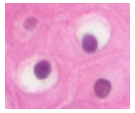
Image Analysis in Life Sciences 2023

Gisele Miranda, Ph.D.



Bioimage Analyst, BioImage Informatics Facility, SciLifeLab
Researcher at KTH Royal Institute of Technology

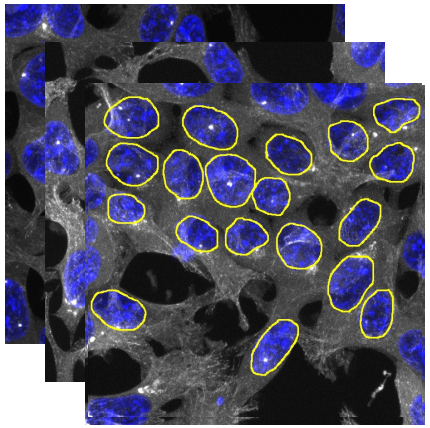
Outline Day 1

Part 1 9:15 – 10:30	Introduction to bioimage analysis & Basics of digital images
	Coffee Break
Part 2 10:45 – 12:00	Image processing and enhancement
	Lunch Break
Part 3 13:00 – 15:00	Workflows & Quantitative Analysis
	Coffee Break
Part 4 15:15 – 17:00	Ensuring image quality

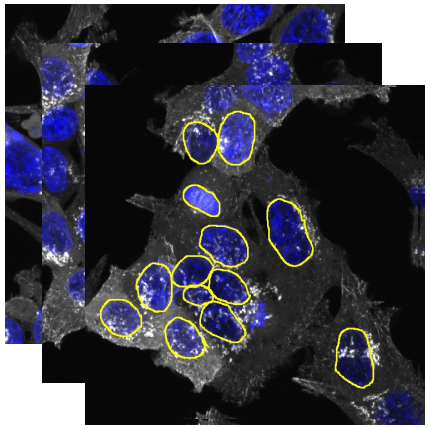
Basic concepts: BioImage Analysis 

What is bioimage analysis?

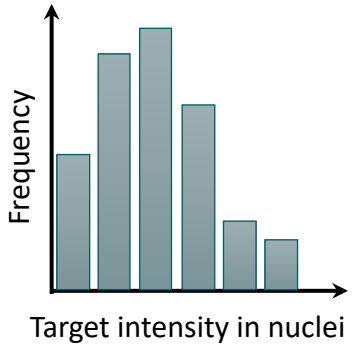
➢Understanding and quantifying microscopy & medical image data

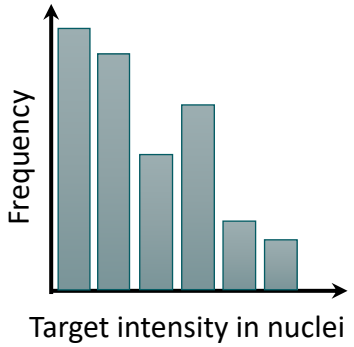


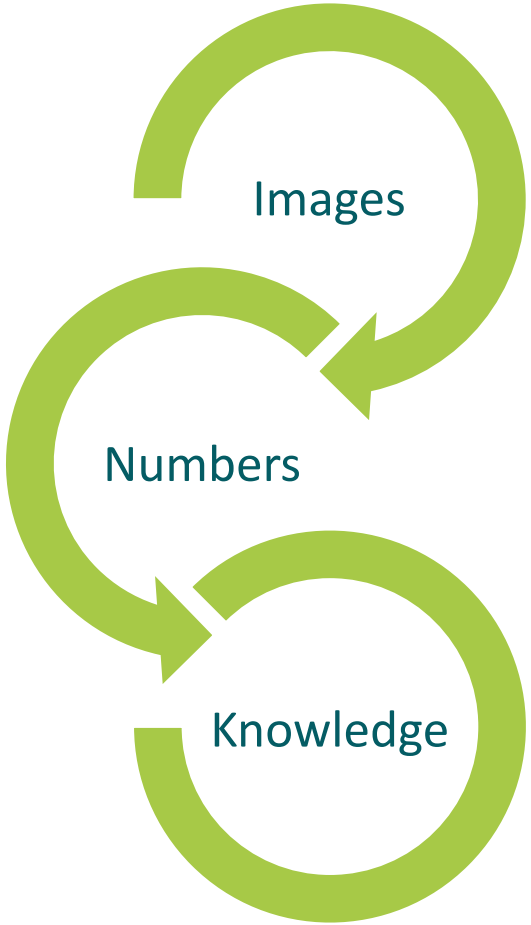
CLASP1



CLASP2







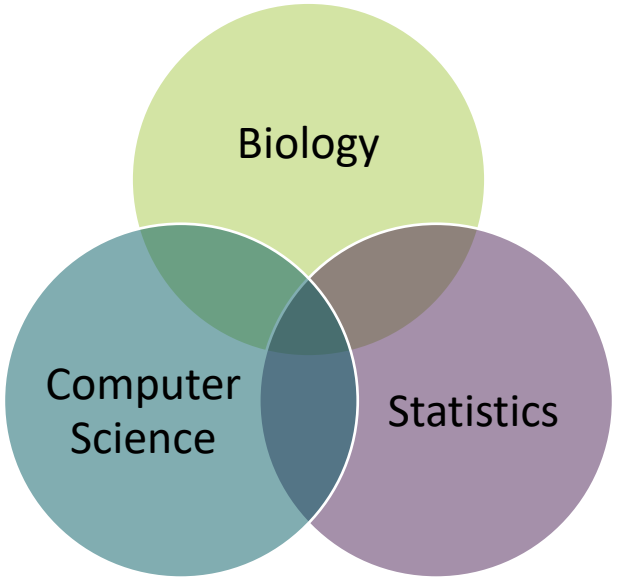
- ✓ Quantitative
- ✓ Objective
- ✓ Reliable
- ✓ Repeatable
- ✓ Reproducible

Image source: https://opencell.czbiohub.org/gallery

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…

Software Tools in the BioImage Analysis ecossystem

Open source software

CellProfiler
cell image analysis software

ImageJ
Image Processing and Analysis in Java

ilastik

Icy

TissUUmaps

Open for Innovation
KNIME

napari

QuPath

ImJoy

Custom solutions

MATLAB

itk

scikit learn

OpenCV

TensorFlow

GitHub

Imaris
BITPLANE

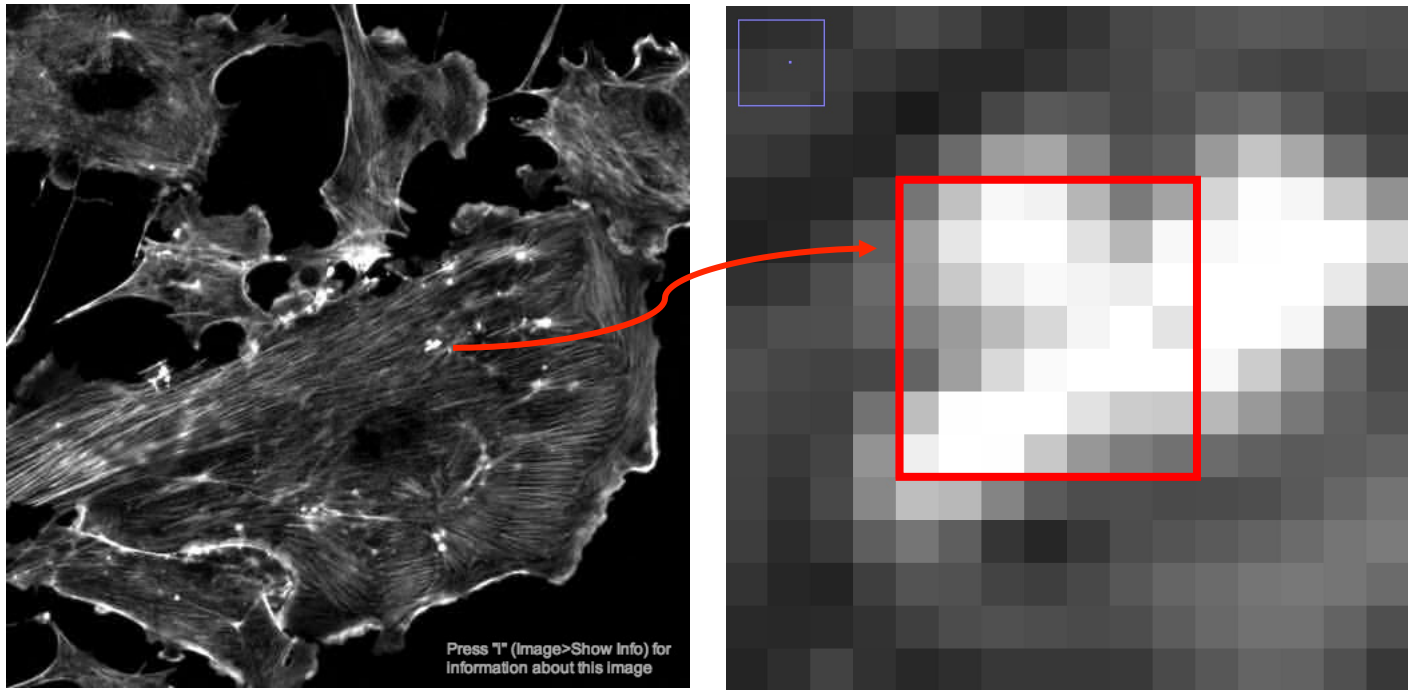
What is your scientific question? 

How does it involve image analysis? 

Basic concepts: Digital images

What is a digital image?

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



Fluorescent cells / Fiji samples

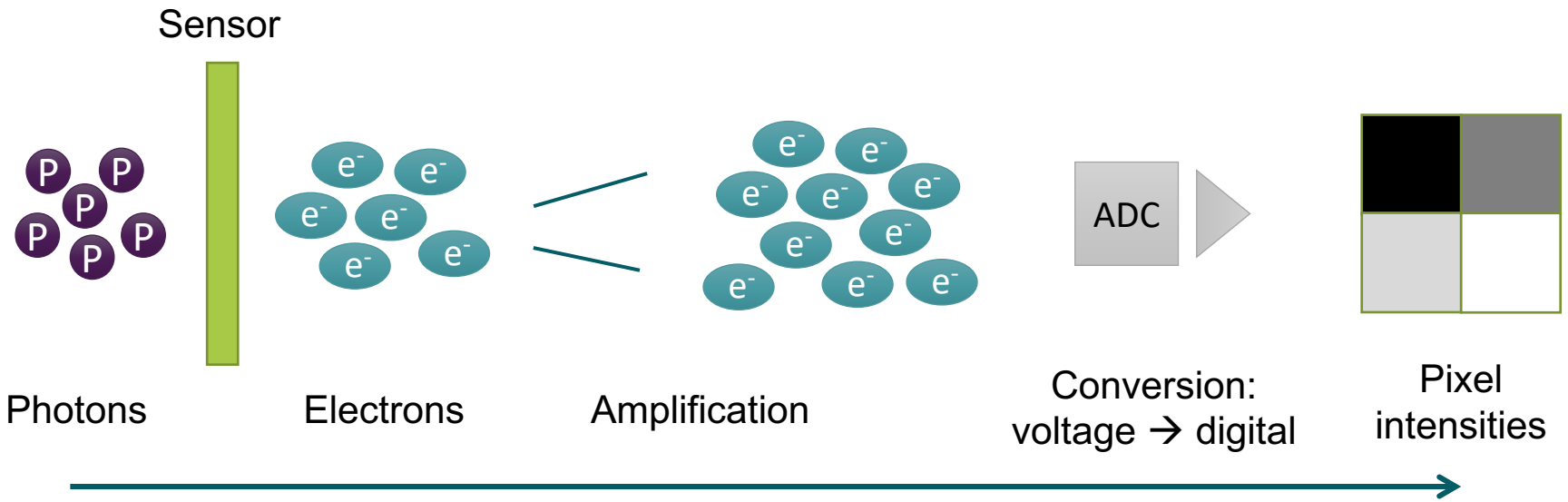
$$\begin{array}{c} \\ 1 \\ 2 \\ 3 \\ \vdots \\ m \end{array} \begin{array}{c} \begin{array}{cccc} 1 & 2 & \dots & n \end{array} \\ \begin{bmatrix} a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \vdots & \vdots \\ a_{m1} &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

Pixel Values

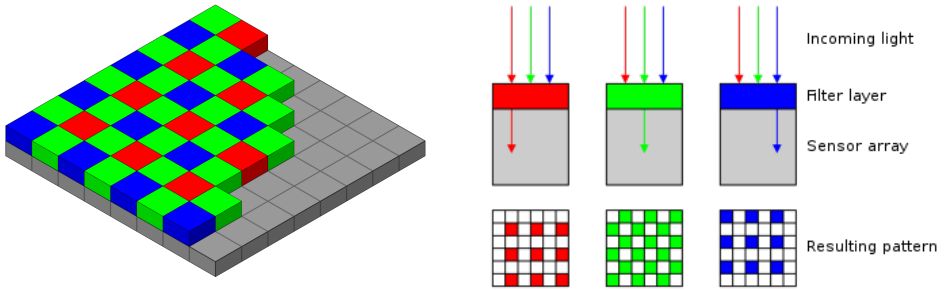
Prefs	314	315	316	317	318	319	320
252	117	193	248	242	182	122	164
253	158	230	255	255	225	183	248
254	152	201	236	249	243	236	255
255	126	155	186	216	245	255	228
256	99	159	217	250	255	255	255
257	190	254	255	255	226	204	201
258	239	255	254	200	151	125	113

How is the pixel value defined?

