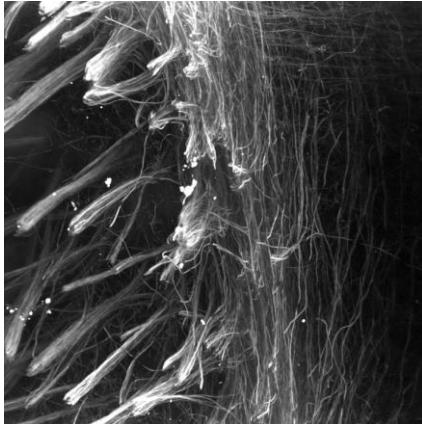
# Nearly all microscopy images aren't colour



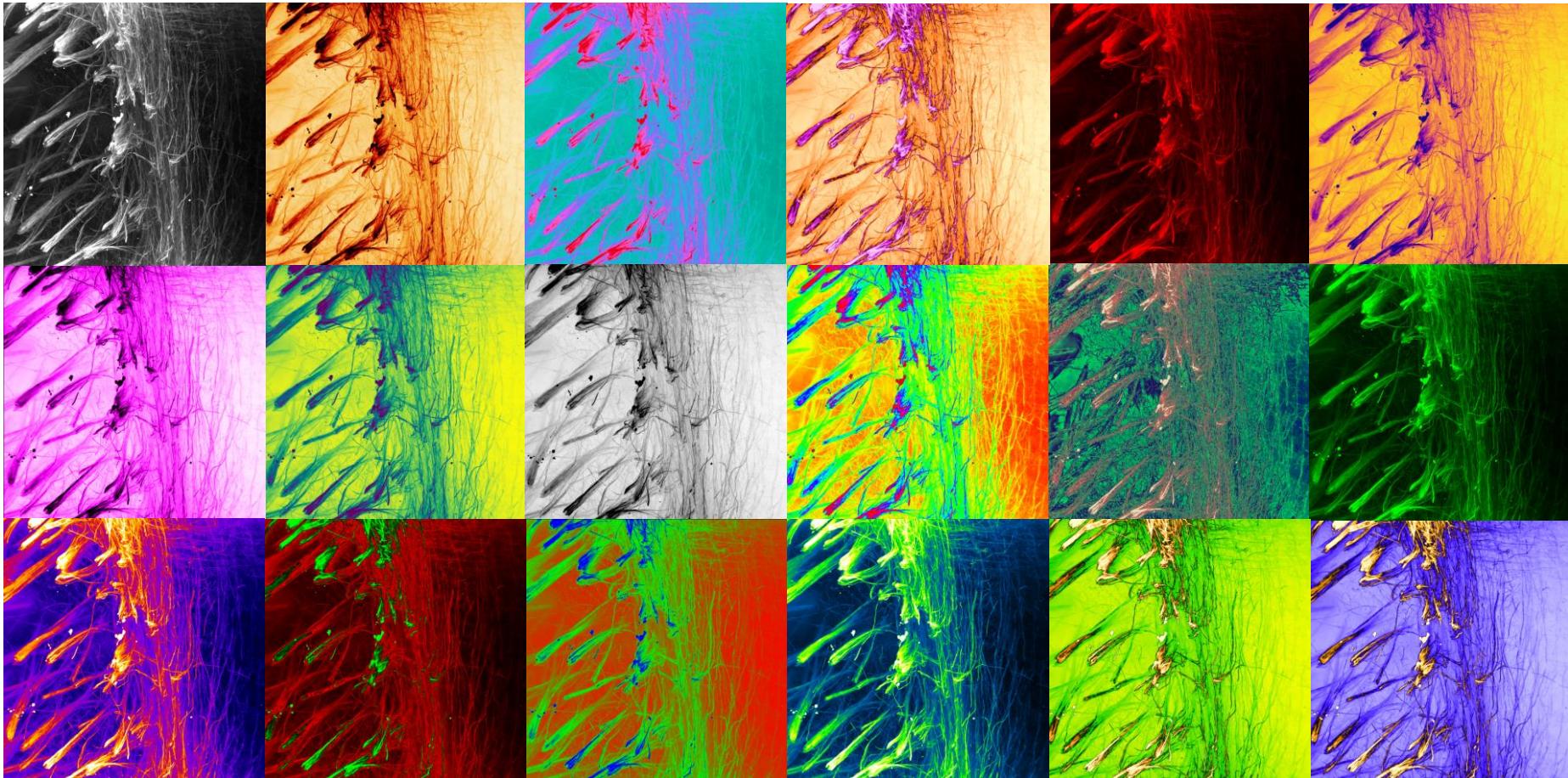
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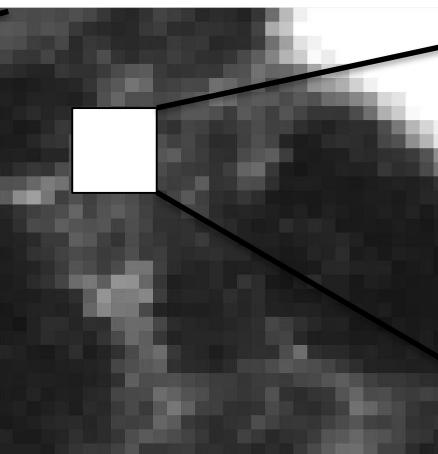
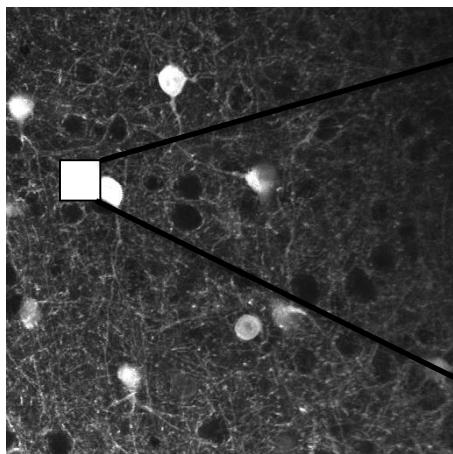
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# Lookup tables (LUTs)



# Images are arrays of numbers



78	64	71	57	43	44
86	93	69	60	48	46
75	64	57	59	55	62
70	65	69	85	56	54
54	68	88	63	46	43
51	51	74	66	43	35

# More complicated images are still arrays of numbers

2D

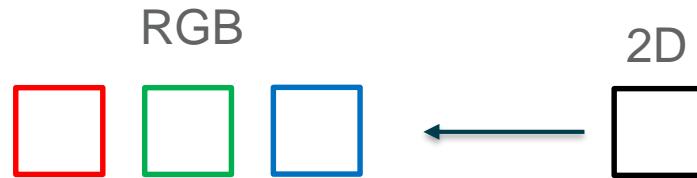
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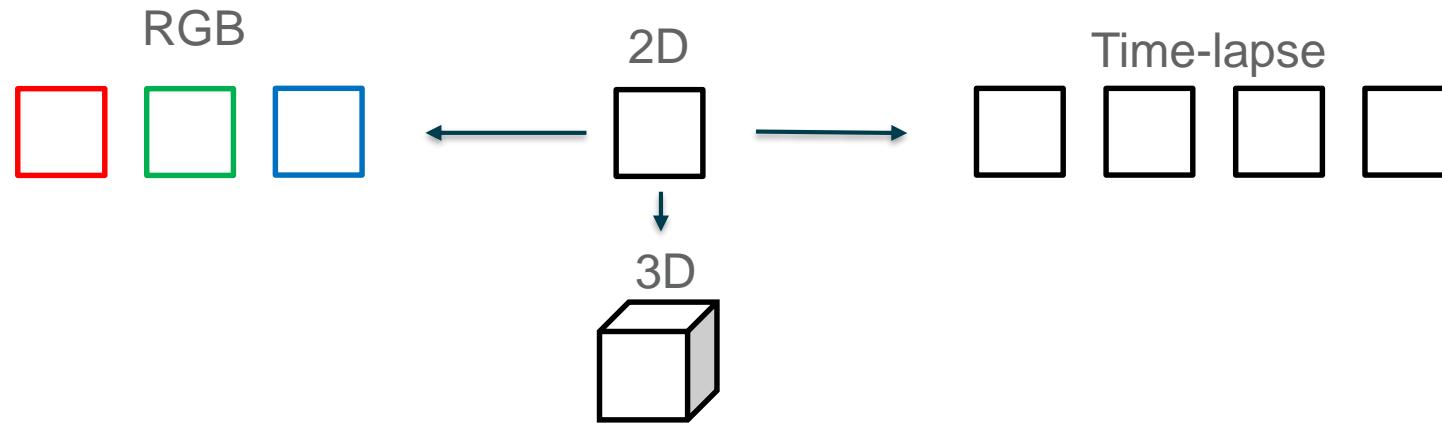
More complicated images are still arrays of numbers



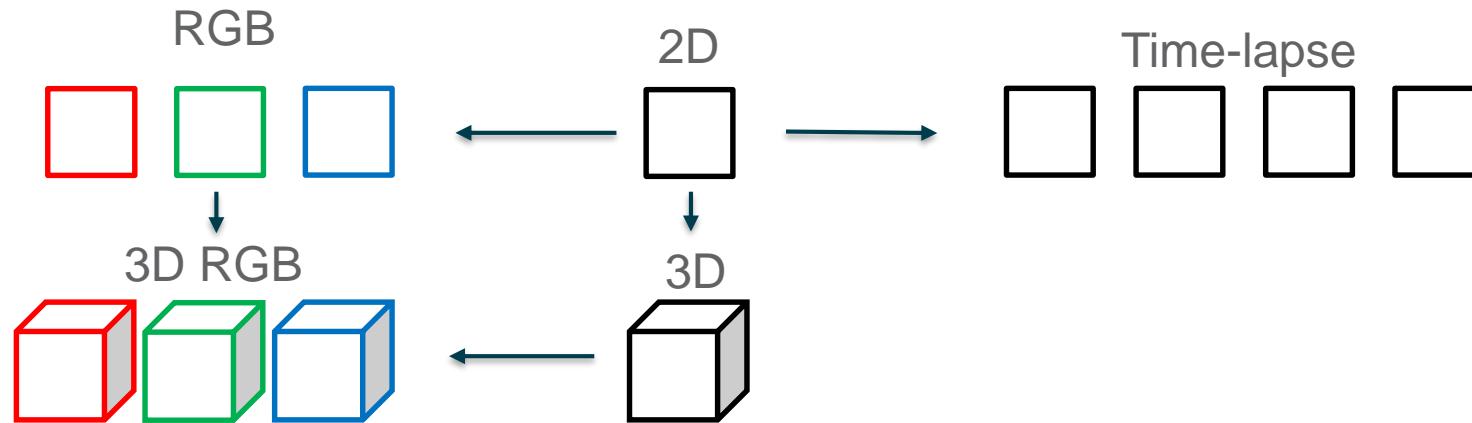
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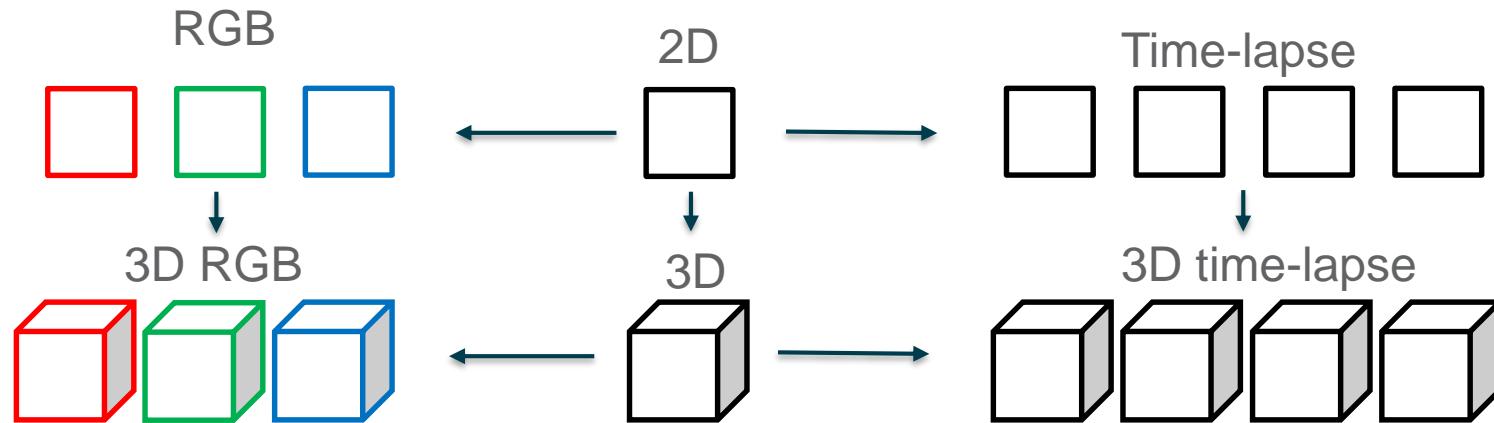
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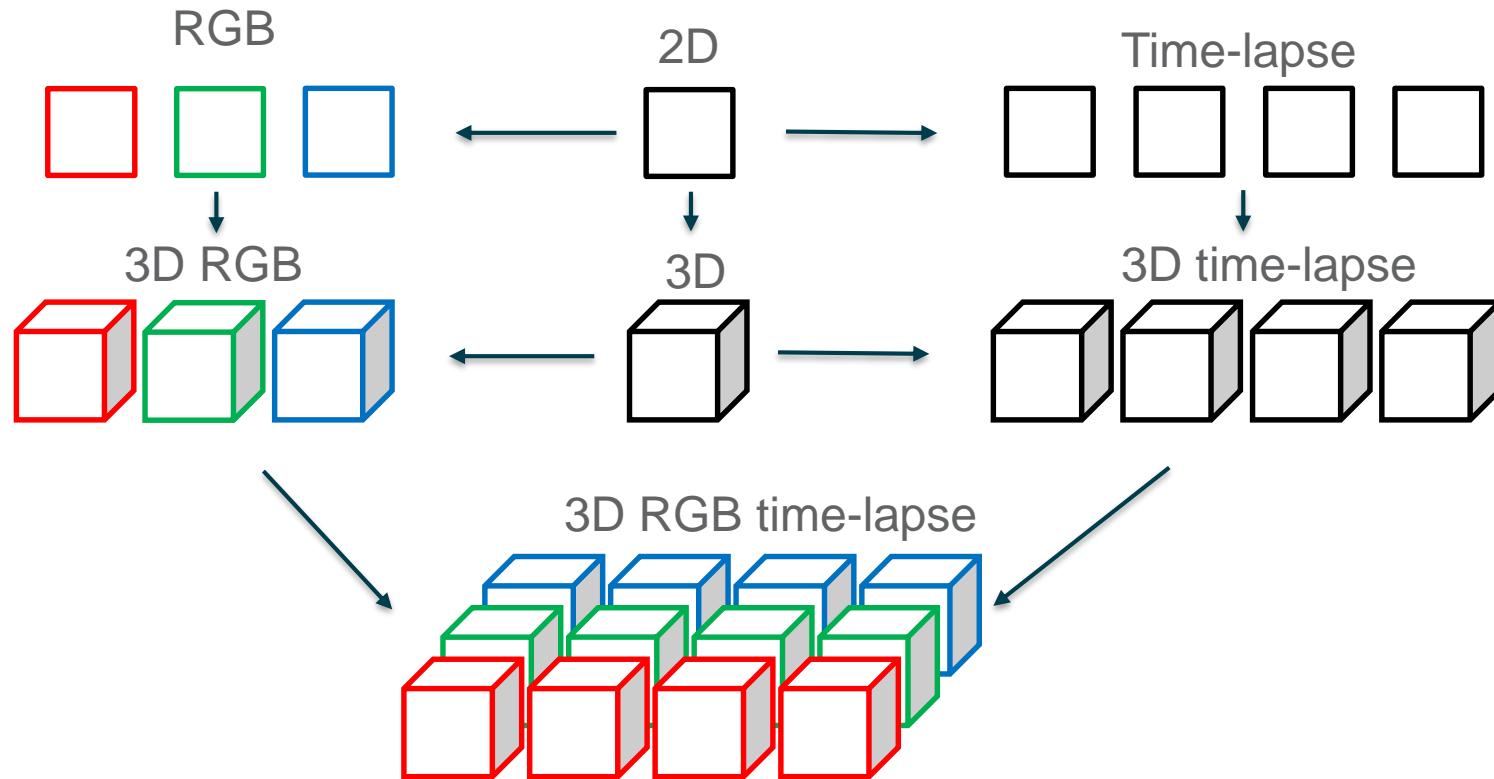
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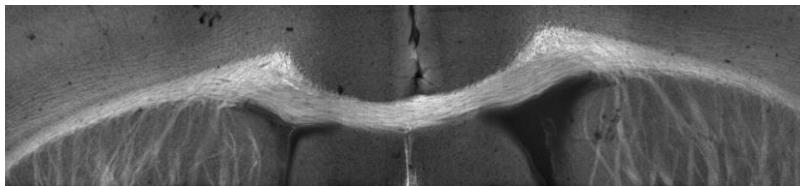
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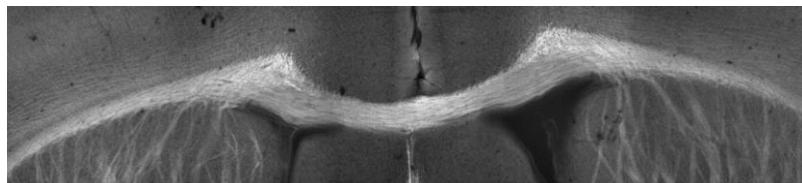
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# Image processing vs analysis



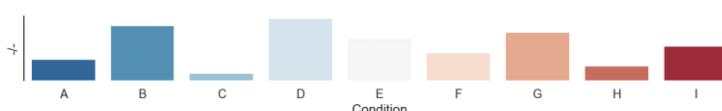
# Image processing vs analysis



Volume

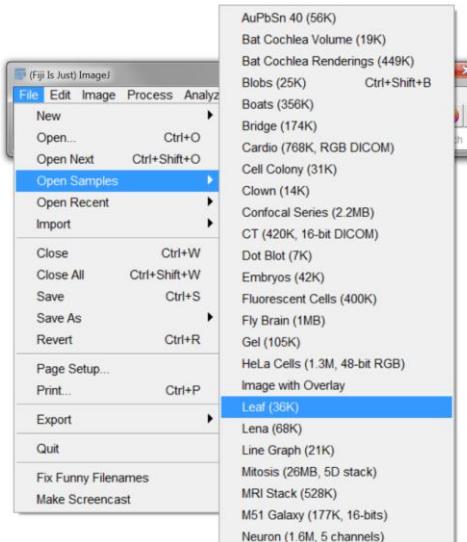
Shape

Texture



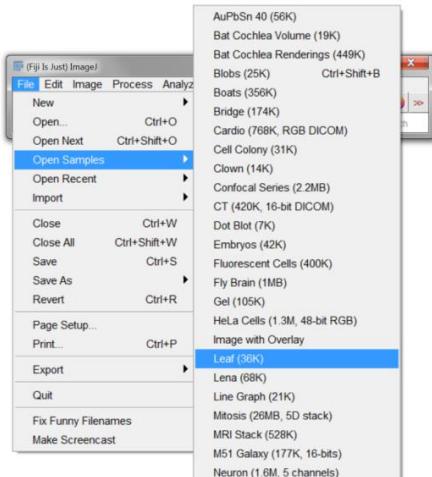
# Histogram

- Open any sample image

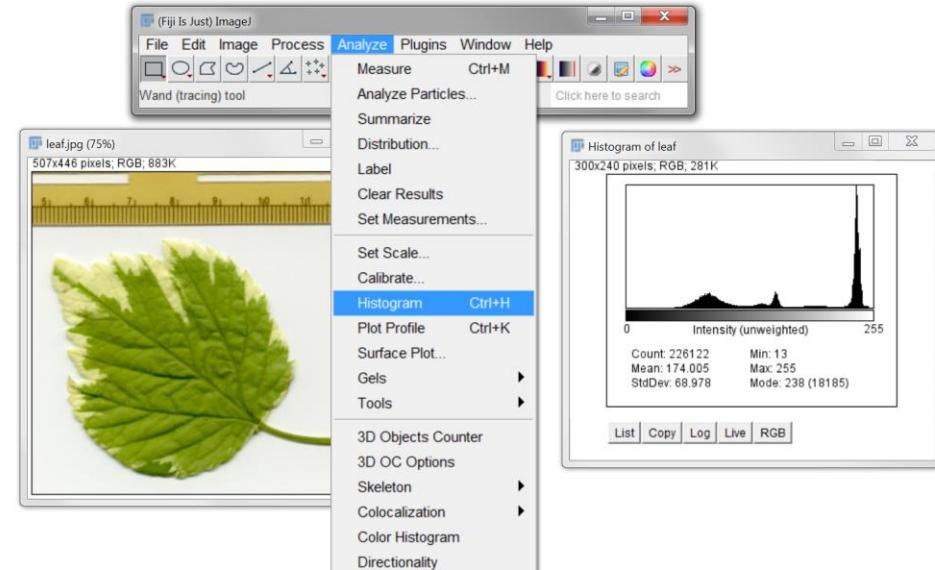


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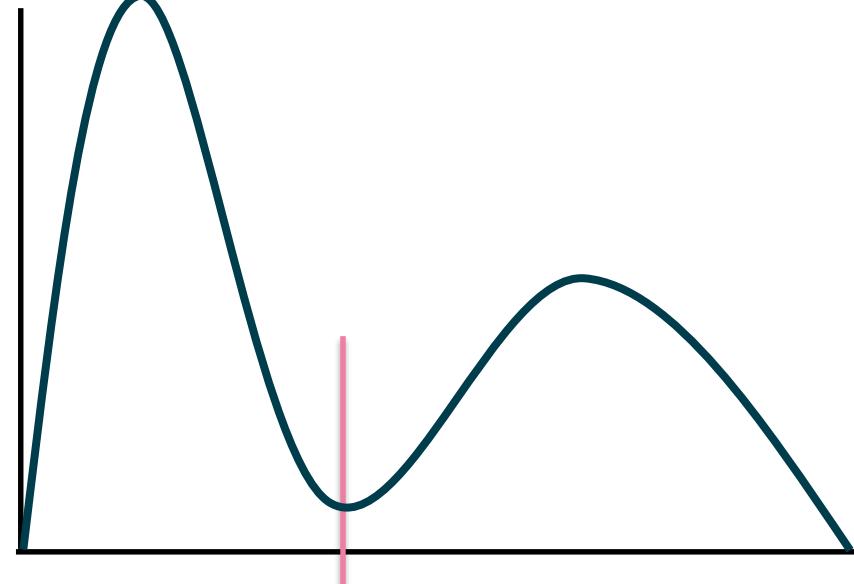
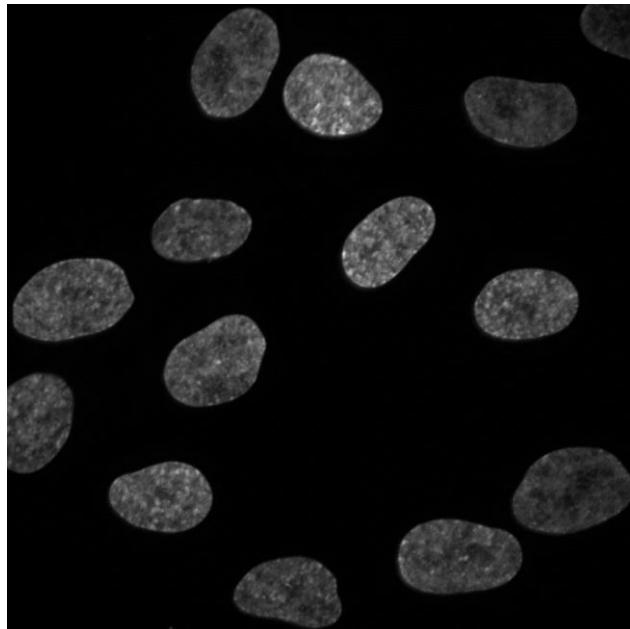
- Open histogram



N.B. – On OSX, tabs are in the menu bar

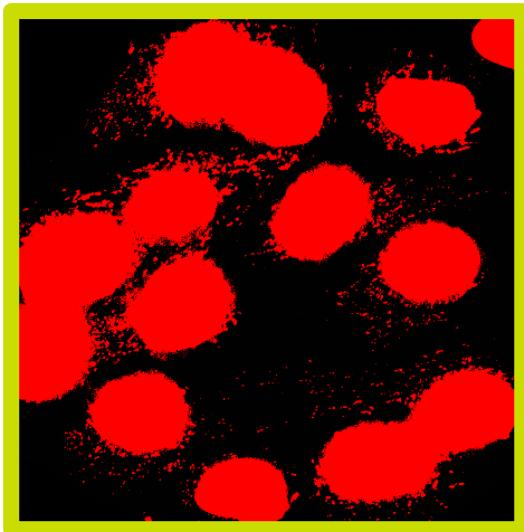
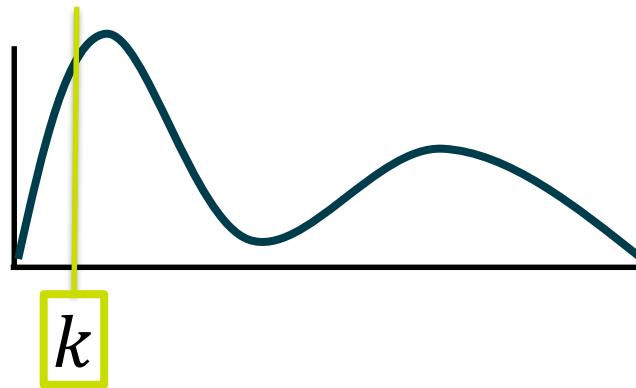


# Thresholding

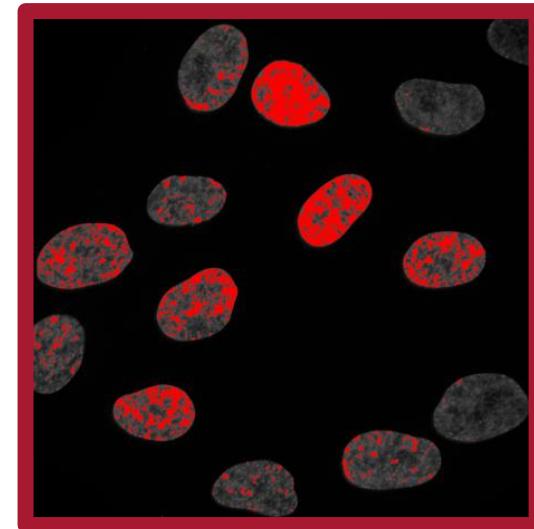
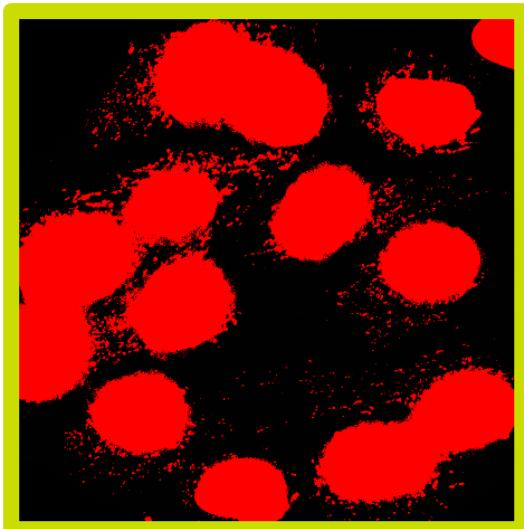
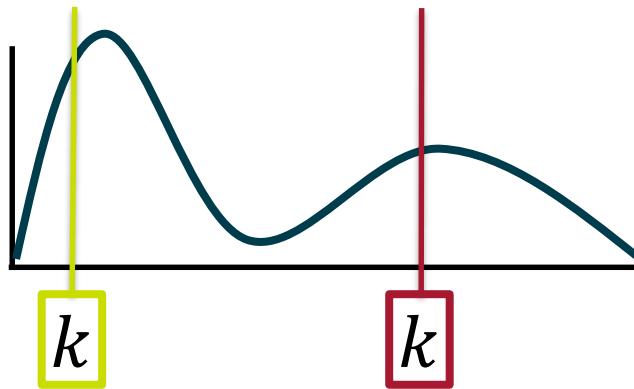


Threshold ( $\kappa$ )

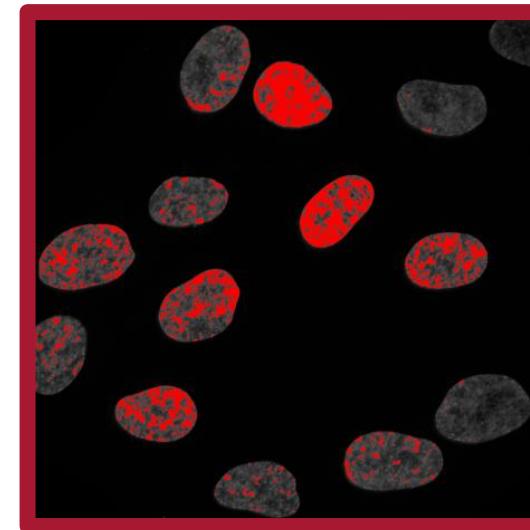
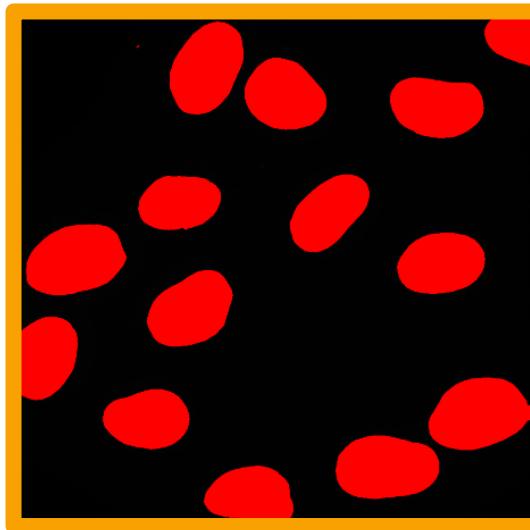
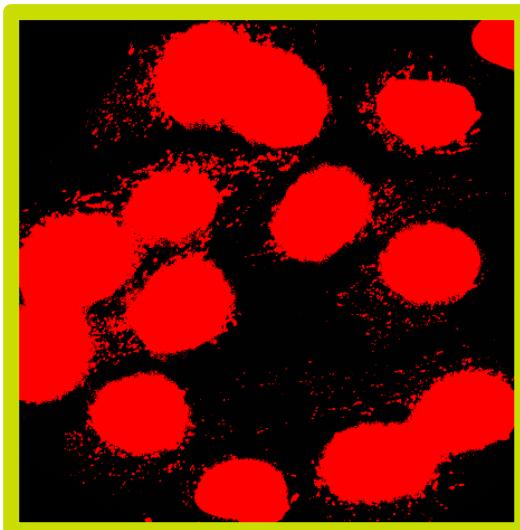
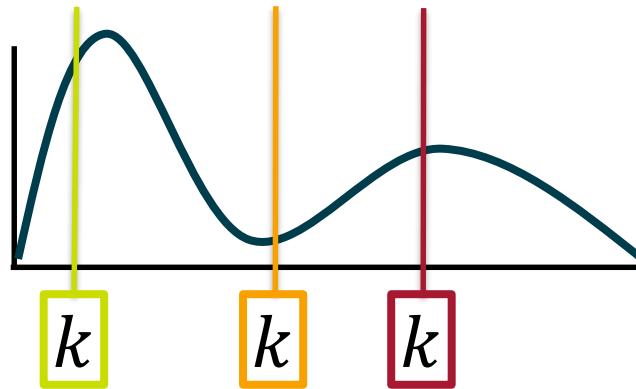
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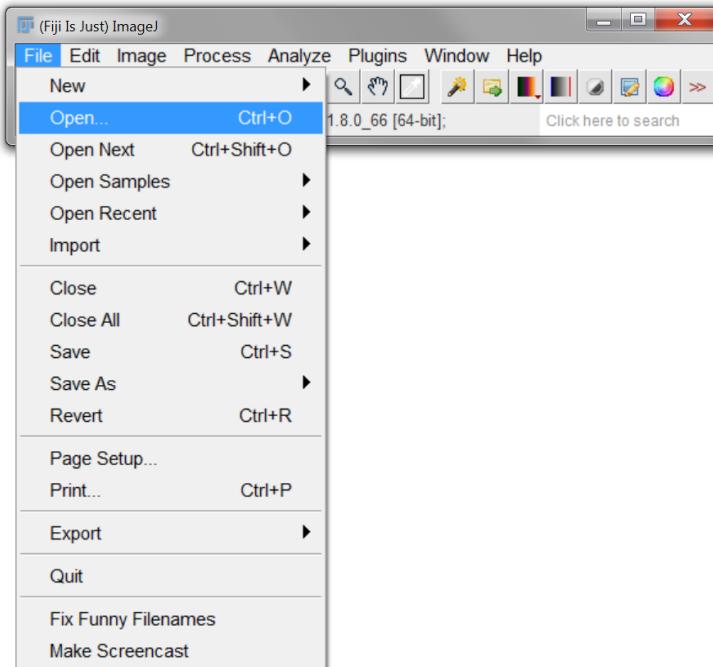


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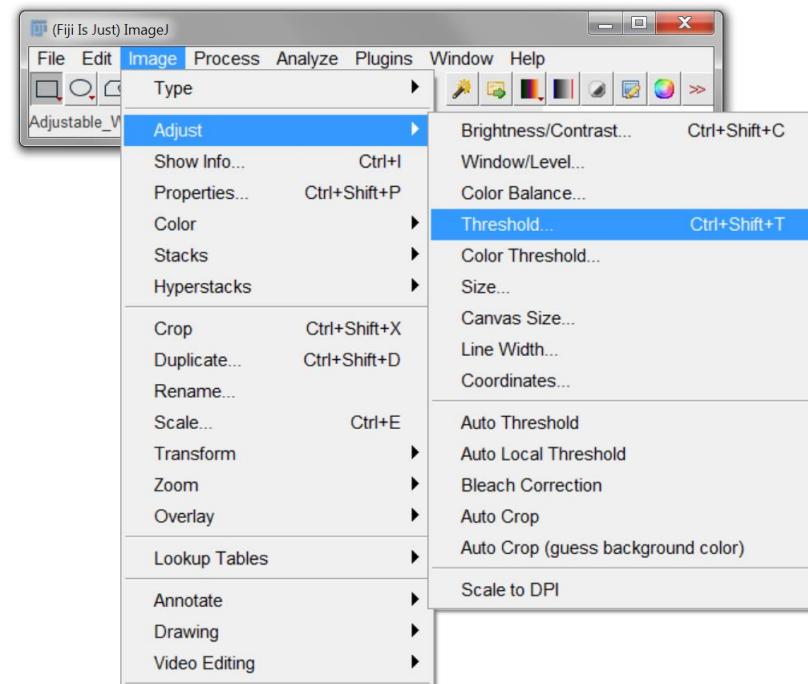
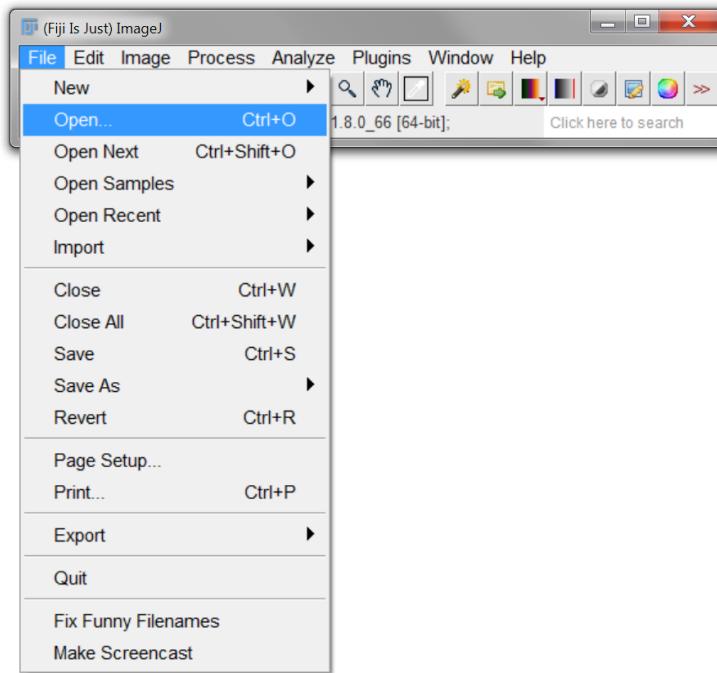
# Thresholding – try it out

