

Introduction to image analysis

Adam Tyson

April 2018

Course website

bit.ly/ICR-analysis

- Slides
- Sample images
- Software

The screenshot shows a website page for 'Introduction to Image Analysis' at the Institute of Cancer Research. The page includes a logo, navigation links for course materials and details, a summary of the course, and a list of aims and contents.

ICR The Institute of Cancer Research

Course Materials

- Course slides (.pdf)
- Sample images (.zip)
- FLIR download (OSX)
- FLIR download (Windows)
- FLIR download (Linux)
- Deconvolution toolbox

Introduction to Image Analysis

Details

April 17th, 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/FLIR
- 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

Aims

- Introduction to fundamental image analysis principles
- Using ImageJ/FIJI (but everything is general)
- Convert biological question into computational problem

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Disclaimer:

- Some parts will be very simple. Some parts won't.
- The aim is to help you understand image analysis for your PhD/postdoc/career, not to be able to use ImageJ by the end of the day.
- Like anything, requires practice.

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Email me whenever – adam.tyson@icr.ac.uk

Questions

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- Which system(s)?
 - Confocal/spinning disk
 - Multiphoton
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 - Any others?

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Questions

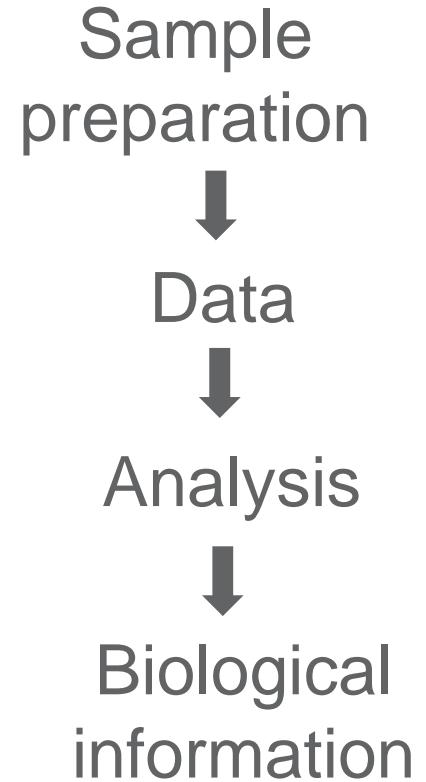
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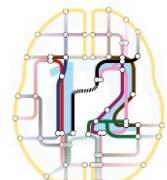
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- A bit / no / lots of experience?
- Any specific aims?

First (and most important lesson)

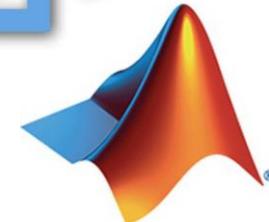
Test your analysis
before you
acquire data!



Software



SlideBook

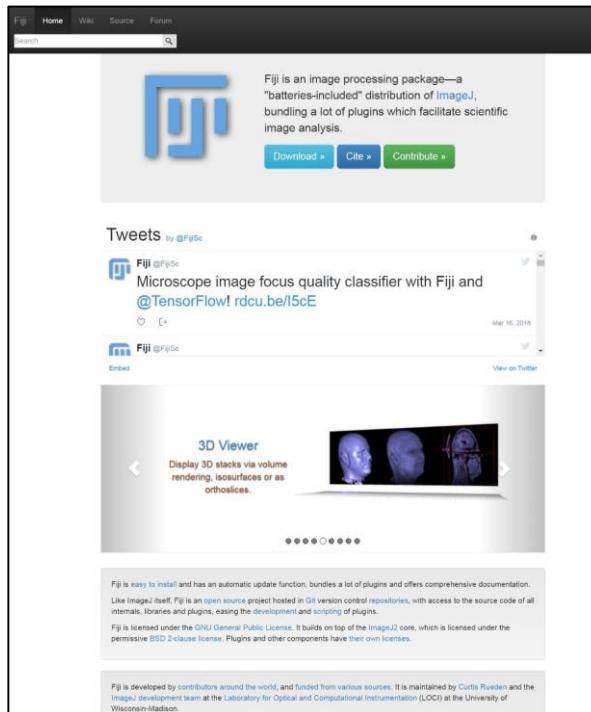


Imaris 9

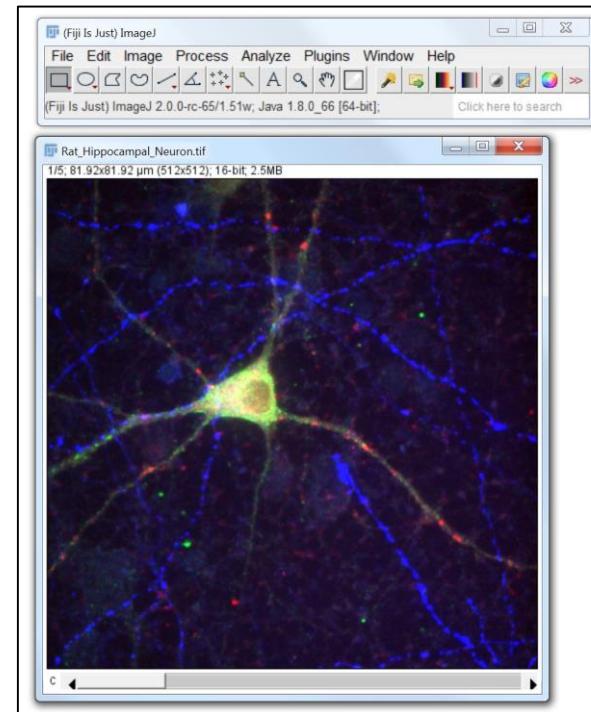
julia



FIJI (FIJI is just ImageJ)

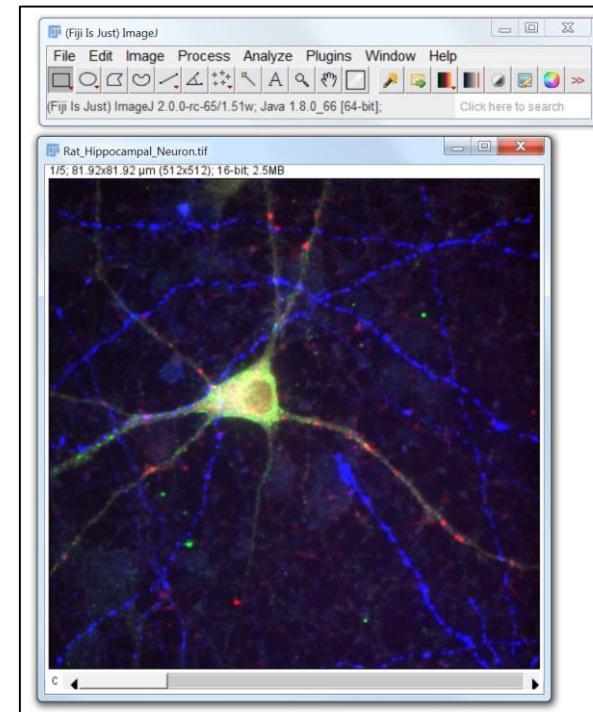
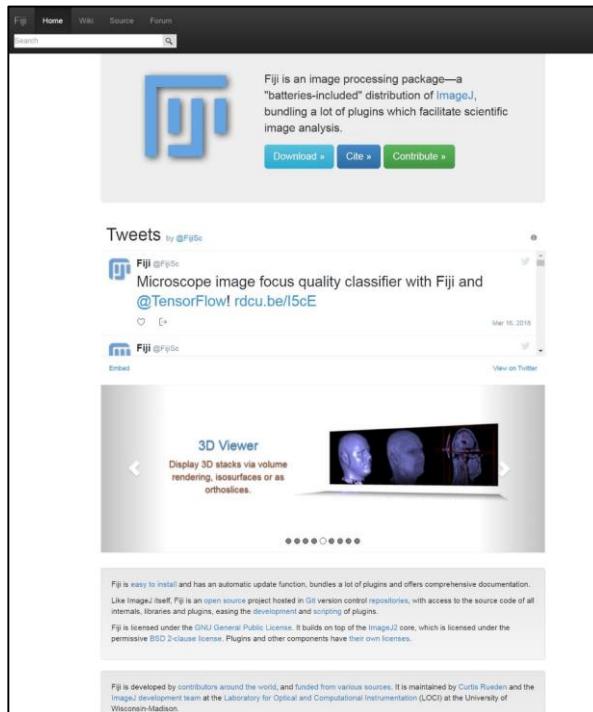


<https://fiji.sc/>



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

FIJI (FIJI is just ImageJ)



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

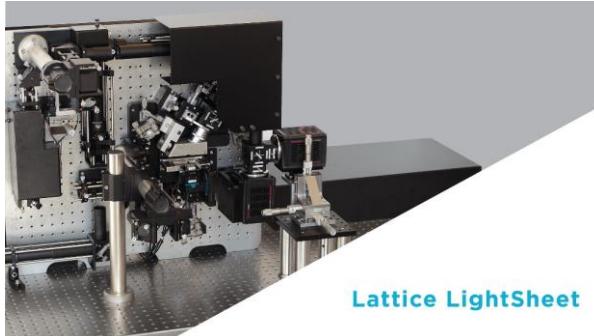
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Why computational image analysis?

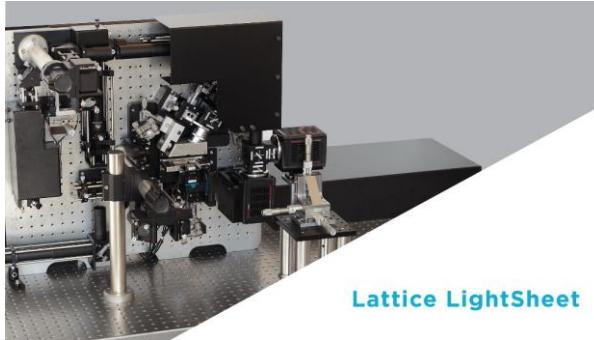
Why computational image analysis?

- 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

Axon Initial Segment Kv1 Channels Control Axonal Action Potential Waveform and Synaptic Efficacy

Maarten H.P. Kole,^{1,2} Johannes J. Letzkus,^{1,2} and Greg J. Stuart^{1,*}

A Selective Filter for Cytoplasmic Transport at the Axon Initial Segment

Ai-hong Song,¹ Dong Wang,¹ Gang C...
AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity

Activity-dependent regulation of the axon initial segment fine-tunes neuronal excitability

Kristian L. Hedstrom,¹ Yasuhiro Ogawa,^{1,2} and Matthew N. Rasband^{1,2}

Matthew S. Grubb¹ & Juan Burrone¹

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

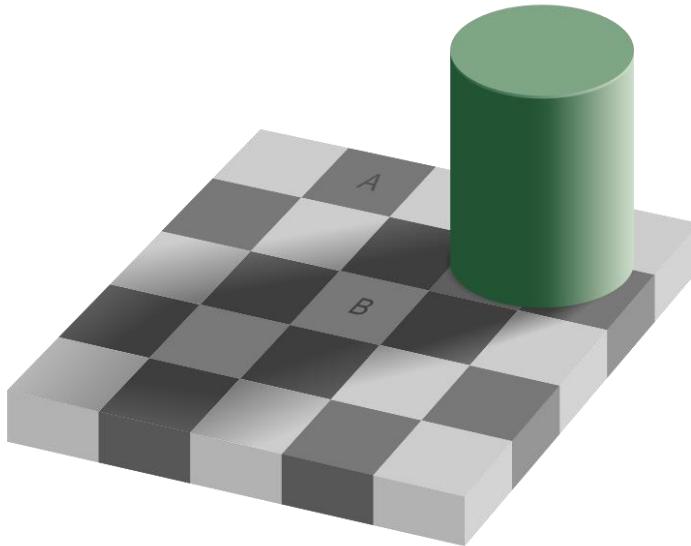
Maarten H P Kole¹, Susanne U Ilshner¹, Björn M Kampa^{1,4}, Stephen R Williams^{1,2}, Peter C Ruben^{1,3} & Greg J Stuart¹

Directs GABAergic Excitation in the Axon Initial Segment

¹Department of Neuroscience, University of Connecticut Health Center, Farmington, CT 06032
²Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030

Why computational image analysis?

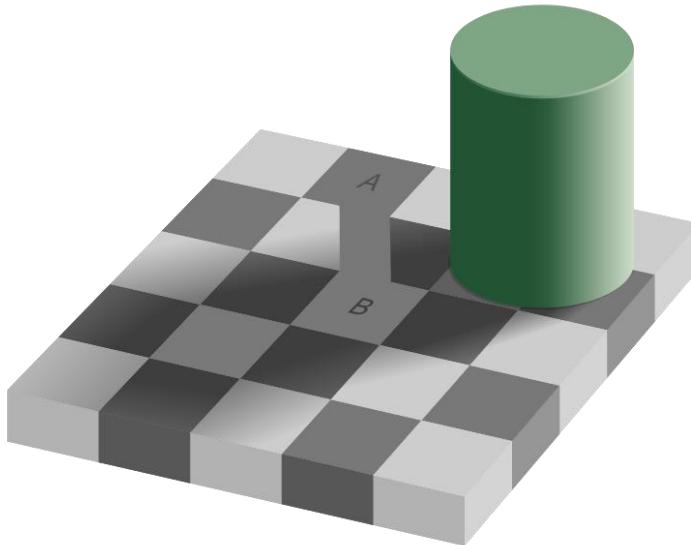
- Visual system relies heavily on context and relative changes.



Checker shadow illusion, Edward Adelson 1995.

Why computational image analysis?

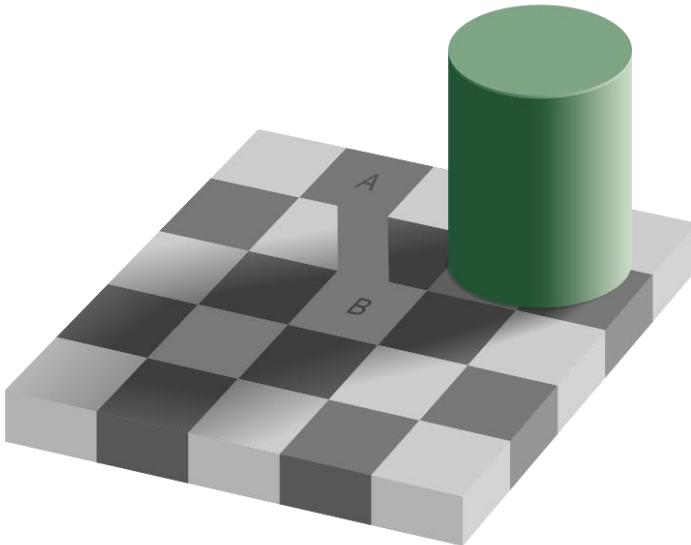
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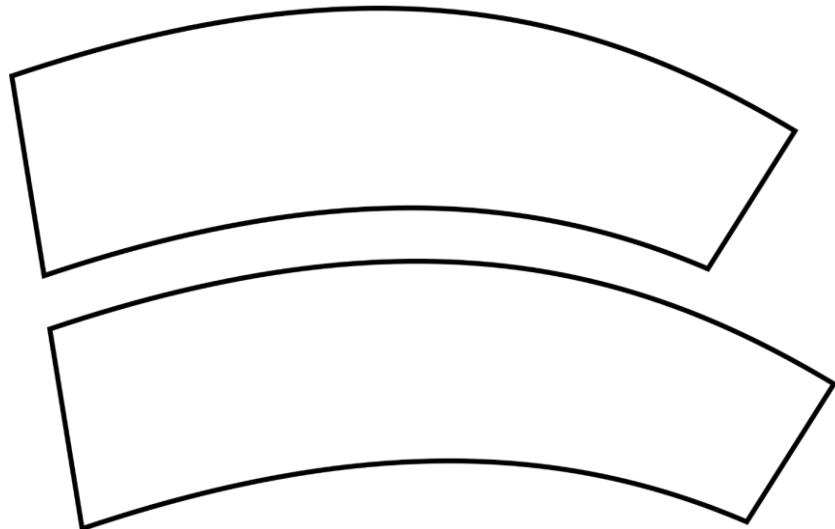
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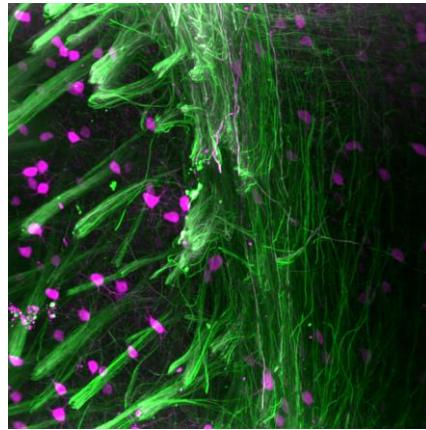


Checker shadow illusion, Edward Adelson 1995.

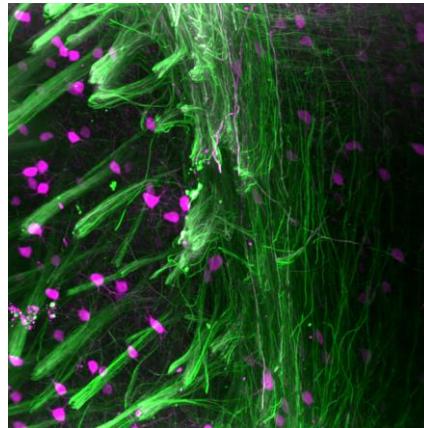
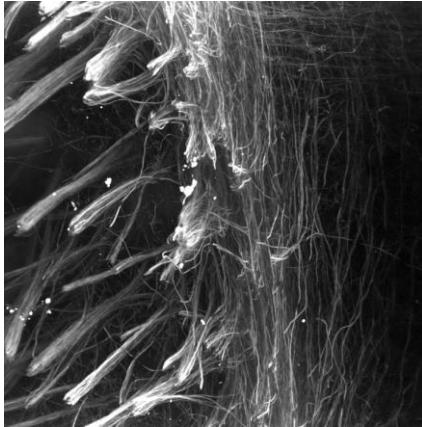


Jastrow illusion, Joseph Jastrow (and others), 1892.

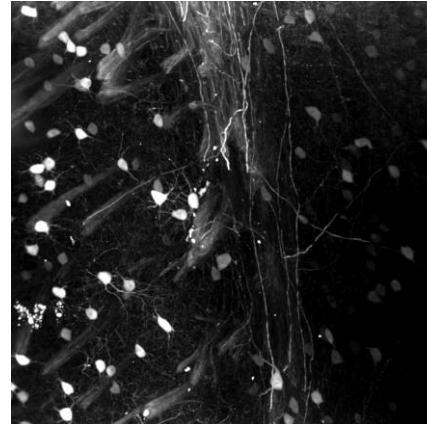
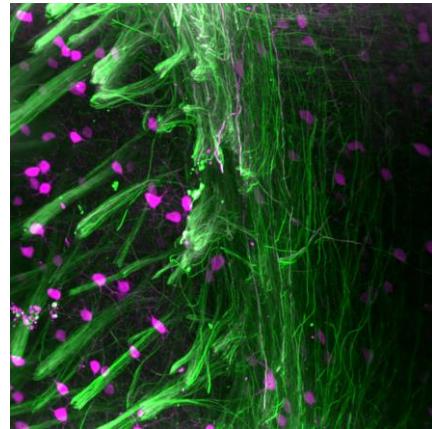
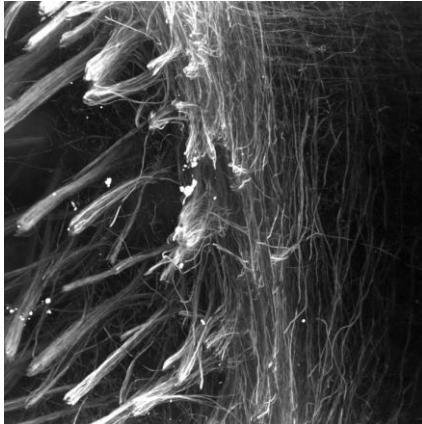
Nearly all microscopy images aren't colour



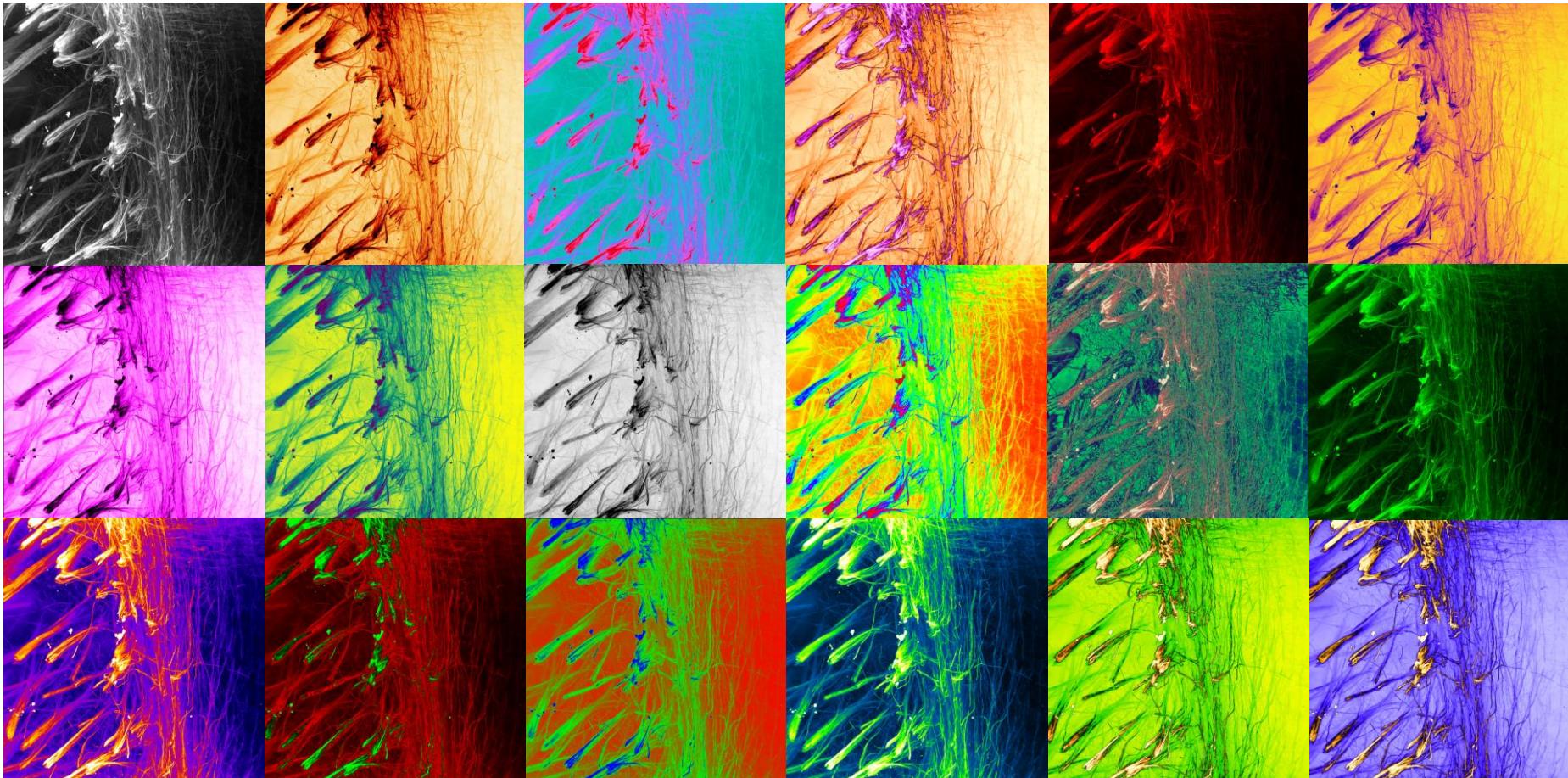
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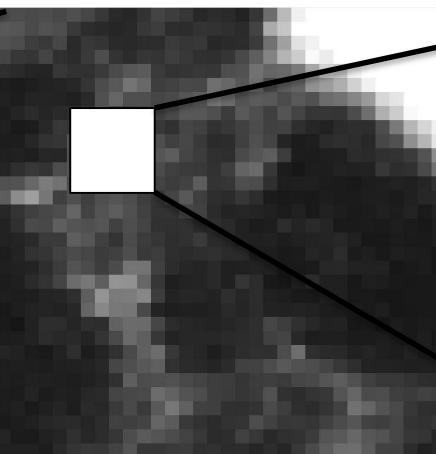
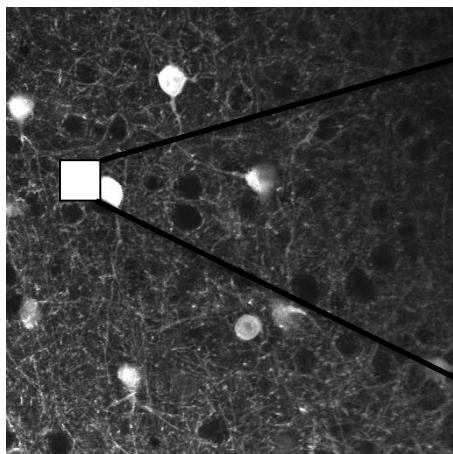
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Lookup tables (LUTs)



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

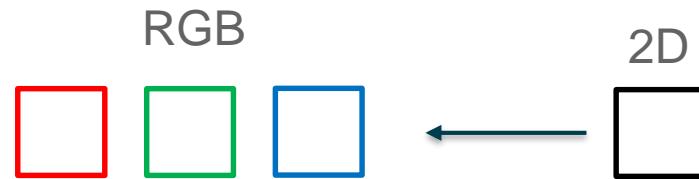
78	64	71	57	43	44
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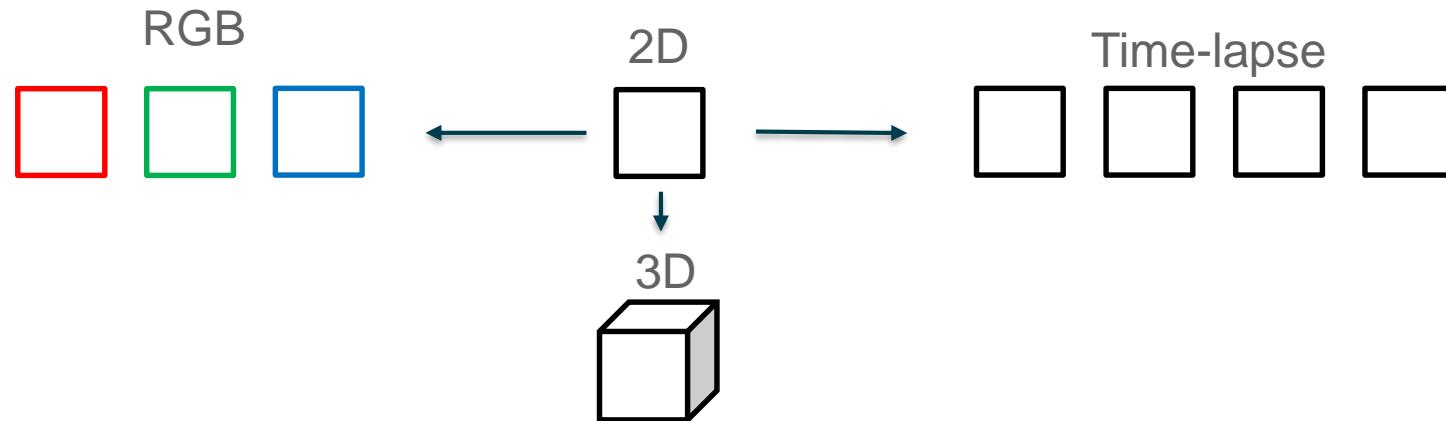
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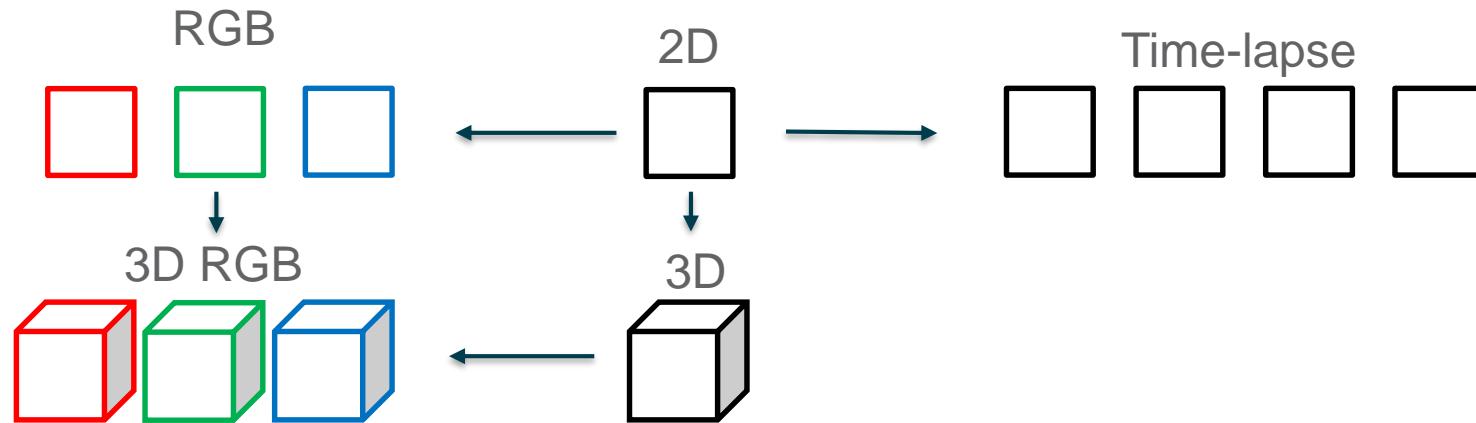
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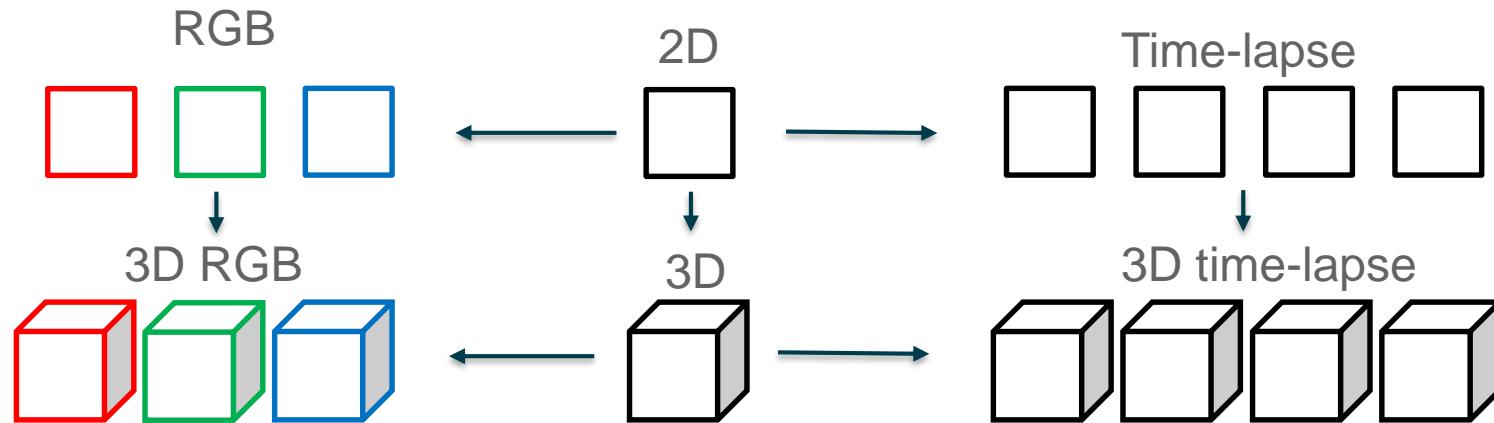
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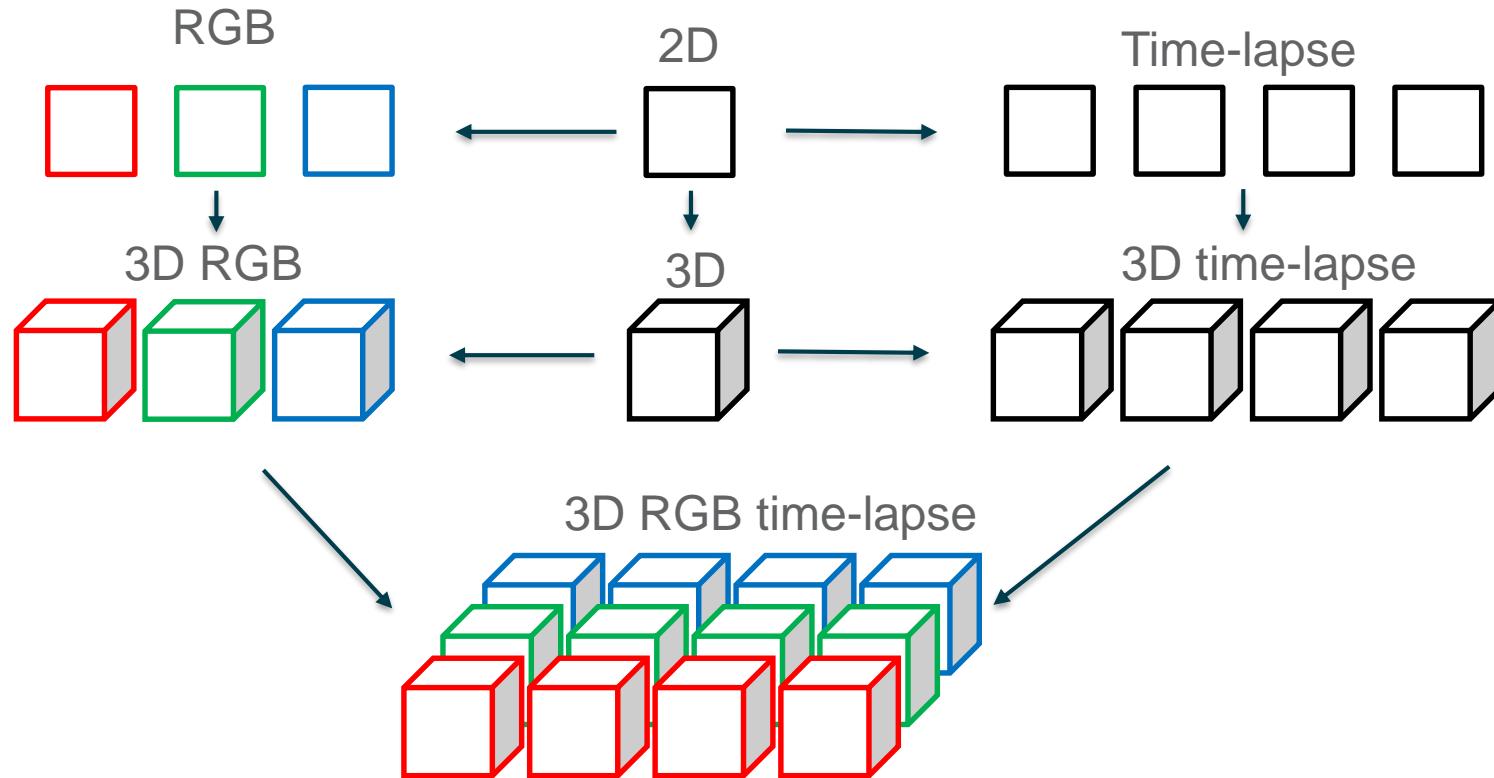


Image processing vs analysis

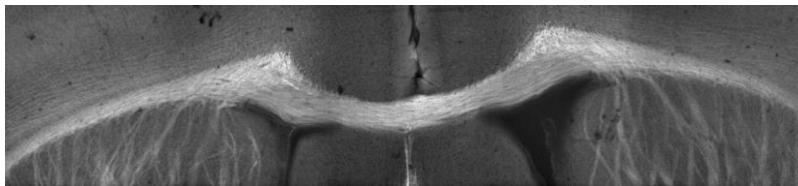
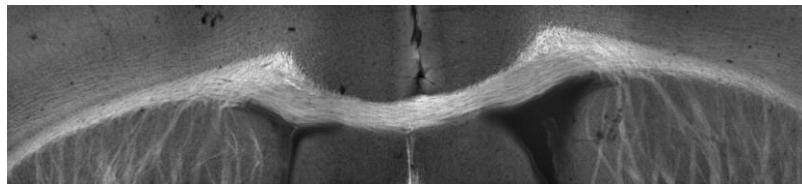