Acquisition of digital images



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

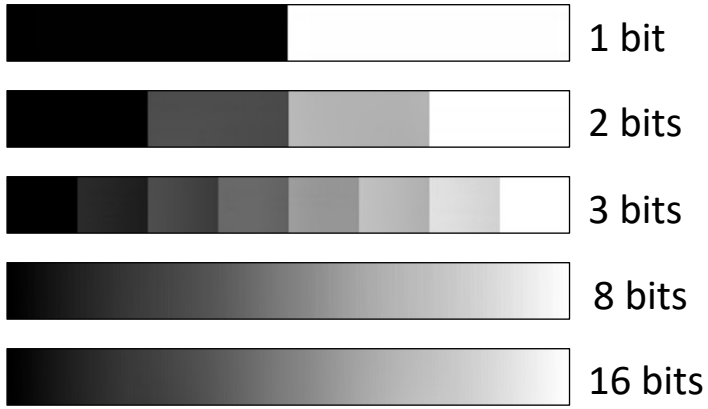


- ✓ Color-cameras (Bayer filter): blue, green (2x) and red filters

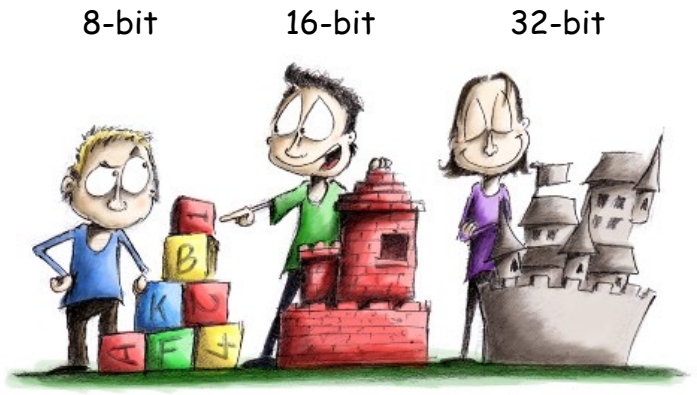
https://en.wikipedia.org/wiki/Bayer_filter

Bit-depth & gray levels

- Bith-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

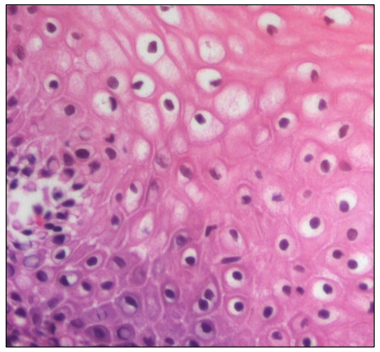


https://petebankhead.gitbooks.io/imagejintro/content/chapters/bit_depths/bit_depths.html

- ✓ 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

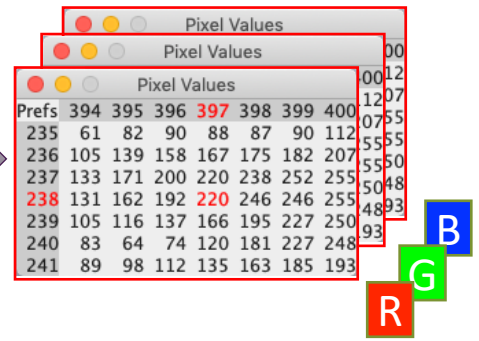
Color Images

- RGB (Ex: stained H&E)

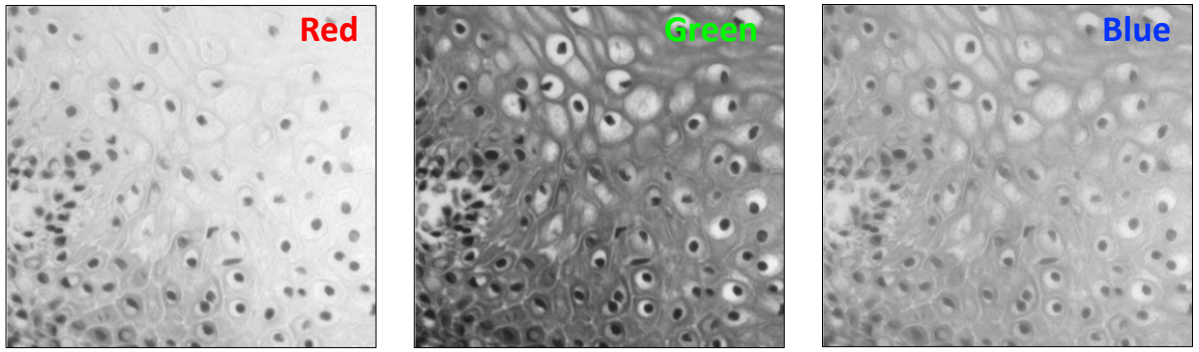


$$\begin{array}{c} \\ 1 \\ 2 \\ 3 \\ \vdots \\ m \end{array} \begin{array}{c} \begin{array}{cccc} 1 & 2 & \dots & n \end{array} \\ \begin{bmatrix} a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ a_{31} & a_{32} & \dots & a_{3n} \\ \vdots & \vdots & \vdots & \vdots \\ a_{m1} &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

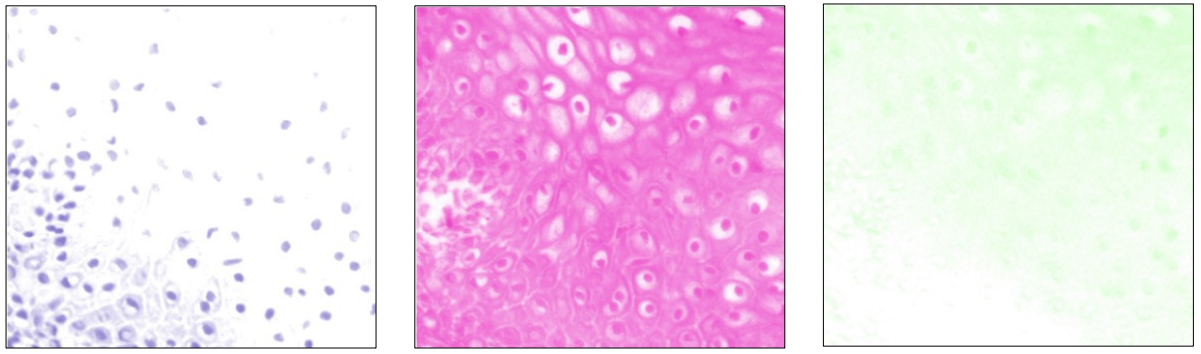
$(a_{mnR}, a_{mnG}, a_{mnB})$

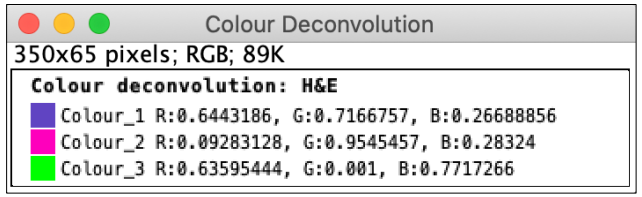


Split channels in RGB space



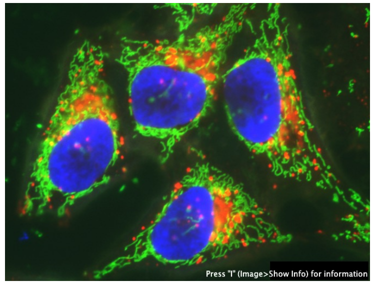
Color Deconvolution – spectral unmixing of the dyes





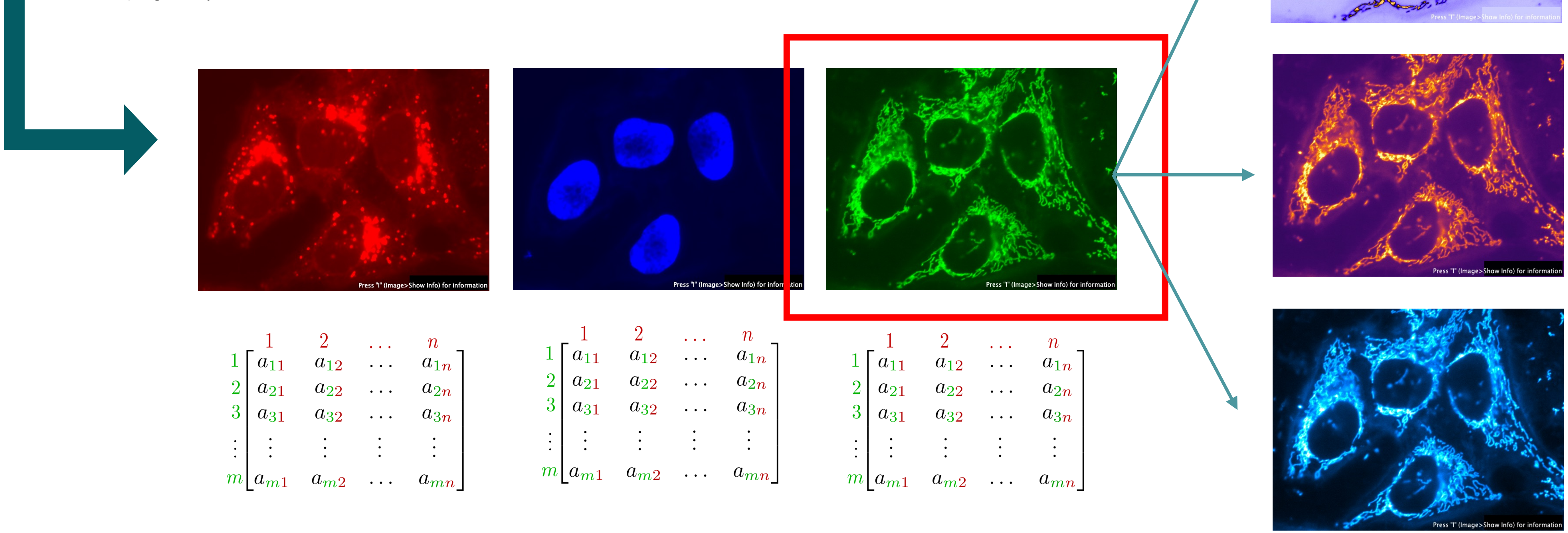
Have a look at: https://imagej.net/plugins/colour-deconvolution for other examples

Fluorescent Images



HeLa Cells / Fiji samples

- ✓ Gray-level images
- ✓ Independent channel representation

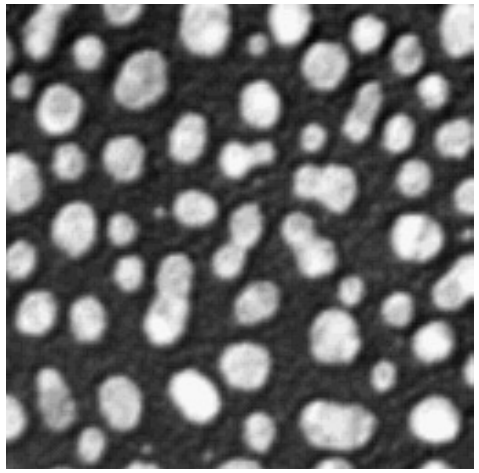


Look-up tables

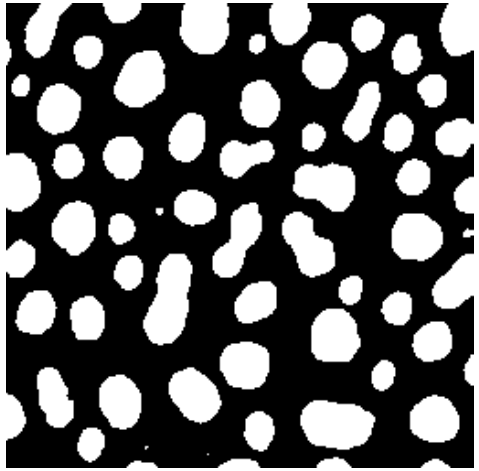
- ✓ Define how the image is displayed
- ✓ Original values are not modified!