- Open DAPI.tif (can drag and drop)



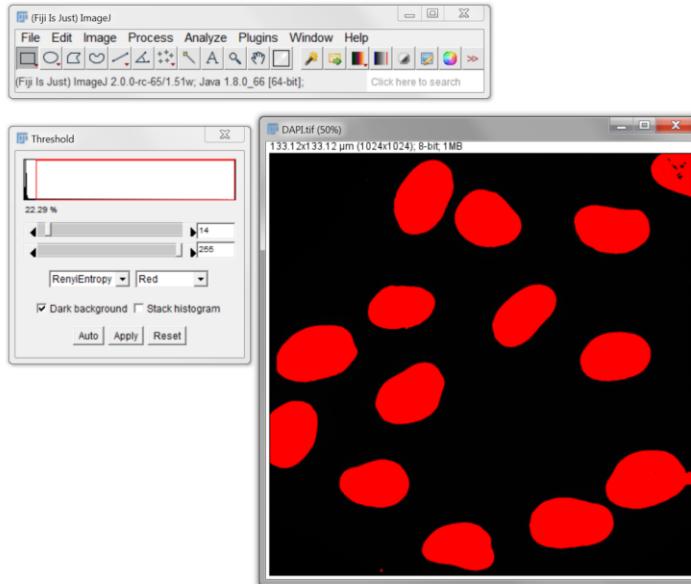
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- Open DAPI.tif (can drag and drop)
- Threshold



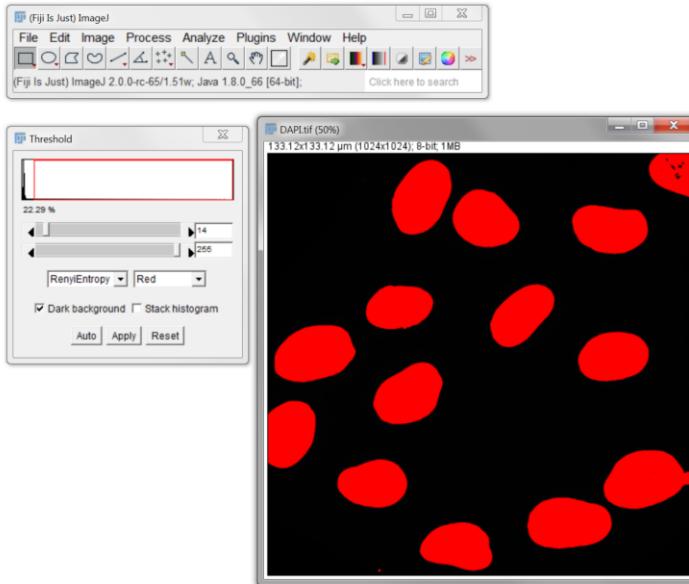
# Manual threshold - problems

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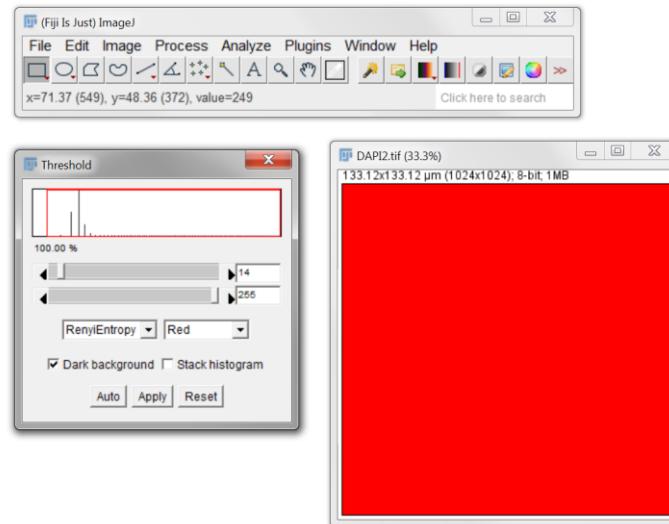


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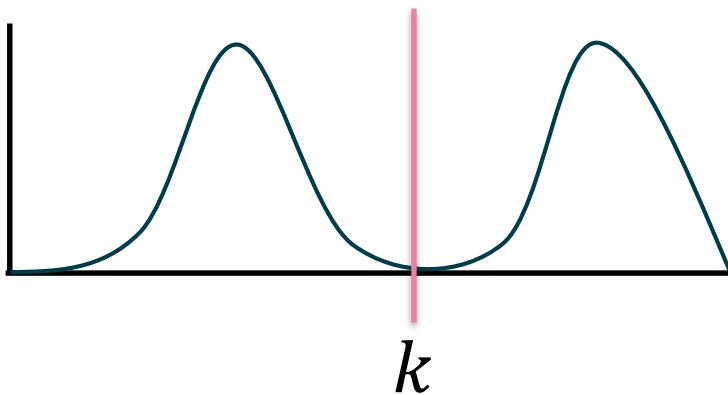


- DAPI\_2.tif



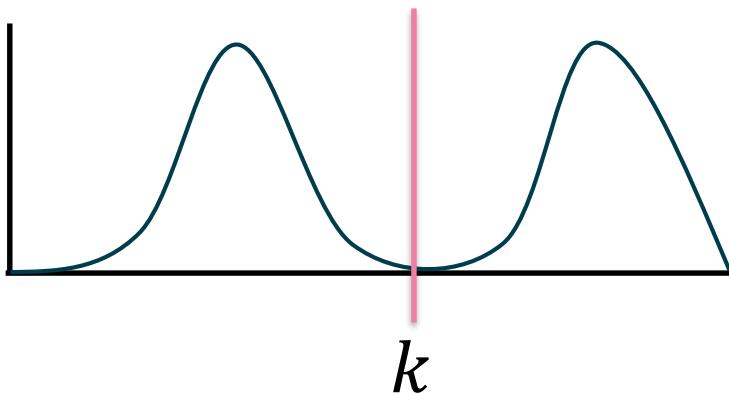
# Automatic thresholding – Equal masses

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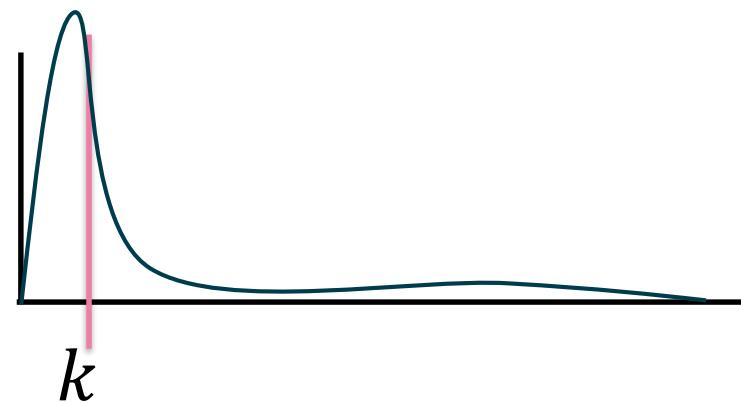


# Automatic thresholding – Equal masses

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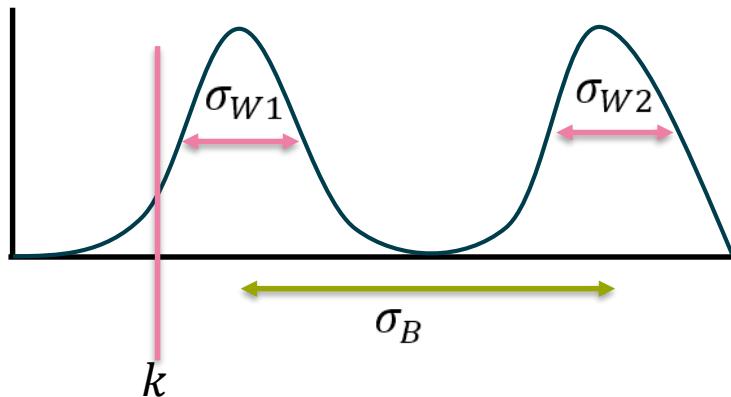


- Real case



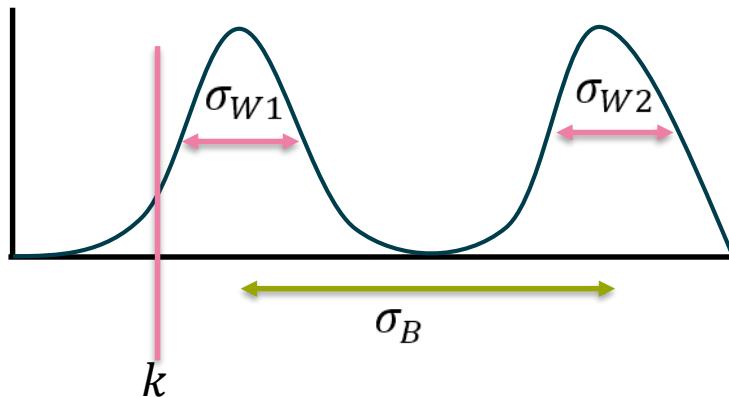
# Automatic thresholding – Otsu's method

- Minimise within class variance ( $\sigma_w$ )
- Maximise between class variance ( $\sigma_B$ )



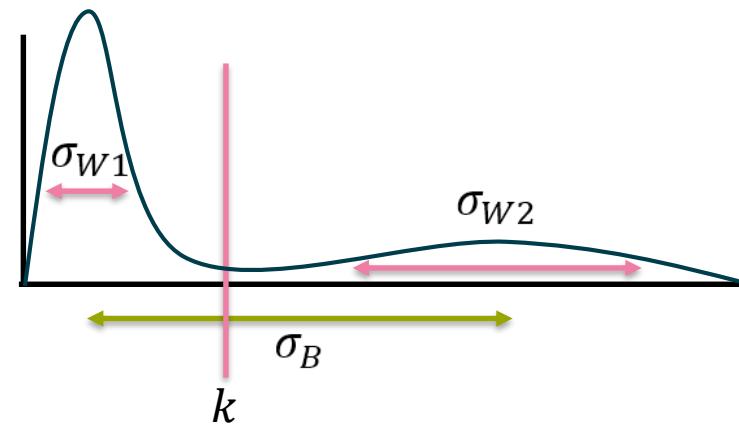
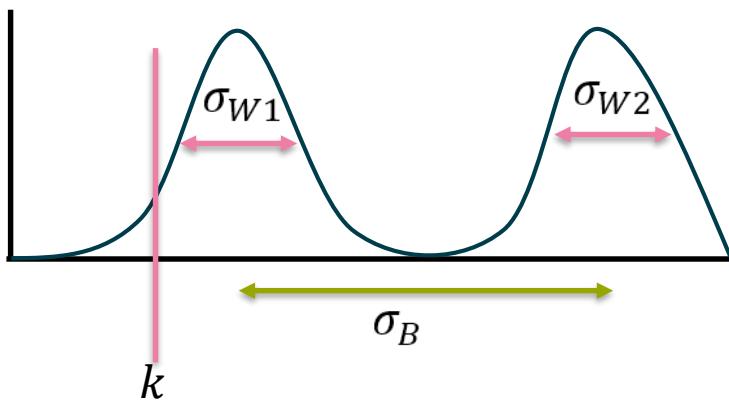
# Automatic thresholding – Otsu's method

- Minimise within class variance ( $\sigma_w$ )
- Maximise between class variance ( $\sigma_B$ )

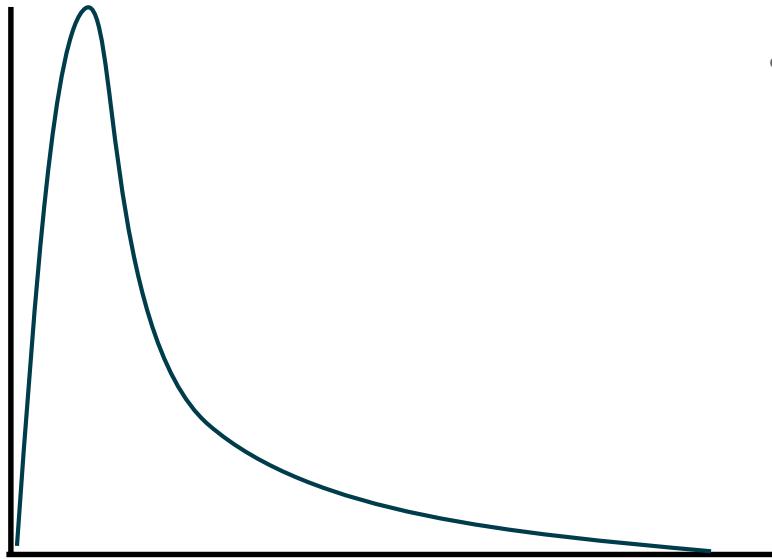


# Automatic thresholding – Otsu's method

- Minimise within class variance ( $\sigma_w$ )
- Maximise between class variance ( $\sigma_B$ )

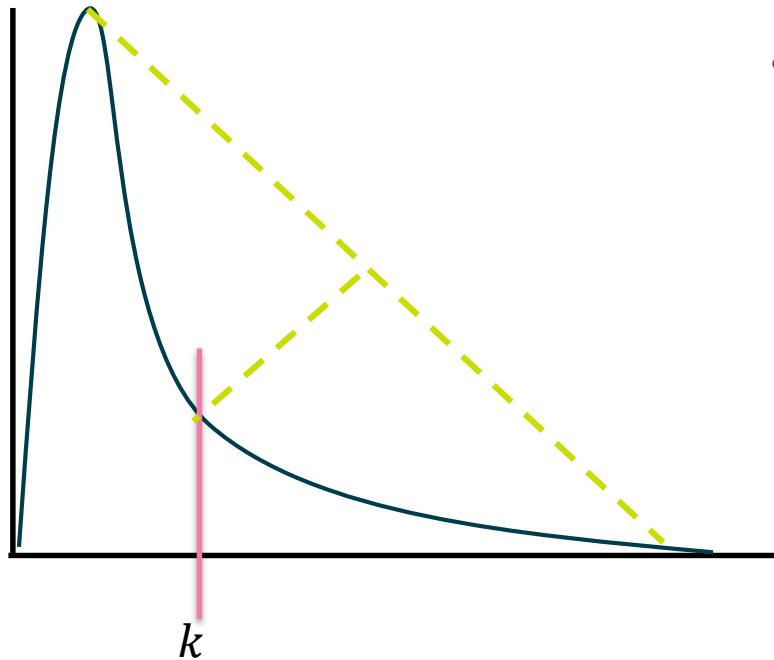


# Unimodal thresholding – Rosin's method



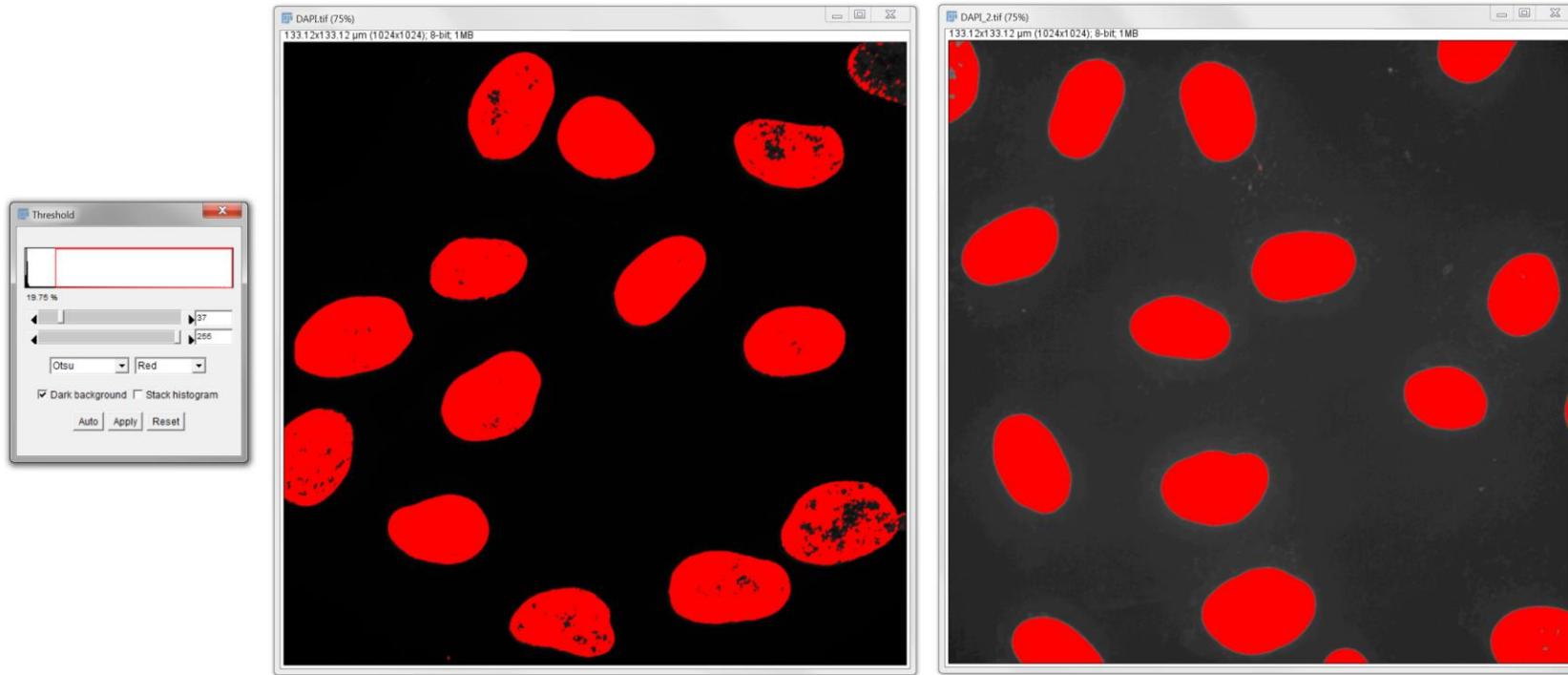
- Use shape of distribution to find threshold

# Unimodal thresholding – Rosin's method

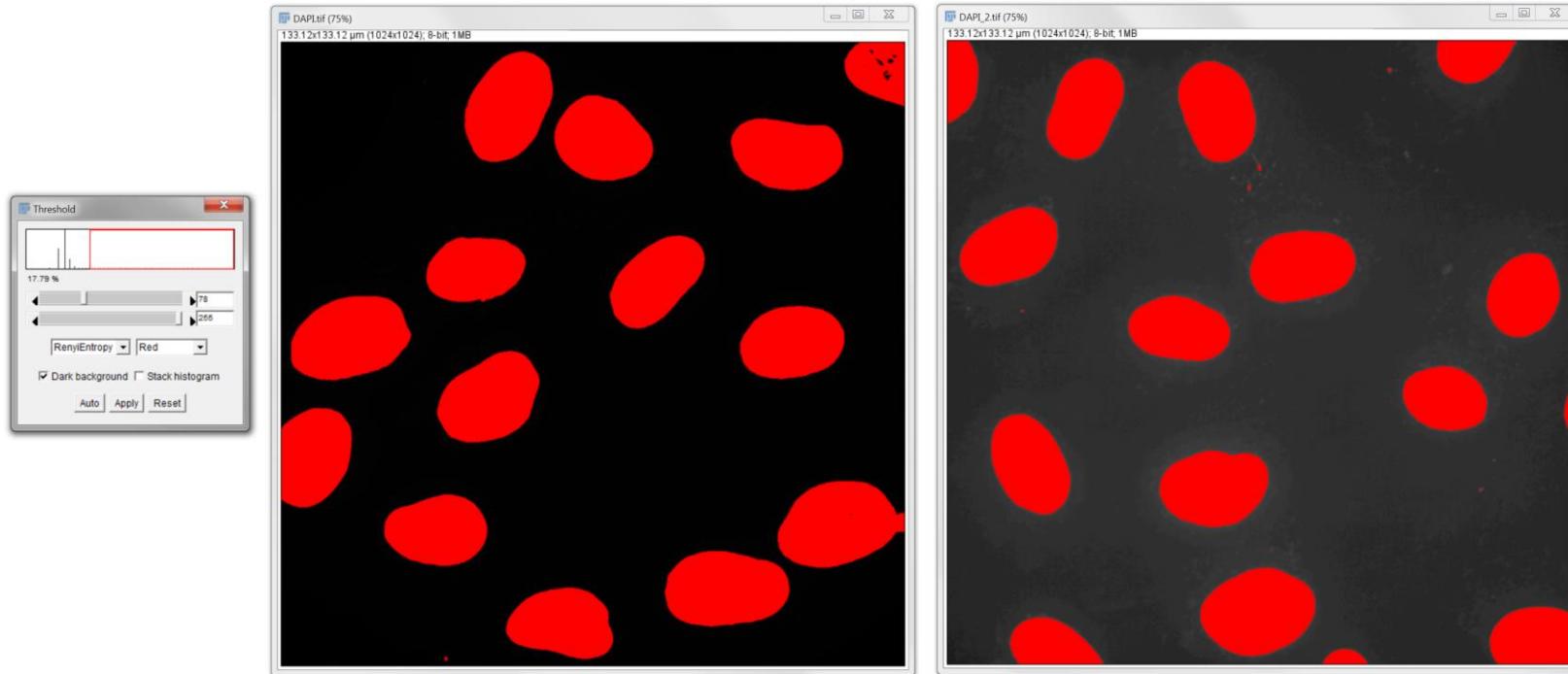


- Use shape of distribution to find threshold

# Try it out – Otsu's method

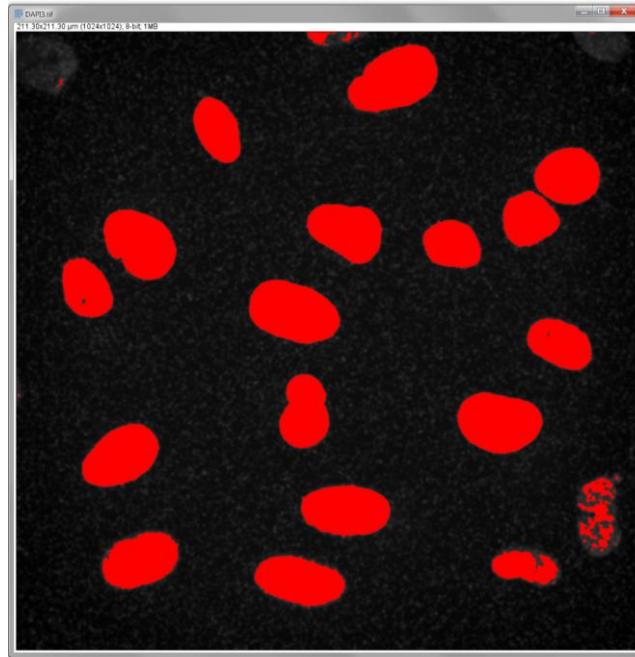


# Many, many other methods

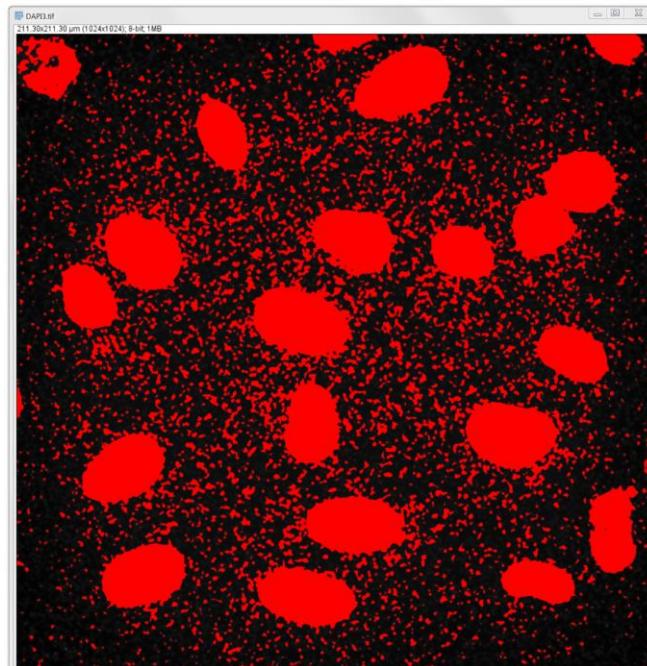


# Not always that simple ...

- Open DAPI\_3.tif
- Pick a threshold

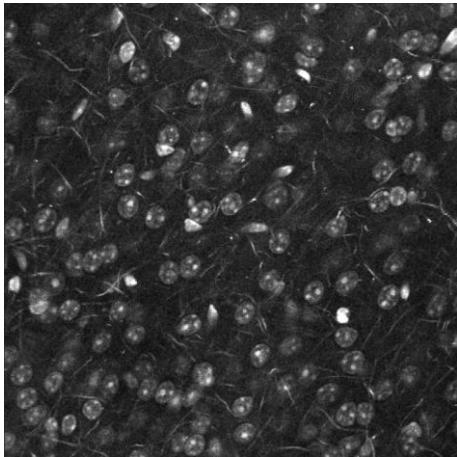


VS



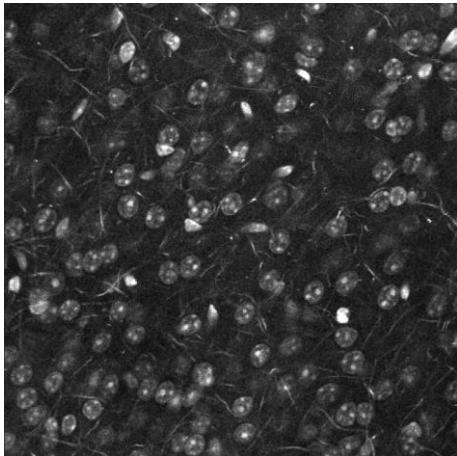
# Background subtraction – e.g. vignetting

Sample

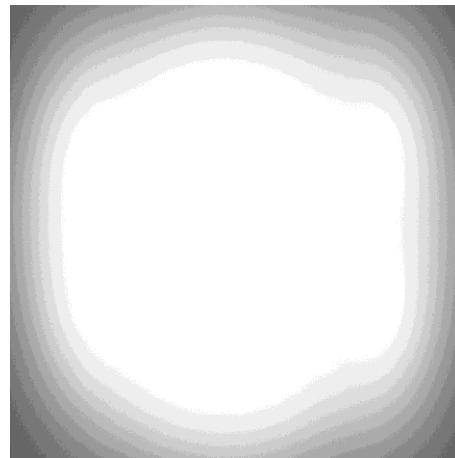


# Background subtraction – e.g. vignetting

Sample

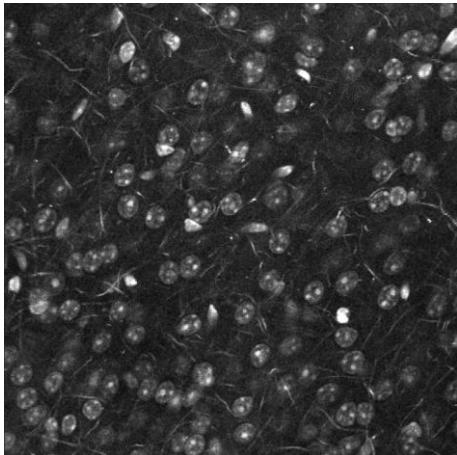


Microscope

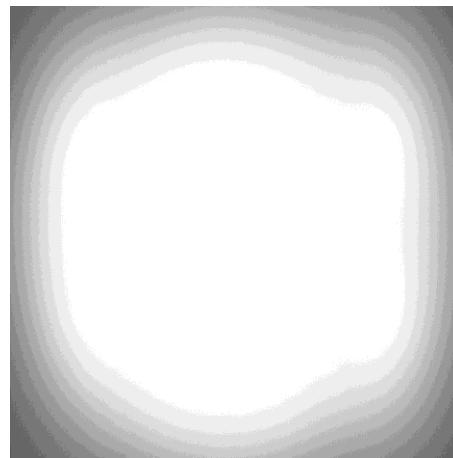


# Background subtraction – e.g. vignetting

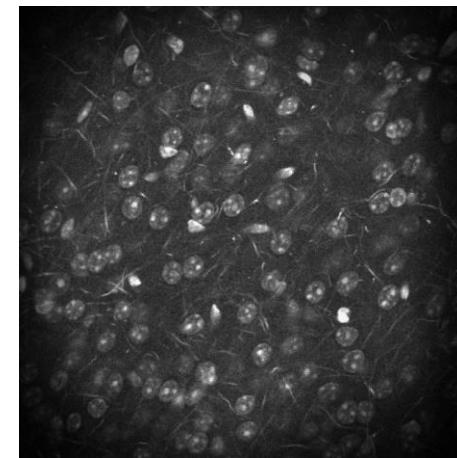
Sample



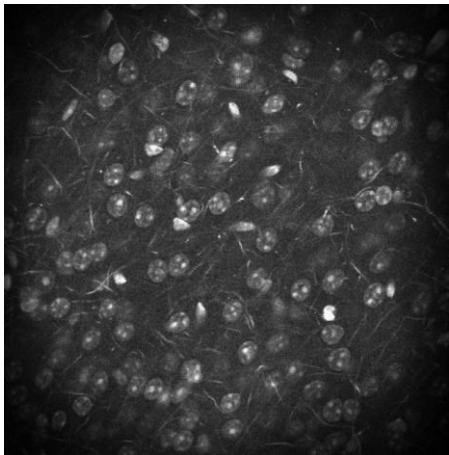
Microscope



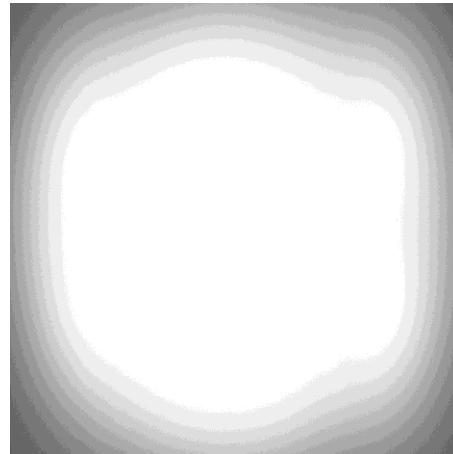
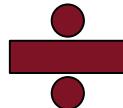
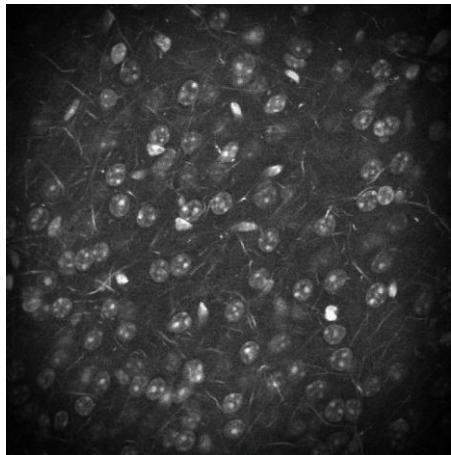
Image



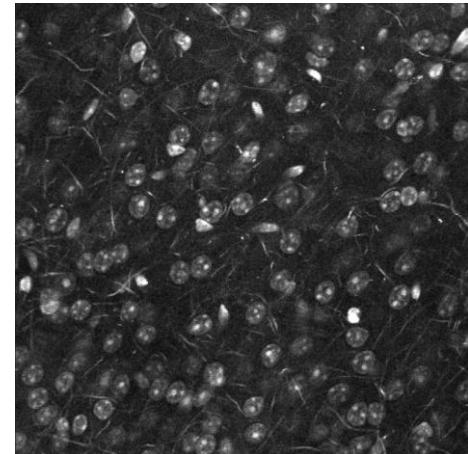
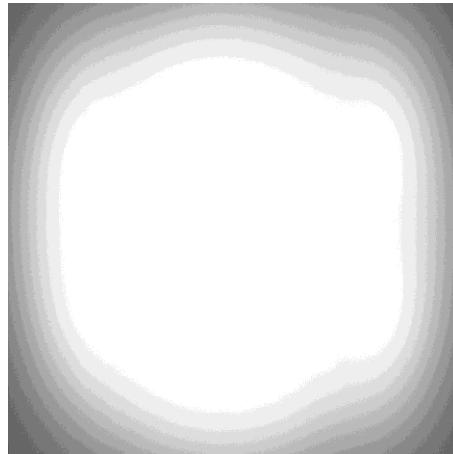
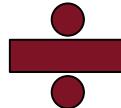
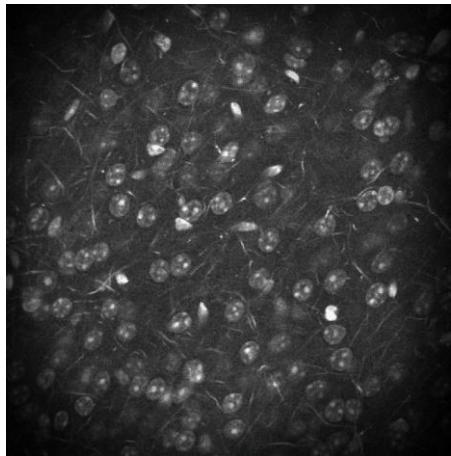
# Background subtraction – e.g. vignetting



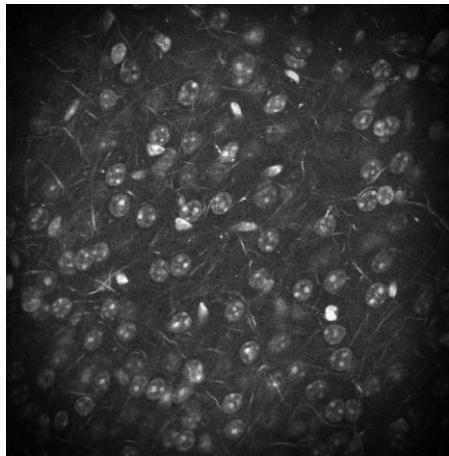
# Background subtraction – e.g. vignetting



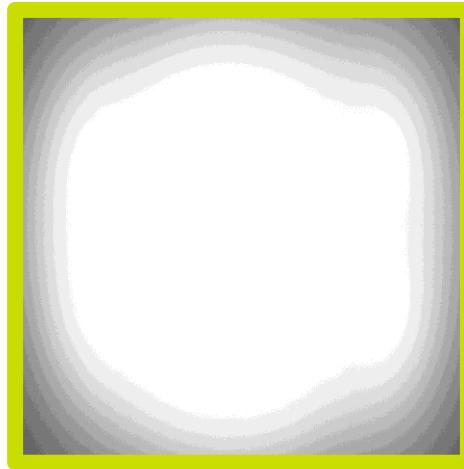
# Background subtraction – e.g. vignetting



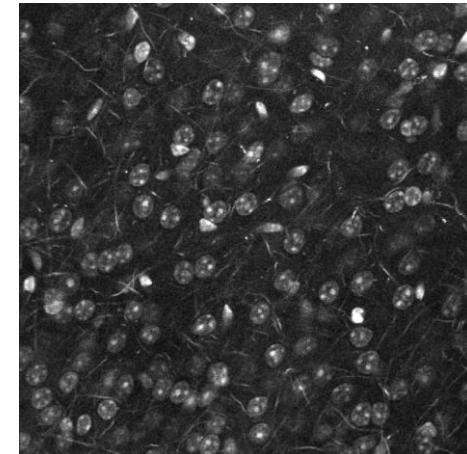
# Background subtraction – e.g. vignetting



$$\div$$



$$\div$$



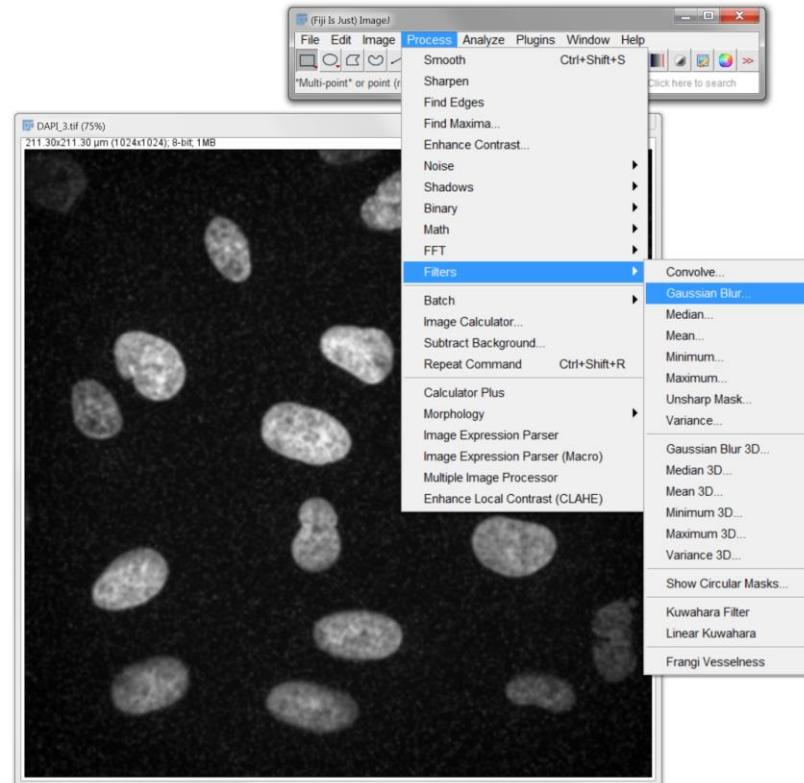
How to  
calculate??