Image processing vs analysis



Volume

Shape

Texture



A

B

C

D

E

F

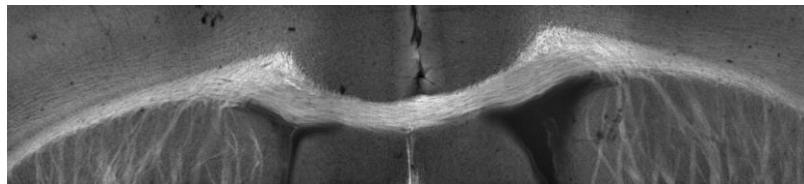
G

H

I

Condition

Image processing vs analysis



Volume

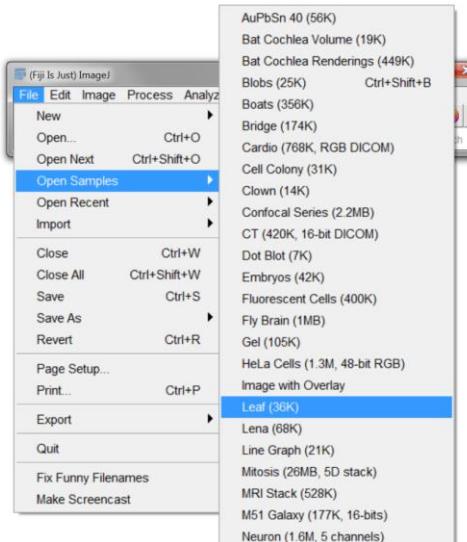
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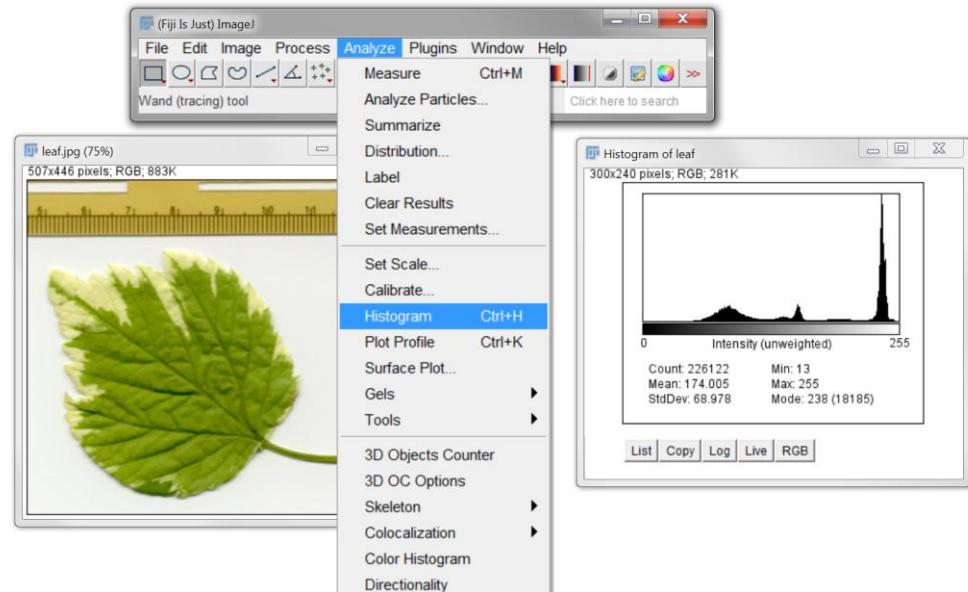
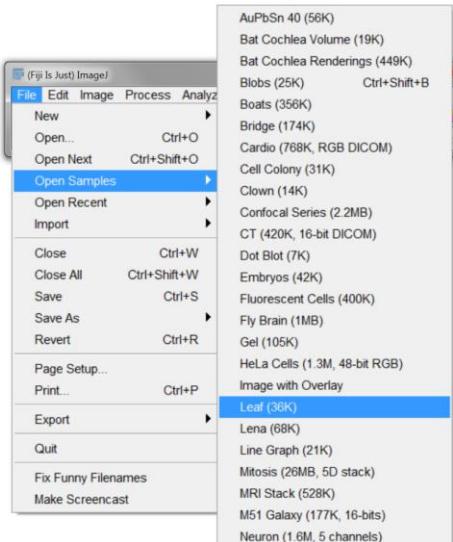
Histogram

- Open any sample image

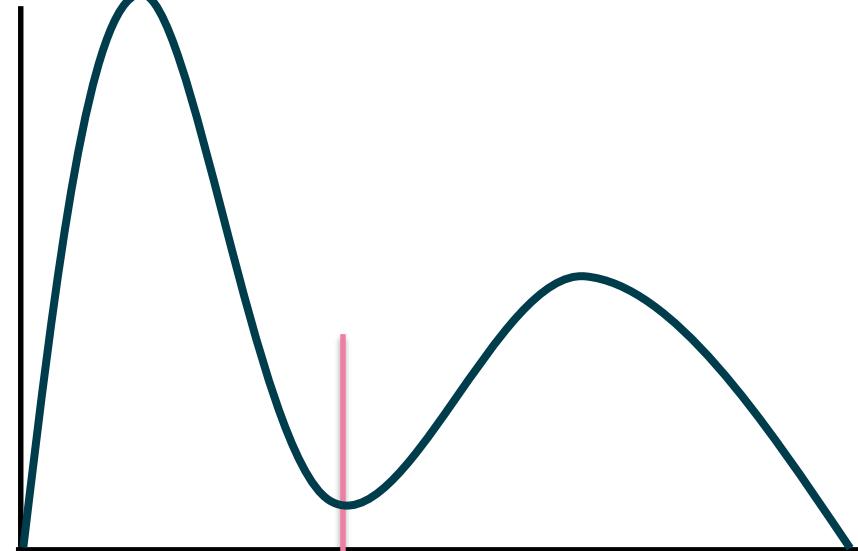
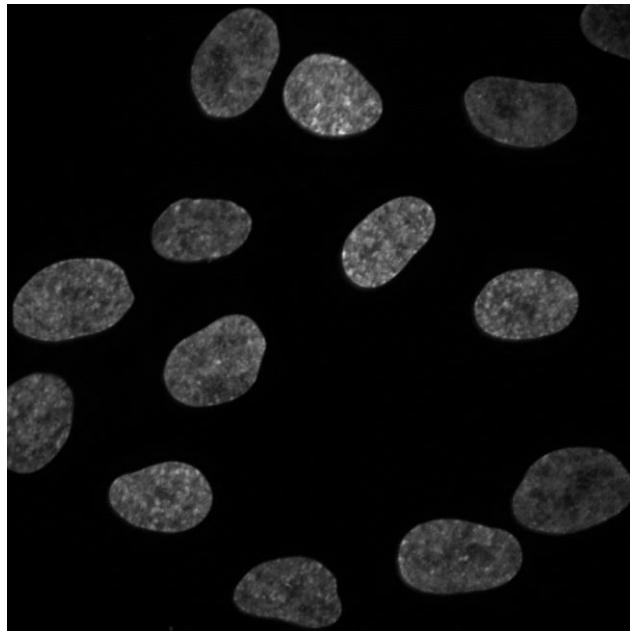


Histogram

- Open any sample image
- Open histogram

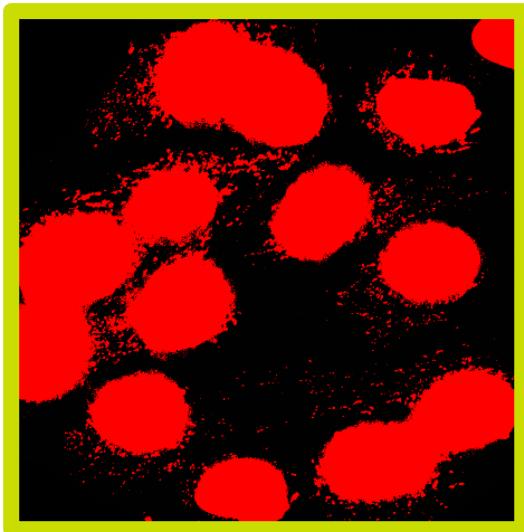
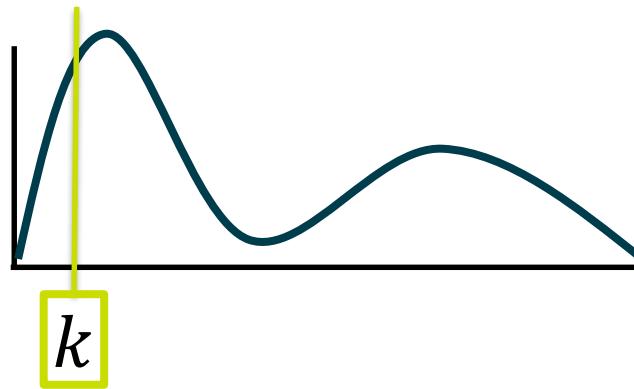


Thresholding

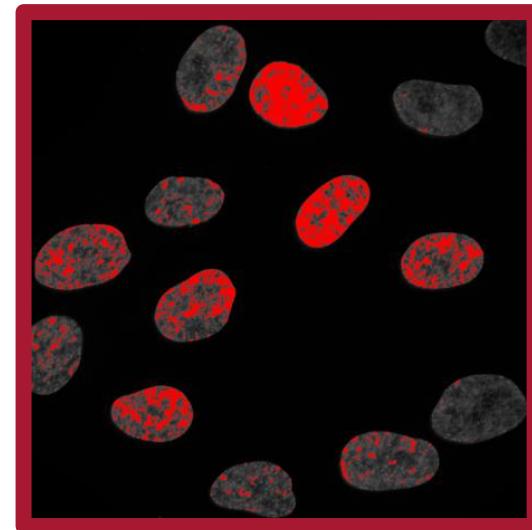
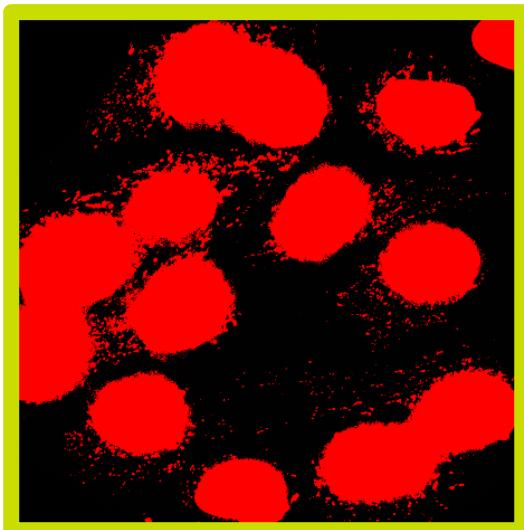
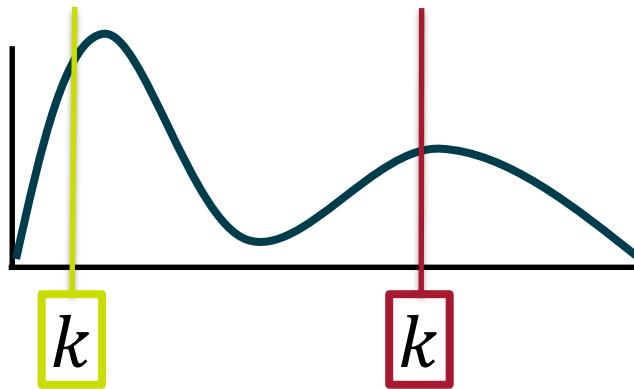


Threshold (κ)

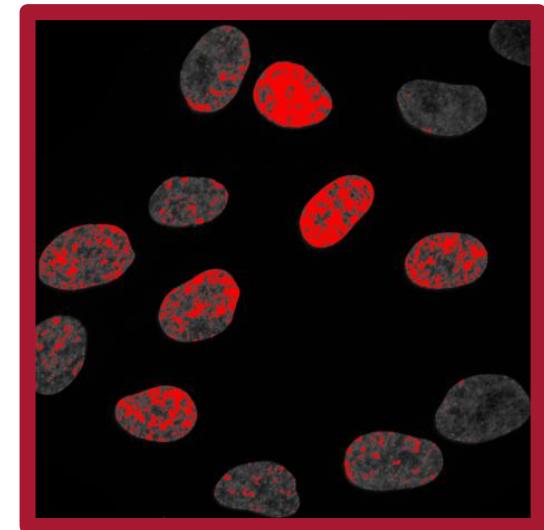
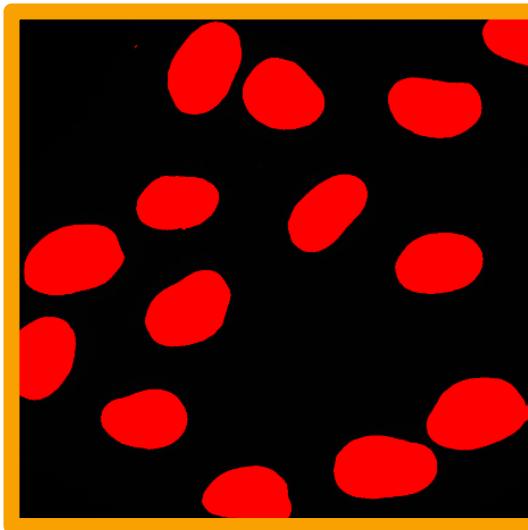
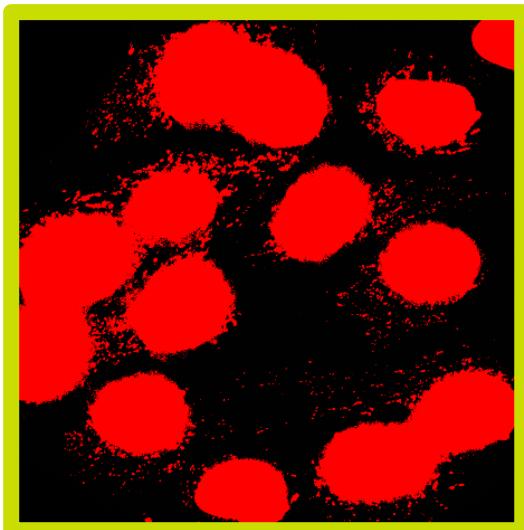
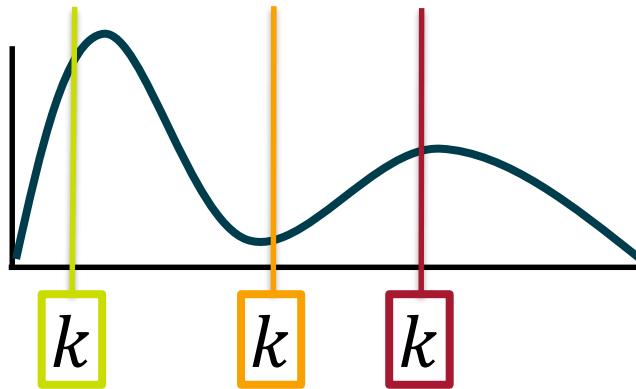
Thresholding



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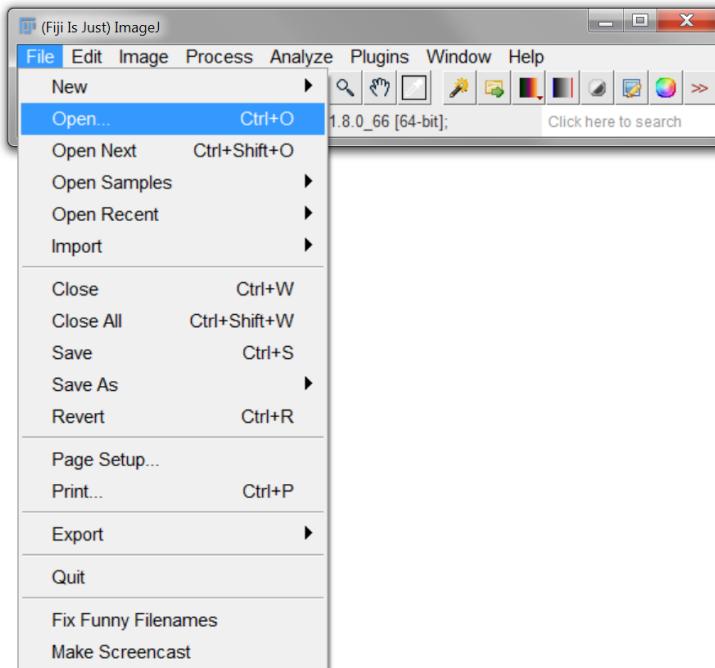


Thresholding



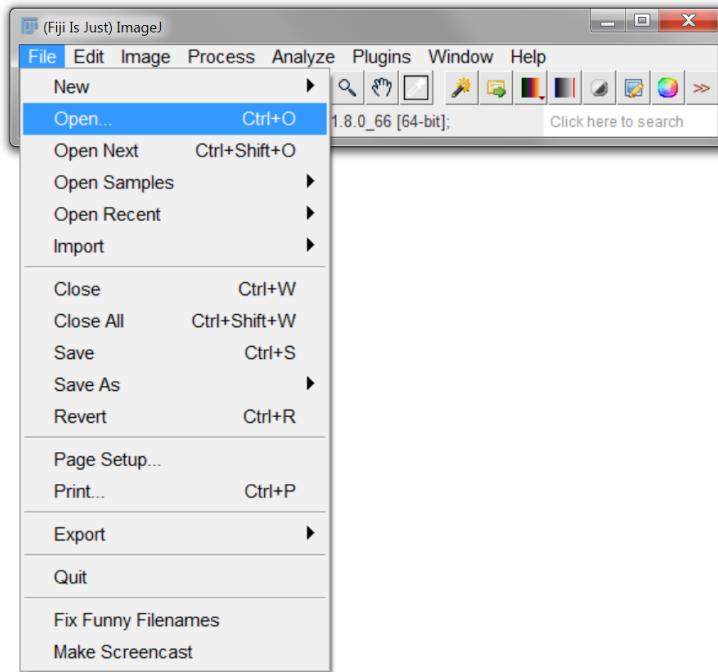
Thresholding – try it out

- Open DAPI.tif

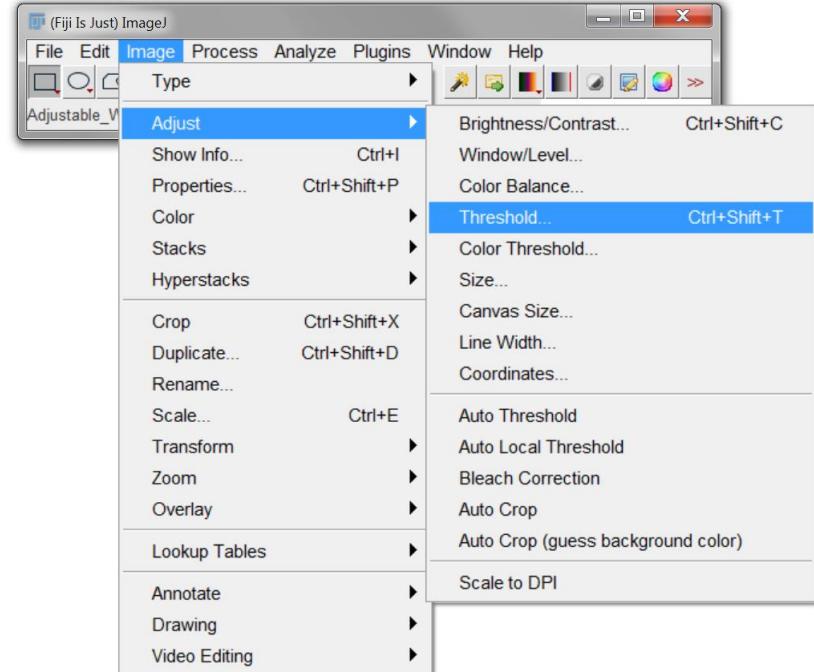


Thresholding – try it out

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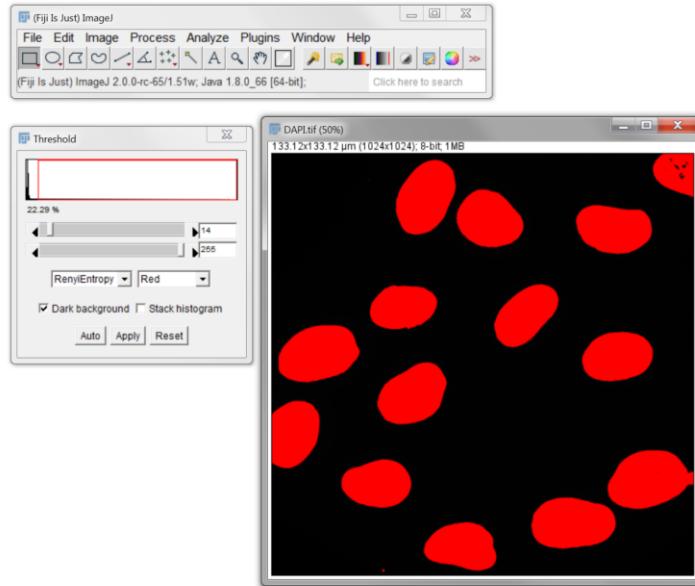


- Threshold



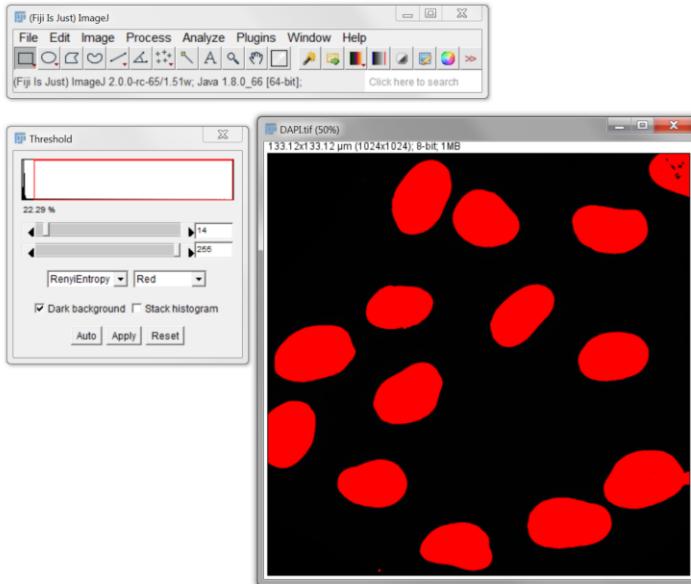
Manual threshold - problems

- DAPI.tif

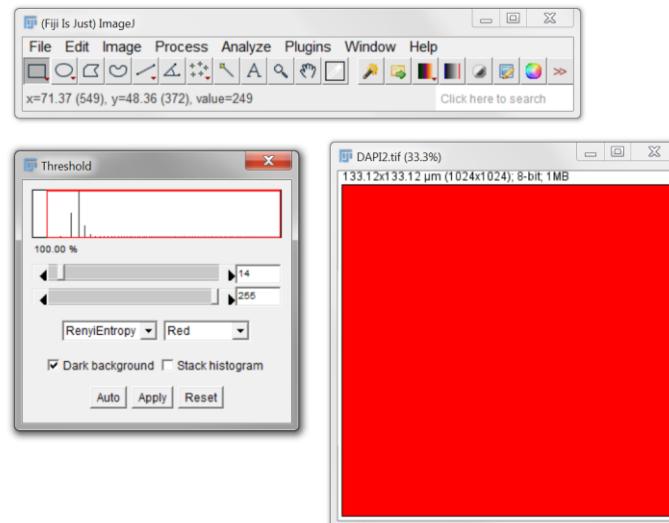


Manual threshold - problems

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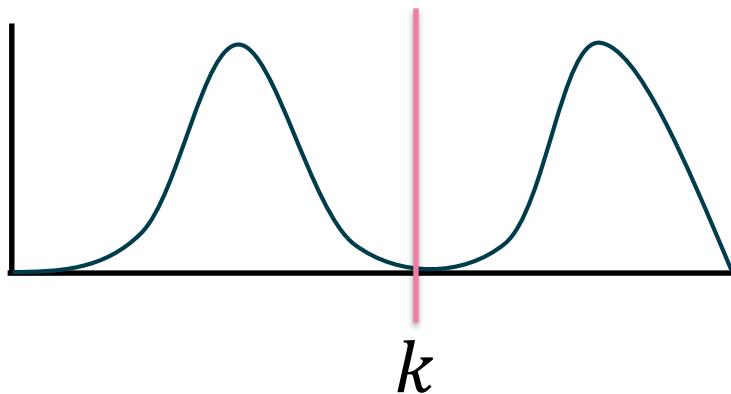


- DAPI_2.tif



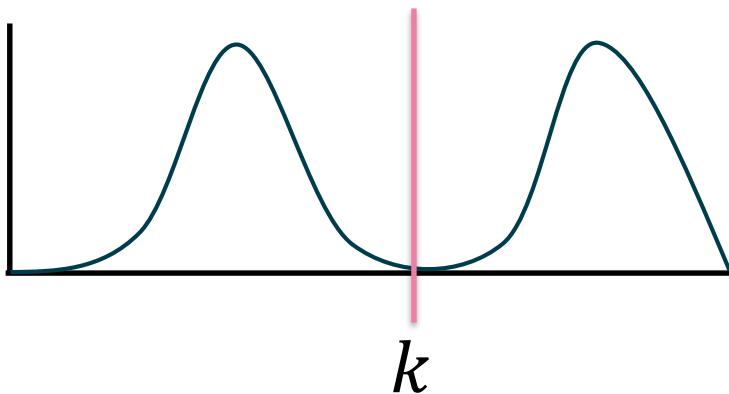
Automatic thresholding – Equal masses

- Ideal case

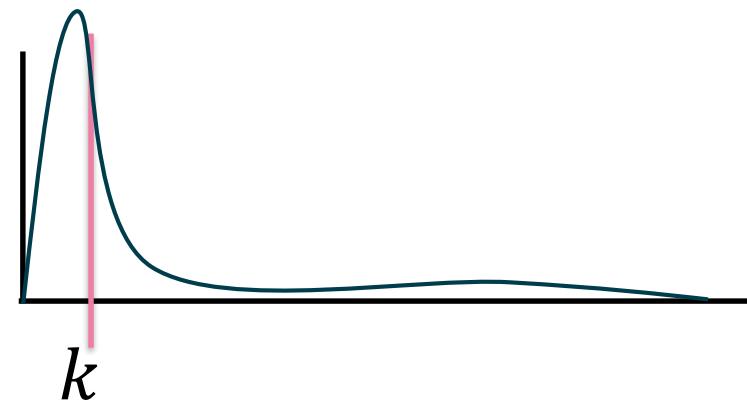


Automatic thresholding – Equal masses

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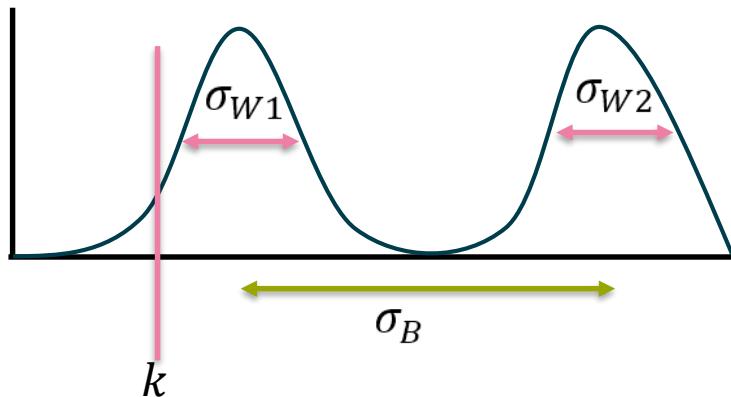


- Real case



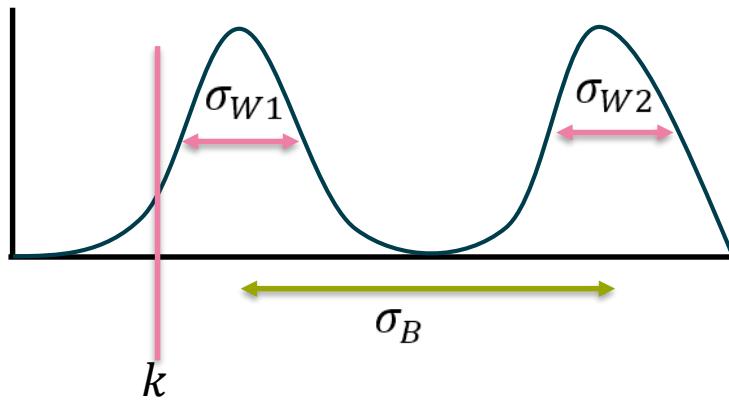
Automatic thresholding – Otsu's method

- Minimise within class variance (σ_w)
- Maximise between class variance (σ_B)



Automatic thresholding – Otsu's method

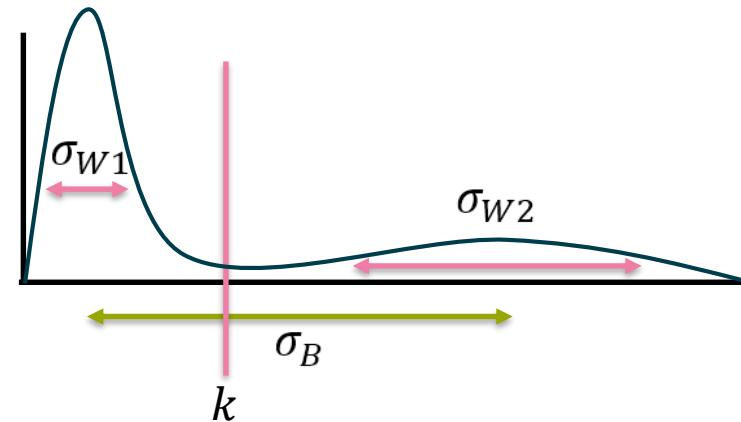
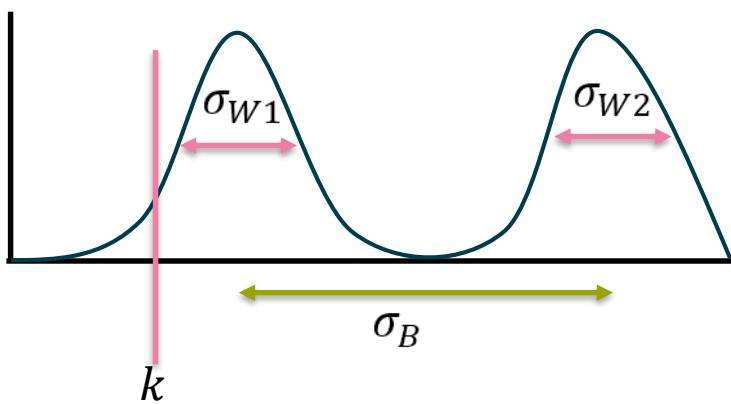
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$$\max_k \left(\frac{\sigma_{W1t} + \sigma_{W2k}}{\sigma_{Bk}} \right)$$

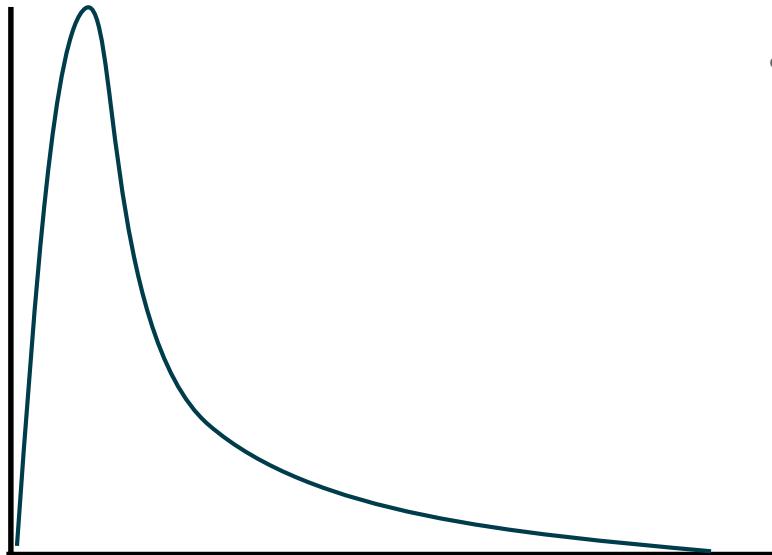
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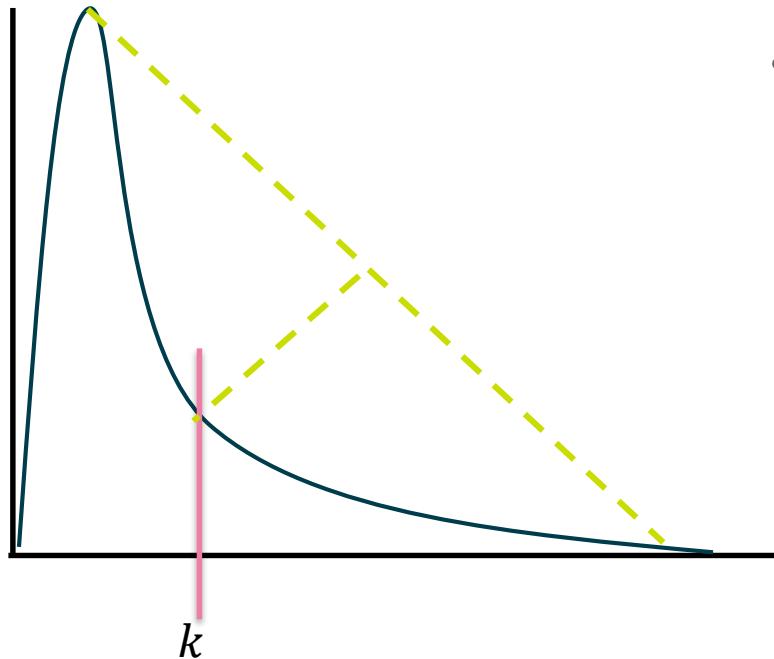
$$\max_k \left(\frac{\sigma_{W1t} + \sigma_{W2k}}{\sigma_{Bk}} \right)$$

Unimodal thresholding – Rosin's method



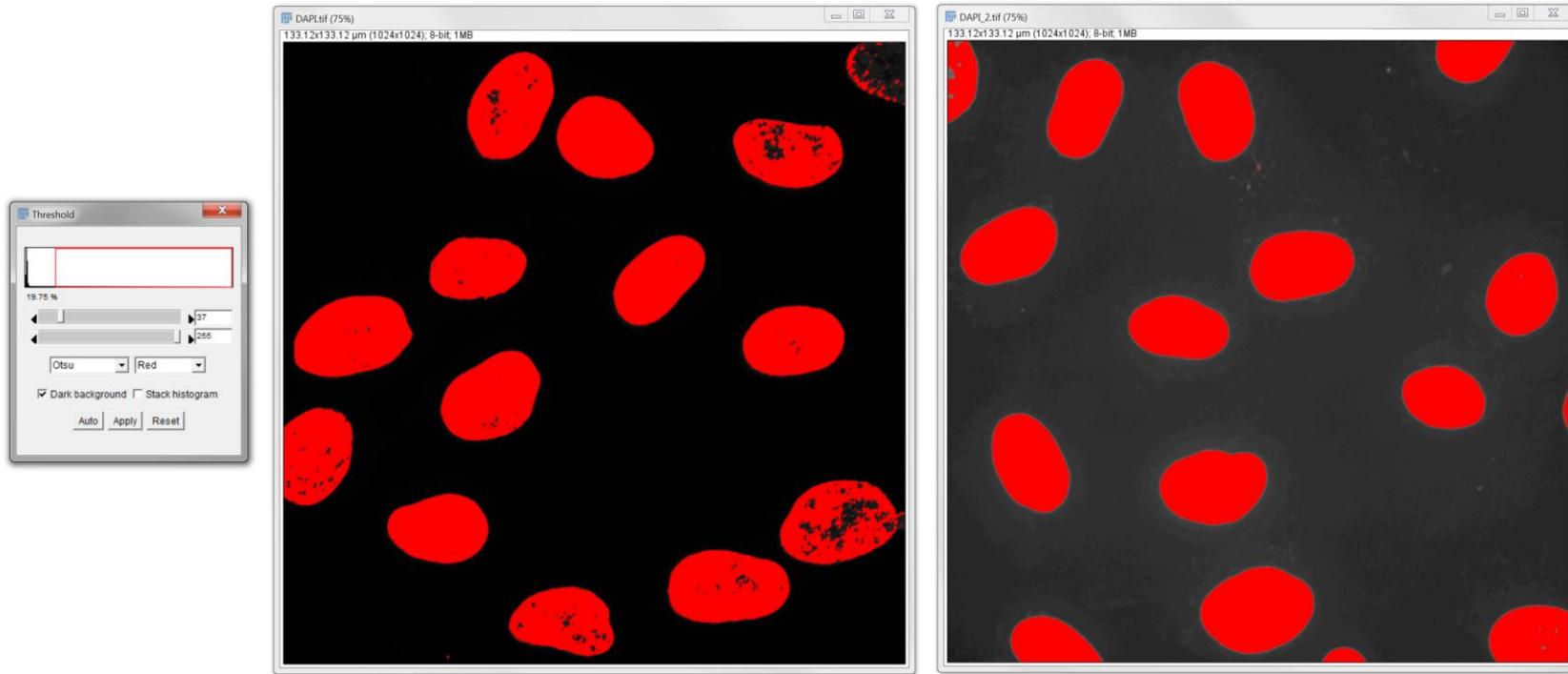
- Use shape of distribution to find threshold

Unimodal thresholding – Rosin's method

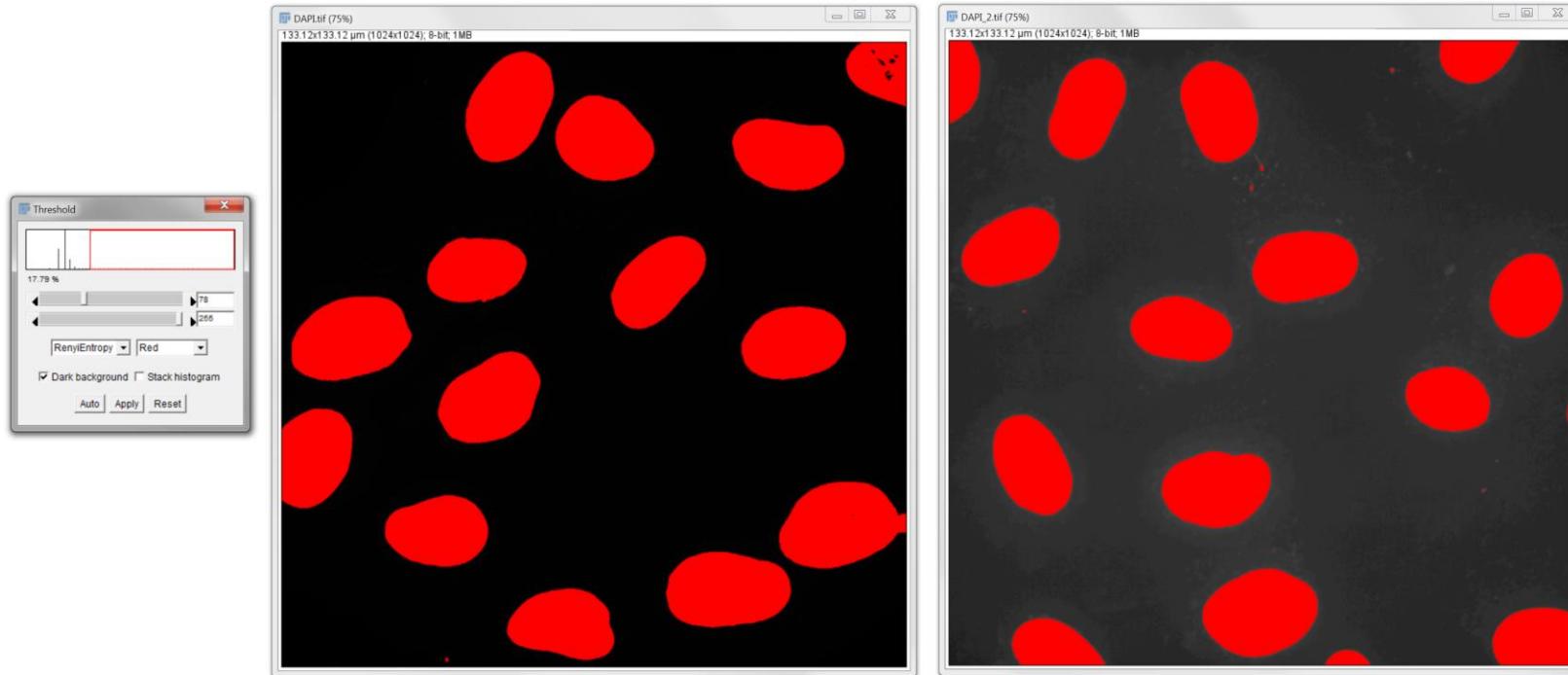


- Use shape of distribution to find threshold

Try it out – Otsu's method

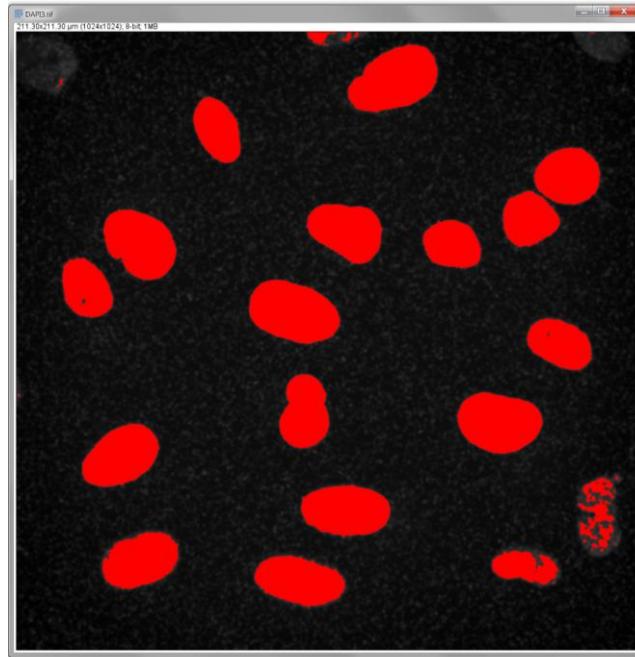


Many, many other methods

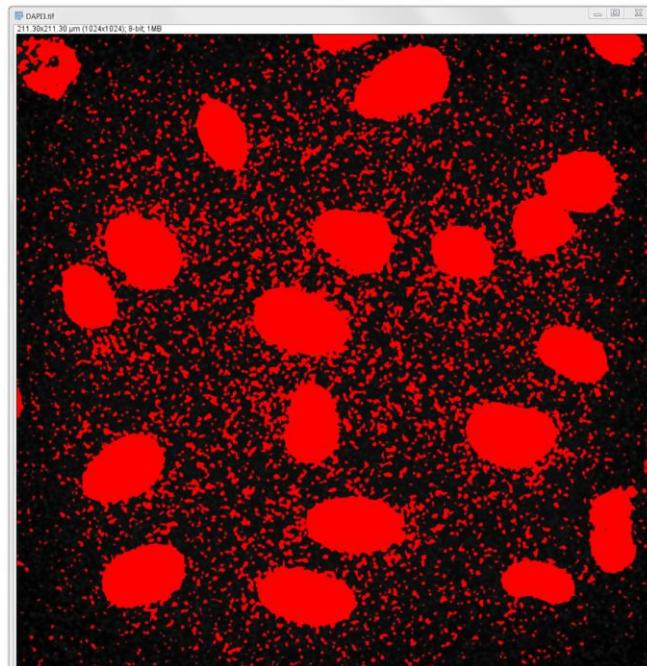


Not always that simple ...

