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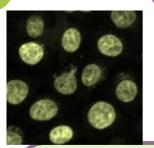
# **Workshop: Data Analysis Strategies for Single-Cell Image-Based Profiling:**

*Day 1: Introduction to Image Analysis*

# Workshop overview



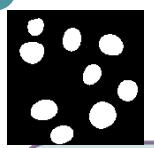
1



## Introduction to Bioimage Analysis

- ❖ Basics of Digital Images
- ❖ File formats & Metadata
- ❖ Fund. Image Processing: filtering, denoising, etc
- ❖ Image Quality and reproducibility
- ❖ Hands-on session using Fiji

2



## Cell Segmentation and Feature extraction

- ❖ Overview of segmentation techniques
- ❖ Introduction to machine and deep learning-based segmentation
- ❖ Feature extraction: measurements
- ❖ Exporting single-cell datasets for analysis

3



## Single Cell Data Analysis

- ❖ Overview of high-content data structure
- ❖ Dimensionality reduction: PCA, LDA, t-SNE, UMAP
- ❖ Clustering methods: K-means, GMM
- ❖ Hands-on session with single-cell datasets

4



## Daily Schedule

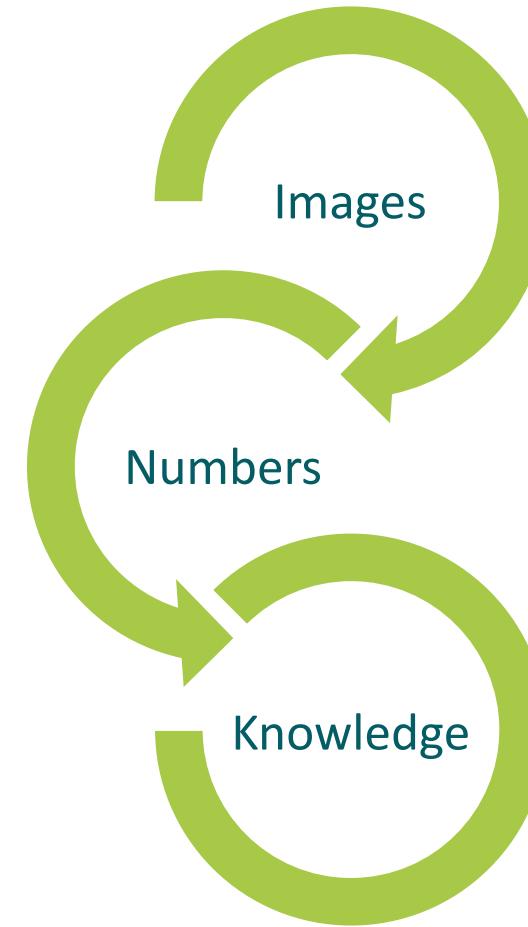
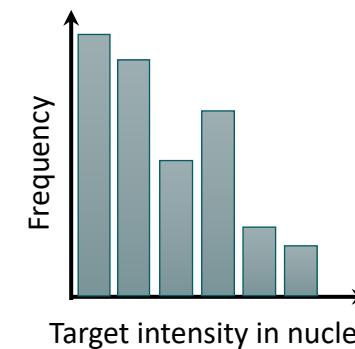
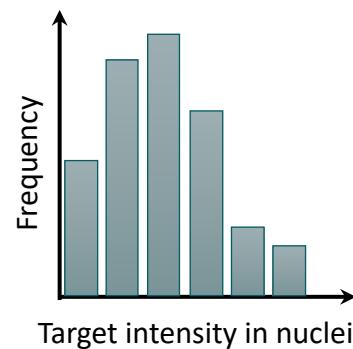
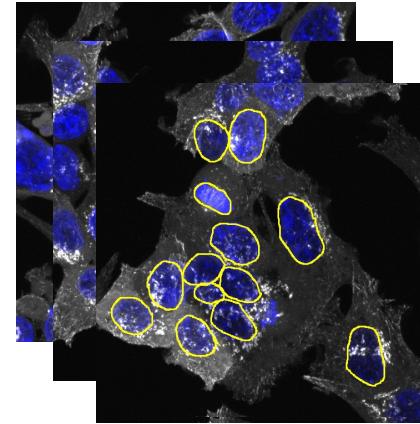
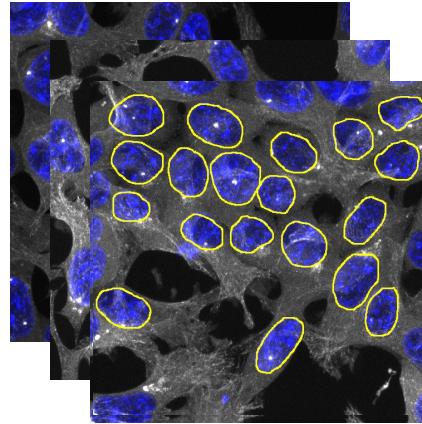
- ⌚ 09:00 – 10:20: First Session
- ☕ 10:20 – 10:40: Break
- ⌚ 10:40 – 12:00: Second Session
- 🍽 12:00 – 13:30: Lunch Break
- ⌚ 13:30 – 14:30: Third Session
- ☕ 14:30 – 14:45: Break
- ⌚ 14:45 – 16:00: Fourth Session

*Basic concepts: BioImage Analysis* 

# What is bioimage analysis?



- Understanding and quantifying microscopy & medical image data



- ✓ Quantitative
- ✓ Objective
- ✓ Reliable
- ✓ Repeatable
- ✓ Reproducible

# What is bioimage analysis?



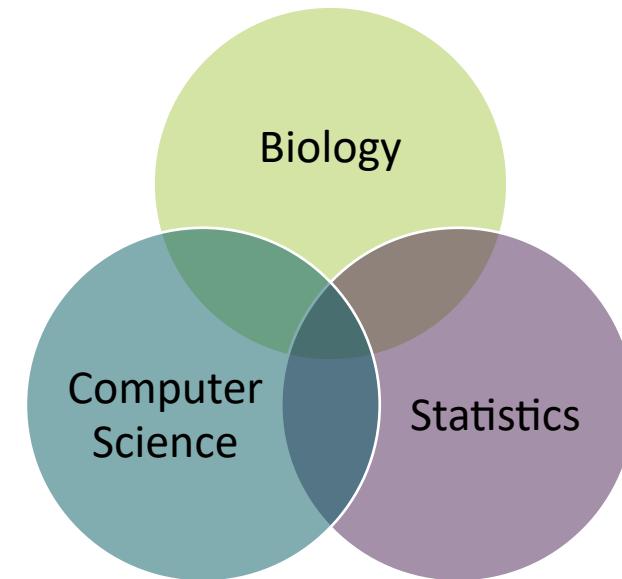
➤ Understanding and quantifying microscopy & medical image data

✓ Great demand due to:

- Advances in microscopy techniques (Ex.: Screening microscopy, EM, Light-sheet, etc)
- More sensitive fluorescent labels and sensors
- High throughput data creation (nanometer resolution, volumes and time-lapses)

✓ Increased storage capacity and faster computing / better algorithms

✓ Faster than humans and less biased approach



Bioimage  
Informatics

*Examples...*

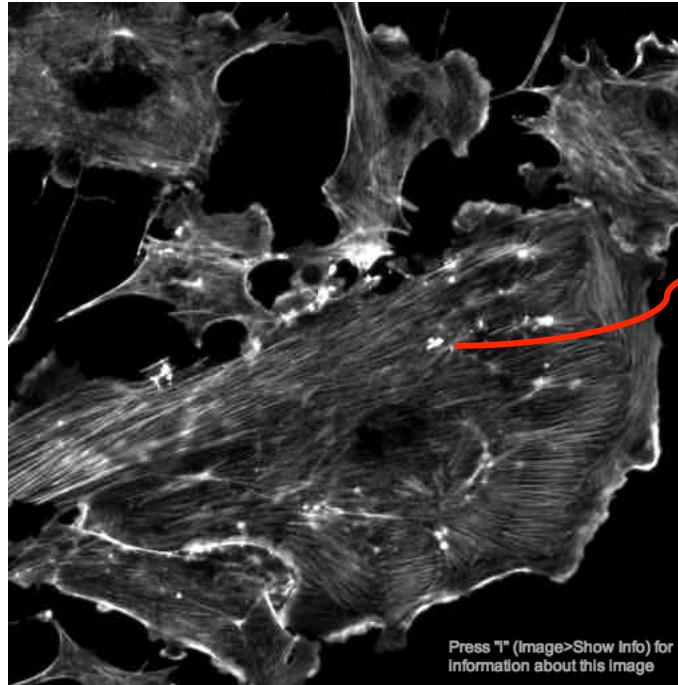
*Basic concepts: Digital images*



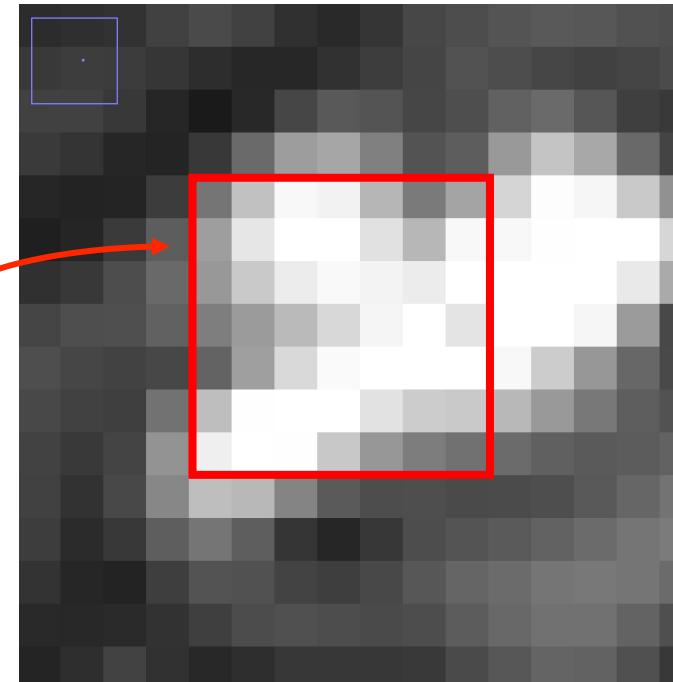
# What is a digital image?



- An image is a matrix of numbers (*pixel*: picture element)



Fluorescent cells / Fiji samples

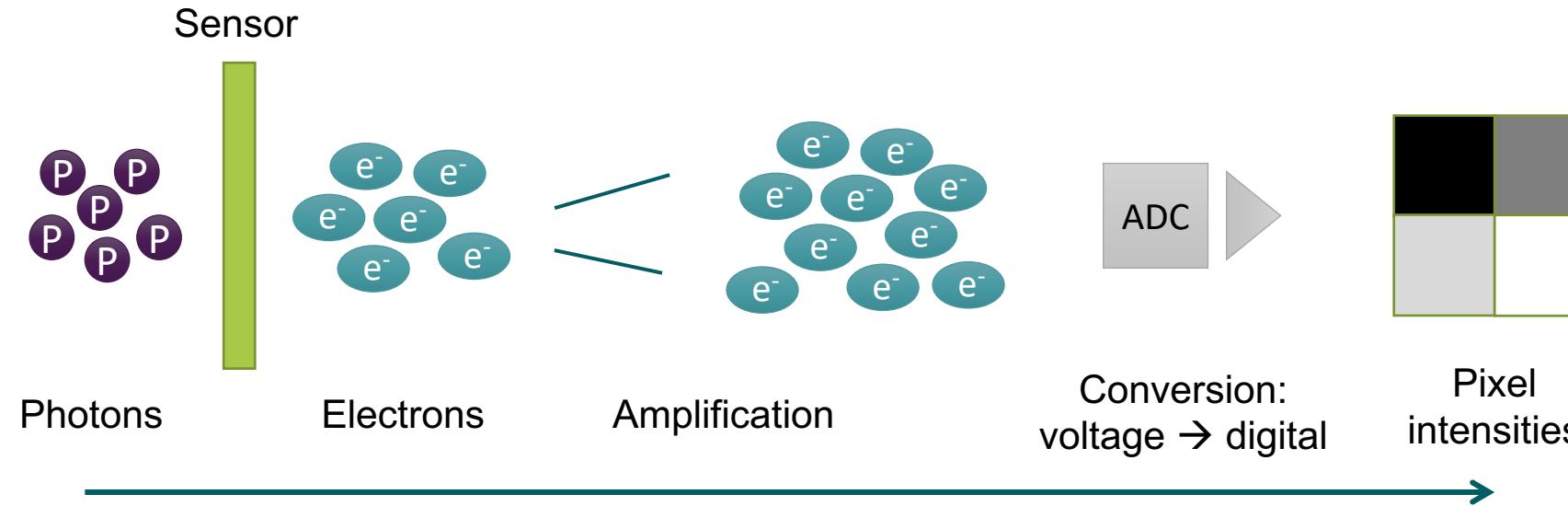


$$\begin{matrix} & 1 & 2 & \dots & n \\ 1 & a_{11} & a_{12} & \dots & a_{1n} \\ 2 & a_{21} & a_{22} & \dots & a_{2n} \\ 3 & a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ m & a_{m1} & a_{m2} & \dots & a_{mn} \end{matrix}$$

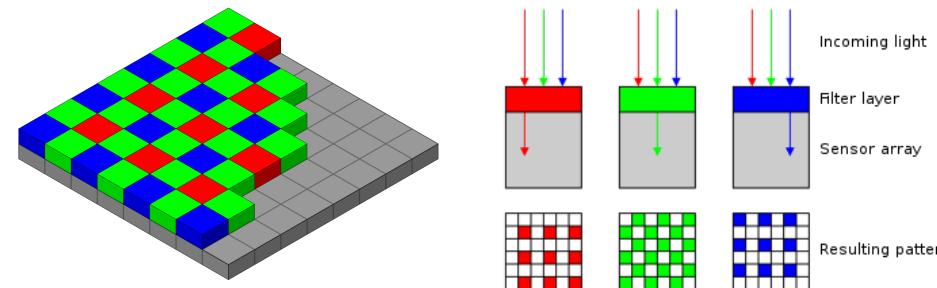
	●	●	●	Pixel Values			
Prefs	314	315	316	317	318	319	320
	252	117	193	248	242	182	122
	253	158	230	255	255	225	183
	254	152	201	236	249	243	236
	255	126	155	186	216	245	255
	256	99	159	217	250	255	255
	257	190	254	255	255	226	204
	258	239	255	254	200	151	125
							113

How is the pixel value defined?

# Acquisition of digital images



- ✓ Sensor types:  
detector-based,  
camera-based  
(CMOS, CCD)

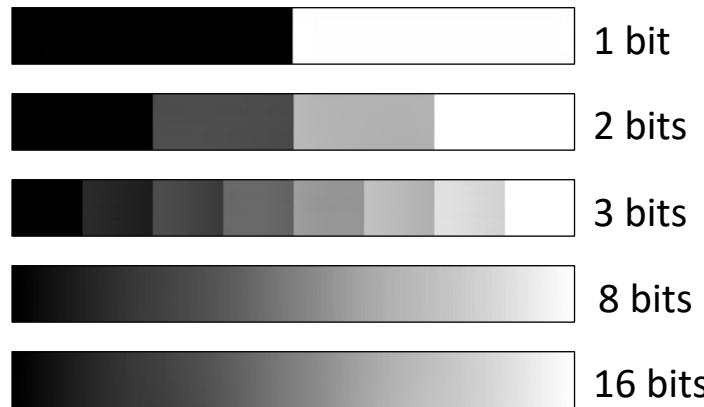


- ✓ Color-cameras  
(Bayer filter):  
blue, green (2x)  
and red filters

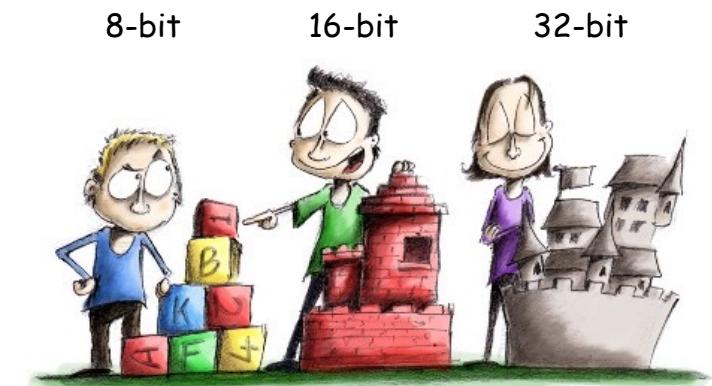
# Bit-depth & gray levels



- Bit-depth corresponds to the number of gray levels



Bit-depth	Gray levels $= 2^n$	Grayscale Values
1-bit	$2^1$	2
2-bit	$2^2$	4
3-bit	$2^3$	8
8-bit	$2^8$	256
16-bit	$2^{16}$	65536



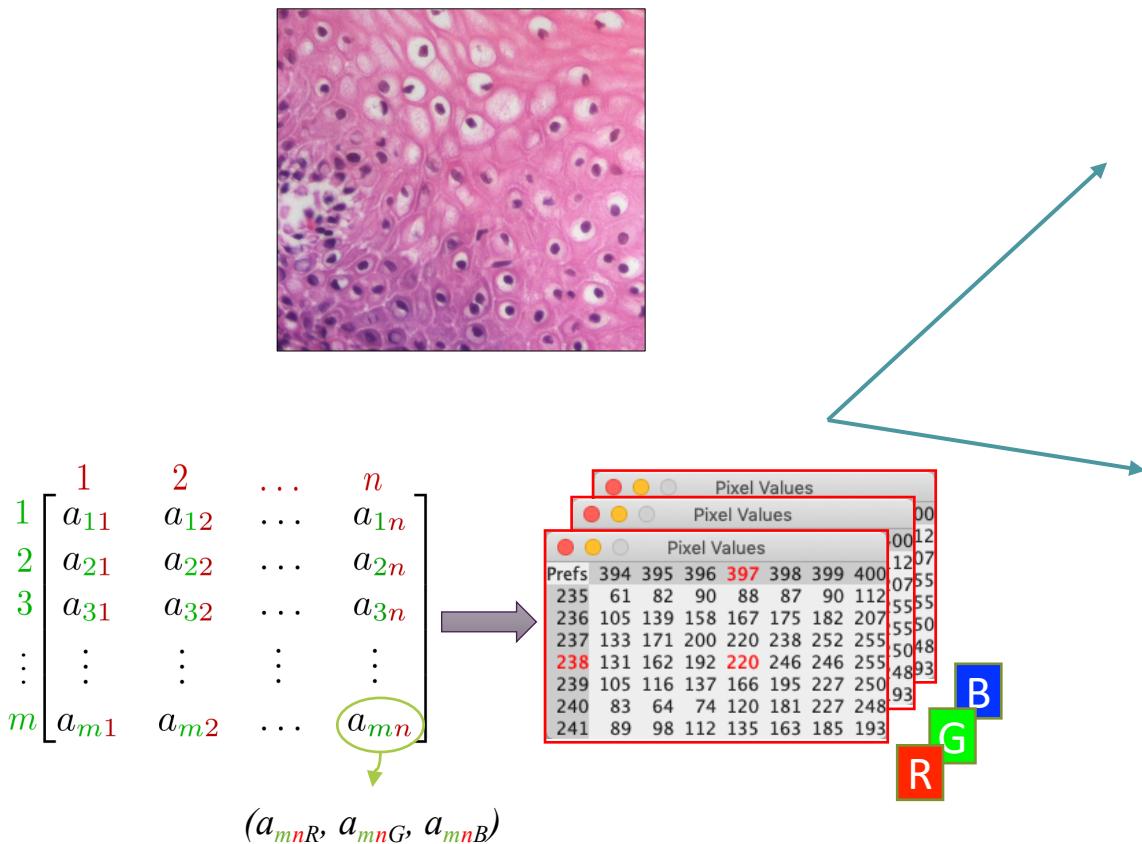
[https://petebankhead.gitbooks.io/imagejintro/content/chapters/bit\\_depths/bit\\_depths.html](https://petebankhead.gitbooks.io/imagejintro/content/chapters/bit_depths/bit_depths.html)

- ✓ Number of **possible** intensity values! Different from the maximum intensity value.
  - ✓ More bits, more fine intensity details, lower risk of saturation, among others
- ✓ More bits are usually better. However, a lower bit-depth is sometimes desirable
  - ✓ E.g.: storage limit, speed, very dim samples, etc

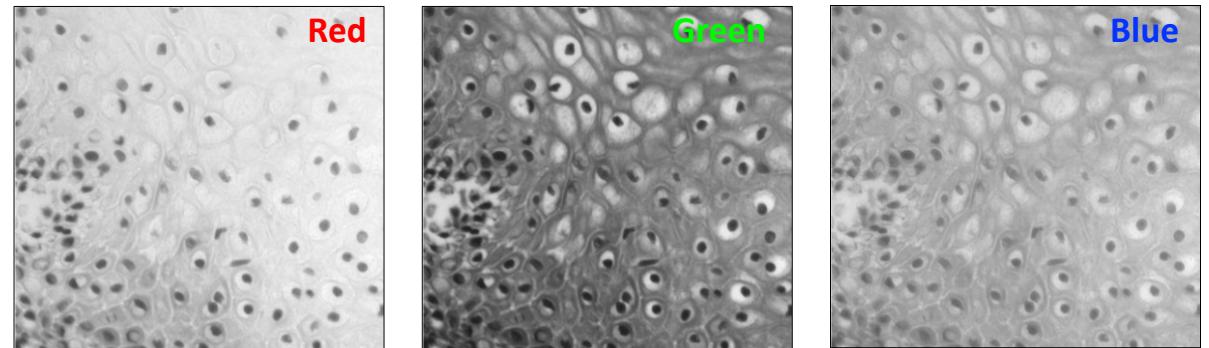


# Color Images

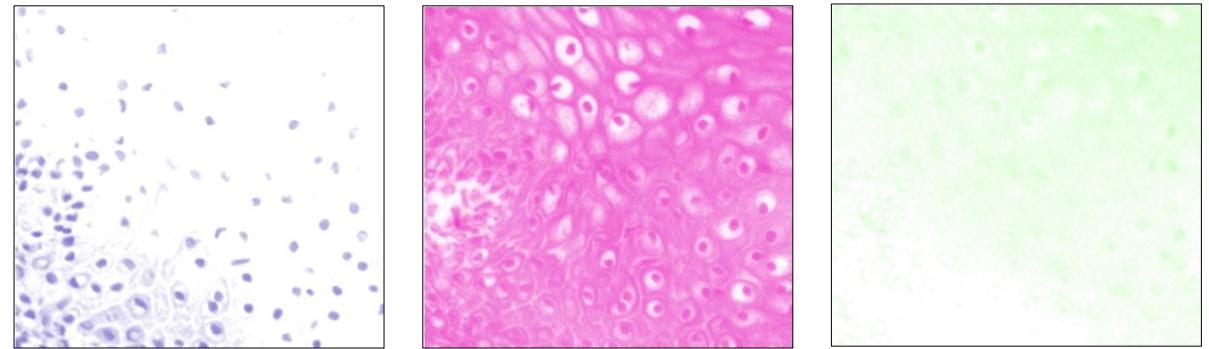
- RGB (Ex: stained H&E)