## Estimate background – Gaussian smoothing

- Seems arbitrary, but smoothing is often a key step in image processing

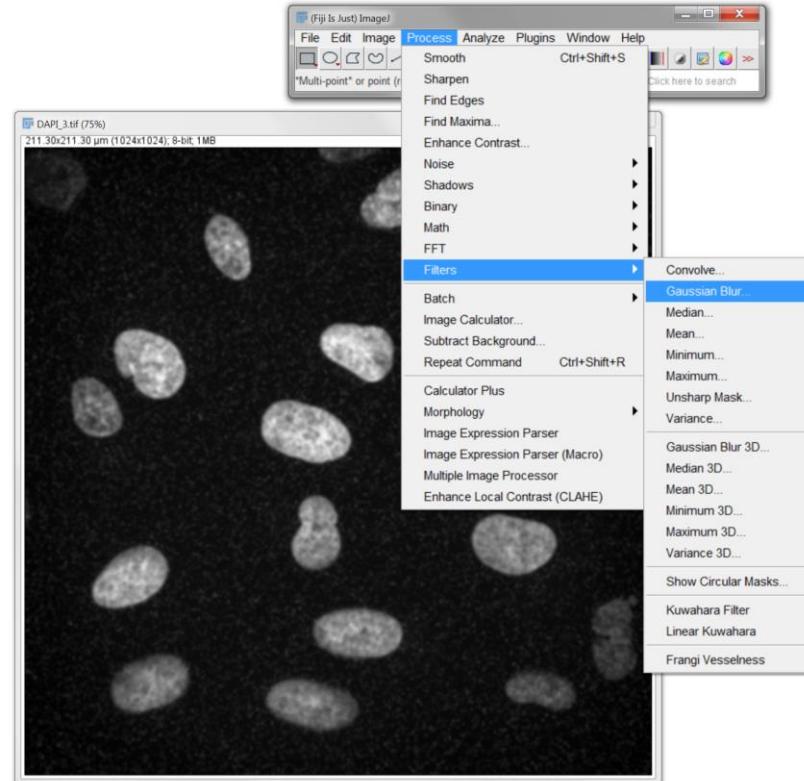
# Estimate background – Gaussian smoothing

- Seems arbitrary, but smoothing is often a key step in image processing



# Estimate background – Gaussian smoothing

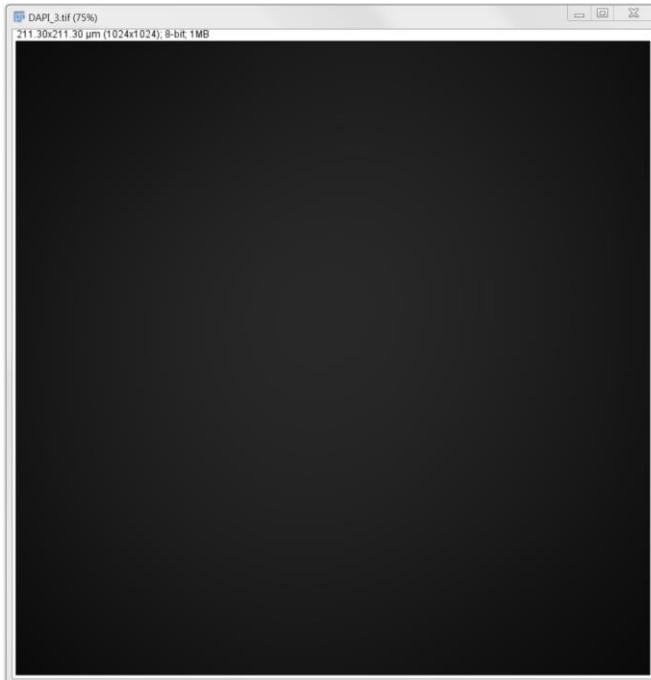
- Seems arbitrary, but smoothing is often a key step in image processing



- Try a value of 200 pixels

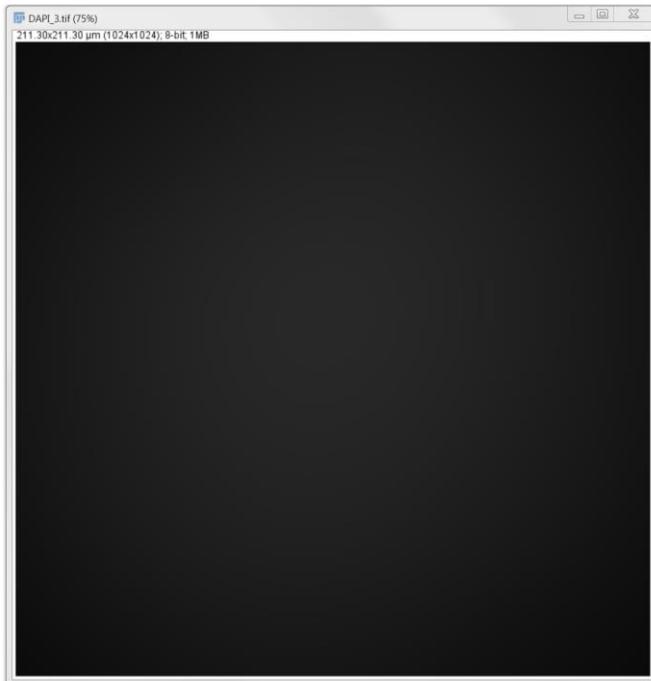
# Estimate background – Gaussian smoothing

- May need to adjust brightness



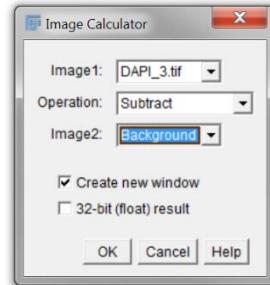
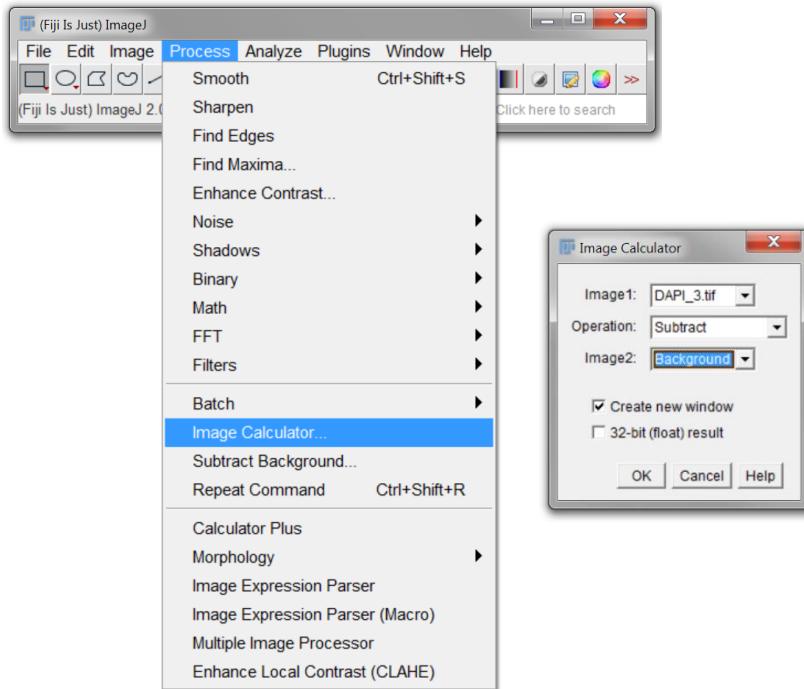
# Estimate background – Gaussian smoothing

- May need to adjust brightness



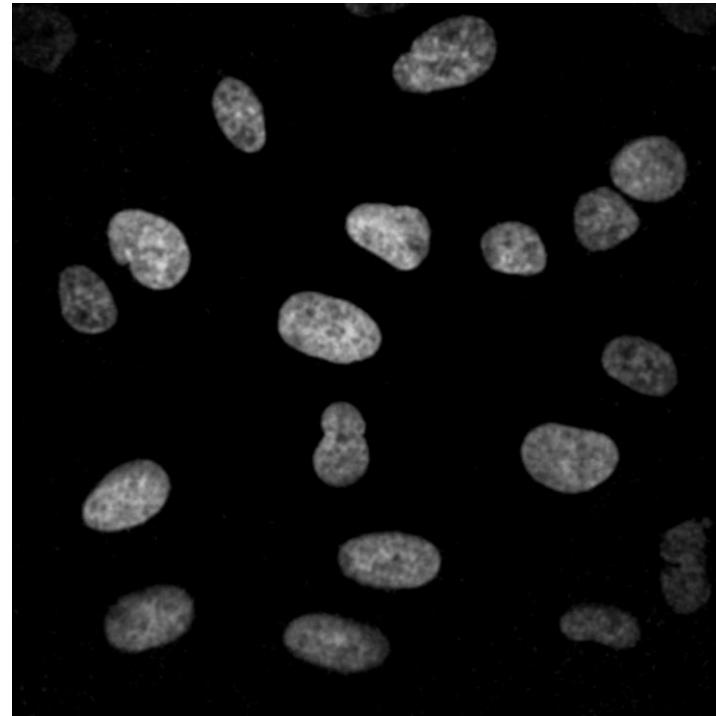
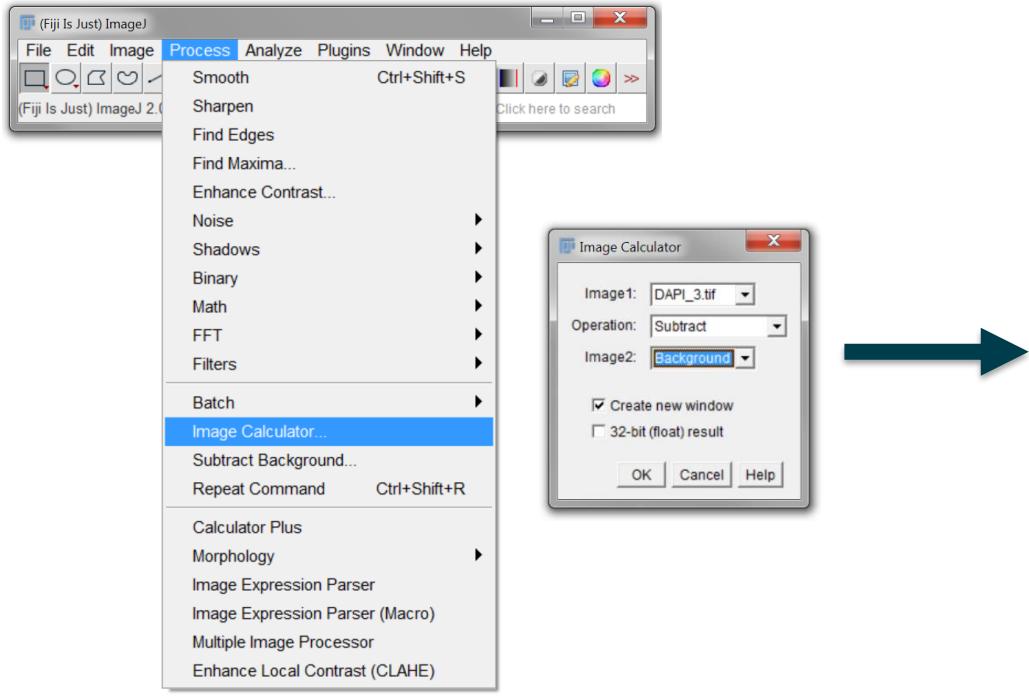
A screenshot of the Fiji software interface. The top menu bar shows "File", "Edit", "Image", "Process", "Analyze", "Plugins", "Window", and "Help". A context menu is open over the black image window, with "Adjust" selected. This menu lists various image processing options like "Show Info...", "Properties...", "Color", "Stacks", etc., along with their keyboard shortcuts. Below this, a "Brightness/Contrast" submenu is open, containing "Ctrl+Shift+C" and other options like "Window/Level...", "Color Balance...", "Threshold...", "Color Threshold...", "Size...", "Canvas Size...", "Line Width...", "Coordinates...", "Auto Threshold", "Auto Local Threshold", "Bleach Correction", "Auto Crop", and "Auto Crop (guess background color)". At the bottom of the "Adjust" menu, there's a "Scale to DPI" option. To the right of the image windows, a small floating dialog box titled "B&amp;C" contains sliders for "Minimum", "Maximum", "Brightness", and "Contrast", with "Auto", "Reset", "Set", and "Apply" buttons at the bottom.

# Estimate background



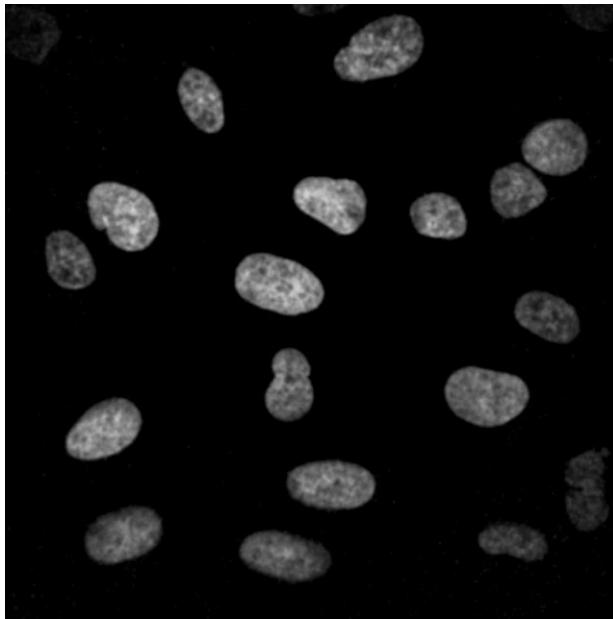
(Open a new DAPI\_3.tif)

# Estimate background

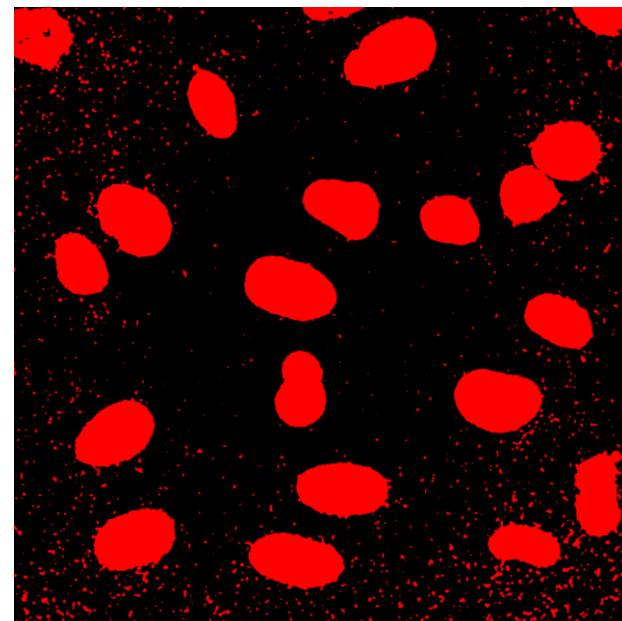
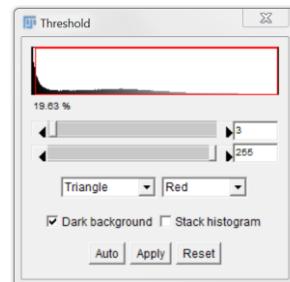
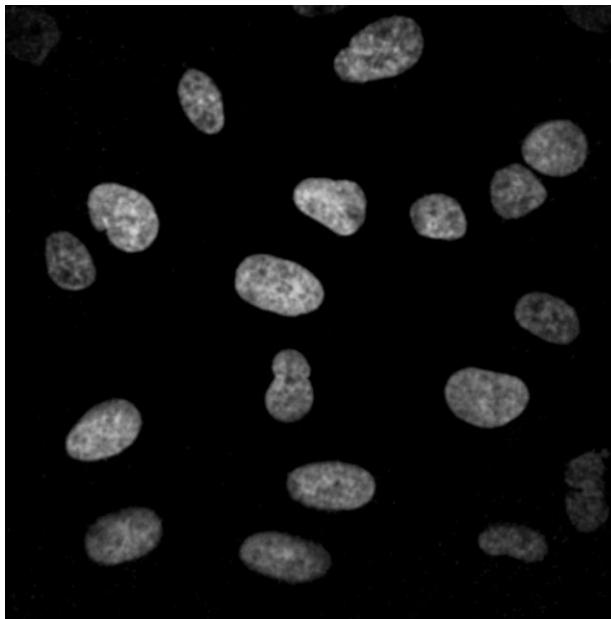


(Open a new DAPI\_3.tif)

# Now try thresholding



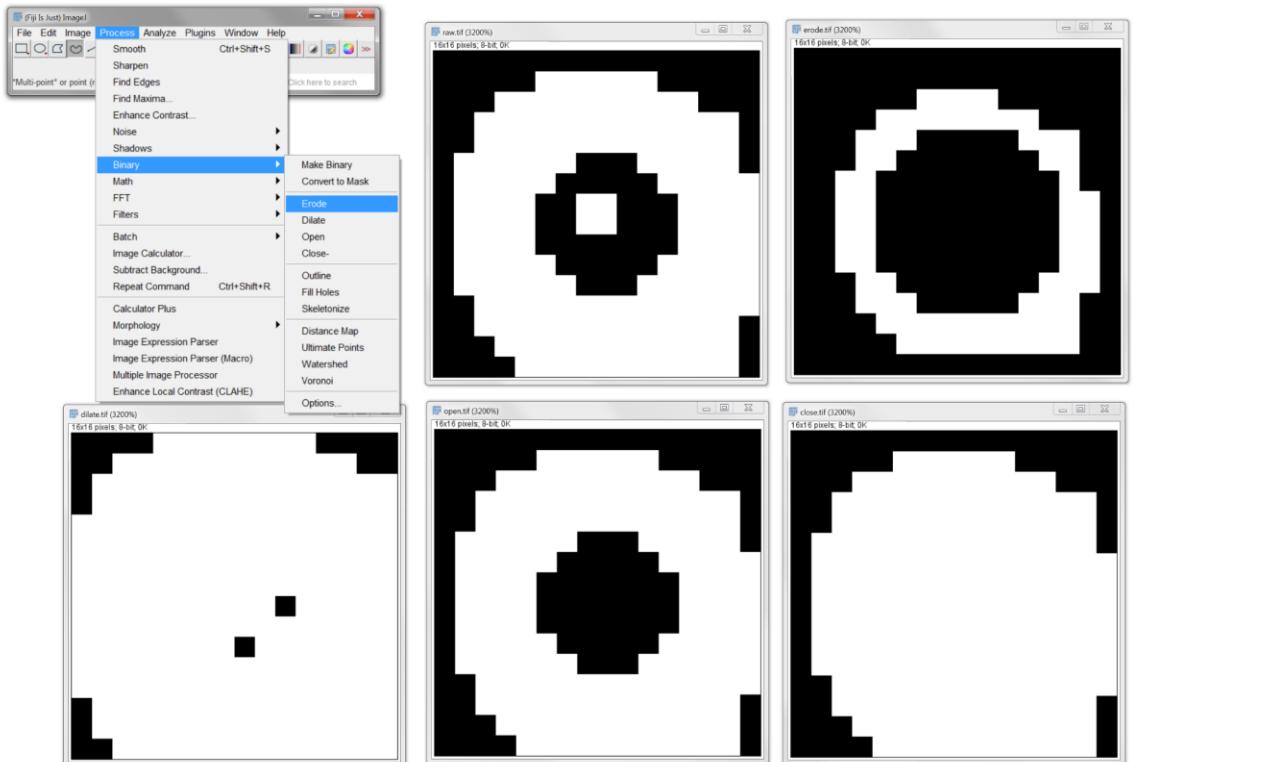
# Now try thresholding



- Better, but not great

# Morphological operations

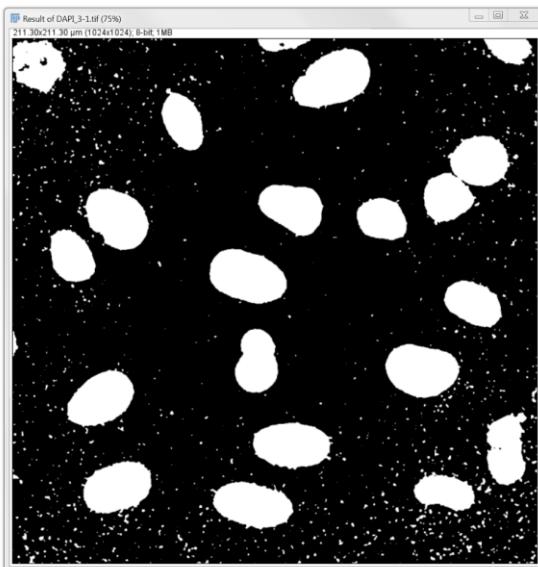
- Processing of (usually binary) images based on shape



# Morphological operations

- Try on the previous example

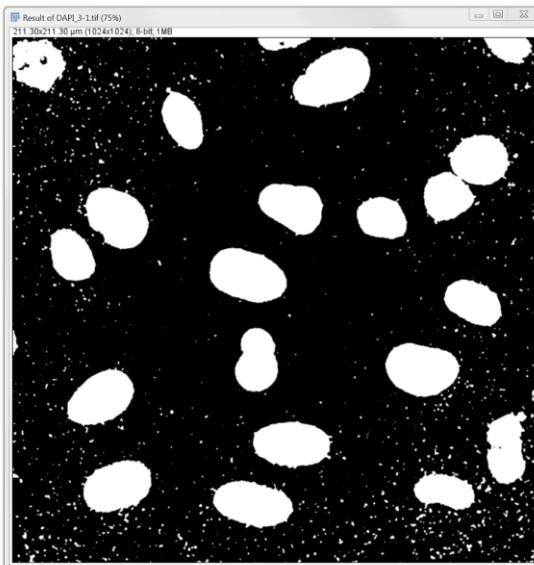
Thresholded



# Morphological operations

- Try on the previous example

Thresholded



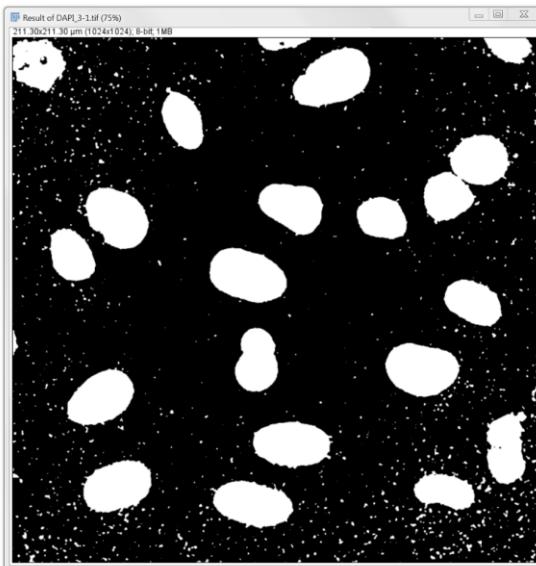
Eroded



# Morphological operations

- Try on the previous example

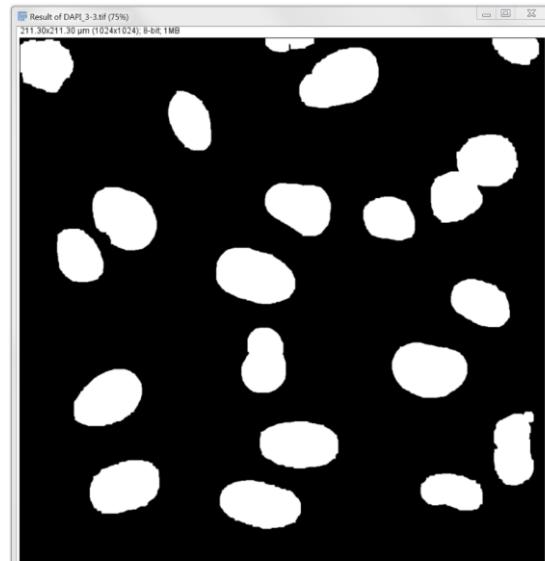
Thresholded



Eroded

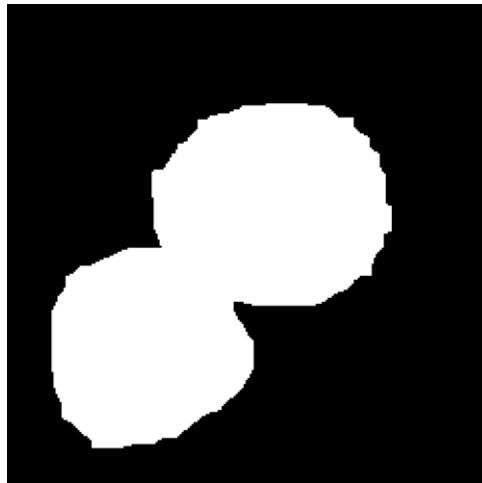


Dilated and  
closed



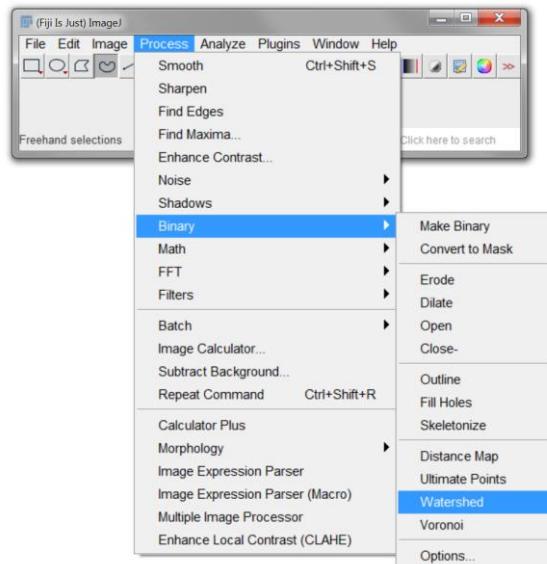
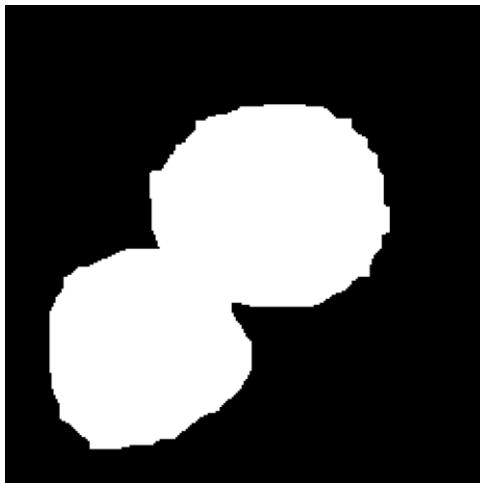
# Watershed – separate objects

- From the previous example



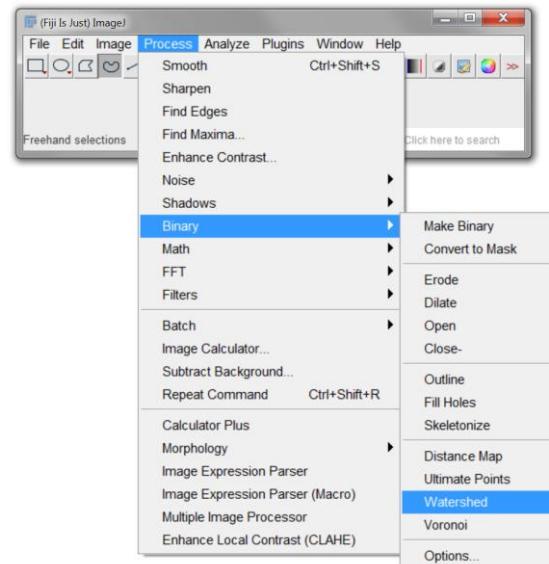
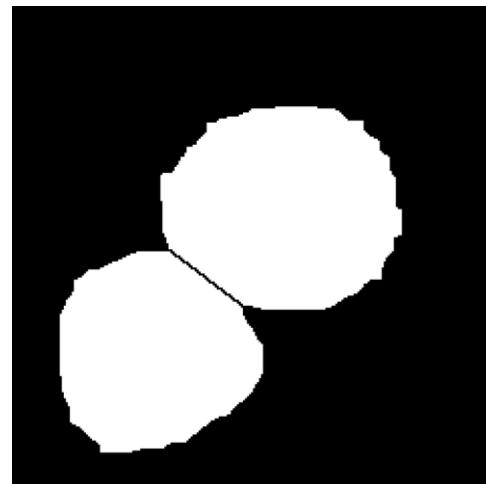
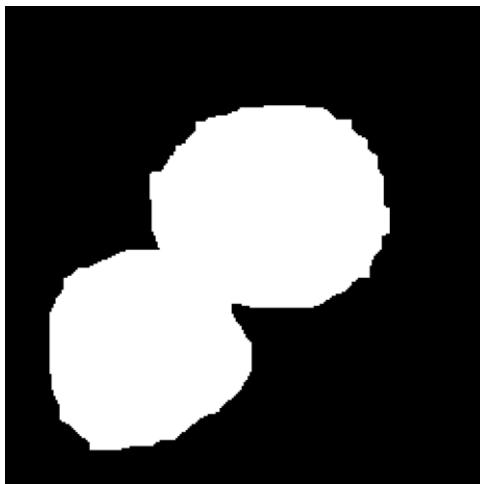
# Watershed – separate objects

- From the previous example

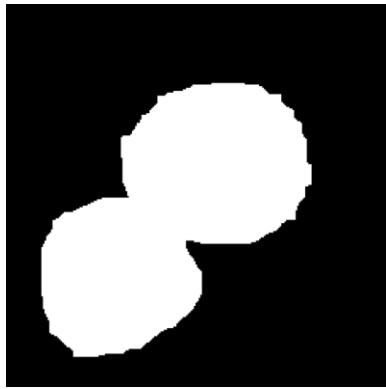


# Watershed – separate objects

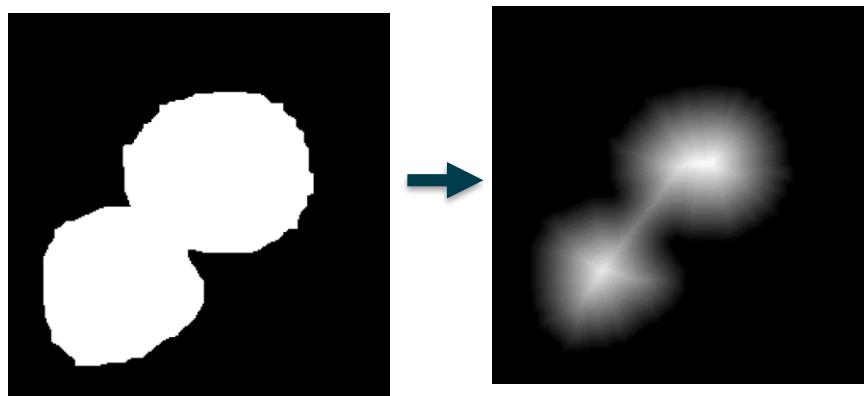
- From the previous example



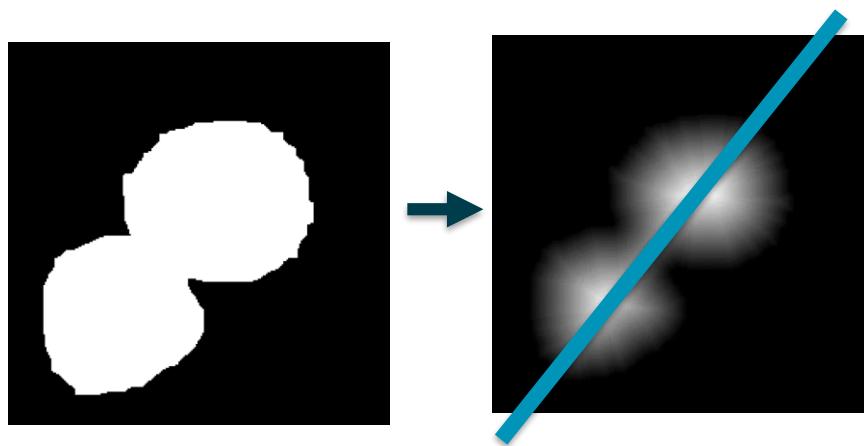
# Watershed



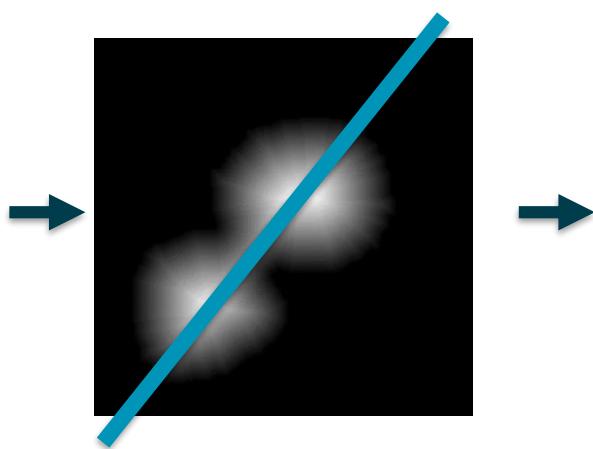
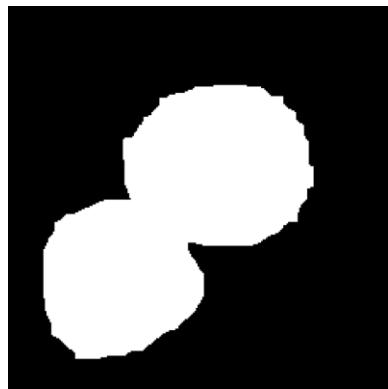
# Watershed



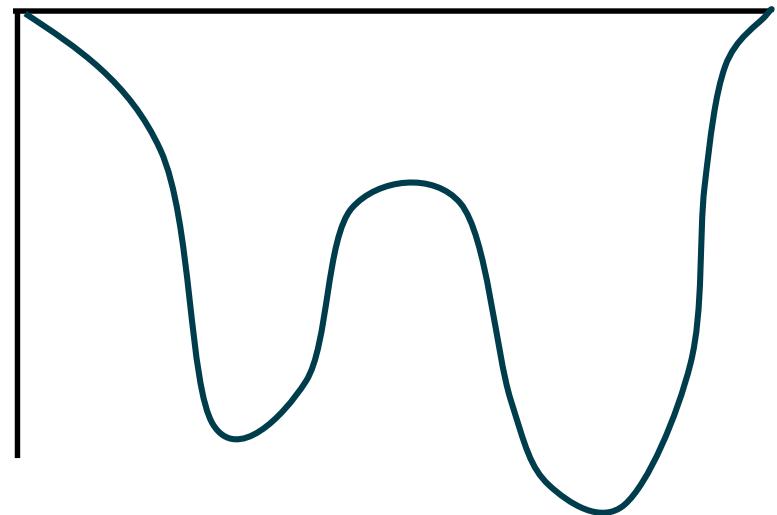
# Watershed



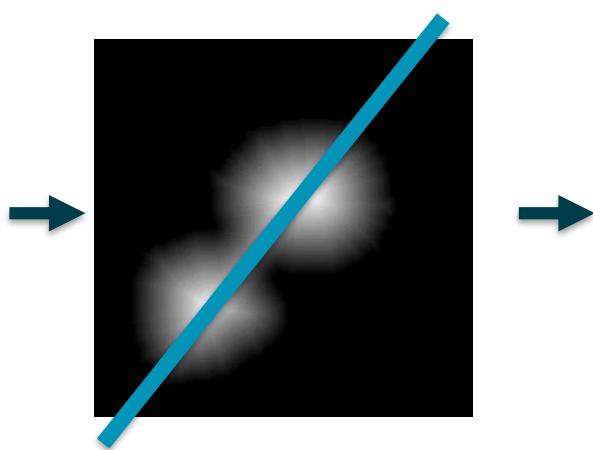
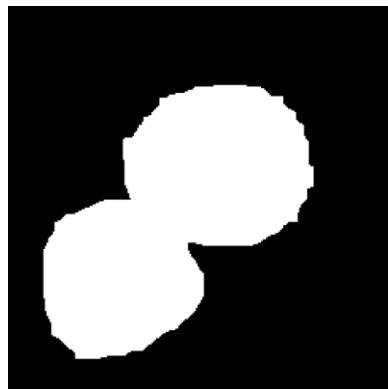
# Watershed