- Open DAPI_3.tif
- Pick a threshold

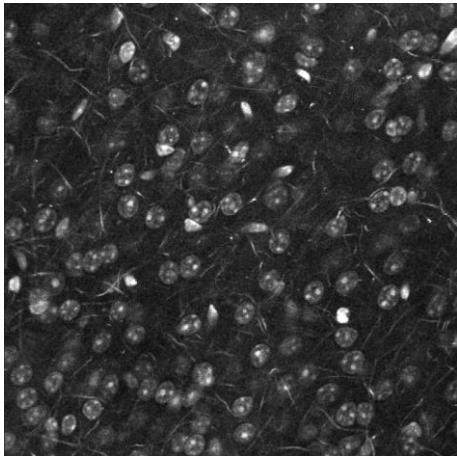


VS



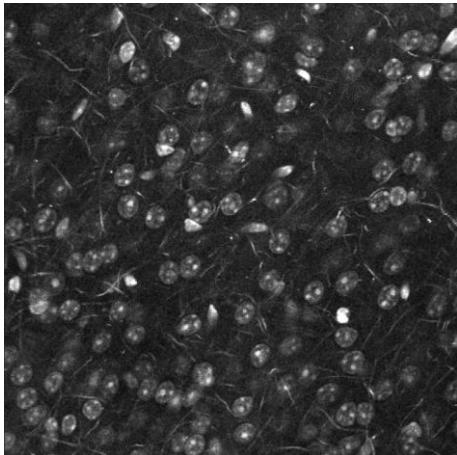
Background subtraction – e.g. vignetting

Sample

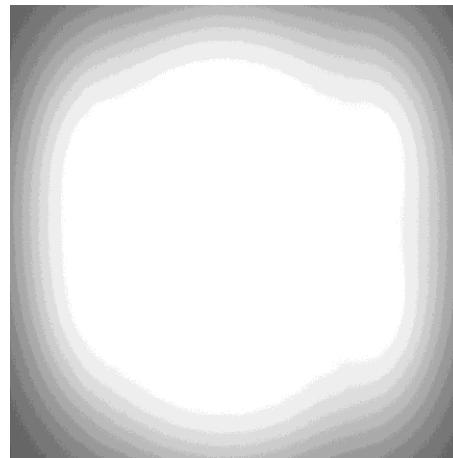


Background subtraction – e.g. vignetting

Sample

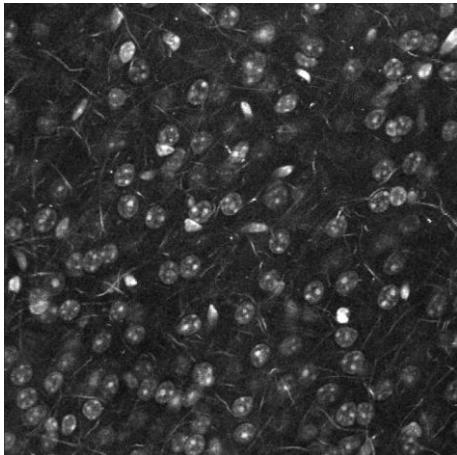


Microscope

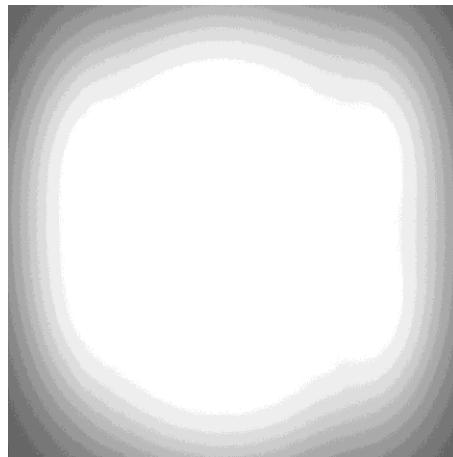


Background subtraction – e.g. vignetting

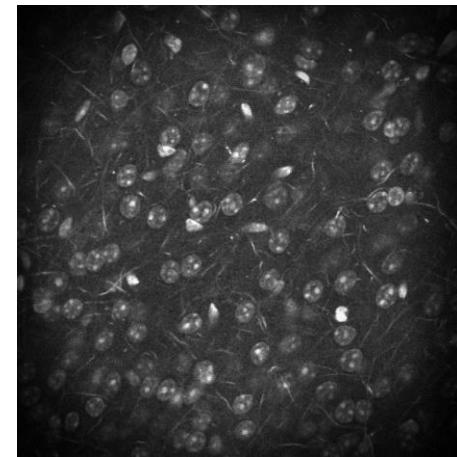
Sample



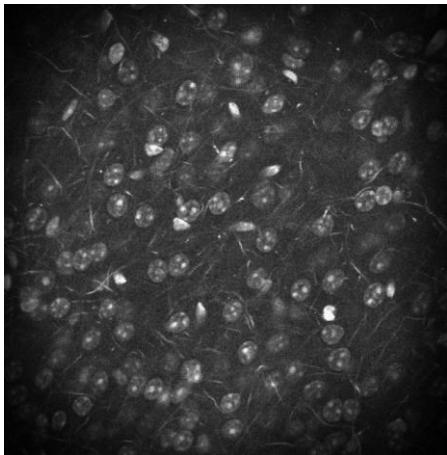
Microscope



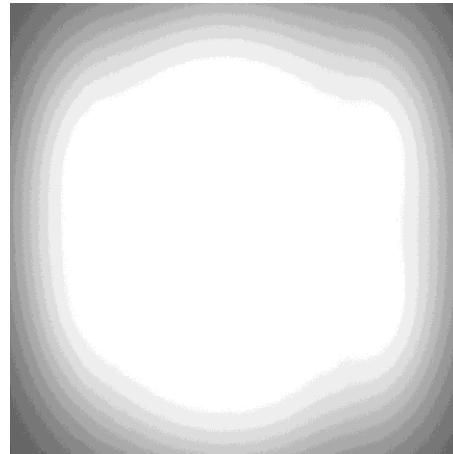
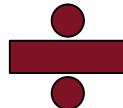
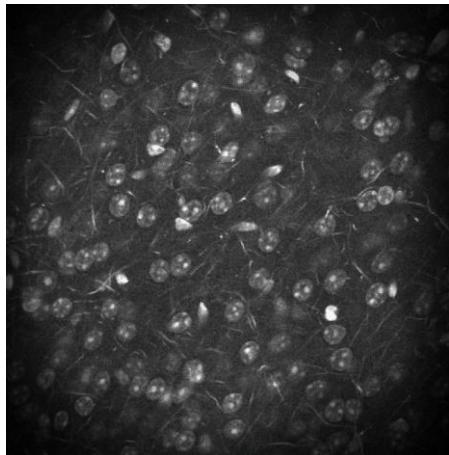
Image



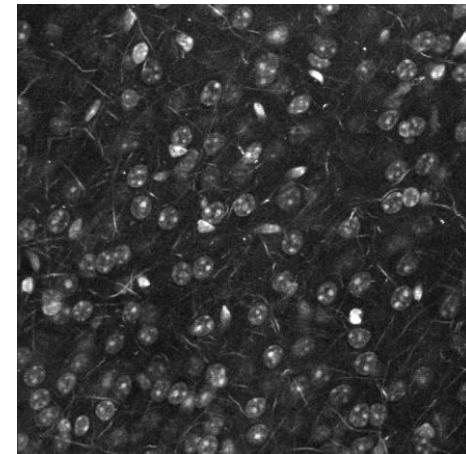
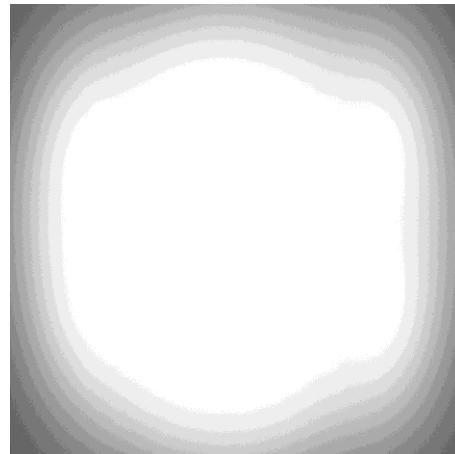
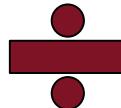
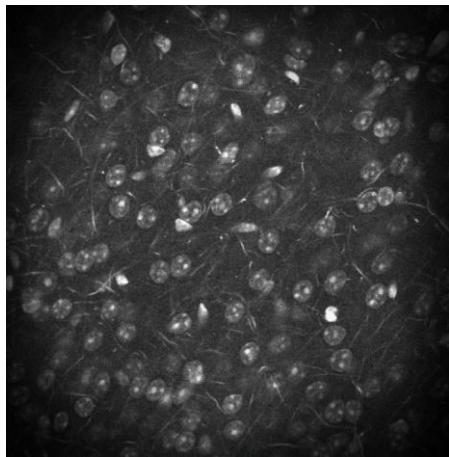
Background subtraction – e.g. vignetting



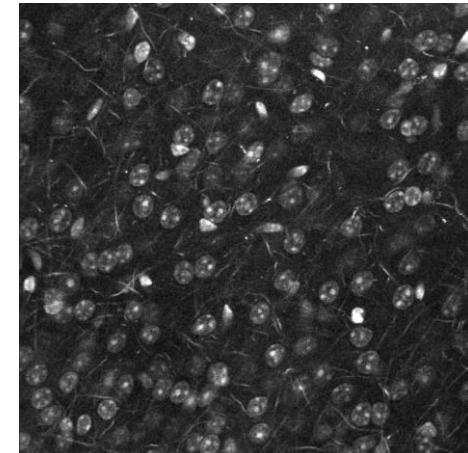
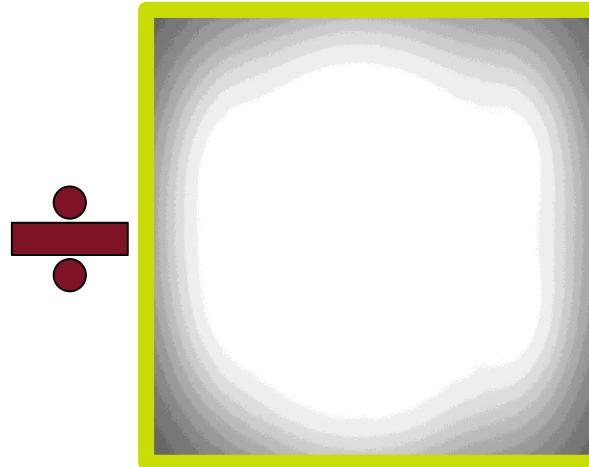
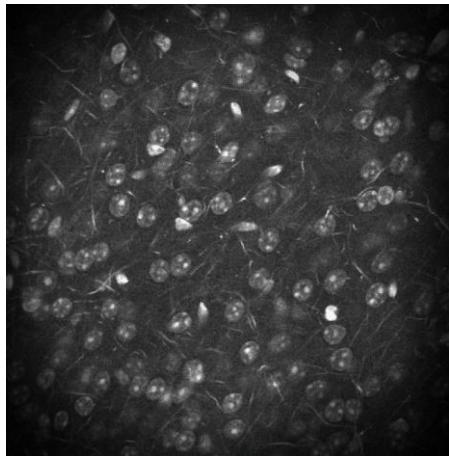
Background subtraction – e.g. vignetting



Background subtraction – e.g. vignetting



Background subtraction – e.g. vignetting



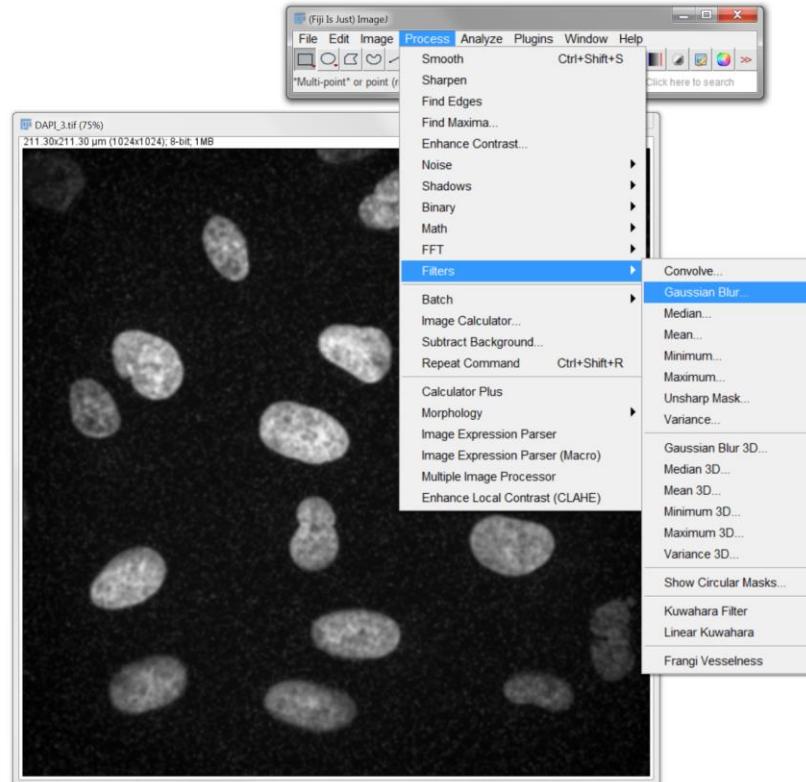
How to
calculate??

Estimate background – Gaussian smoothing

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

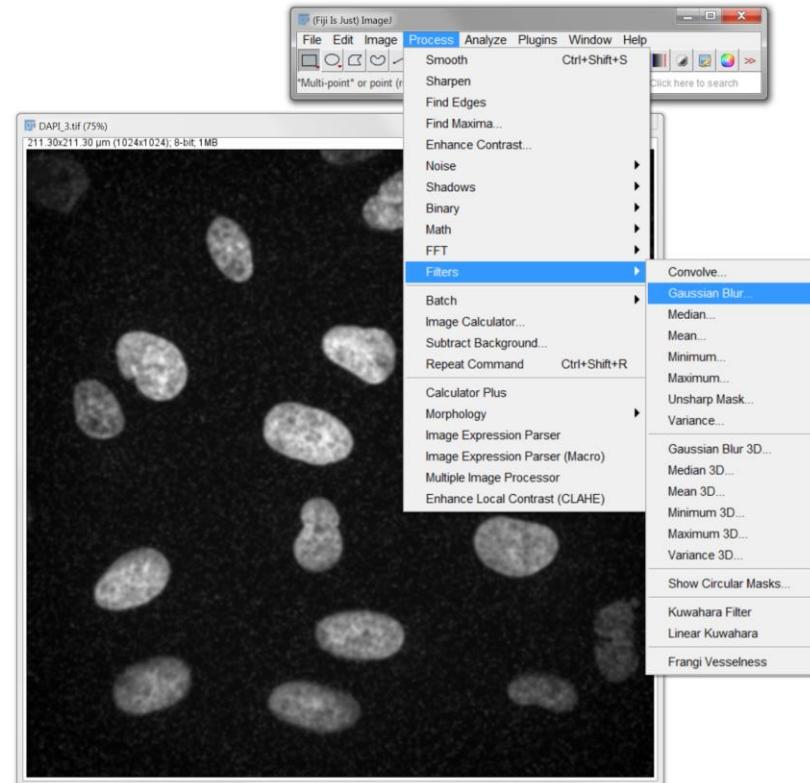
Estimate background – Gaussian smoothing

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



Estimate background – Gaussian smoothing

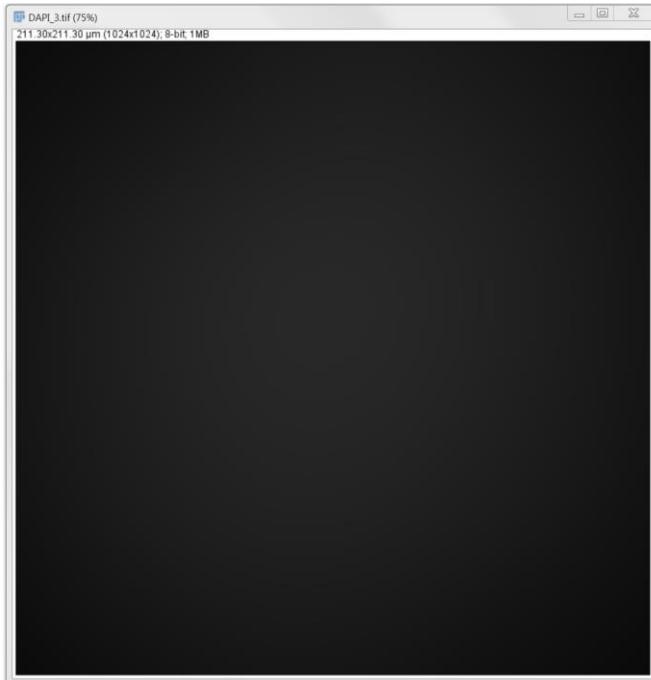
- Seems arbitrary, but smoothing is often a key step in image processing



- Try a value of 200 pixels

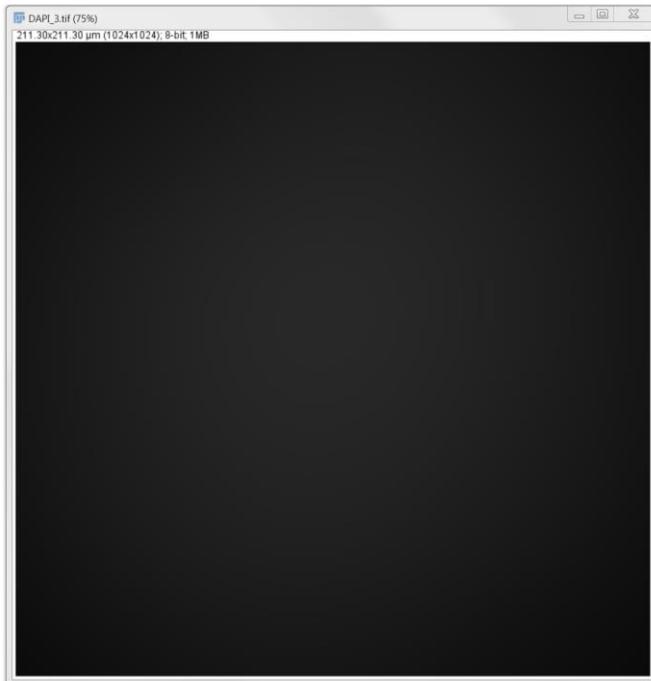
Estimate background – Gaussian smoothing

- May need to adjust brightness



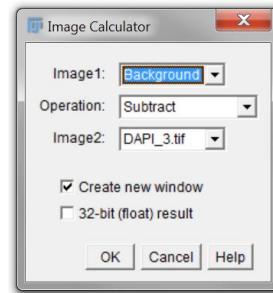
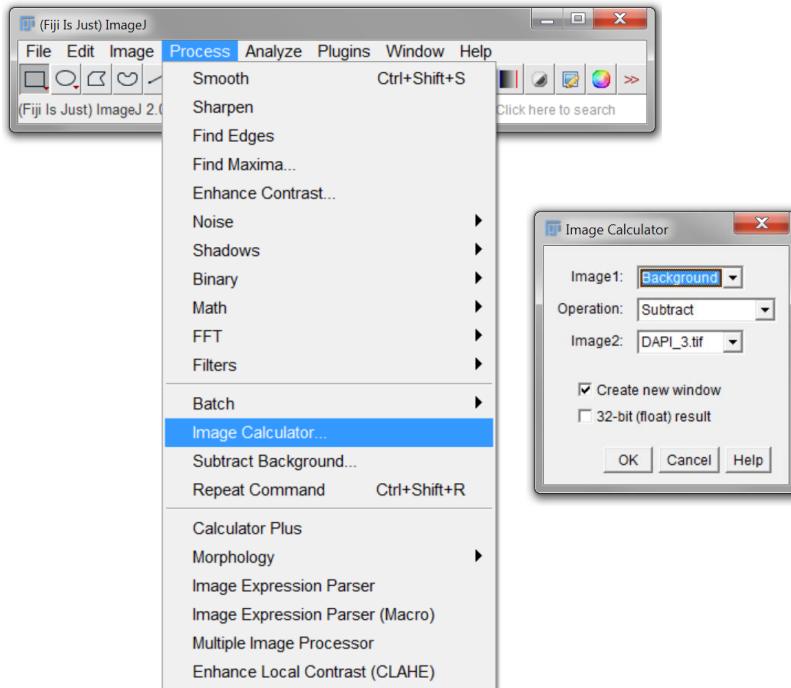
Estimate background – Gaussian smoothing

- May need to adjust brightness

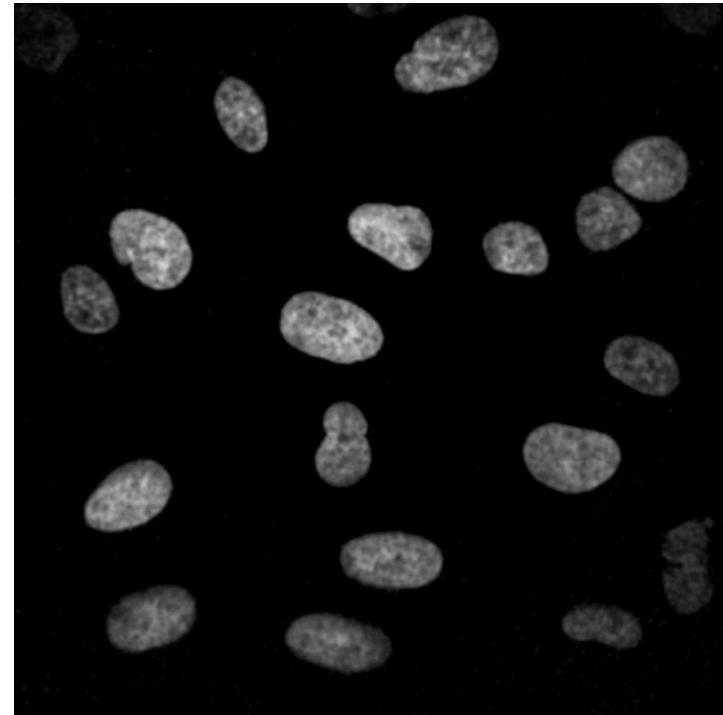
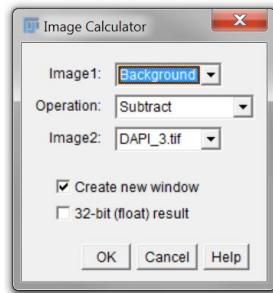
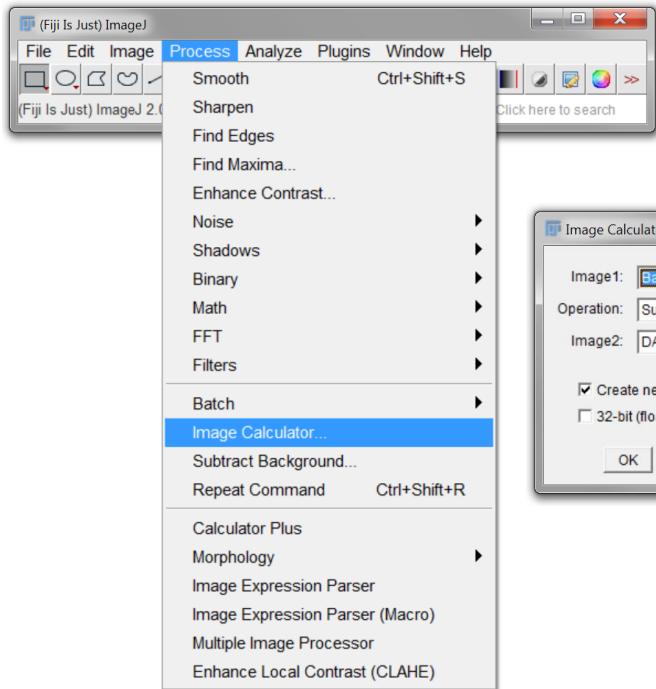


A screenshot of the Fiji software interface. The top menu bar shows "File", "Edit", "Image", "Process", "Analyze", "Plugins", "Window", and "Help". A context menu is open over the black image window, with "Adjust" selected. This reveals a submenu with options like "Multi-point...", "Show Info...", "Properties...", "Color", "Stacks", "Hyperstacks", "Crop", "Duplicate...", "Rename...", "Scale...", "Transform", "Zoom", "Overlay", "Lookup Tables", "Annotate", "Drawing", "Video Editing", "Axes", "Threshold", "Convert", and "Convolve". Below this, the "Image" menu is expanded, showing "Brightness/Contrast" (selected), "Window/Level...", "Color Balance...", "Threshold...", "Color Threshold...", "Size...", "Canvas Size...", "Line Width...", "Coordinates...", "Auto Threshold", "Auto Local Threshold", "Bleach Correction", "Auto Crop", "Auto Crop (guess background color)", and "Scale to DPI". In the bottom right corner, a small floating dialog box titled "B&C" contains sliders for "Minimum", "Maximum", "Brightness", and "Contrast", with "Auto", "Reset", "Set", and "Apply" buttons below.

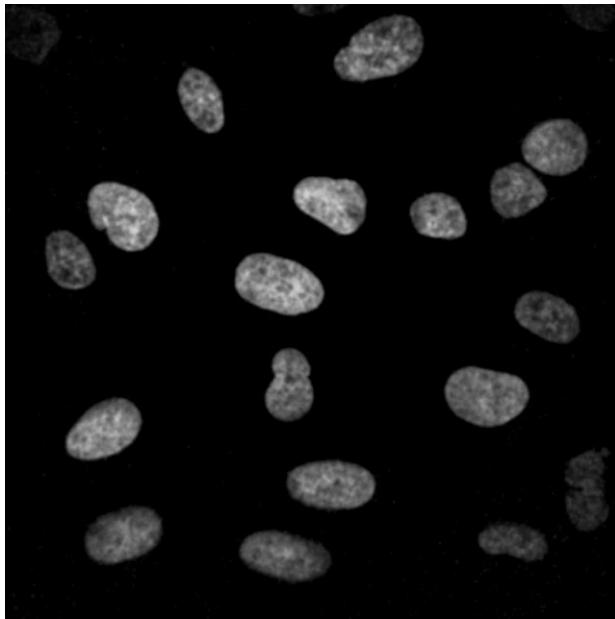
Estimate background



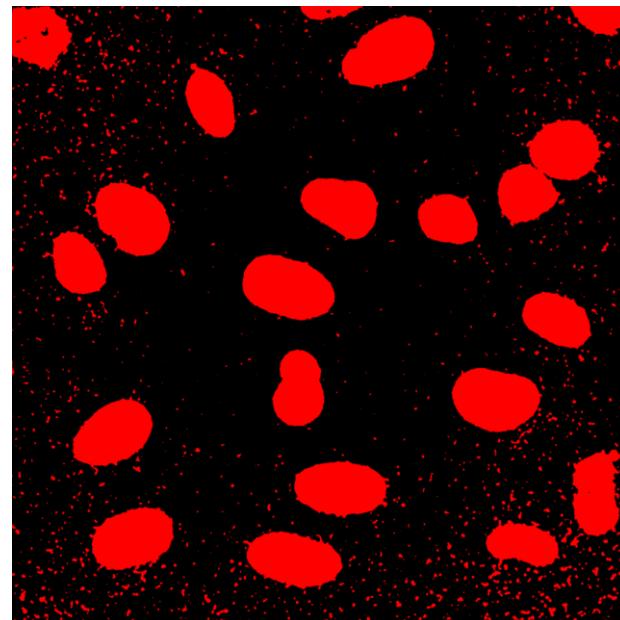
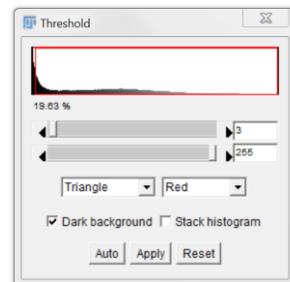
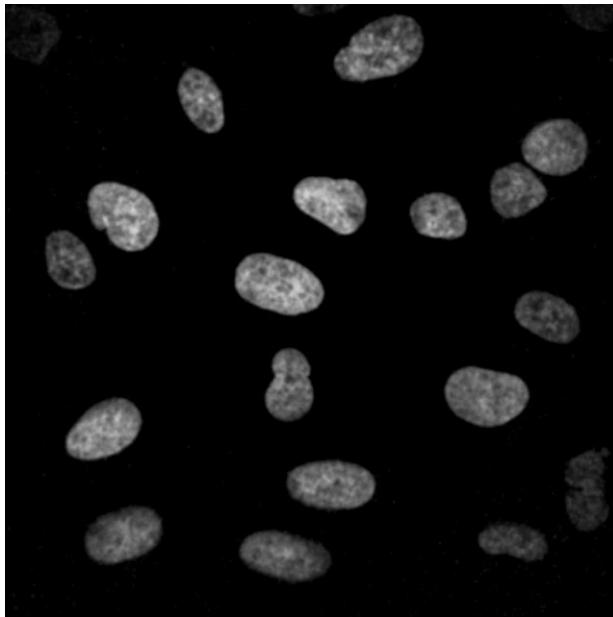
Estimate background



Now try thresholding



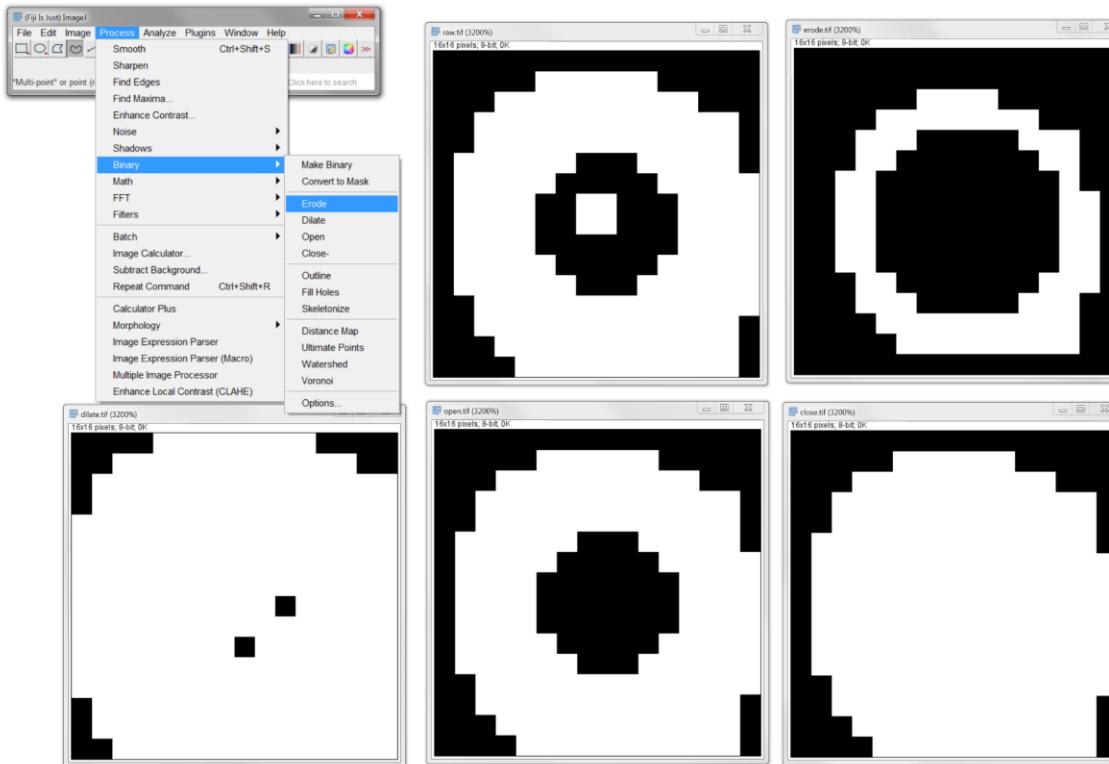
Now try thresholding



- Better, but not great

Morphological operations

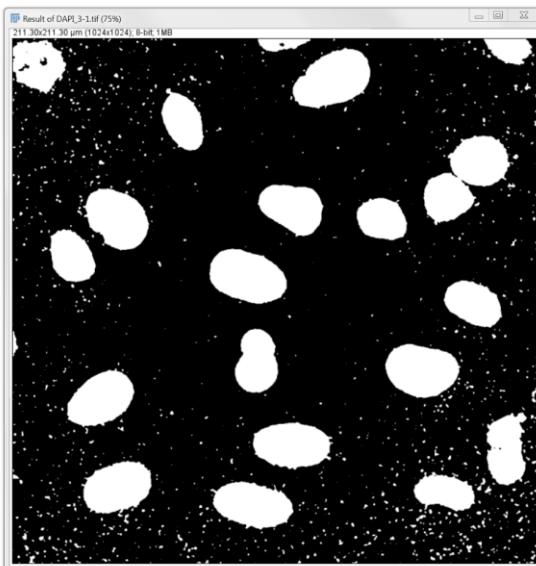
- Processing of (usually binary) images based on shape