Split channels in RGB space



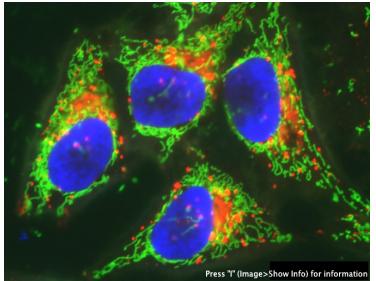
Color Deconvolution – spectral unmixing of the dyes



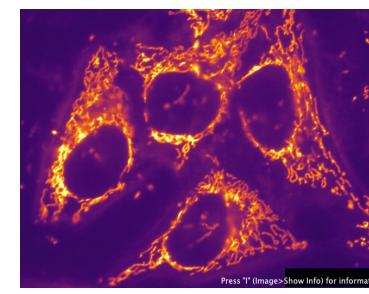
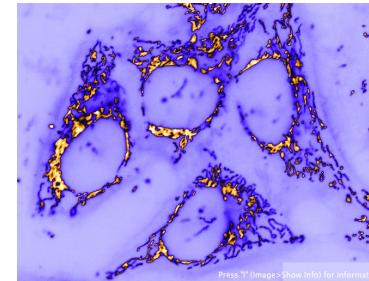
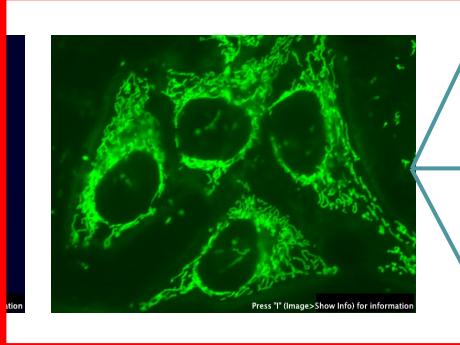
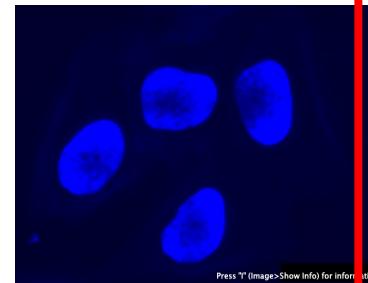
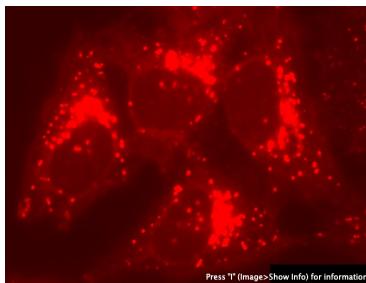
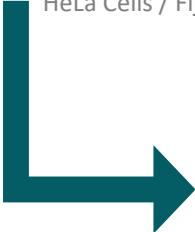
Colour Deconvolution  
350x65 pixels; RGB; 89K  
Colour deconvolution: H&E  
Colour\_1 R:0.6443186, G:0.7166757, B:0.26688856  
Colour\_2 R:0.09283128, G:0.9545457, B:0.28324  
Colour\_3 R:0.63595444, G:0.001, B:0.7717266



# Fluorescent Images



HeLa Cells / Fiji samples



$$1 \begin{bmatrix} 1 & 2 & \dots & n \\ a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \ddots & \vdots \\ m & a_{m1} & a_{m2} & \dots & a_{mn} \end{bmatrix}$$

$$1 \begin{bmatrix} 1 & 2 & \dots & n \\ a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \ddots & \vdots \\ m & a_{m1} & a_{m2} & \dots & a_{mn} \end{bmatrix}$$

$$1 \begin{bmatrix} 1 & 2 & \dots & n \\ a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \ddots & \vdots \\ m & a_{m1} & a_{m2} & \dots & a_{mn} \end{bmatrix}$$

- ✓ Gray-level images
- ✓ Independent channel representation

## Look-up tables

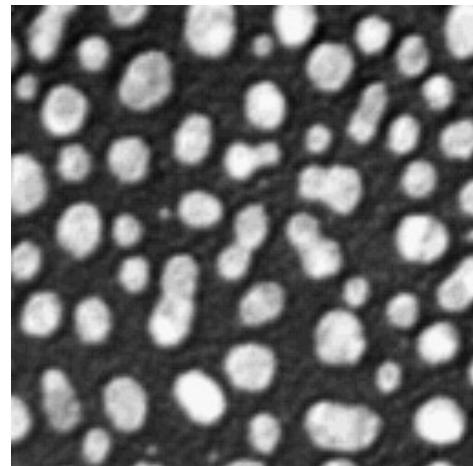
- ✓ Define how the image is displayed
- ✓ Original values are not modified!

# Look-up table (LUT)

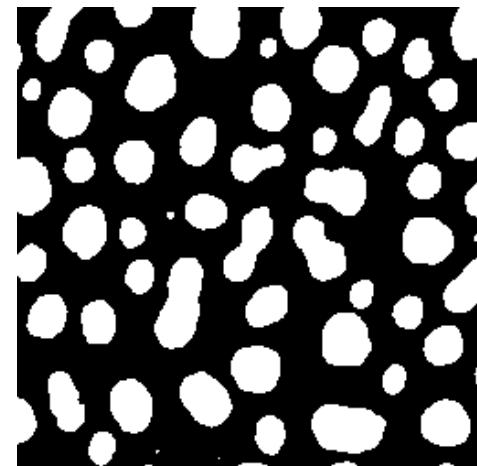


## ➤ Others examples

- ✓ Labeling: to highlight instance-based segmentation (Glasbey LUT)
- ✓ Detection of artifacts: saturation & under exposure (HiLo LUT)



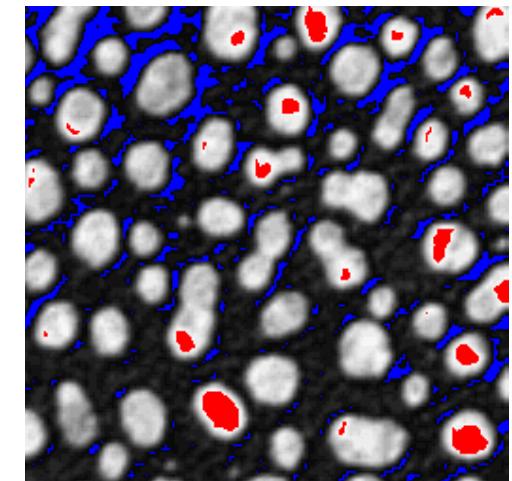
Fiji samples / Blobs



Blobs - thresholded

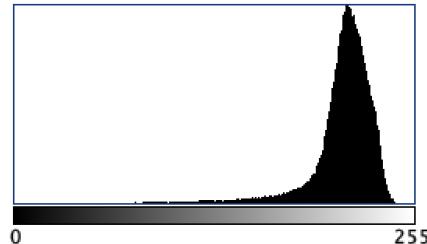
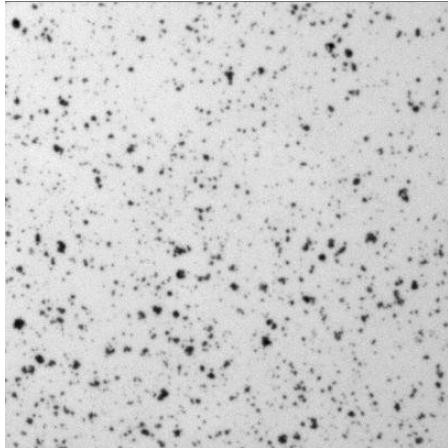


Glasbey LUT



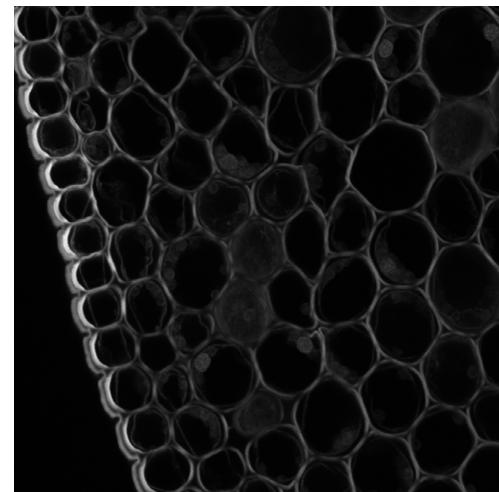
HiLo LUT

# Histograms

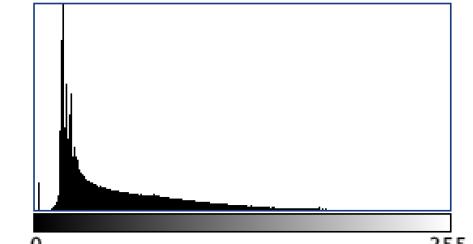
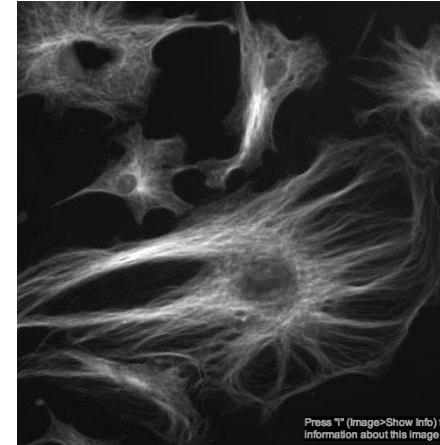


Fiji sample images: *Cell colony*

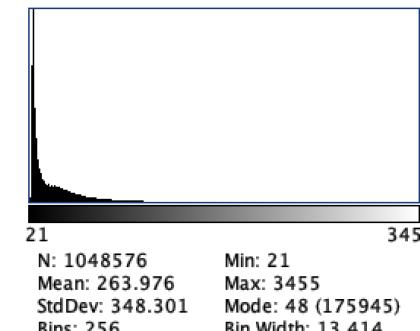
- The pixel order does not affect the histogram



Source: LCI Microscopy course 2023



Fiji sample images: *Fluorescent cells*



Basic measurements derived from histograms:

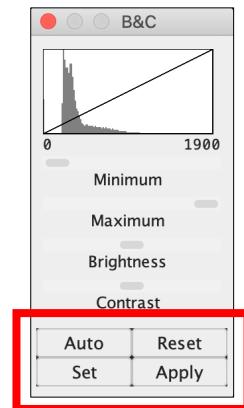
- Mean
- Variance
- Standard Deviation
- Min, Max, Mode



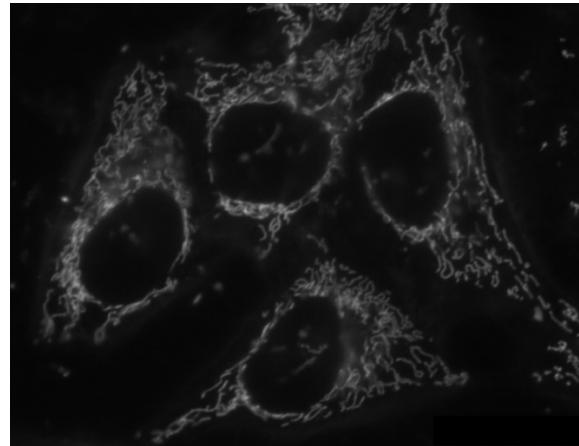
# Brightness & Contrast

## ➤ Changing pixel values

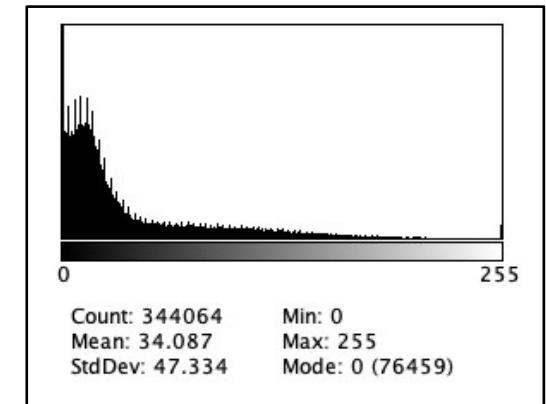
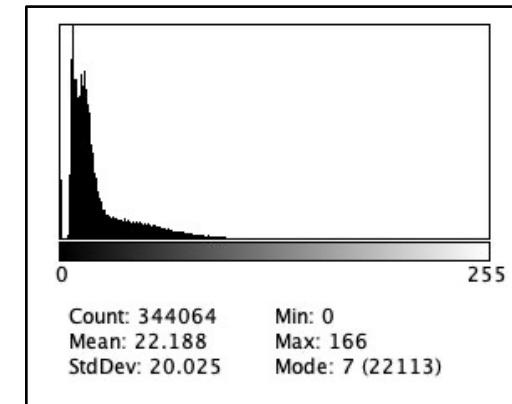
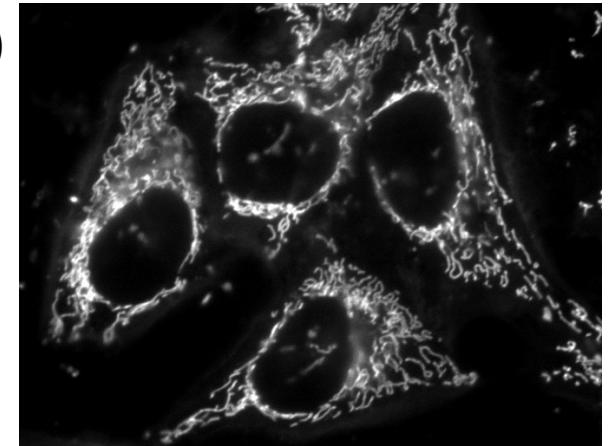
- ✓ Brightness: “amount of light”
- ✓ Contrast: Maximum difference in intensity



A)



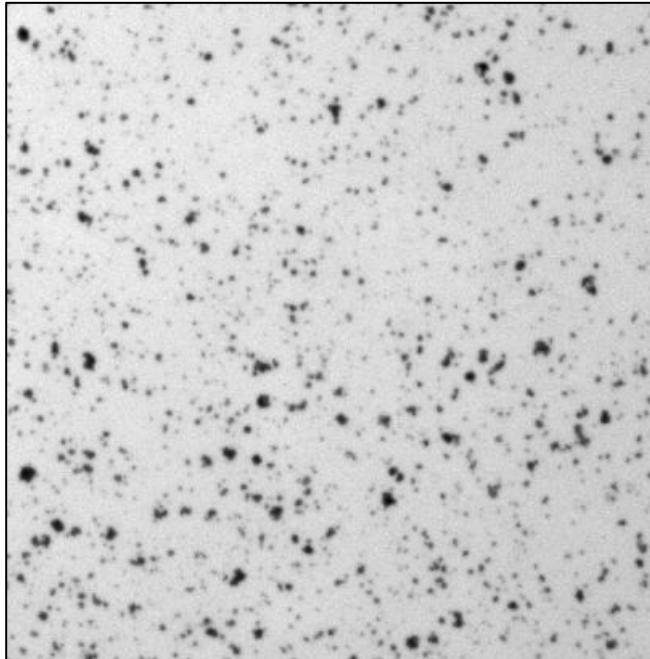
B)



# Image dimensions

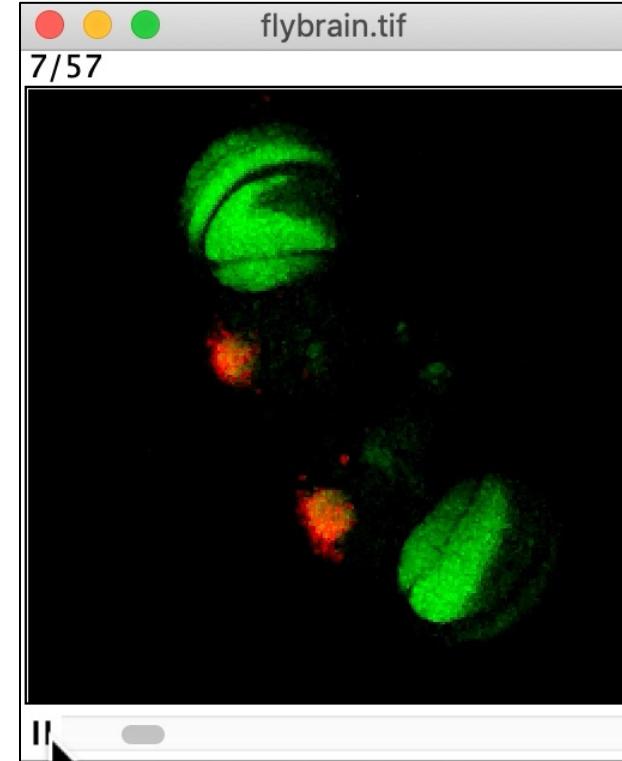


2D image



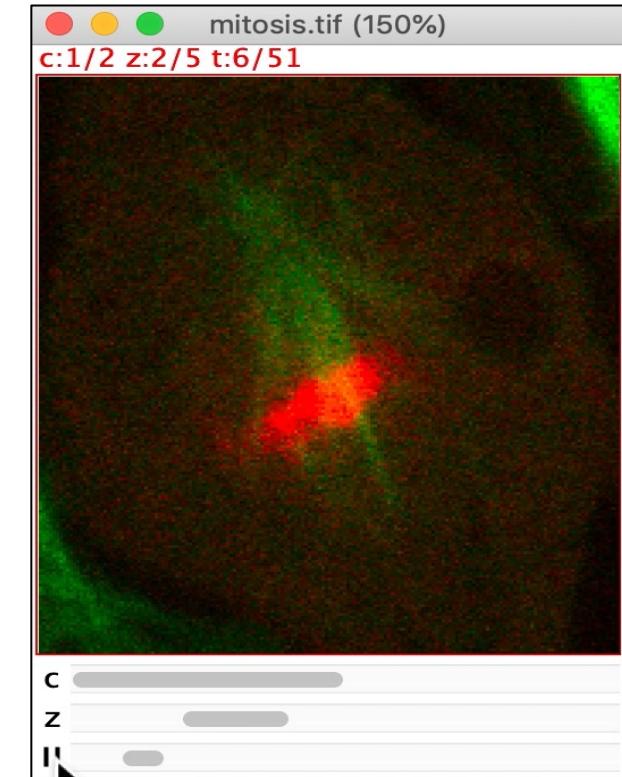
Cell colony / Fiji samples

3D image / Stack



Fly brain / Fiji samples

5D image / HyperStack



Mitosis / Fiji samples

An image may have at most 5 dimensions!

# Image Metadata



## ➤ Experimental

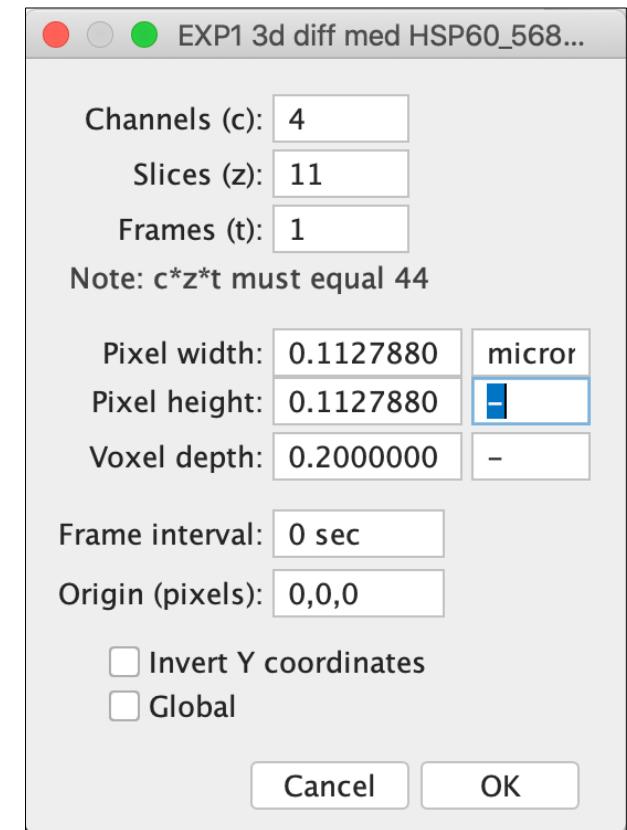
- Ex: protocols, probes, treatments, etc

## ➤ Image Acquisition

- Ex.: parameters, instrument settings, (excitation power, filters, gain, exposure time, etc)

## ➤ Analytic

- Outputs from processing and analysis techniques

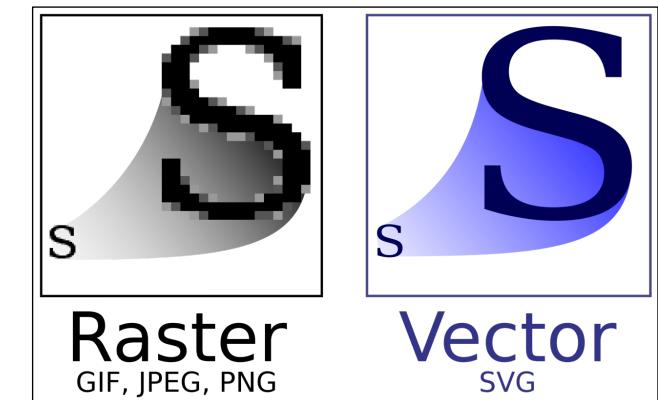


# File formats

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- Microscopy file formats
  - Proprietary formats: Ex.: ND2 (Nikon), LIF or SCN (Leica), OIB or OIF (Olympus), CZI or ZVI (Zeiss), etc
  - Store metadata
- General file formats (JPEG, PNG, MPEG, AVI, TIFF, etc)
  - JPEG lossy compression
  - TIFF is a lossless format and stores metadata
  - PNG used for transfer and display (no stacks possible)
- File formats for figures (publications)
  - Bitmaps (BMP)
  - Vector images (SVG, PDF, EPS etc)