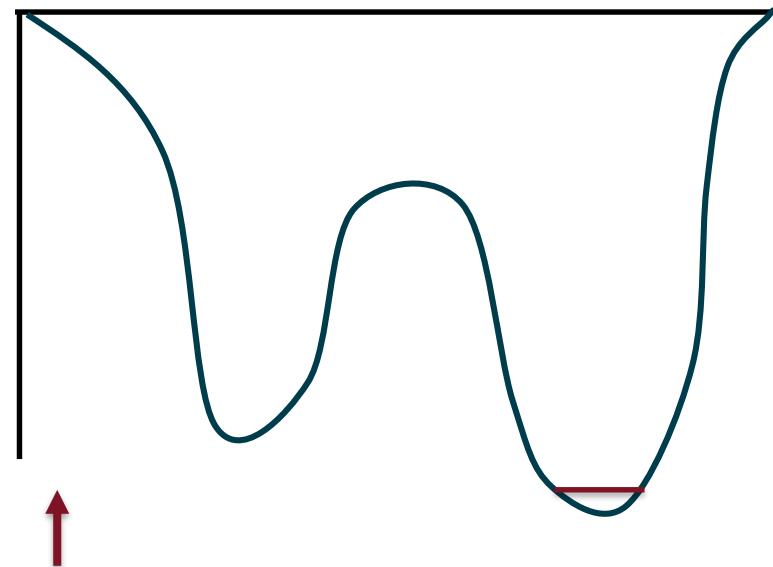
Negative intensity profile



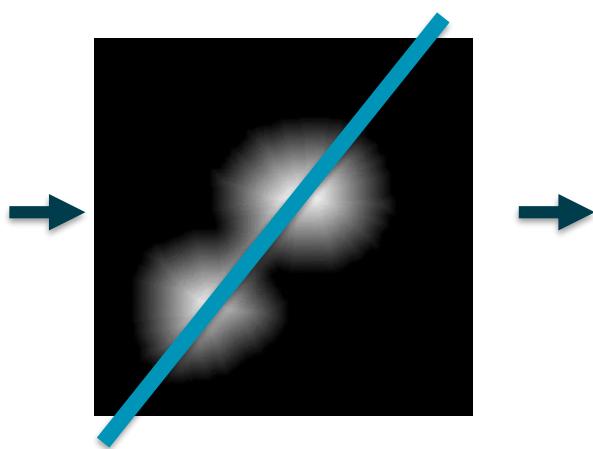
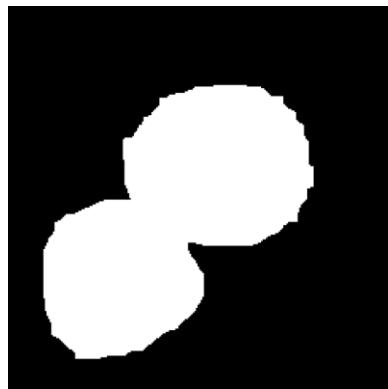
# Watershed



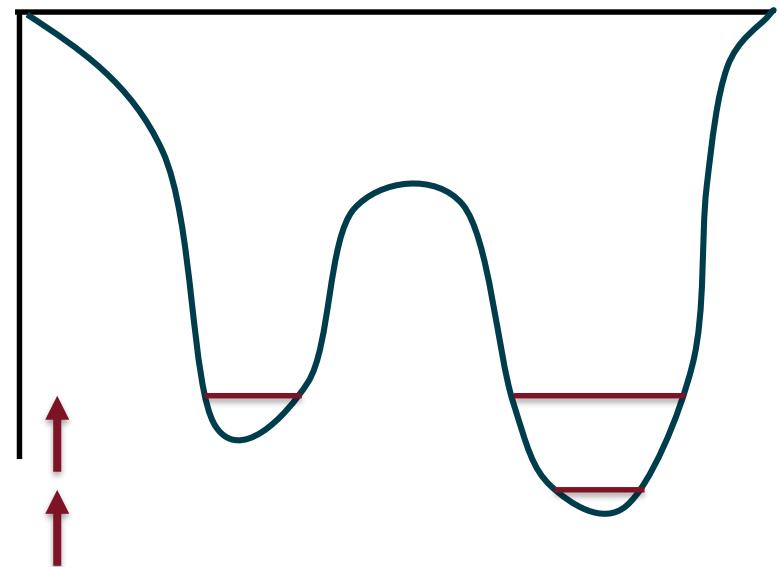
Negative intensity profile



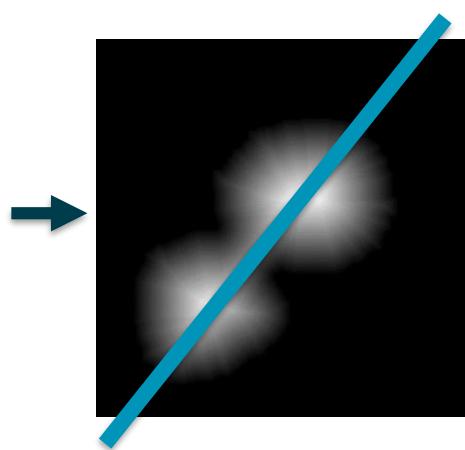
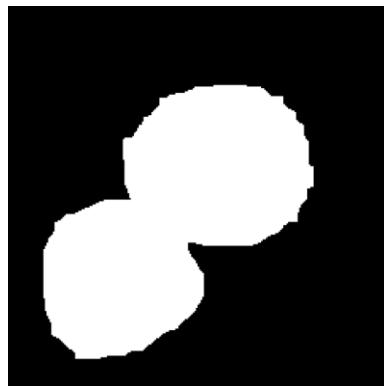
# Watershed



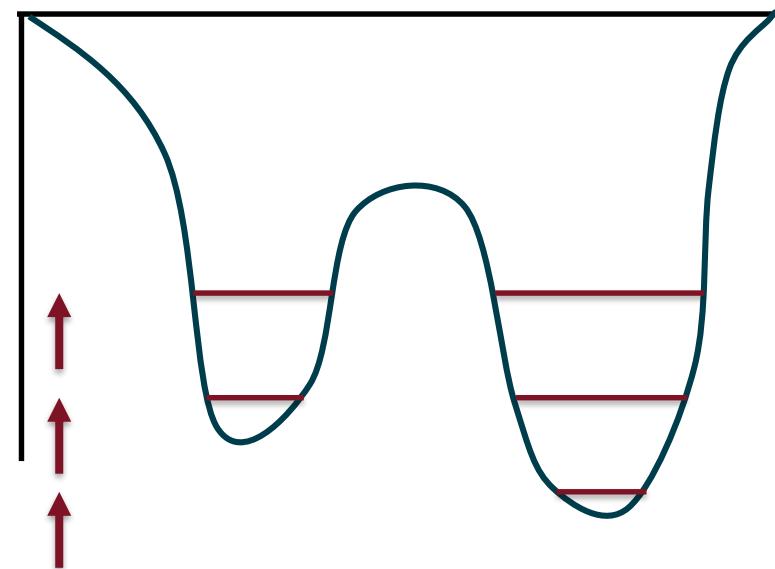
Negative intensity profile



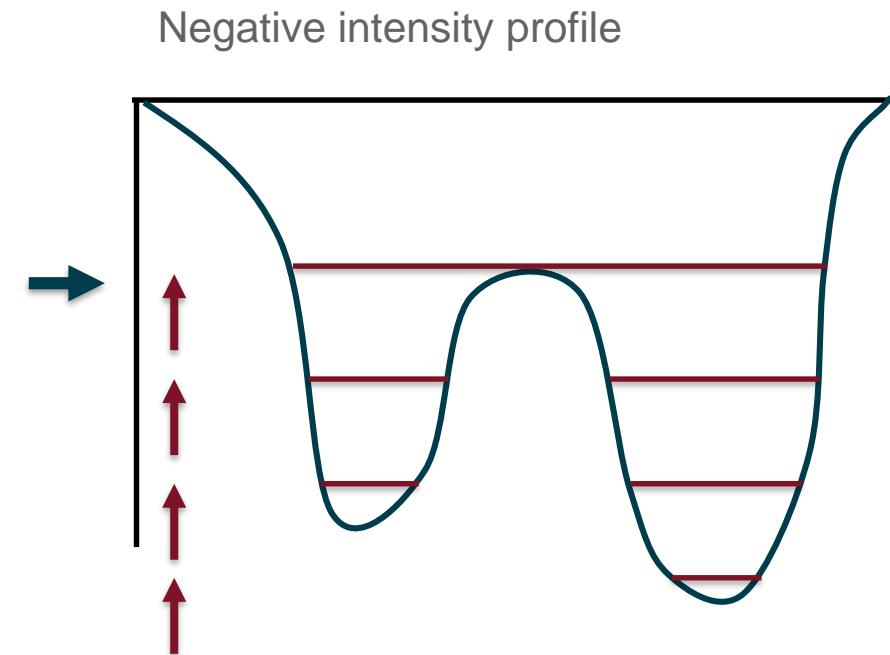
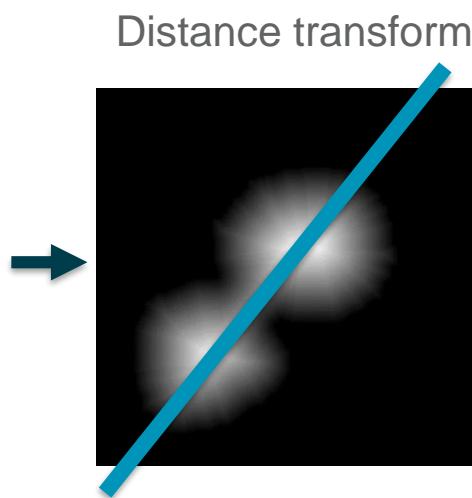
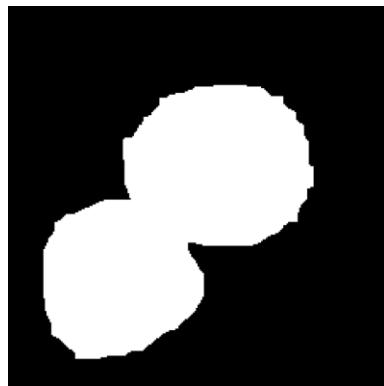
# Watershed



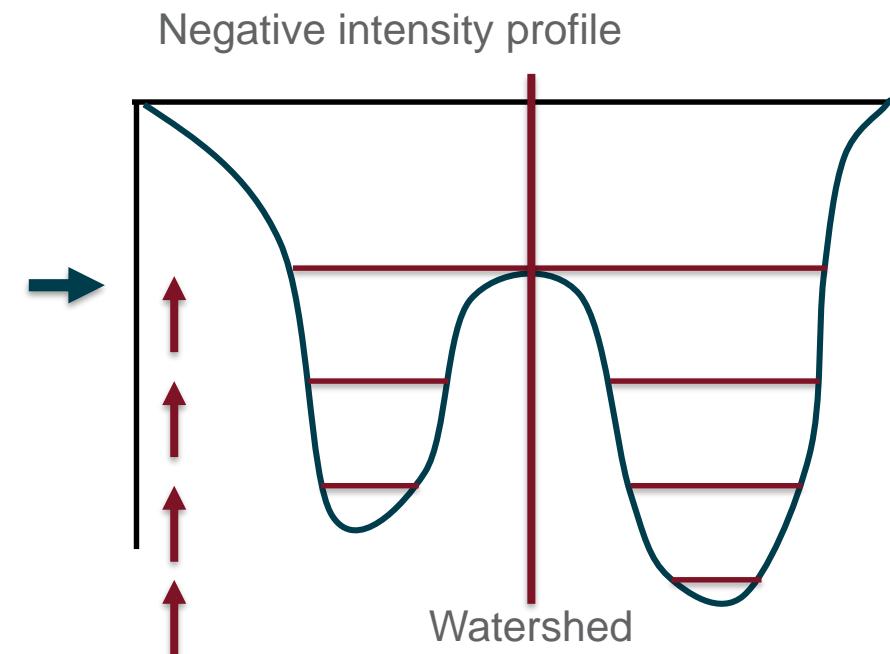
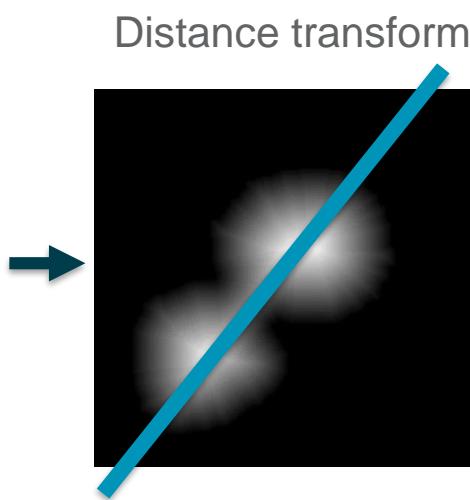
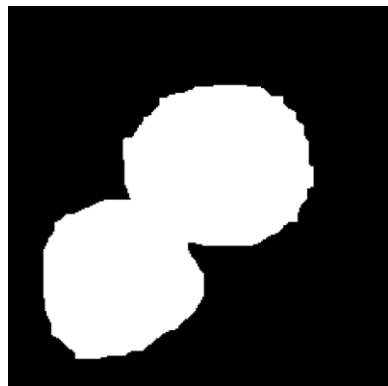
Negative intensity profile



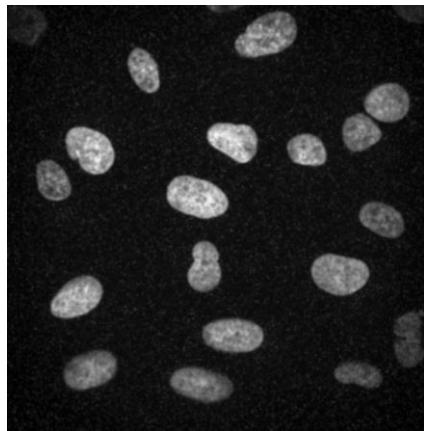
# Watershed



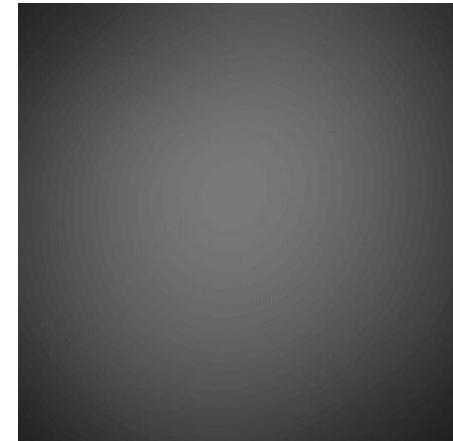
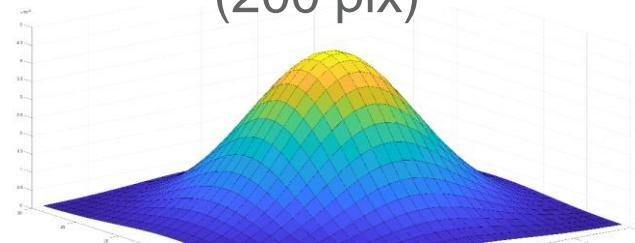
# Watershed



# Filters - Gaussian

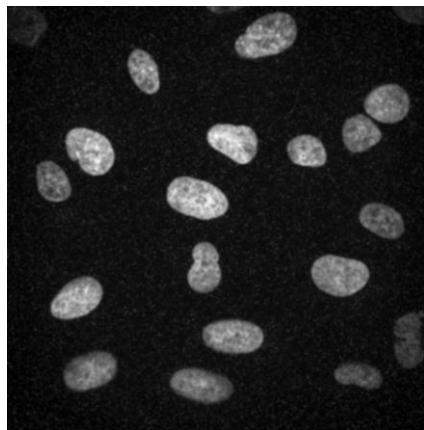


Large kernel  
(200 pix)

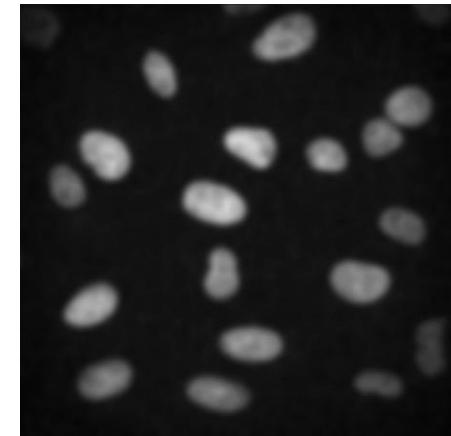
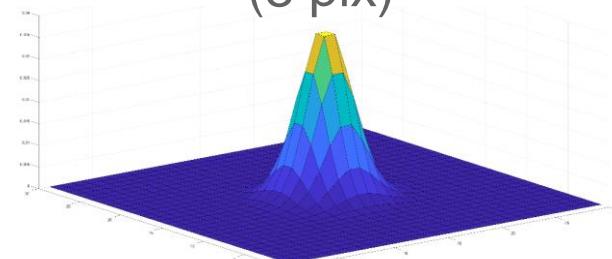


- Correct inhomogeneous illumination

# Filters - Gaussian



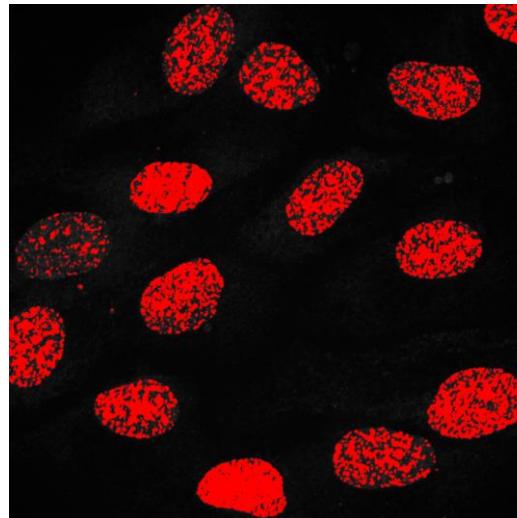
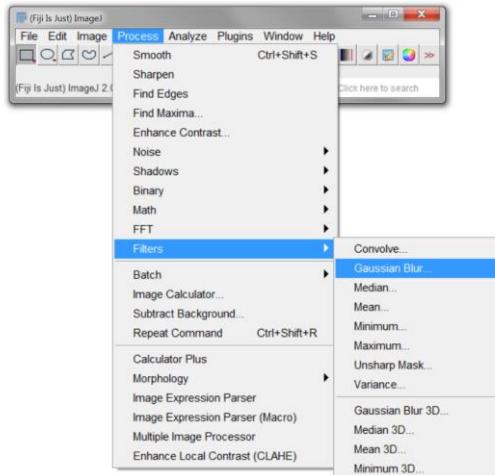
Small kernel  
(8 pix)



- Smooth intensity variations for thresholding

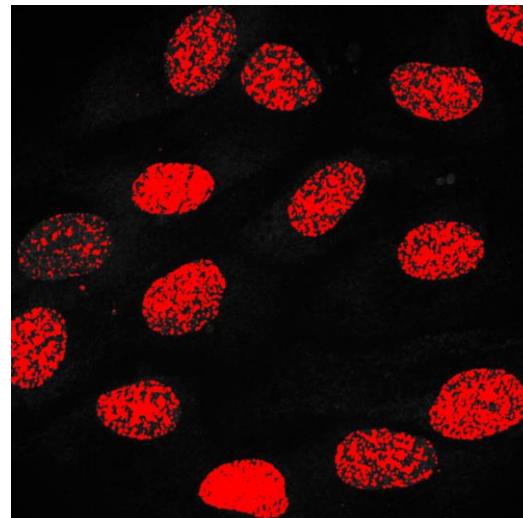
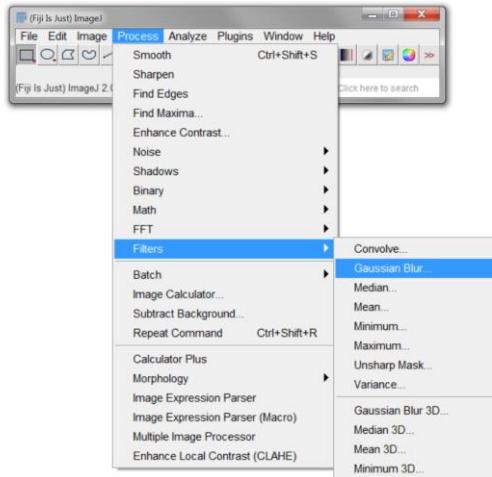
# Filters - Gaussian

- Open foci.tif & try thresholding (to segment whole nuclei)
- Apply Gaussian smoothing (use preview) & try again

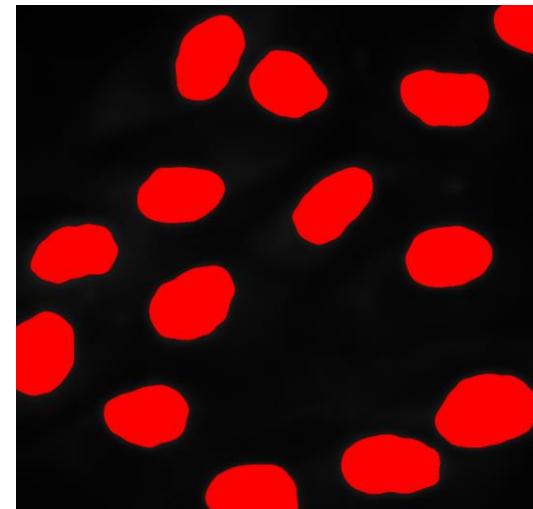


# Filters - Gaussian

- Open foci.tif & try thresholding
- Apply Gaussian smoothing (use preview) & try again

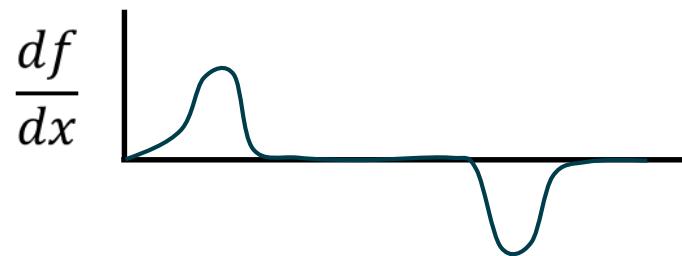
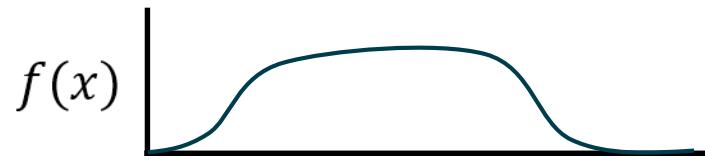


VS



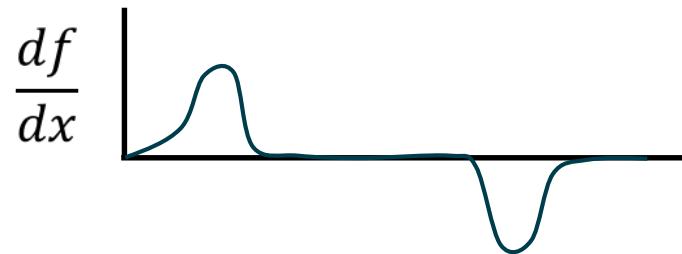
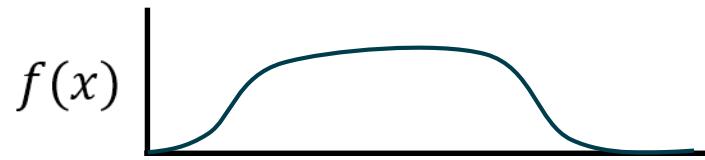
# Filters - Gradient

- Edge detection



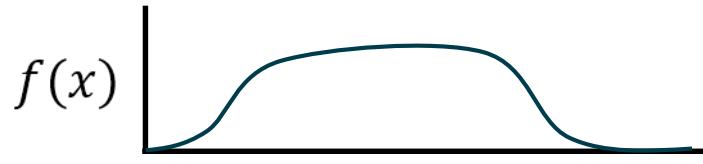
# Filters - Gradient

- Edge detection
- Approximate derivative:  
The Sobel operator

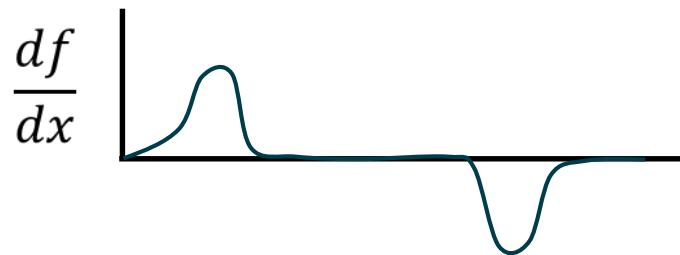


# Filters - Gradient

- Edge detection
- Approximate derivative:  
The Sobel operator

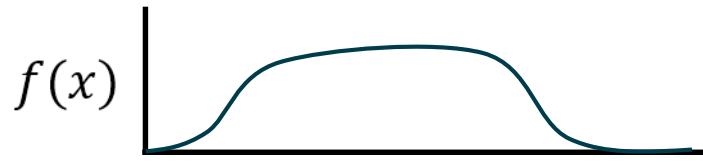


$$G_x = \begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * I$$

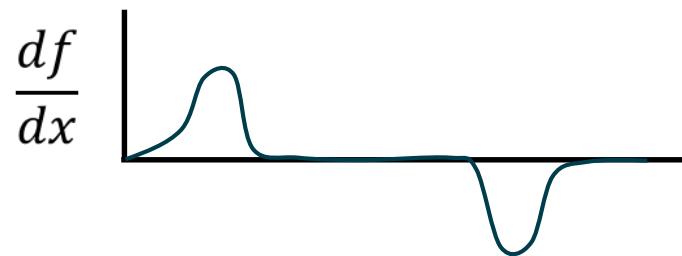


# Filters - Gradient

- Edge detection
- Approximate derivative:  
The Sobel operator

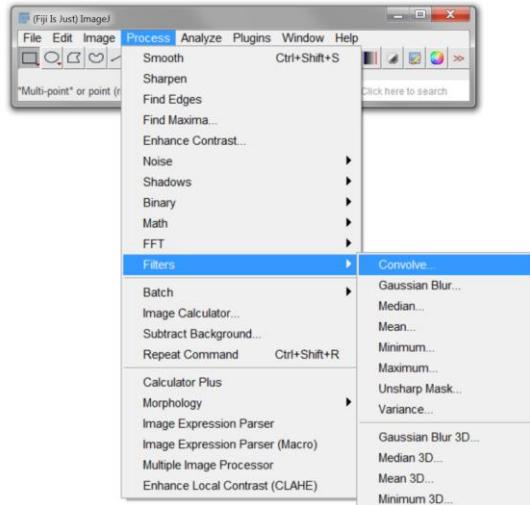


$$G_x = \begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * I$$

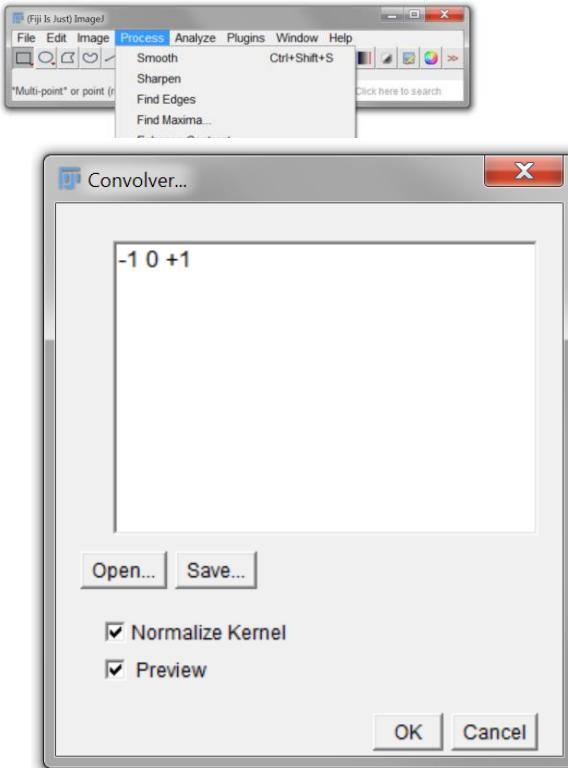


$$G_y = \begin{bmatrix} +1 & +2 & +1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{bmatrix} * I$$

# Filters - Gradient



# Filters - Gradient



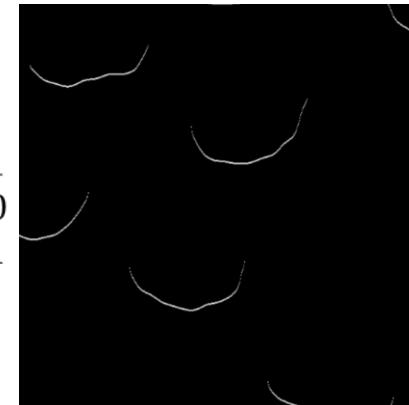
-1 0 +1



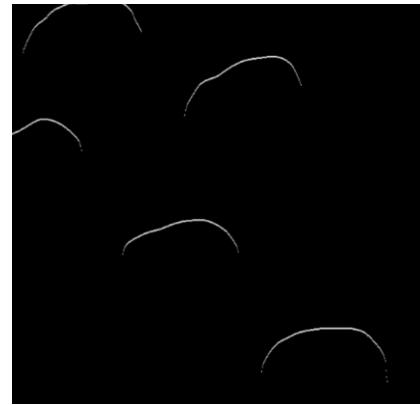
+1 0 -1



+1  
0  
-1

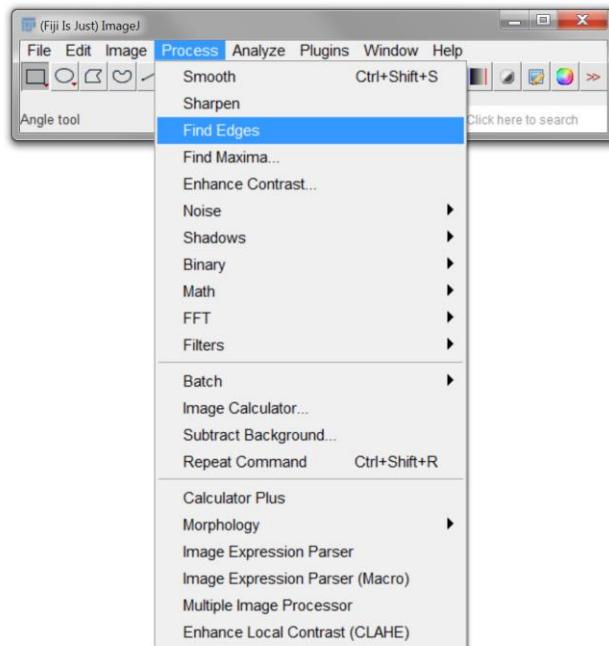


-1  
0  
+1



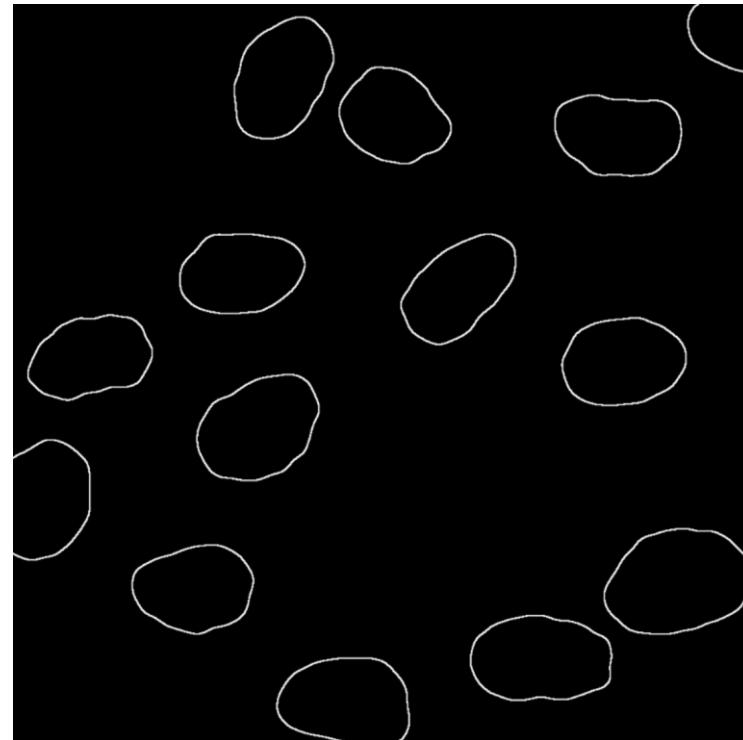
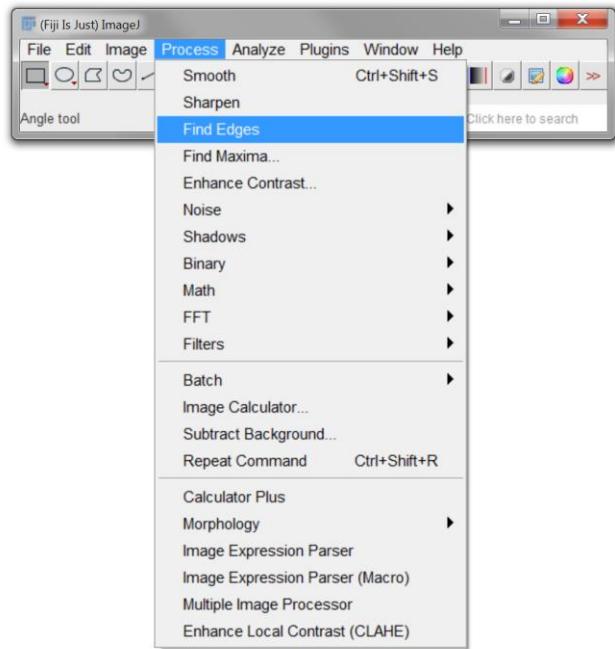
# Filters - Gradient

- Combine

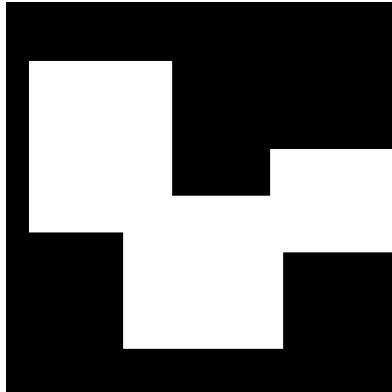
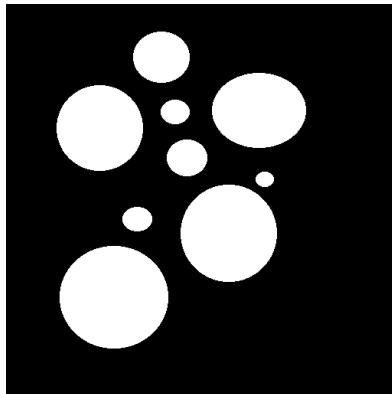