Look-up table (LUT)

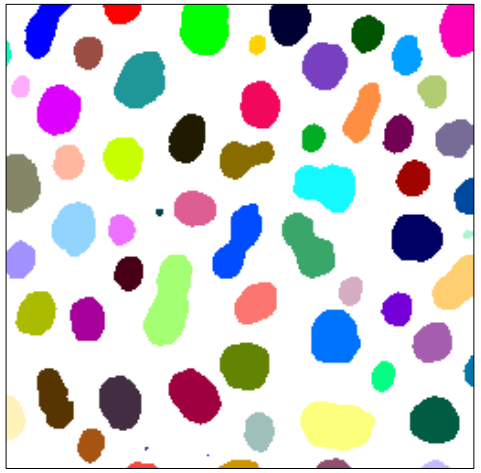
- Others examples
  - ✓ Labeling: to highlight instance-based segmentation (Glasbey LUT)
  - ✓ Detection of artifacts: saturation & under exposure (HiLo LUT)



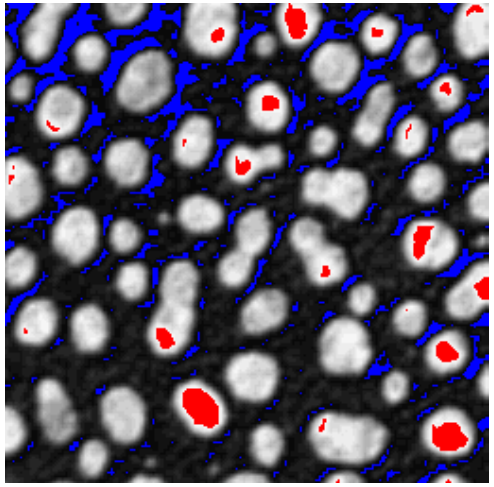
Fiji samples / Blobs



Blobs - thresholded

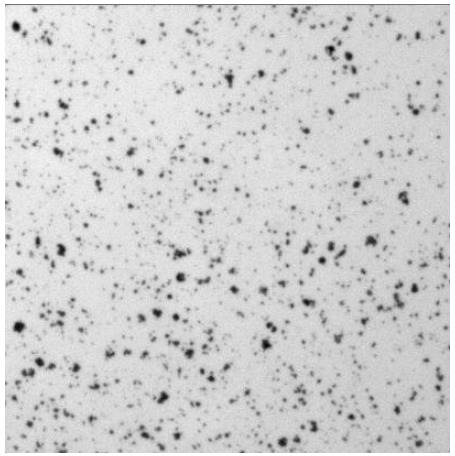


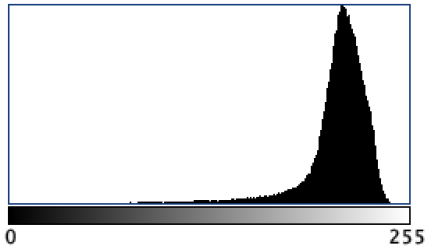
Glasbey LUT



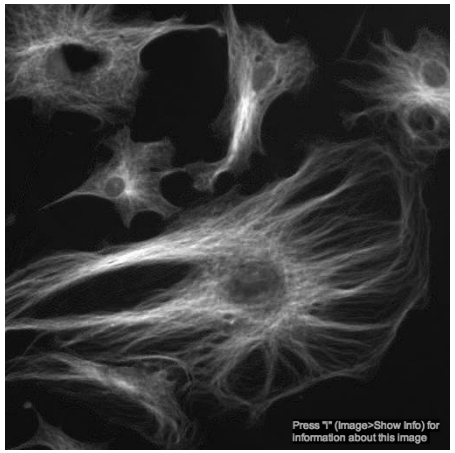
HiLo LUT

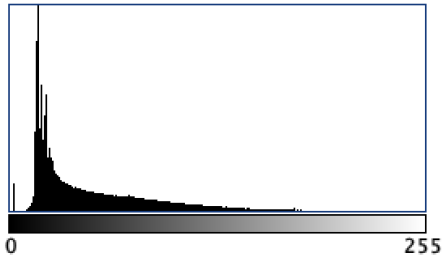
Histograms





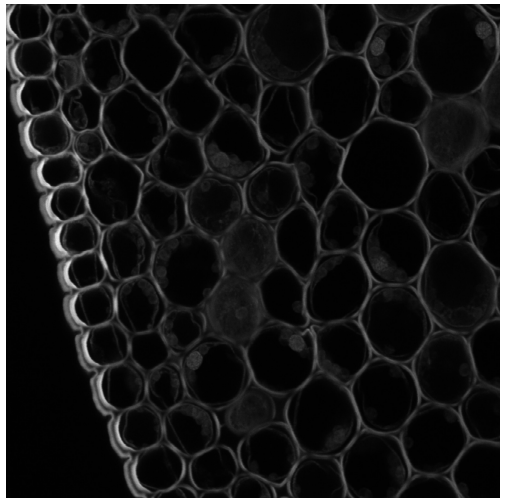
Fiji sample images: *Cell colony*

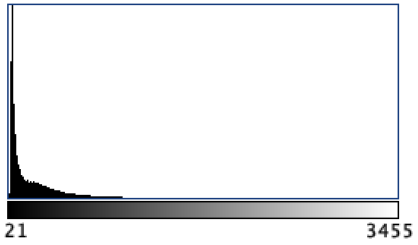




Fiji sample images: *Fluorescent cells*

- The pixel order does not affect the histogram





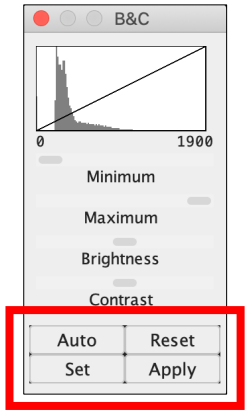
Source: LCI Microscopy course 2023

Basic measurements derived from histograms:

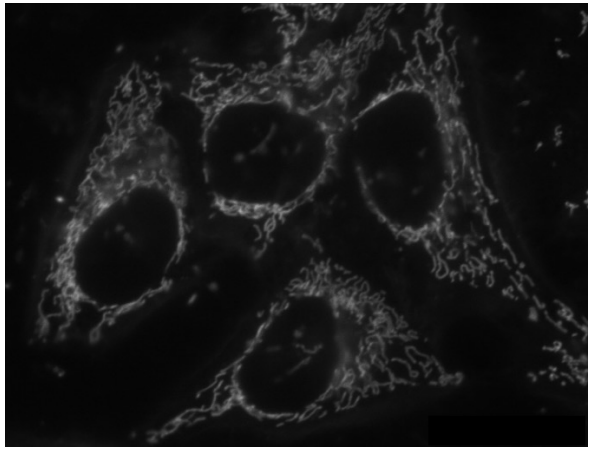
- Mean
- Variance
- Standard Deviation
- Min, Max, Mode

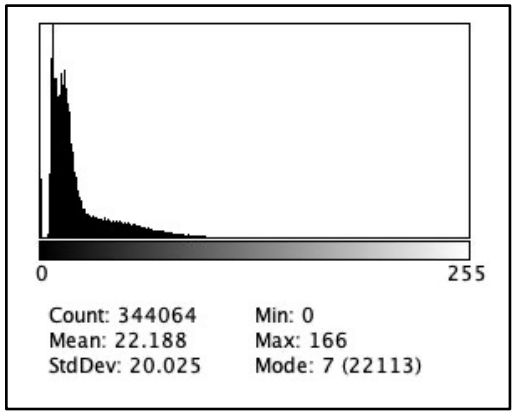
Brightness & Contrast

- Changing pixel values
  - ✓ Brightness: “amout of light”
  - ✓ Contrast: Maximum difference in intensity

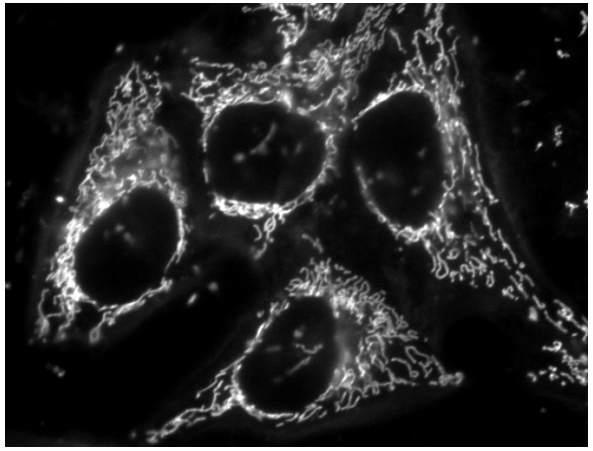


A)





B)



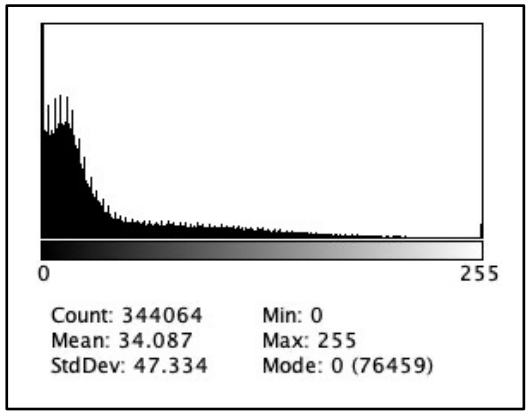
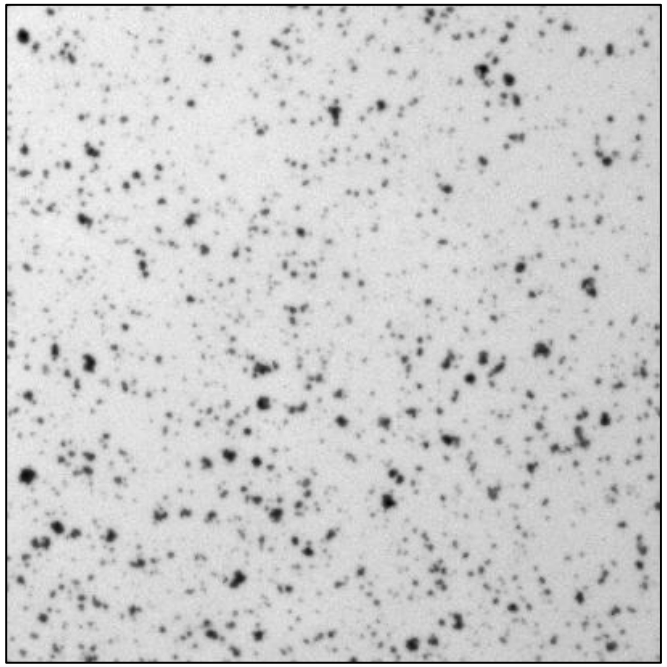