Morphological operations

- Try on the previous example

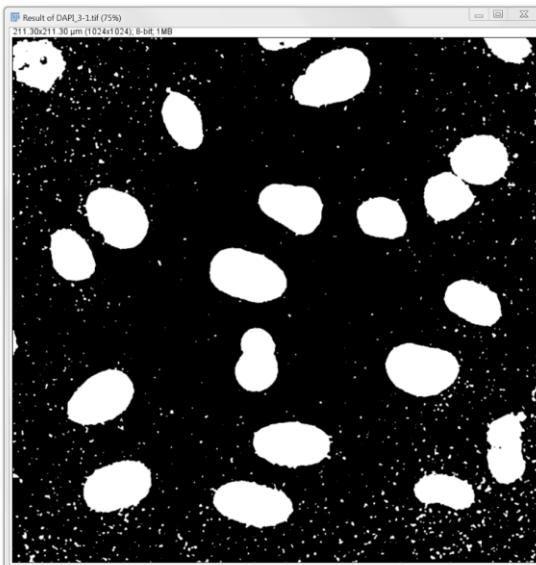
Thresholded



Morphological operations

- Try on the previous example

Thresholded



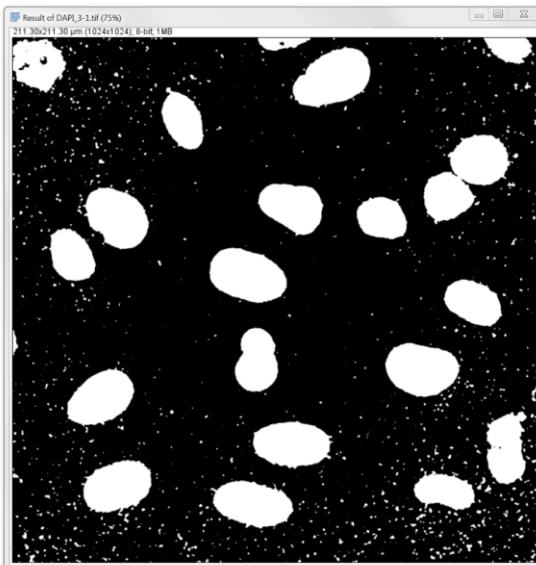
Eroded



Morphological operations

- Try on the previous example

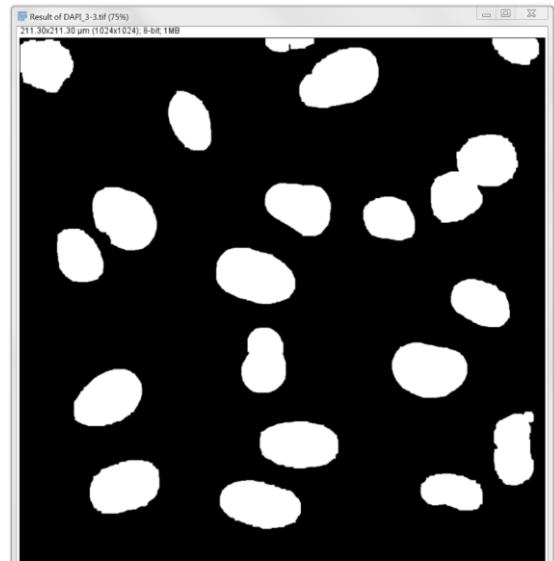
Thresholded



Eroded

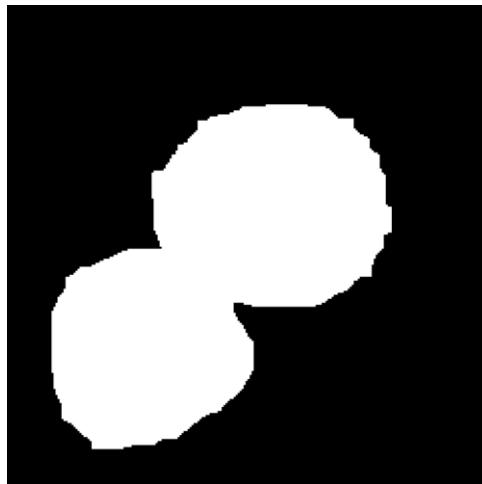


Dilated and
closed



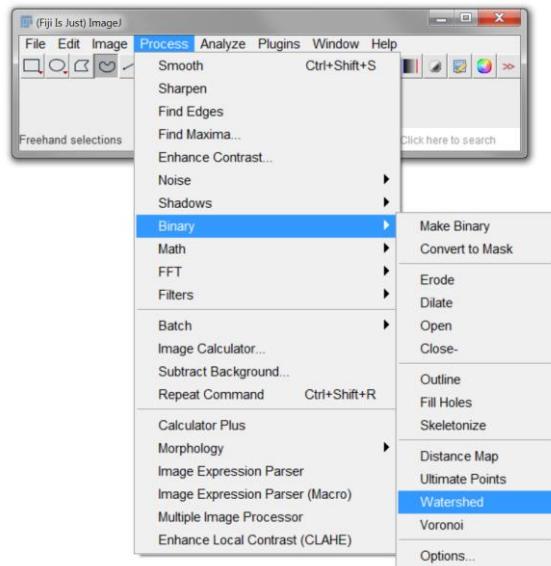
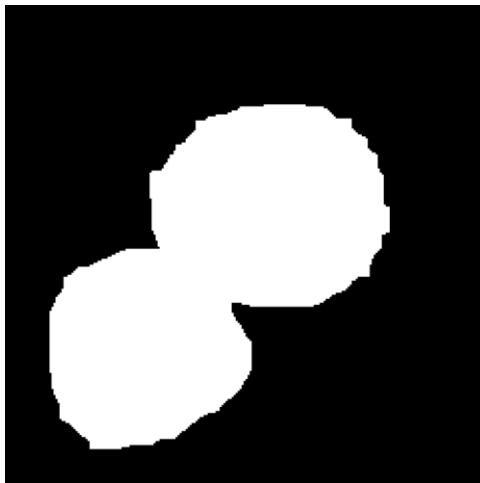
Watershed – separate objects

- From the previous example



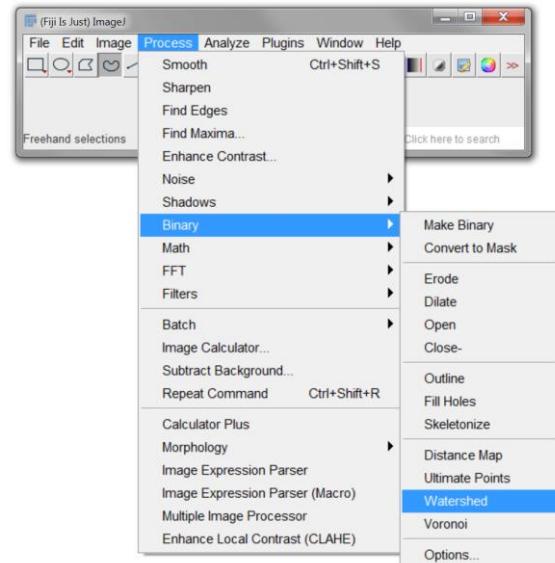
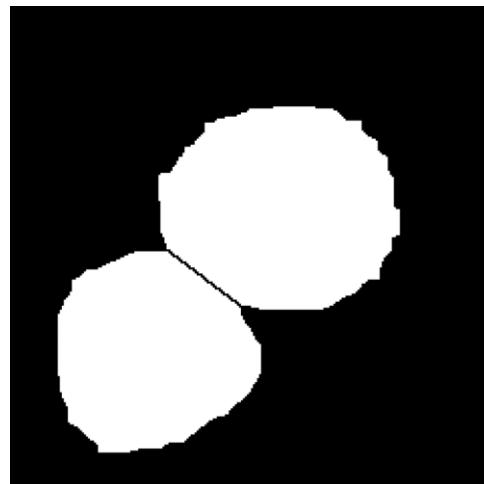
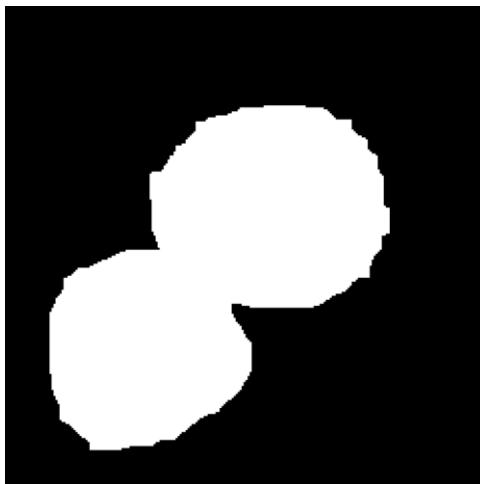
Watershed – separate objects

- From the previous example

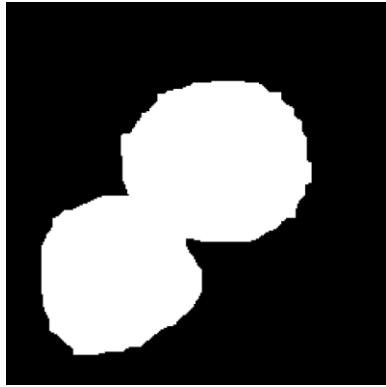


Watershed – separate objects

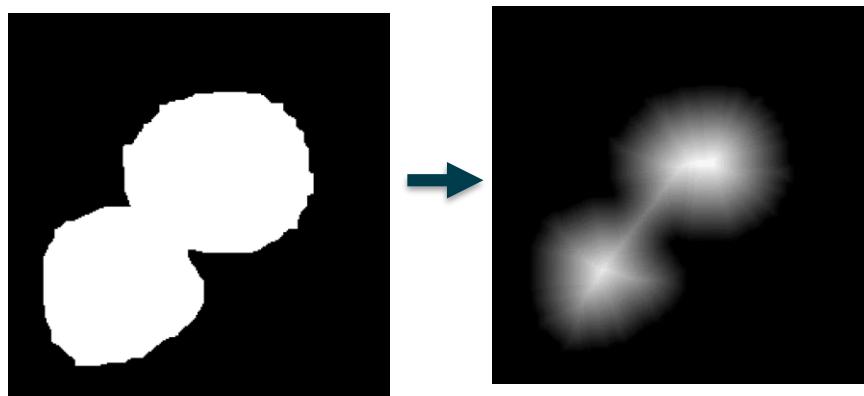
- From the previous example



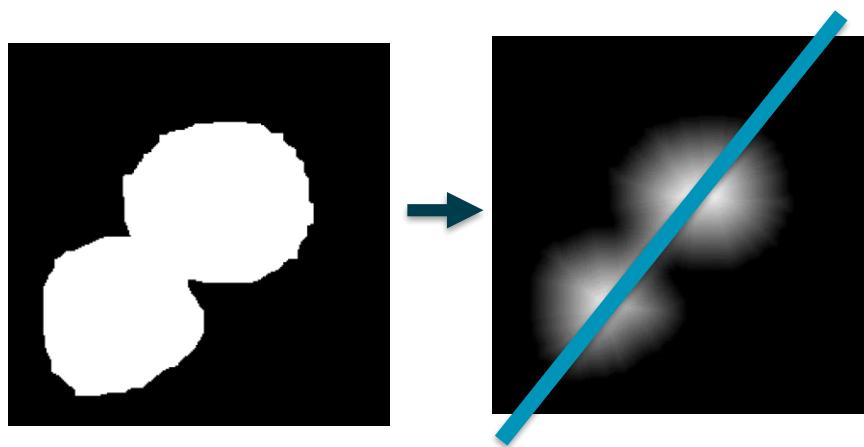
Watershed



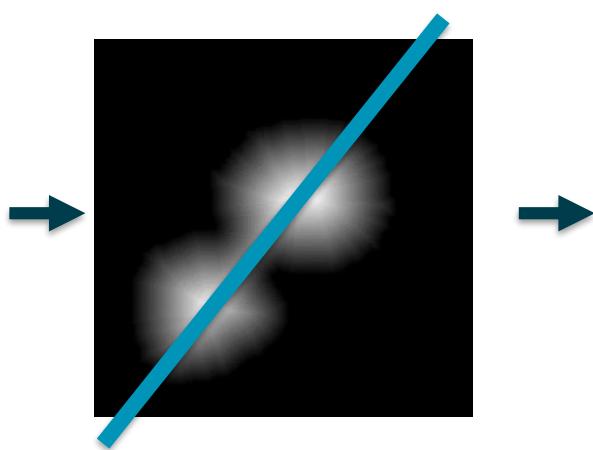
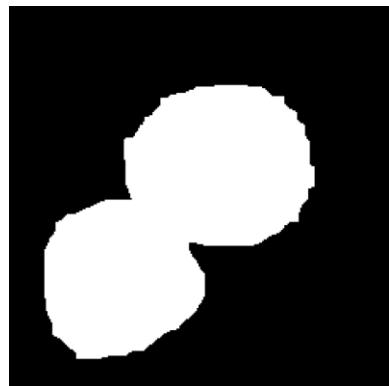
Watershed



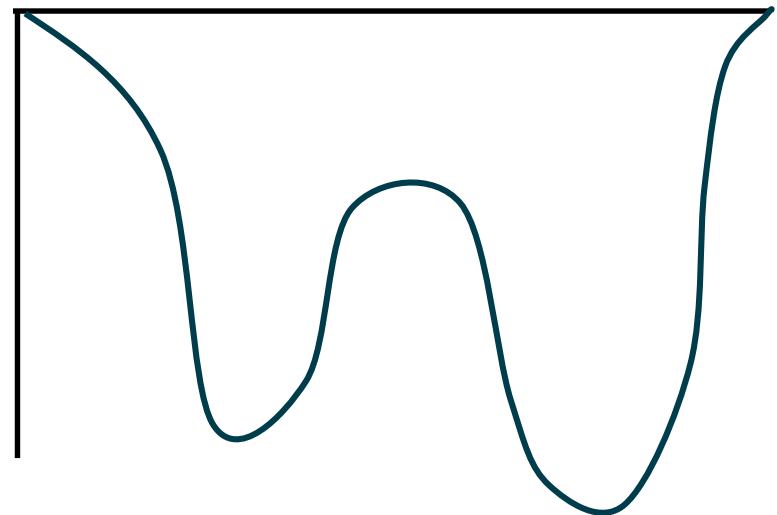
Watershed



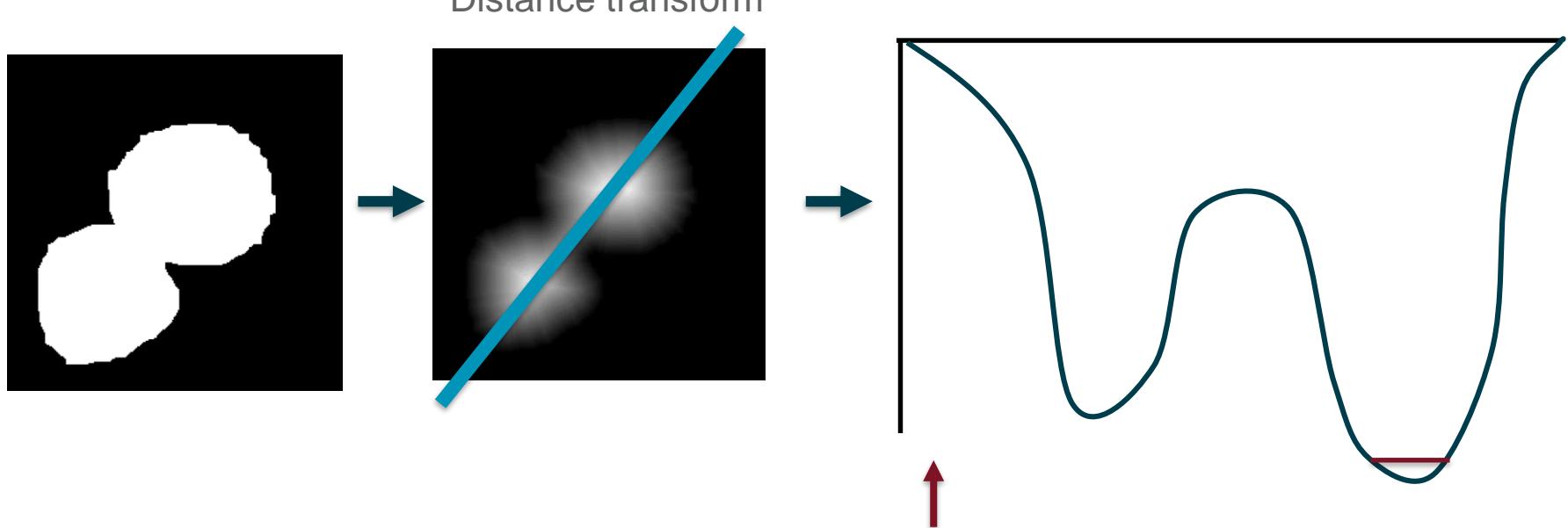
Watershed



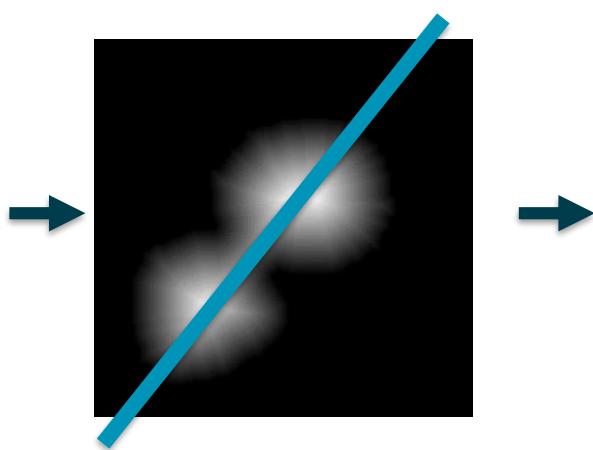
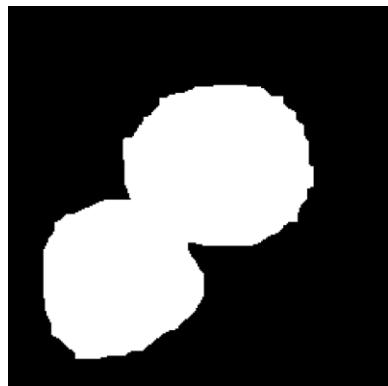
Negative intensity profile



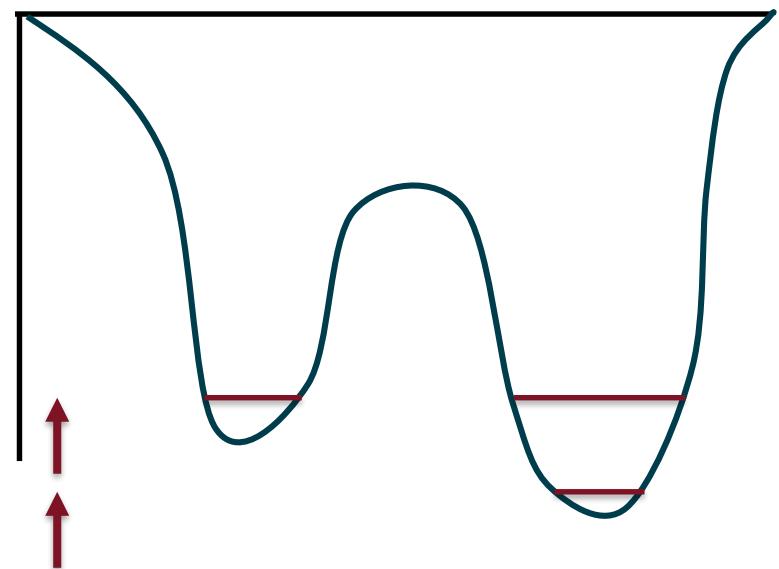
Watershed



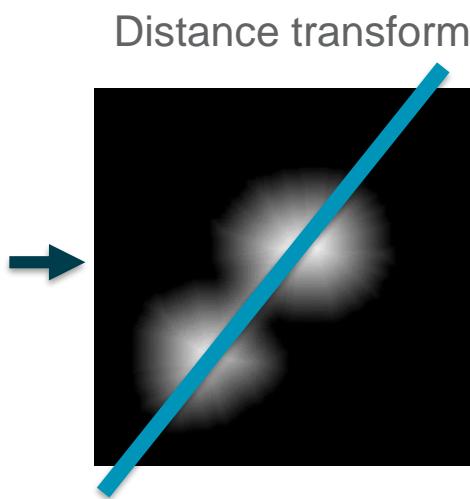
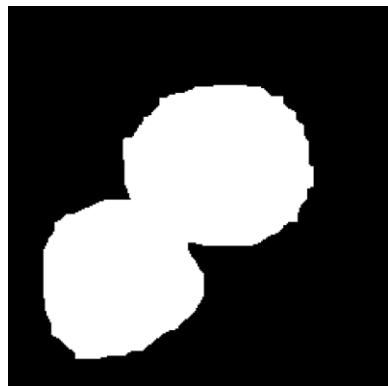
Watershed



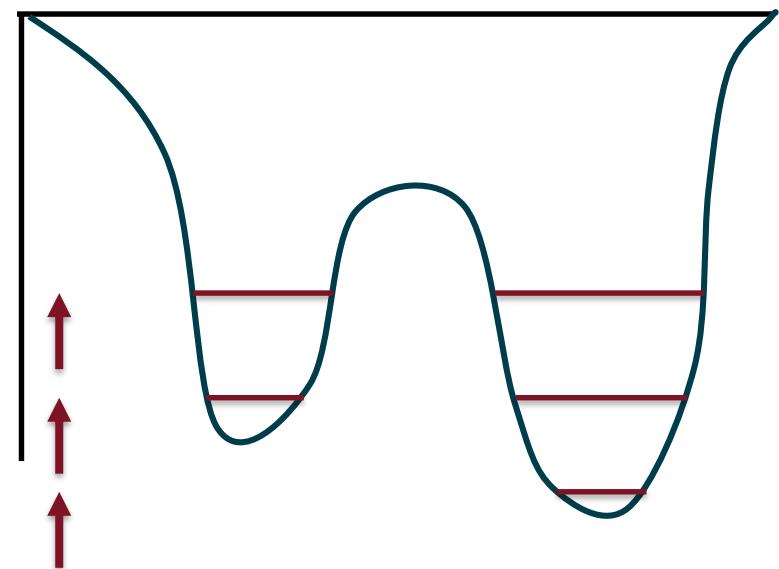
Negative intensity profile



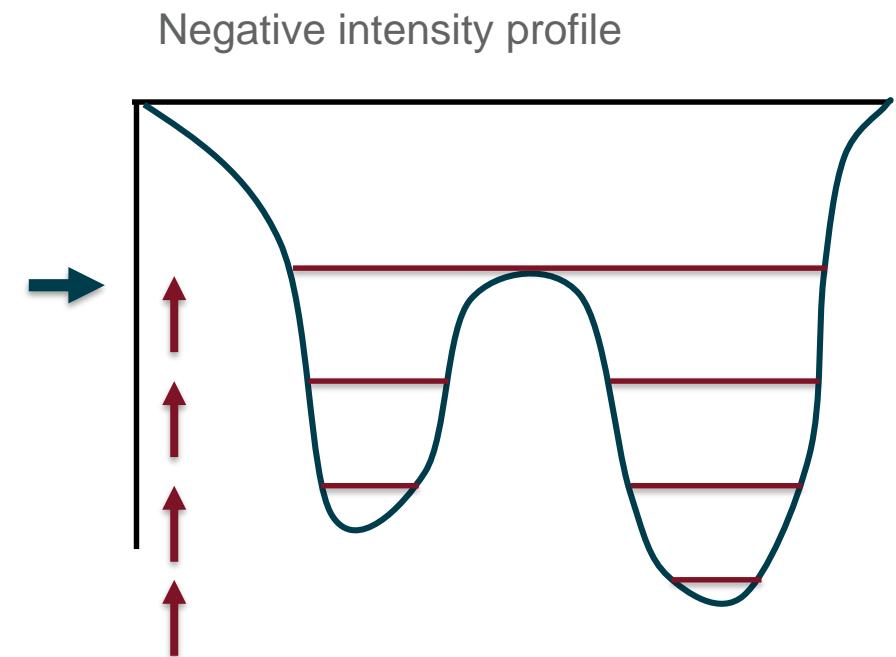
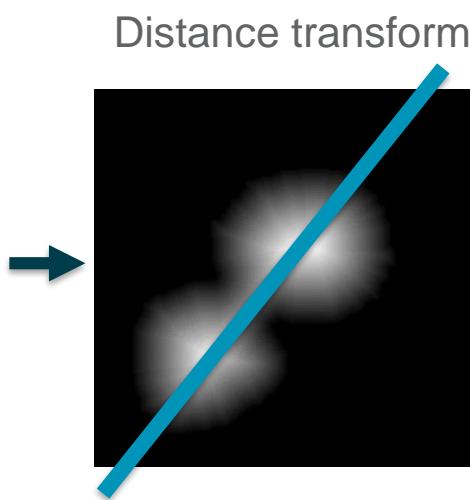
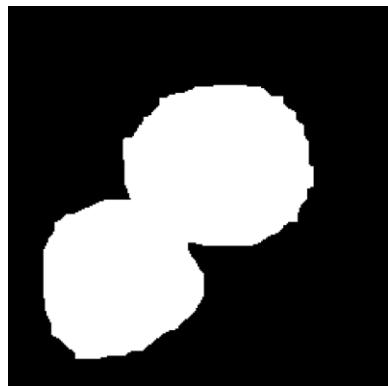
Watershed



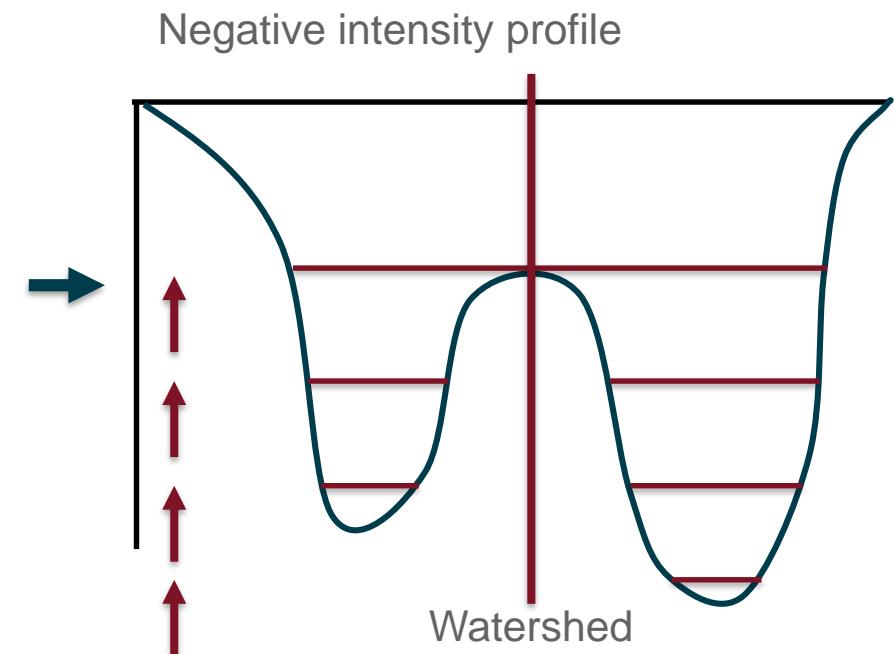
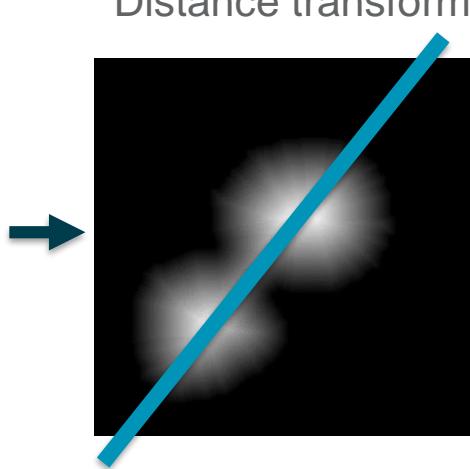
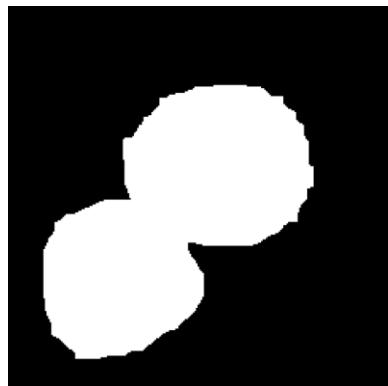
Negative intensity profile



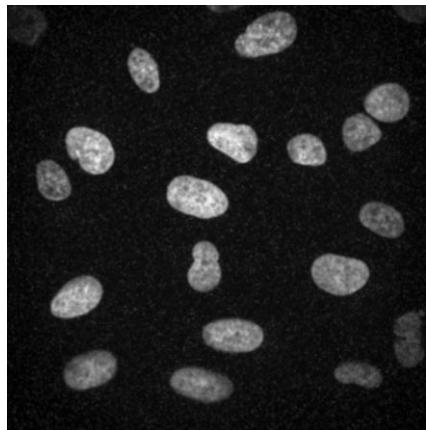
Watershed



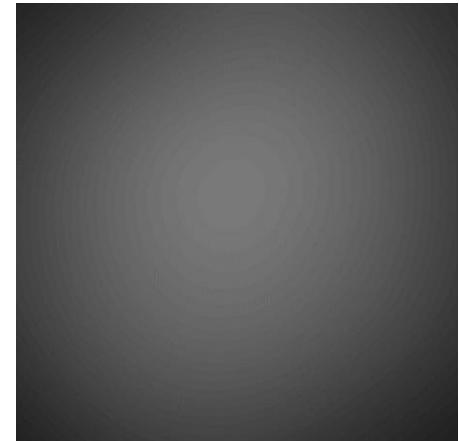
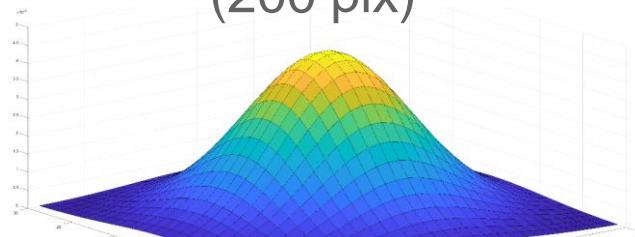
Watershed



Filters - Gaussian

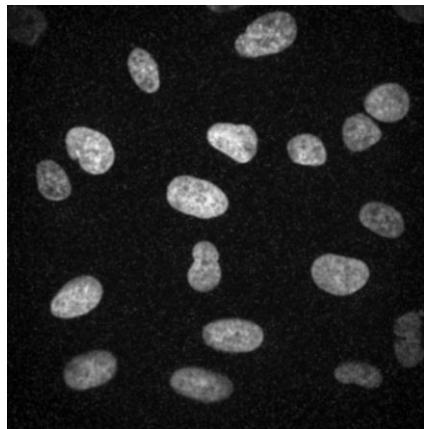


Large kernel
(200 pix)

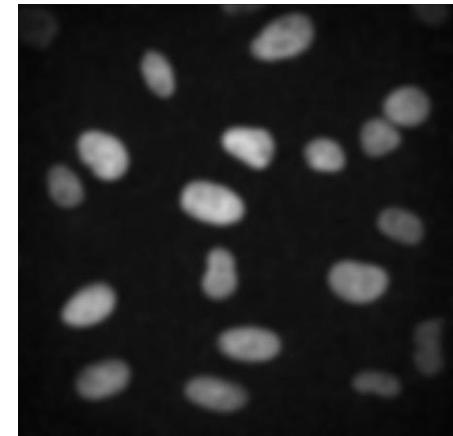
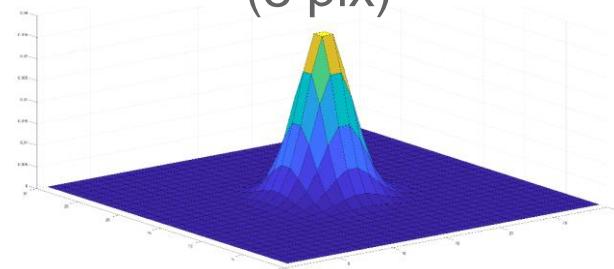


- Correct inhomogeneous illumination

Filters - Gaussian



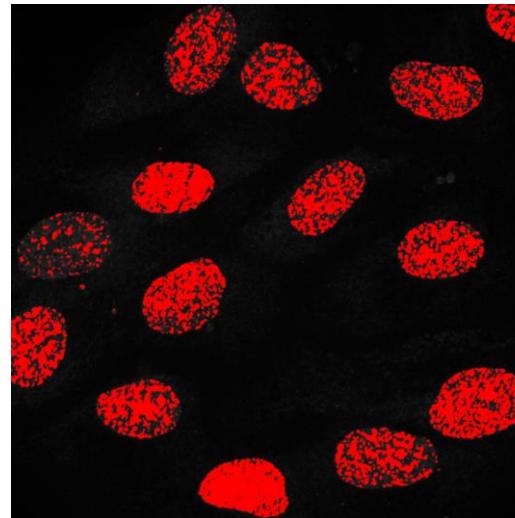
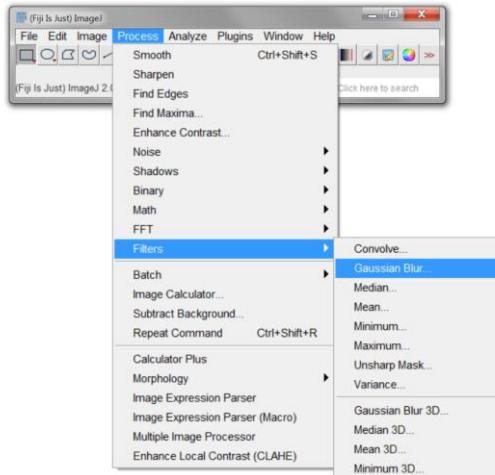
Small kernel
(8 pix)



- Smooth intensity variations for thresholding

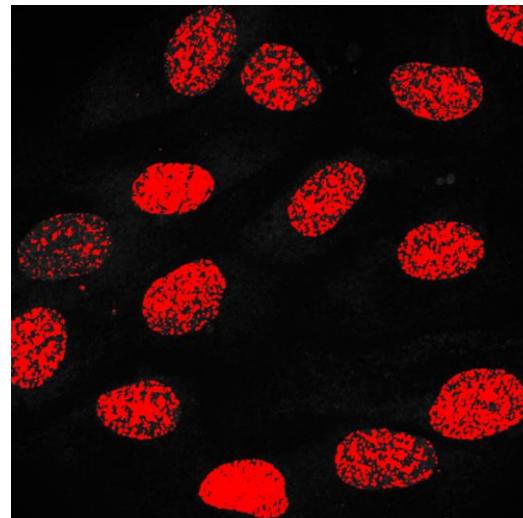
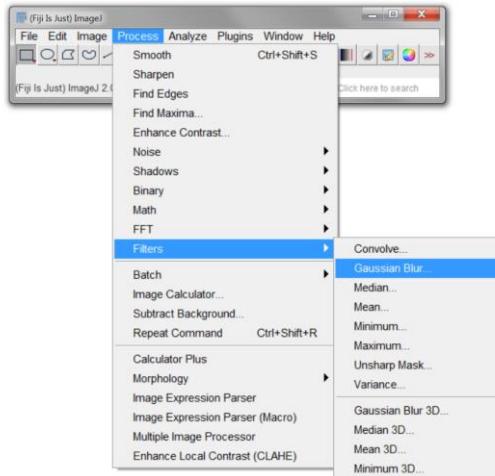
Filters - Gaussian

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

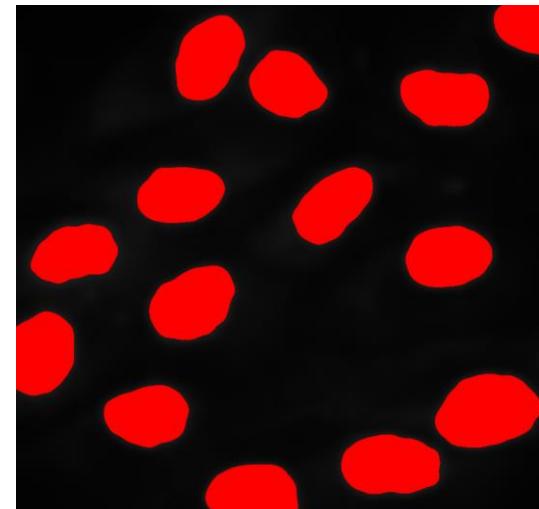


Filters - Gaussian

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

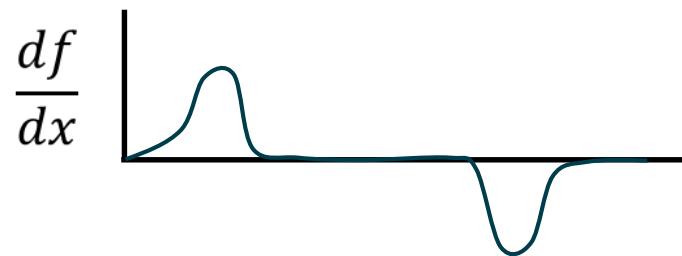
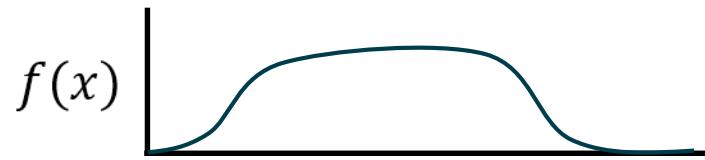


VS



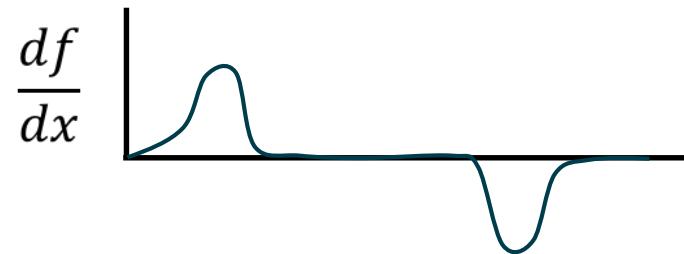
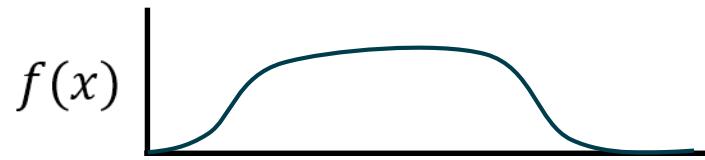
Filters - Gradient

- Edge detection



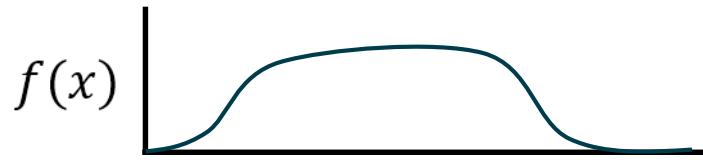
Filters - Gradient

- Edge detection
- Approximate derivative:
The Sobel operator

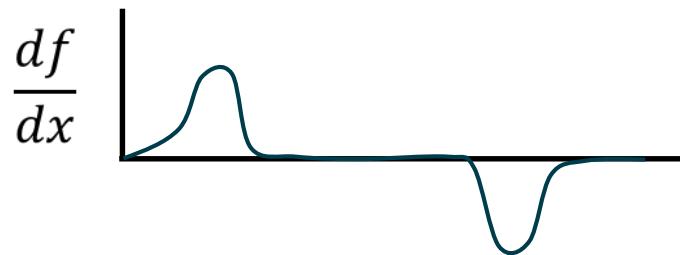


Filters - Gradient

- Edge detection
- Approximate derivative:
The Sobel operator

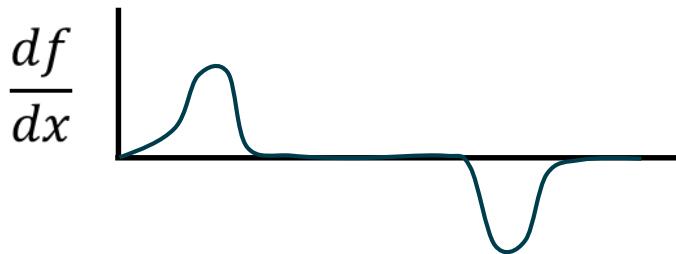
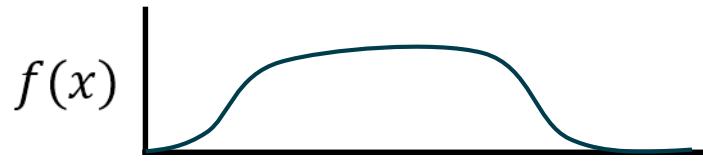


$$G_x = \begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * I$$



Filters - Gradient

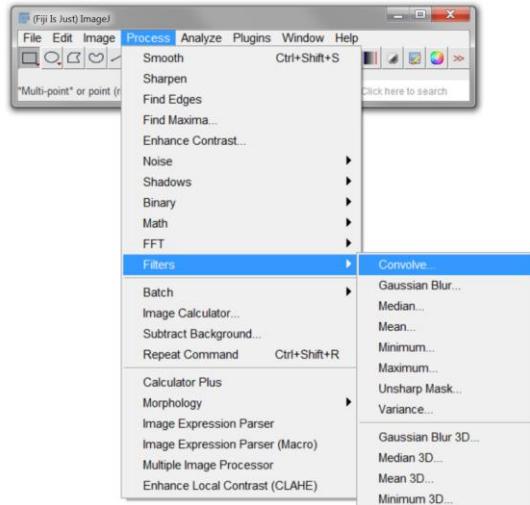
- Edge detection
- Approximate derivative:
The Sobel operator