# Filters - Gradient

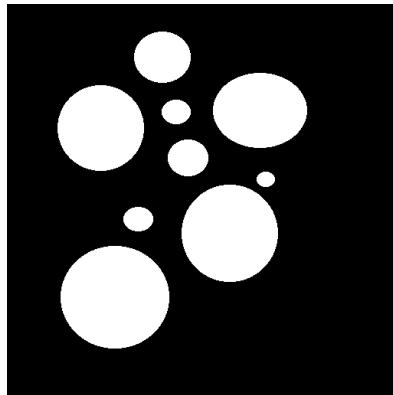
- Combine



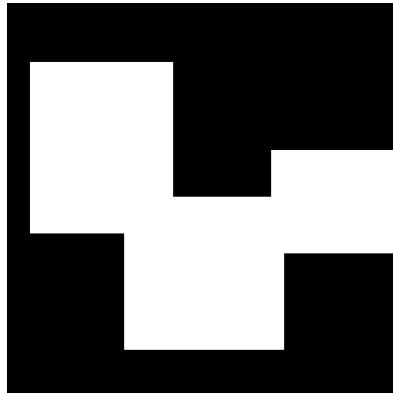
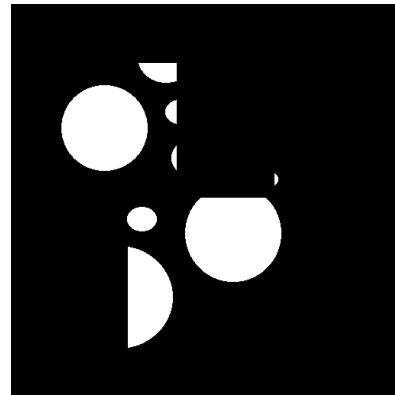
# Boolean image mathematics



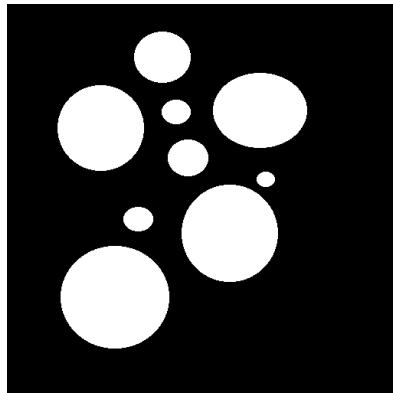
# Boolean image mathematics



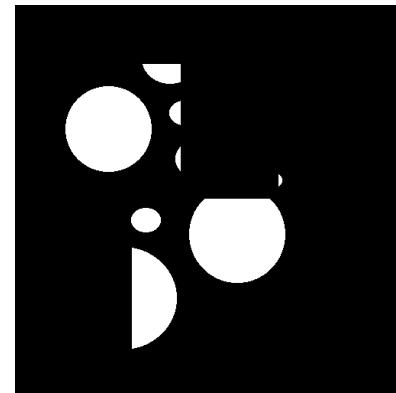
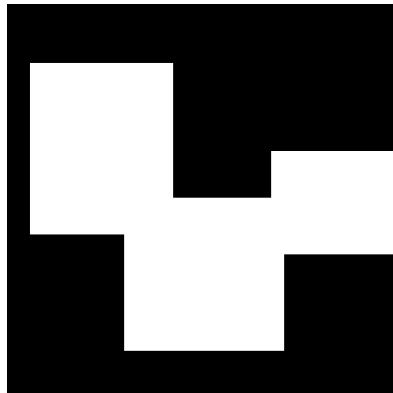
AND



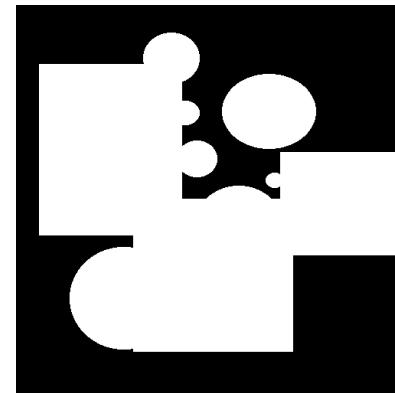
# Boolean image mathematics



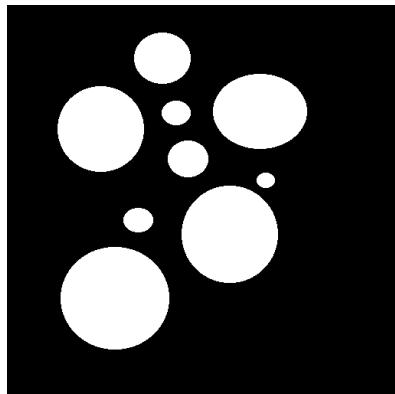
AND



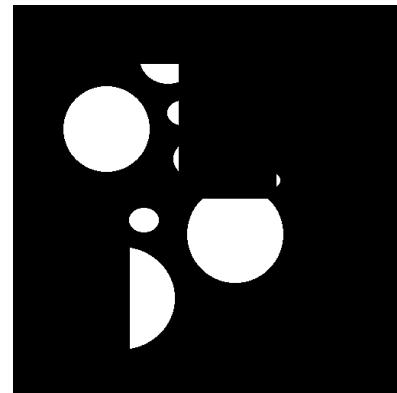
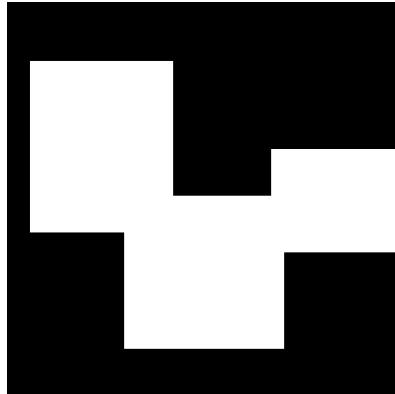
OR



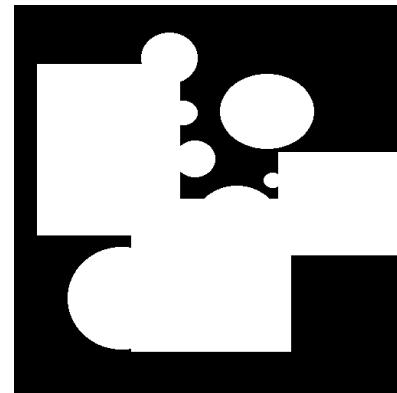
# Boolean image mathematics



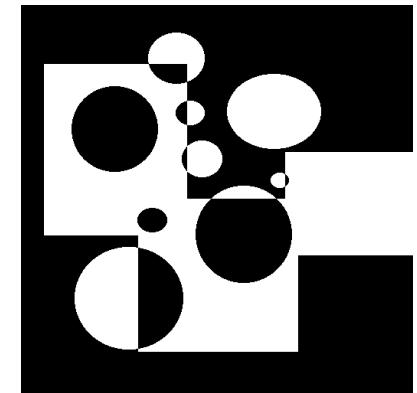
AND



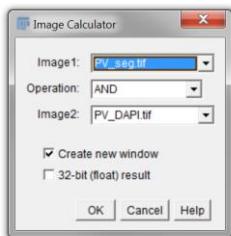
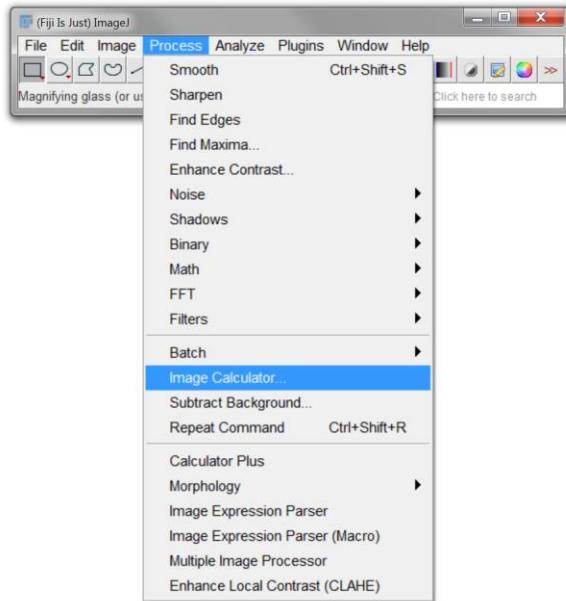
OR



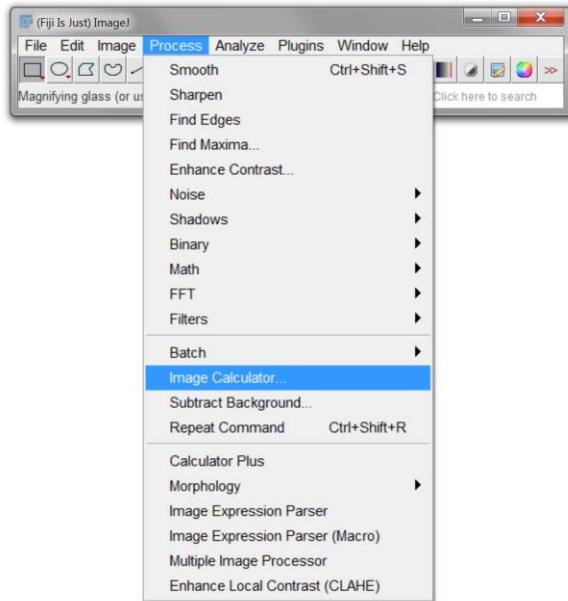
XOR



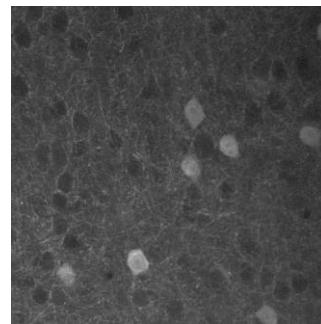
# Boolean image mathematics - masking



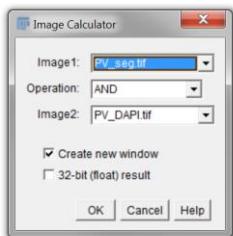
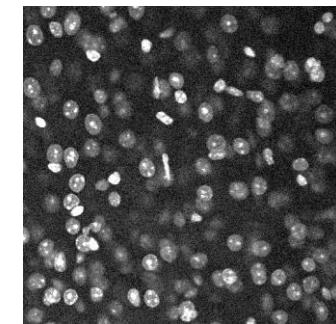
# Boolean image mathematics - masking



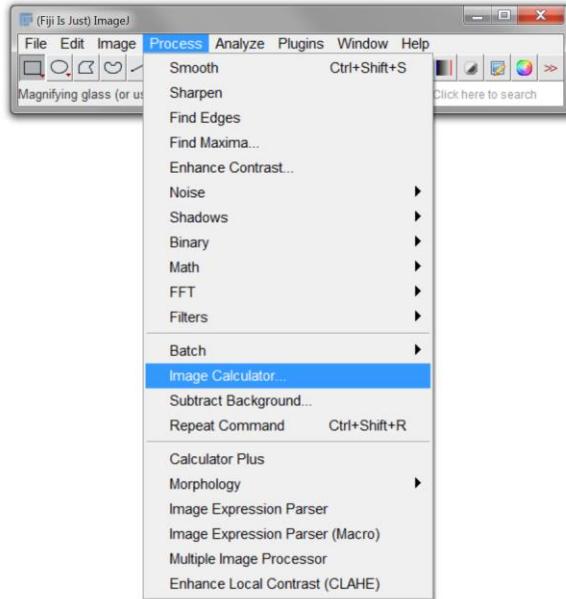
PV.tif



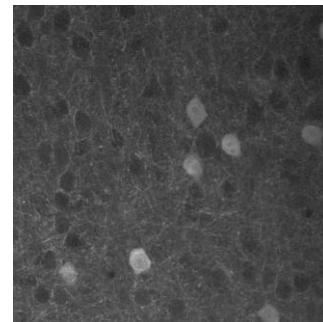
PV\_DAPI.tif



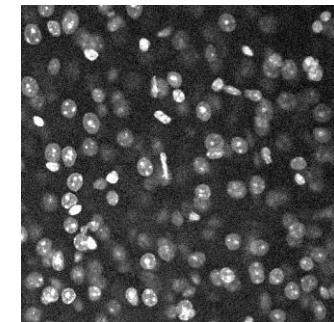
# Boolean image mathematics - masking



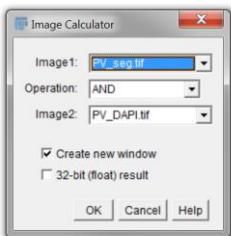
PV.tif



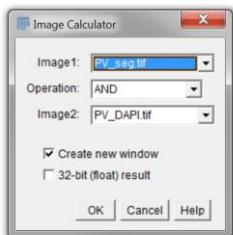
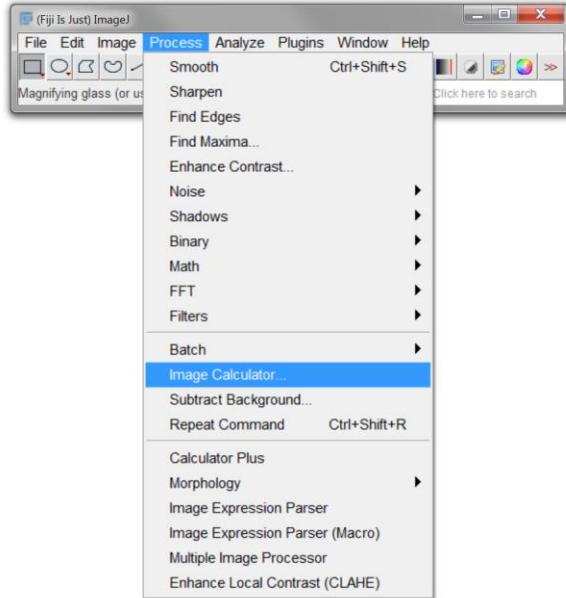
PV\_DAPI.tif



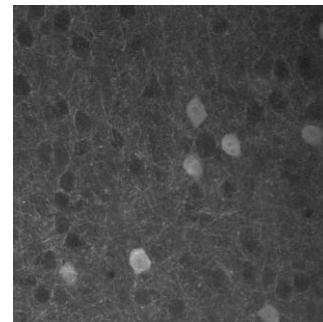
PV\_seg.tif



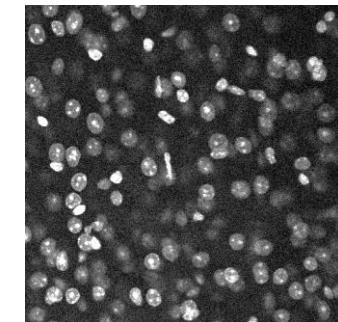
# Boolean image mathematics - masking



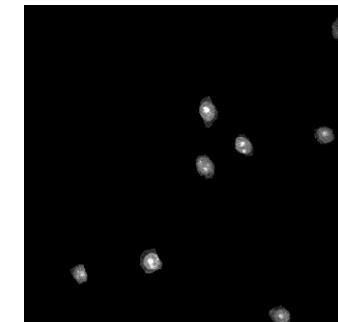
PV.tif



PV\_DAPI.tif

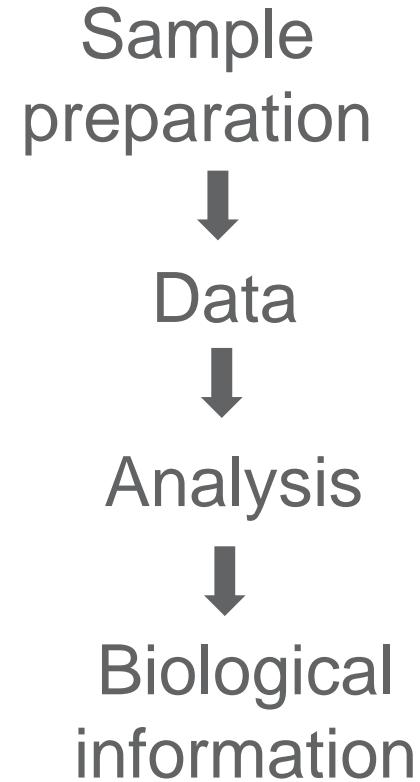


PV\_seg.tif



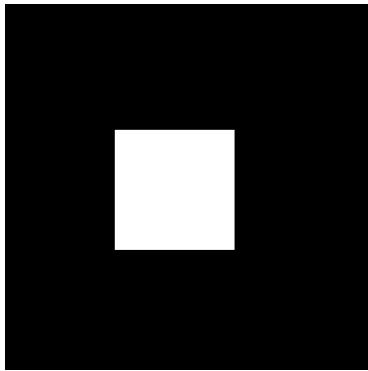
# First (and most important lesson) – again!

Test your analysis  
**before** you  
acquire data!



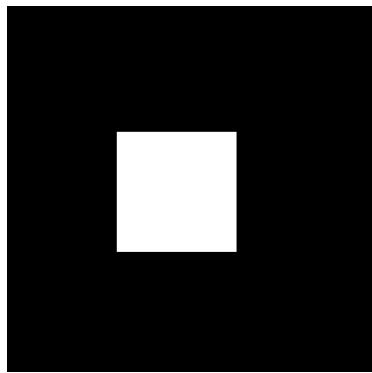
# Deconvolution

Sample



# Deconvolution

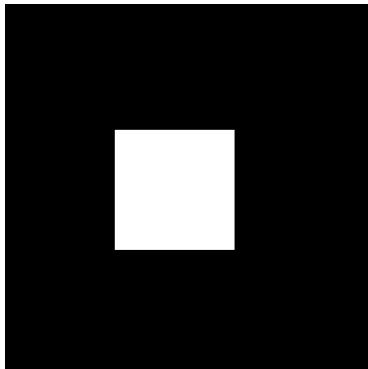
Sample



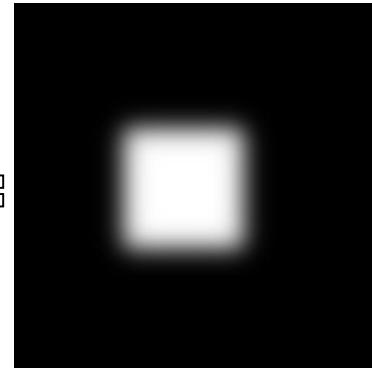
PSF

# Deconvolution

Sample

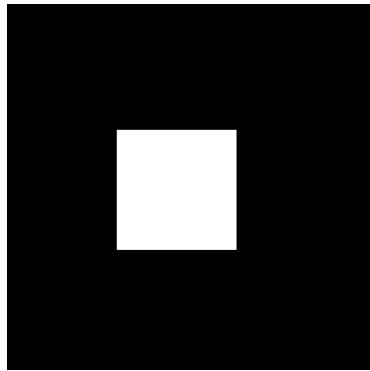


Blurred

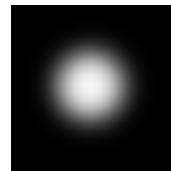


# Deconvolution

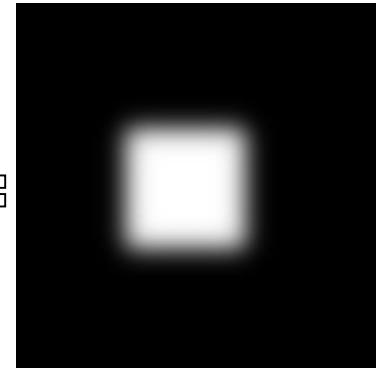
Sample



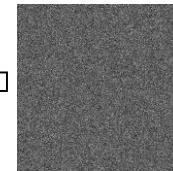
PSF



Blurred

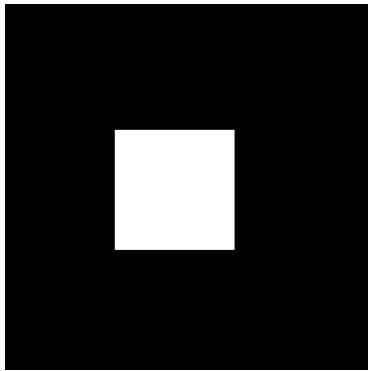


Noise



# Deconvolution

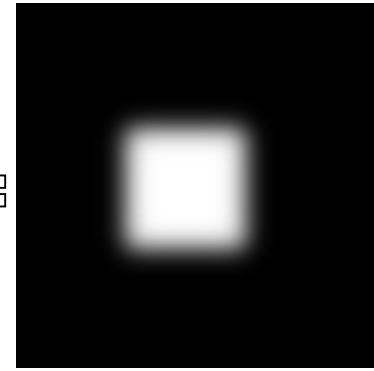
Sample



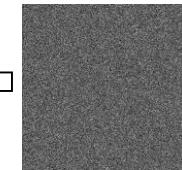
PSF



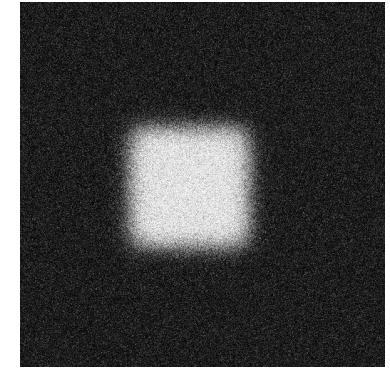
Blurred



Noise

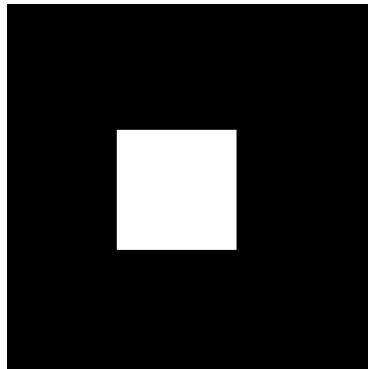


Blurred & noisy



# Deconvolution

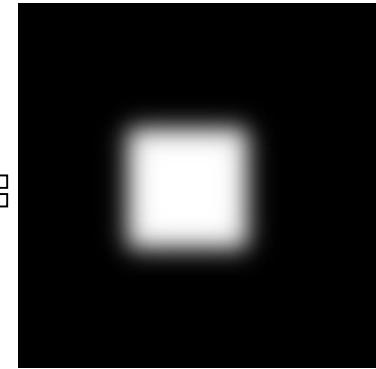
Sample



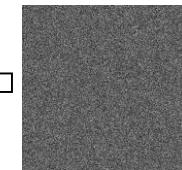
PSF



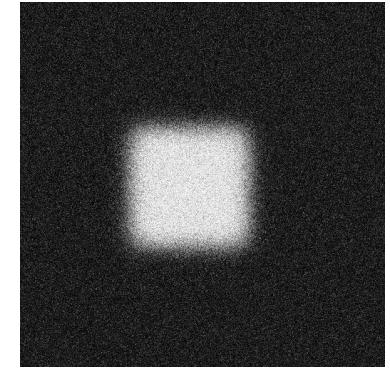
Blurred



Noise



Blurred & noisy



?



# Deconvolution – how?

Constrained iterative deconvolution  
e.g. Richardson-Lucy

# Deconvolution – how?

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Image

Operation

Raw Image

# Deconvolution – how?

Constrained iterative deconvolution  
e.g. Richardson-Lucy

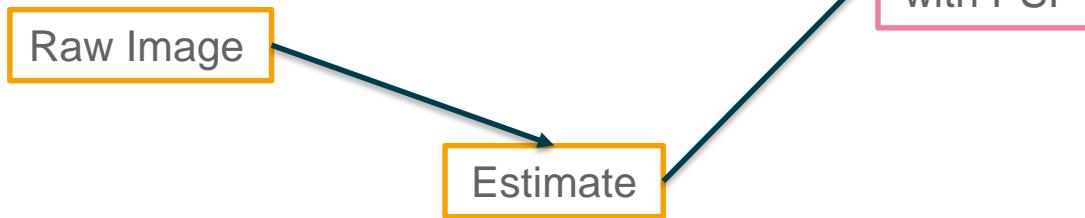
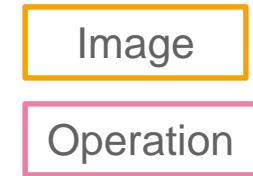
Image

Operation



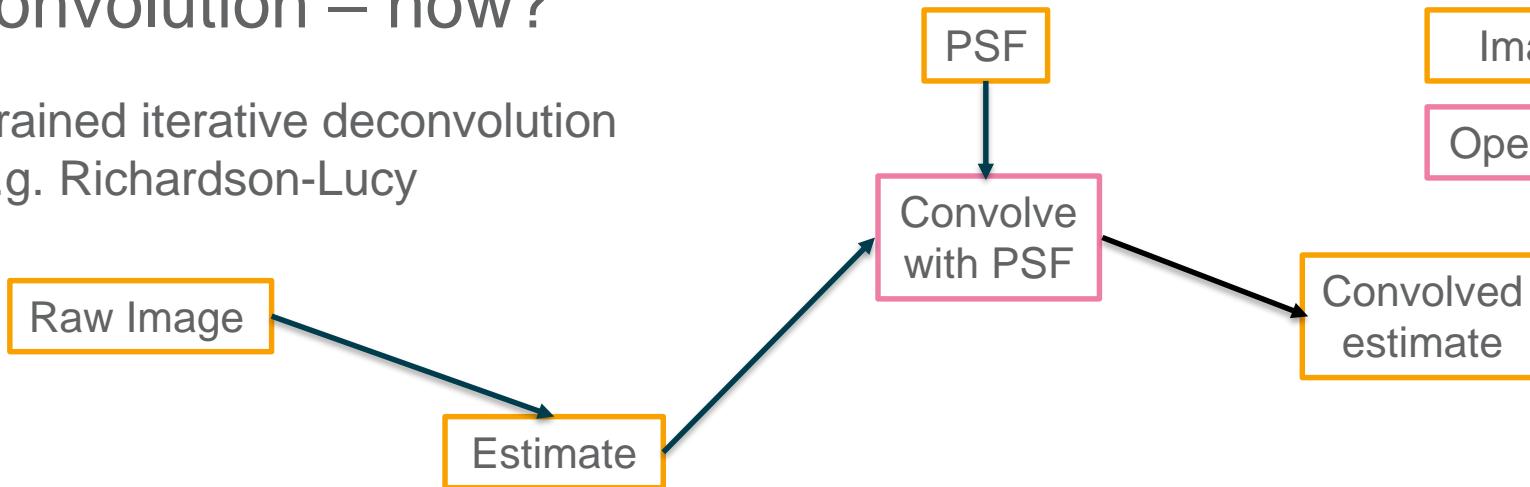
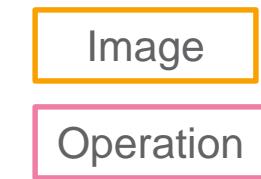
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Constrained iterative deconvolution  
e.g. Richardson-Lucy



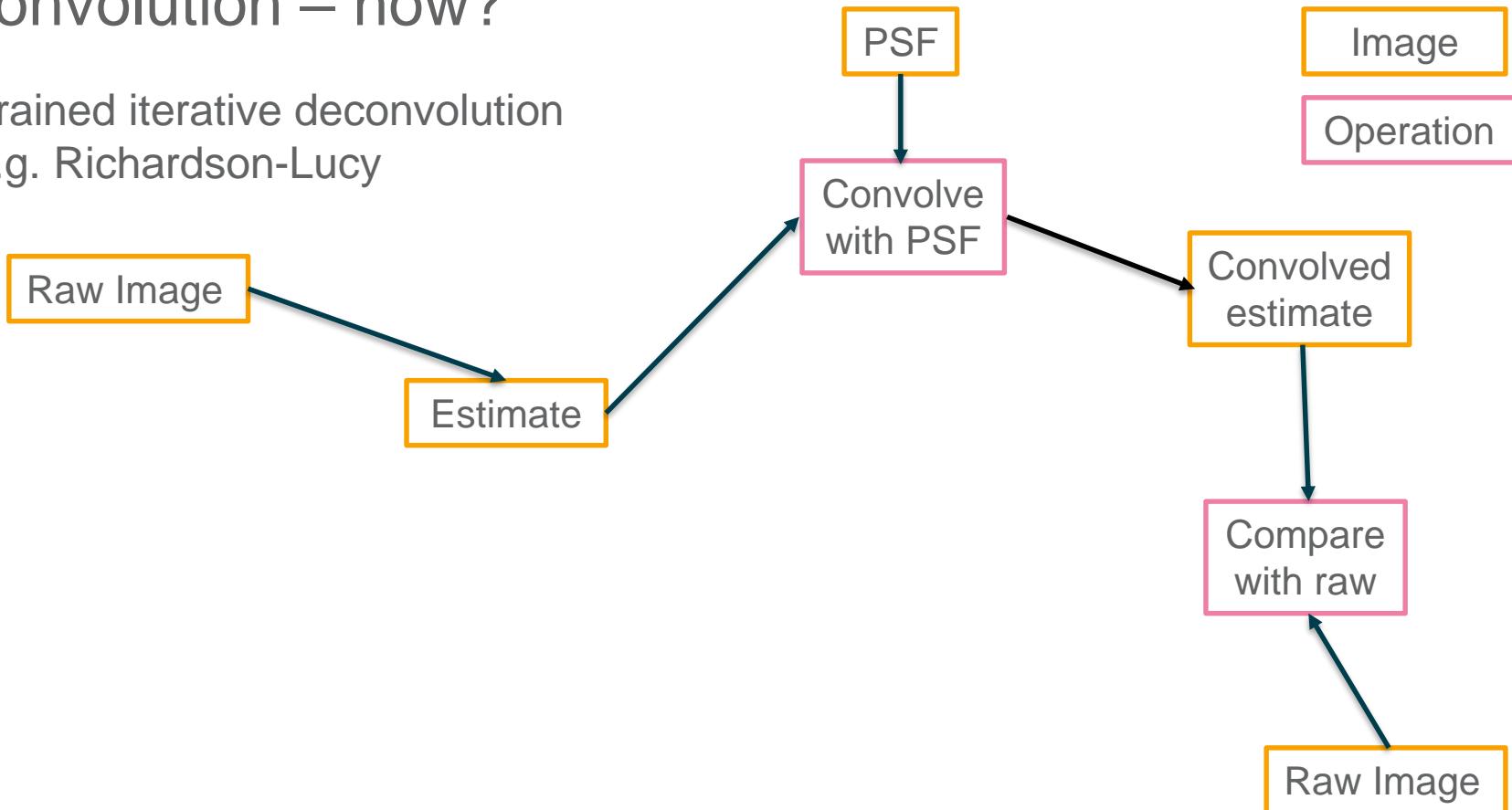
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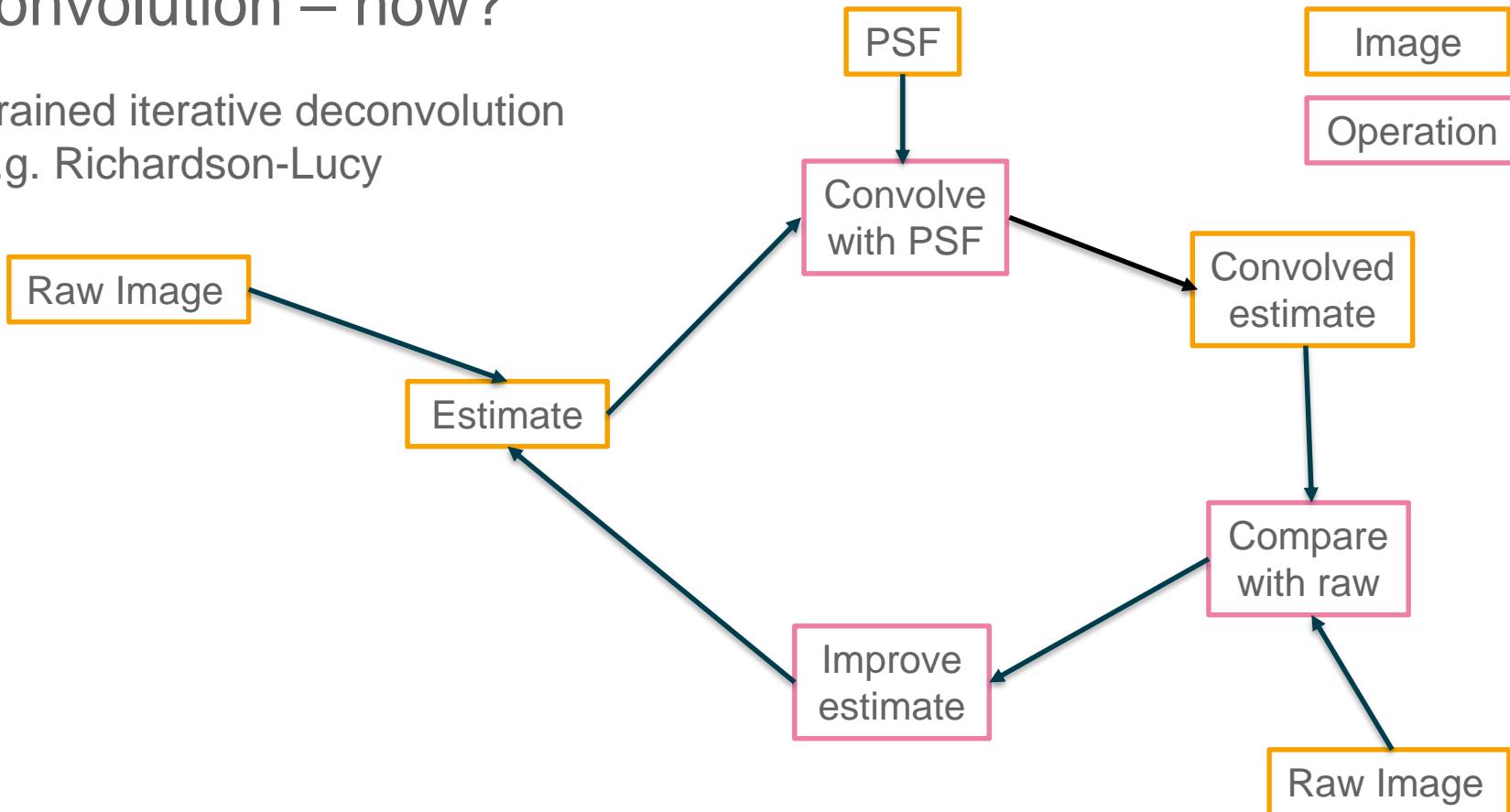
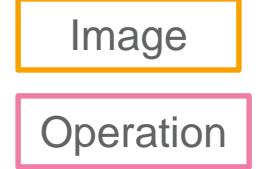
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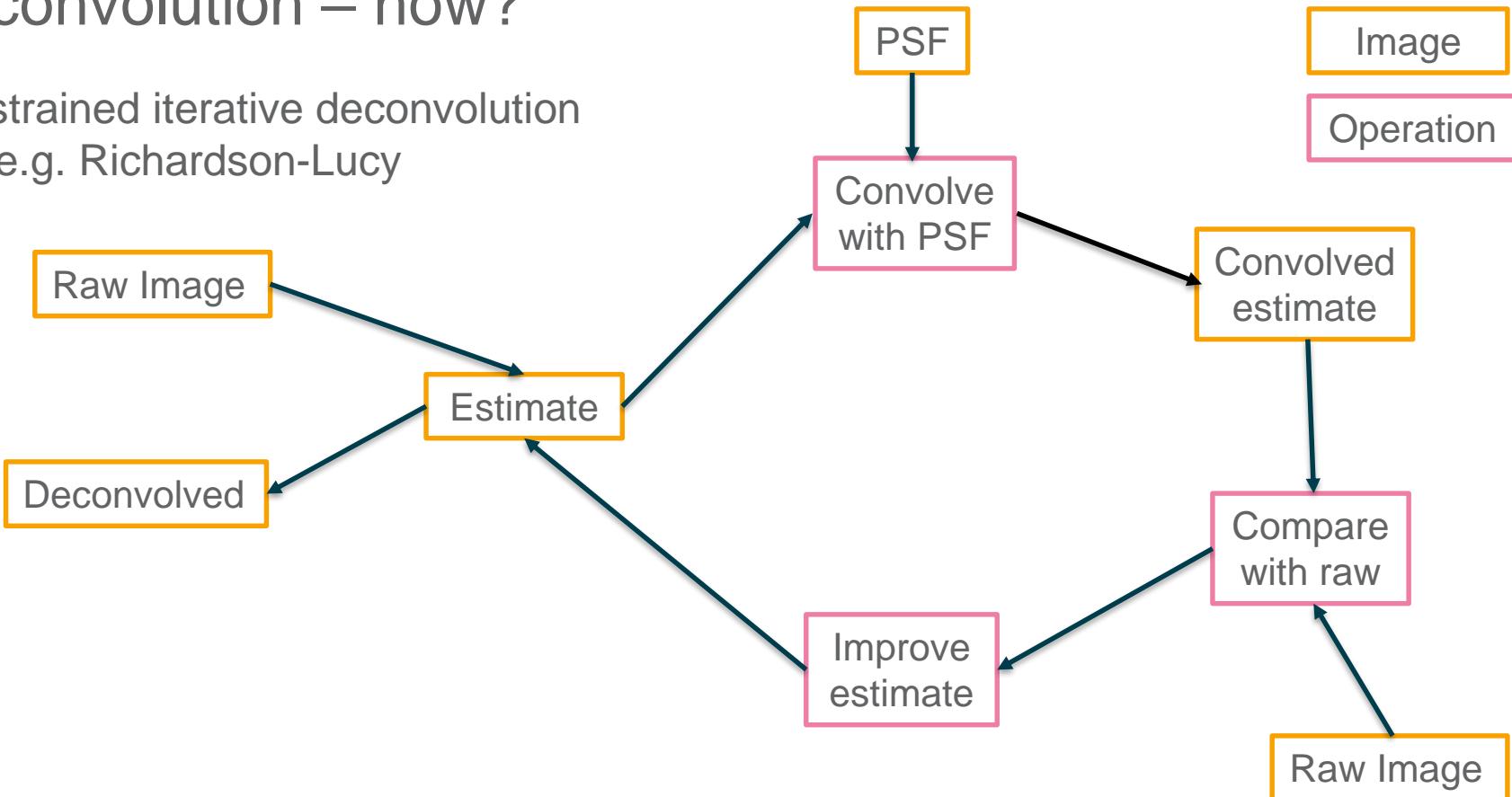
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Constrained iterative deconvolution  
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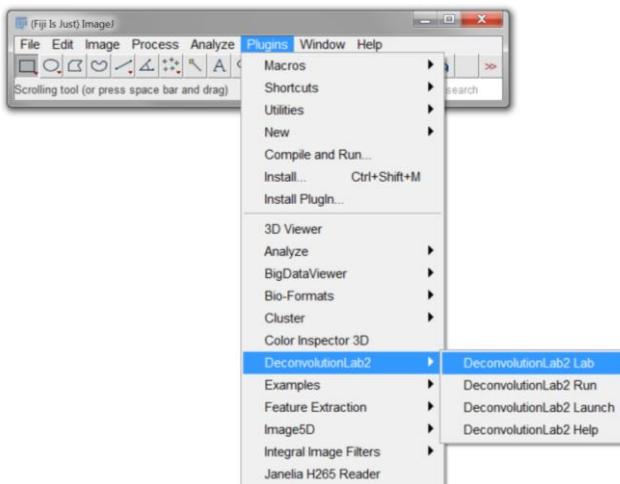
# Deconvolution – how?