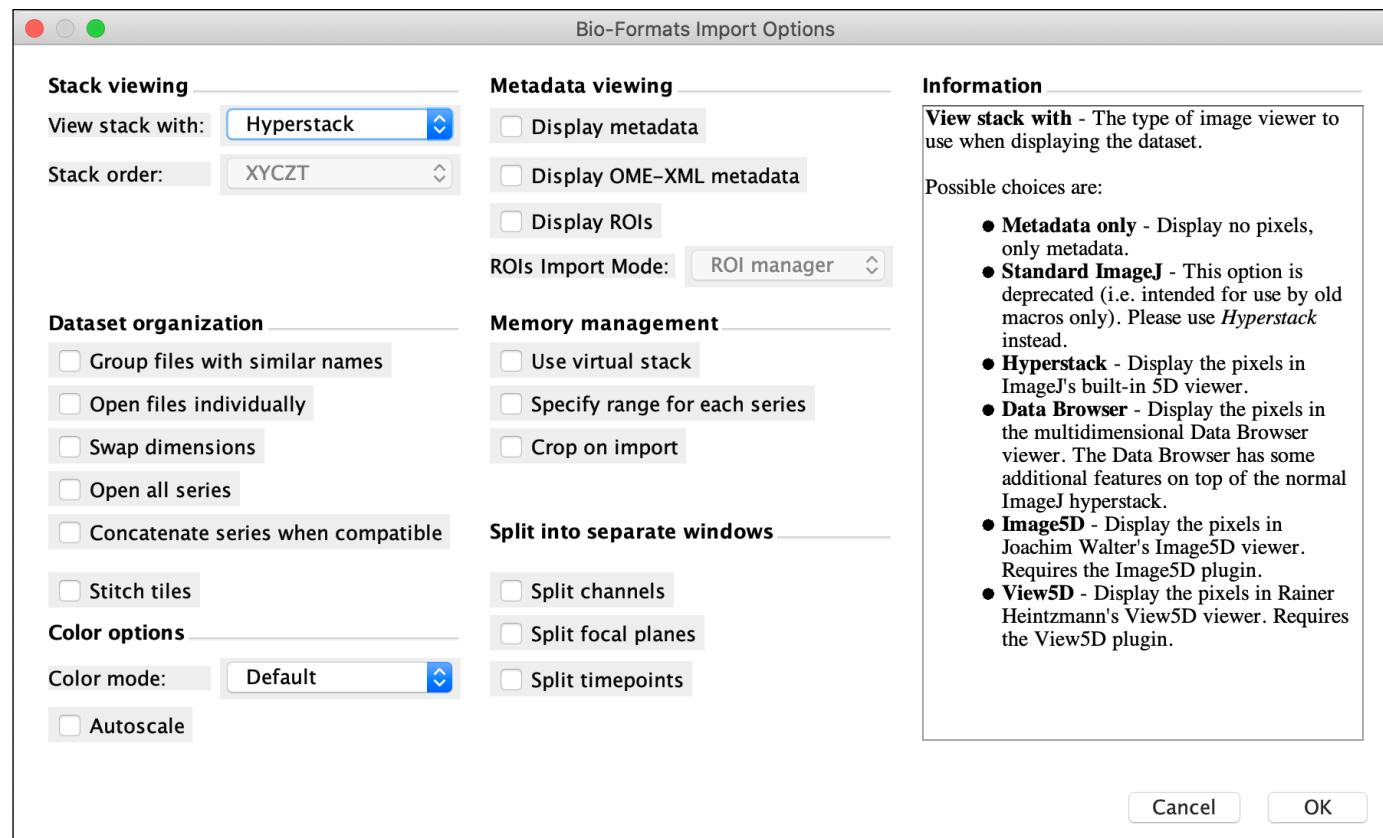


Demonstration of differences  
between bitmap and SVG images

# File formats



- LOCI Bio-Formats plugin\*



Import Options of the Bio-Formats plugin – Fiji interface

*Good practice:  
always keep original  
files and refer to the  
acquisition software  
for the original  
meta*

# Practical Exercises

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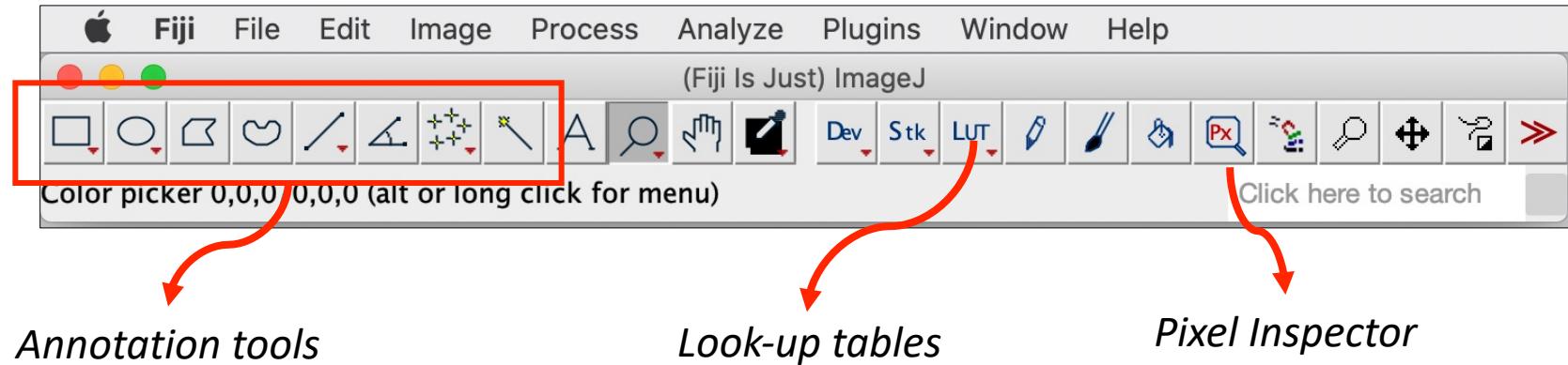
Let's explore some tools to inspect image data in Fiji!

- ❑ Image files to be used in this session are located in `../data_day1/part1/`
  - Open the file “**nuclei1.tif**” in Fiji
  - What is the image dimension?
  - Inspect pixel values with the *Pixel Inspector* tool
  - Modify the LUT
  - Inspect the metadata. What tags can you interpret?
  - Analyze the histogram
- Now repeat the steps above for the “`humanMCF7_cells.tif`”, “`CIN_1_HE.tif`” and Fiji/sample images “Mitosis”

# Practical Exercises



- Fiji tools

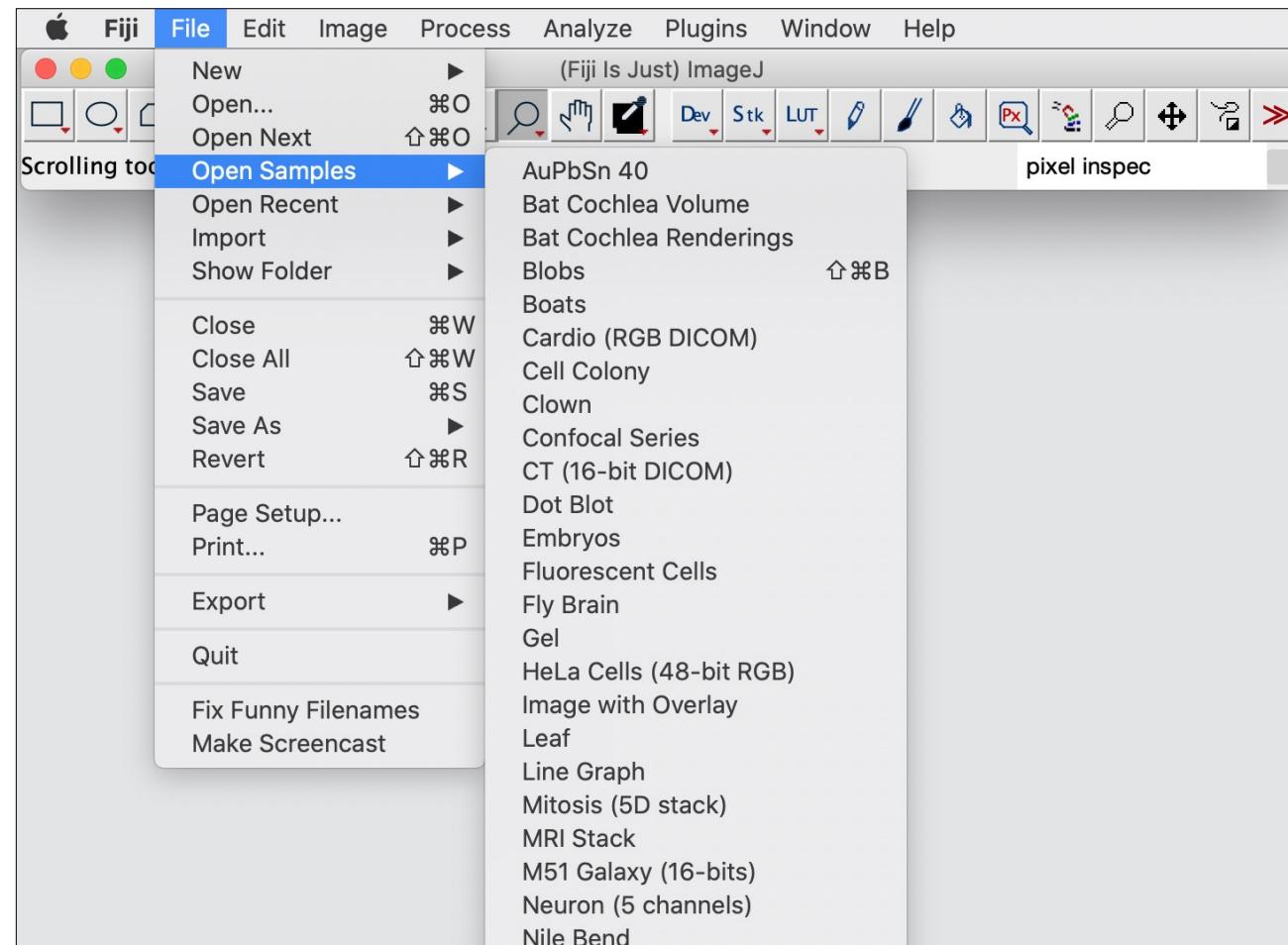


- Loading an image: go to "File->Open", then browse the image of interest. If the Bio-Formats plugin interface appears, check the import options and press "OK"

# Practical Exercises



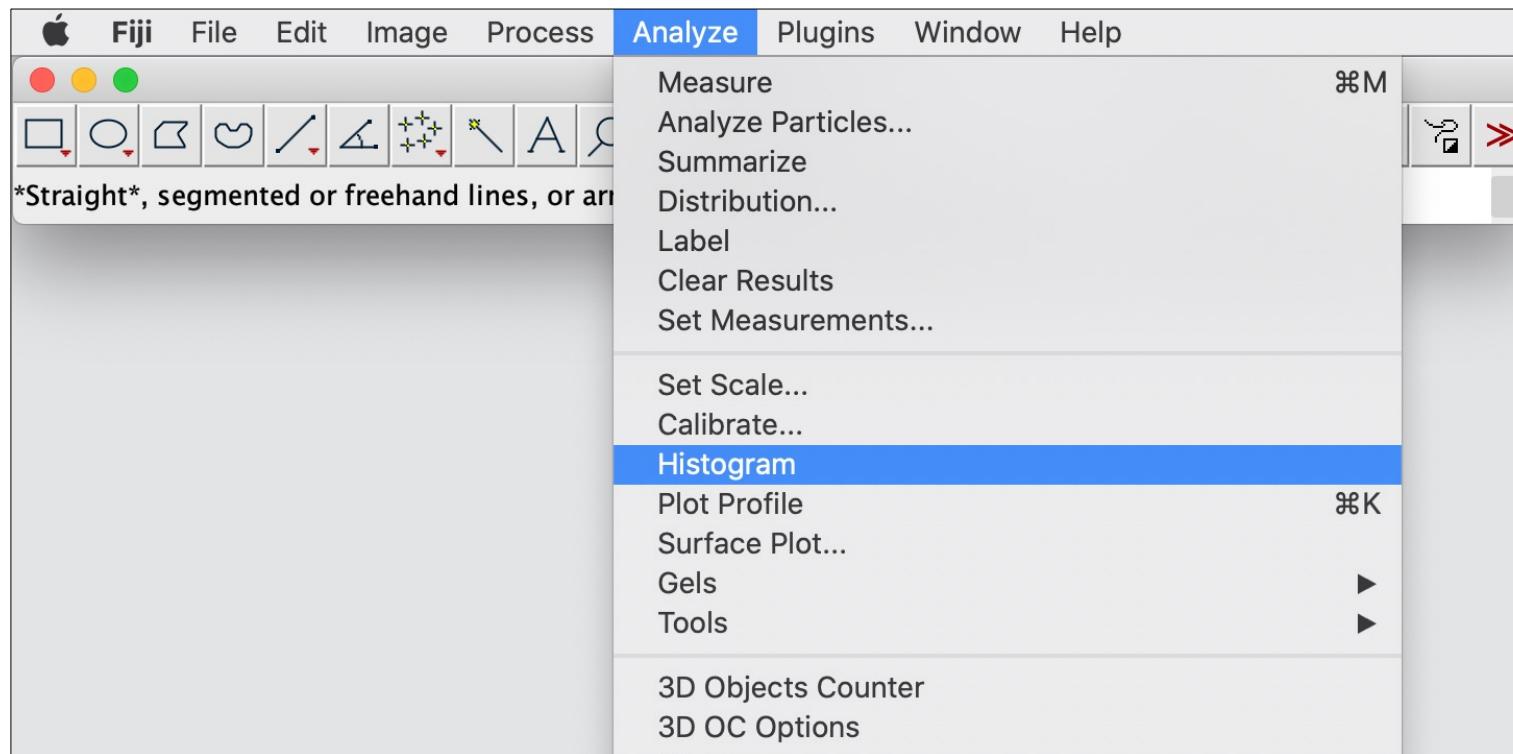
- Loading Fiji sample images



# Practical Exercises



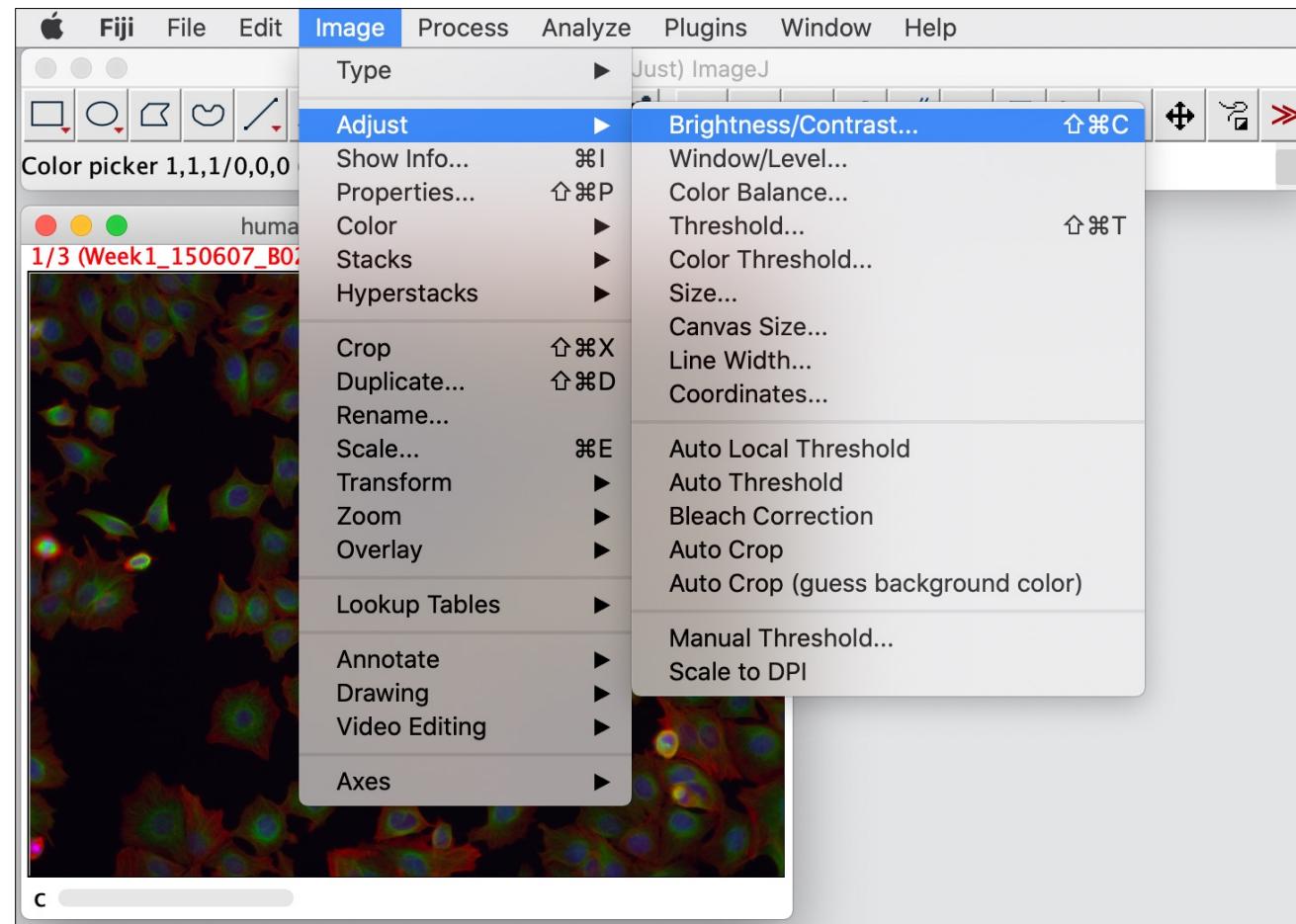
- Generating the histogram



# Practical Exercises



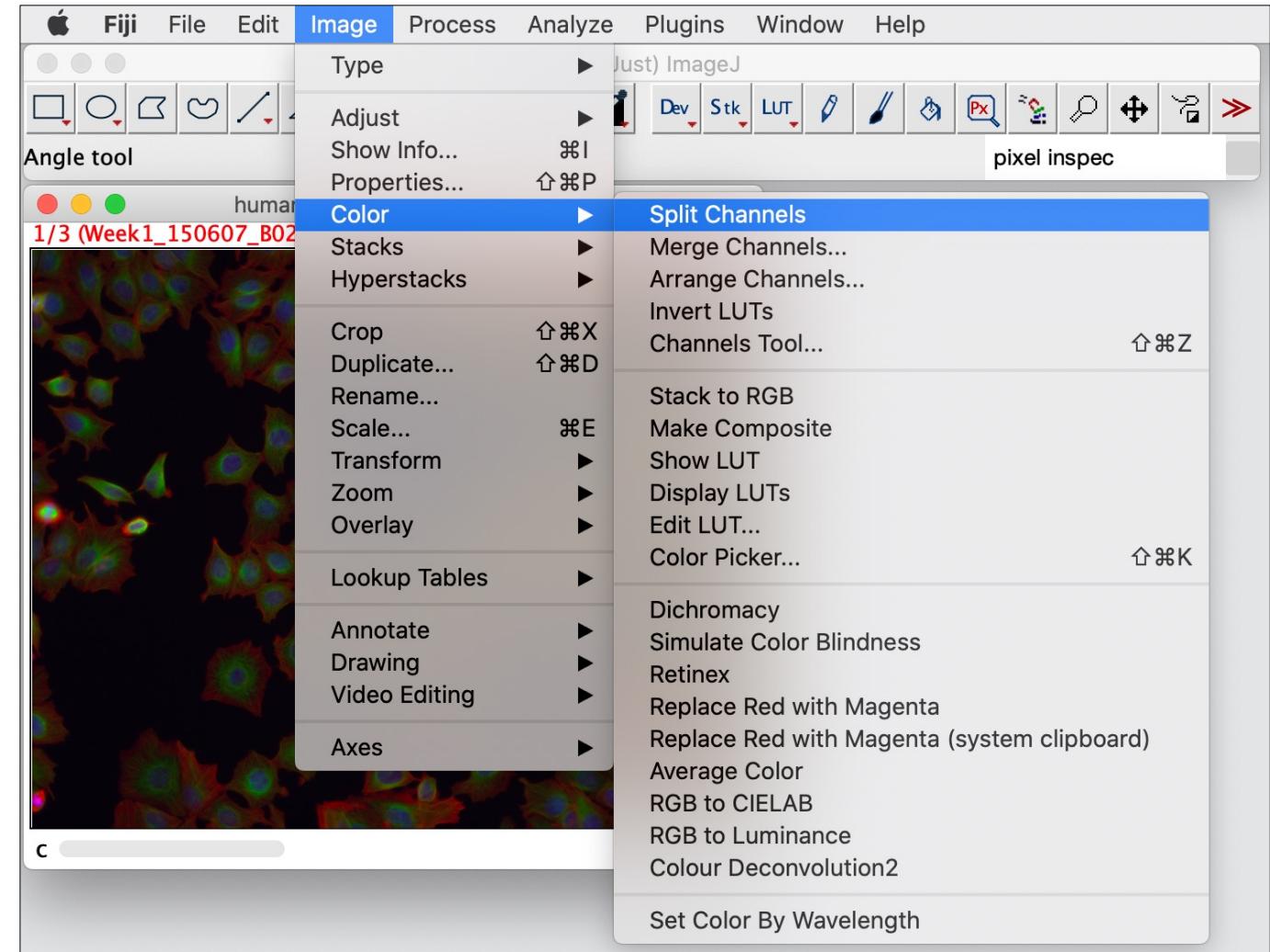
- Brightness & Contrast



# Practical Exercises



- Splitting and merging channels / channel tool



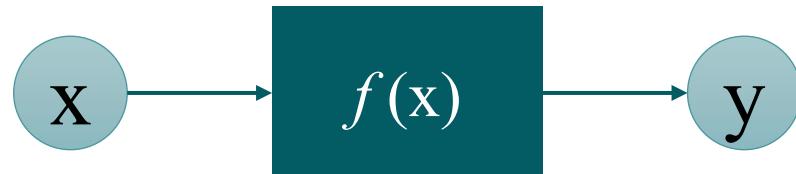
*Image processing and enhancement*





# Image Enhancement

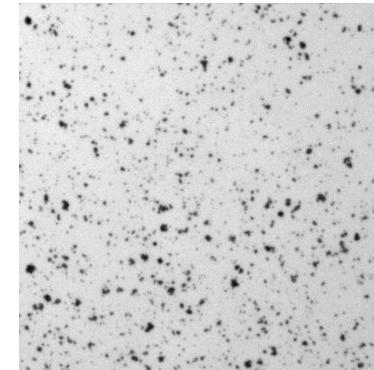
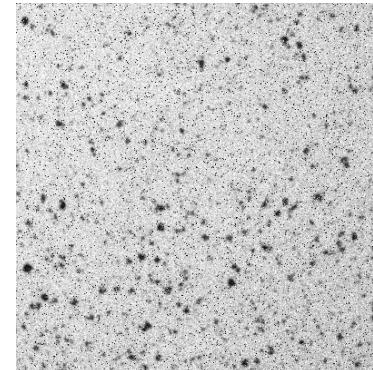
- Image operations designed to modify or enhance image properties, such as edges, corners and blobs.