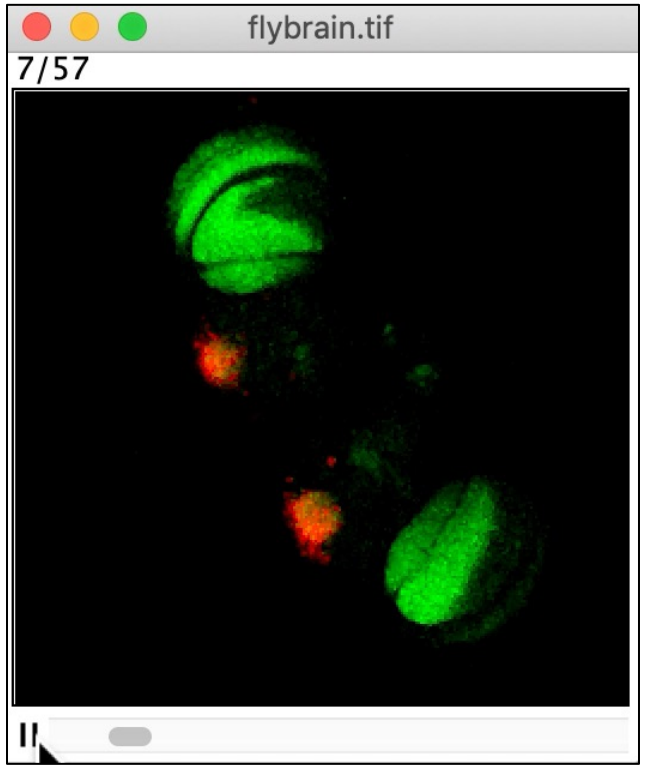
Image dimensions

2D image



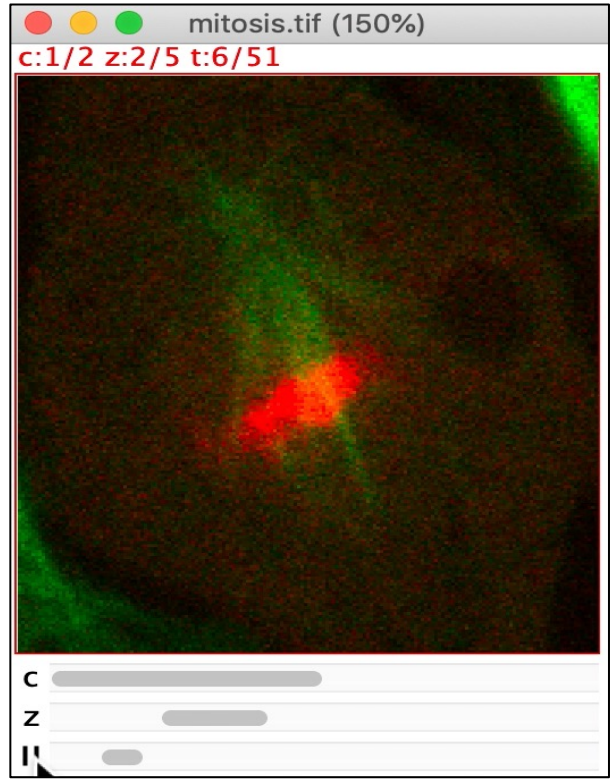
Cell colony / Fiji samples

3D image / Stack



Fly brain / Fiji samples

5D image / HyperStack

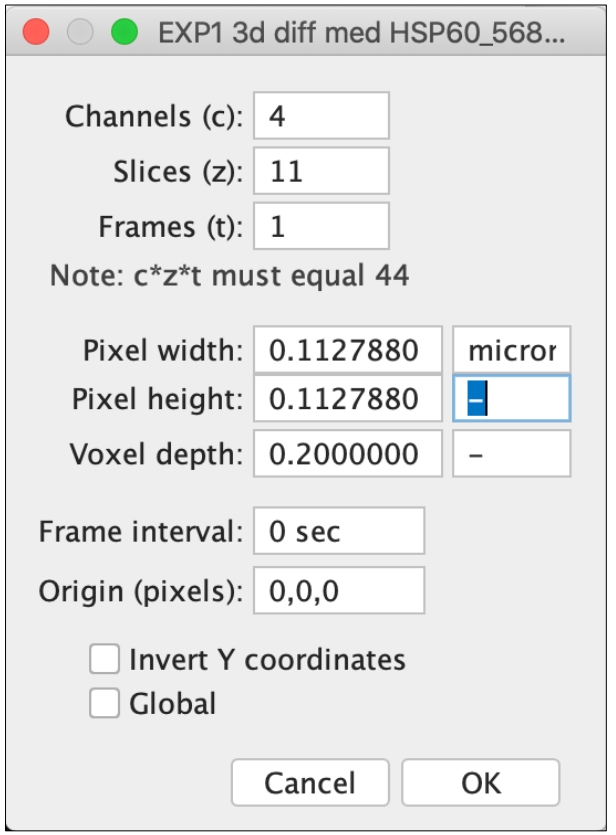


Mitosis / Fiji samples

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



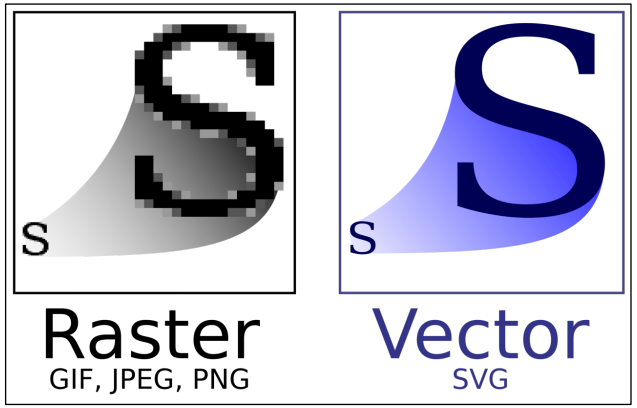
https://www.ibiology.org/techniques/metadata/

File formats

- Microscopy file formats
  - Proprietary formats: Ex.: ND2 (Nikon), LIF or SCN (Leica), OIB or OIF (Olympus), CZI or ZVI (Zeiss), etc
  - Store metada

- 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)

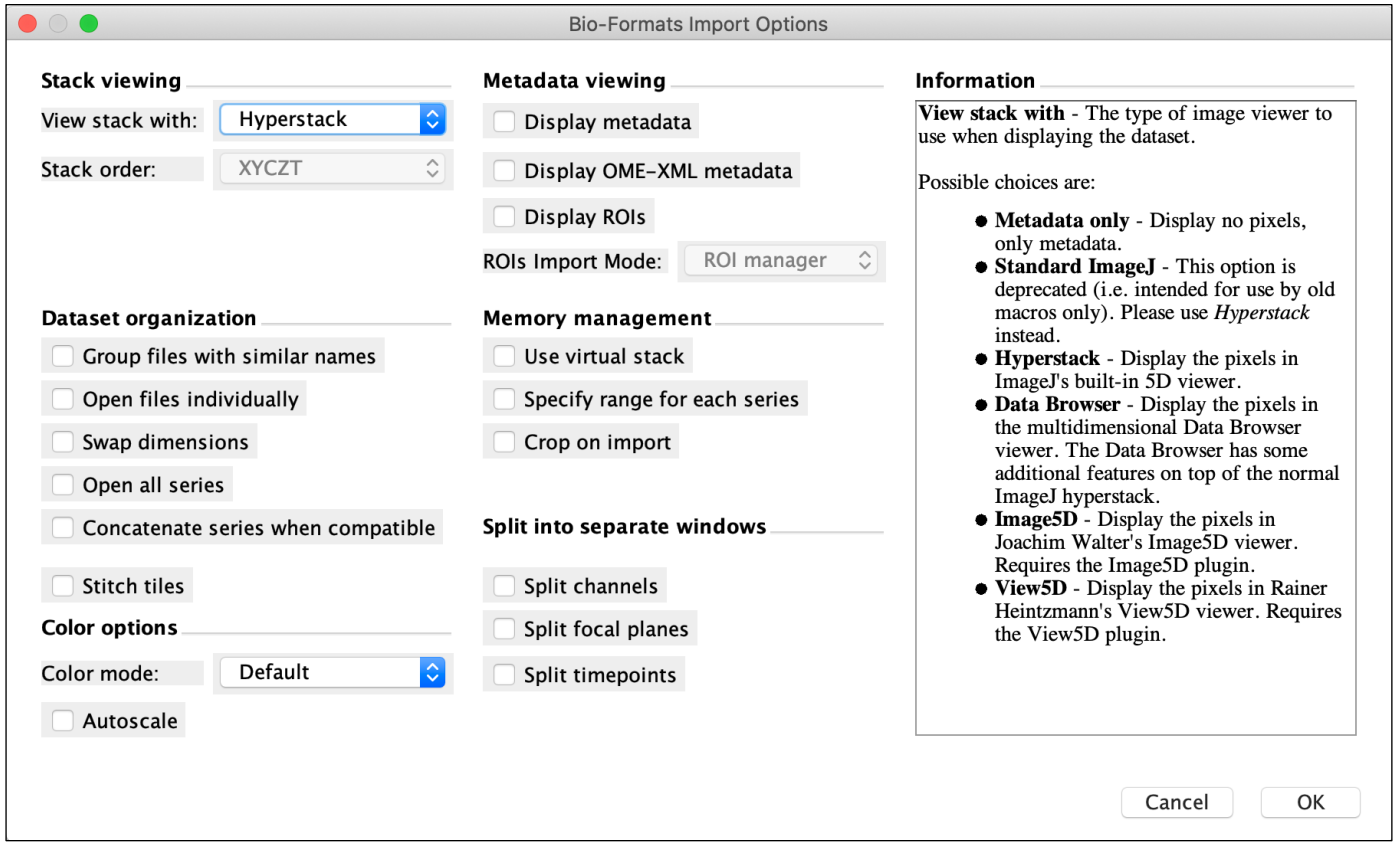
- File formats for figures (publications)
  - Bitmaps (BMP)
  - Vector images (SVG, PDF, EPS etc)



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 metada

* https://imagej.net/formats/bio-formats

Practical Exercises

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/

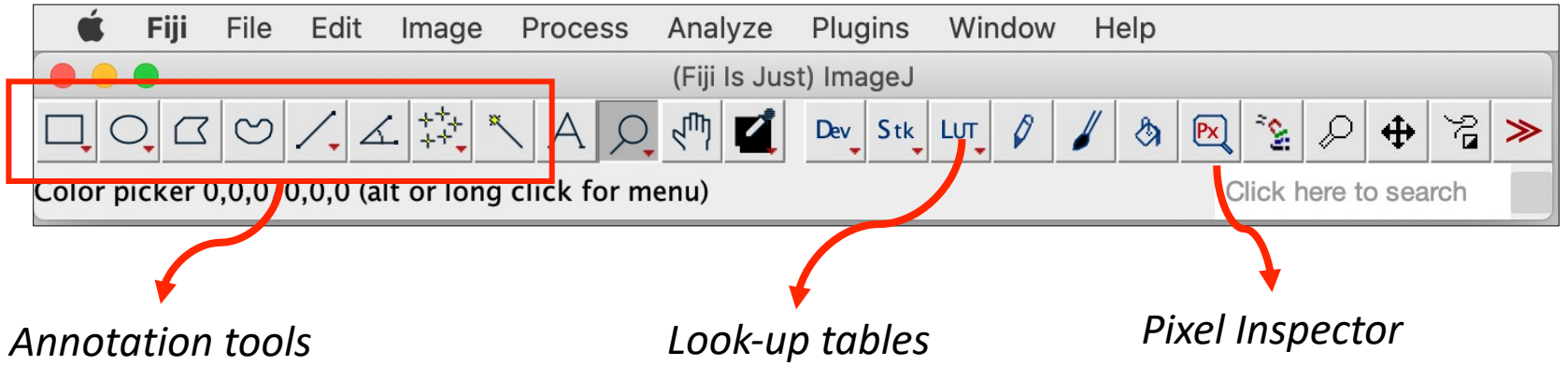
- Open the file "**nuclei1.tif**" in Fiji
- What is the image dimension?
- Inspect pixel values with the *Pixel Inspector* tool
- Modify the LUT
- Inspect the metadata. Which tags can you interpret?
- Analyze the histogram

- Now repeat the steps above for the "humanMCF7_cells.tif", "CIN_1_HE.tif" and Fiji/sample images "Mitosis"

Some image files used in these exercises were obtained from Broad Bioimage Benchmark Collection: https://bbbc.broadinstitute.org/image_sets

Practical Exercises

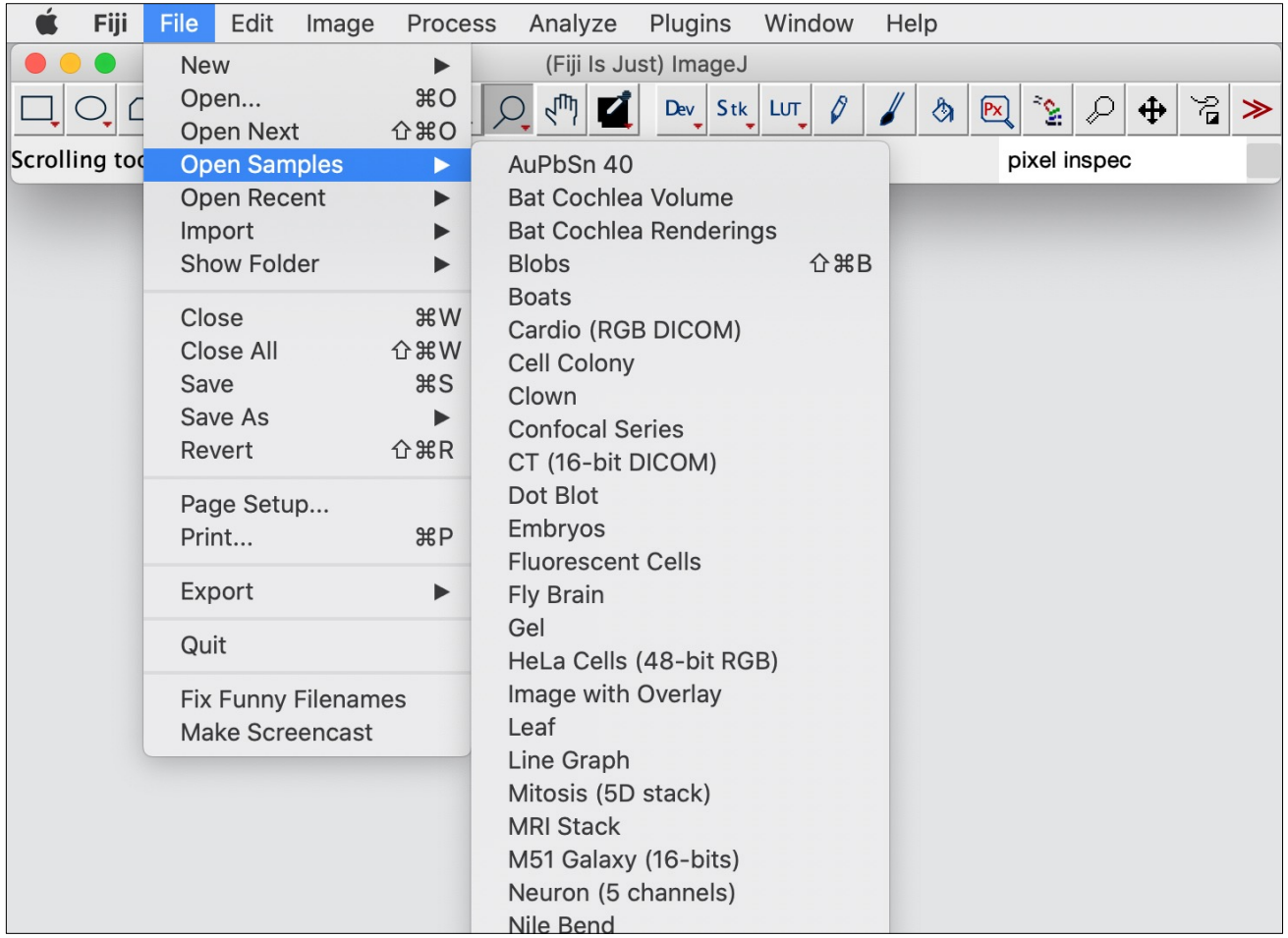
- Fiji tools



- 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"