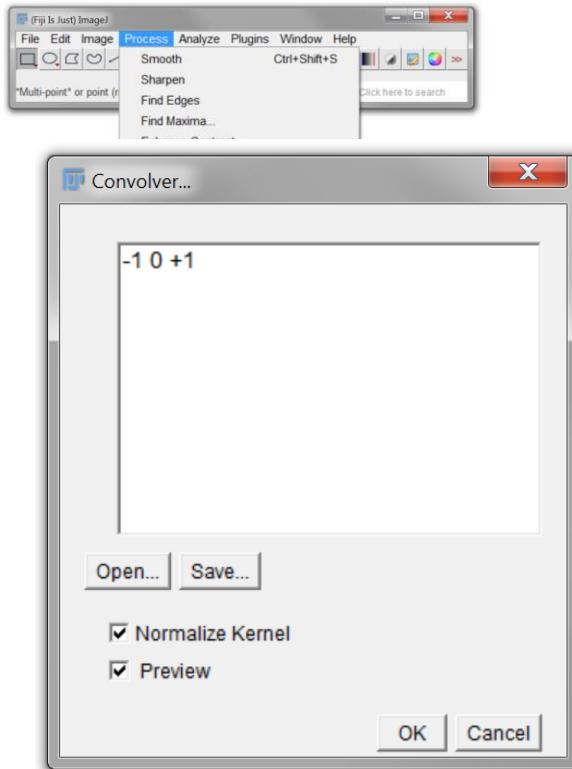
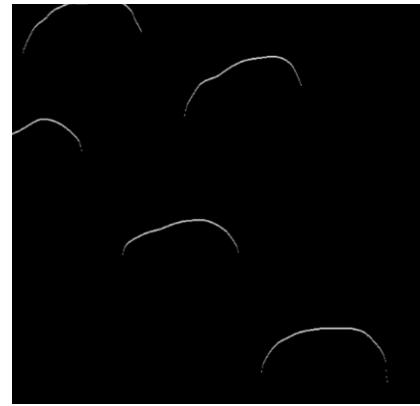
$$G_x = \begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * I$$

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

Filters - Gradient

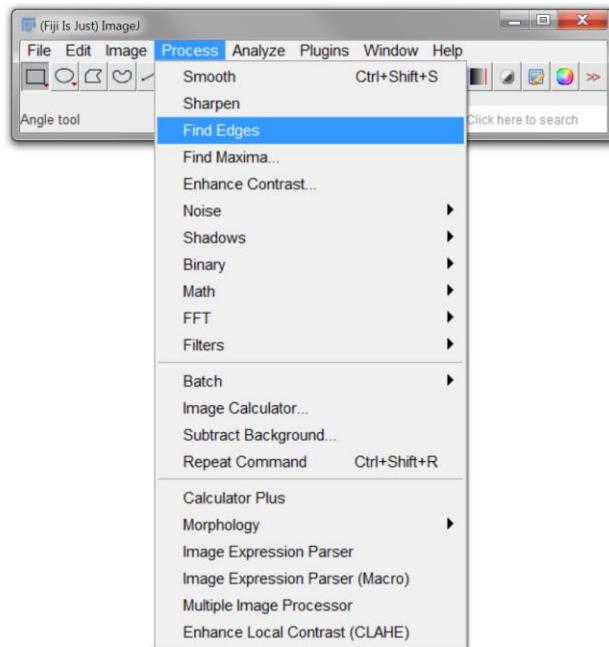


Filters - Gradient

 $-1 \ 0 \ +1$  $+1 \ 0 \ -1$  $+1 \ 0 \ -1$  $-1 \ 0 \ +1$ 

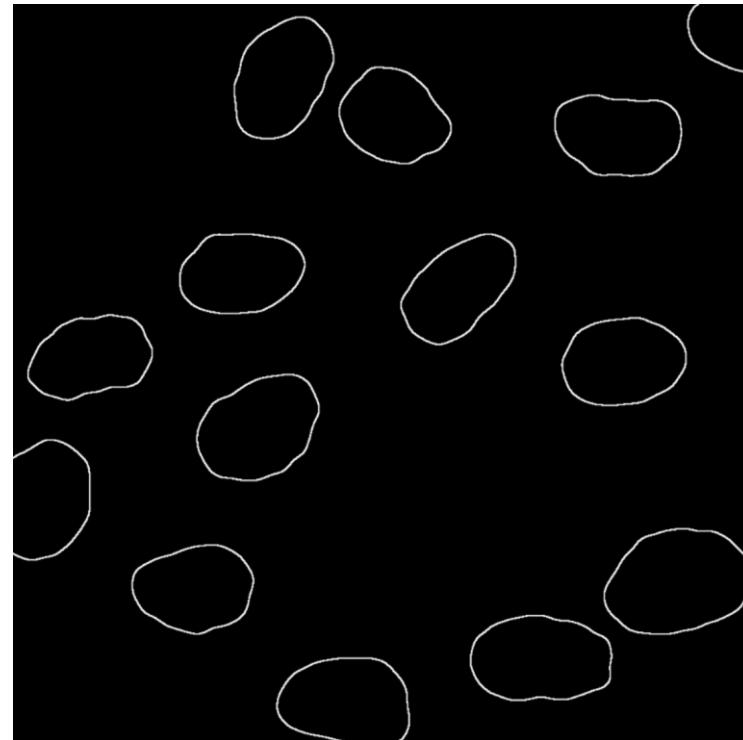
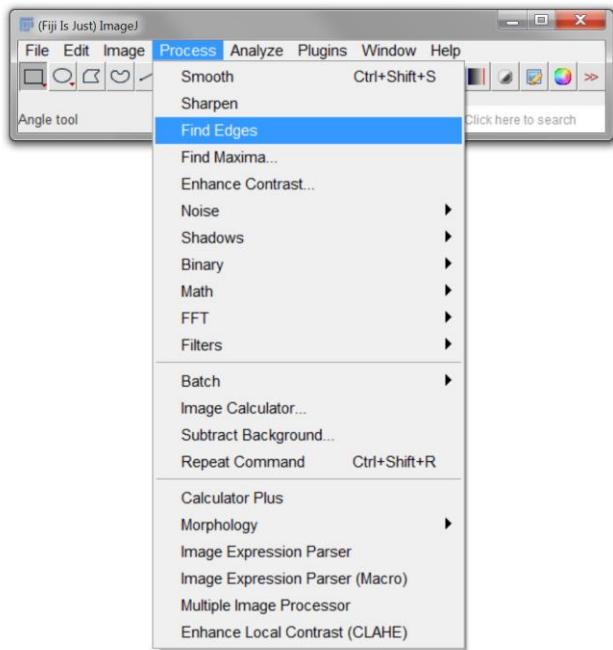
Filters - Gradient

- Combine

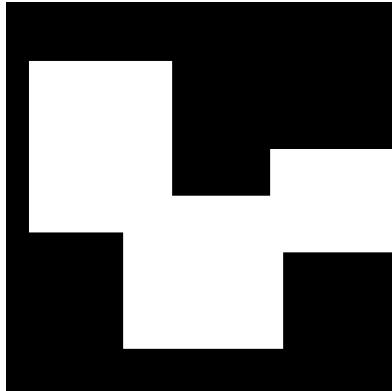
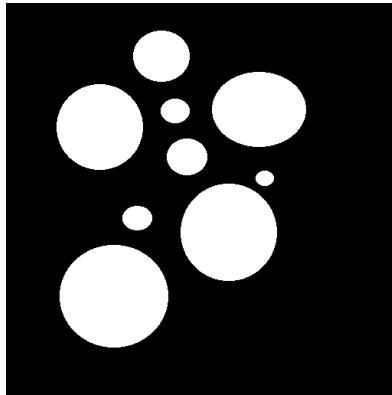


Filters - Gradient

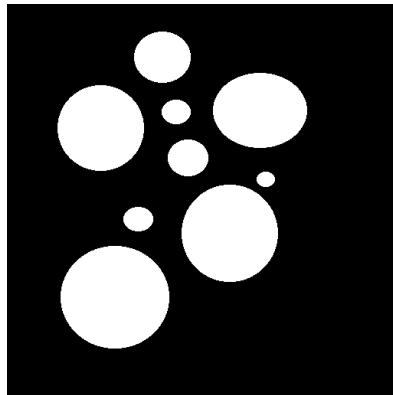
- Combine



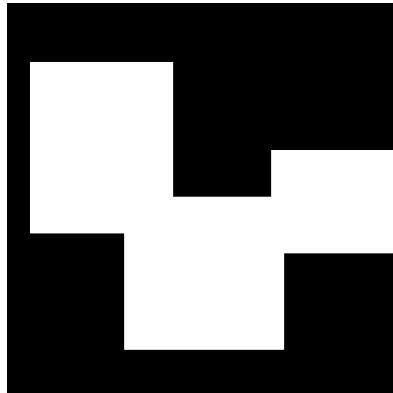
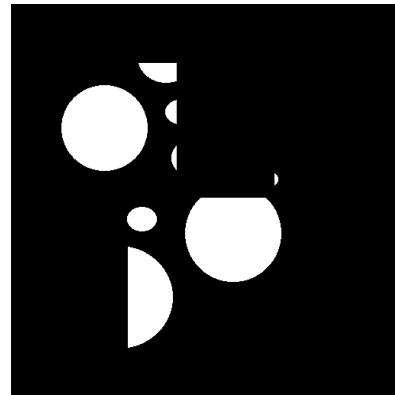
Boolean image mathematics



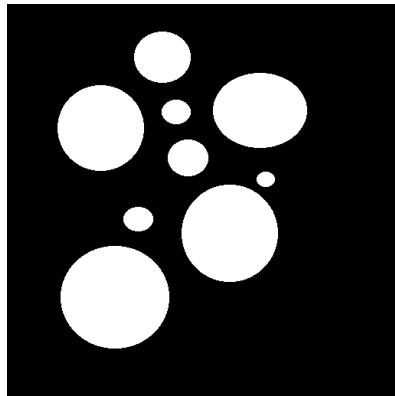
Boolean image mathematics



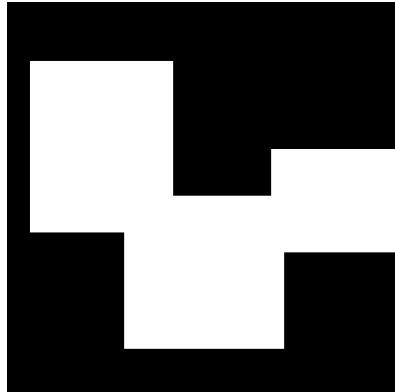
AND



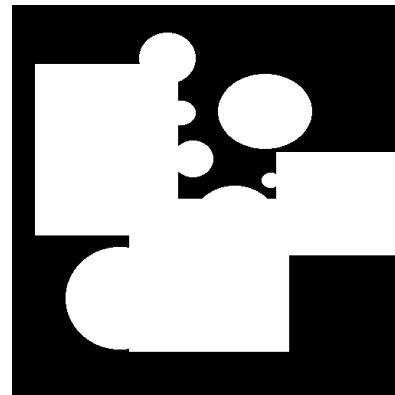
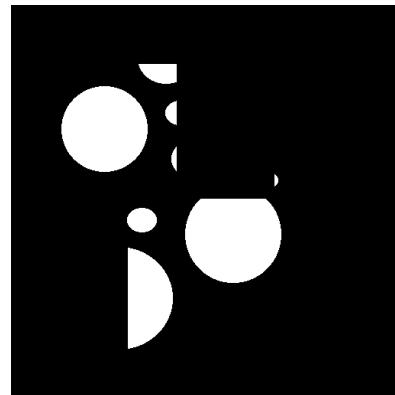
Boolean image mathematics



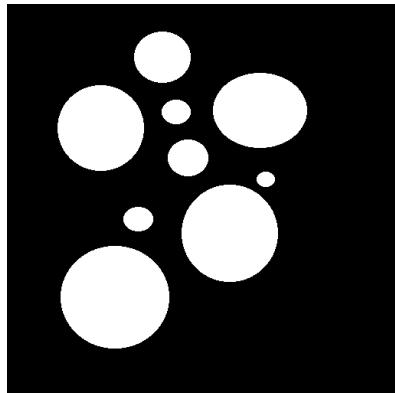
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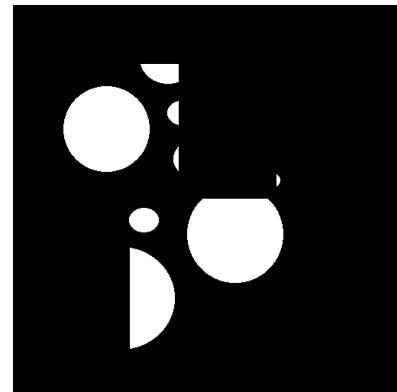
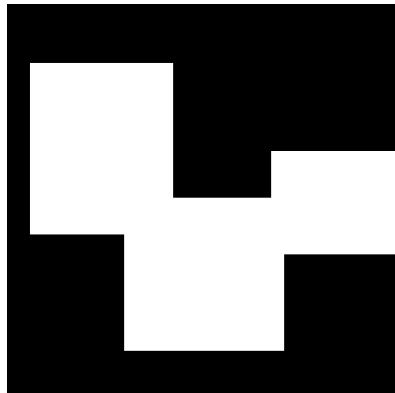
OR



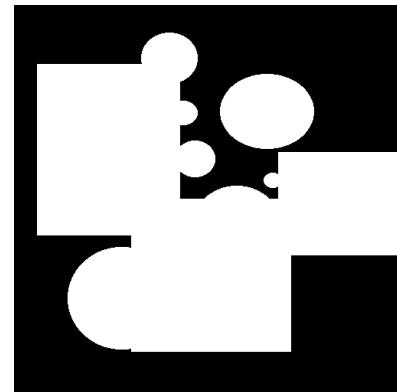
Boolean image mathematics



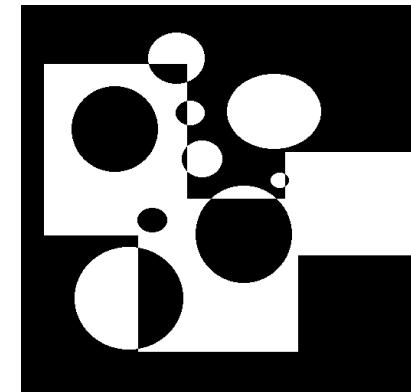
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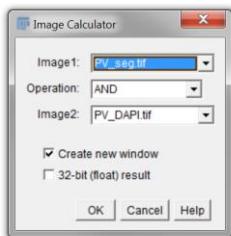
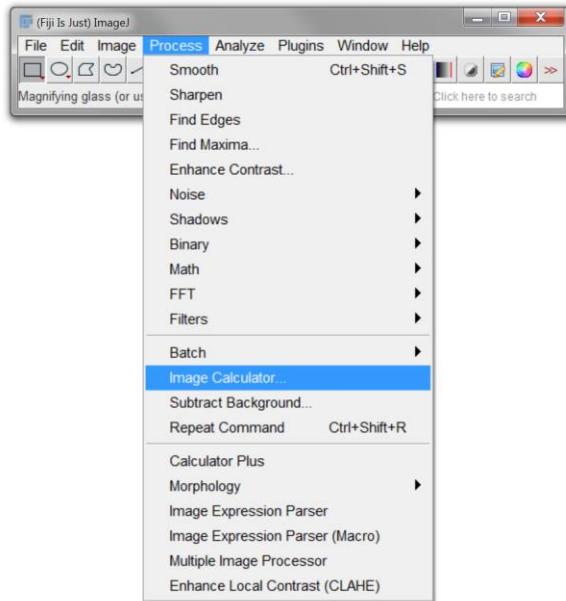
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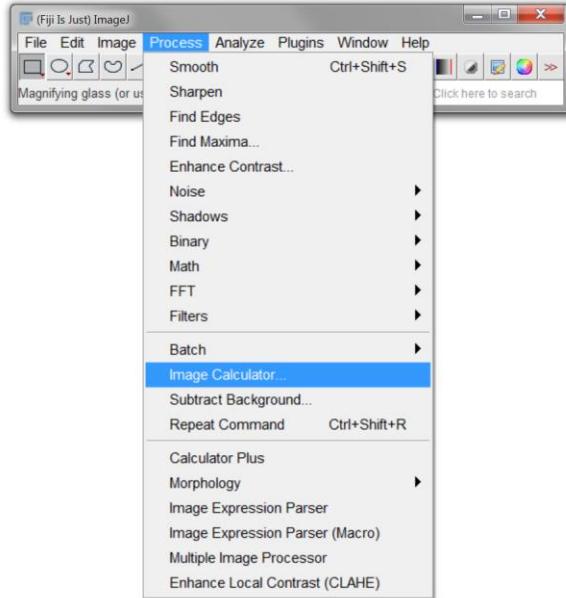
XOR



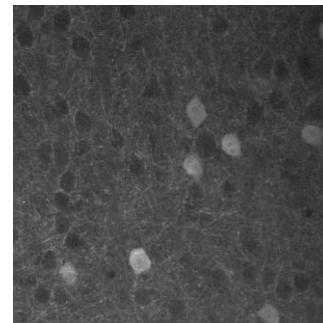
Boolean image mathematics - masking



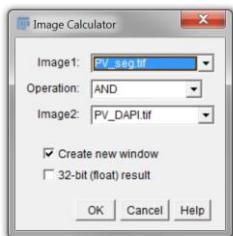
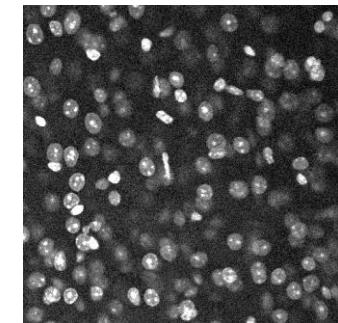
Boolean image mathematics - masking



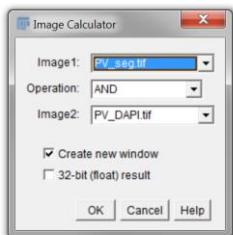
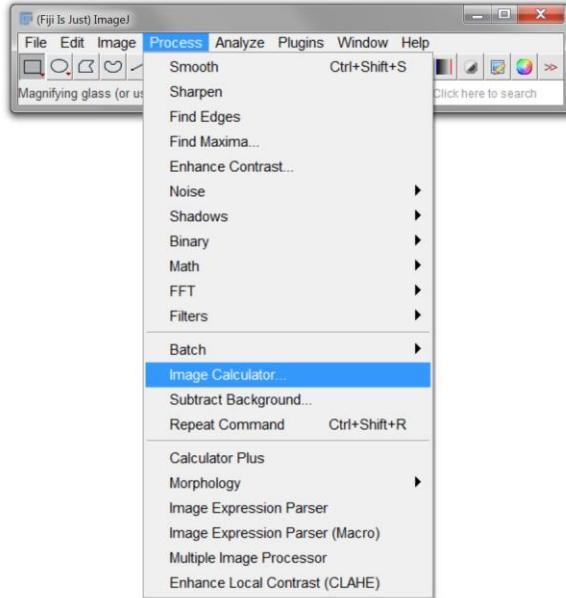
PV.tif



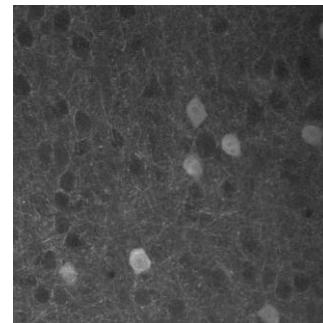
PV_DAPI.tif



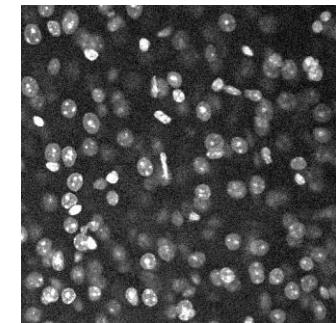
Boolean image mathematics - masking



PV.tif



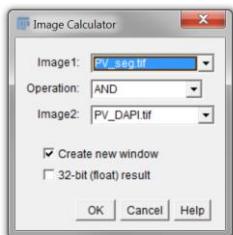
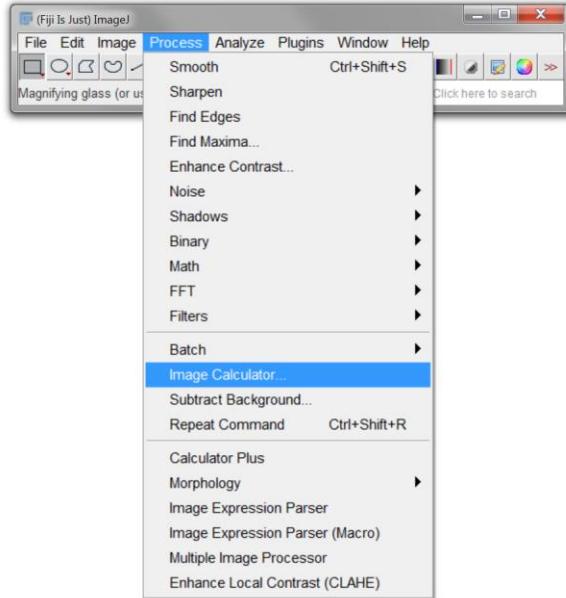
PV_DAPI.tif



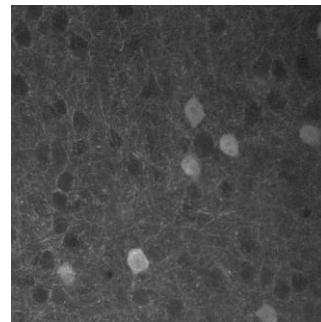
PV_seg.tif



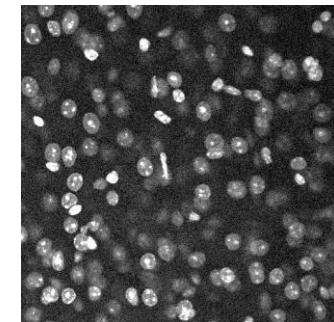
Boolean image mathematics - masking



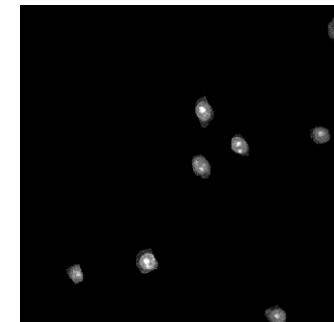
PV.tif



PV_DAPI.tif

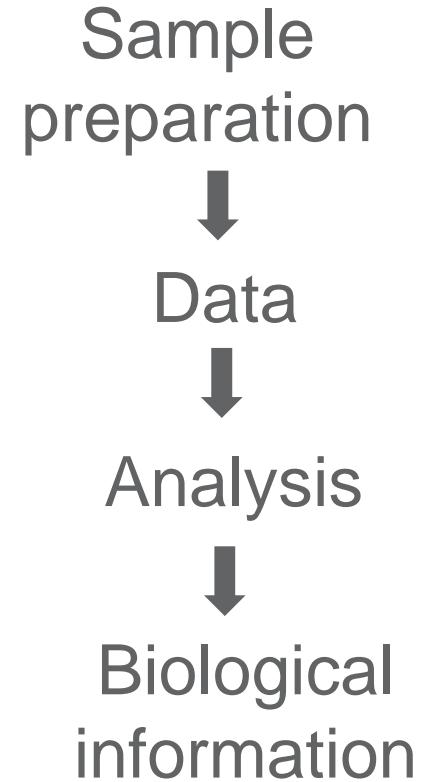


PV_seg.tif



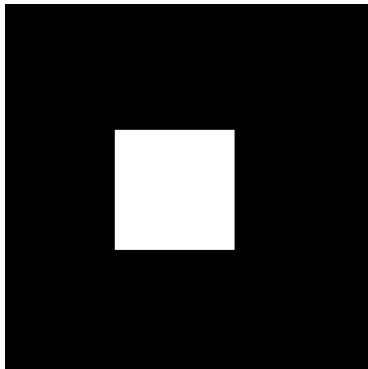
First (and most important lesson) – again!

Test your analysis
before you
acquire data!



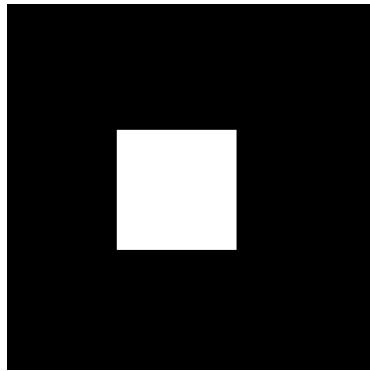
Deconvolution

Sample



Deconvolution

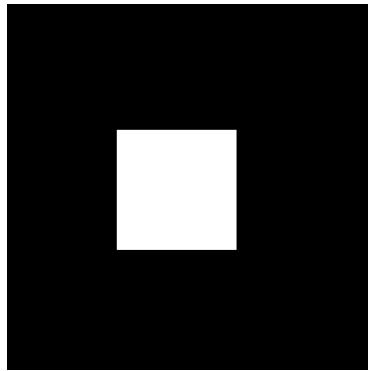
Sample



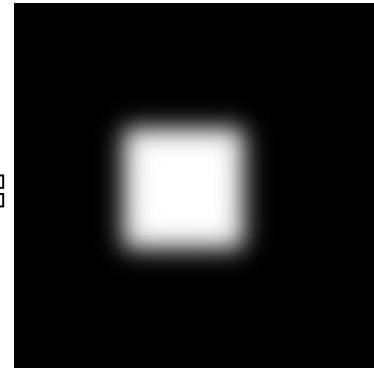
PSF

Deconvolution

Sample

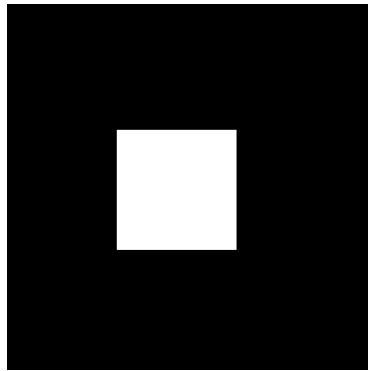


Blurred



Deconvolution

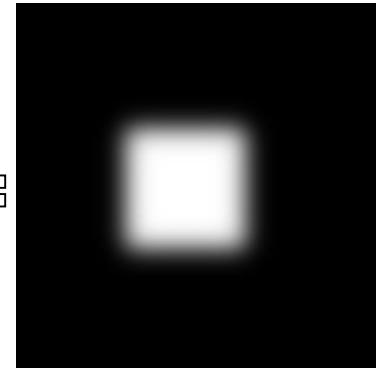
Sample



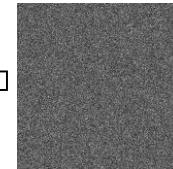
PSF



Blurred

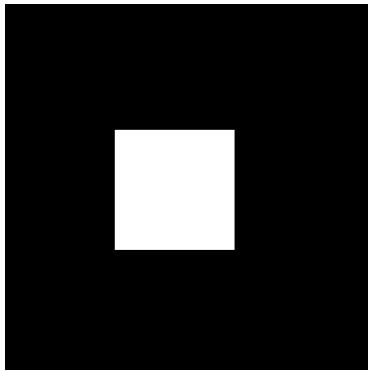


Noise



Deconvolution

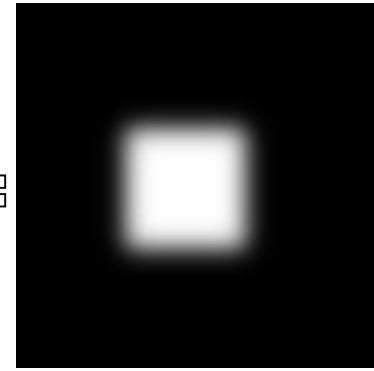
Sample



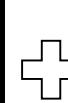
PSF



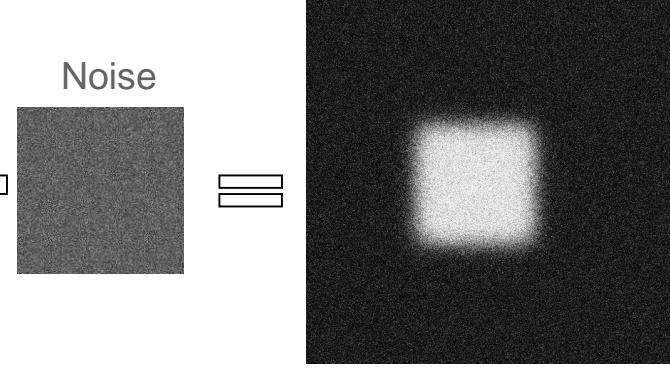
Blurred



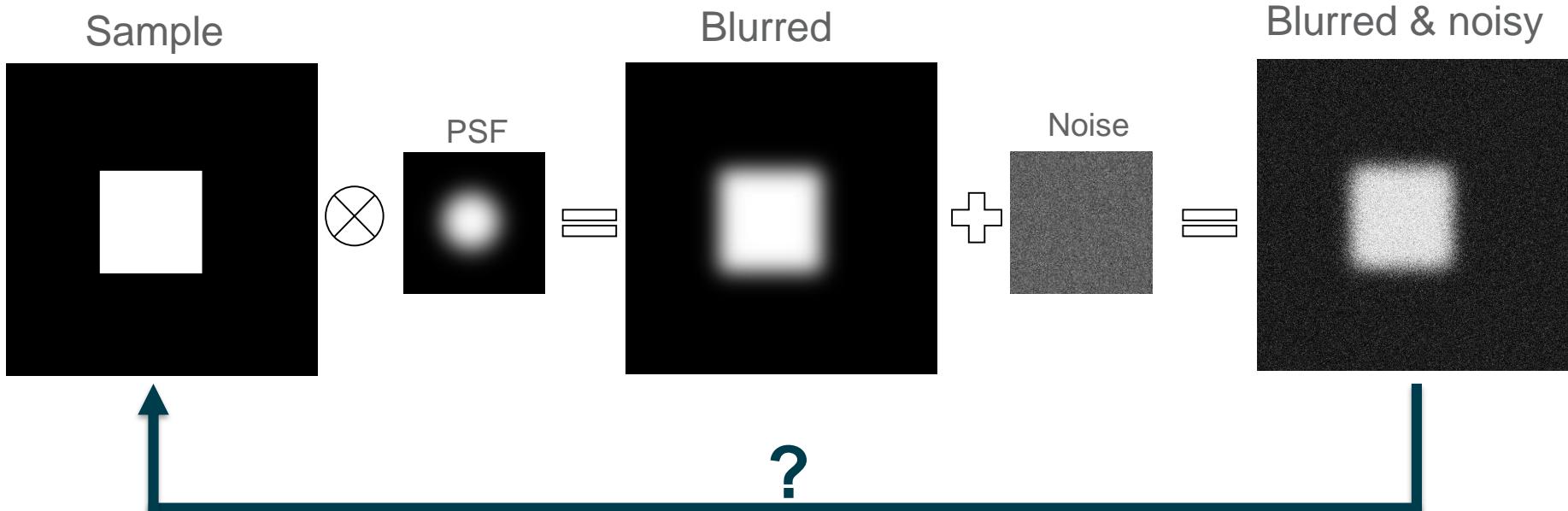
Noise



Blurred & noisy



Deconvolution



Deconvolution – how?

Constrained iterative deconvolution
e.g. Richardson-Lucy

Deconvolution – how?

Constrained iterative deconvolution
e.g. Richardson-Lucy

Image

Operation

Raw Image

Deconvolution – how?

Constrained iterative deconvolution
e.g. Richardson-Lucy

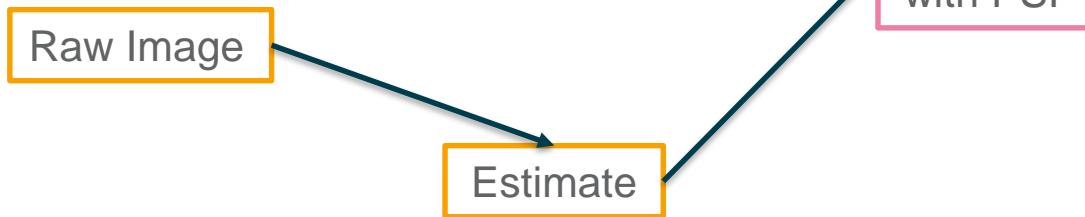
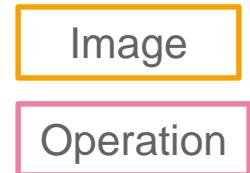
Image

Operation



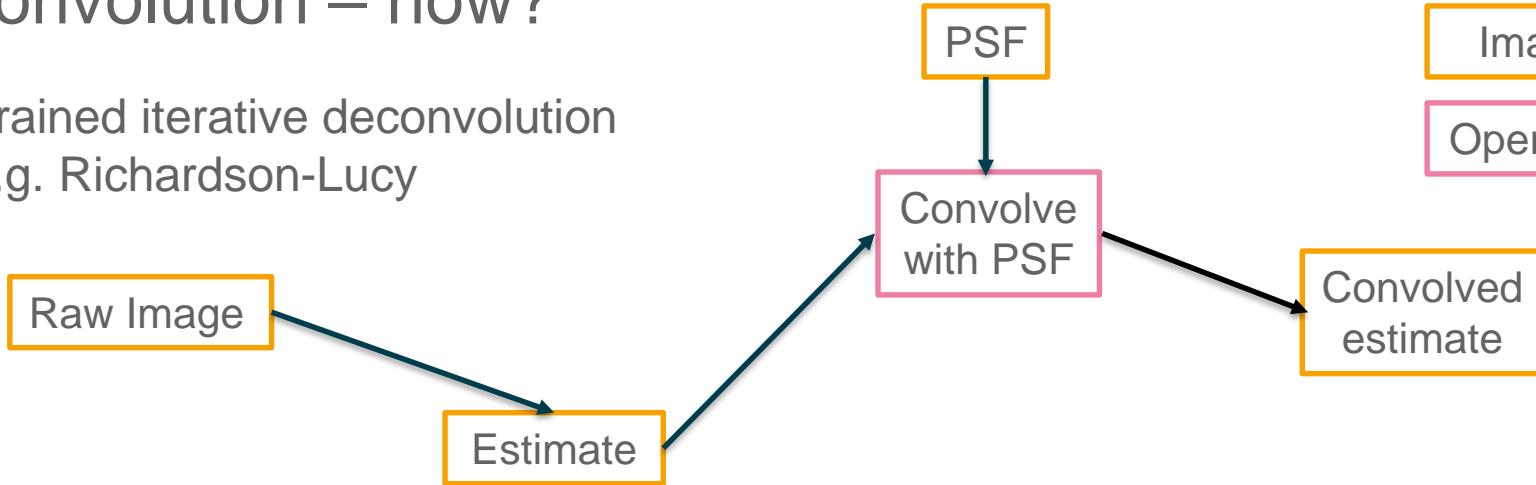
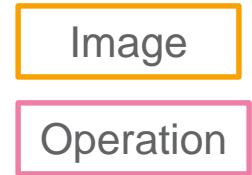
Deconvolution – how?