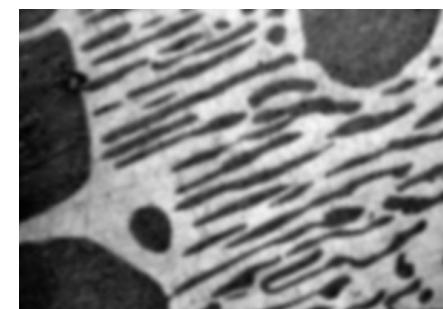
Edge detection



De-noising



Gaussian blur

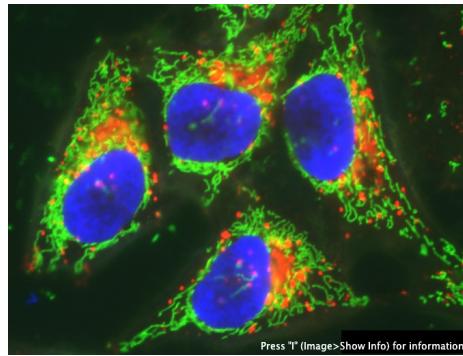


✓ Can be performed in the spatial domain and frequency domain

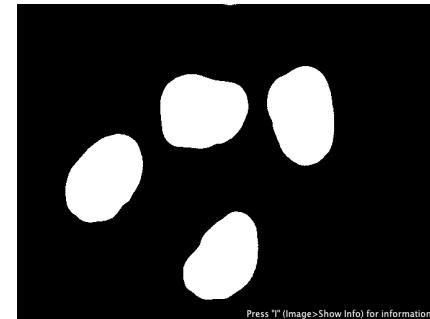
# Basic arithmetics



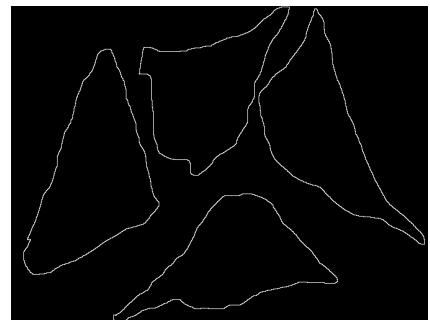
- Standard operations:  $+, -, *, /$
- Logical operators (binary images): AND, OR, XOR, etc



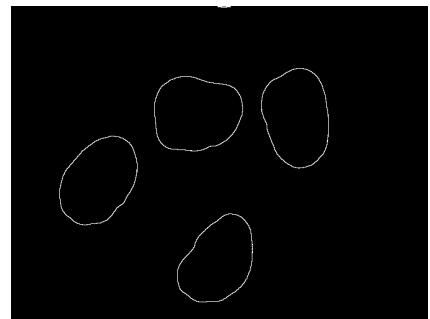
A



B



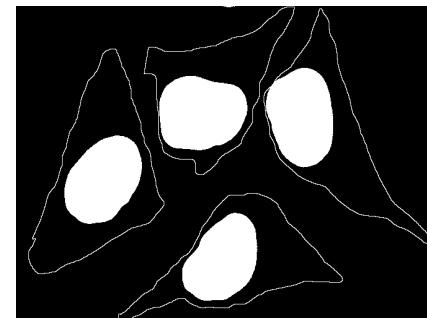
C



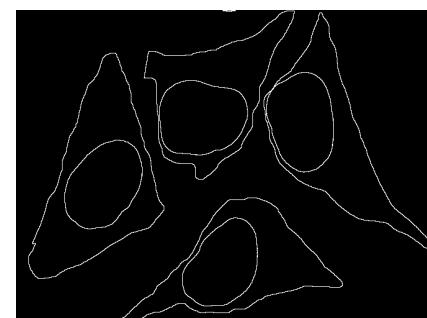
D



Subtraction  
(A - B)



Add  
(C + B)

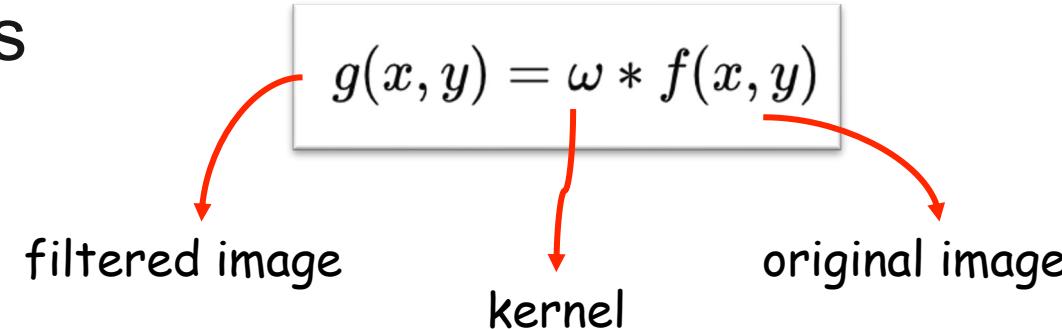


Add  
(C + D)

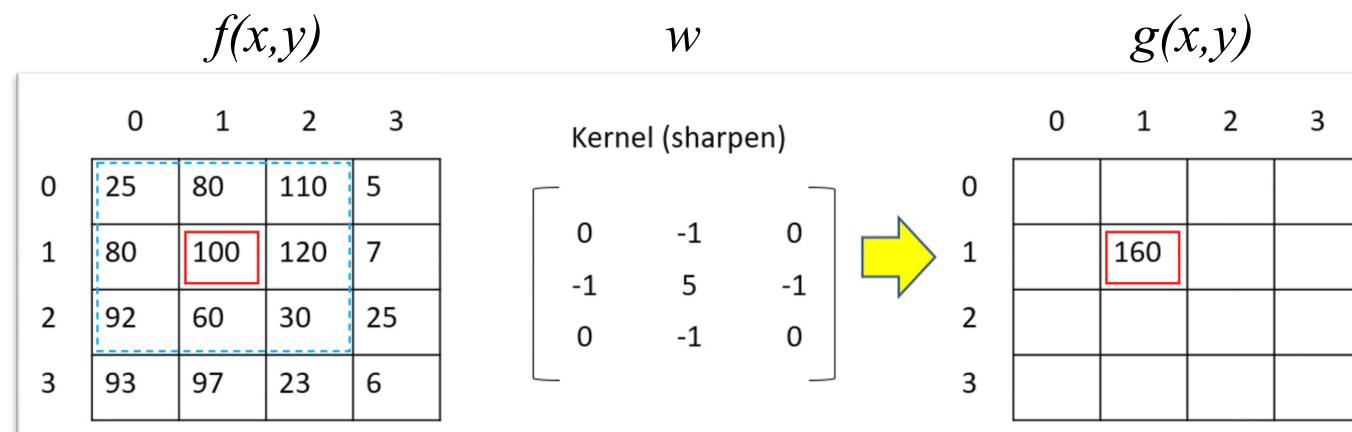
# Convolution filters



- Also known as kernels



- Ex: sharpen

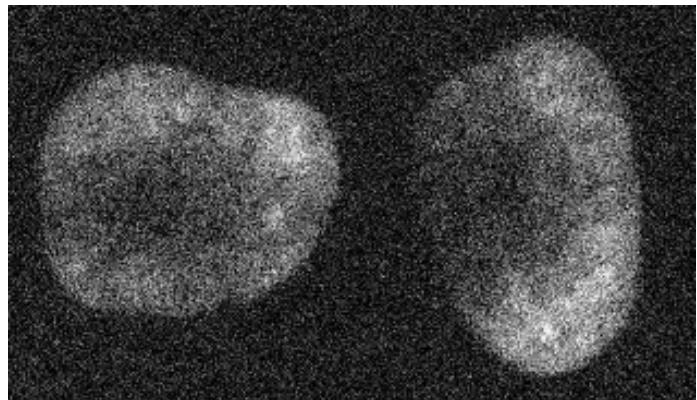


Adapted from <https://medium.com/@ianormy/convolution-filters-4971820e851f>

Each pixel in the original image is multiplied by the corresponding value in the kernel, and then summed

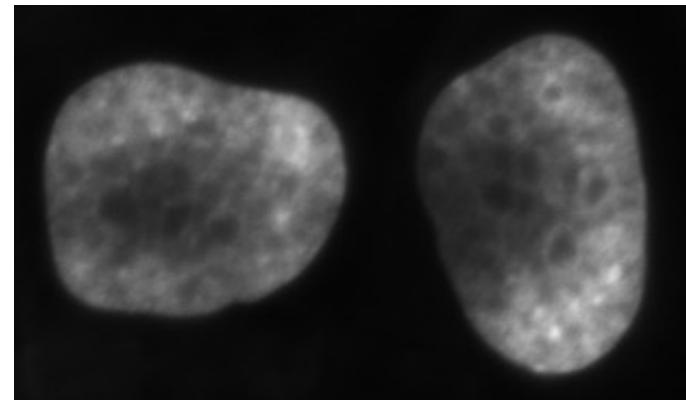


# The mean filter



$$* \frac{1}{9} \begin{array}{|c|c|c|} \hline +1 & +1 & +1 \\ \hline +1 & \textcolor{green}{+1} & +1 \\ \hline +1 & +1 & +1 \\ \hline \end{array} =$$

Kernel



10	2	3	
1	<b>23</b>	43	
7	13	9	



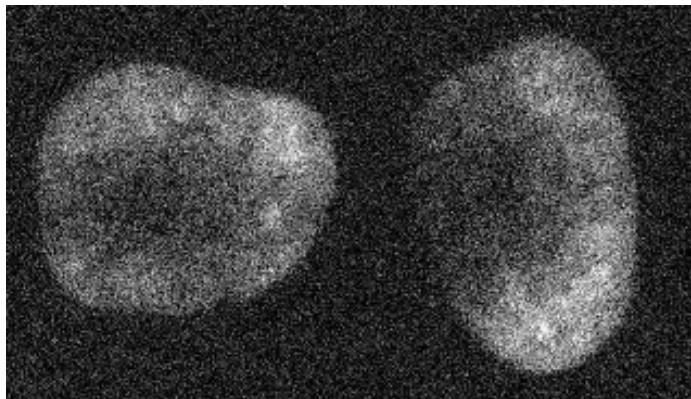
	<b>12</b>		

Sum:  $10 + 2 + 3 + 1 + 23 + 43 + 7 + 13 + 9 = 111$

Average:  $111/9 \sim 12$

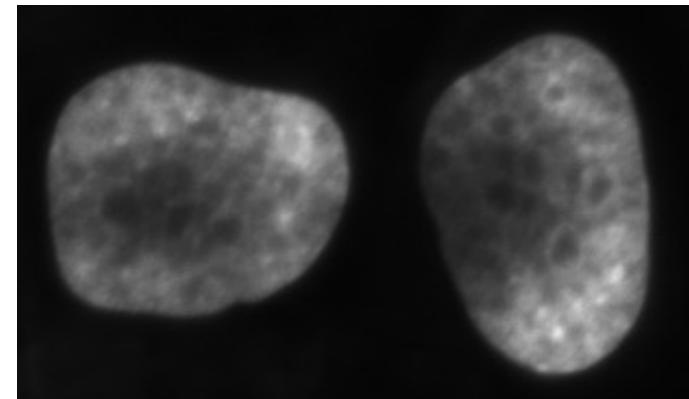


# The mean filter



$$* \frac{1}{9} \begin{array}{|c|c|c|} \hline +1 & +1 & +1 \\ \hline +1 & \textcolor{green}{+1} & +1 \\ \hline +1 & +1 & +1 \\ \hline \end{array} =$$

Kernel



	2	3	5
	23	<b>43</b>	4
	13	9	2

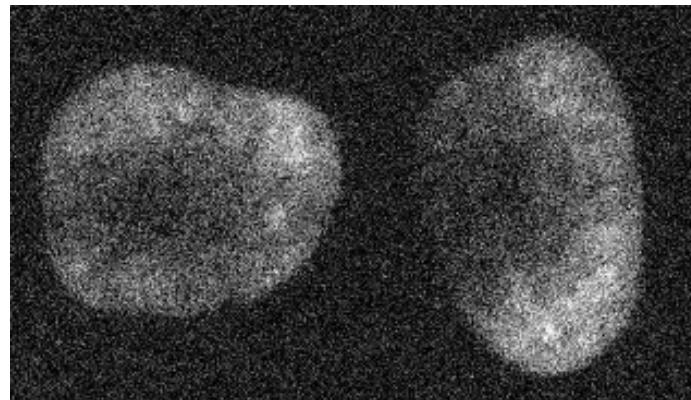


	<b>12</b>	<b>12</b>	

Sum:  $2 + 3 + 5 + 23 + 43 + 4 + 13 + 9 + 2 = 104$

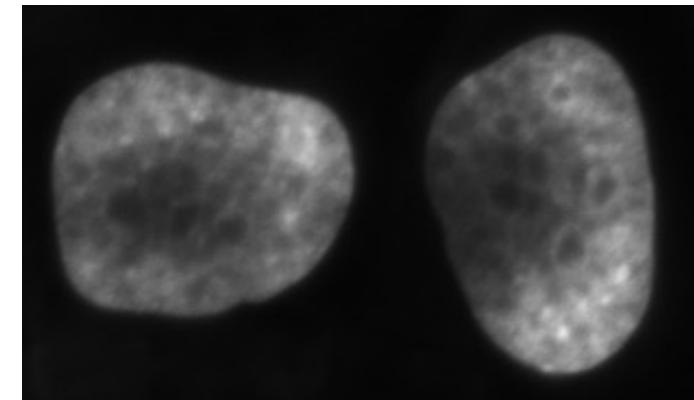
Average:  $104/9 \sim 12$

# The median filter



$$* \begin{array}{|c|c|c|} \hline +1 & +1 & +1 \\ \hline +1 & \textcolor{green}{+1} & +1 \\ \hline +1 & +1 & +1 \\ \hline \end{array} =$$

Kernel



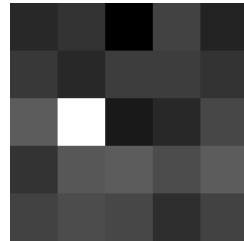
10	2	3	
1	<b>23</b>	43	
7	13	9	



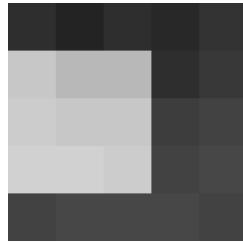
		<b>9</b>	

Sort: 1, 2, 3, 7, **9**, 10, 13, 23, 43

# Mean vs median filter – noise reduction



*Unfiltered*



*Mean  
filtered*



*Median  
filtered*



*salt and pepper noise*



*mean-filtered*



*median-filtered*

# Mean vs median filter – smoothing effect



## ✓ Median filter

- Noise reduction
- No new values created → sharp edges are preserved
- More time consuming in order to sort values



*original*



*median-filtered*

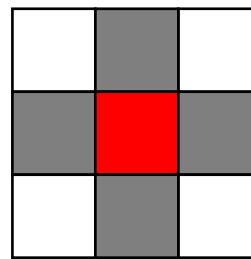


*mean-filtered*

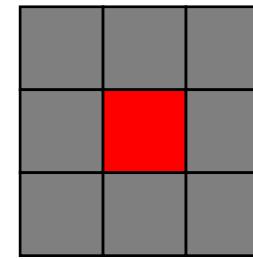
# Neighborhood size



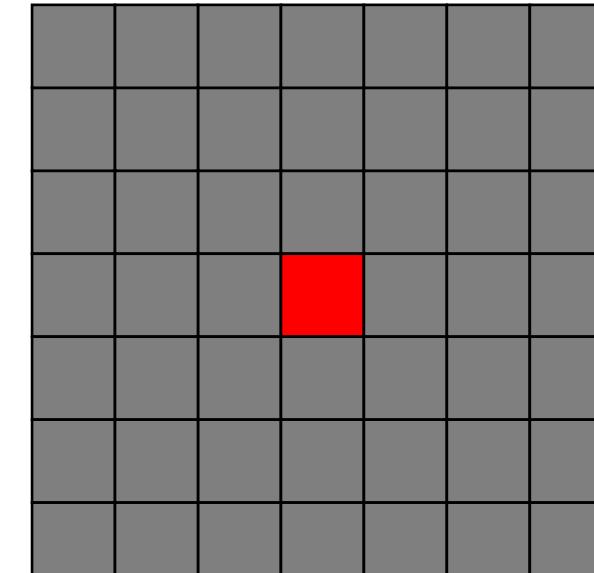
- Kernel: different sizes and shapes



4-connected



8-connected,  
(3x3)



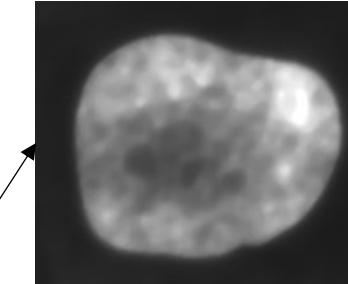
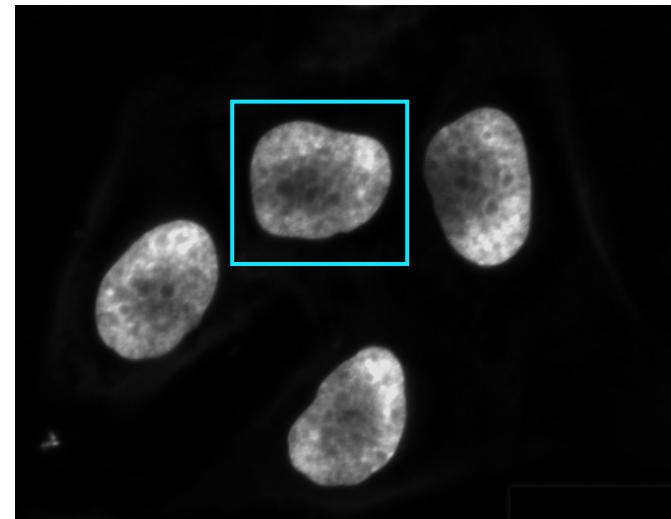
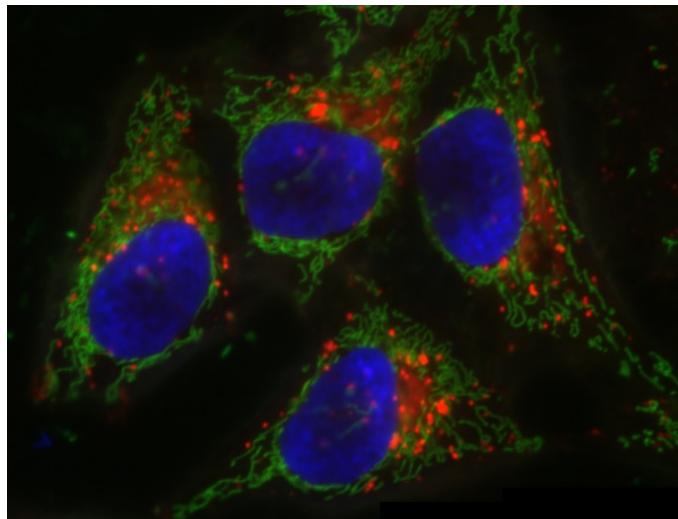
5x5

*What happens as the neighborhood size increases?*

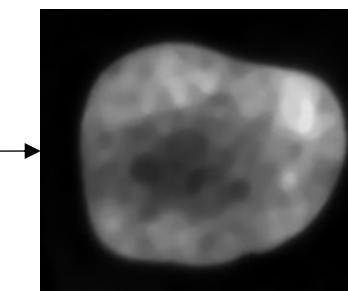
# Convolution filters



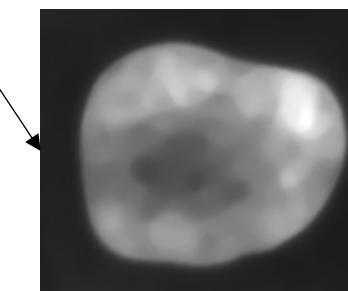
- Let's have a look at the result of the median filter applied to the HeLa cells example:



$r=2$



$r=4$



$r=6$

# Gaussian Smoothing



- ✓ Used to “blur” images and remove detail and noise

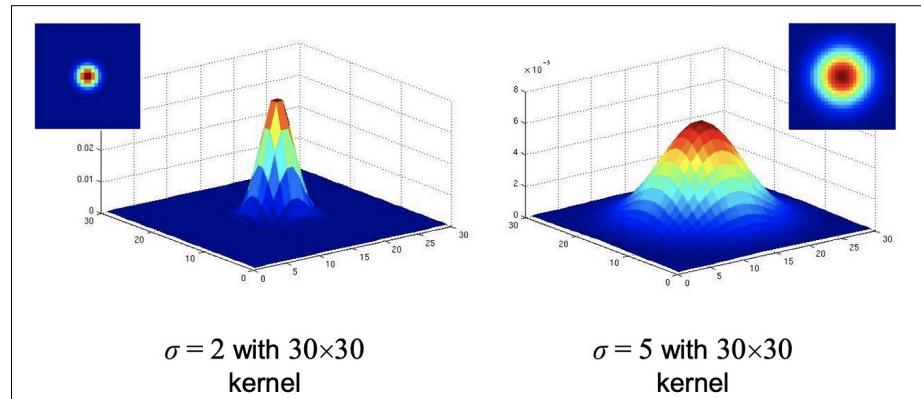
- Bell-shaped kernel

- ✓ Computationally efficient:

- Larger filters (e.g. 2D) can be implemented using smaller 1D filters

- ✓ Rotationally symmetric

- ✓ The degree of smoothing is given by the standard deviation  $\sigma$



[https://medium.com/jun94-devpblog/cv-2-gaussian-and-median-filter-separable-2d-filter-2d11ee022c66#:~:text=Gaussian%20kernel%2C%20as%20its%20name,\(pixels\)%20in%20an%20image](https://medium.com/jun94-devpblog/cv-2-gaussian-and-median-filter-separable-2d-filter-2d11ee022c66#:~:text=Gaussian%20kernel%2C%20as%20its%20name,(pixels)%20in%20an%20image)



original

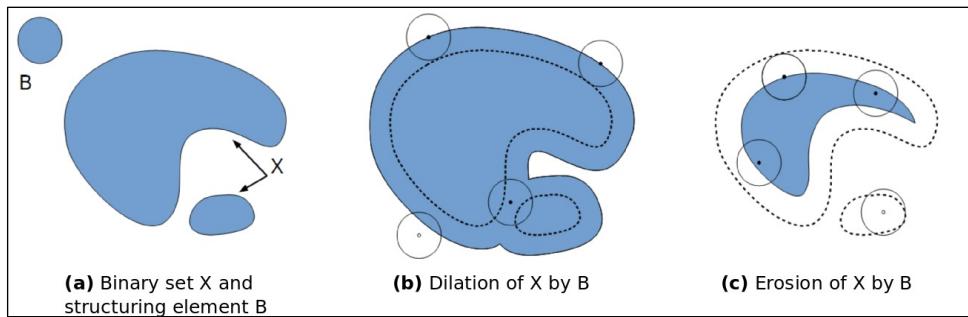


gaussian-filtered  $\sigma=2$

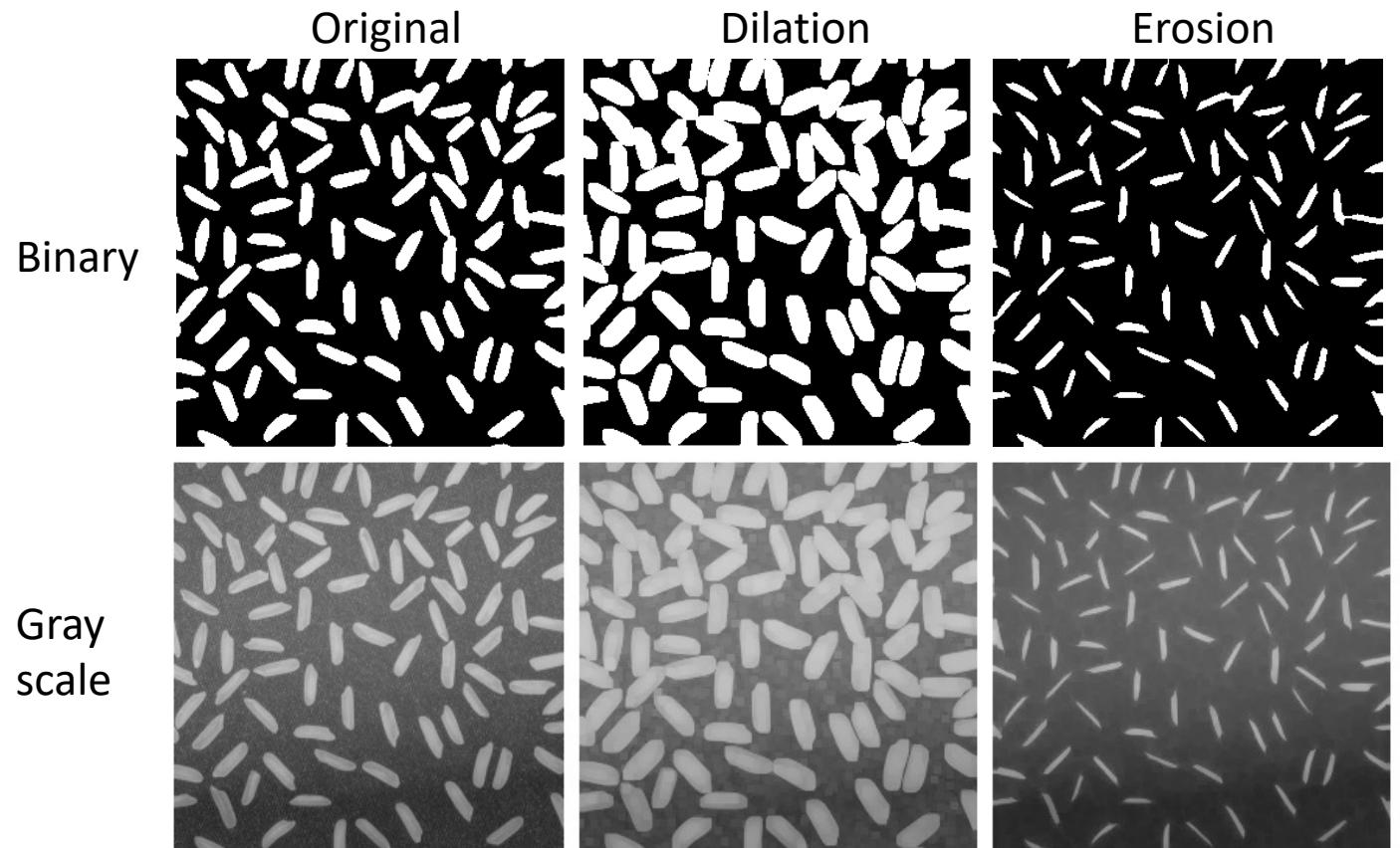
# Morphological filters



- Describe shapes by using another shape as test probe
  - Dilation and erosion are the most basic operations