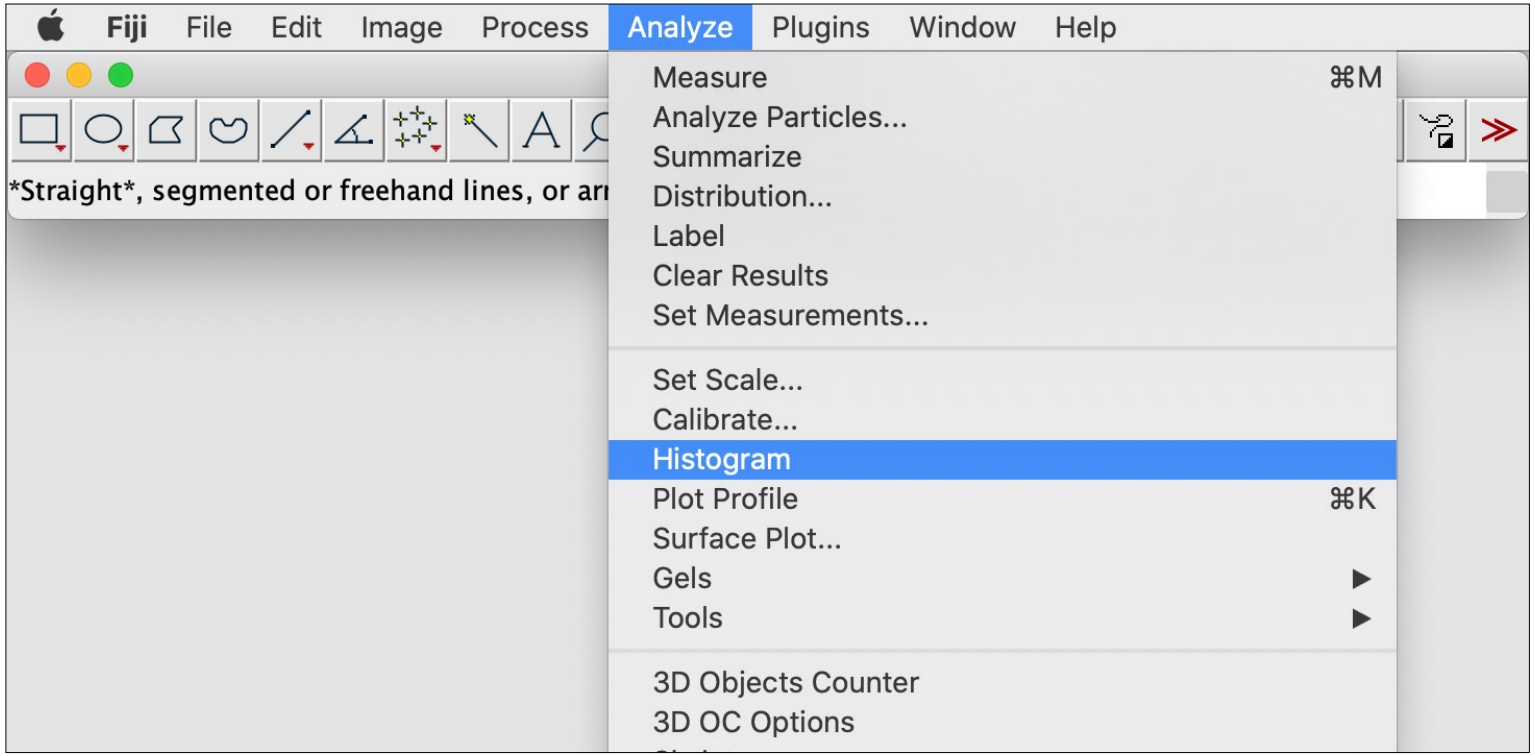
Practical Exercises

- Loading Fiji sample images



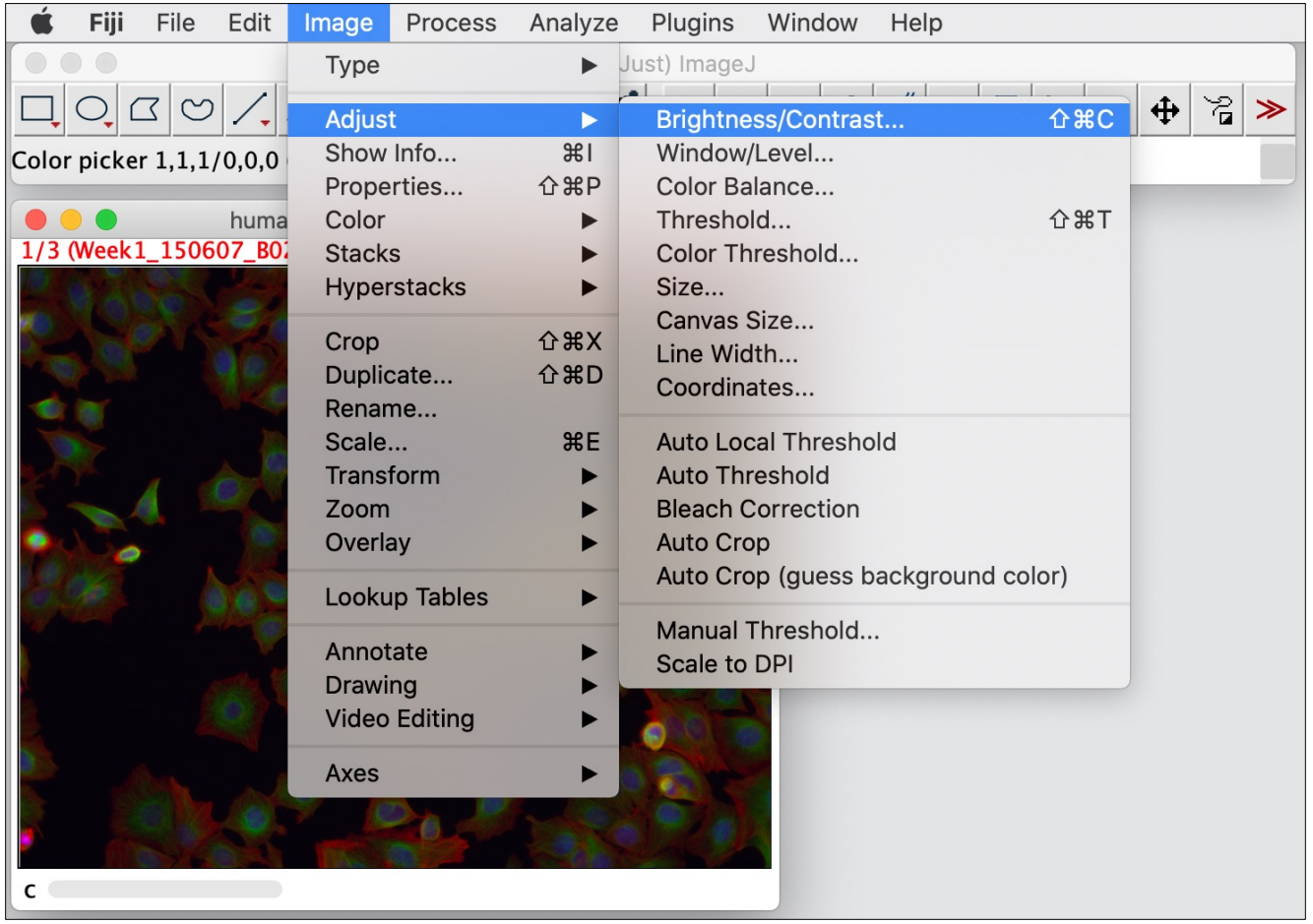
Practical Exercises

- Generating the histogram



Practical Exercises

- Brightness & Contrast



Practical Exercises

- Splitting and merging channels / channel tool

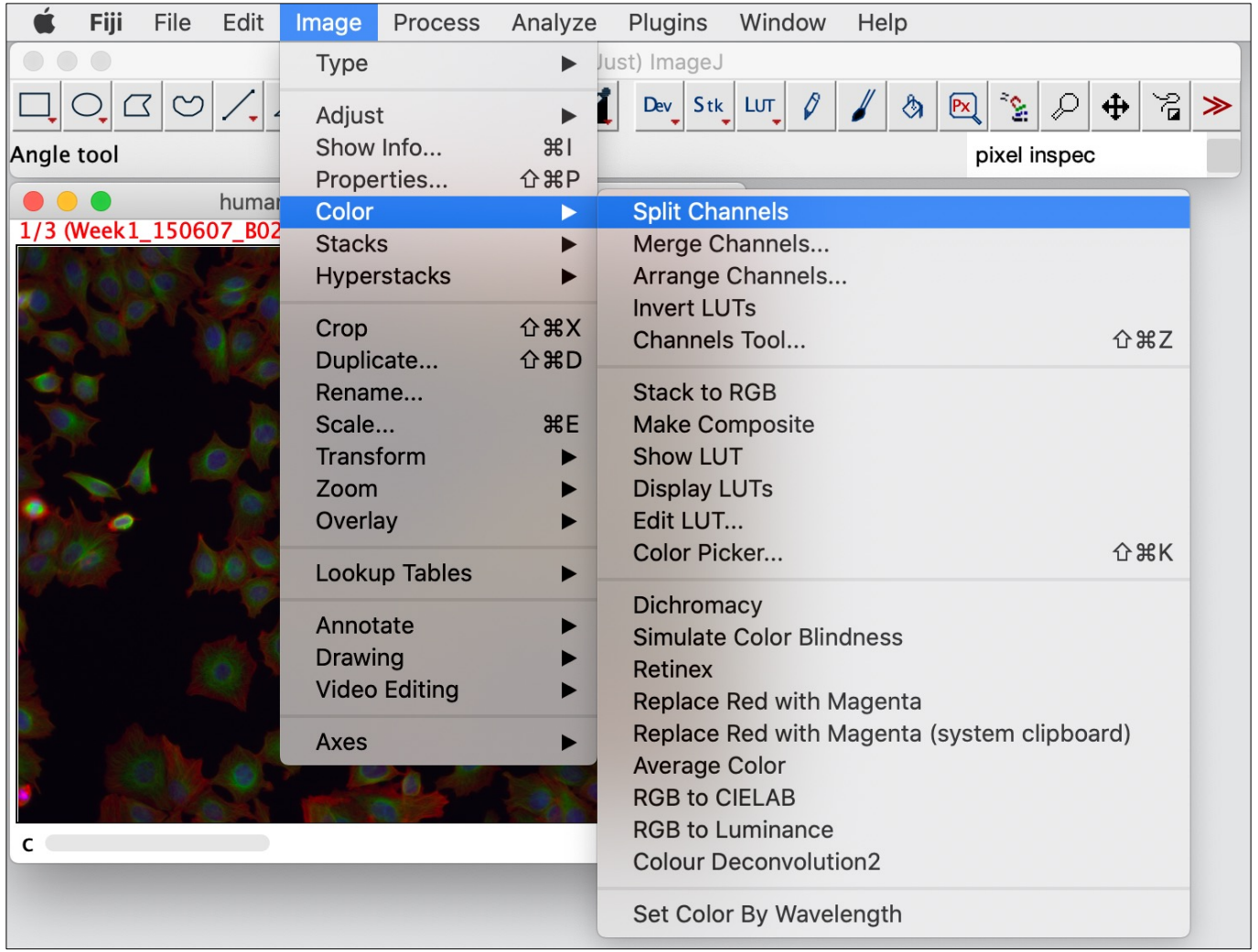
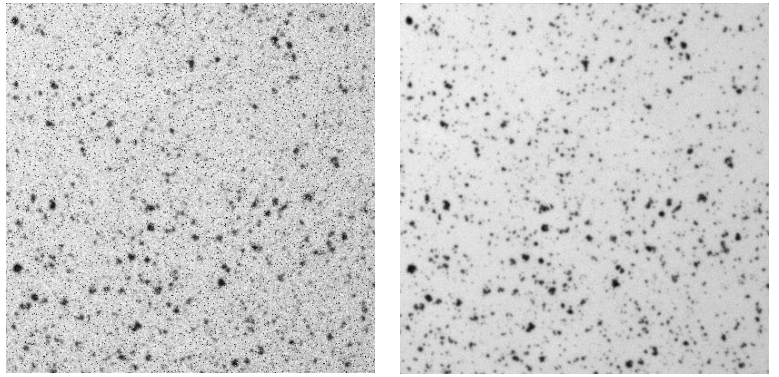


Image processing and enhancement 

Image Enhancement

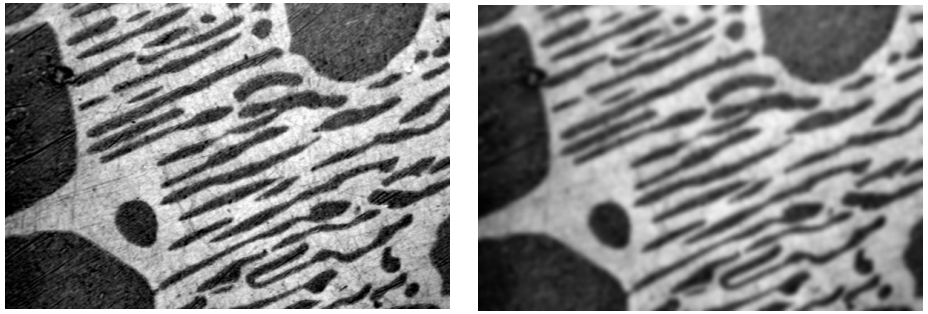
- Image operations designed to modify or enhance image properties, such as edges, corners and blobs.