Constrained iterative deconvolution  
e.g. Richardson-Lucy



# Deconvolution – try it out

## DeconvolutionLab2

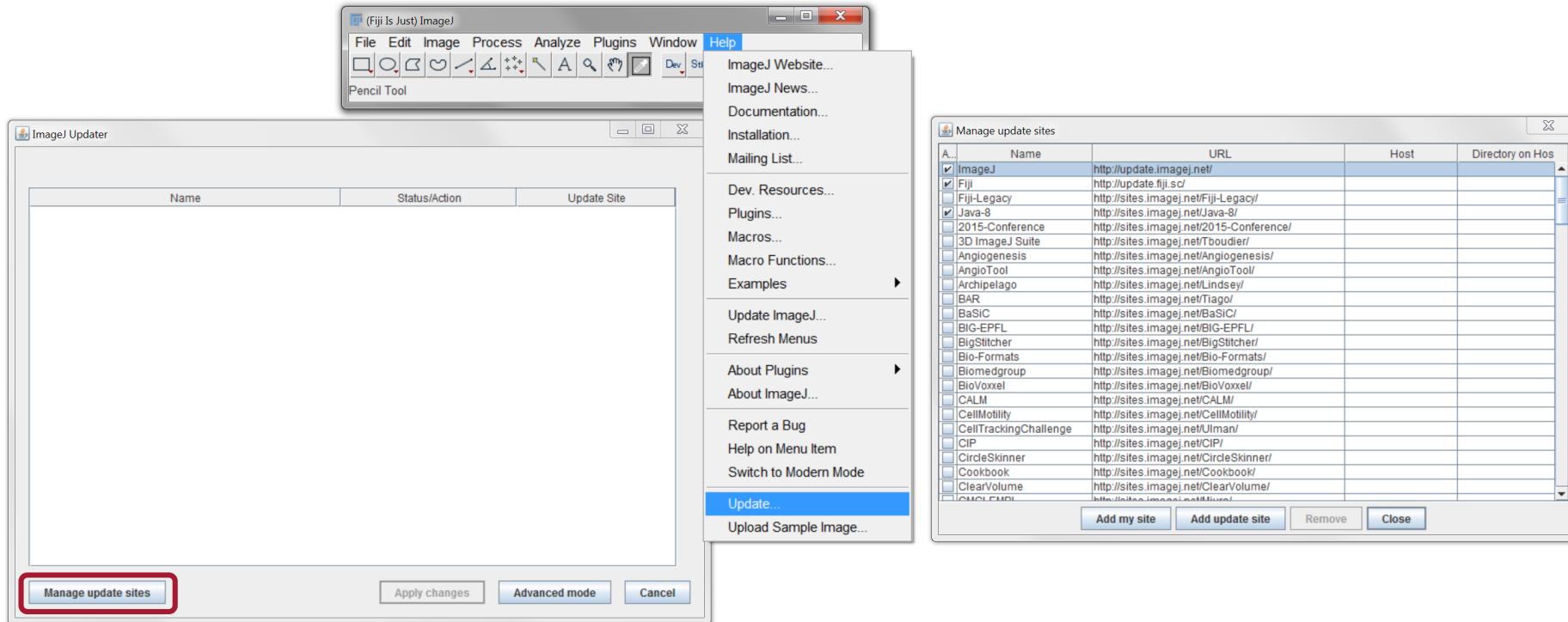


# Deconvolution – BUT

Not installed with ImageJ/FIJI

# Installing plugins

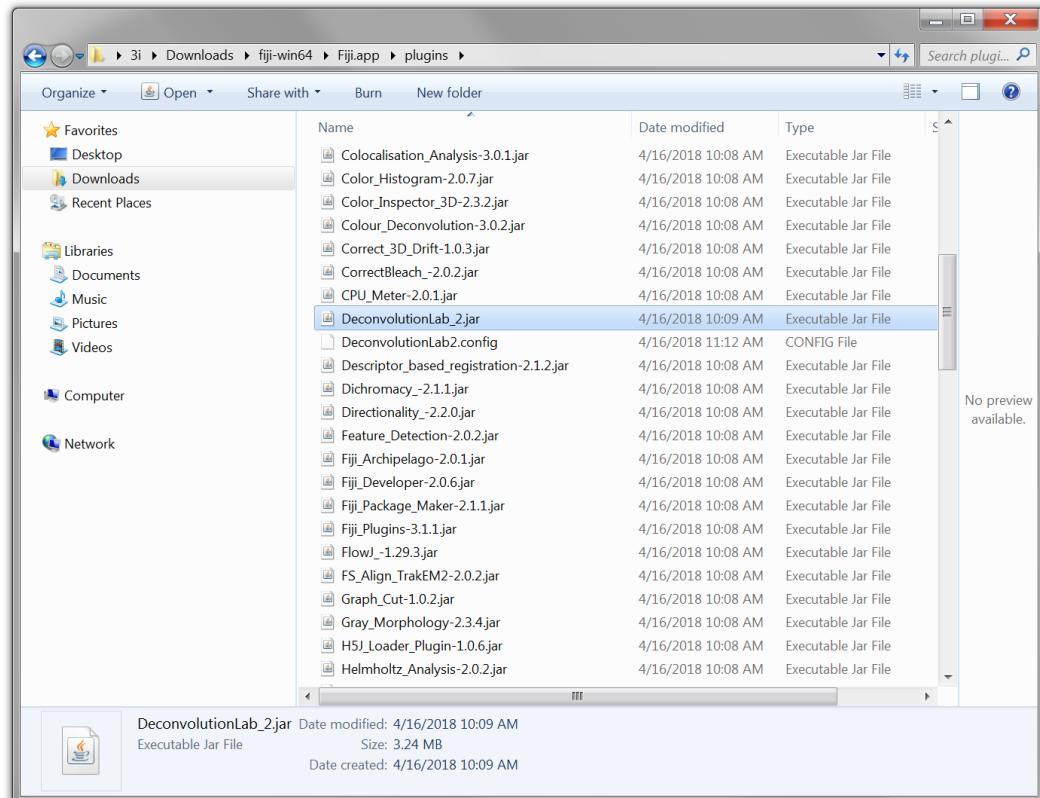
Easy way:



# Installing plugins

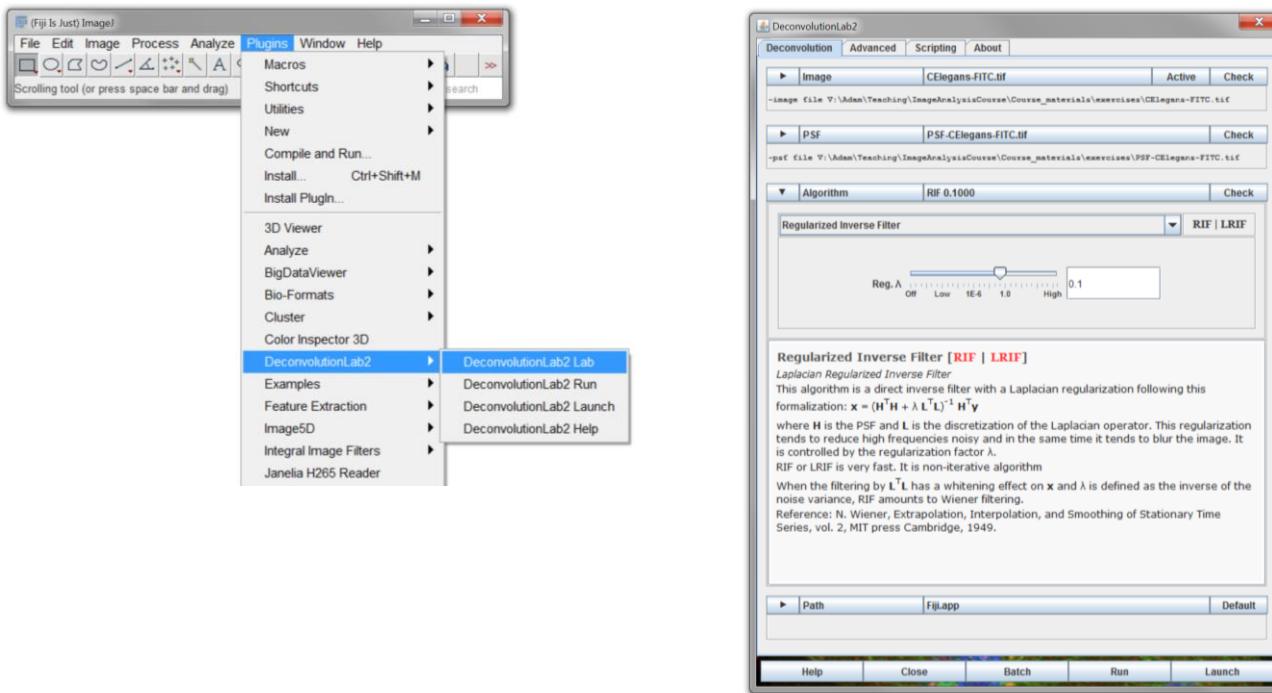
Hard way:

- Download .jar file and copy into plugins folder
- On OSX – right click > “Show package contents”



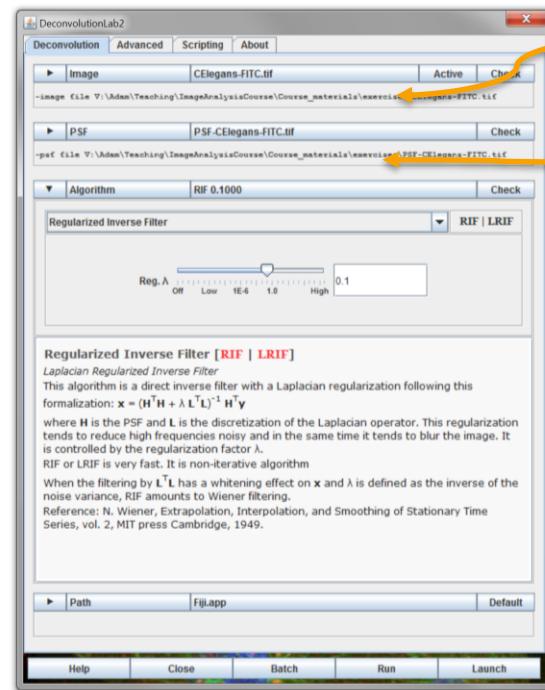
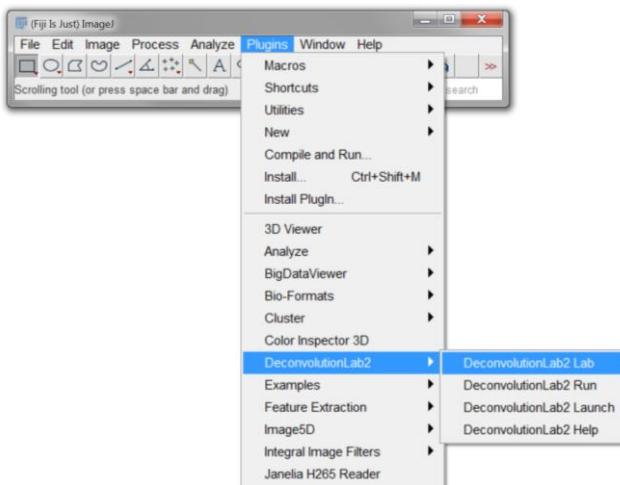
# Deconvolution – try it out

## DeconvolutionLab2



# Deconvolution – try it out

## DeconvolutionLab2



Drag and drop

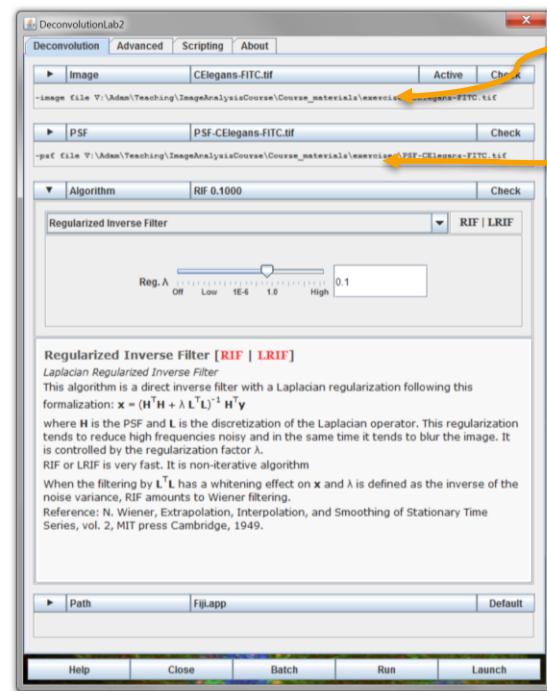
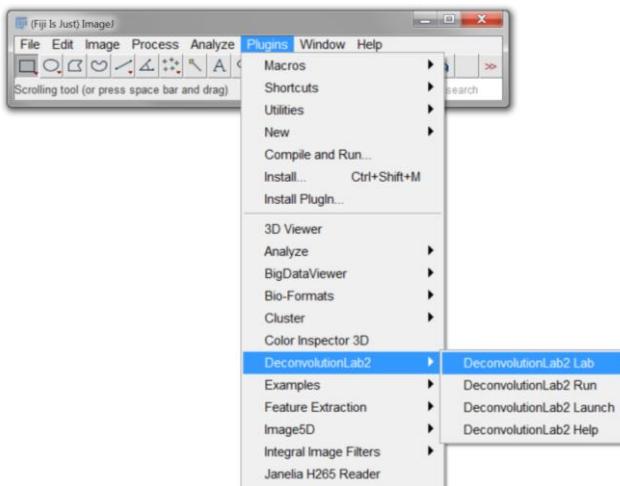
CElegans-FITC.tif

PSF-CElegans-FITC.tif

C. Elegans embryo  
- microtubules

# Deconvolution – try it out

## DeconvolutionLab2

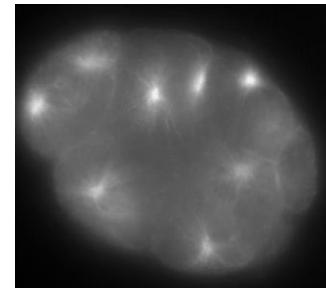


Drag and drop

CElegans-FITC.tif

PSF-CElegans-FITC.tif

C. Elegans embryo  
- microtubules



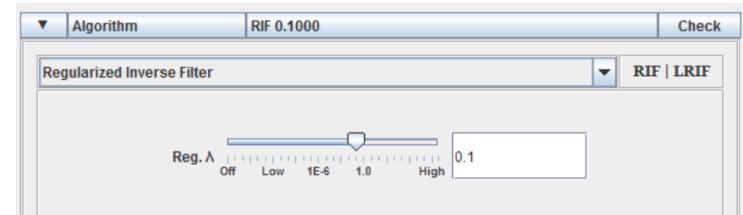
# Deconvolution – try it out (hopefully)

Deconvolution is computationally intense – may be very slow on these machines

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- Try “Regularized Inverse Filter”



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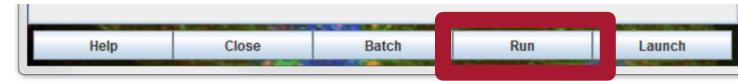
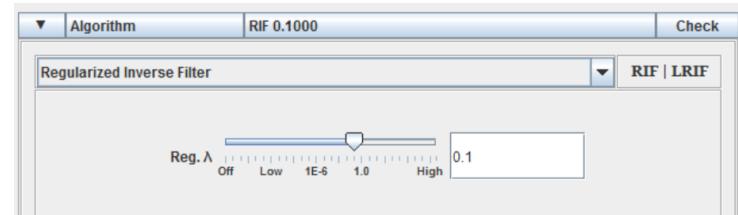
- Try “Regularized Inverse Filter”



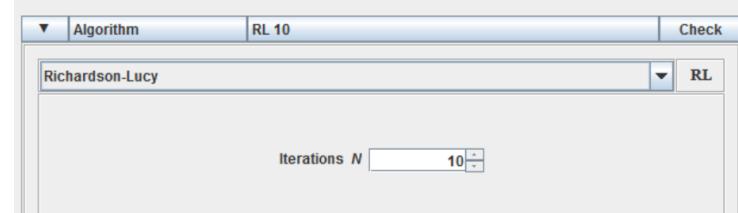
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Deconvolution is computationally intense – may be very slow on these machines

- Try “Regularized Inverse Filter”



- Richardson-Lucy – start with 10 iterations - compare



# Deconvolution – notes

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GIGA – Garbage In, Garbage Out

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- Not always necessary

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# Deconvolution – notes

## GIGA – Garbage In, Garbage Out

- Not always necessary
- Only an estimate of fluorophore distribution
- 100's of algorithms and options – try them out
- Can be very slow
- Requires a good quality measured PSF (estimated PSF's can work for standard microscopes)
  - Routine to measure each day on light-sheet systems (e.g. lattice)

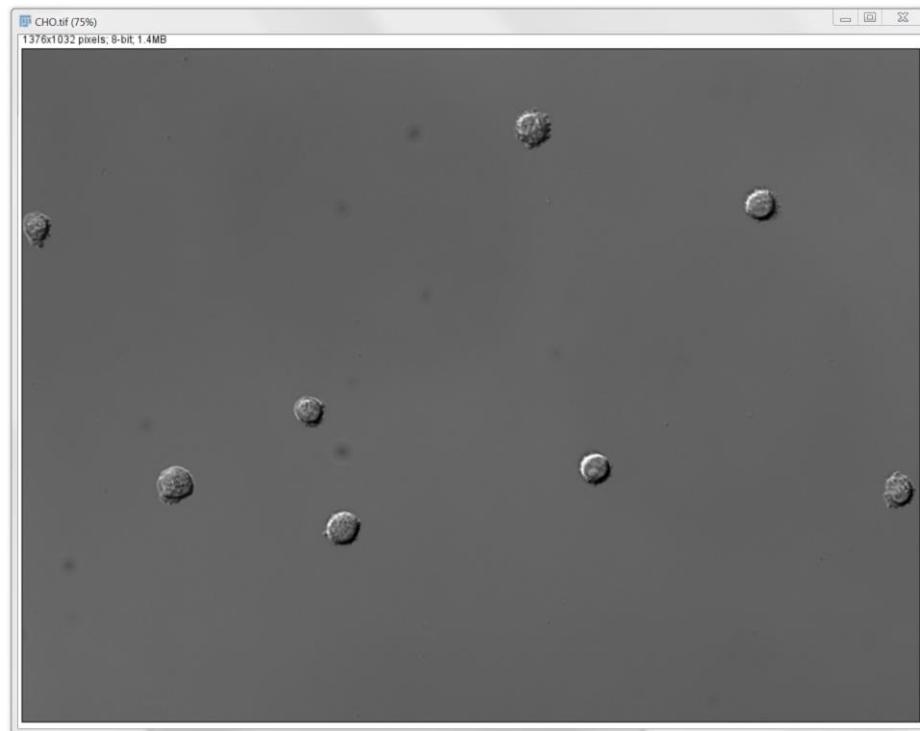
# Machine Learning

- Often intensity alone can't be used for segmentation
- Need “context”
- Train the computer to “recognize” structures

# Machine Learning

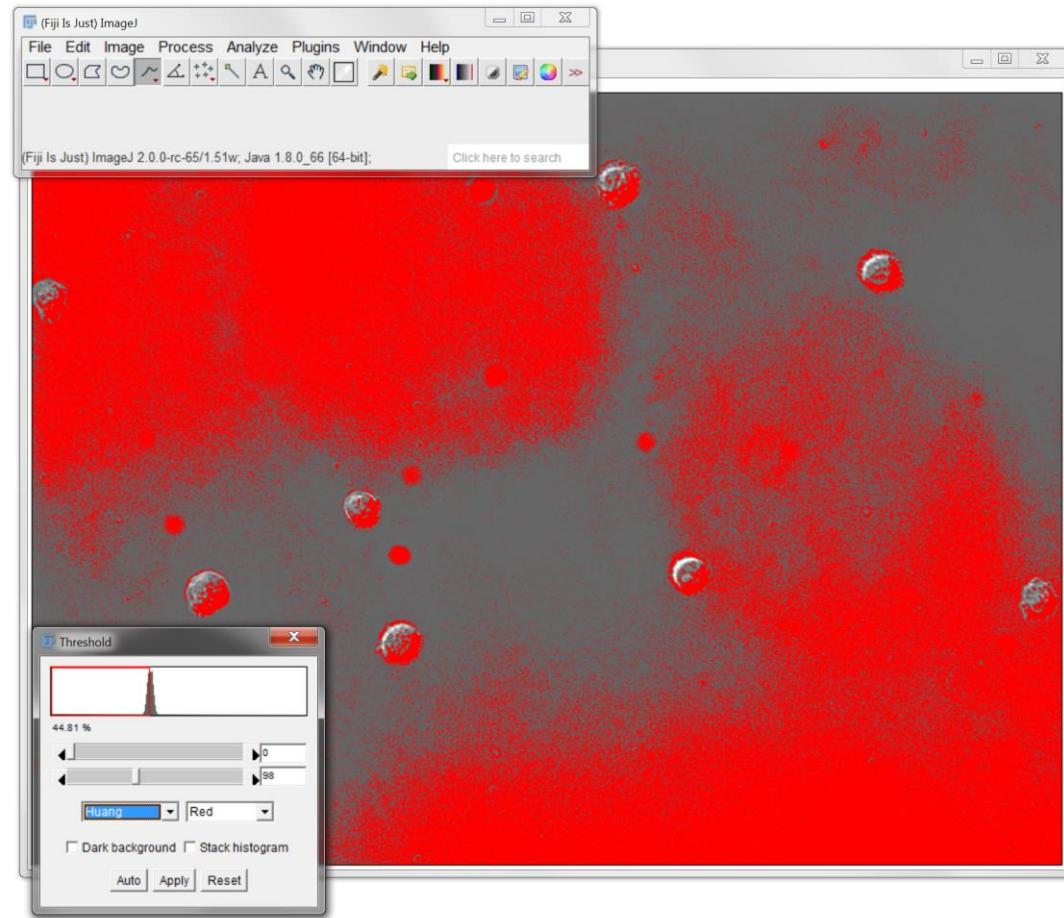
- Often intensity alone can't be used for segmentation
- Need “context”
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- Open CHO.tif

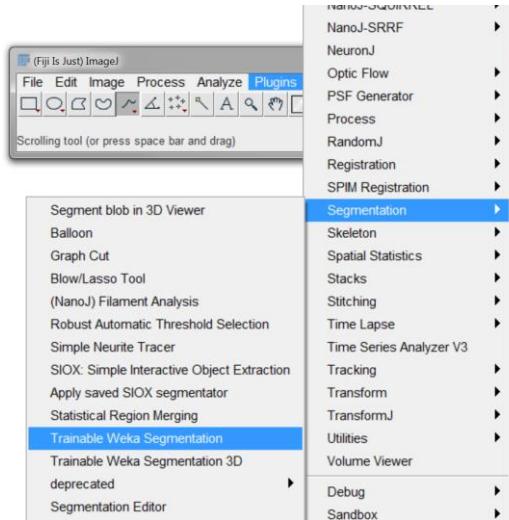


# Machine Learning

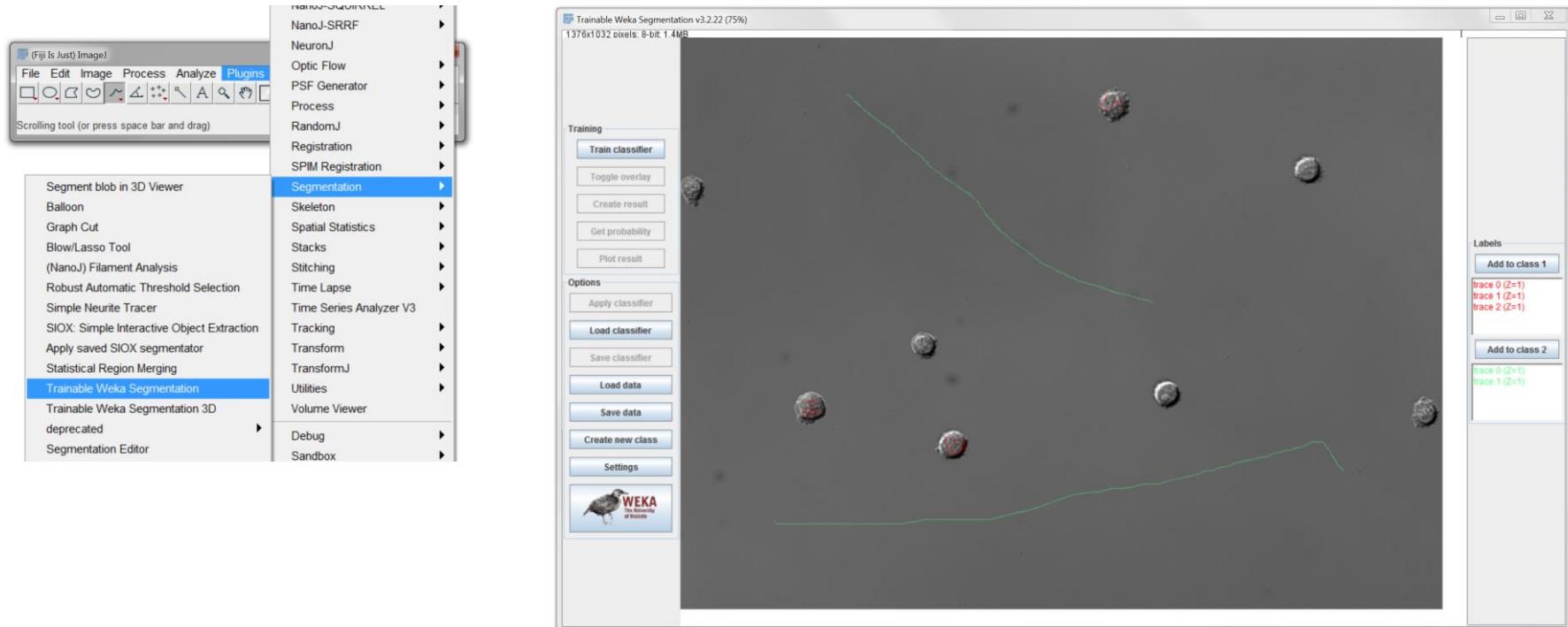
- Try to threshold!



# Machine Learning – Trainable Weka Segmentation



# Machine Learning – Trainable Weka Segmentation

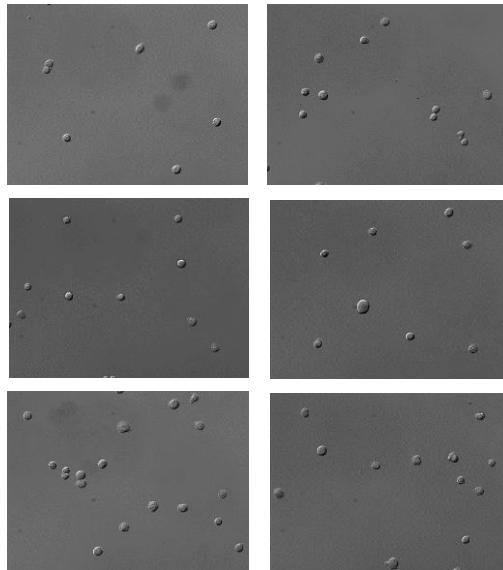


# Machine Learning – Trainable Weka Segmentation



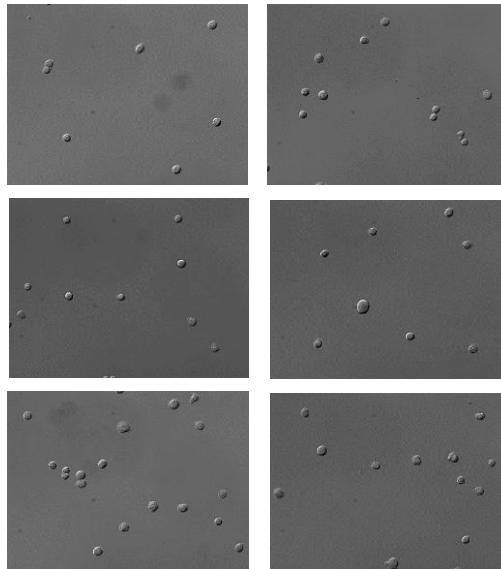
# Machine Learning – Trainable Weka Segmentation

- Lots of training data

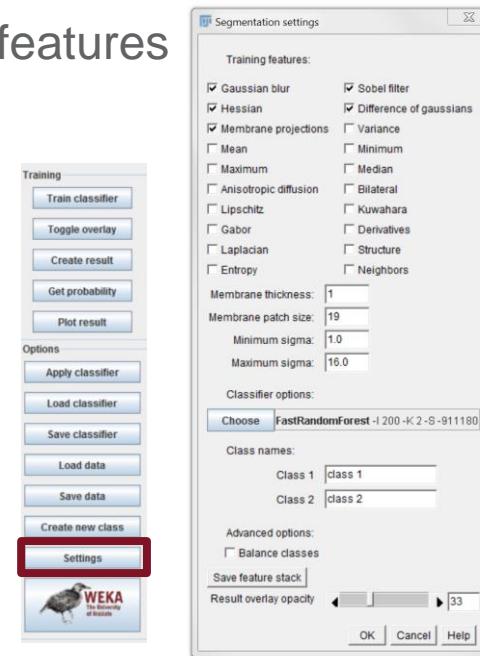


# Machine Learning – Trainable Weka Segmentation

- Lots of training data

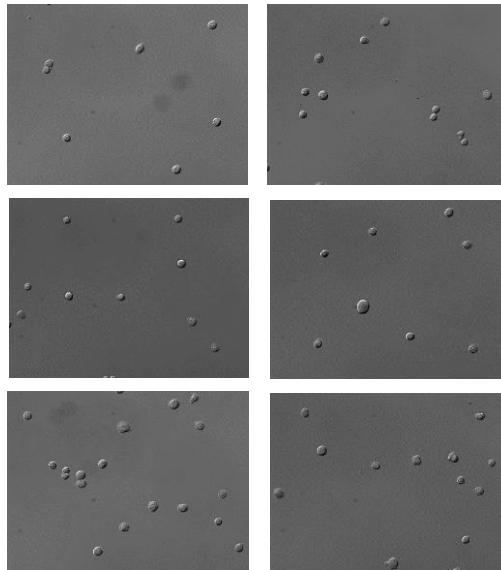


- Choose features carefully

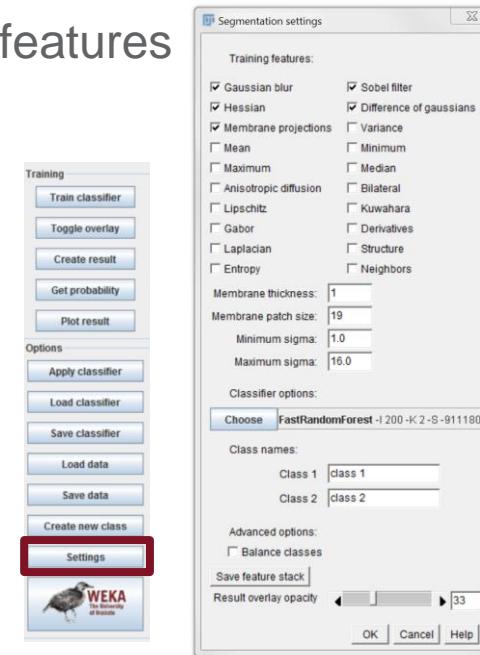


# Machine Learning – Trainable Weka Segmentation

- Lots of training data



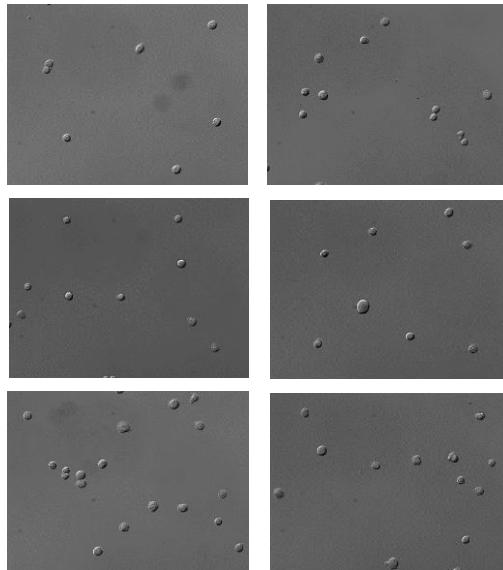
- Choose features carefully



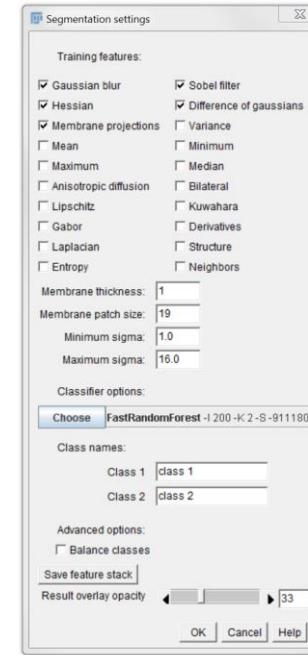
- Will be slow!