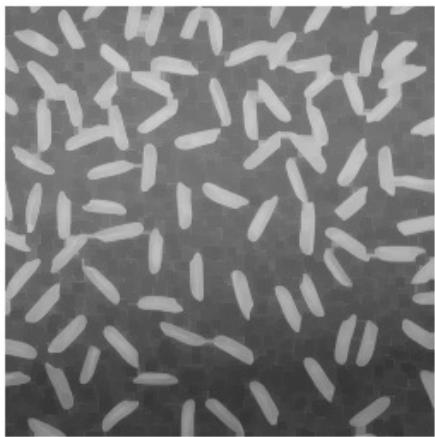
Morphological dilation and erosion on a binary set



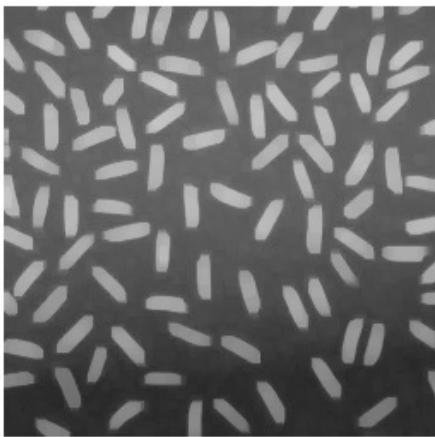
# Morphological filters: gray level images



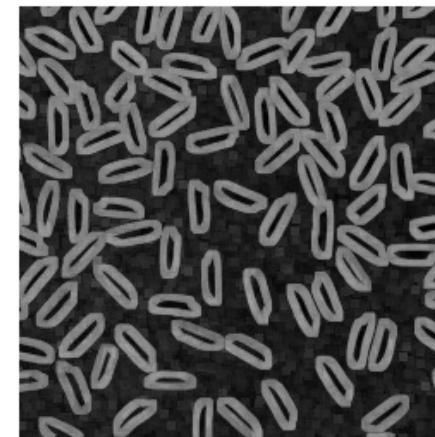
- Dilation and erosion combined to create other morphological filters



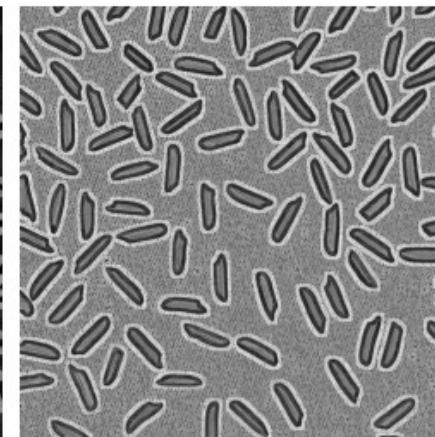
Closing



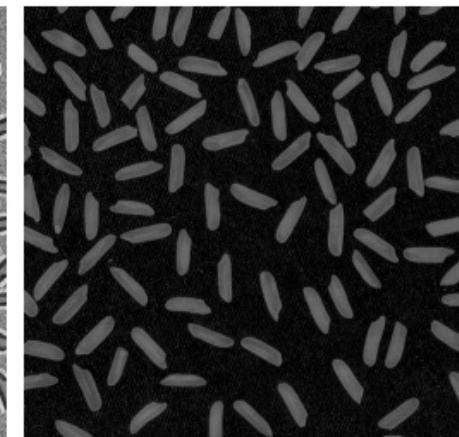
Opening



Gradient



Laplacian



Top-hats

**Dilation + Erosion**

Removes dark structures smaller than the structuring element

**Erosion + Dilation**

Removes bright structures smaller than the structuring element

**Dilation - Erosion**

Boundary detection

**Original image – 1/5(Dilation+Erosion)**

Edge enhancement

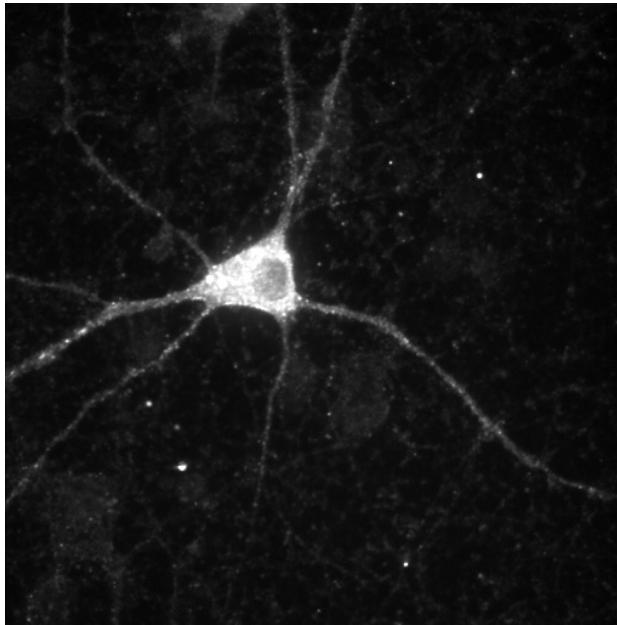
**Original image - Opening**

Enhance bright or dark structures

# Filtering in the frequency domain

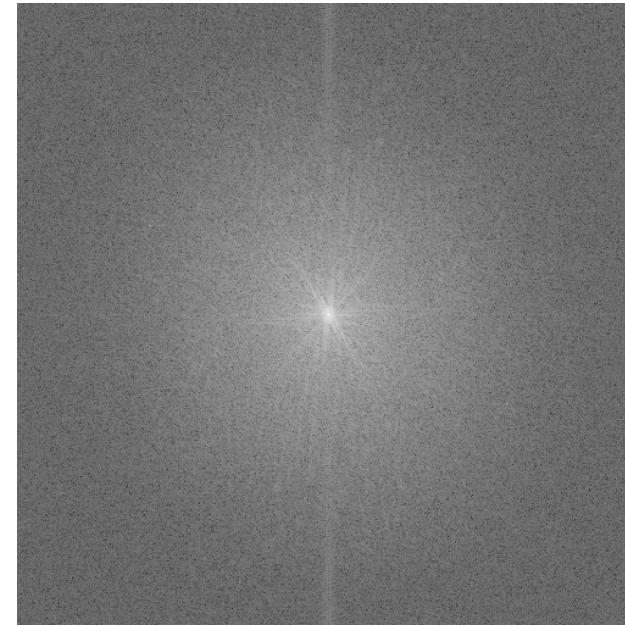


Original Image



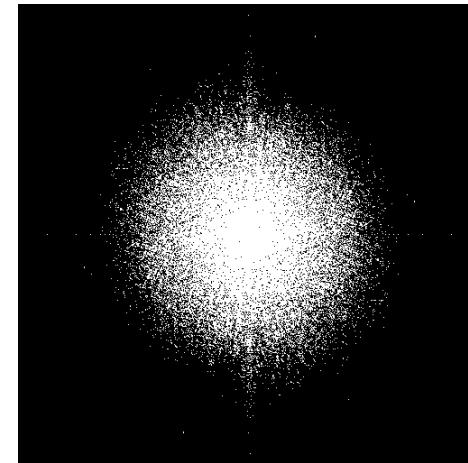
Rat Hippocampal Neuron  
Fiji samples

Frequency domain

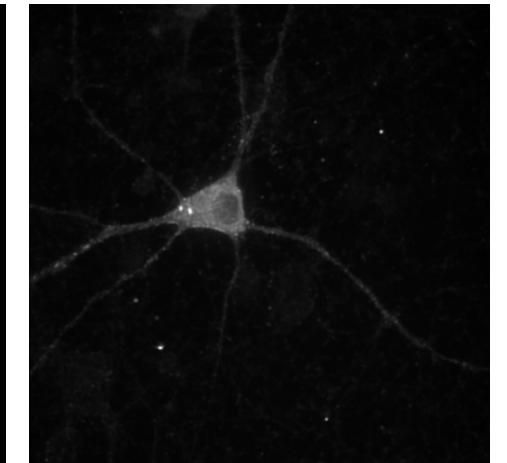
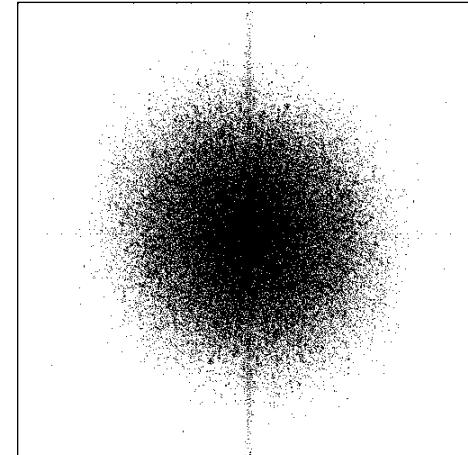


Fourier  
Transform

Low-pass filter



High-pass filter



# Uneven Illumination

---

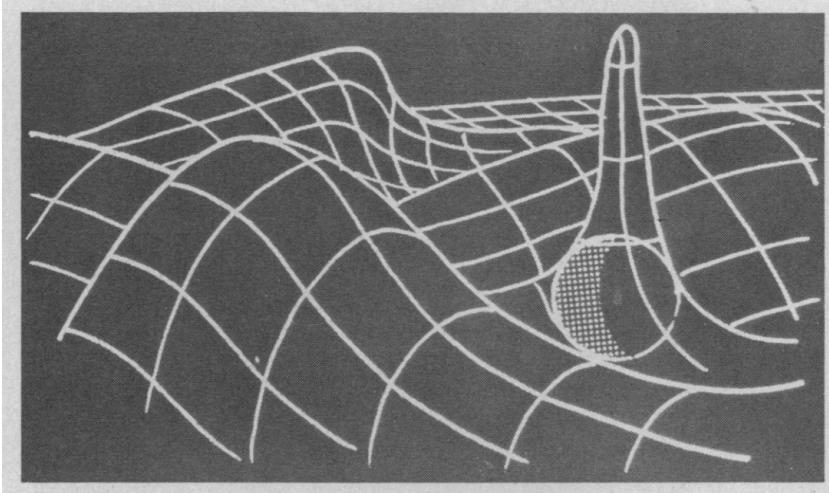


- ✓ Due to different factors, e.g.:
  - ❖ microscope settings;
  - ❖ sample artifacts (samples that are not flat);
  - ❖ shading or vignetting (attenuation of the pixel intensity from the center of the optical axis to the edges).
  
- ✓ Consequences:
  - ❖ discontinuities in whole slide images;
  - ❖ background bleaching in time-lapse fluorescent images ;
  - ❖ compromise of downstream analysis.



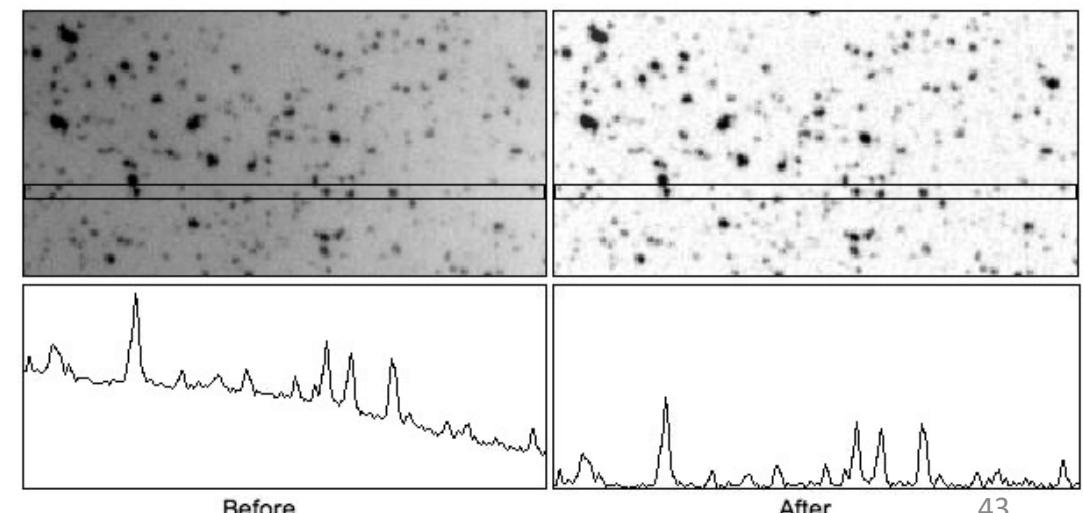
# Background subtraction

- Rolling-ball algorithm

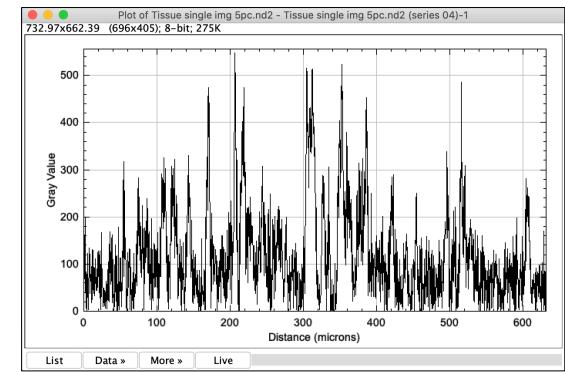
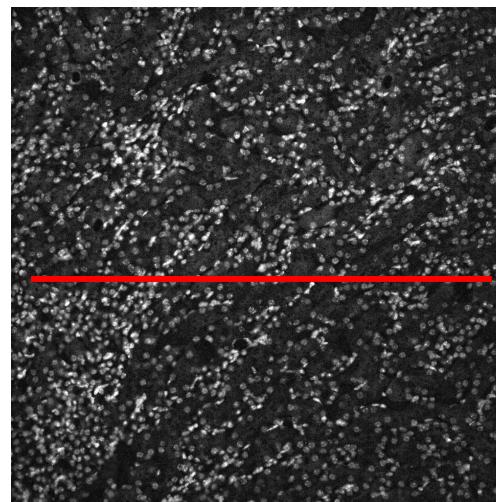
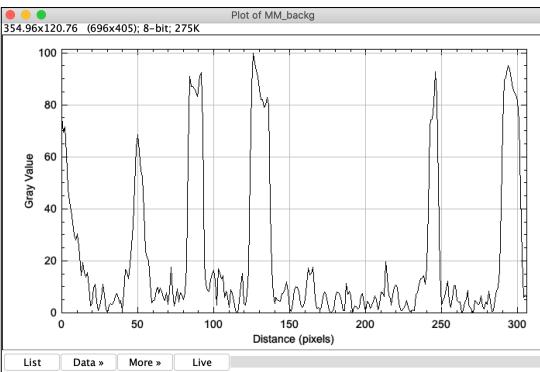
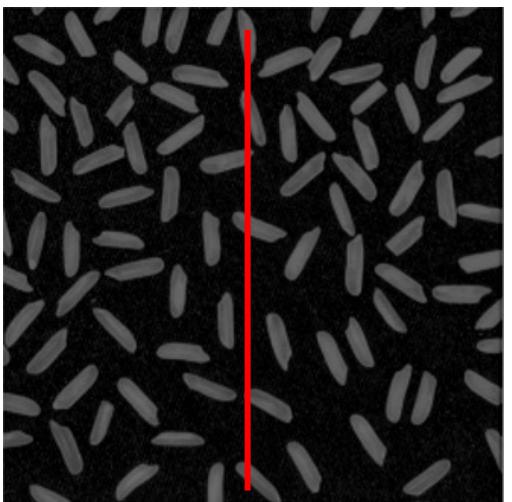
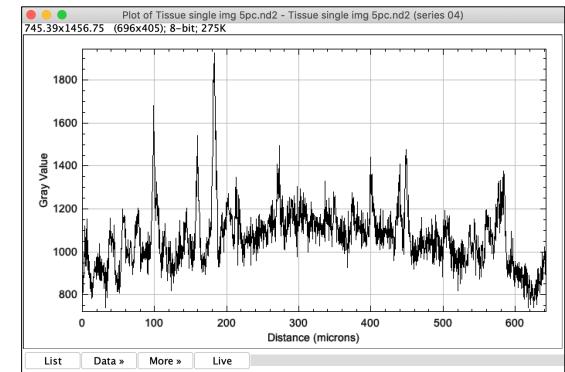
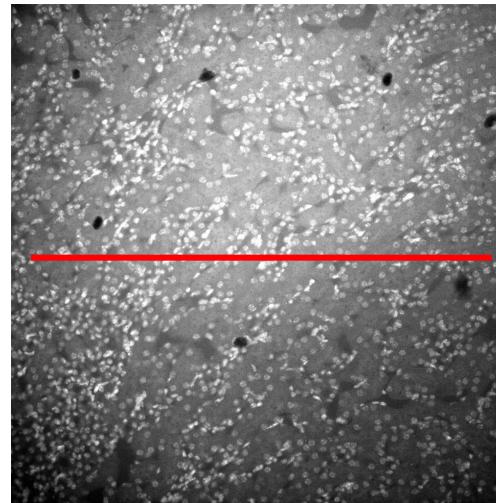
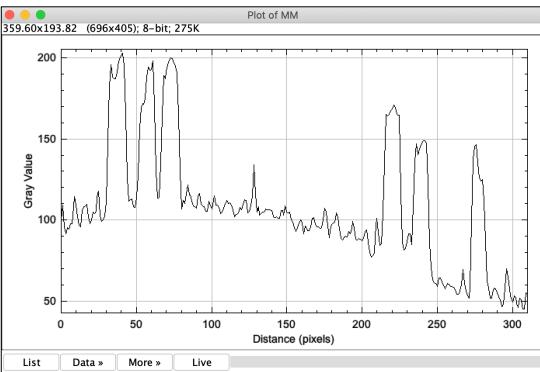
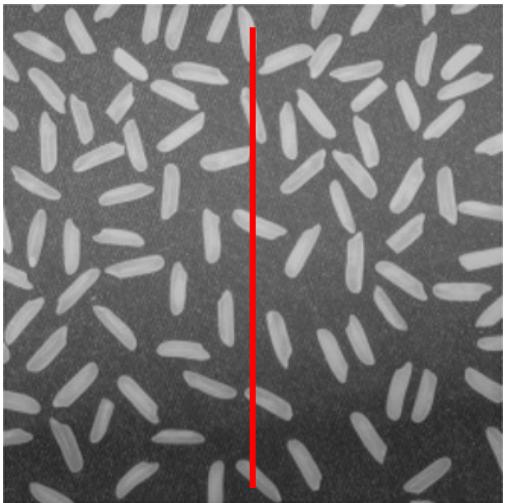


Sternberg, Stanley R. "Biomedical image processing." Computer 1 (1983): 22-34. DOI:10.1109/MC.1983.1654163

- ✓ 3D surface with the pixel values of the image being the height
- ✓ A ball rolling over the back side of the surface creates the background
- ✓ The rolling-ball is equivalent to eroding and dilating by a spherical structuring element



# Background correction



# Practical Exercises

---



- Image files to be used in this session are located in  
..../data\_day1/part2/
  1. Open the file “Nuclei\_no\_avg.nd2”
    - Take a moment to analyze the interface of the Bio-formats plugin
    - Why was it loaded?
  2. Subtract background (Process / Subtract Background...)
    - Use different values for the radius parameter
  3. Apply mean, median and gaussian filters (Process / Filters) – compare final results
    - Use different values for radius / sigma

Tip: Always duplicate the reference image (in Fiji: *Image / Duplicate...*) before applying any filter. In this way, you keep both, the original and processed images opened.