De-noising



Edge dection

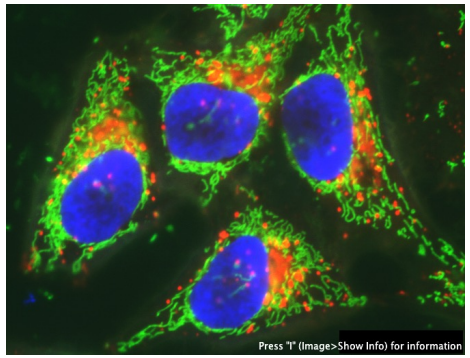
Gaussian blur

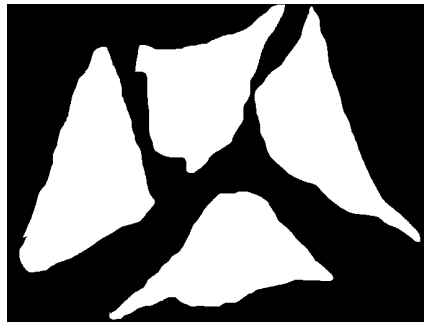


✓ Can be performed in the spatial domain and frequency domain

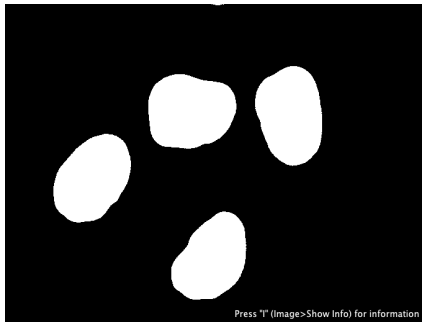
Basic arithmetics

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

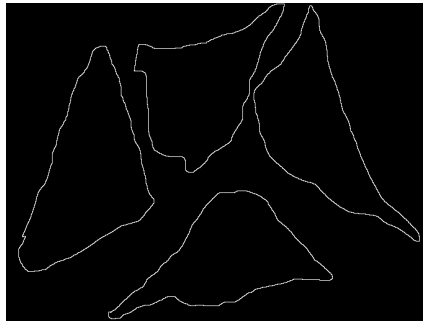




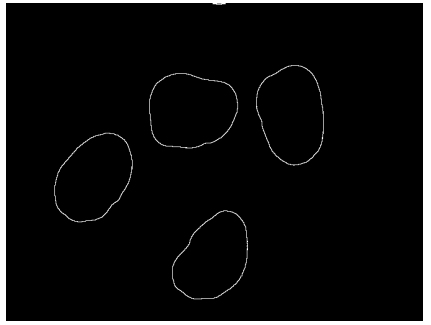
A



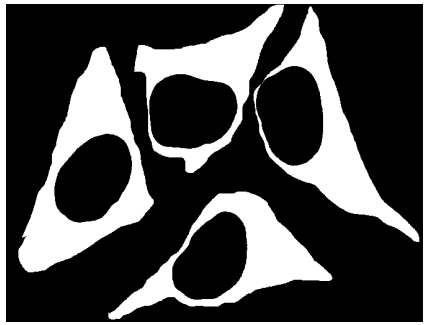
B



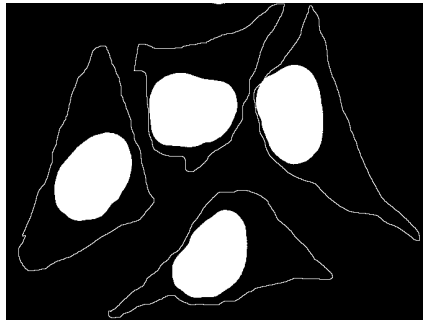
C



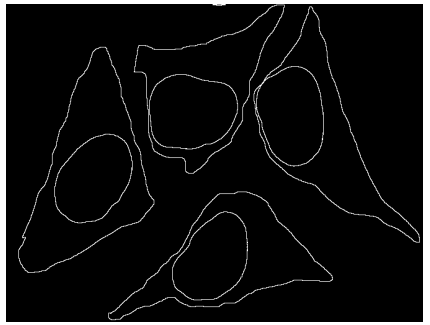
D



Subtraction
(A – B)



Add
(C + B)



Add
(C + D)