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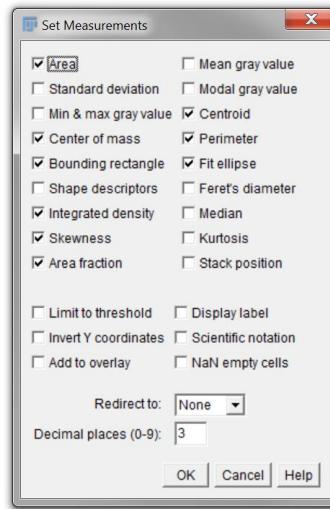
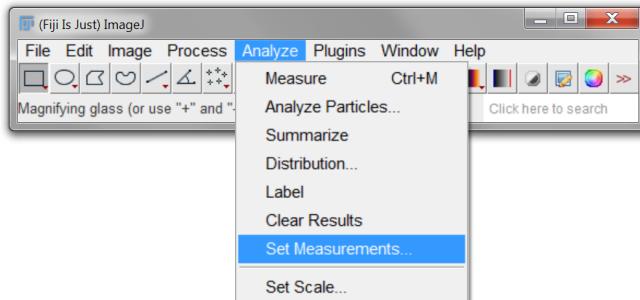


# Analysis (rather than processing)

- Measure segmented objects

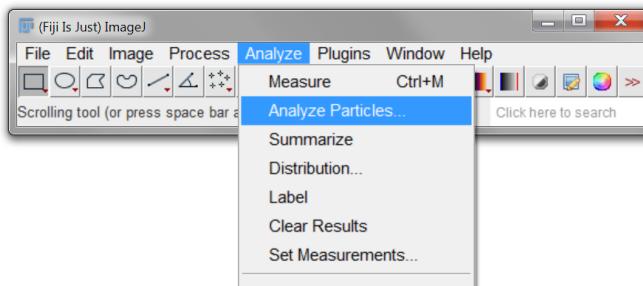
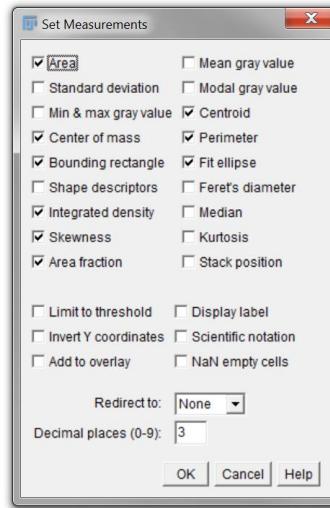
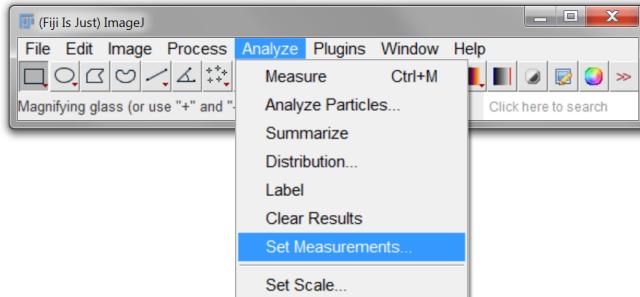
# Analysis (rather than processing)

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- Measure segmented objects



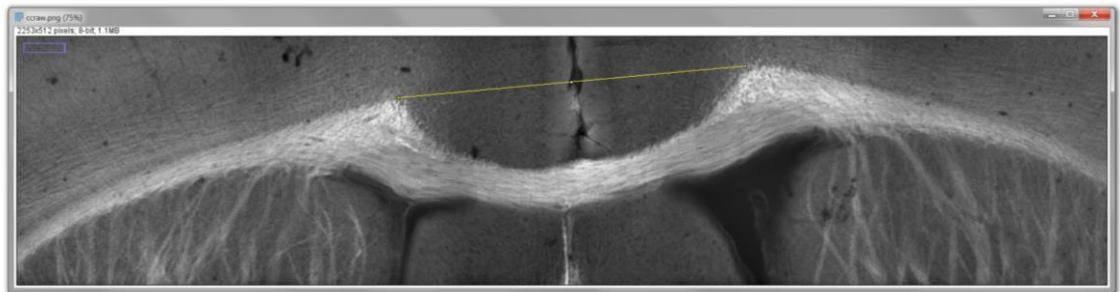
Results

	Area	X	Y	XM	YM	Perim.	BX	BY	Width	Height	Major	Minor	Angle	IntDen	Skew	%Area	RawIntDen
1	306.543	48.743	13.836	48.743	13.836	68.090	39.650	2.210	18.330	23.140	24.526	16.018	63.026	78678.556	NaN	100	4659553
2	304.589	68.498	20.756	68.498	20.756	65.769	57.980	10.920	21.710	18.980	21.734	17.844	152.580	77670.132	NaN	100	4595865
3	228.302	107.863	23.952	107.863	23.952	60.395	96.720	17.680	22.100	13.130	22.568	12.881	173.225	58217.046	NaN	100	3444795
4	282.872	80.092	50.905	80.092	50.905	65.496	69.940	41.080	20.670	20.150	24.379	14.773	43.674	72192.424	NaN	100	4266190
5	214.275	41.559	48.428	41.559	48.428	56.616	31.720	41.730	20.150	13.260	20.445	13.344	9.375	54640.160	NaN	100	3233145
6	348.884	14.667	63.056	14.667	63.056	72.447	1.950	53.950	25.350	18.070	26.180	16.968	17.103	88965.334	NaN	100	5264220
7	274.118	109.123	64.044	109.123	64.044	63.476	98.150	56.160	22.230	15.600	22.567	15.466	9.578	69900.103	NaN	100	4136100
8	309.152	44.635	75.758	44.635	75.758	67.754	33.540	65.780	21.970	19.370	23.901	16.469	33.564	78833.698	NaN	100	4664715
9	236.566	6.737	88.163	6.737	88.163	59.730	0.130	78.390	14.170	20.150	20.893	14.416	74.310	60324.392	NaN	100	3569490
10	296.629	118.505	102.967	118.505	102.967	66.404	106.990	94.900	23.660	16.510	23.782	15.881	15.163	75640.358	NaN	100	4475760
11	259.973	33.502	104.096	33.502	104.096	62.283	22.100	96.330	21.970	15.340	21.982	15.058	1.484	66293.050	NaN	100	3922665
12	322.215	94.661	116.663	94.661	116.663	70.553	82.160	108.680	25.480	15.470	26.381	15.551	0.507	82164.942	NaN	100	4861830
13	225.852	60.121	123.999	60.121	123.999	59.095	49.010	117.000	20.930	13.910	21.251	13.532	174.469	57592.168	NaN	100	3407820

# Analysis

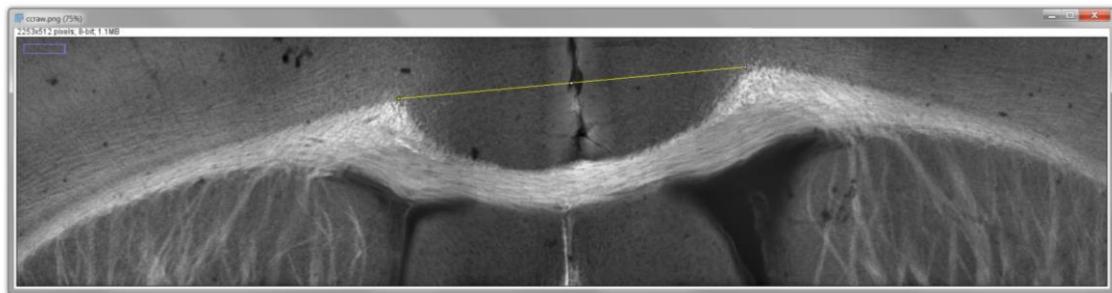
# Analysis

- Measure other objects
  - Draw, then measure



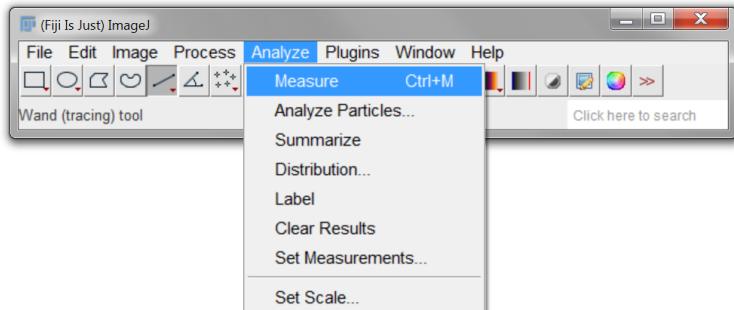
# Analysis

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  - Draw, then measure



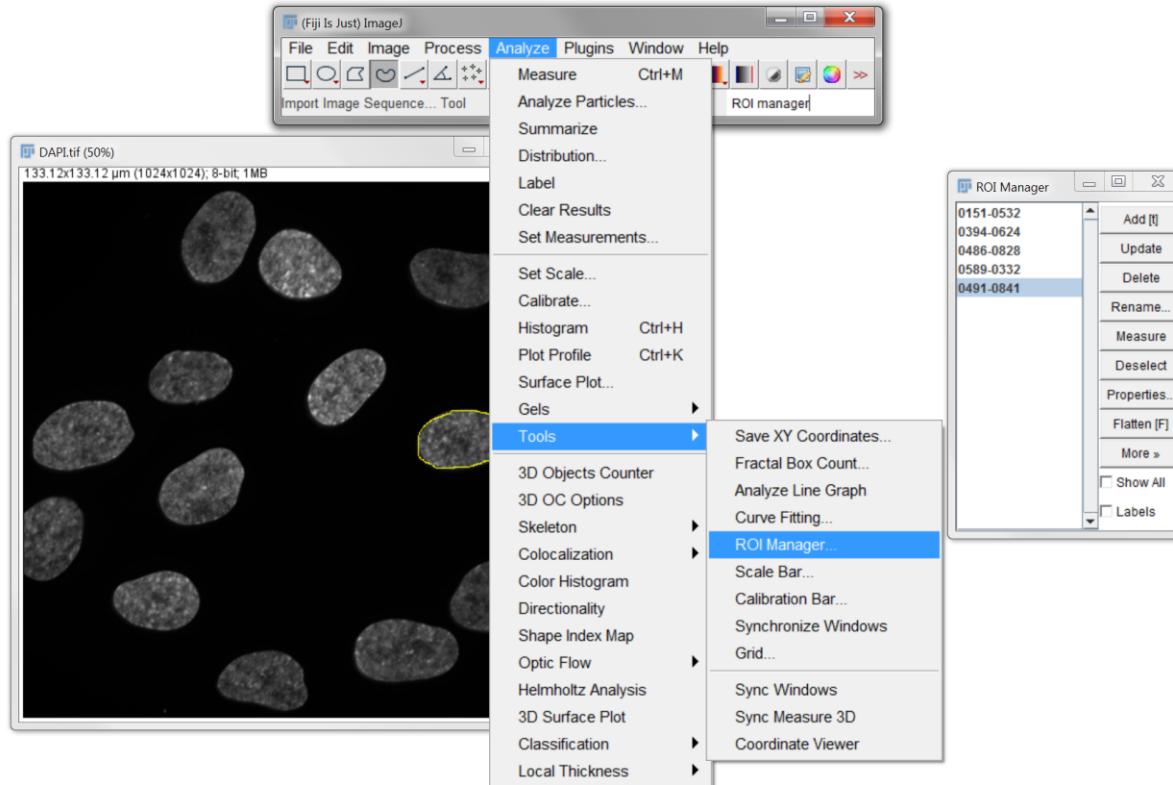
Results

	Area	X	Y	XM	YM	Perim.	BX	BY	Width	Height	Major	Minor	Angle	IntDen	Skew	%Area	RawIntDen	Length
1	720	1146	93	0	0	719.035	788	60	716	66	0	0	5.267	53522.083	0	0	53522.083	719.035



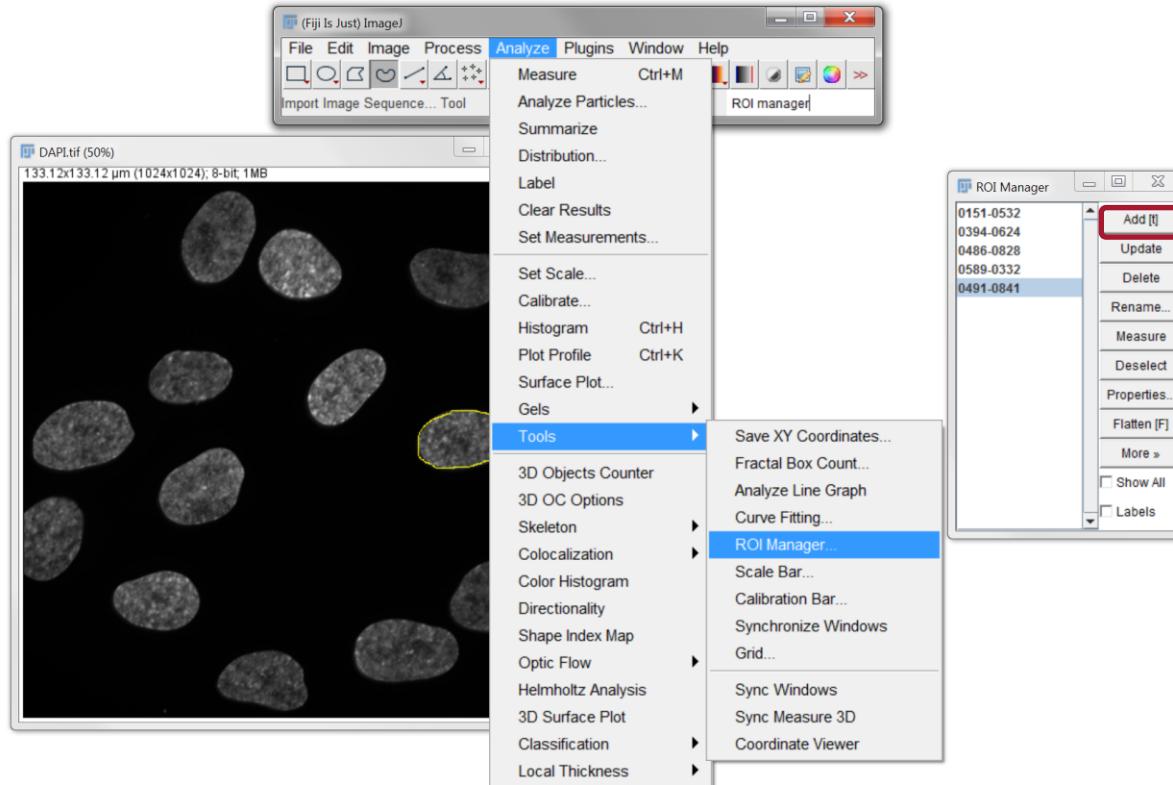
# Analysis

- Multiple objects – ROI manager



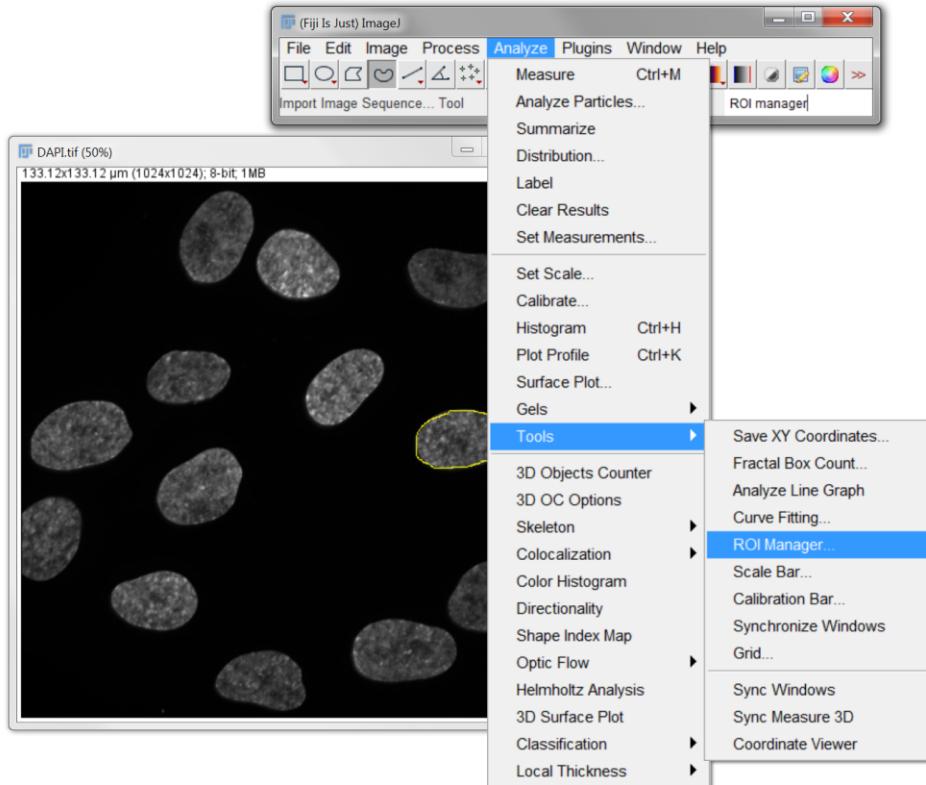
# Analysis

- Multiple objects – ROI manager

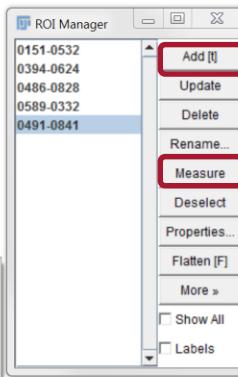


# Analysis

- Multiple objects – ROI manager

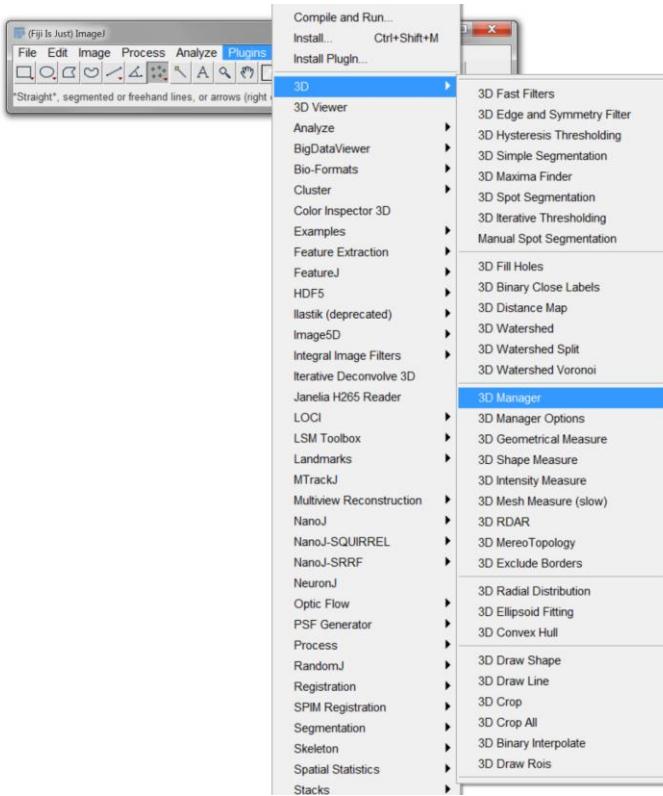


	Area	X	Y	XM	YM	Perim.	BX	BY	Width	Height
1	255.376	109.189	63.968	109.143	63.873	60.619	98.410	56.680	21.840	14.300
2	165.738	69.346	21.431	69.315	21.524	50.334	60.840	11.960	16.770	15.340
3	250.424	80.426	50.511	79.884	50.827	62.513	70.460	42.250	21.320	18.070
4	185.866	107.138	63.003	107.647	63.155	51.511	98.150	56.030	19.110	14.430
5	325.612	43.476	76.691	44.307	75.962	68.361	31.850	67.210	22.620	18.850
6	255.376	109.189	63.968	109.143	63.873	60.619	98.410	56.680	21.840	14.300

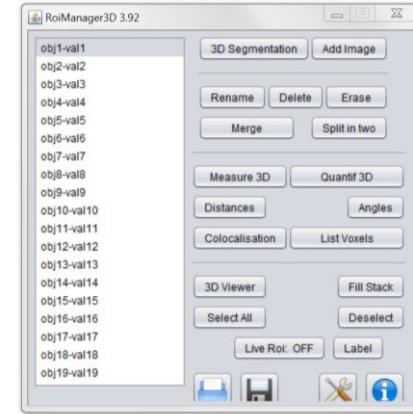
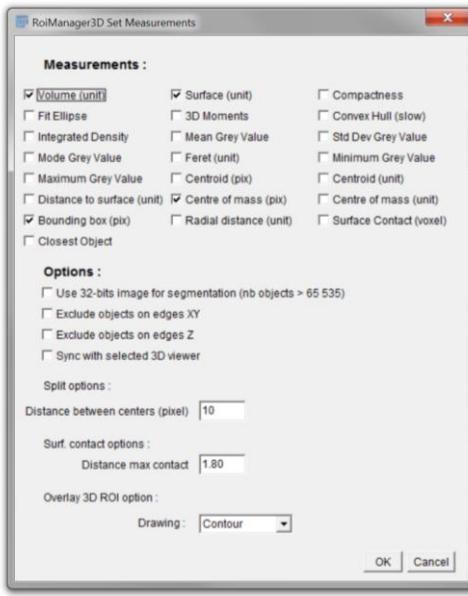
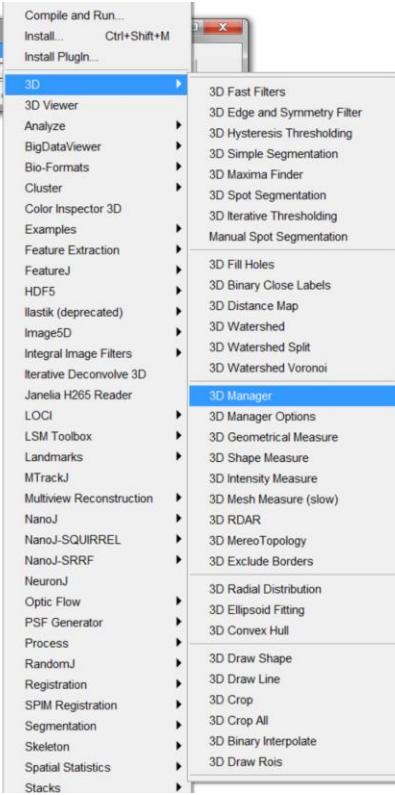
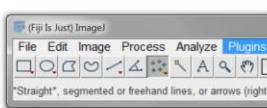


# Analysis – 3D

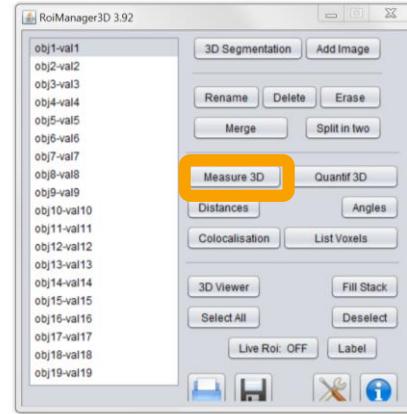
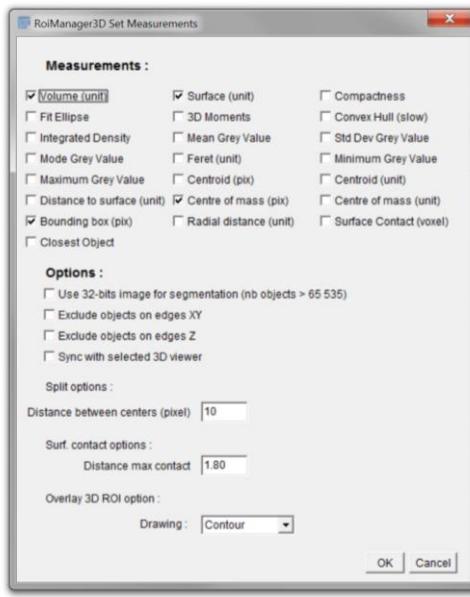
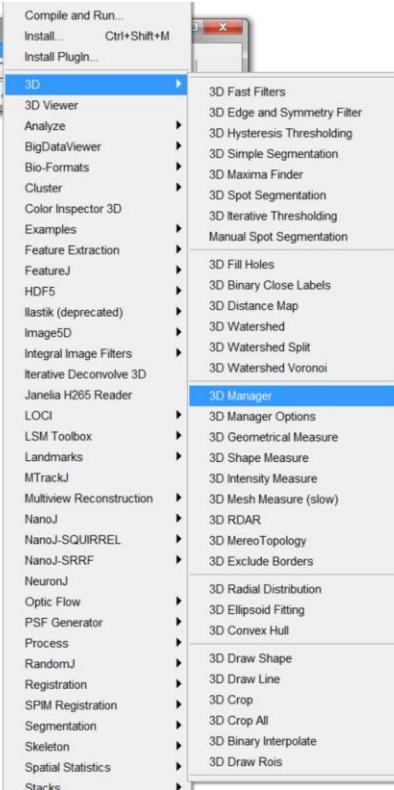
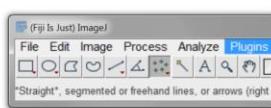
# Analysis – 3D



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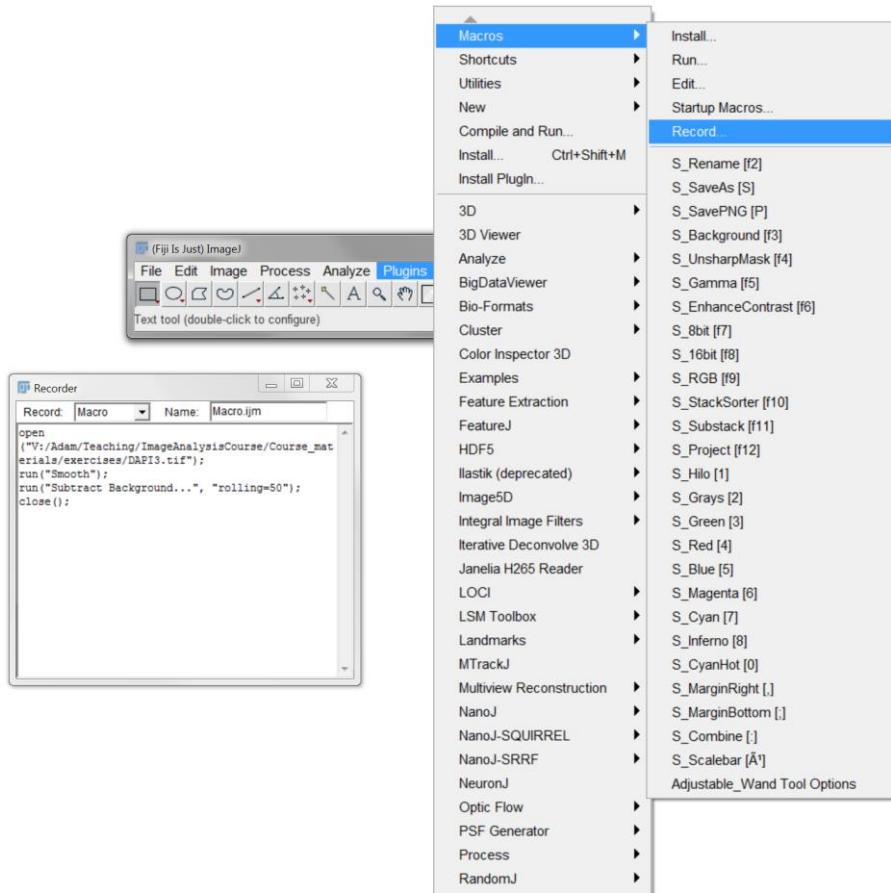
Nb	Obj	Type	Label	Xmin (pix)	Ymin (pix)	Zmin (pix)	Xmax (pix)	Ymax (pix)	Zmax (pix)	VolBound...	RatioVolbox	Vol (unit)	Vol (pix)
0	1	0	obj1-val1	1939	0	0	2021	8	3	2,988	0.201	2,543	602
1	2	0	obj2-val2	1861	33	0	1889	108	5	13,224	0.335	18,742	4436
2	3	0	obj3-val3	1481	256	0	1839	478	10	880,627	0.665	2,472,567	585223
3	4	0	obj4-val4	604	28	0	892	388	10	1,147,619	0.66	3,201,553	757764
4	5	0	obj5-val5	1897	101	0	1988	175	5	41,400	0.109	19,008	4499
5	6	0	obj6-val6	483	640	0	803	848	10	737,979	0.684	2,132,881	504824
6	7	0	obj7-val7	2009	130	0	2047	155	0	1,014	0.738	3.16	748
7	8	0	obj8-val8	1509	864	0	1851	1101	10	897,974	0.735	2,789,632	660268
8	9	0	obj9-val9	1079	633	0	1394	937	10	1,060,180	0.626	2,802,493	663312
9	10	0	obj10-val10	29	830	0	423	1106	10	1,203,565	0.72	3,660,117	866300
10	11	0	obj11-val11	2032	167	0	2047	195	0	464	0.619	1,213	287
11	12	0	obj12-val12	2009	174	0	2020	180	0	84	0.714	0.253	60
12	13	0	obj13-val13	893	171	0	1225	453	10	1,036,629	0.63	2,757,074	652562
13	14	0	obj14-val14	743	1795	0	1086	2022	10	862,752	0.637	2,321,591	549489
14	15	0	obj15-val15	0	1203	0	225	1526	8	659,016	0.526	1,464,334	346588
15	16	0	obj16-val16	514	1011	0	852	1314	10	1,133,616	0.641	3,070.4	726722

# Macros - record

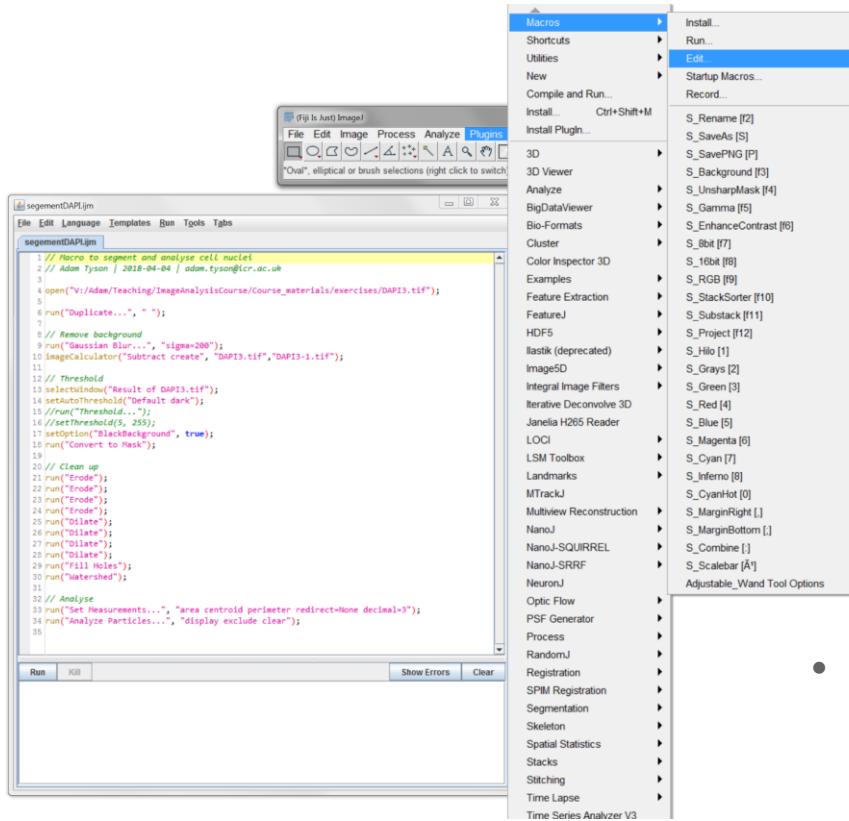
- Automated
- Quicker
- Reproducible (sharing)
- Less chance for bias/mistakes
- Works with most ImageJ plugins
- No coding required (but possible)

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# Macros – edit & run



- Use comments (lines starting with “//”) for notes/explanations

# Macros – edit & run

The image shows three main windows illustrating the use of macros in Fiji:

- Macro Editor:** A screenshot of the Fiji interface showing the "segmentDAPI.jm" macro script. The script performs various operations including opening files, applying Gaussian blur, subtracting backgrounds, thresholding, and performing morphological operations like erosion and dilation.
- Macros Submenu:** A screenshot of the "File" menu open to the "Macros" submenu. It includes options like "Edit", "Run...", and "Install...". Below the submenu is a list of available macros, each preceded by a small icon and its name.
- Plugins Submenu:** A screenshot of the "File" menu open to the "Plugins" submenu. It includes options like "Run", "Edit", and "Install...". Below the submenu is a list of available plugins, each preceded by a small icon and its name.

# How to make your analysis easier – plan!

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- Use the correct method
  - Widefield
  - Confocal
  - Multiphoton
  - TIRF
  - Super resolution (PALM/STORM/SRRF)
  - Light sheet (iSPIM/diSPIM/Lattice)
  - Something else – flow cytometry, biochemistry?

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- Use the correct method
  - Widefield
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- Choose dyes carefully (SNR!)
  - mNeonGreen vs EGFP
  - Alexa 488 vs FITC

# Resolution

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- Resolution depends on:
  - Wavelength of light
  - Refractive index of media ( $n$ )
  - NA of objective
  - (Width of light sheet)

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$$\text{Axial resolution} = \frac{0.88\lambda_{ex}}{n - \sqrt{n^2 - NA^2}}$$

# Resolution

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  - Wavelength of light
  - Refractive index of media (n)
  - NA of objective
  - (Width of light sheet)
- Nyquist theorem
  - Sampling frequency (how many pixels) should be  $\leq$  resolution
  - Modern acquisition software will do this for you.

$$\text{Lateral resolution} = \frac{0.51\lambda_{ex}}{NA}$$

$$\text{Axial resolution} = \frac{0.88\lambda_{ex}}{n - \sqrt{n^2 - NA^2}}$$

# Resolution – However!

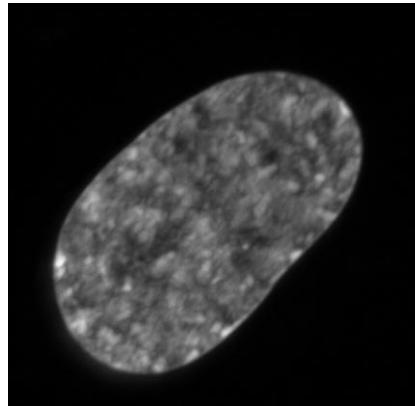
# Resolution – However!

- What do you want to image?

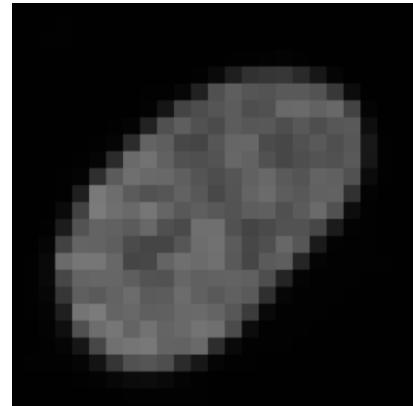
# Resolution – However!

- What do you want to image?

15.4 pix  $\mu\text{m}^{-1}$



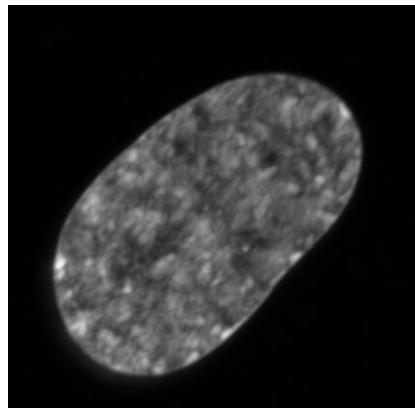
0.916 pix  $\mu\text{m}^{-1}$



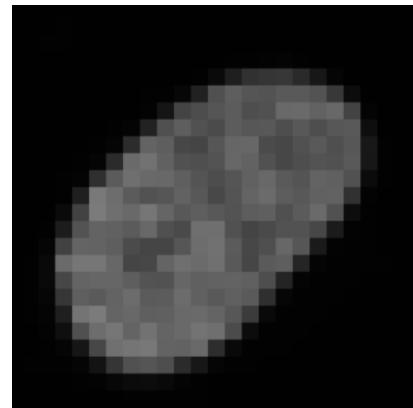
# Resolution – However!

- What do you want to image?