# Practical Exercises

---



- Image files to be used in this session are located in  
..../data\_day1/part2/

4. Can you think of a kernel that detects edges?

- Edge detection is a very important pre-processing step for any object detection or recognition process.
- Simple edge detection kernels are based on approximation of gradient images

Customized kernels can be implemented in Fiji using Process / Filters / Convolve

# Practical Exercises



- Image files to be used in this session are located in  
..../data\_day1/part2/

4. Can you think of a kernel that detects edges?

- Edge detection is a very important pre-processing step for any object detection or recognition process.
- Simple edge detection kernels are based on approximation of gradient images

Prewitt operator

For  $I_x(x, y)$

$$\begin{bmatrix} -1 & 0 & 1 \\ -1 & 0 & 1 \\ -1 & 0 & 1 \end{bmatrix}$$

For  $I_y(x, y)$

$$\begin{bmatrix} -1 & -1 & -1 \\ 0 & 0 & 0 \\ 1 & 1 & 1 \end{bmatrix}$$

Sobel operator

For  $I_x(x, y)$

$$\begin{bmatrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{bmatrix}$$

For  $I_y(x, y)$

$$\begin{bmatrix} -1 & -2 & -1 \\ 0 & 0 & 0 \\ 1 & 2 & 1 \end{bmatrix}$$

Customized kernels can be implemented in Fiji using Process / Filters / Convolve

# Practical Exercises

---



- Image files to be used in this session are located in  
..../data\_day1/part2/

## 5. Correcting uneven illumination

- Open files “tissue\_uneven\_illumination.tif” and “math\_morphology\_example.png”
- Use the straight line tool to annotate these images (example on slide #52)
- Then, plot the intensity profile using “Analyze/Plot Profile”
- In the next step, create a copy of the reference images (Image / Duplicate...),
- Apply the rolling ball algorithm (Process / Subtract Background...) and generate the intensity profile again
- Compare the corrected and uncorrected plots for each image

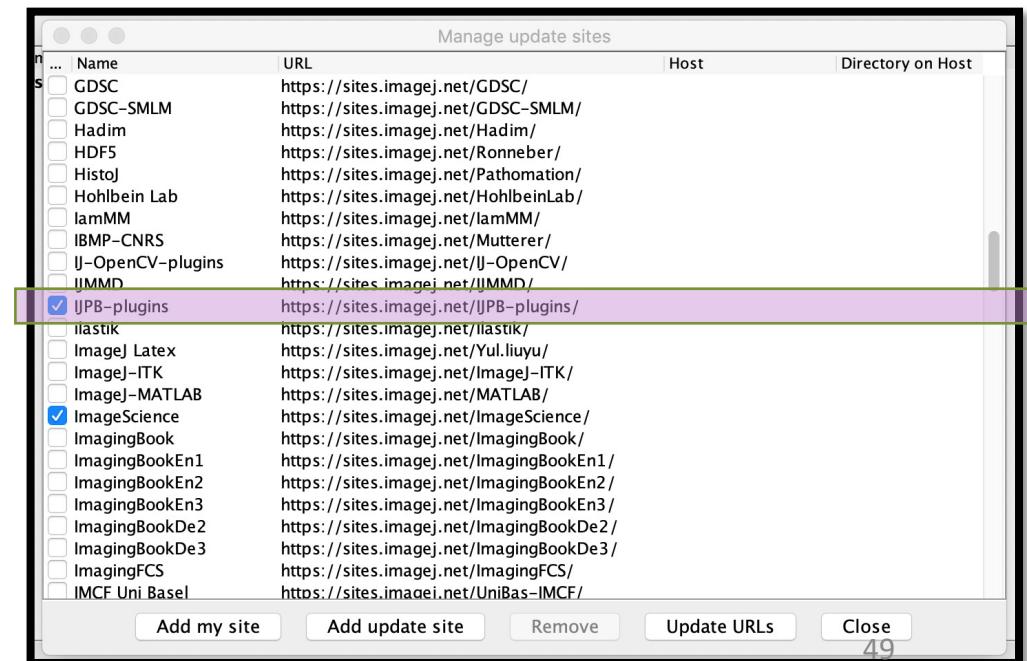
# Practical Exercises



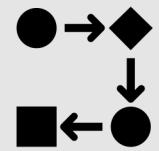
- Image files to be used in this session are located in  
..../data\_day1/part2/

## 6. Morphological filters

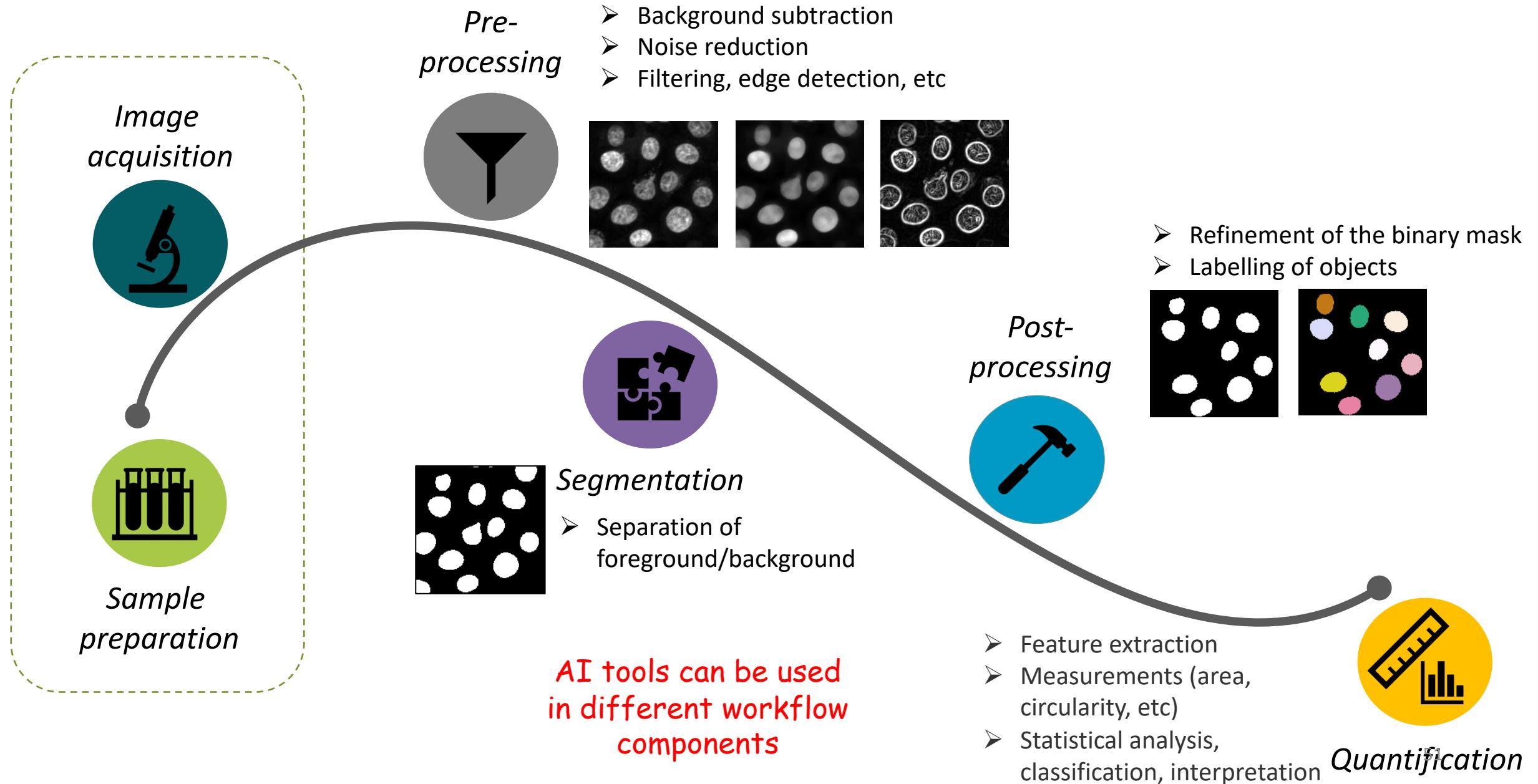
- Install the **MorphoLibJ plugin**: <https://imagej.net/MorphoLibJ>.
  - Go to Help / Update... / Manage update sites.
  - Select IJPB-plugins as shown in the figure
  - Click on “Close” and then “Apply changes”.
  - You will be requested to restart Fiji.
- Using files “Nuclei\_no\_avg.nd2” and “Nuclei\_no\_avg\_binary.tif”, try out the morphological filters described in slides 47 And 48 (Plugins / MorphoLibJ / Filtering / Morphological Filters)

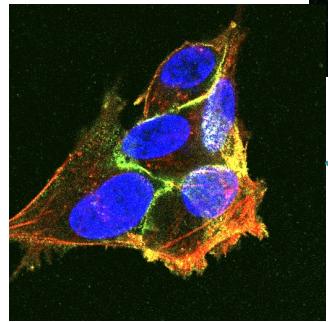
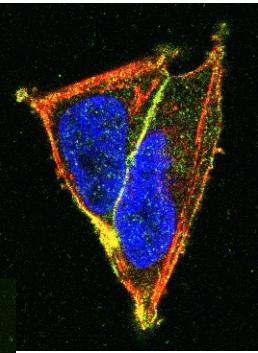
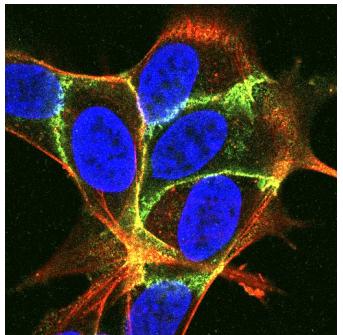


# *Image Analysis Workflow*



# The pathway towards image quantification

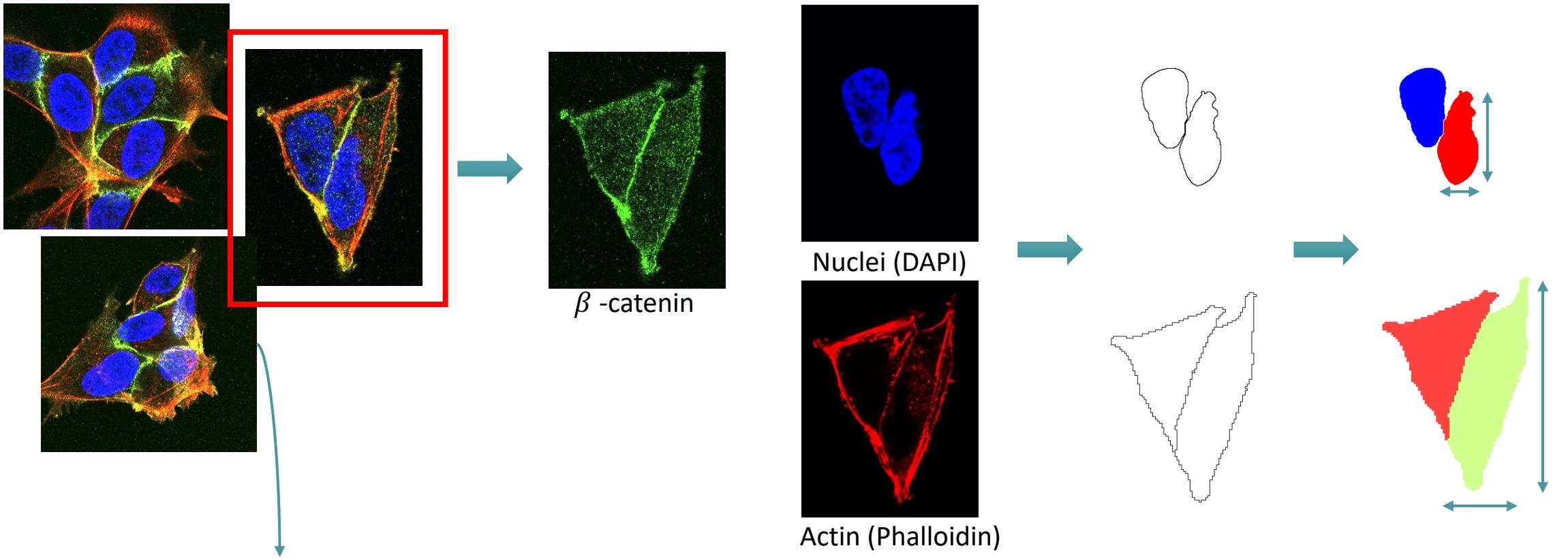




3-channel image dataset

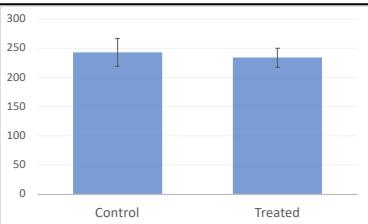
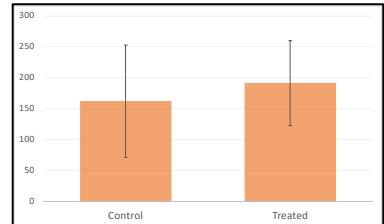
- Nuclei (DAPI - blue)
- $\beta$ -catenin (GFP - green)
- Actin (Phalloidin - red)

✓ How to quantify the  $\beta$ -catenin translocated from the cell membrane to the cell cytoplasm and nucleus ?



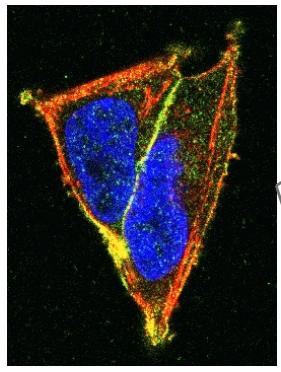
- ✓ How to quantify the  $\beta$ -catenin translocated from the cell membrane to the cell cytoplasm and nucleus ?

?

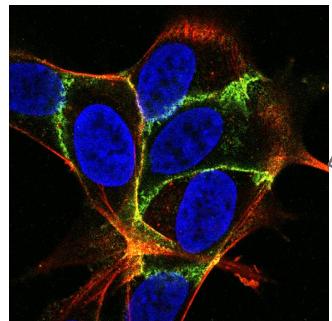
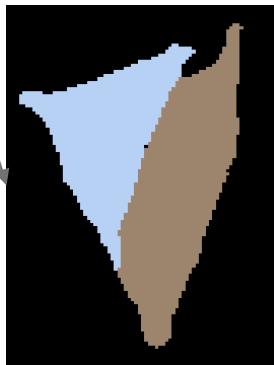
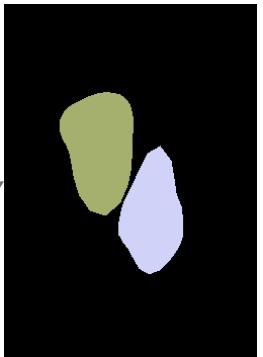


ROI	$M_1$	$M_2$	...	...	$M_n$
Blue	20	102	...	...	0.34
Red	25	198	...	...	0.29
Green	58	240	...	...	0.72
Yellow	51	211	...	...	0.49

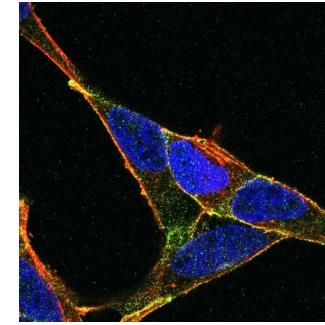
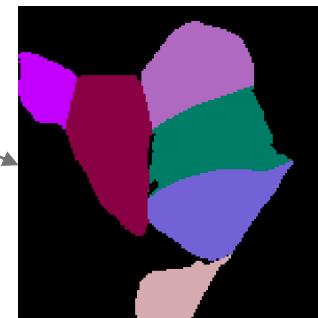
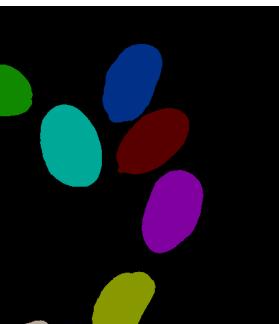
# Other images of the dataset



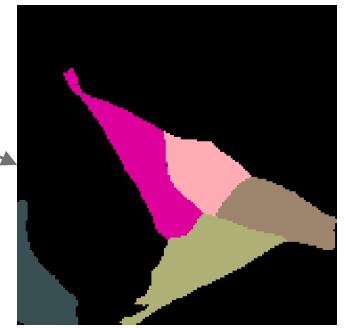
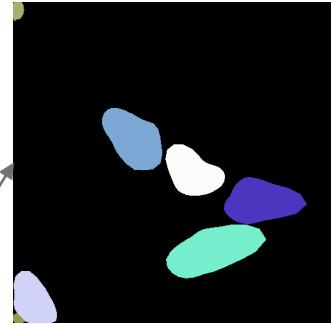
Example #1



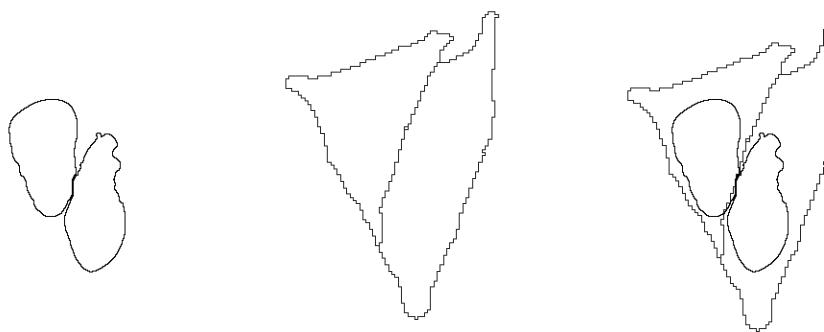
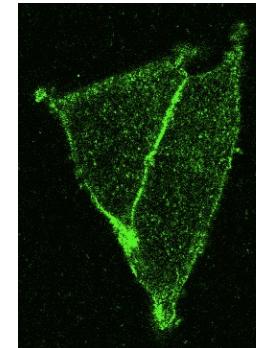
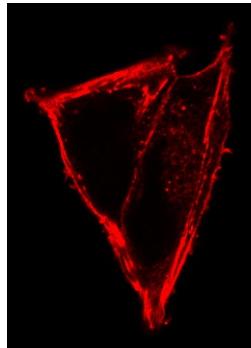
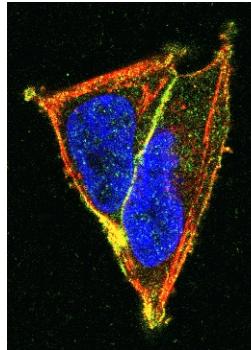
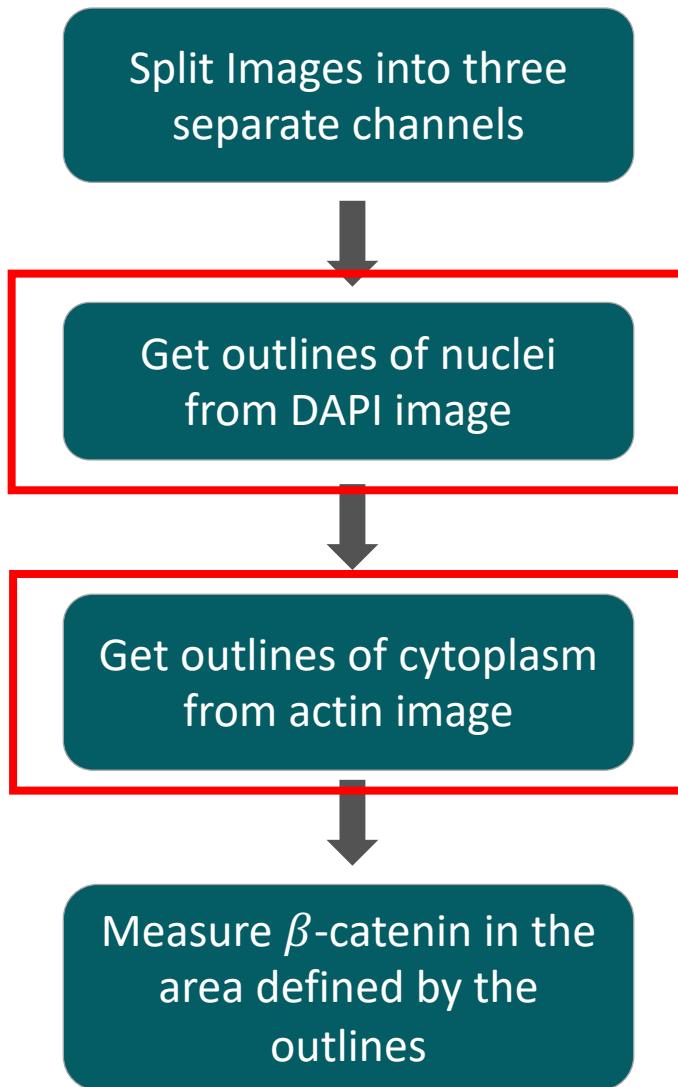
Example #3



Example #2



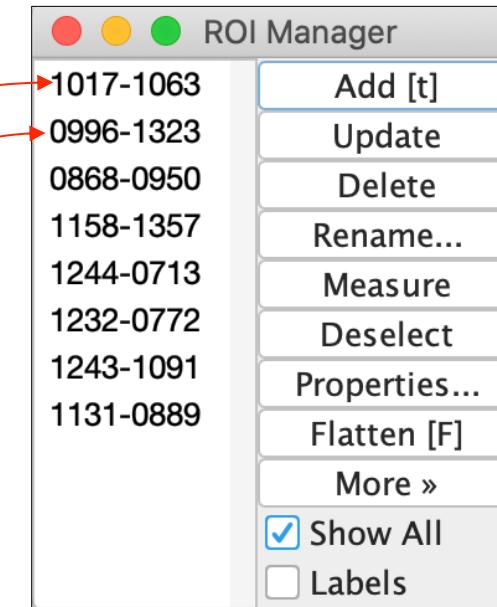
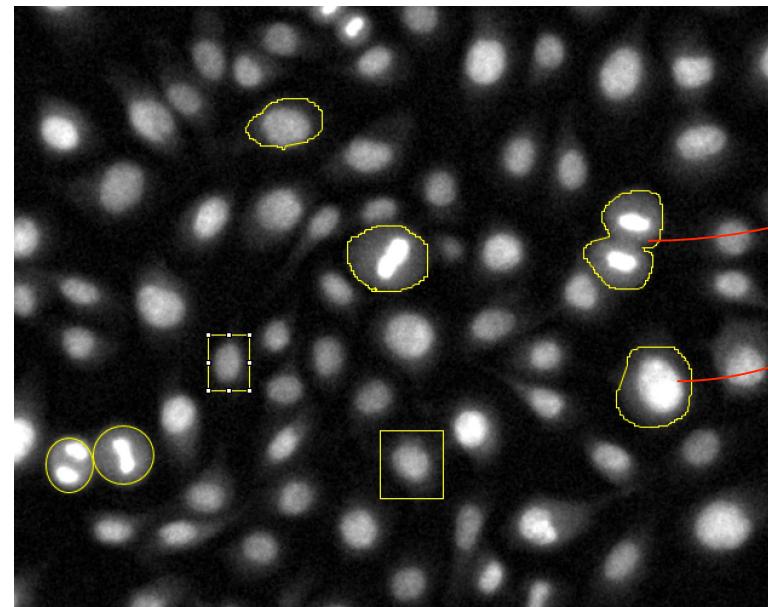
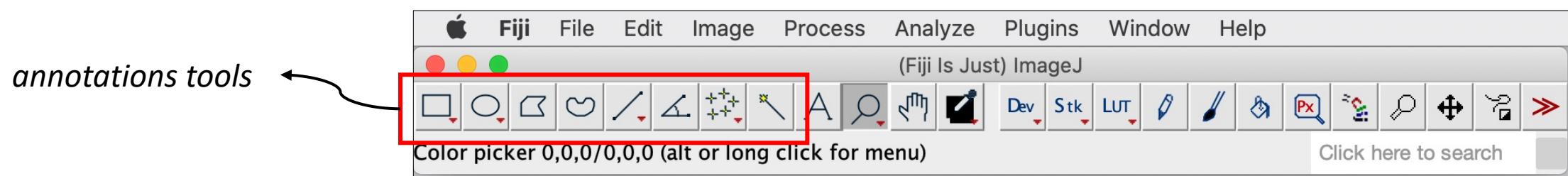
# Summarizing the workflow...