Constrained iterative deconvolution
e.g. Richardson-Lucy



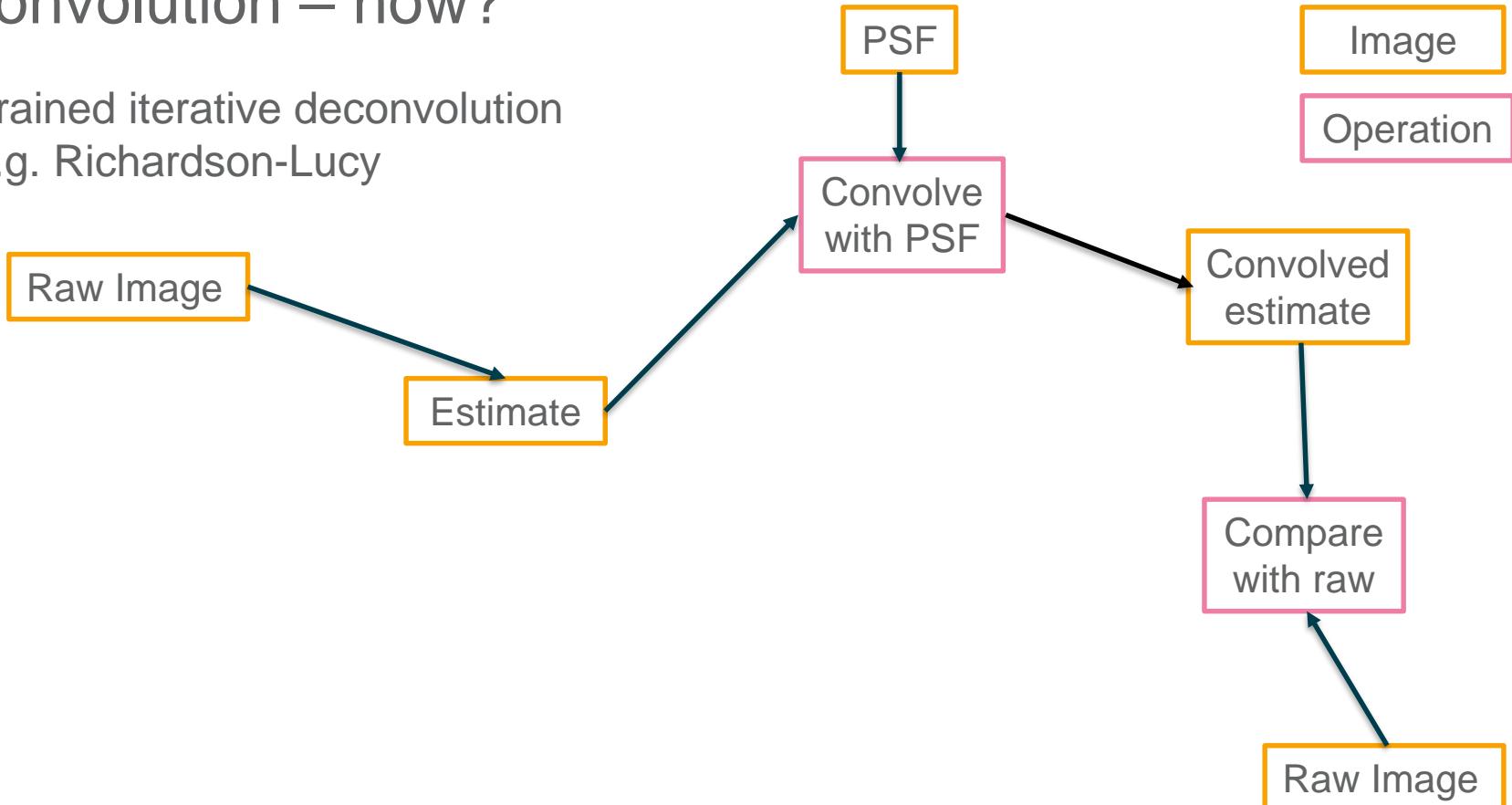
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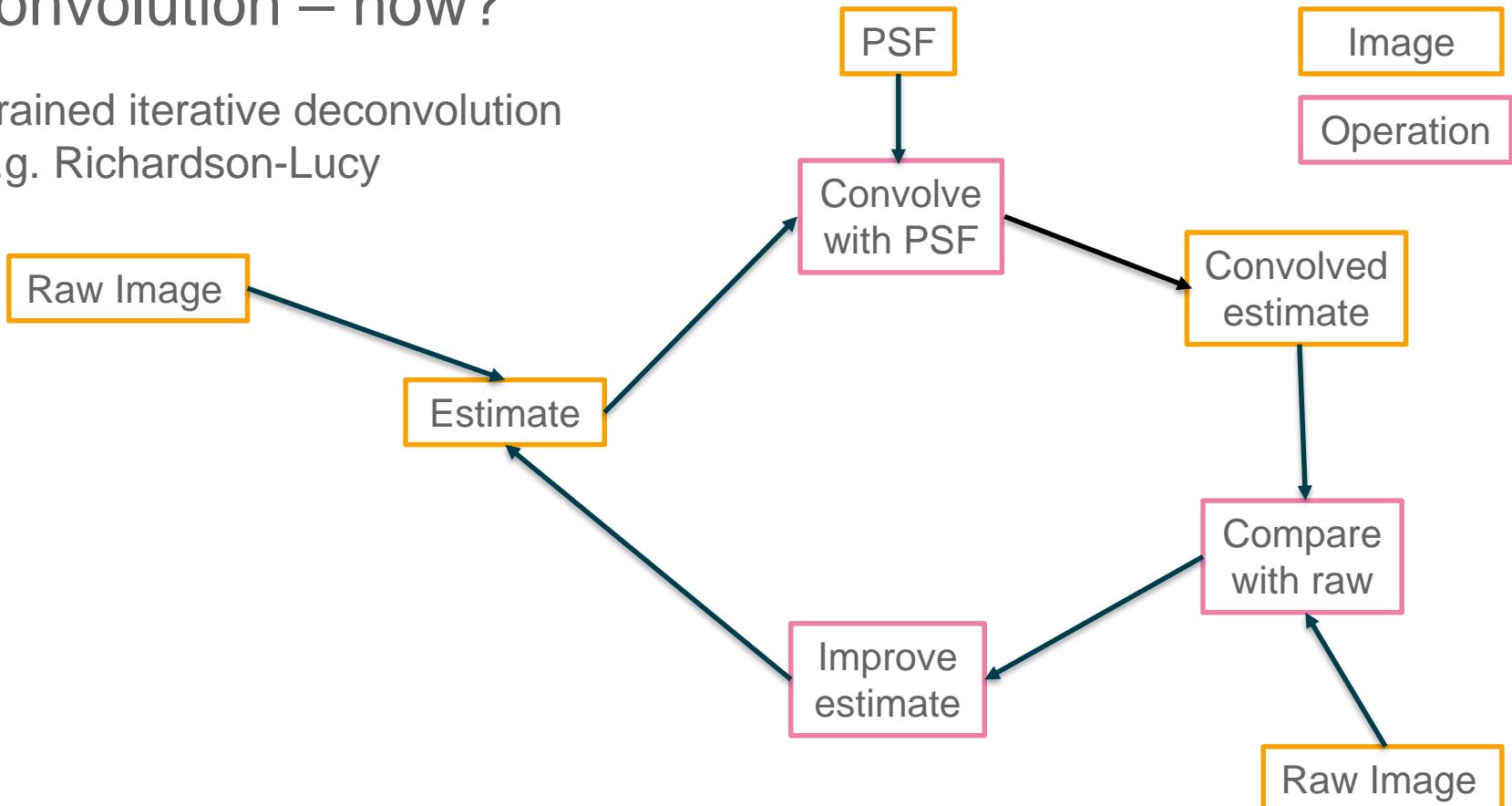
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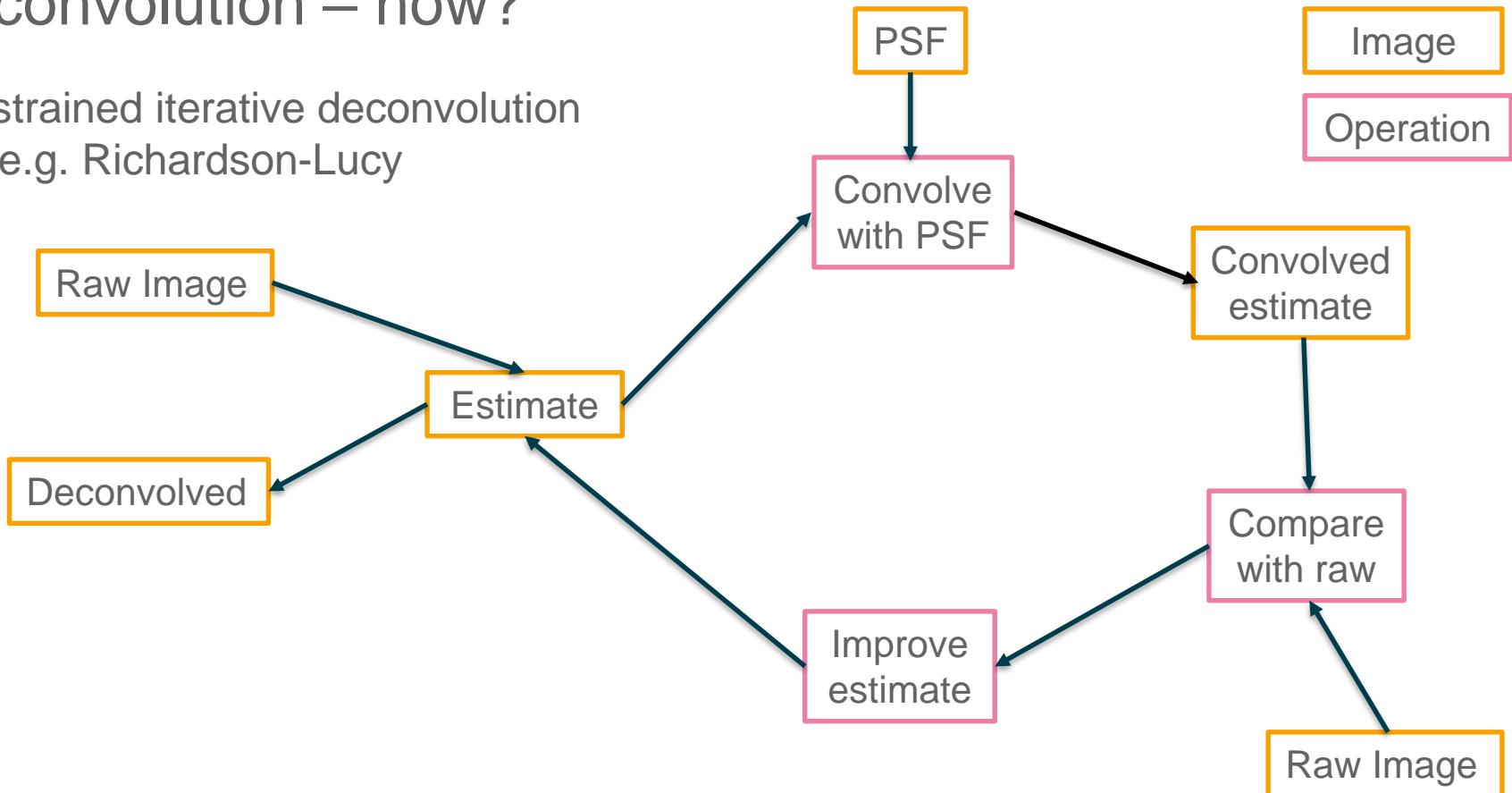
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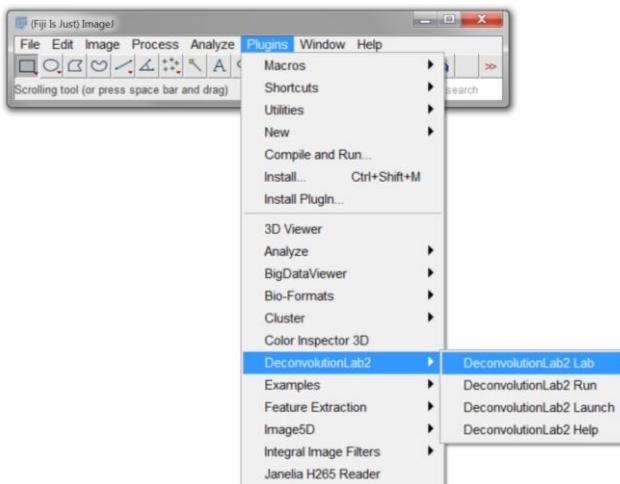
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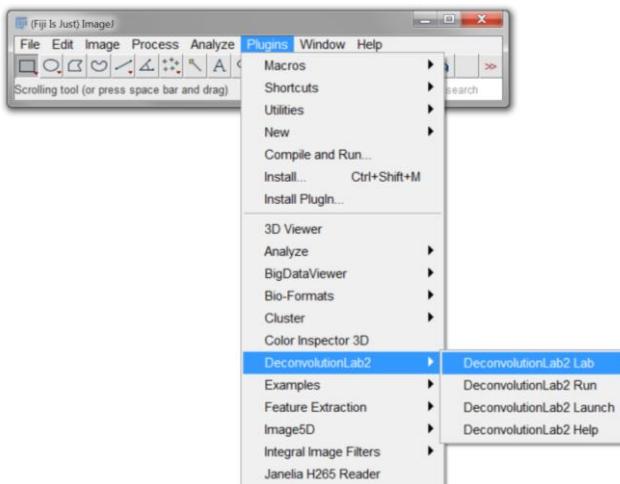
Deconvolution – try it out

DeconvolutionLab2



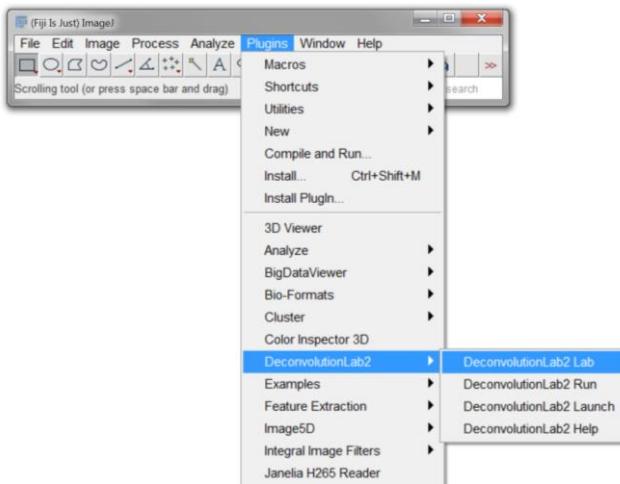
Deconvolution – BUT

DeconvolutionLab2



Deconvolution – BUT

DeconvolutionLab2

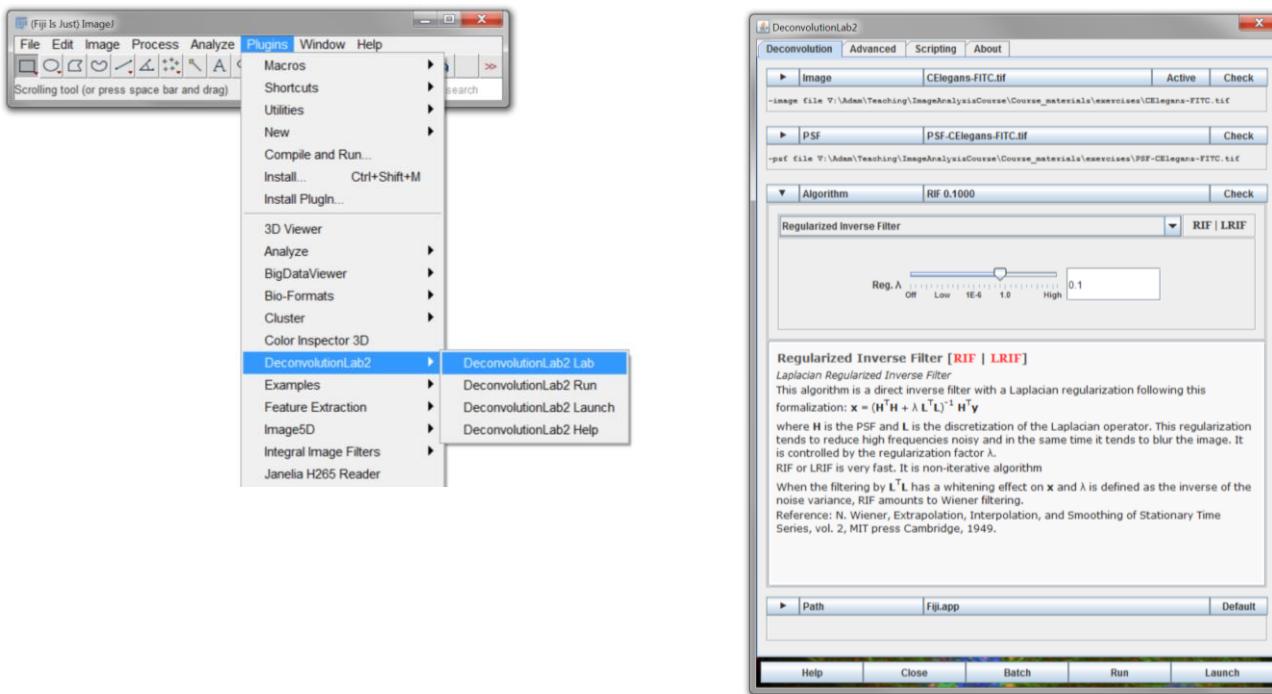


- Plugin is free, but must be manually installed
- Download .jar file and copy into plugins folder



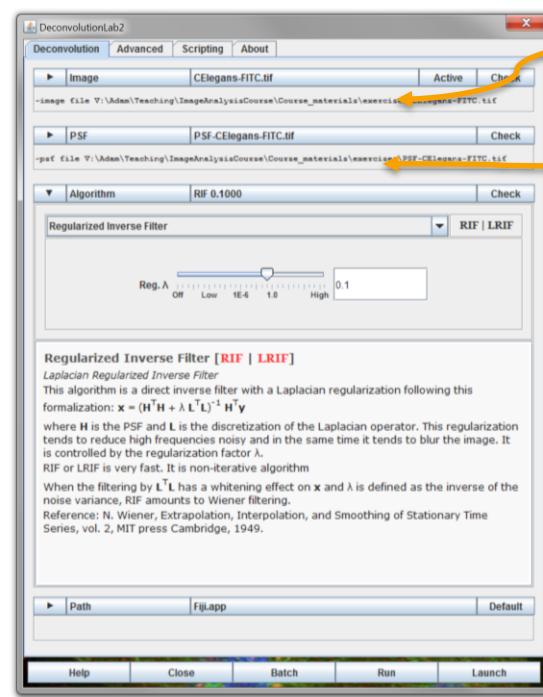
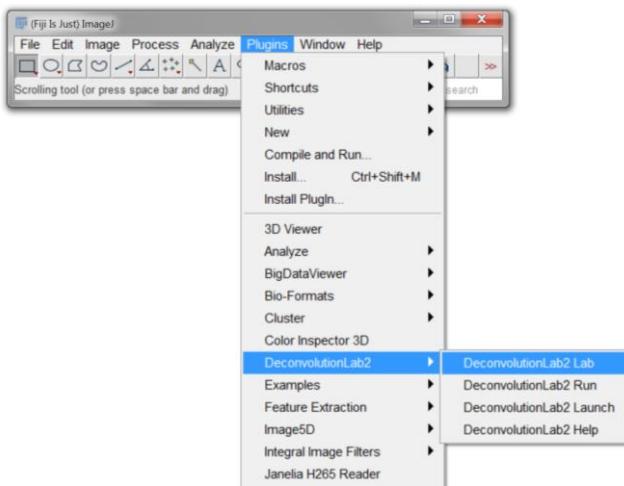
Deconvolution – try it out

DeconvolutionLab2



Deconvolution – try it out

DeconvolutionLab2



Drag and drop

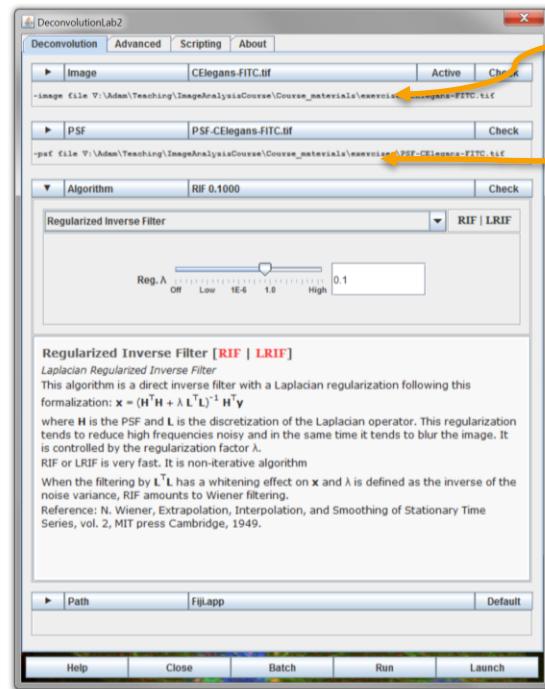
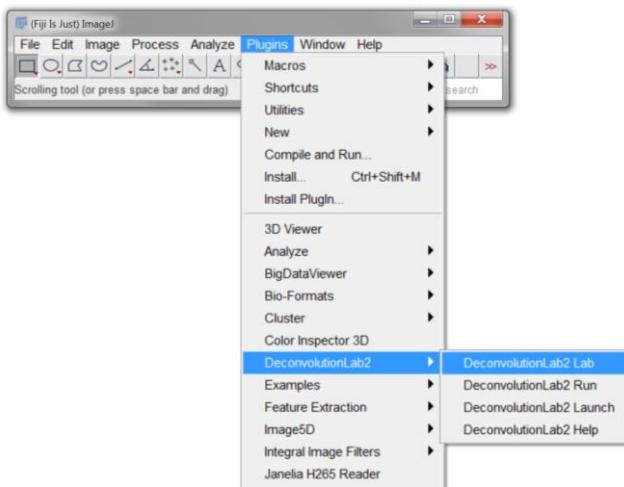
CElegans-FITC.tif

PSF-CElegans-FITC.tif

C. Elegans embryo
- microtubules

Deconvolution – try it out

DeconvolutionLab2

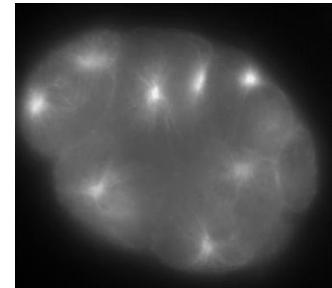


Drag and drop

CElegans-FITC.tif

PSF-CElegans-FITC.tif

C. Elegans embryo
- microtubules



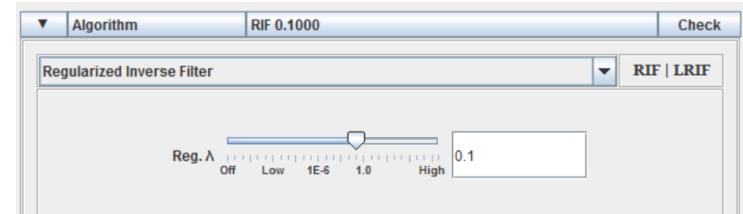
Deconvolution – try it out (hopefully)

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

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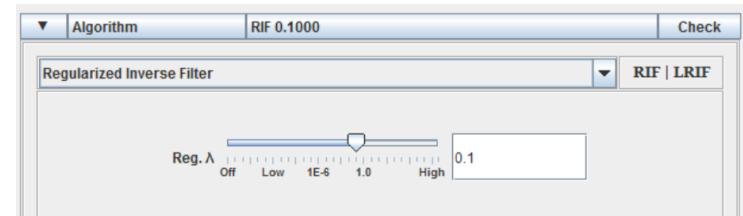
- Try “Regularized Inverse Filter”



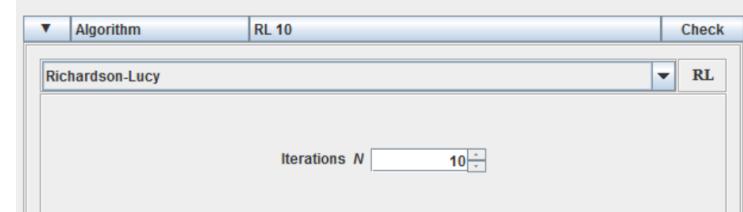
Deconvolution – try it out (hopefully)

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

Deconvolution – notes

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- Only an estimate of fluorophore distribution

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- Can be very slow

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)

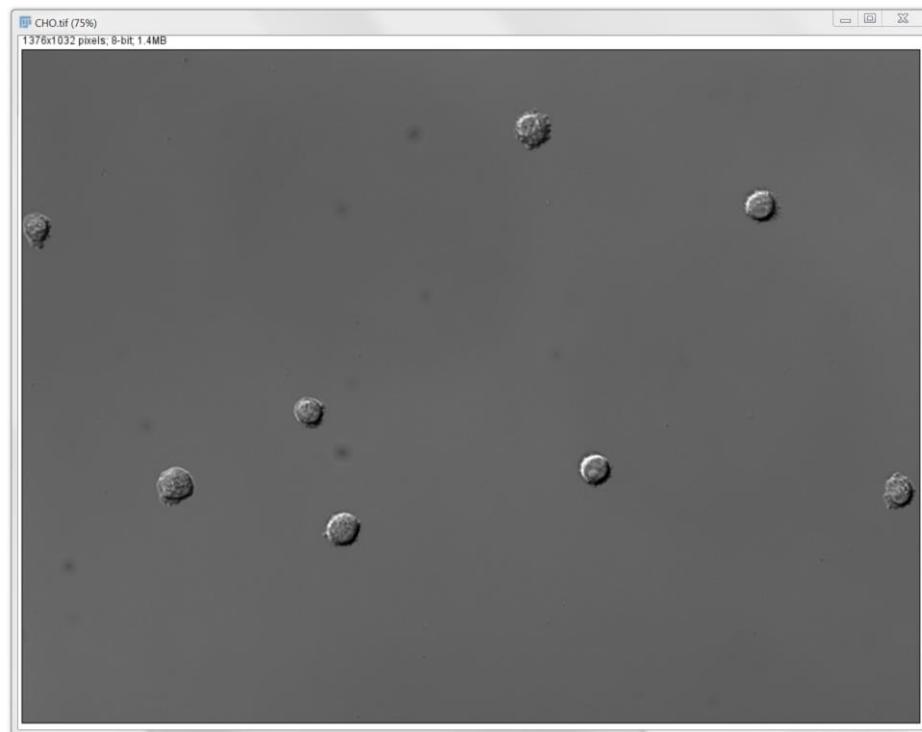
Machine Learning

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

Machine Learning

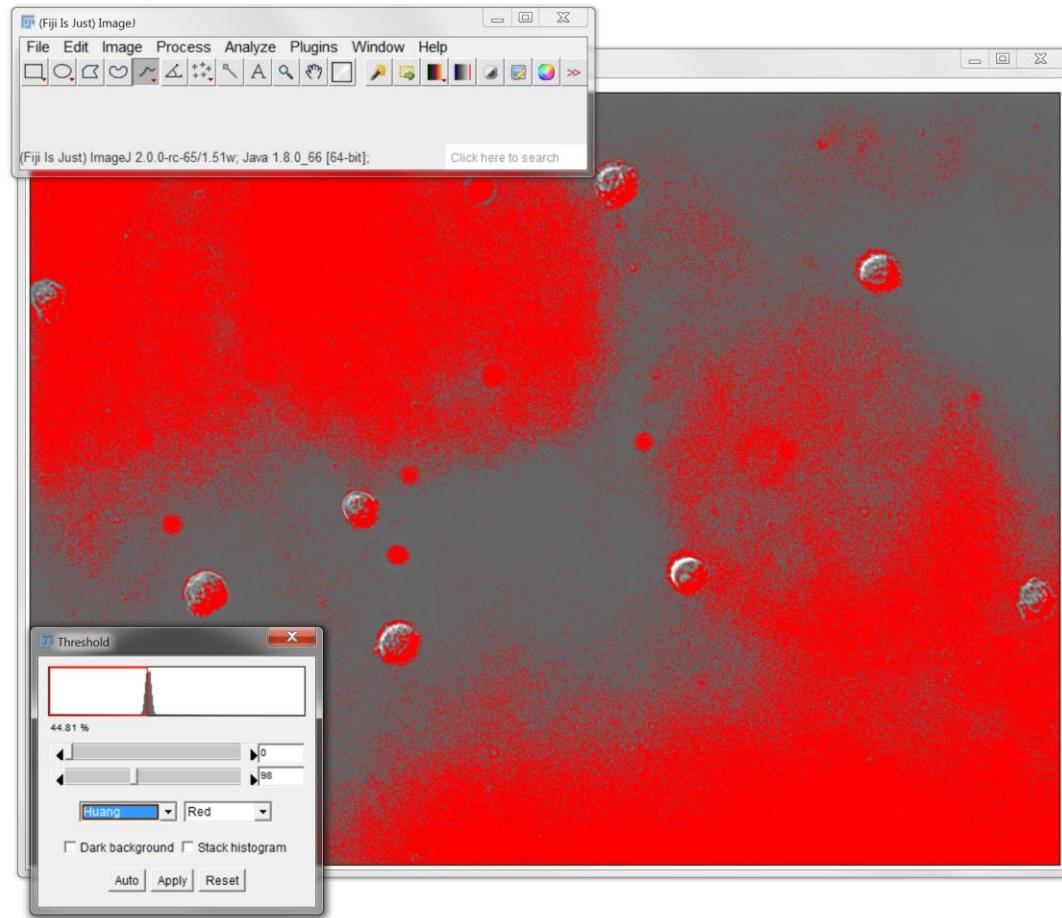
- Often intensity alone can't be used for segmentation
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- Open CHO.tif

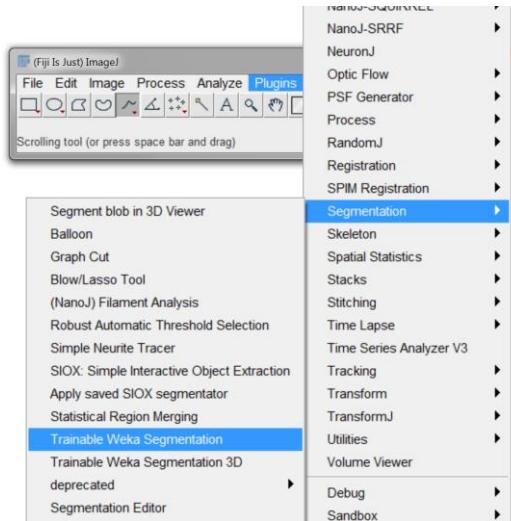


Machine Learning

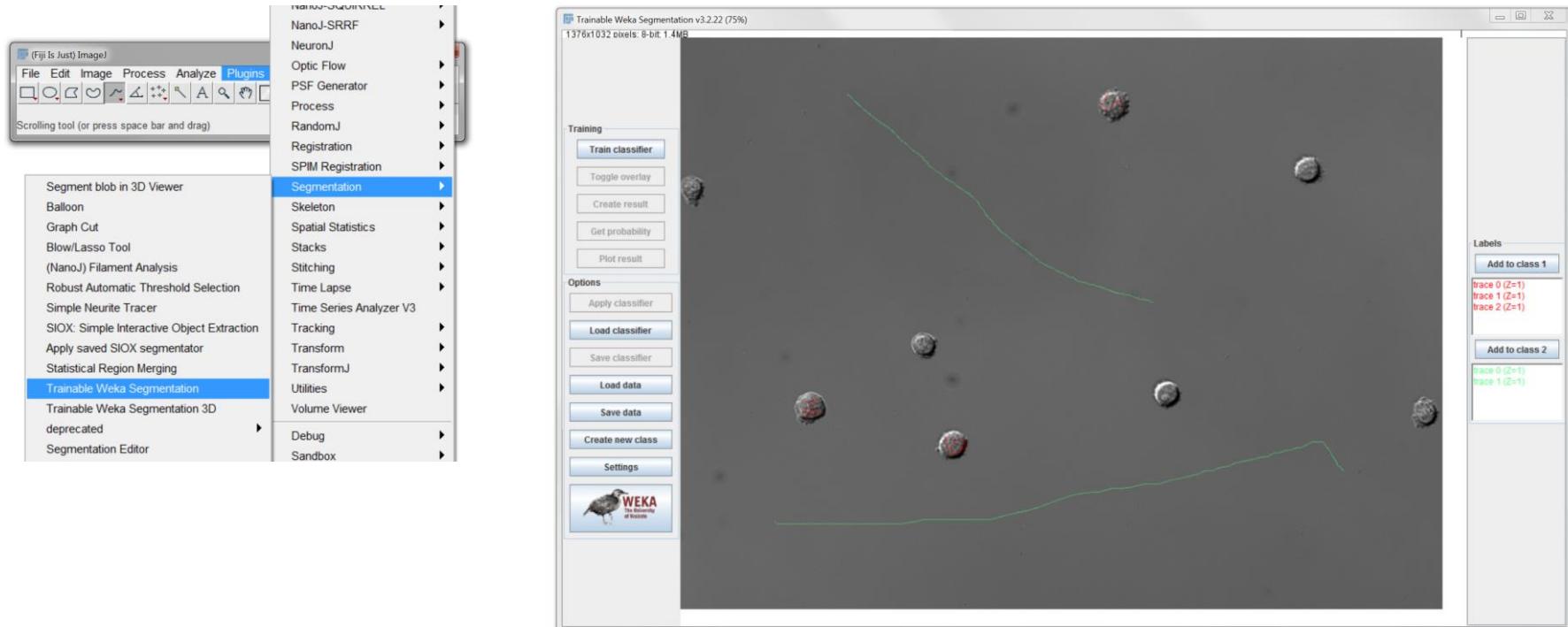
- Try to threshold!



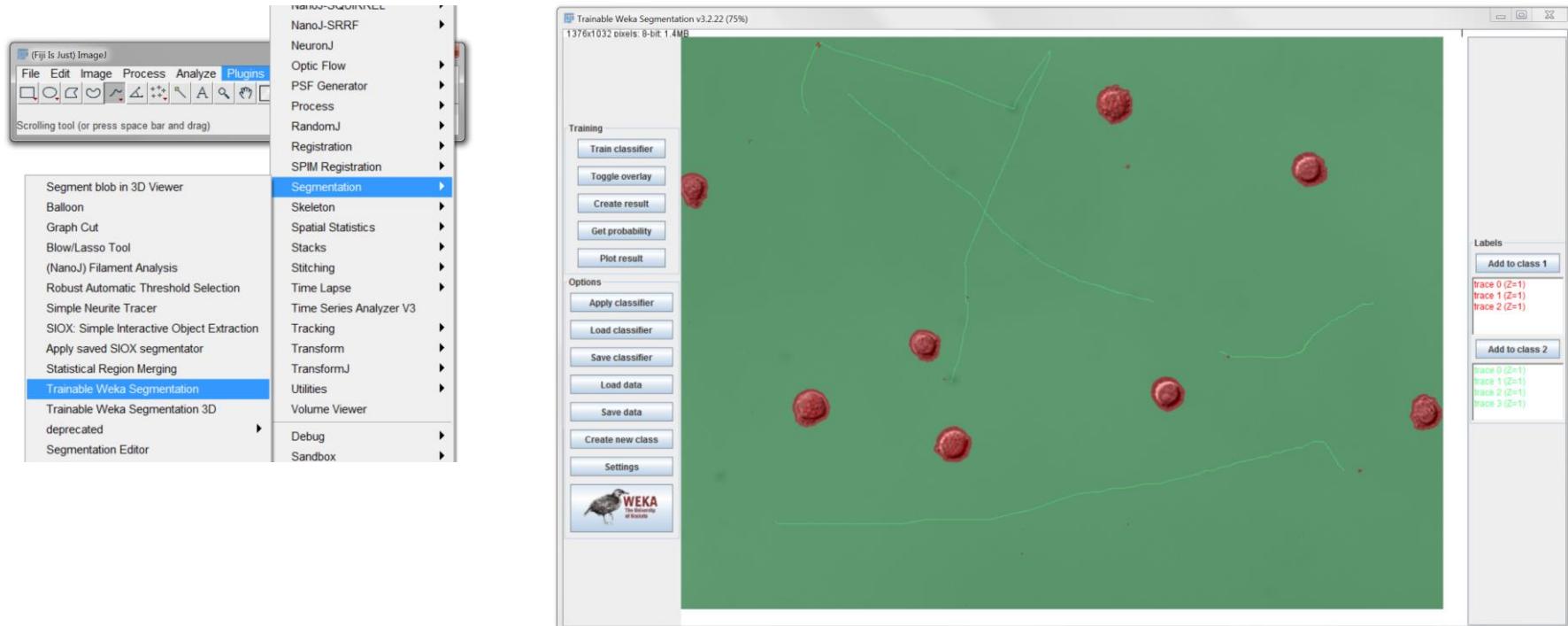
Machine Learning – Trainable Weka Segmentation



Machine Learning – Trainable Weka Segmentation

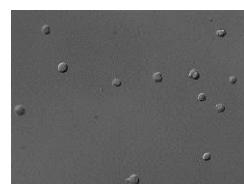
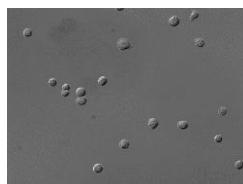
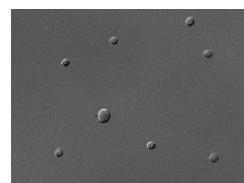
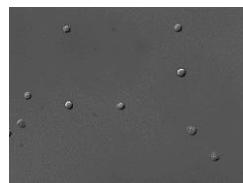
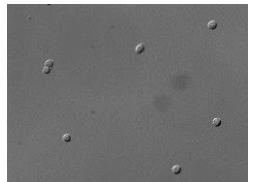


Machine Learning – Trainable Weka Segmentation



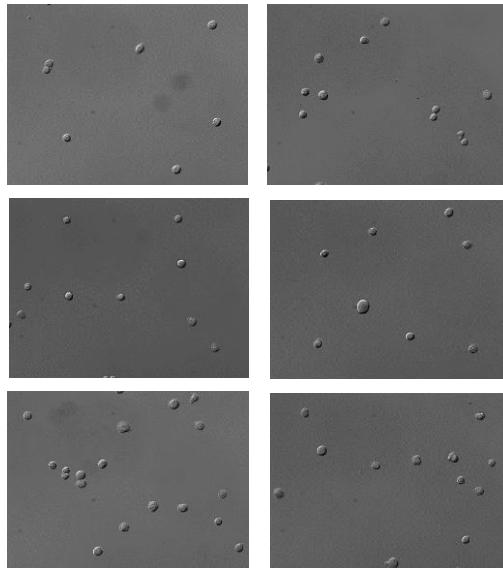
Machine Learning – Trainable Weka Segmentation

- Lots of training data

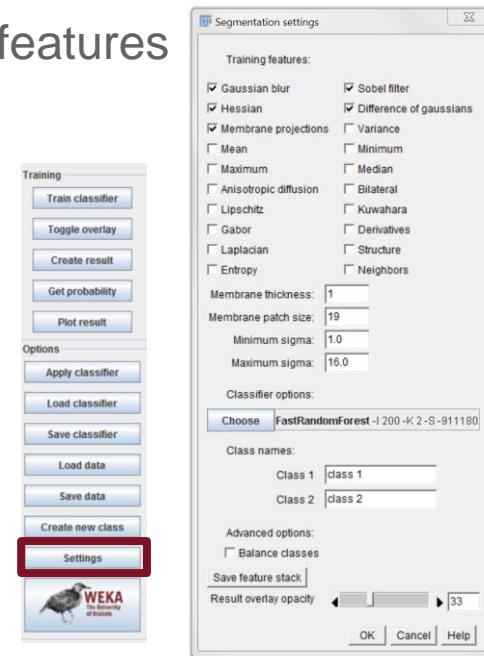


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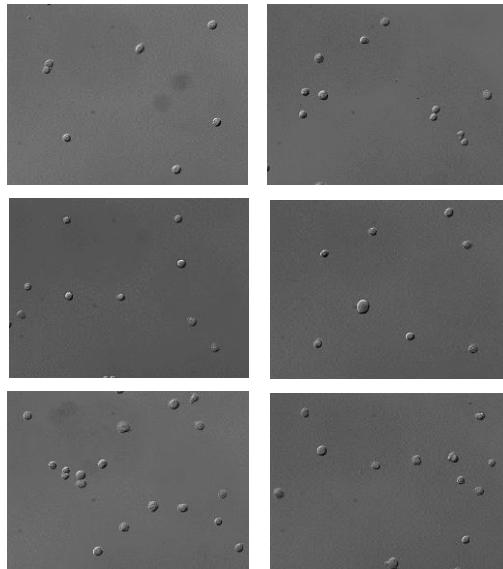


- Choose features carefully

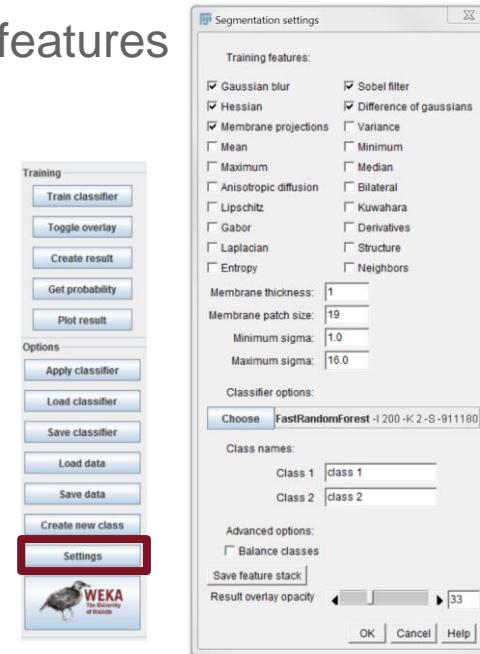


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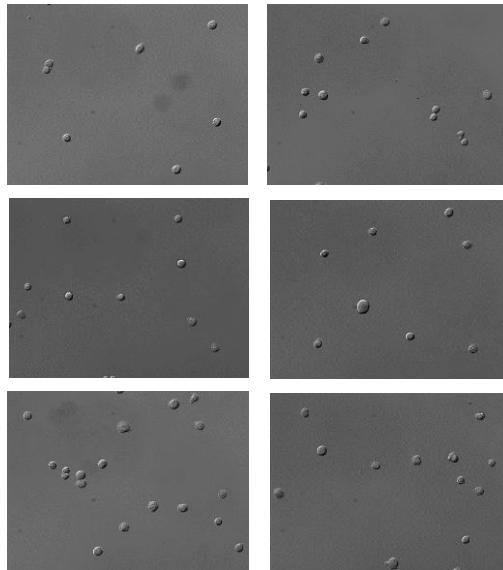
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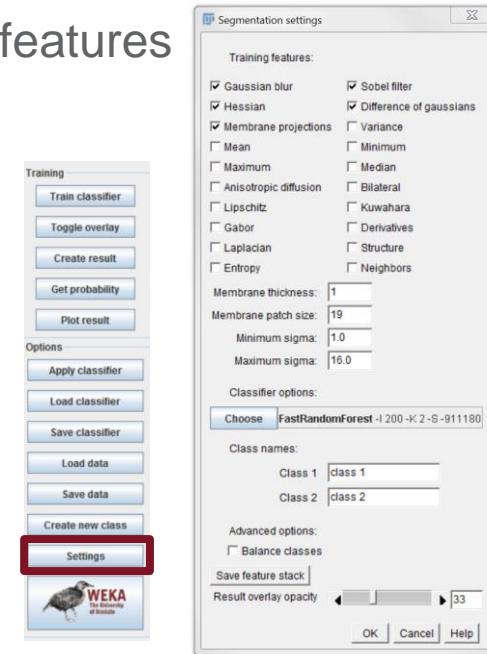
- Will be slow!