15.4 pix  $\mu\text{m}^{-1}$



0.916 pix  $\mu\text{m}^{-1}$



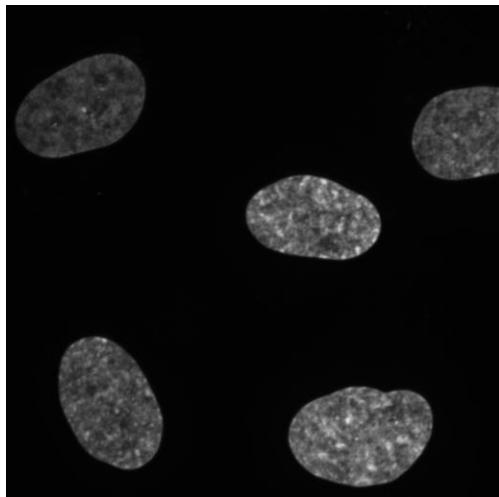
- Same information – 300 x less data
  - Also maybe quicker and more gentle

# Resolution – 3D

- If you want to analyse in 3D:
  - Isotropic resolution

# Resolution – 3D

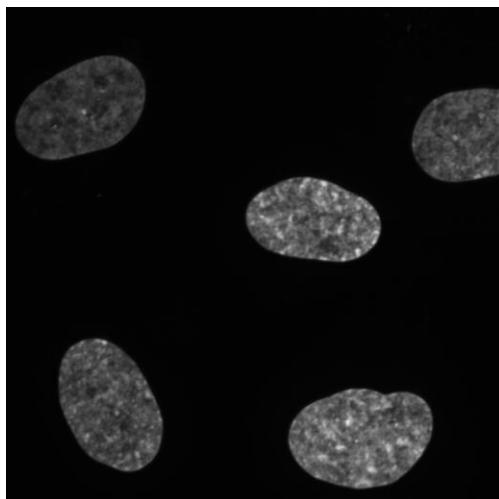
- If you want to analyse in 3D:
  - Isotropic resolution



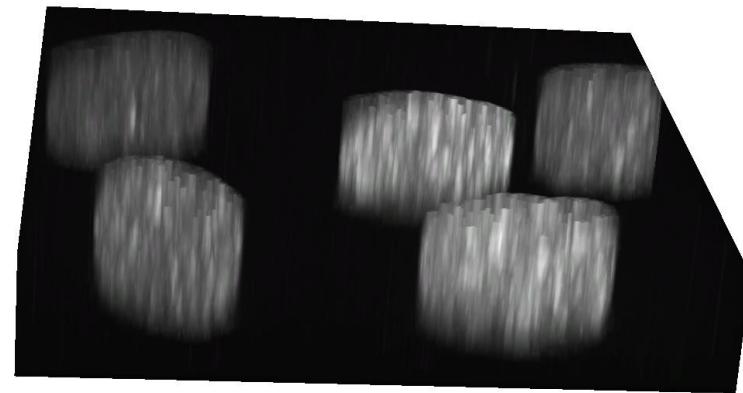
2D

# Resolution – 3D

- If you want to analyse in 3D:
  - Isotropic resolution



2D



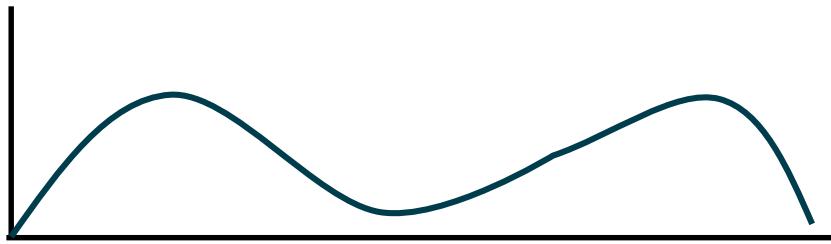
3D

## Note – 3D analysis

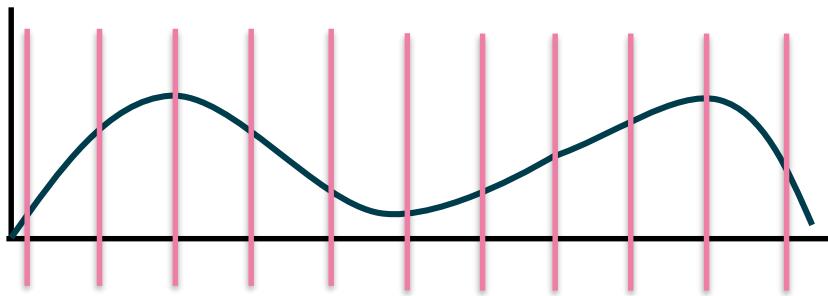
- Analysis almost always exactly the same as 2D:
  - Takes longer
  - Need to view in 3D to check
  - 3D not necessarily supported by software package

# Resolution – temporal

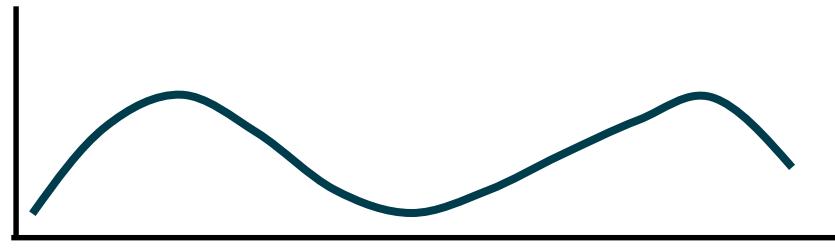
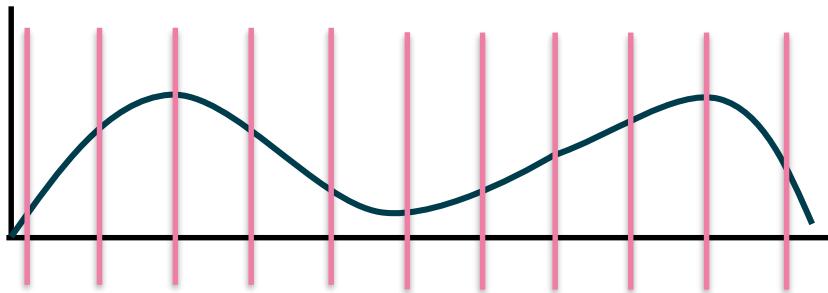
## Resolution – temporal



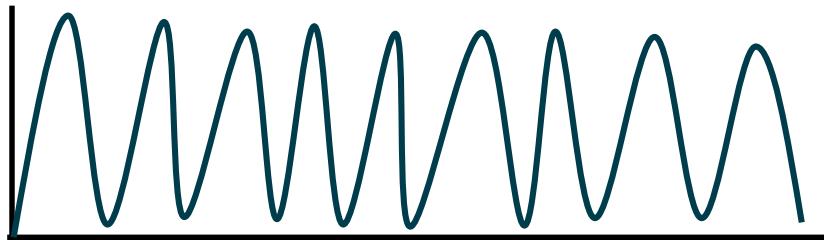
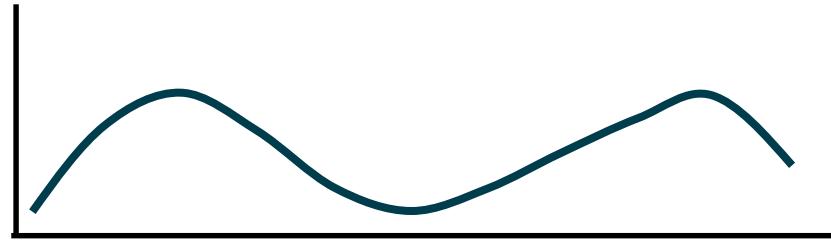
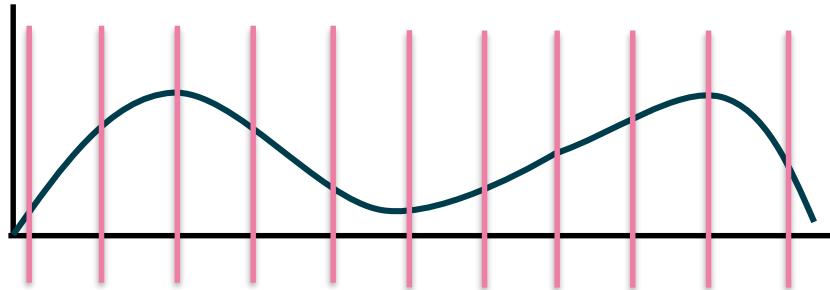
# Resolution – temporal



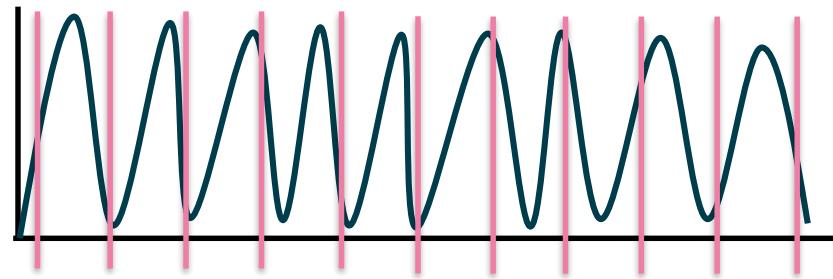
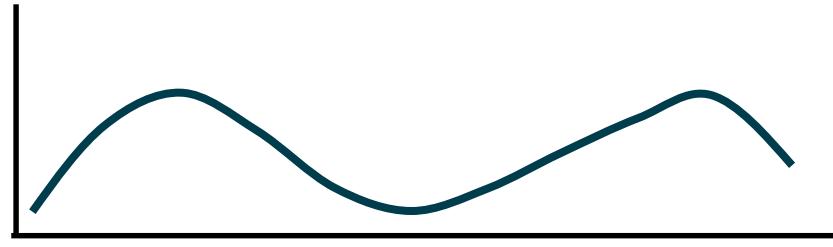
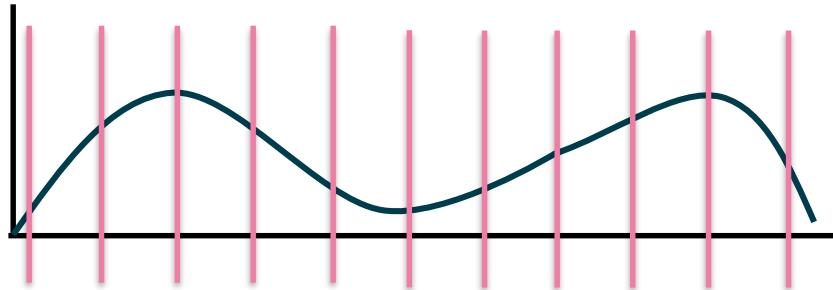
## Resolution – temporal



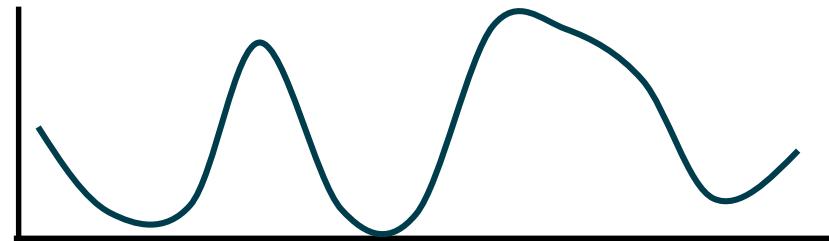
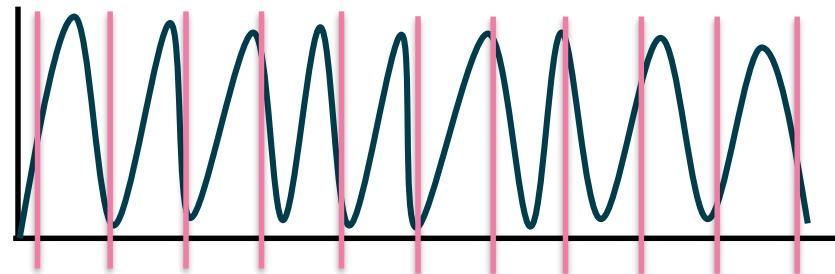
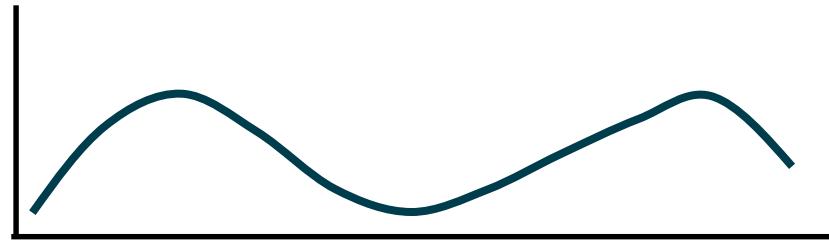
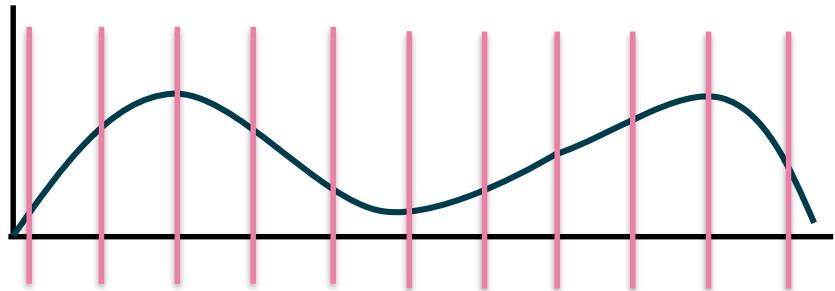
## Resolution – temporal



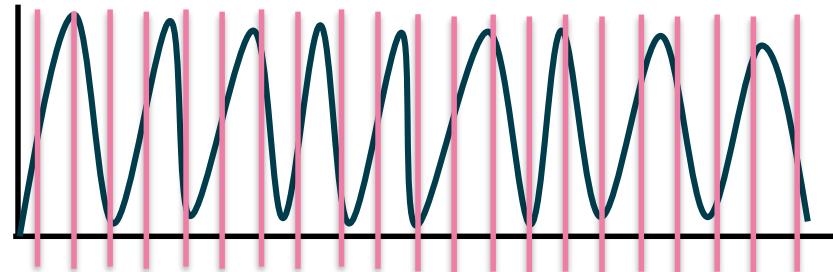
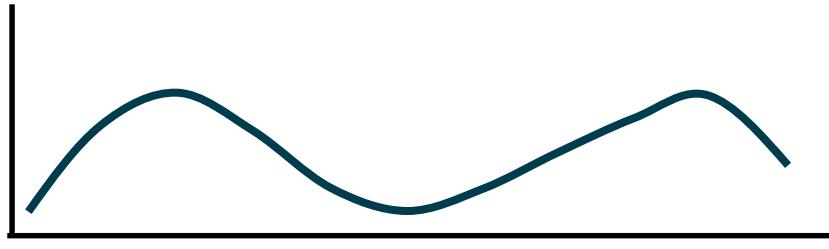
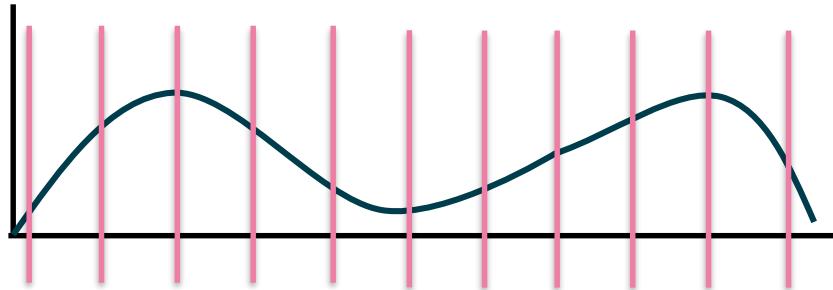
## Resolution – temporal



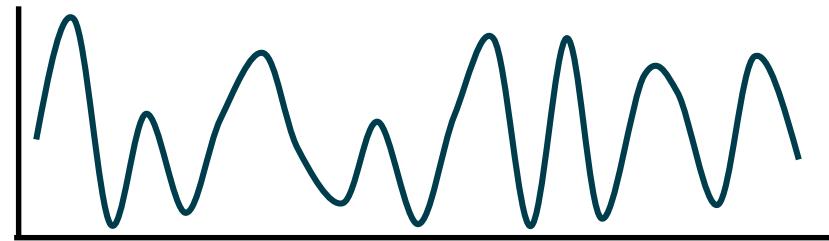
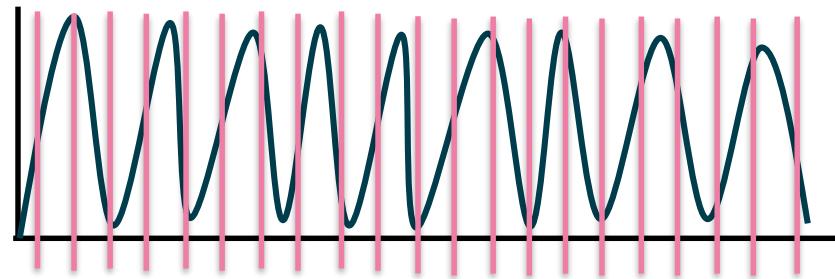
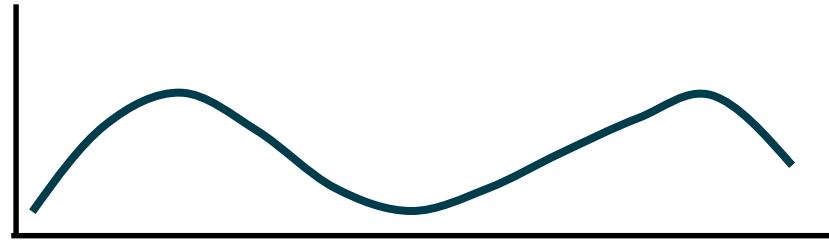
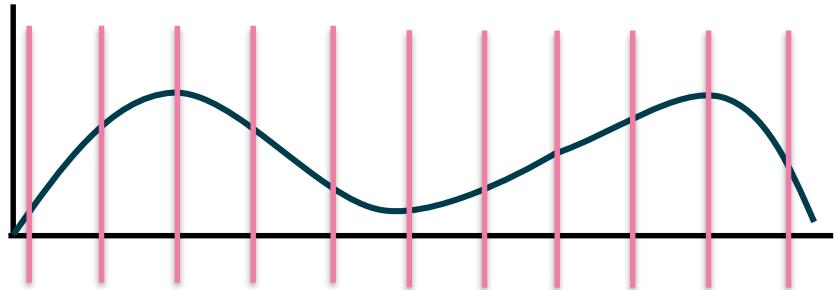
## Resolution – temporal



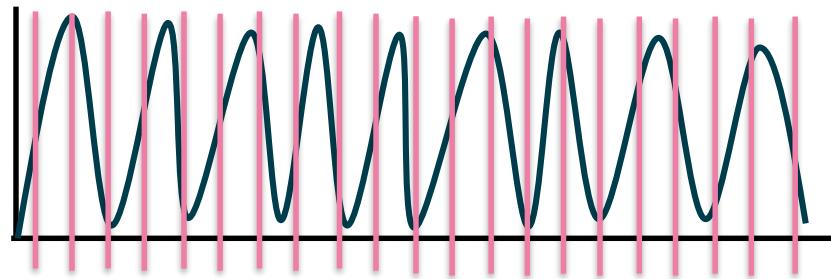
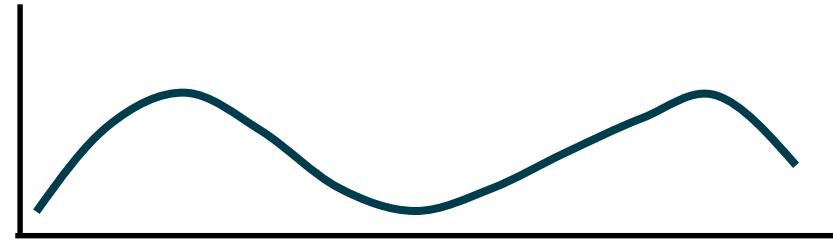
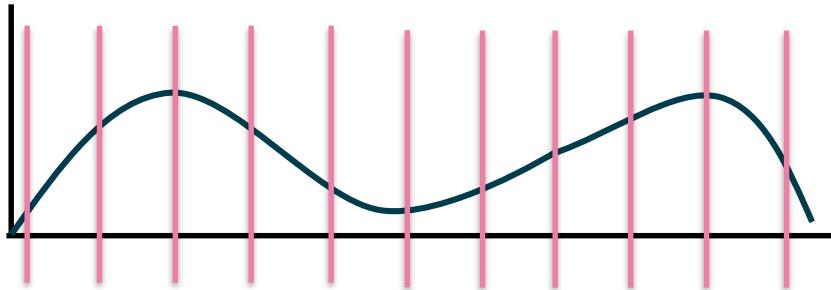
## Resolution – temporal



## Resolution – temporal



# Resolution – temporal



- Accurate sampling vs photobleaching & data size

# Signal to noise ratio (SNR)

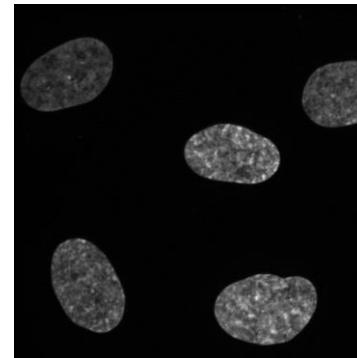
- Resolution irrelevant if you can't detect structures of interest

# Signal to noise ratio (SNR)

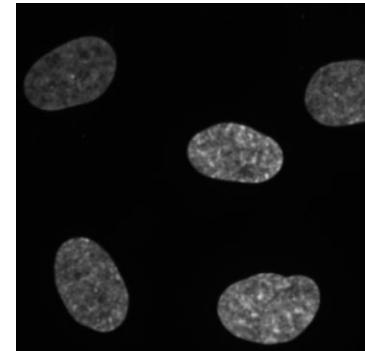
- Resolution irrelevant if you can't detect structures of interest

High  
SNR

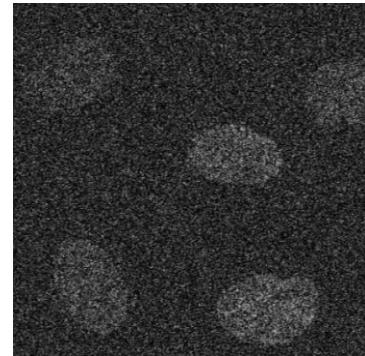
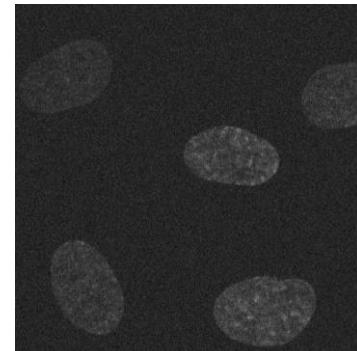
High resolution



Low resolution



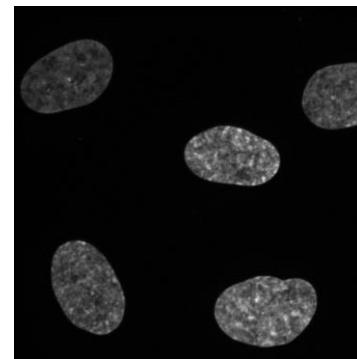
Low  
SNR



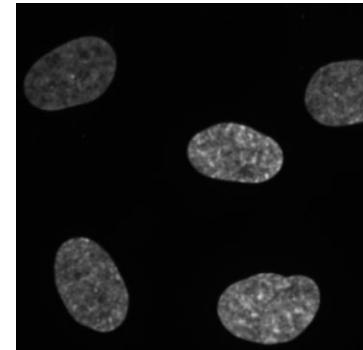
# Signal to noise ratio (SNR)

- Resolution irrelevant if you can't detect structures of interest

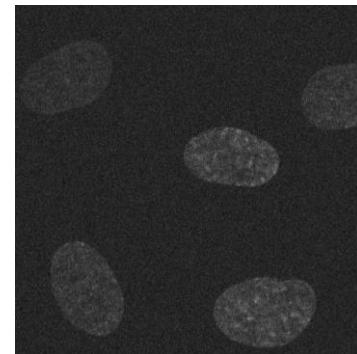
High  
SNR



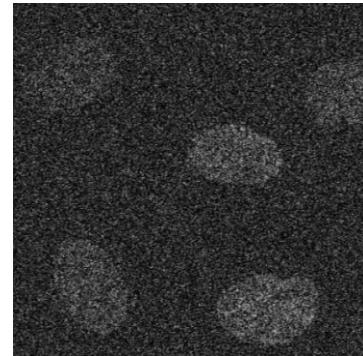
Low resolution



Low  
SNR



- Contrast often the most important aspect of a good image



# Analysis as a method

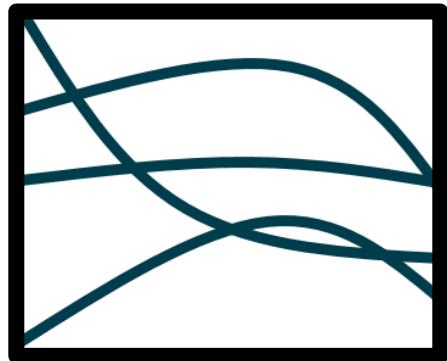
# Analysis as a method

“True” image



# Analysis as a method

“True” image



Observed  
widefield image

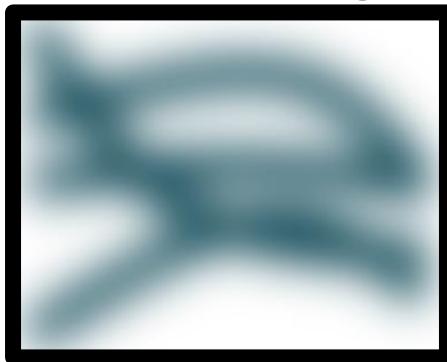


# Analysis as a method

“True” image



Observed  
widefield image



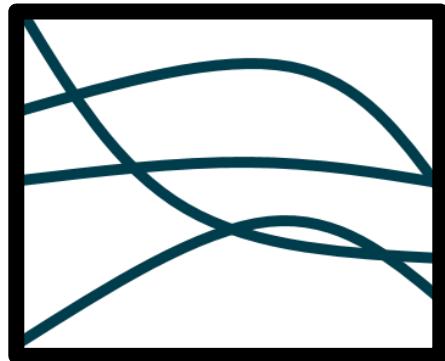
STORM/PALM  
aquisition



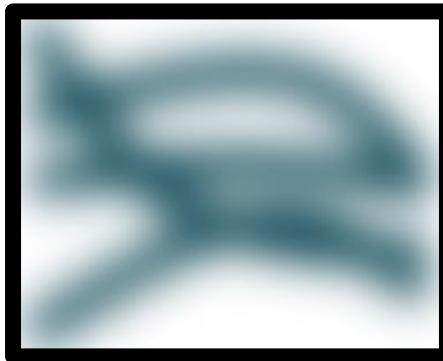
STORM/  
PALM

# Analysis as a method

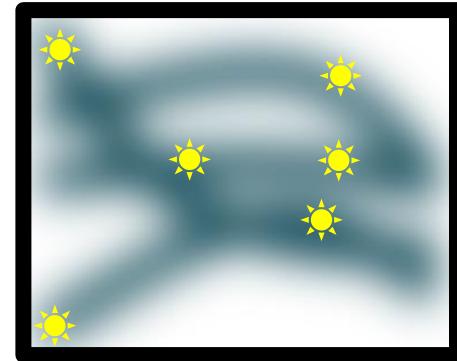
“True” image



Observed  
widefield image



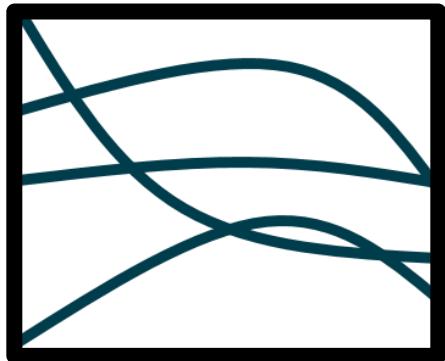
STORM/PALM  
aquisition



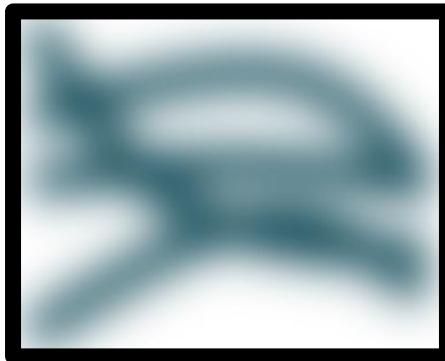
STORM/  
PALM

# Analysis as a method

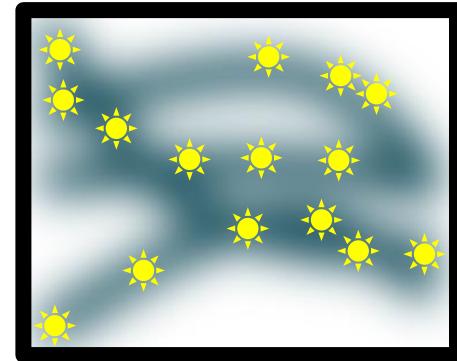
“True” image



Observed  
widefield image



STORM/PALM  
aquisition



STORM/  
PALM

# Analysis as a method

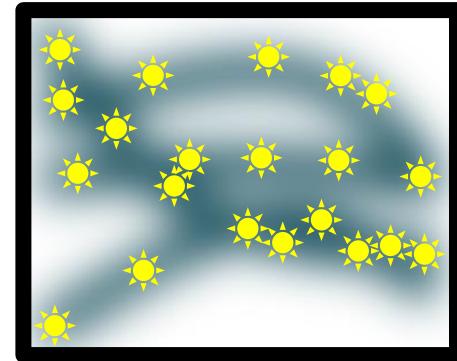
“True” image



Observed  
widefield image



STORM/PALM  
aquisition



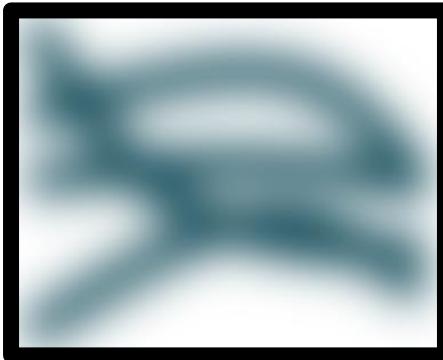
STORM/  
PALM

# Analysis as a method

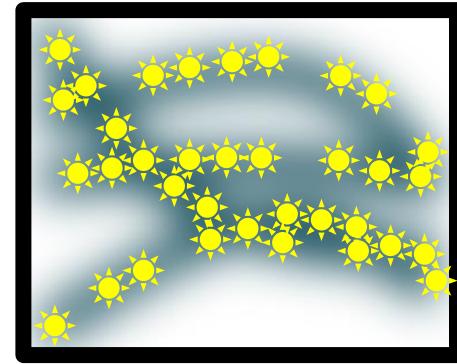
“True” image



Observed  
widefield image



STORM/PALM  
aquisition



STORM/  
PALM

# Analysis as a method

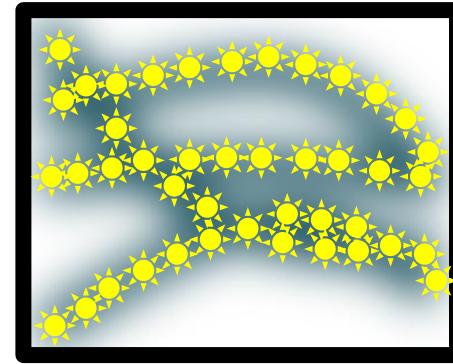
“True” image



Observed  
widefield image



STORM/PALM  
aquisition



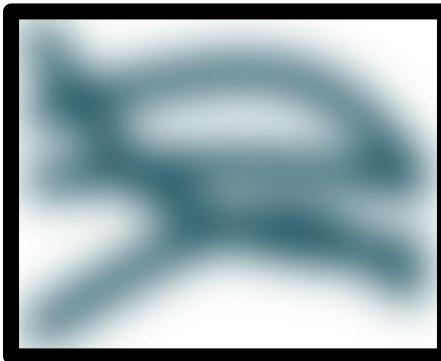
STORM/  
PALM

# Analysis as a method

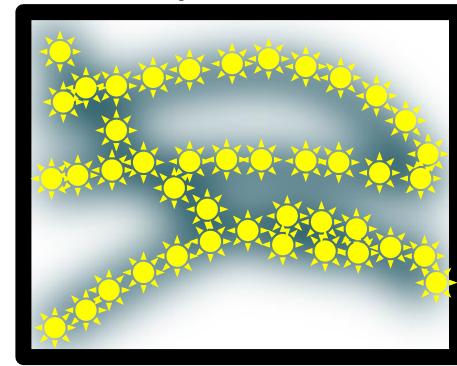
“True” image



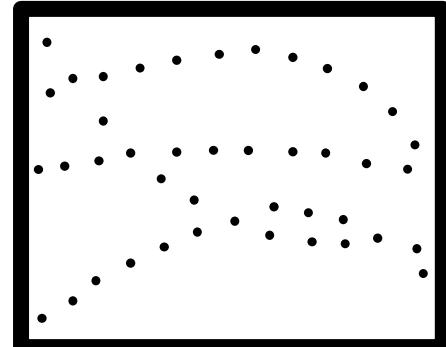
Observed  
widefield image



STORM/PALM  
aquisition



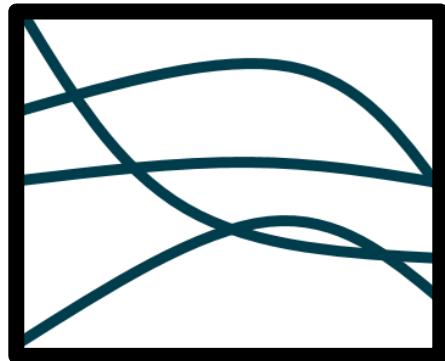
Localised fluorophores



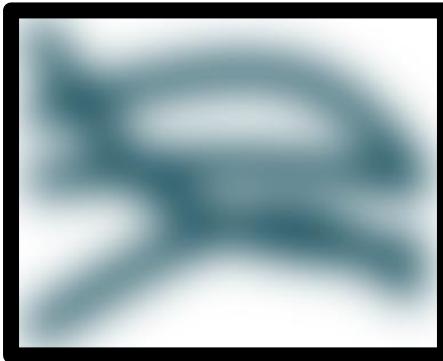
STORM/  
PALM

# Analysis as a method

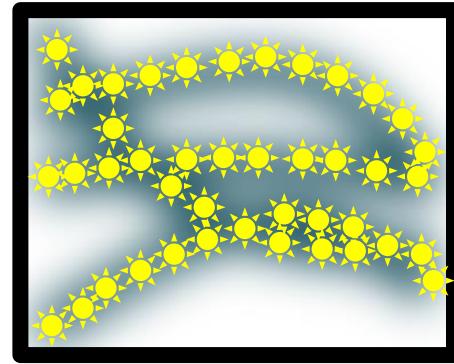
“True” image



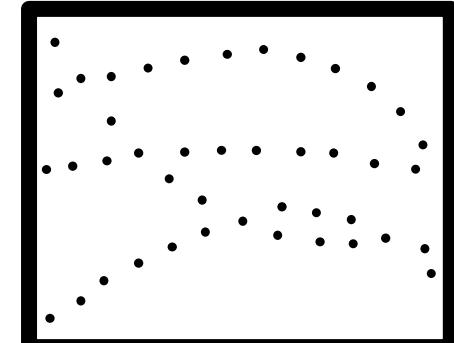
Observed widefield image



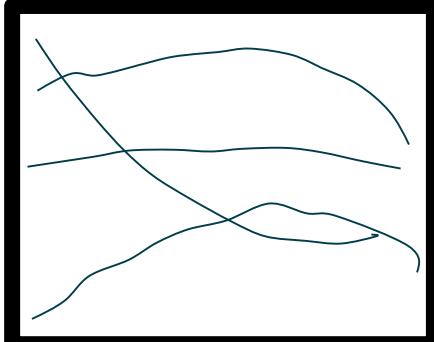
STORM/PALM aquisition



Localised fluorophores



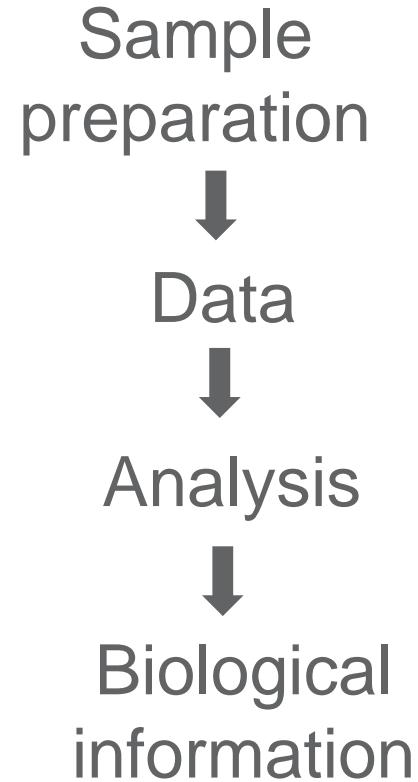
Reconstructed image



STORM/  
PALM

# First (and most important lesson) – again!

Test your analysis  
**before** you  
acquire data!



Any other courses?

## Any other courses?

- General – more advanced

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- 3D

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- General – more advanced
- 3D
- Big datasets

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- General – more advanced
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- General – more advanced
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- Writing custom scripts – MATLAB / Python etc

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- Anything else?