# Region of Interest (ROI)



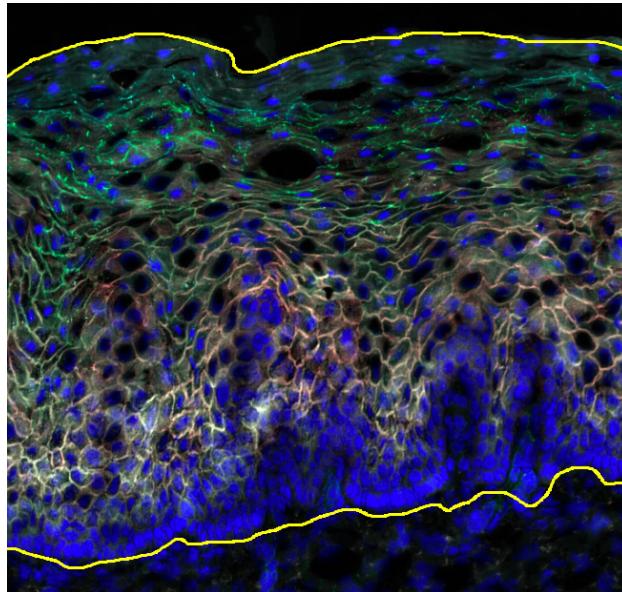
- Can be annotated manually



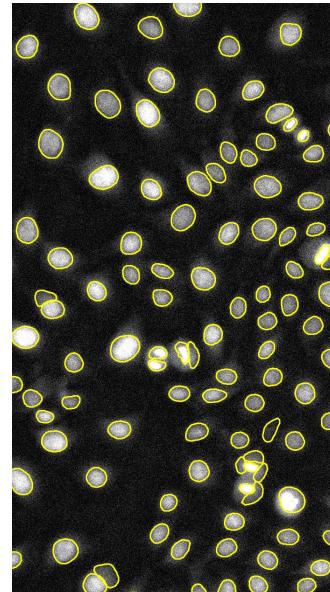
# Region of Interest (ROI)



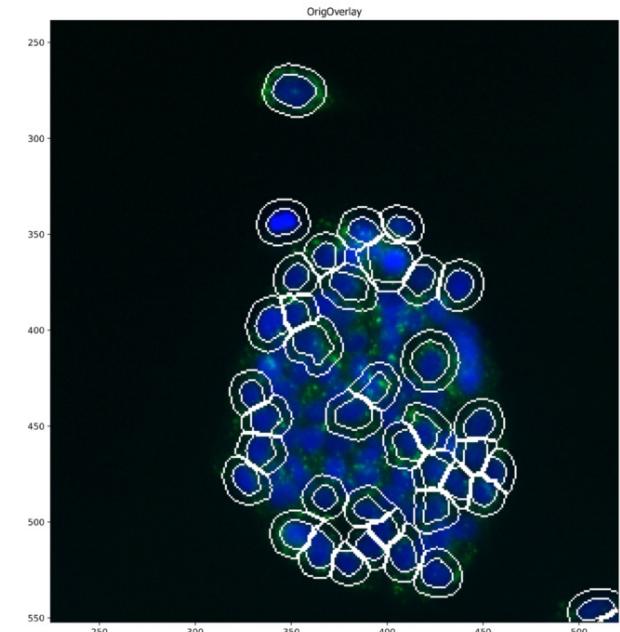
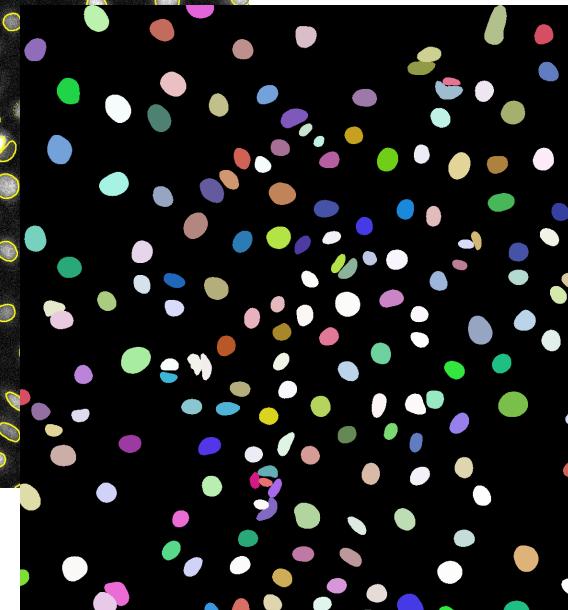
- Can be obtained via segmentation
  - ROIs may correspond to cells, nuclei, filamentous structures, parts of a tissue, etc



ROI: epithelial tissue

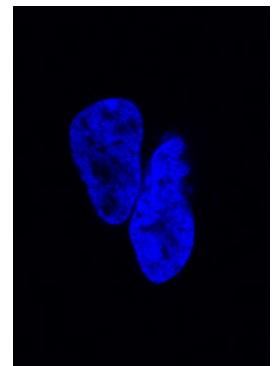


Nuclei

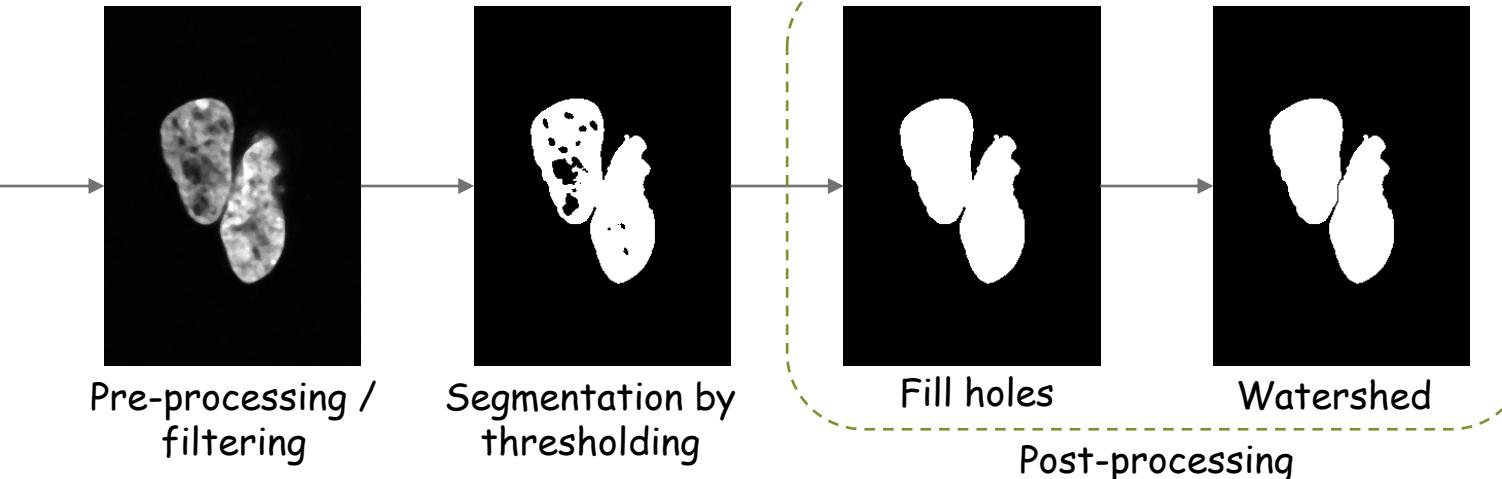


Cell membrane

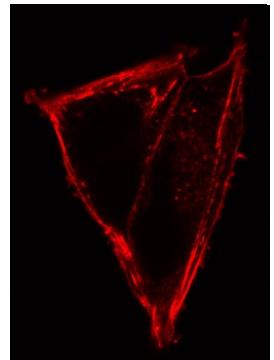
Get outlines of nuclei  
from DAPI image



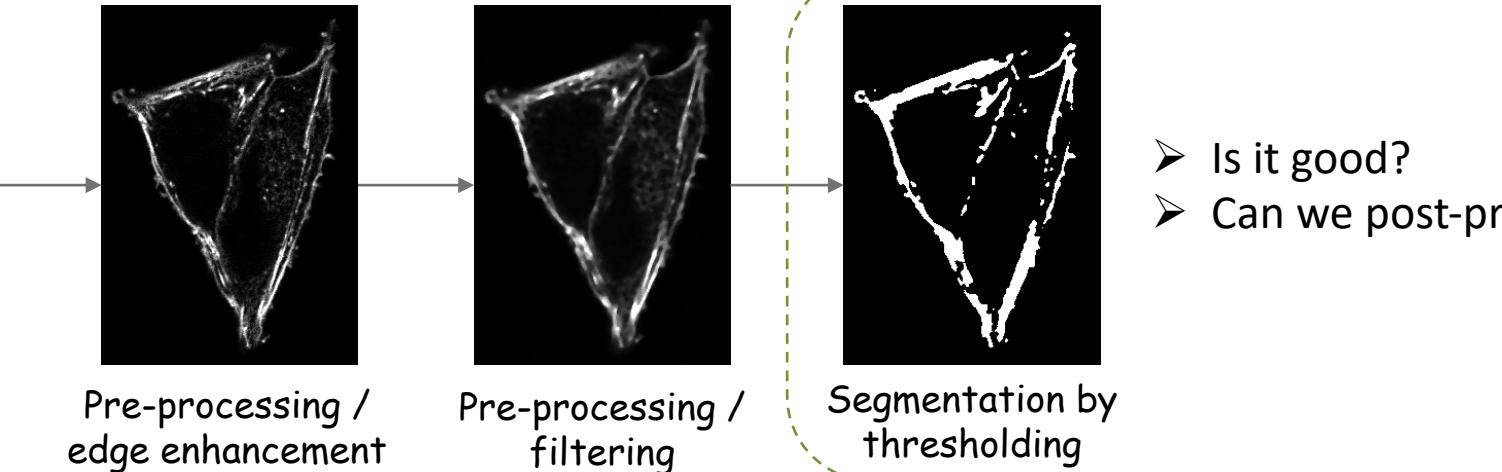
Nuclei



Get outlines of cytoplasm  
from actin image

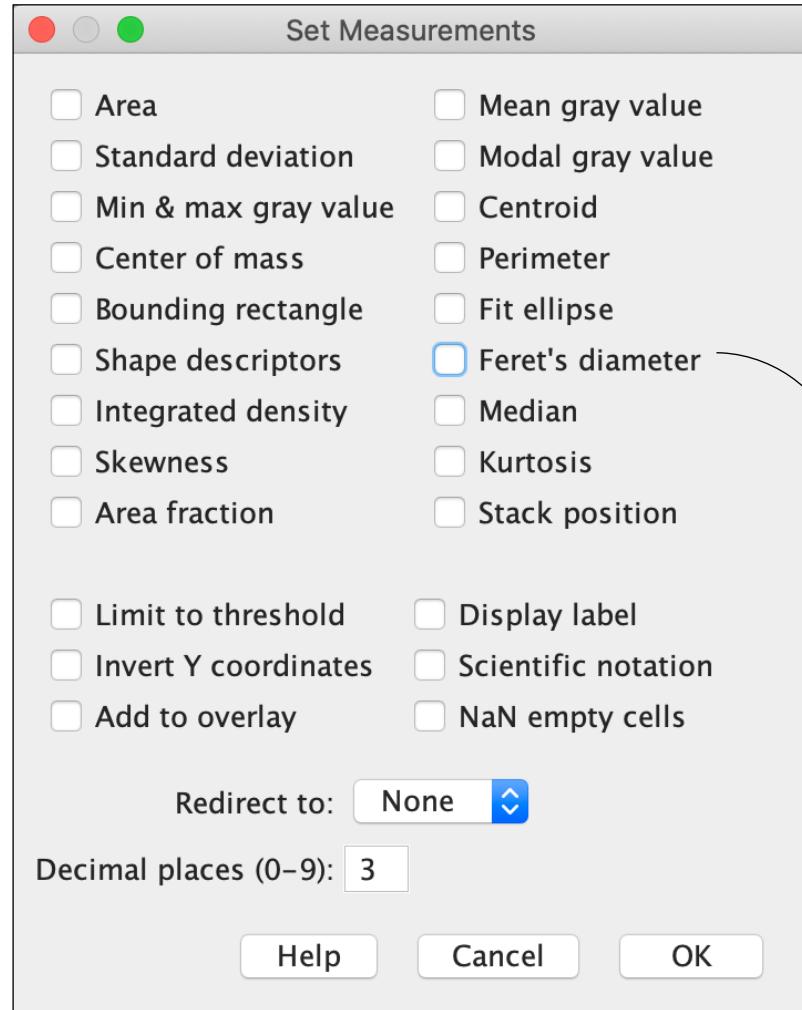


Actin



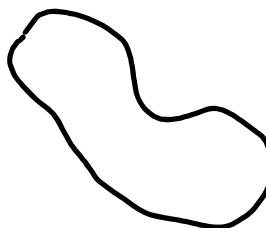
- Is it good?
- Can we post-process it?

# Image quantification: 2D measurements

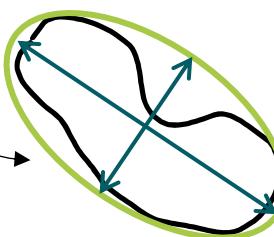


## ✓ Shape-related measurements

$$Circ = \frac{4\pi * area}{perimeter^2}$$



$$Round = \frac{4 * area}{\pi * major\ axis^2}$$



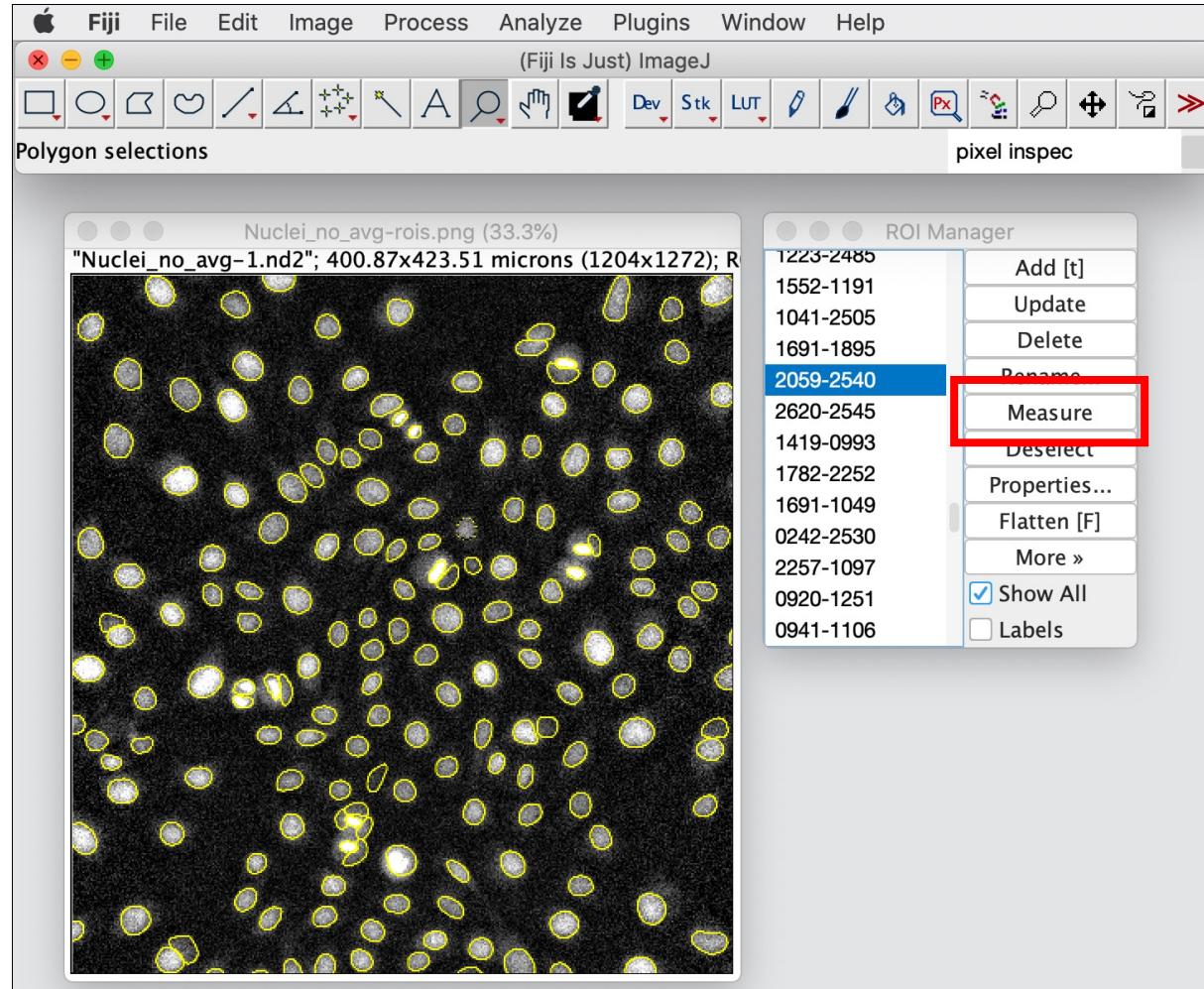
$$AR \text{ (aspect ratio)} = \frac{major\ axis}{minor\ axis}$$

## ✓ Intensity-related measurements

$$Int.\ Density = Area * Mean\ pixel\ intensity$$

$$Raw\ Int.\ Density = Sum\ of\ pixel\ intensity\ in\ a\ ROI$$

# The Analyze Particle tool in Fiji



- Analyze / Tools / ROI Manager
- Use “Add”, “Update” or “Delete” to manage the ROIs
- For binary masks, use “Analyze / Analyze Particles...” to automatically add ROIs to the ROI Manager

# The Analyze Particle tool in Fiji



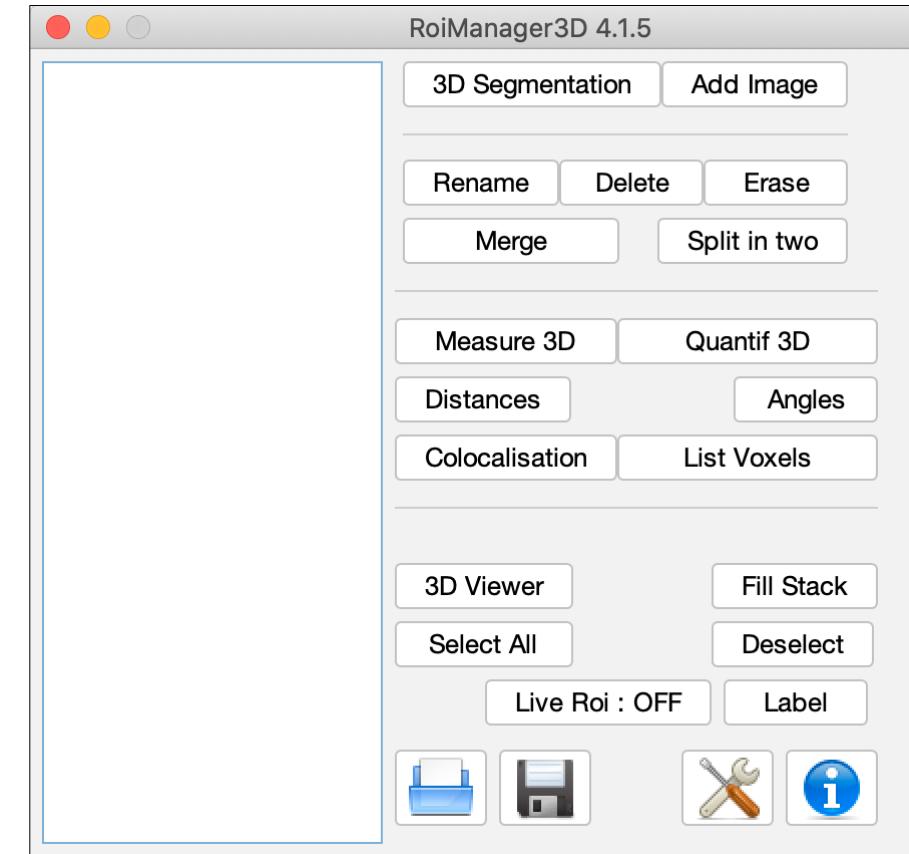
	Results								
	Area	Mean	StdDev	Min	Max	X	Y	Perim.	
1	105.4206	34454.7750	8865.1751	10194	56283	48.2751	4.0970	120.6690	
2	86.3540	37523.8344	8260.7674	11565	57740	120.4246	3.2057	117.4975	
3	79.1486	32648.8389	8772.7896	5911	55941	499.5517	3.3905	105.8406	
4	124.4872	33918.3713	10150.2458	7024	61509	581.7036	4.9095	128.6690	
5	148.9855	29496.6228	7826.9600	3769	48916	753.9481	6.6703	136.4680	
6	132.9120	25714.4003	8461.8830	2056	47974	683.9993	12.7198	132.1249	
7	211.5063	47225.0708	14355.8328	8909	65535	408.1226	18.5821	199.7229	
8	130.4732	22842.6364	7356.6866	2398	44718	805.6511	20.5952	134.6102	
9	319.0330	44253.3846	9831.1143	9337	65535	296.7596	24.7274	200.8944	
10	287.9944	41869.6547	9801.7653	10194	65535	541.1541	25.8225	192.0660	
11	264.4937	43476.5386	8661.3806	11907	65535	647.9540	25.4295	182.7523	
12	119.2771	25850.5251	8126.0661	0	48059	779.1815	24.9737	121.6396	
13	233.1225	44914.7194	11617.2974	13963	65535	213.0602	27.9568	170.8528	
14	239.1085	37753.0547	9896.0957	9252	65535	459.5779	28.0787	173.3381	
15	137.0135	24377.0995	8529.0612	0	50287	883.7876	28.2941	132.7107	
16	191.4420	14198.6925	6173.9970	0	35380	394.7219	33.0017	169.3381	
17	80.8114	12720.4403	5599.3513	0	32982	191.5002	32.8887	100.3259	
18	96.7741	27667.9966	7653.4332	6853	46774	849.6799	34.9491	110.8112	
19	153.1979	34128.8806	10517.8217	3940	62623	251.5548	38.1289	138.1249	
..	...	...	...	...	...	...	...	...	

- Results Table: calculated measures for each ROI
  - Each line corresponds to a single ROI and each column to a specific measure
  - Table can be saved as a .csv file

# Image quantification: 3D measurements



- 3D ImageJ Suite
  - Measurements extended to 3D images:
    - Volume (number of voxels)
    - Centroid (x,y,z)
    - Compactness: ratio between volume and surface (in voxel or units )
    - Sphericity
    - etc





# Let's practice in Fiji

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- Image files to be used in this session are located in  
..../data\_day1/part3/
- **Manually adding ROIs to ROI Manager**
  - Open file “Nuclei\_no\_avg.nd2”
  - Open the ROI Manager (*Analyze / Tools / ROI Manager*)
  - Use the annotation tools to segment the nuclei
    - For annotated object, add it to the ROI Manager
    - At the end, export (save) the annotations as a *.zip* file via ROI Manager
  - Go to *Analyze / Set Measurements...* to select different measurements
  - Press the “Measure” button via ROI Manager interface
  - Export results to csv

# Let's practice in Fiji

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- Image files to be used in this session are located in  
..../data\_day1/part3/
- **Reading ROIs from file**
  - Open files “Nuclei\_no\_avg.nd2” and “*Nuclei\_no\_avg\_RoiSet.zip*” (drag and drop in the Fiji main interface)
  - The ROI Manager (*Analyze / Tools / ROI Manager*) will be loaded automatically
  - Generate the measurements again and save the Results table containing the measurements of all ROIs

# Let's practice in Fiji

---



- Image files to be used in this session are located in  
..../data\_day1/part3/
- Generating ROIs from a binary mask
  - Open files “Nuclei\_no\_avg\_binary.tif”
  - Run “Analyze / Analyze Particles...”
    - Inspect the options available through the interface
    - Under “Show”, select “Masks”
    - Check the following options: “Add to Manager”, “Display results”, “Clear results”, “Exclude on edges” and press the “OK” button
    - Inspect the results, what happened to the intensity measures?
      - Remember to redirect the measures to the original image!