Convolution filters

- Also known as kernels

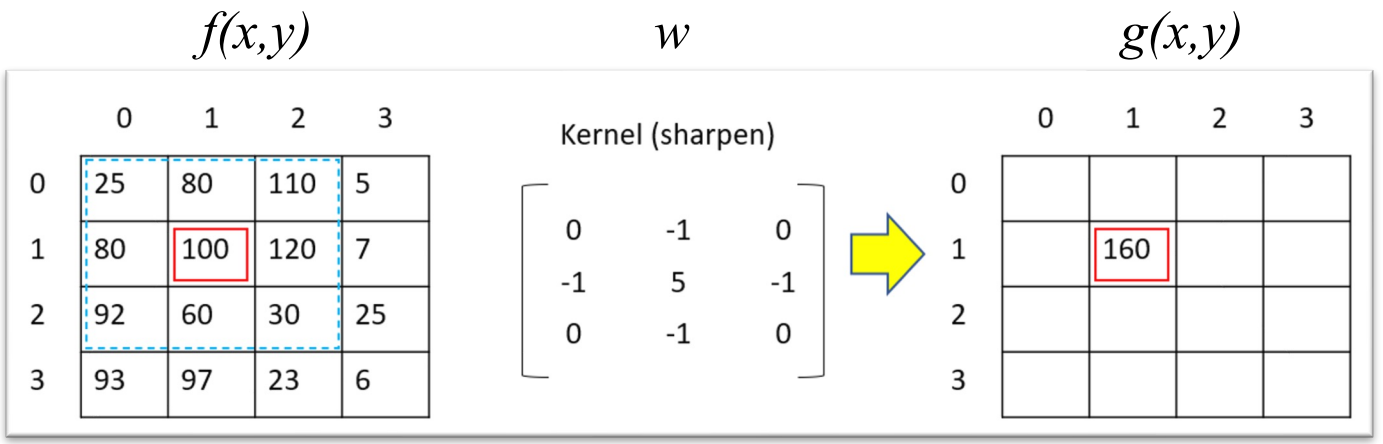
$$g(x, y) = \omega * f(x, y)$$

filtered image

kernel

original image

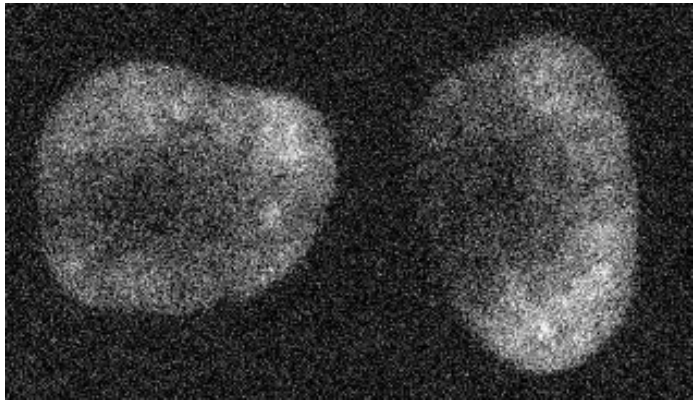
- Ex: sharpen



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

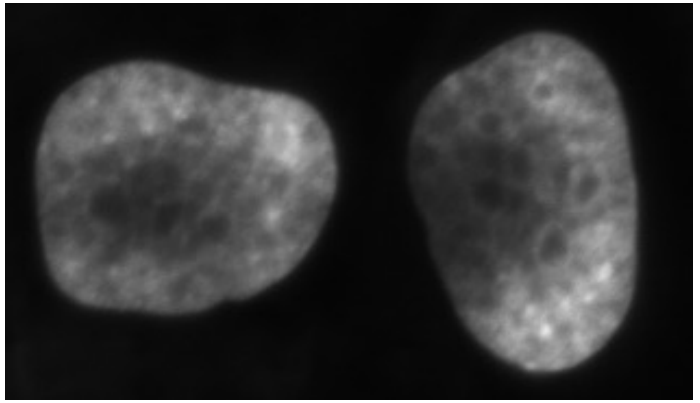


* $\frac{1}{9}$

+1	+1	+1
+1	+1	+1
+1	+1	+1

Kernel

=



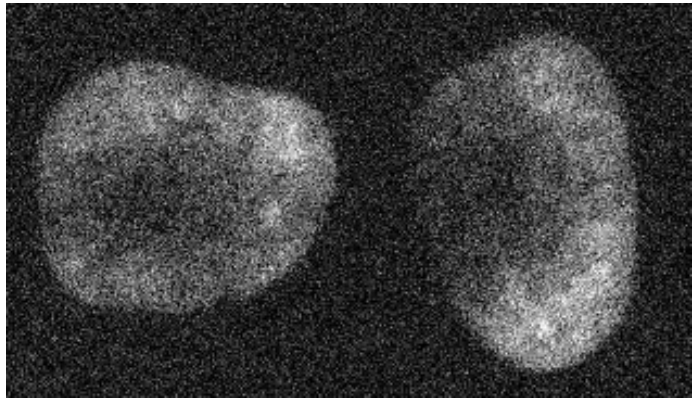
10	2	3	
1	23	43	
7	13	9	

	12		

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

Average: 111/9 ~ 12

The mean filter

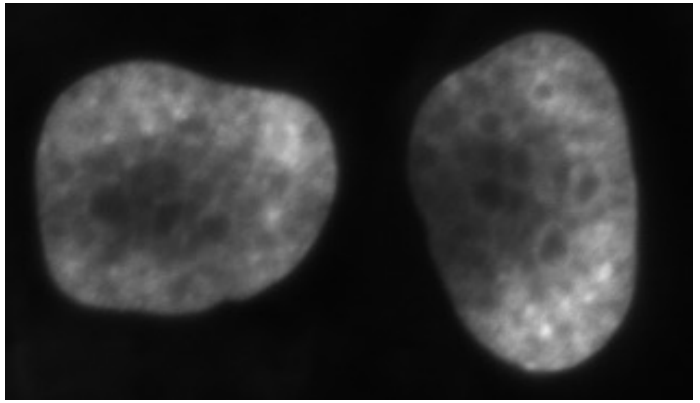


$*\ \frac{1}{9}$

+1	+1	+1
+1	+1	+1
+1	+1	+1

Kernel

$=$



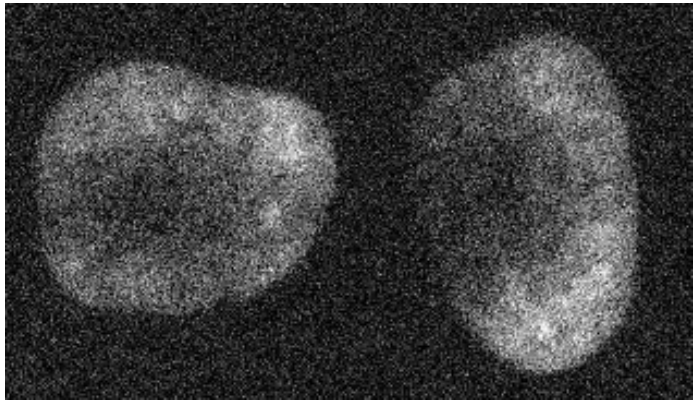
	2	3	5
	23	43	4
	13	9	2

	12	12	

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

Average: 104/9 ~ 12

The median filter

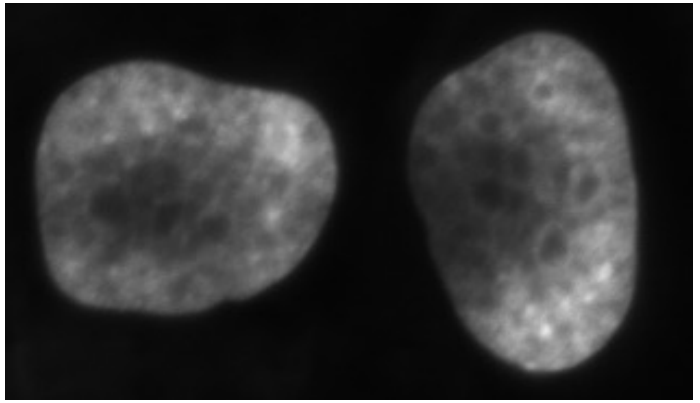


*

+1	+1	+1
+1	+1	+1
+1	+1	+1

Kernel

=



10	2	3	
1	23	43	
7	13	9	

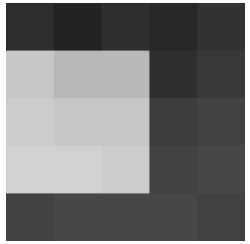
→

	9		

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

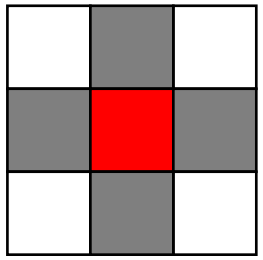
original

median-filtered

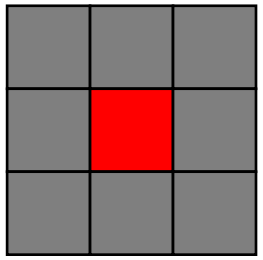
mean-filtered

Neighborhood size

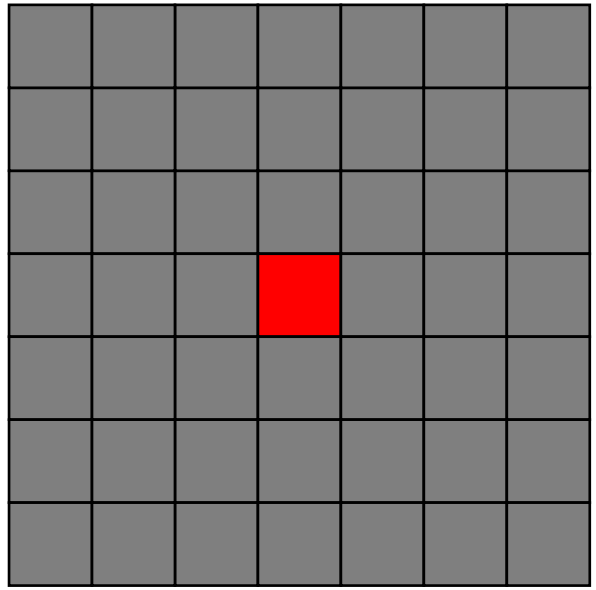
- Kernel: different sizes and shapes



4-connected



8-connected,
(*3x3)*

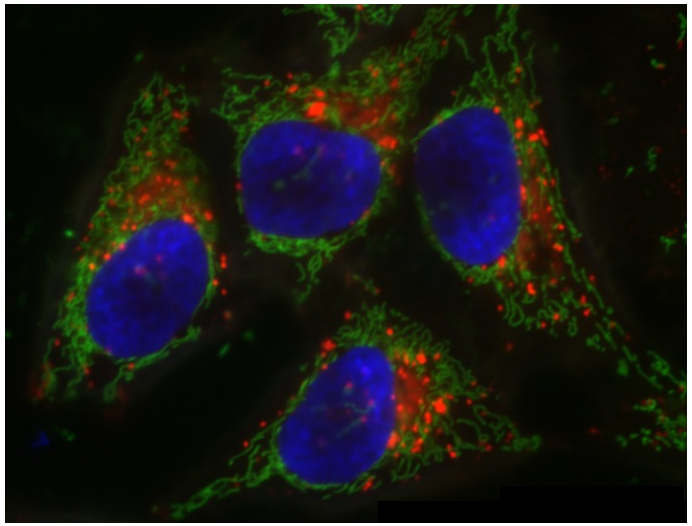


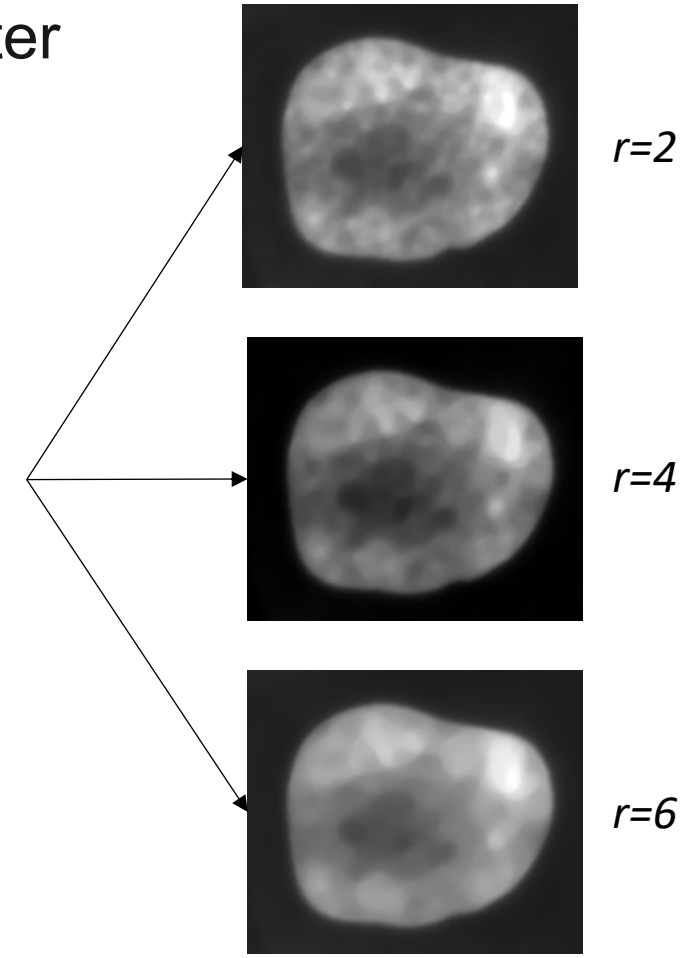
5x5

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:





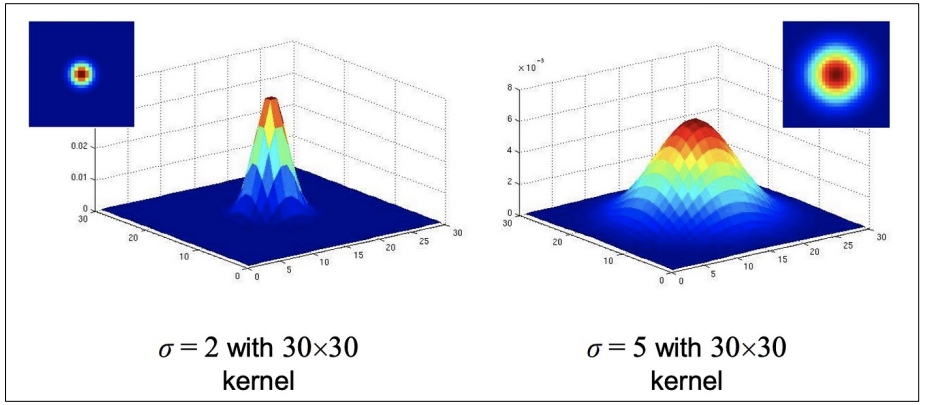
Gaussian Smoothing

✓ 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 σ



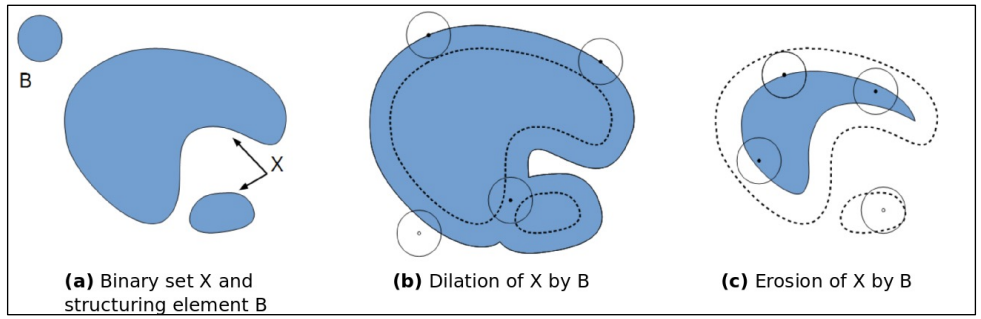
https://medium.com/jun94-devpblog/cv-2-gaussian-and-median-filter-separable-2d-filter-2d11ee022c66#:~:text=Gaussian%20kernel%2C%20as%20its%20name,(pixels)%20in%20an%20image .

original

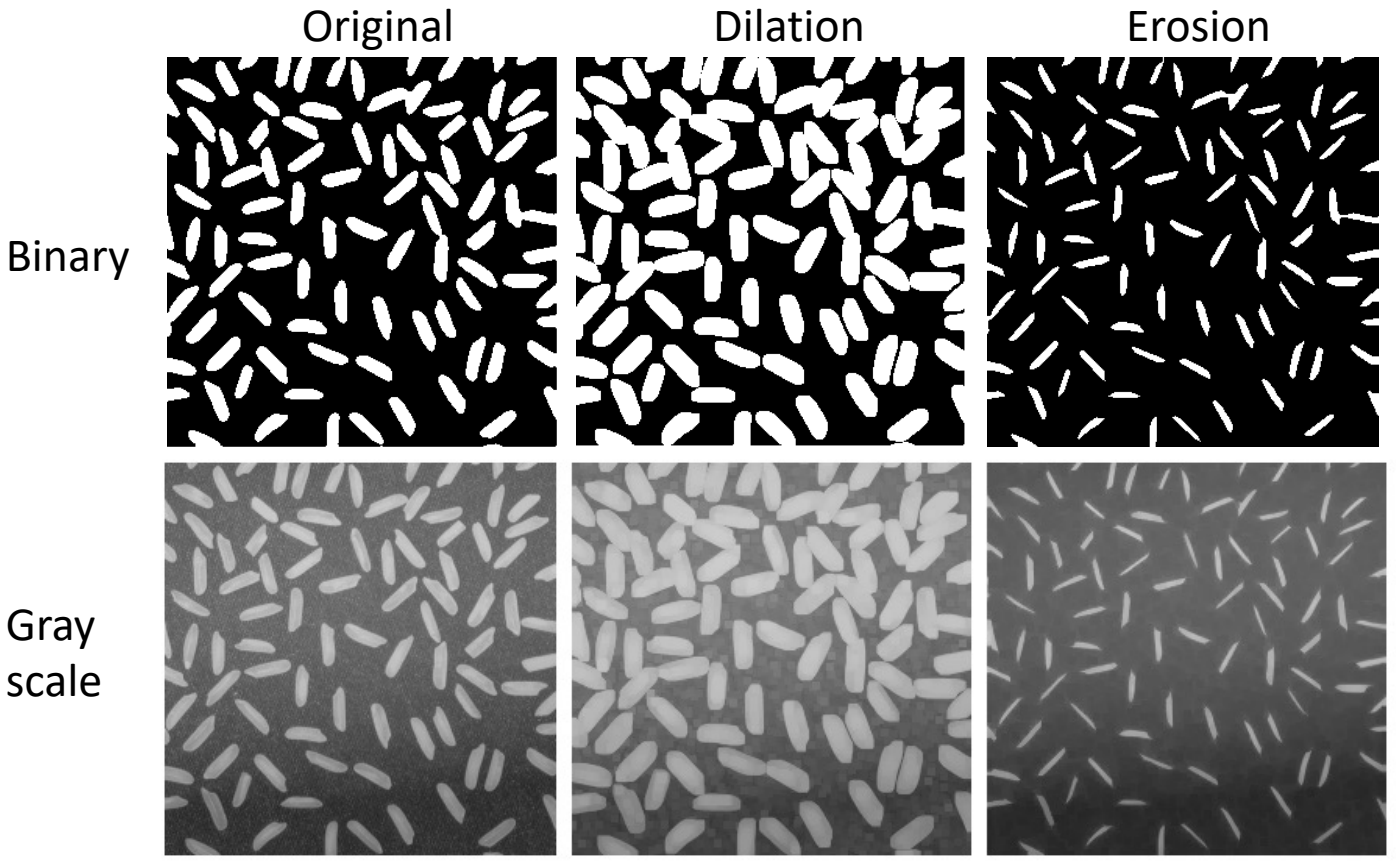
gaussian-filtered σ=2

Morphological filters

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



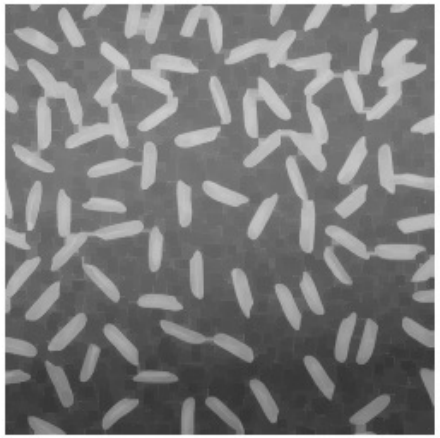
Morphological dilation and erosion on a binary set



https://imagej.net/plugins/morpholibj

Morphological filters: gray level images

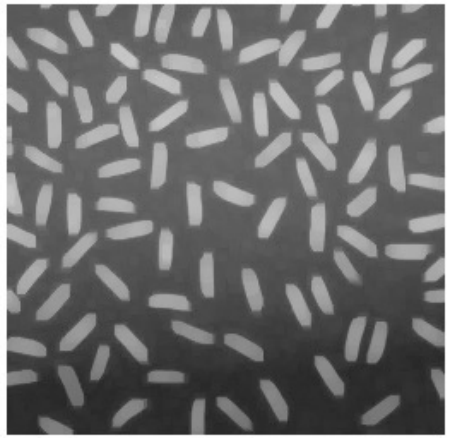
- Dilation and erosion combined to create other morphological filters