Machine Learning – Trainable Weka Segmentation

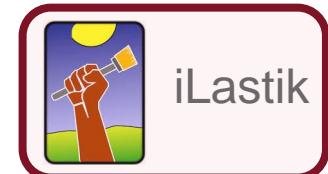
- Lots of training data



- Choose features carefully



- Will be slow!

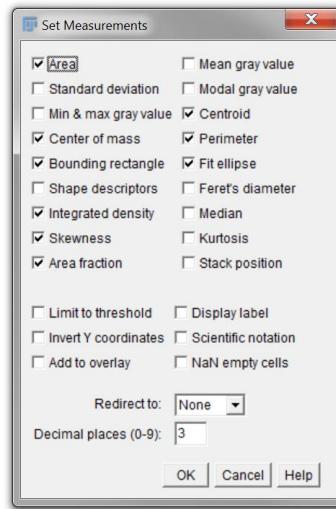
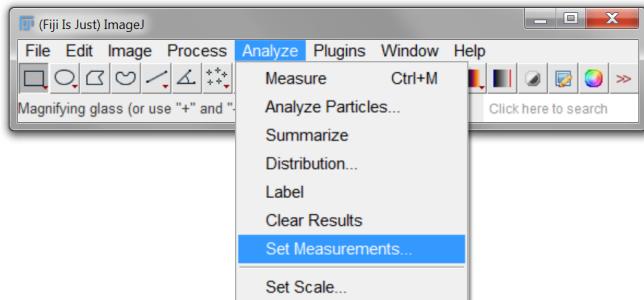


Analysis (rather than processing)

- Measure segmented objects

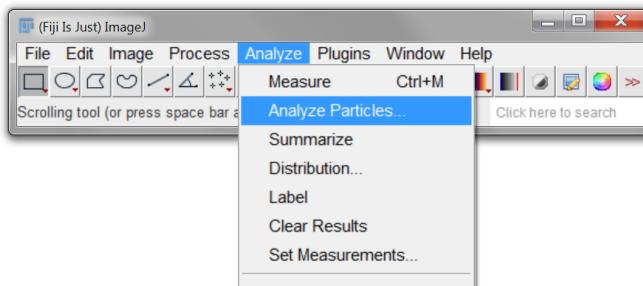
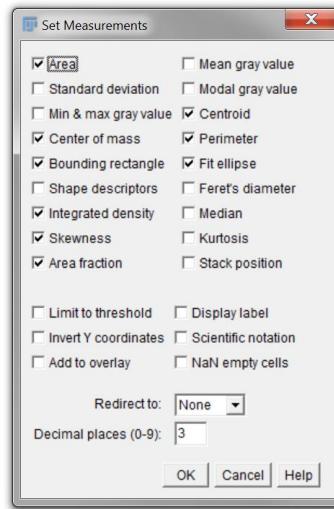
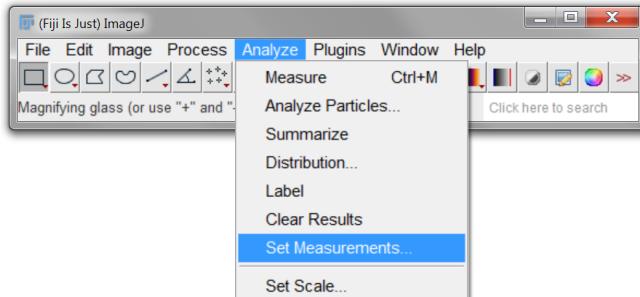
Analysis (rather than processing)

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Analysis (rather than processing)

- Measure segmented objects

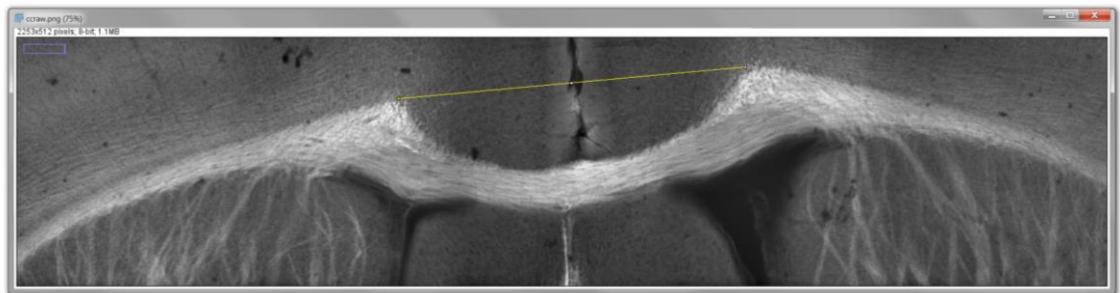


	Area	X	Y	XM	YM	Perim.	BX	BY	Width	Height	Major	Minor	Angle	IntDen	Skew	%Area	RawIntDen
1	306.543	48.743	13.836	48.743	13.836	68.090	39.650	2.210	18.330	23.140	24.526	16.018	63.026	78678.556	NaN	100	4659553
2	304.589	68.498	20.756	68.498	20.756	65.769	57.980	10.920	21.710	18.980	21.734	17.844	152.580	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

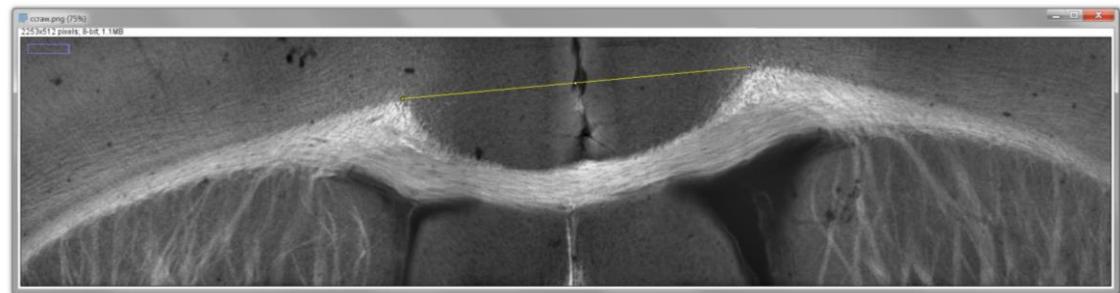
Analysis

- Measure other objects
 - Draw, then measure



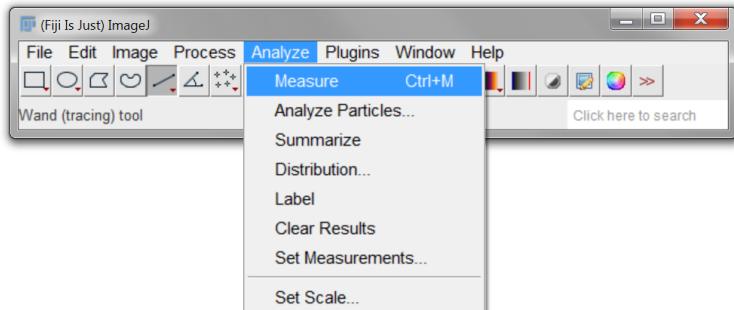
Analysis

- Measure other objects
 - Draw, then measure



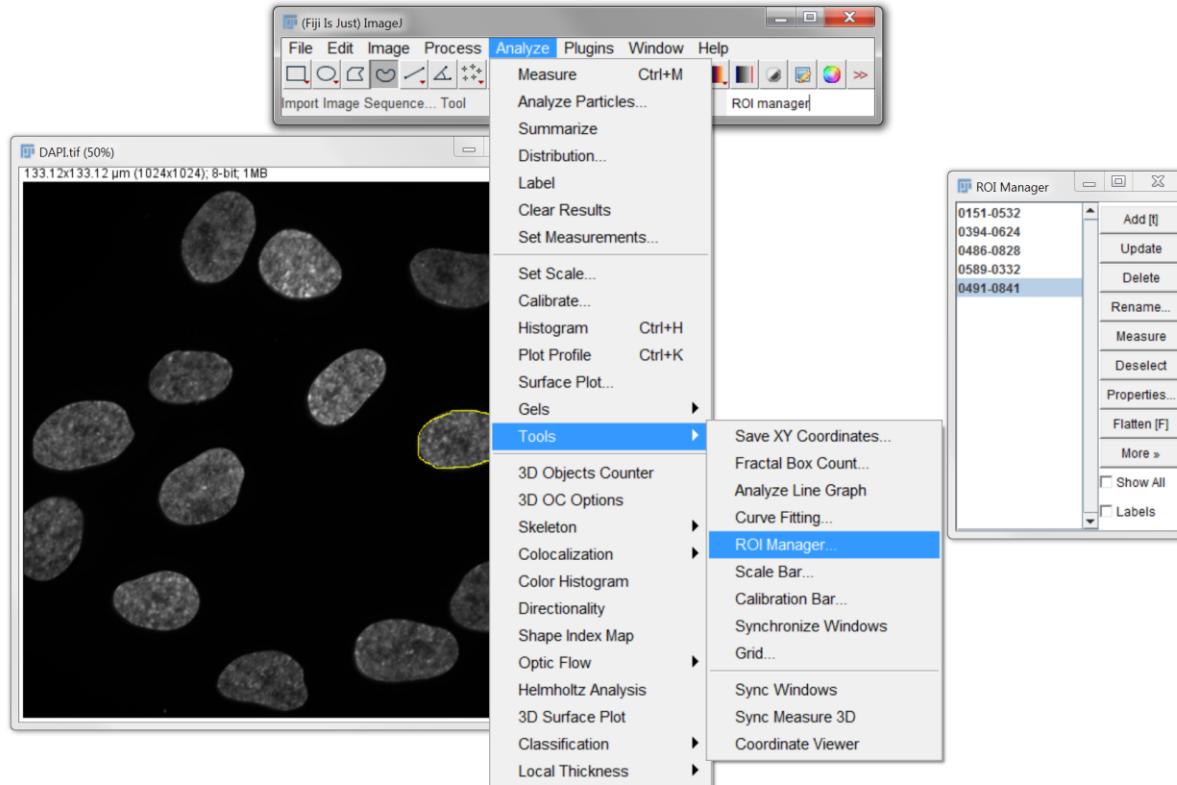
Results

	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



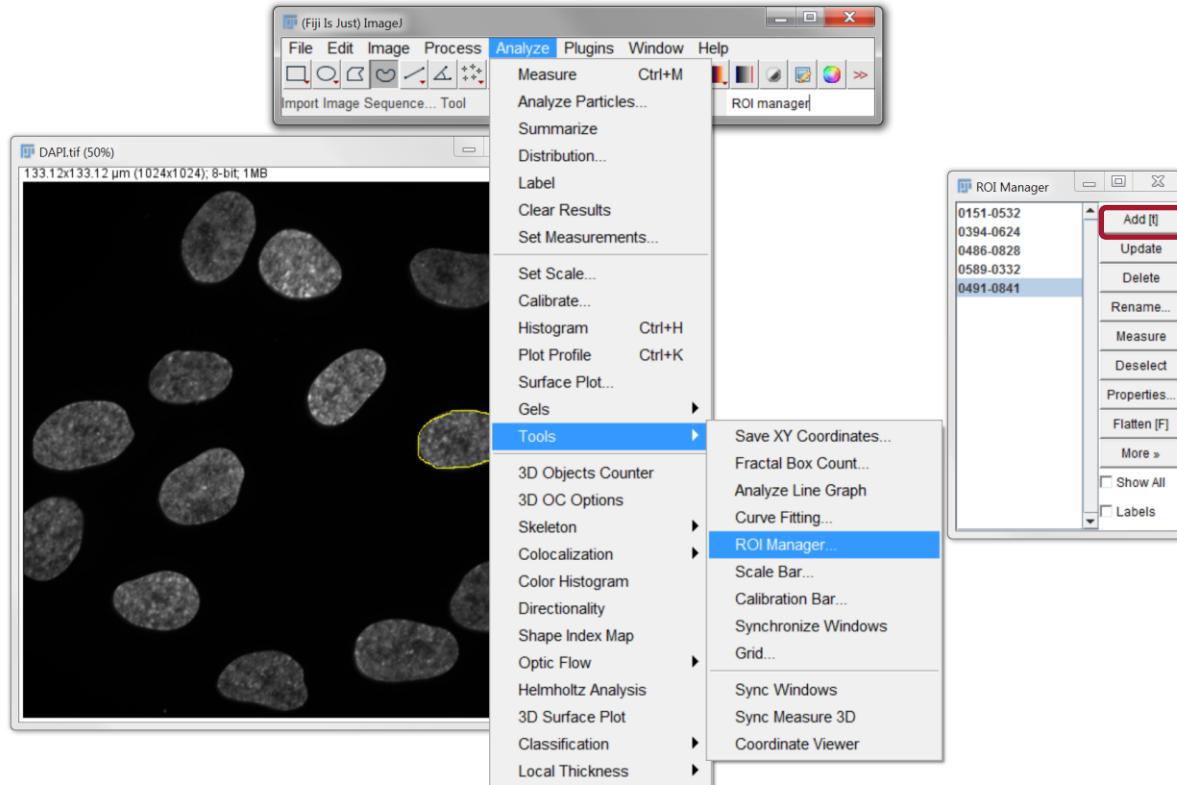
Analysis

- Multiple objects – ROI manager



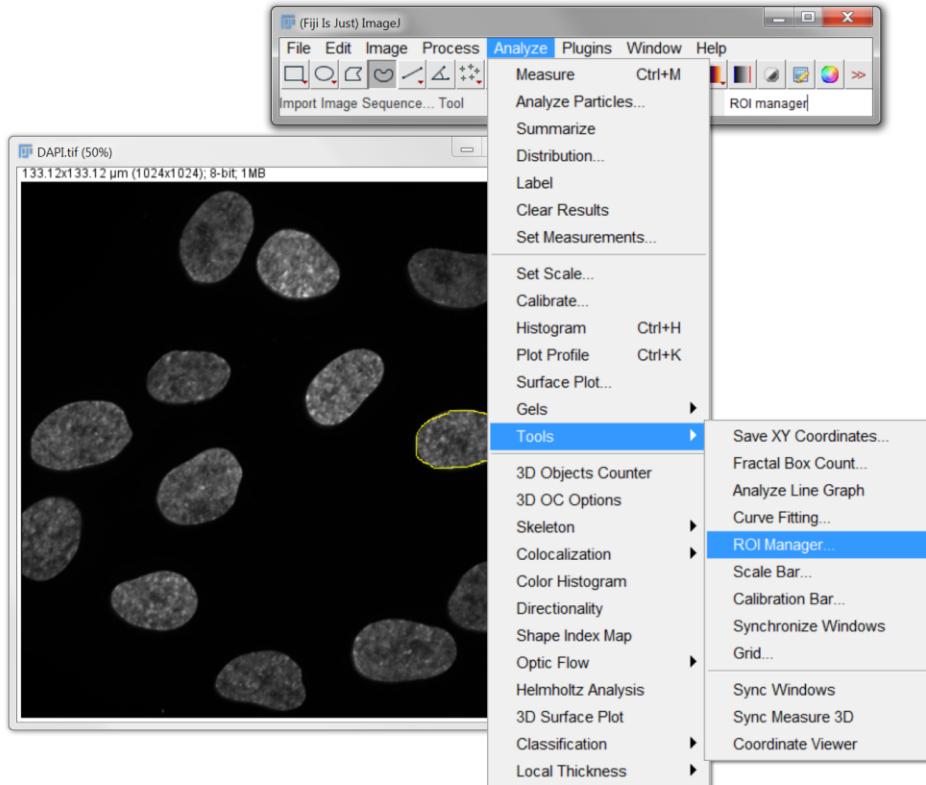
Analysis

- Multiple objects – ROI manager



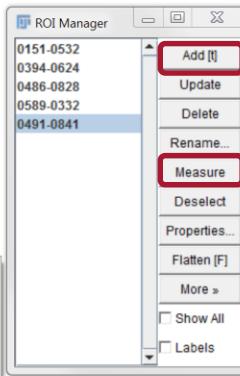
Analysis

- Multiple objects – ROI manager



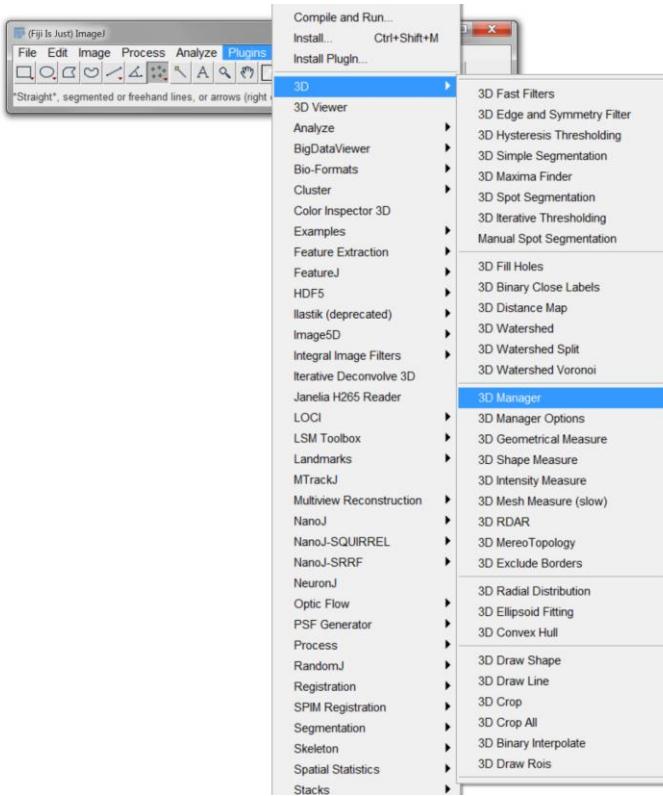
Results

	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

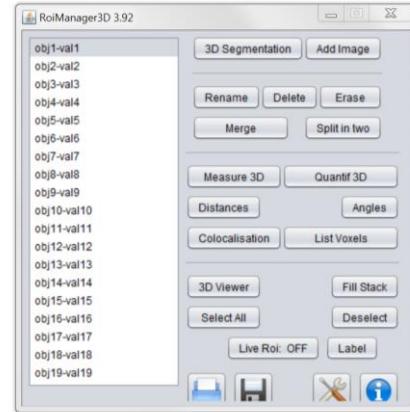
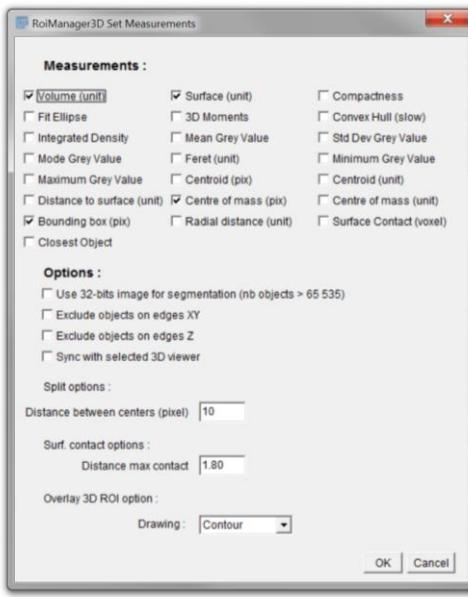
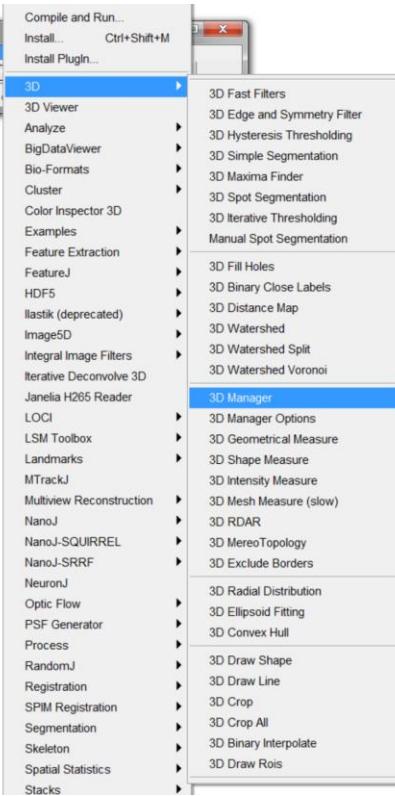
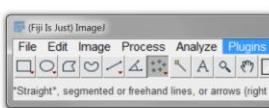


Analysis – 3D

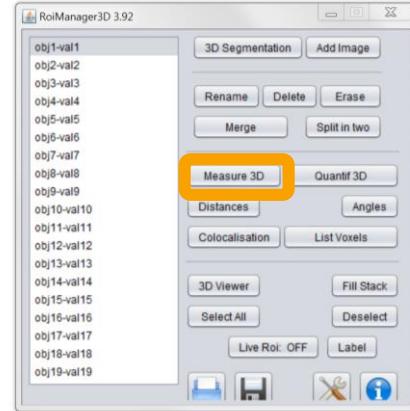
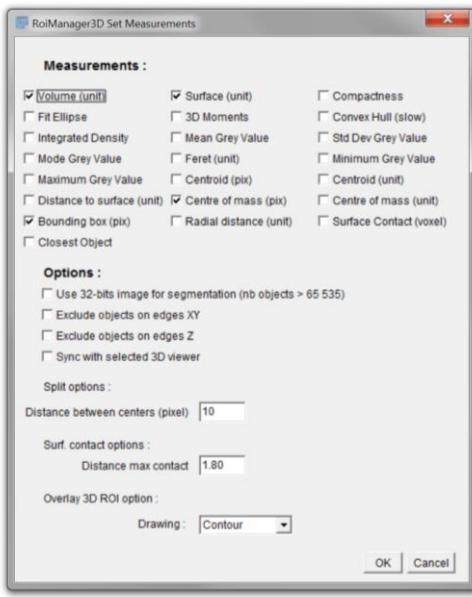
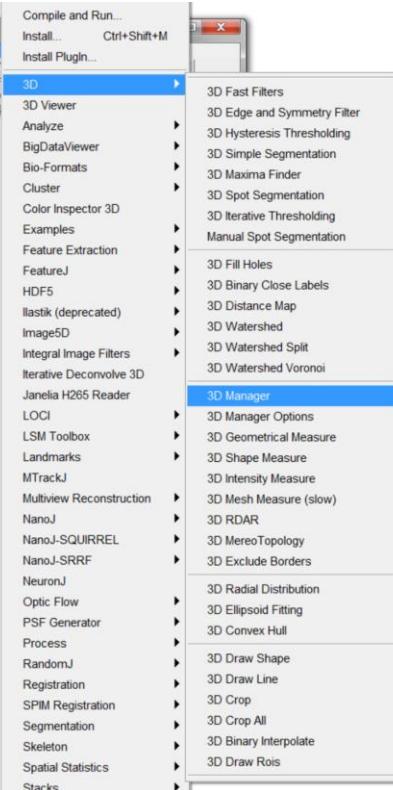
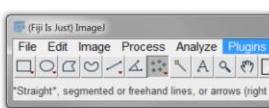
Analysis – 3D



Analysis – 3D



Analysis – 3D



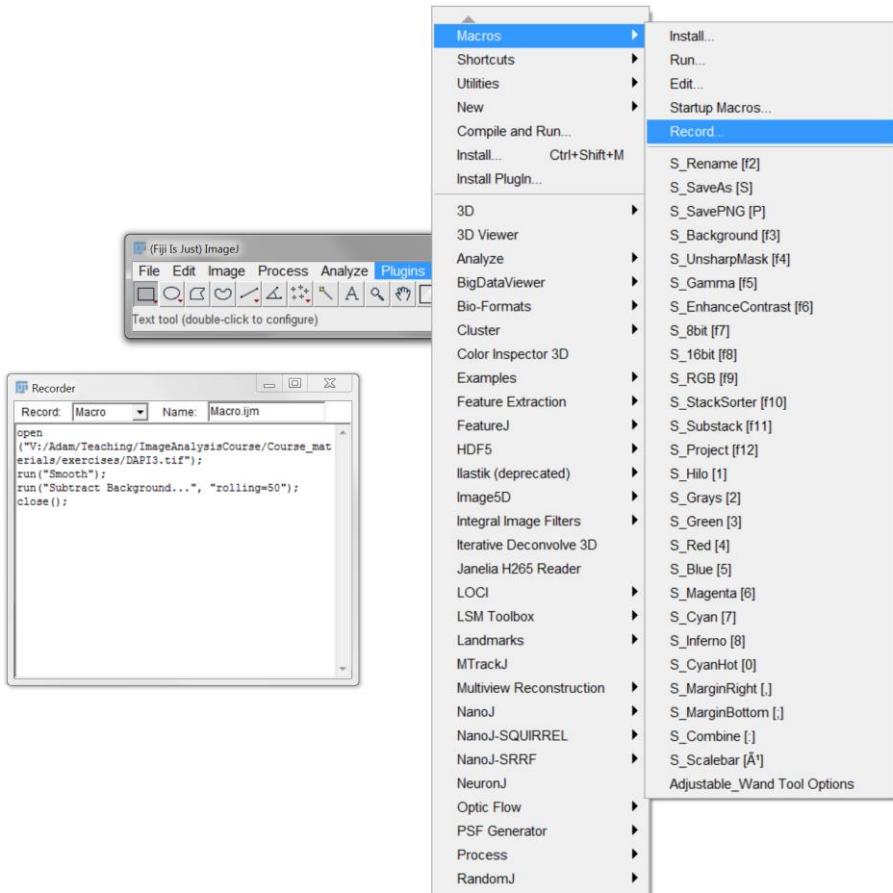
Nb	Obj	Type	Label	Xmin (pix)	Ymin (pix)	Zmin (pix)	Xmax (pix)	Ymax (pix)	Zmax (pix)	VolBound...	RatioVolbox...	Vol (unit)	Vol (pix)
0	1	0	obj1-val1	1939	0	0	2021	8	3	2,988	0.201	2,543	602
1	2	0	obj2-val2	1861	33	0	1889	108	5	13,224	0.335	18,742	4436
2	3	0	obj3-val3	1481	256	0	1839	478	10	880,627	0.665	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

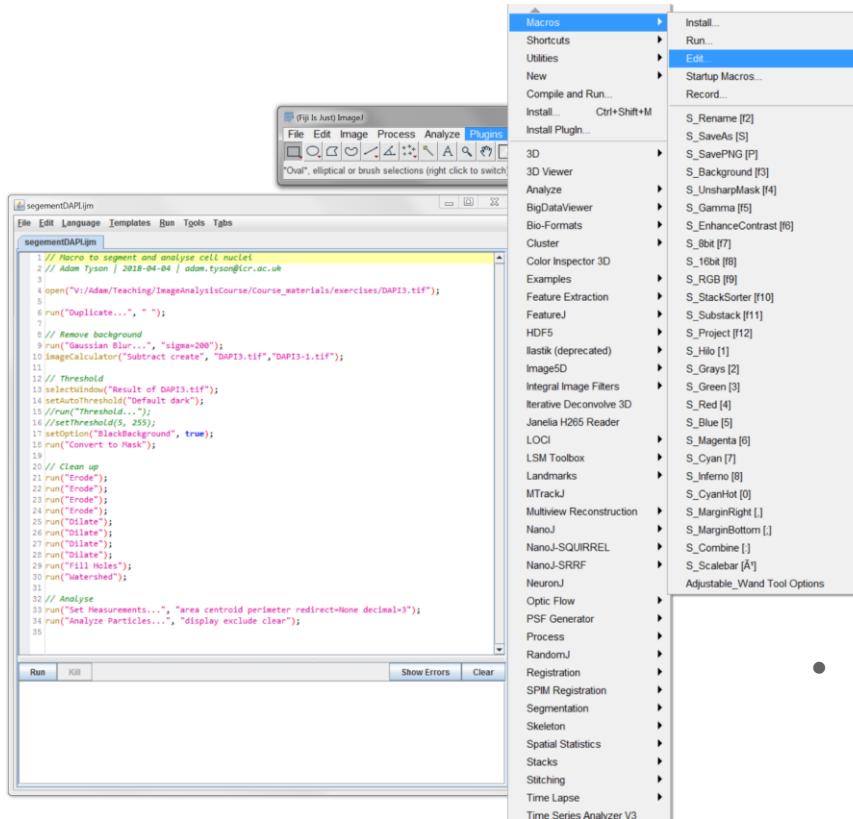
- Automated
- Quicker
- Reproducible (sharing)
- Less chance for bias/mistakes
- Works with most ImageJ plugins
- No coding required (but possible)

Macros - record

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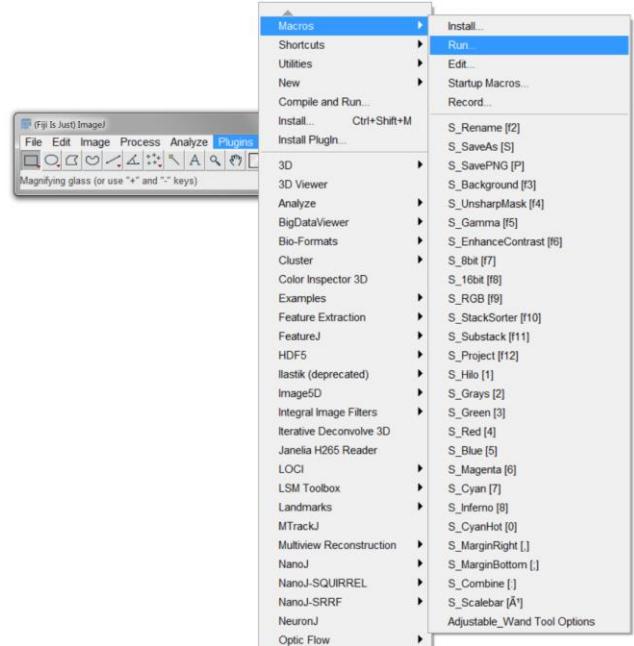
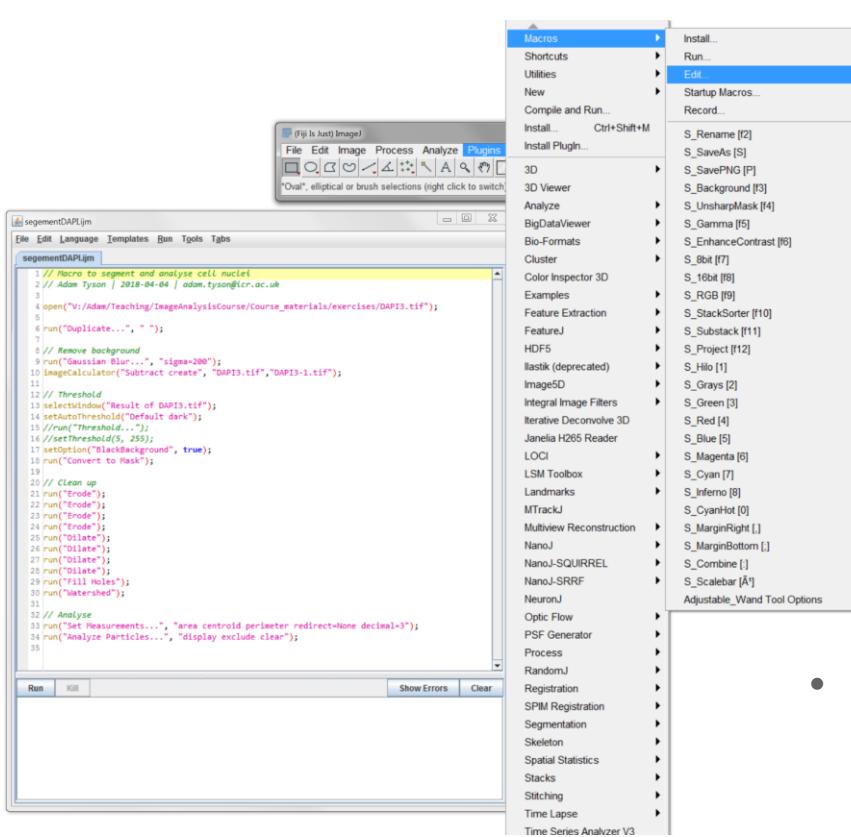


Macros – edit & run



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

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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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 - Super resolution (PALM/STORM/SRRF)
 - Light sheet (iSPIM/diSPIM/Lattice)
 - Something else – flow cytometry, biochemistry?
- Choose dyes carefully (SNR!)
 - mNeonGreen vs EGFP
 - Alexa 488 vs FITC

Resolution

Resolution

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

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

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

Resolution

- Resolution depends on:
 - 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!

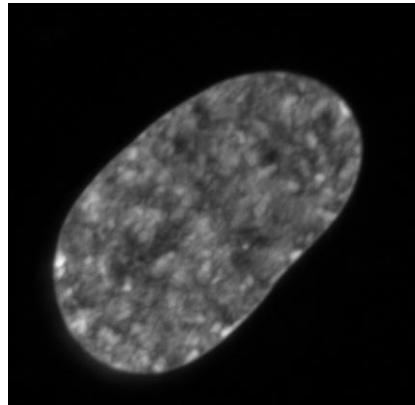
Resolution – However!

- What do you want to image?

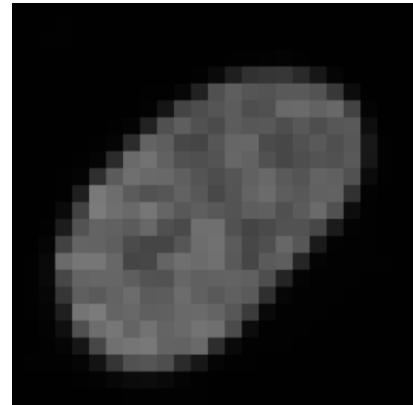
Resolution – However!

- What do you want to image?

15.4 pix μm^{-1}



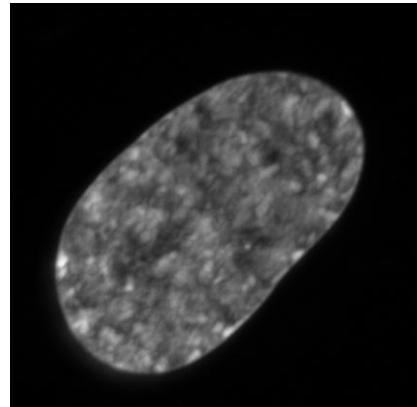
0.916 pix μm^{-1}



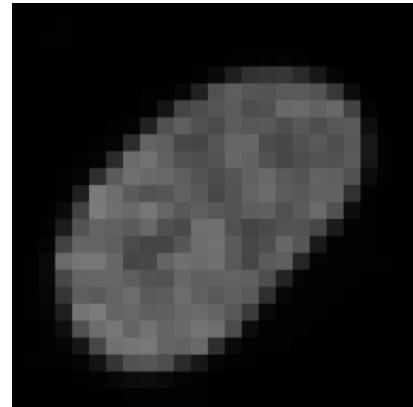
Resolution – However!