# Let's practice in Fiji

---



- Image files to be used in this session are located in  
..../data\_day1/part3/

## □ Dot Counting

- Open “Cell\_Colony” from Fiji samples
- Run “Edit / Invert”
- Subtract Background
- Run “Find Maxima”

*Ensuring image quality for image analysis*



# Image artifacts

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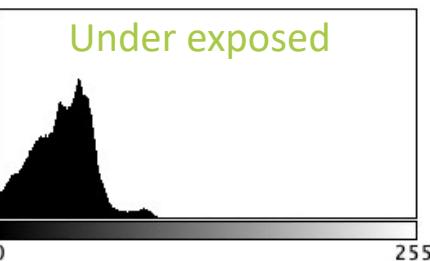
- Some image artifacts cannot be corrected by image analysis
  - Ex: saturation, bleedthrough, etc

*Let's have a look at some examples...*

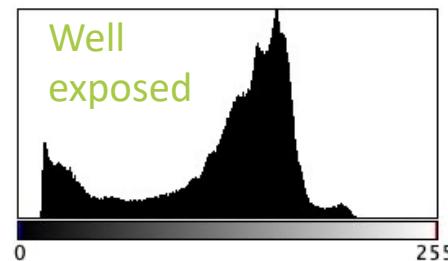


# Saturation

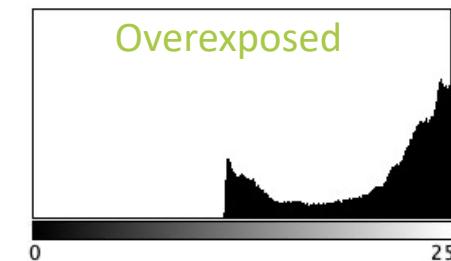
- Histograms help to identify saturation



Count: 397987  
Mean: 29.183  
Max: 112  
StdDev: 23.387



Count: 397987  
Mean: 122.848  
Max: 220  
StdDev: 46.984



Count: 397987  
Mean: 222.028  
Max: 255  
StdDev: 43.466

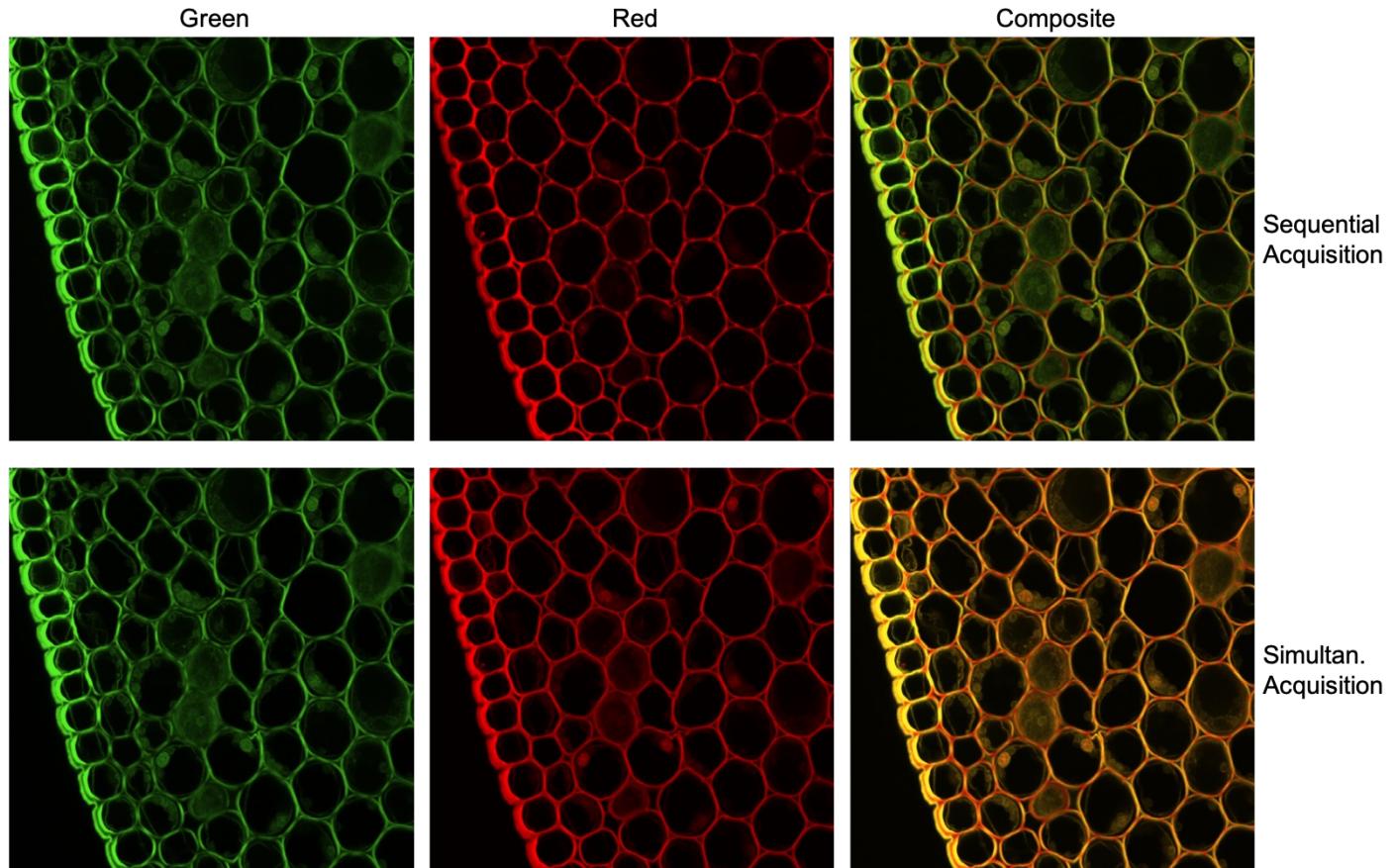
Low contrast at the edges

Objects appear larger



# Bleedthrough

- ✓ Compromise intensity and shape measures
- ✓ Compromises co-localization analysis





# Bit conversion

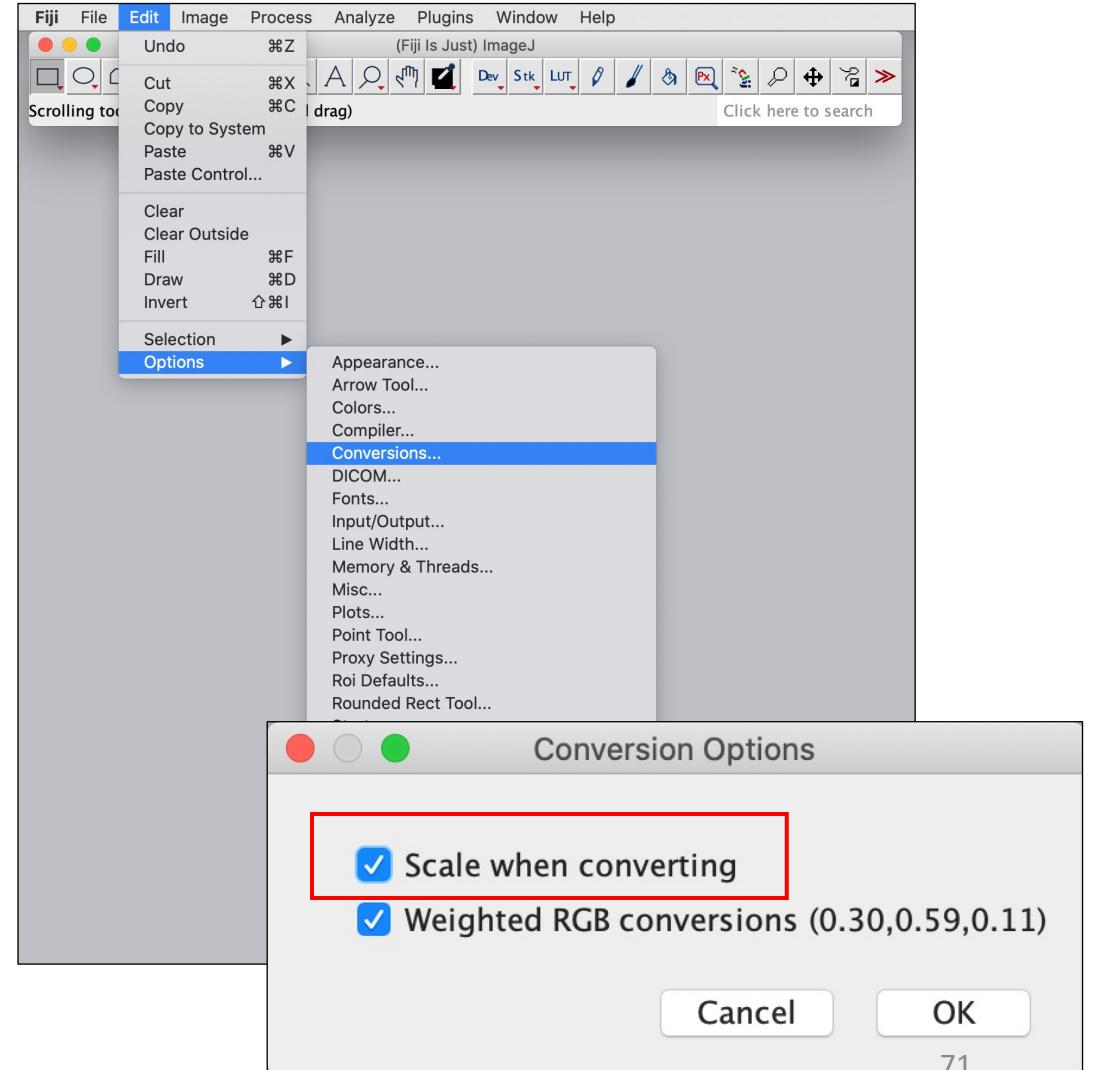
- ***Increasing*** the bit-depth does not change the pixel values

Ex: 8bit → 16 bit

- When *decreasing* bit-depths, scale can (or not) be applied to the pixel values

Ex: 8bit ← 16 bit

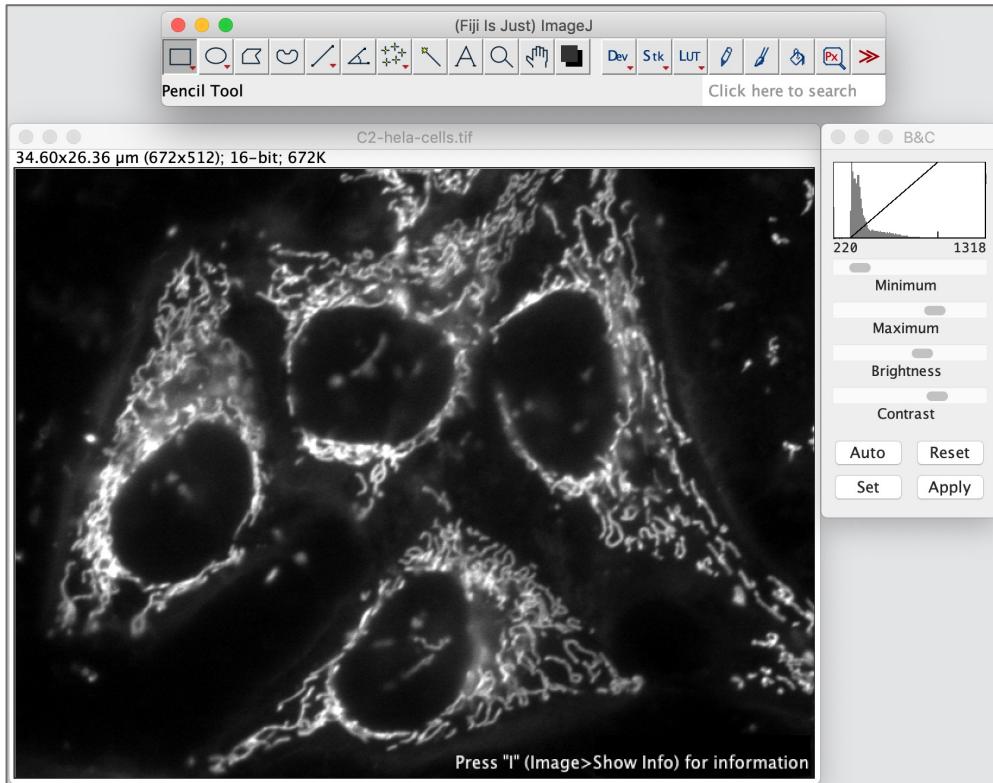
*Good practice: Conversions should be applied with caution! Ex.: specific plugin in Fiji that was developed for specific types of images*





# Image operations

- Some operations can be irreversible:
  - Contrast enhancement, if saved



*Good practice: always create  
a copy of the original image  
when working in Fiji*

# Image artifacts

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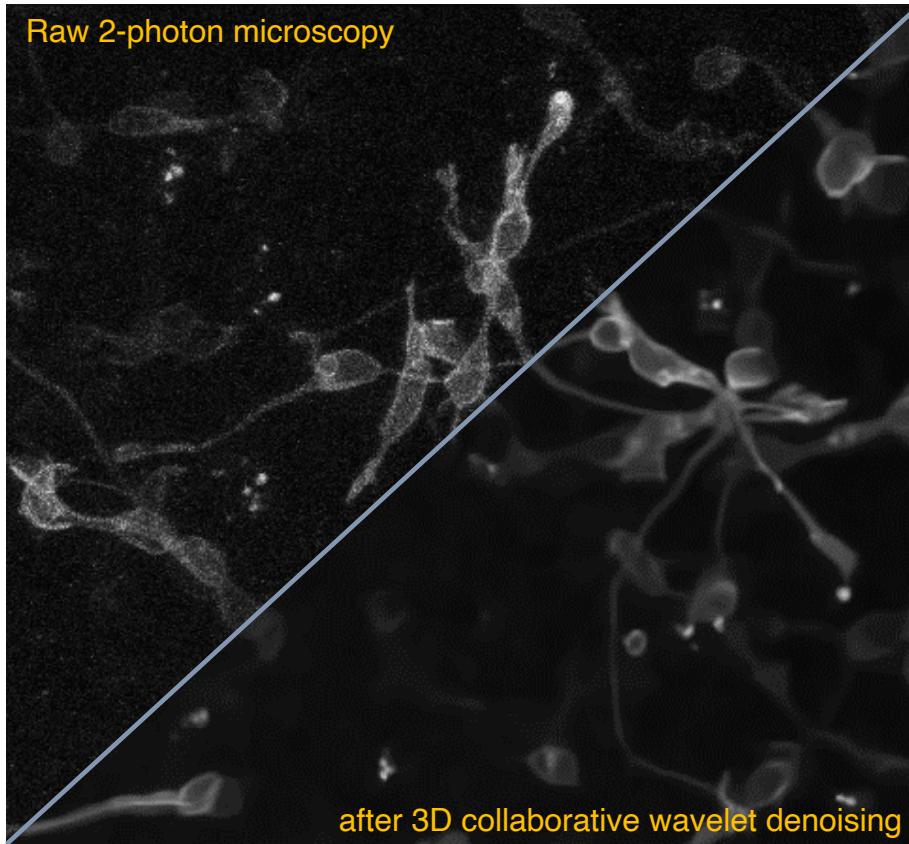
- Some image artifacts cannot be corrected by image analysis
  - Ex: saturation, bleedthrough, etc
- Others can be corrected, but sometimes it's better to correct them while acquiring the image

*Examples...*

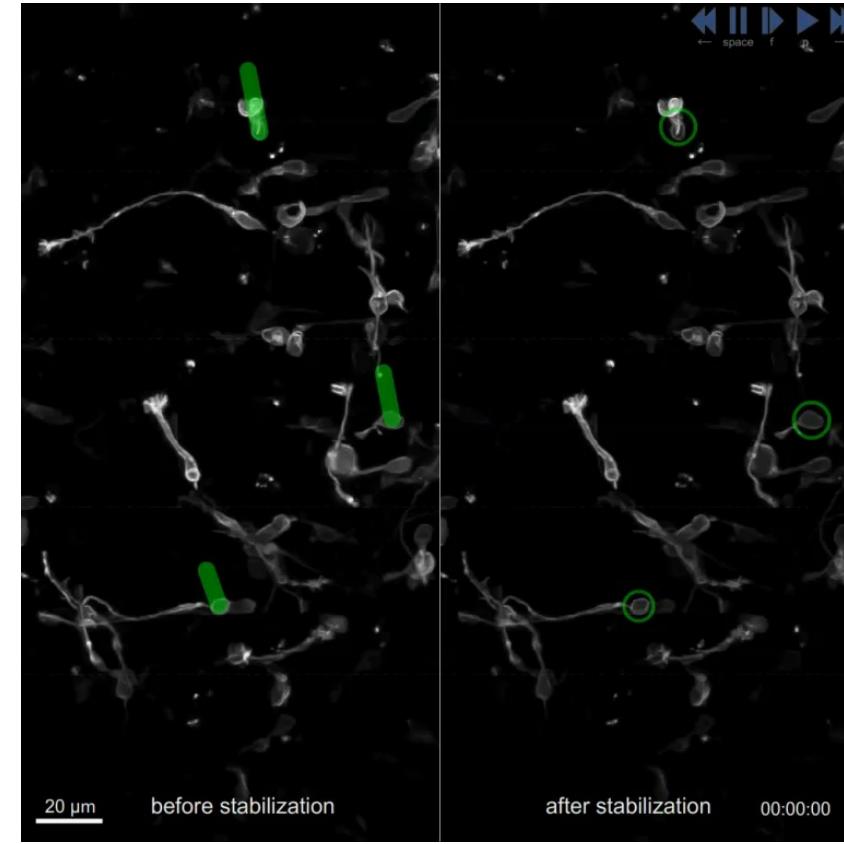
# Image processing and enhancement



Denoising



Stabilization

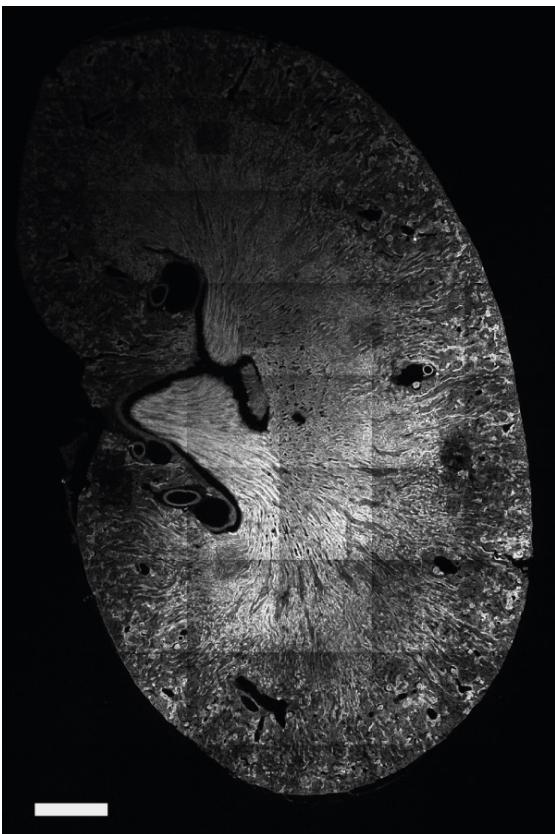


K. Smith *et al.* General Constraints for Batch Multiple-Target Tracking Applied to Large-Scale Videomicroscopy, CVPR (2008)

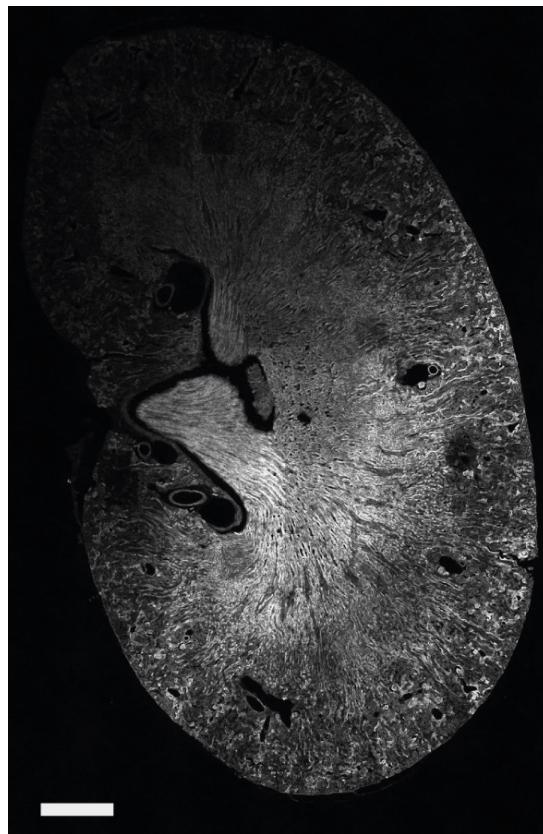


# Illumination correction

Original test image mosaic

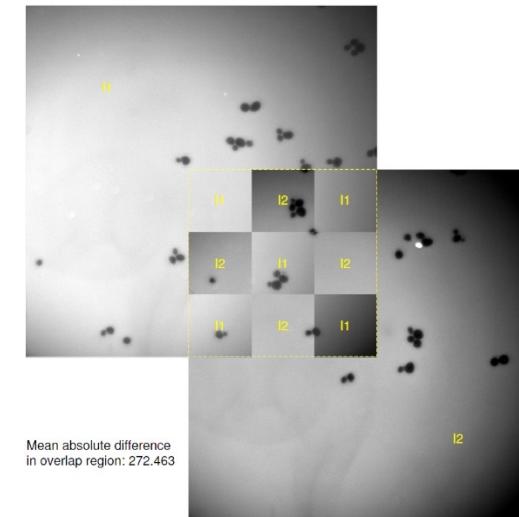


Corrected image

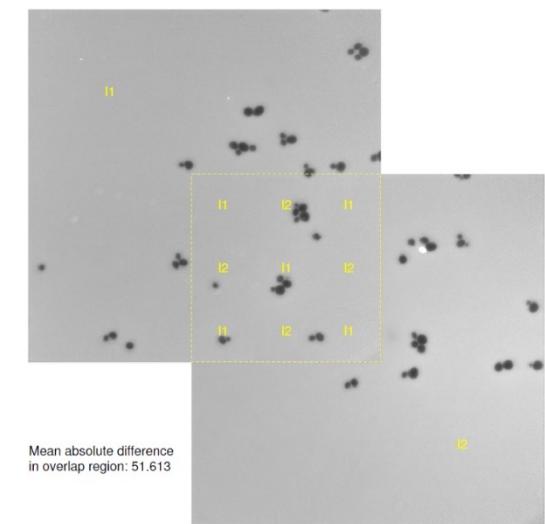


Fluorescent marked mouse kidney section. Confocal microscope  
with glass slide air interface.

Original test image pair



Corrected pair



# Practical Exercises

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- Image files to be used in this session are located in  
..../data\_day1/part4/
  - Open a sample image
  - Extract the measures by using the “*ImageArtifacts.ijm*” script (drag and drop the file on the Fiji main interface)
  - Press the “Run” button
  - Save the Results from the “Log” window
  
- ❖ Reference image “sequential.nd2” (with no artifacts)
- ❖ Alternatively, you can manually annotate ROIs and extract area and intensity measurements using the Analyze Particles tool, as we did before

*Concluding Remarks*



# Useful links

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- Active discussion forum: <http://forum.image.sc/>
- Bioimage Data Analysis: <https://analyticalscience.wiley.com/do/10.1002/was.00050003>
- Bioimage Workflows: <https://link.springer.com/book/10.1007/978-3-030-22386-1>
- Analyzing fluorescence microscopy images <https://petebankhead.gitbooks.io/imagej-intro/content/>
- NEUBIAS Academy: <https://www.youtube.com/channel/UC-oy7UpEhRfHQ-5ePCviKFg>
- Some images used in this presentation can be found at CZI Biohub (OpenCell target gallery): <https://opencell.czbiohub.org/gallery>