- What do you want to image?

15.4 pix μm^{-1}



0.916 pix μm^{-1}



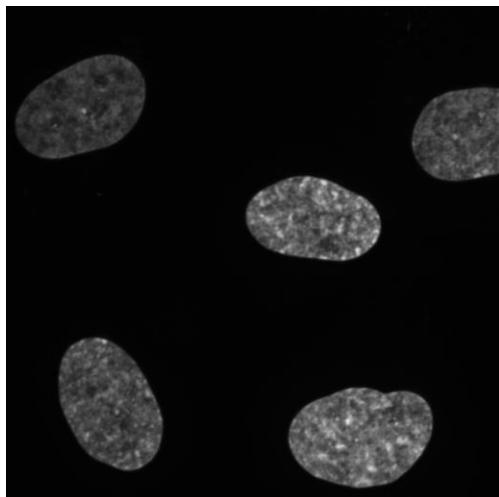
- Same information – 300 x less data
 - Also maybe quicker and more gentle

Resolution – 3D

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

Resolution – 3D

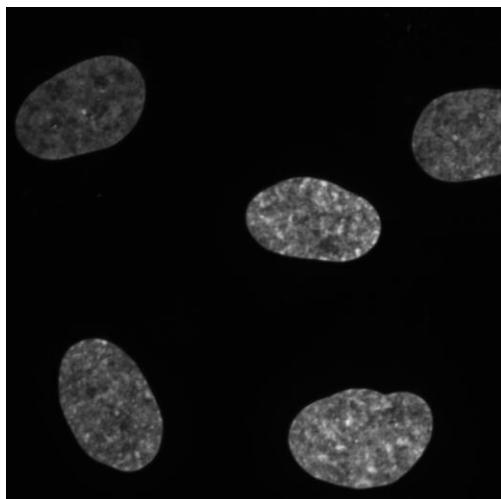
- If you want to analyse in 3D:
 - Isotropic resolution



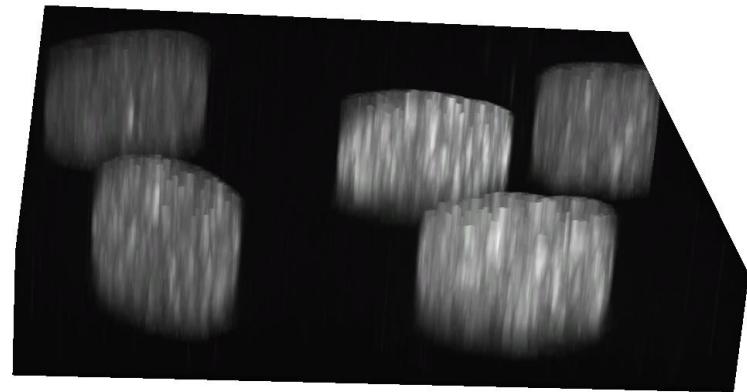
2D

Resolution – 3D

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



2D



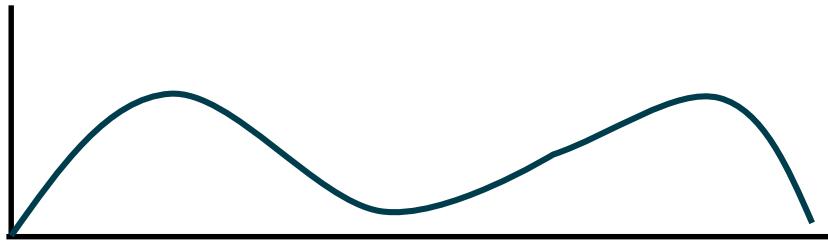
3D

Note – 3D analysis

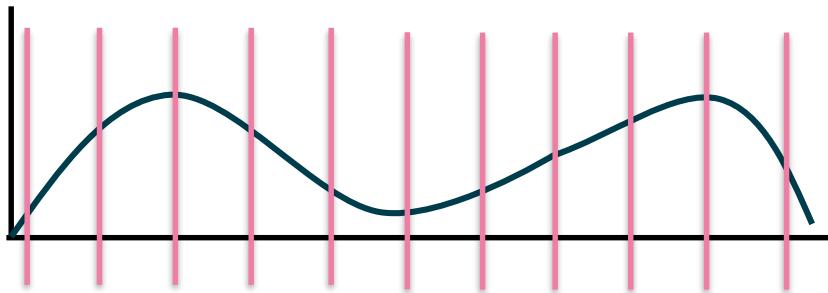
- Analysis almost always exactly the same as 2D:
 - Takes longer
 - Need to view in 3D to check
 - 3D not necessarily supported by software package

Resolution – temporal

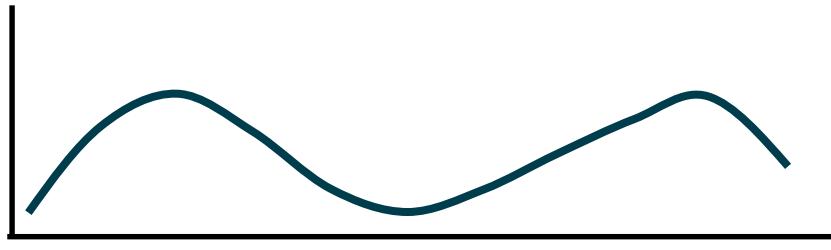
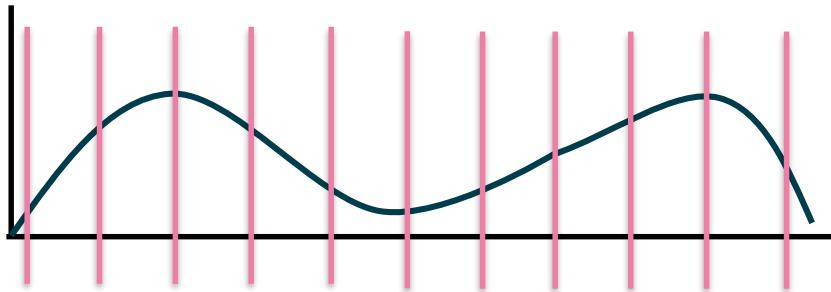
Resolution – temporal



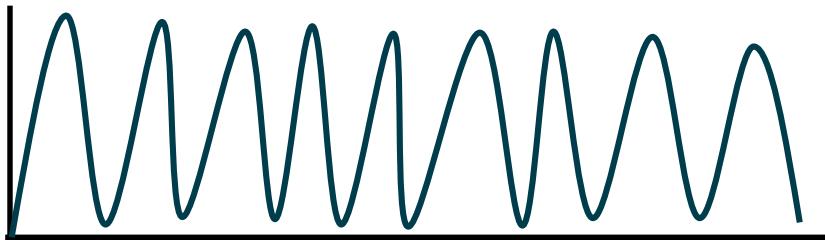
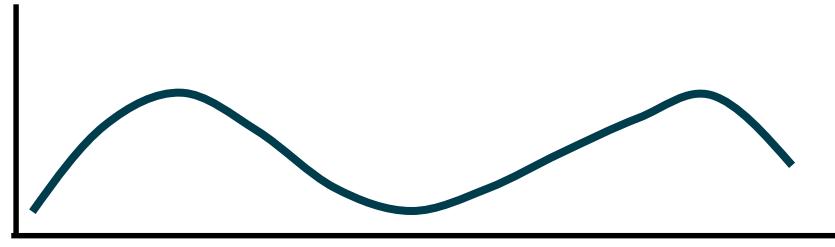
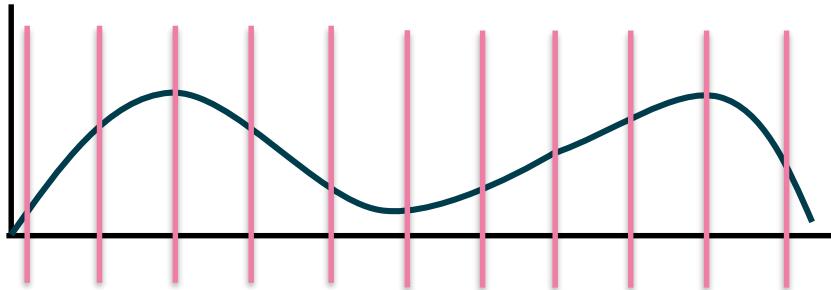
Resolution – temporal



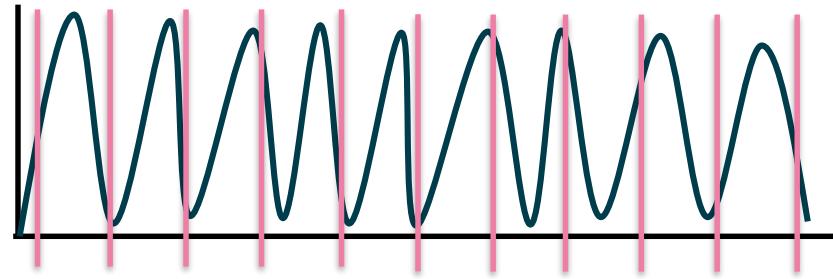
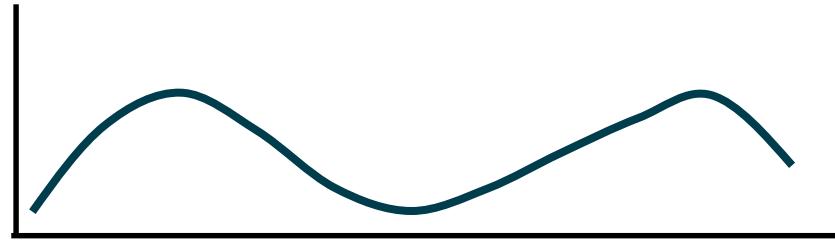
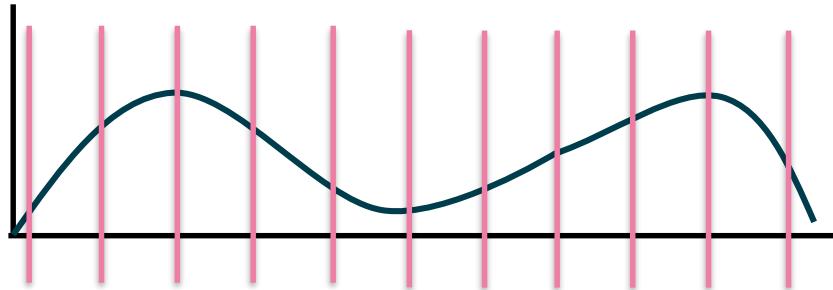
Resolution – temporal



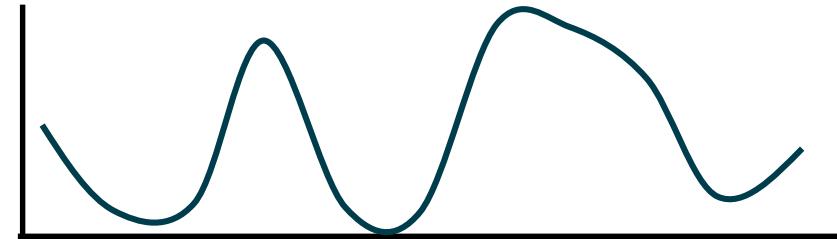
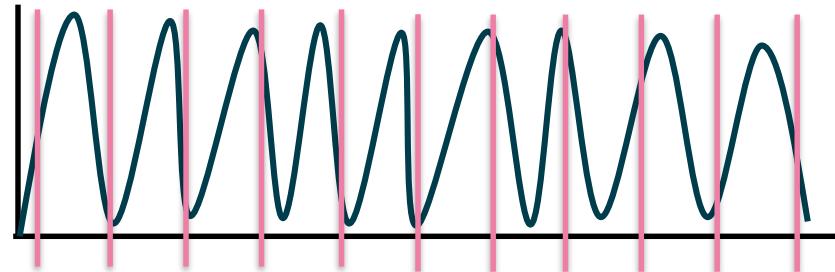
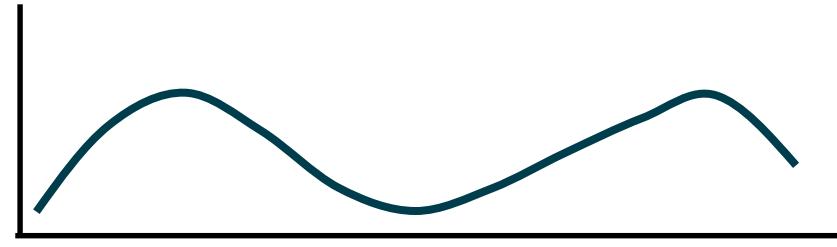
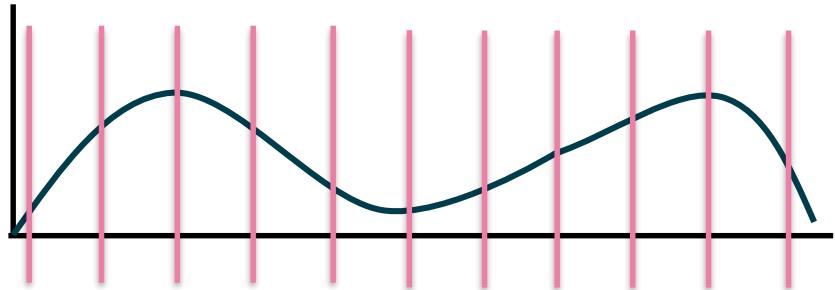
Resolution – temporal



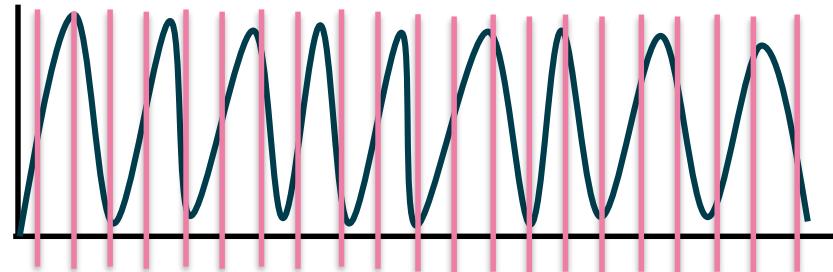
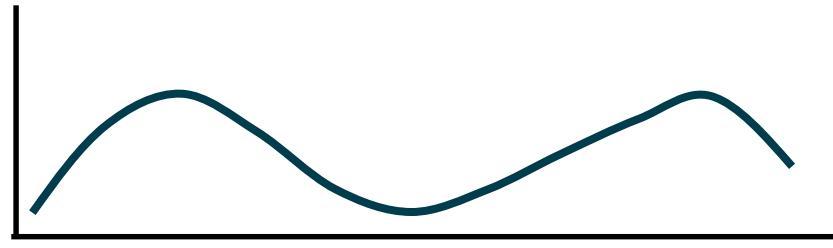
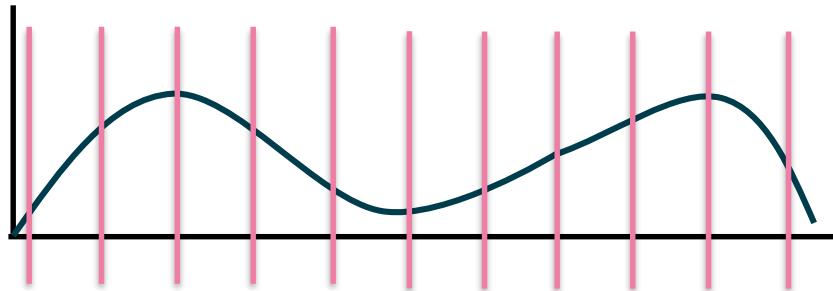
Resolution – temporal



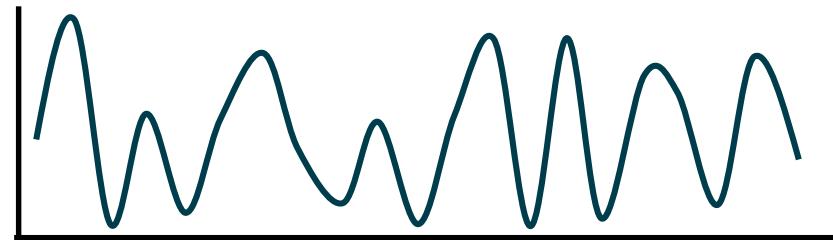
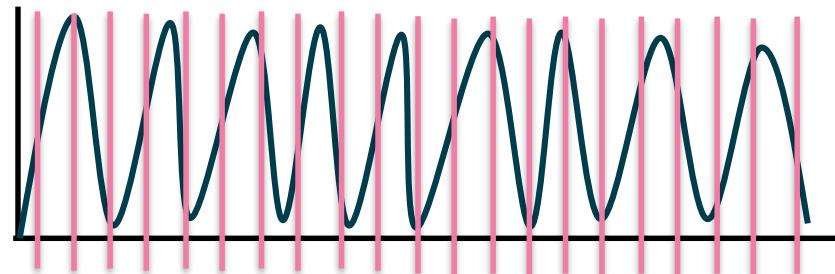
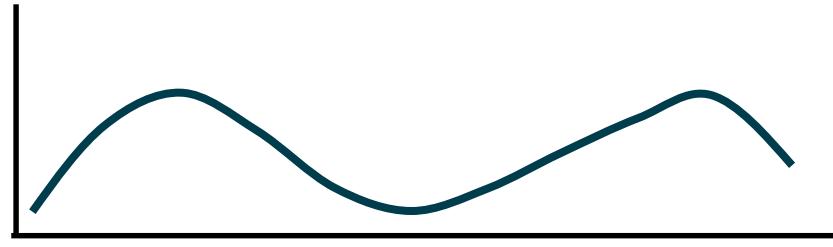
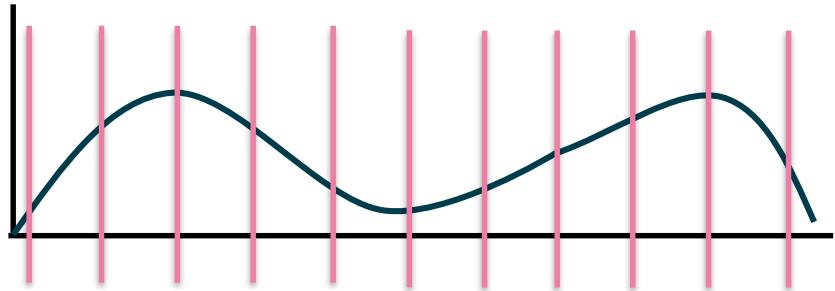
Resolution – temporal



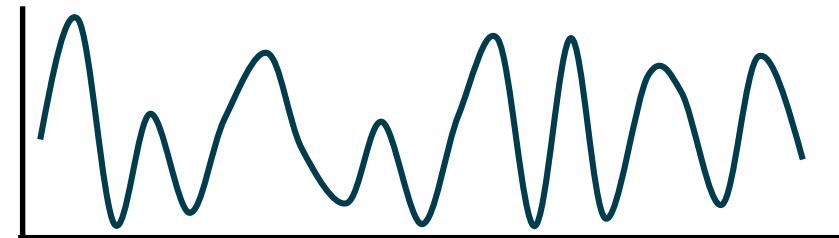
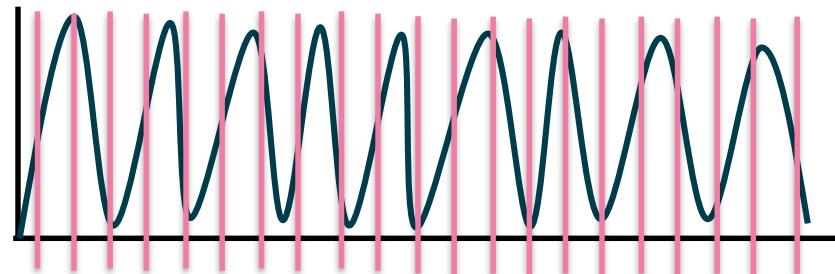
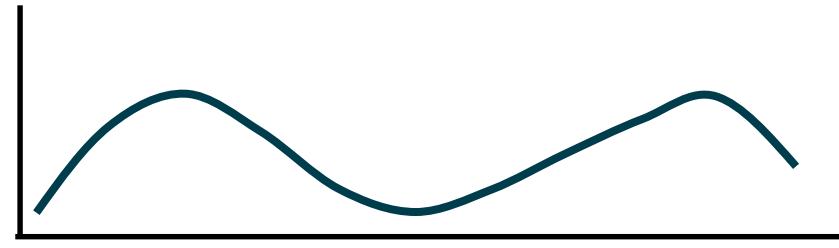
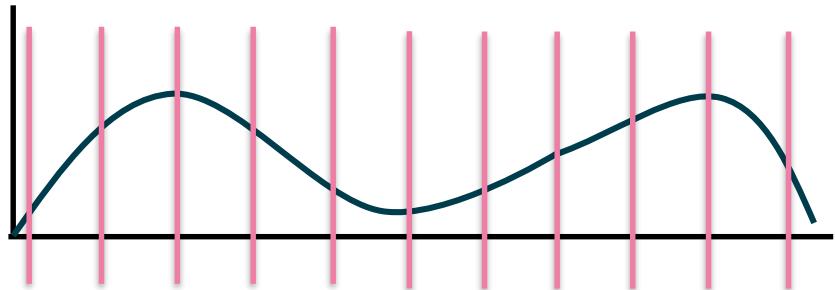
Resolution – temporal



Resolution – temporal



Resolution – temporal



- Accurate sampling vs photobleaching & data size

Signal to noise ratio (SNR)

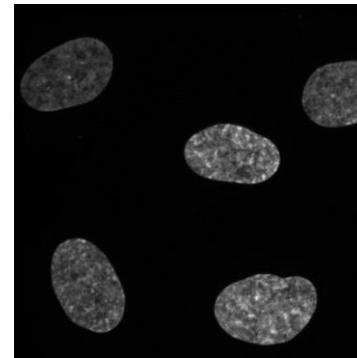
- Resolution irrelevant if you can't detect structures of interest

Signal to noise ratio (SNR)

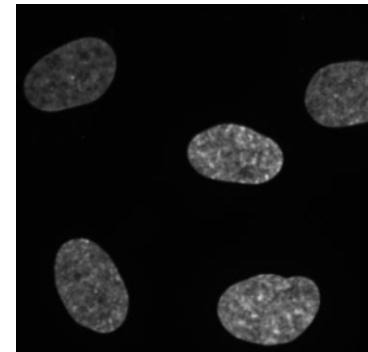
- Resolution irrelevant if you can't detect structures of interest

High
SNR

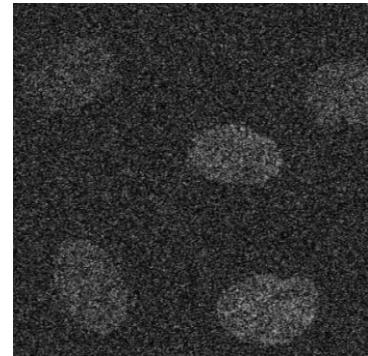
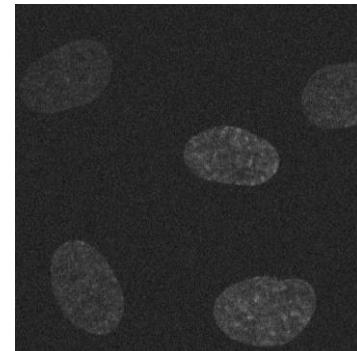
High resolution



Low resolution



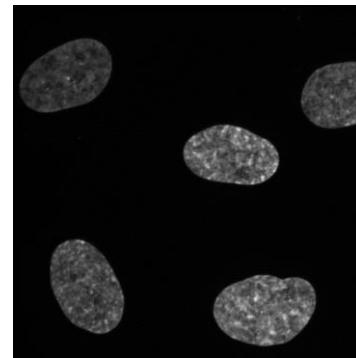
Low
SNR



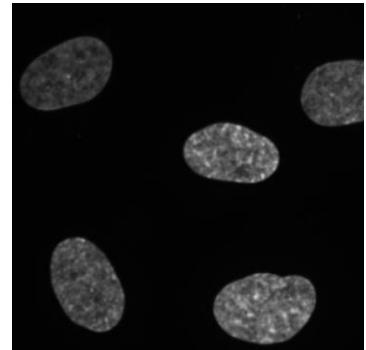
Signal to noise ratio (SNR)

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

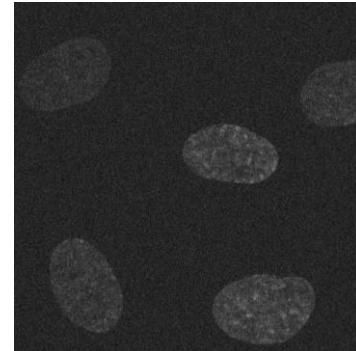
High
SNR



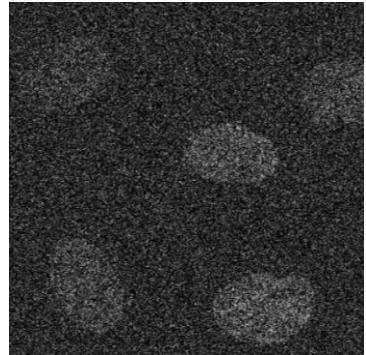
Low resolution



Low
SNR



- Contrast often the most important aspect of a good image



Analysis as a method

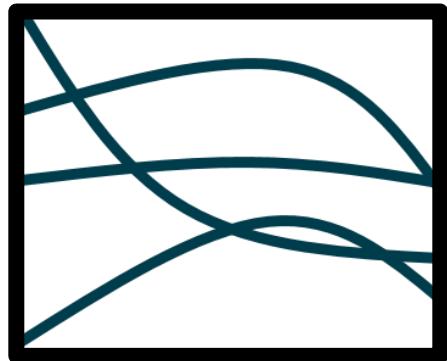
Analysis as a method

“True” image



Analysis as a method

“True” image



Observed
widefield image



Analysis as a method

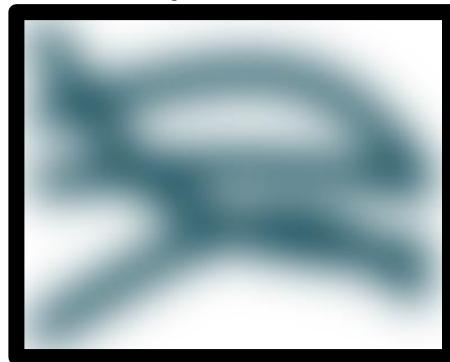
“True” image



Observed
widefield image



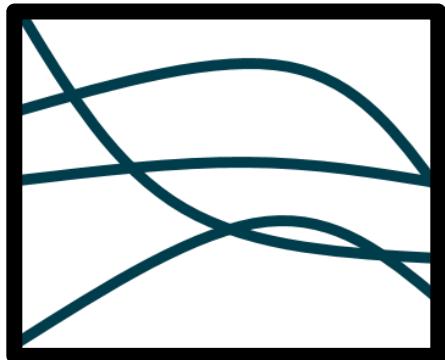
STORM/PALM
aquisition



STORM/
PALM

Analysis as a method

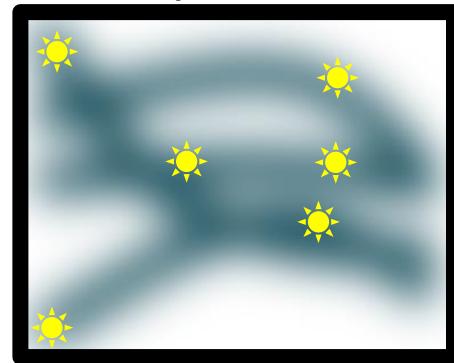
“True” image



Observed
widefield image



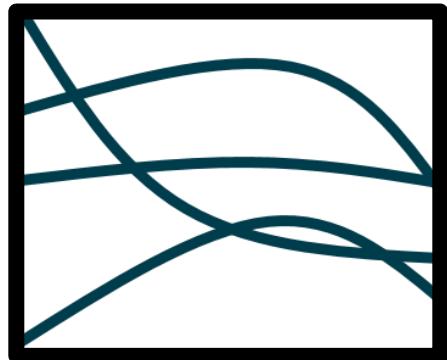
STORM/PALM
aquisition



STORM/
PALM

Analysis as a method

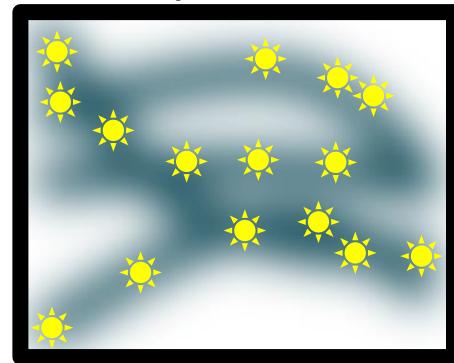
“True” image



Observed
widefield image



STORM/PALM
aquisition



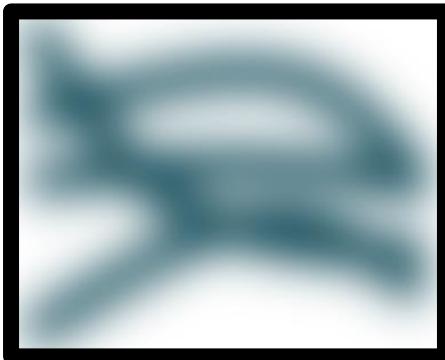
STORM/
PALM

Analysis as a method

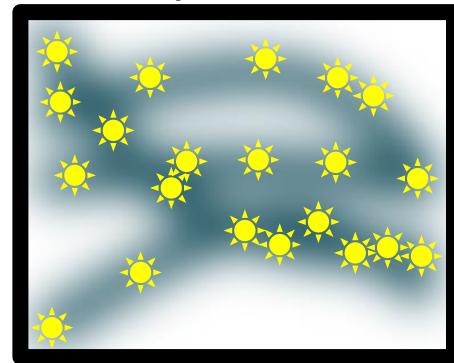
“True” image



Observed
widefield image



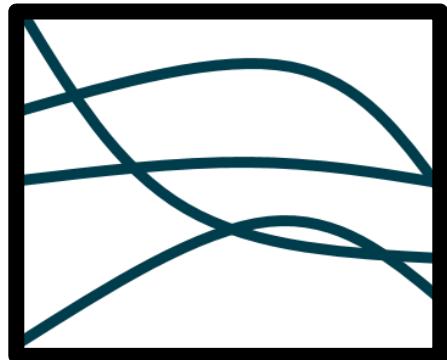
STORM/PALM
aquisition



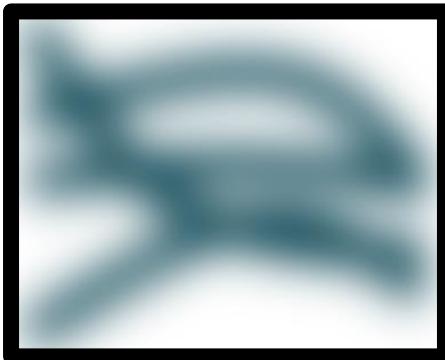
STORM/
PALM

Analysis as a method

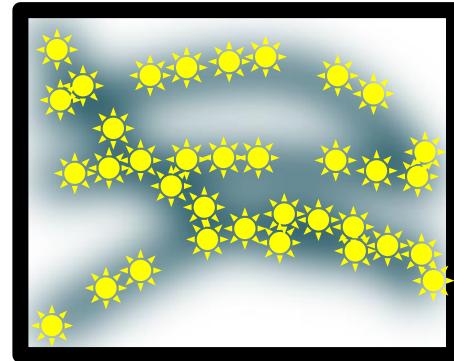
“True” image



Observed
widefield image



STORM/PALM
aquisition



STORM/
PALM

Analysis as a method

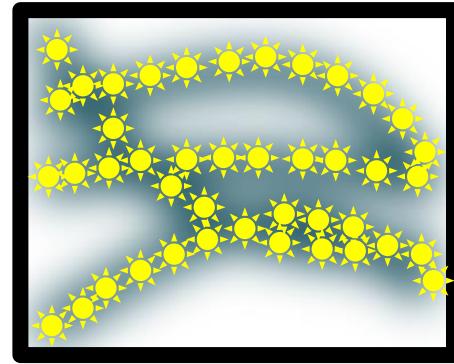
“True” image



Observed
widefield image



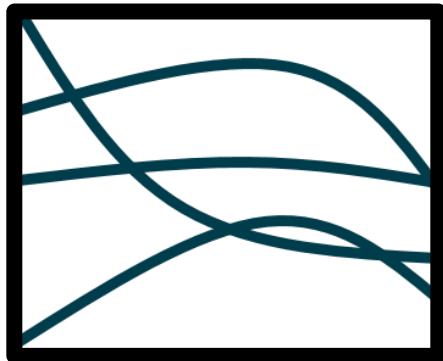
STORM/PALM
aquisition



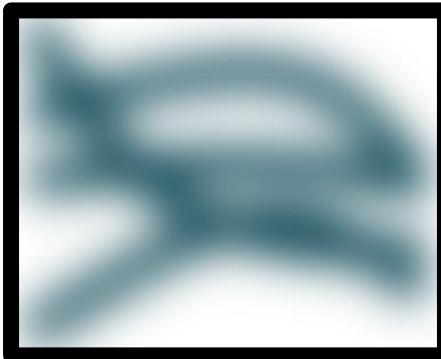
STORM/
PALM

Analysis as a method

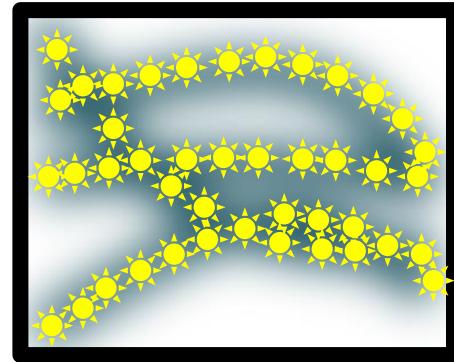
“True” image



Observed
widefield image

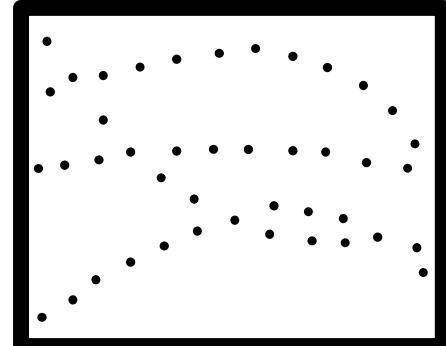


STORM/PALM
aquisition



Localised fluorophores

STORM/
PALM

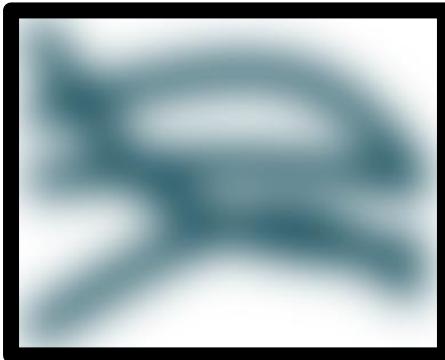


Analysis as a method

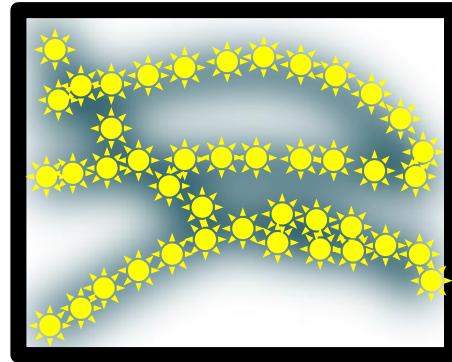
“True” image



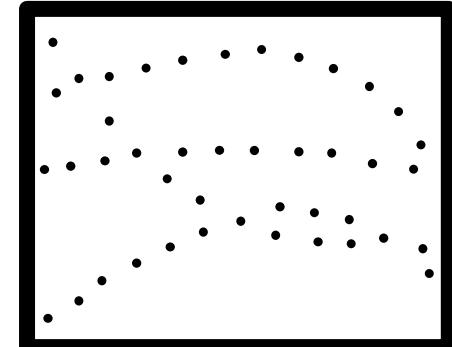
Observed widefield image



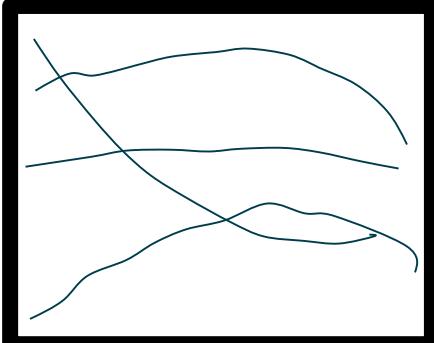
STORM/PALM aquisition



Localised fluorophores



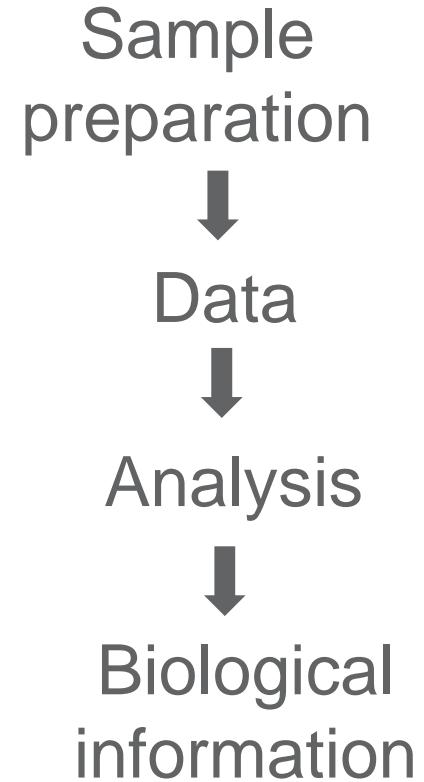
Reconstructed image



STORM/
PALM

First (and most important lesson) – again!

Test your analysis
before you
acquire data!



Any other courses?

Any other courses?

- General – more advanced

Any other courses?

- General – more advanced
- 3D

Any other courses?

- General – more advanced
- 3D
- Big datasets

Any other courses?

- General – more advanced
- 3D
- Big datasets
- Lightsheet processing

Any other courses?

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- Super resolution

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- Writing custom scripts – MATLAB / Python etc

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- Anything else?