Closing

Dilation + Erosion

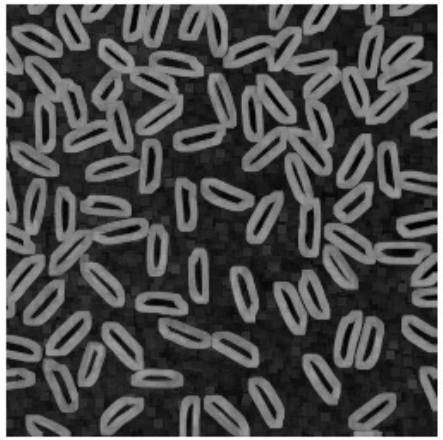
Removes dark structures smaller than the structuring element



Opening

Erosion + Dilation

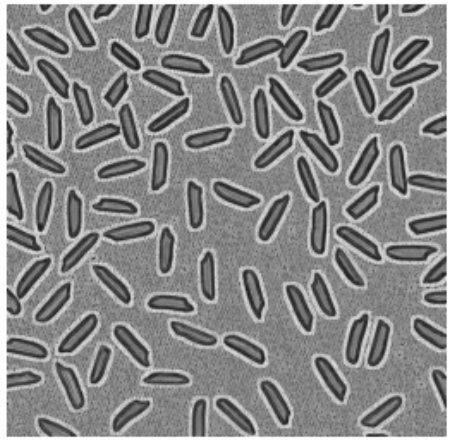
Removes bright structures smaller than the structuring element



Gradient

Dilation - Erosion

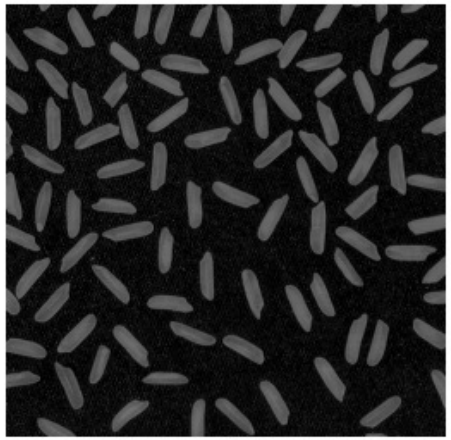
Boundary detection



Laplacian

Original image – 1/5(Dilation+Erosion)

Edge enhancement



Top-hats

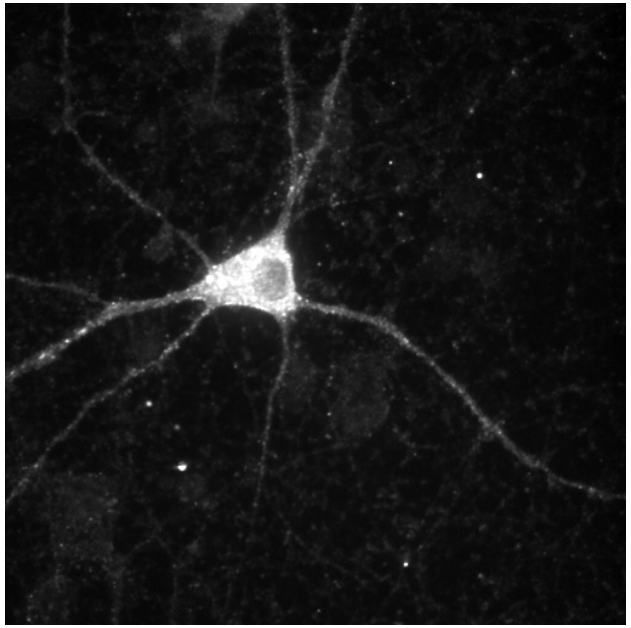
Original image - Opening

Enhance bright or dark structures

https://imagej.net/plugins/morpholibj

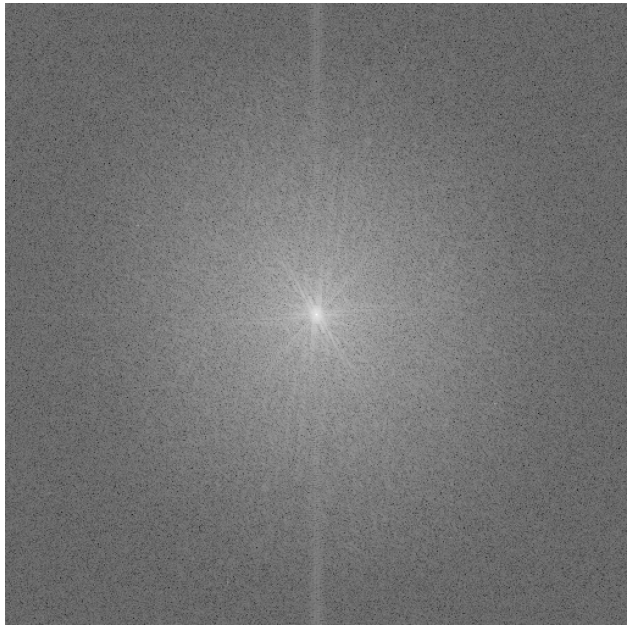
Filtering in the frequency domain

Original Image



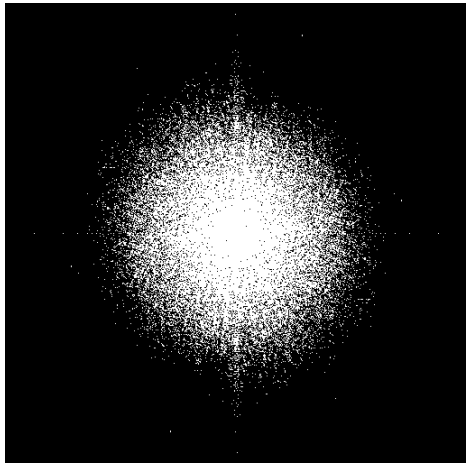
Rat Hippocampal Neuron
Fiji samples

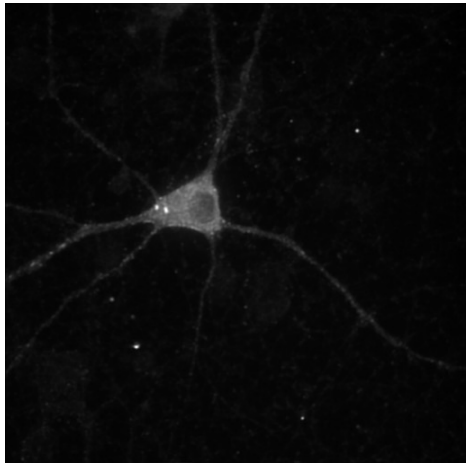
Frequency domain



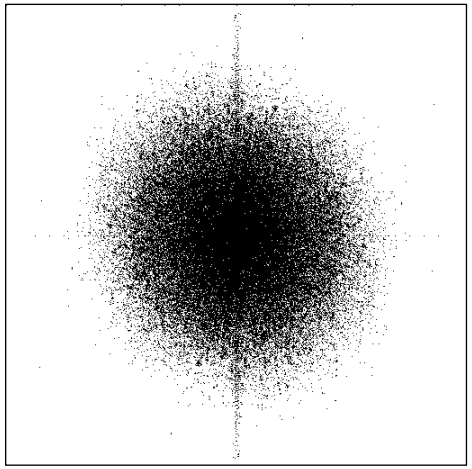
Fourier Transform

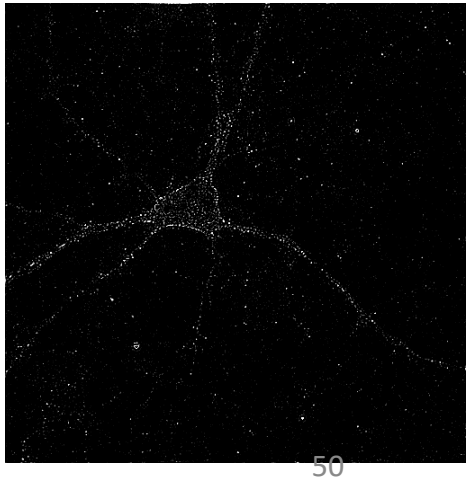
Low-pass filter





High-pass filter





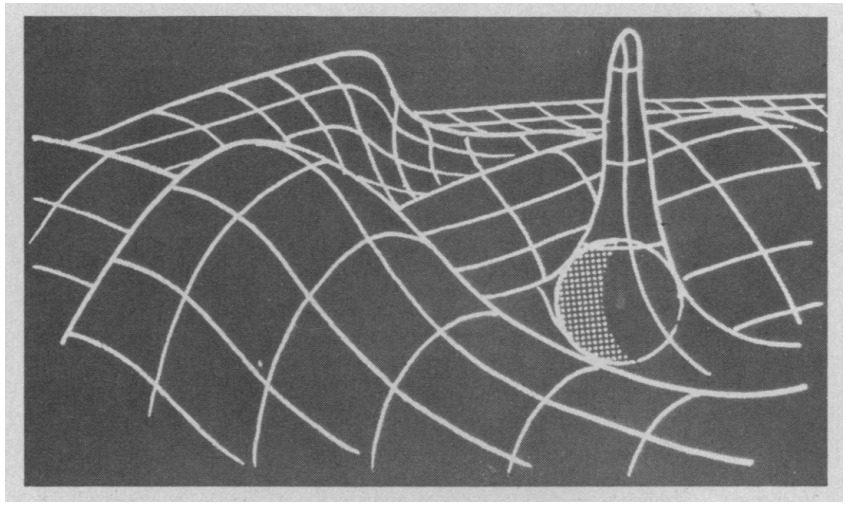
Uneven Illumination

- ✓ 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.

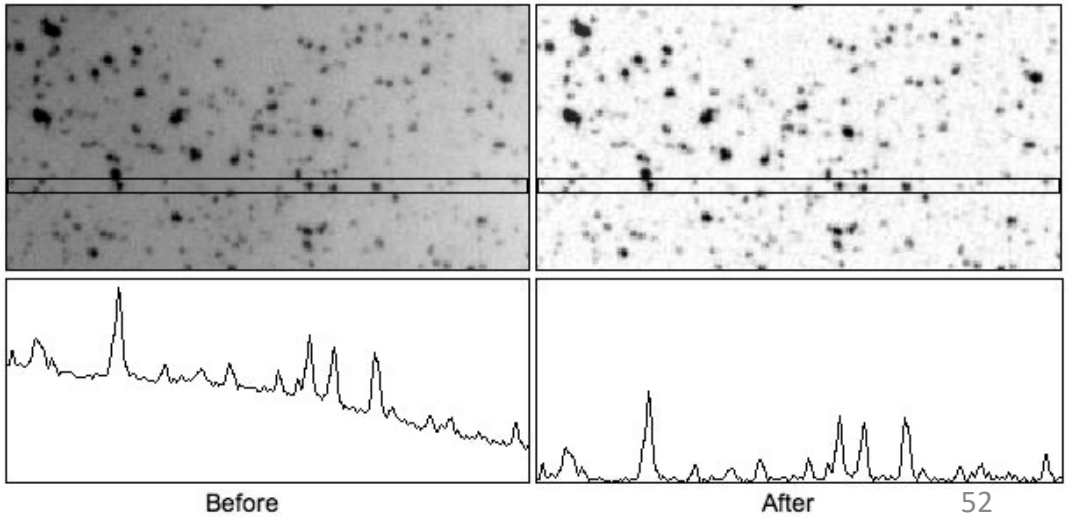
Background subtraction

- Rolling-ball algorithm



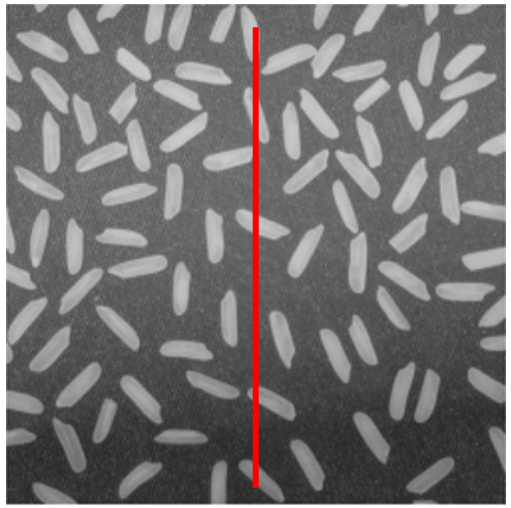
Sternberg, Stanley R. “Biomedical image processing.” Computer 1 (1983): 22-34. DOI:10.1109/MC.1983.1654163

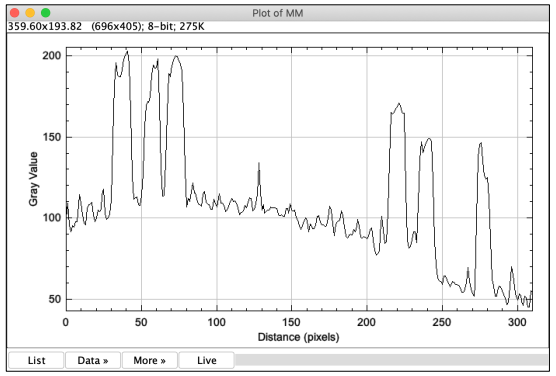
- ✓ 3D surface with the pixel values of the image being the height
- ✓ A ball rolling over the back side of the surface creates the background
- ✓ The rolling-ball is equivalent to eroding and dilating by a spherical structuring element

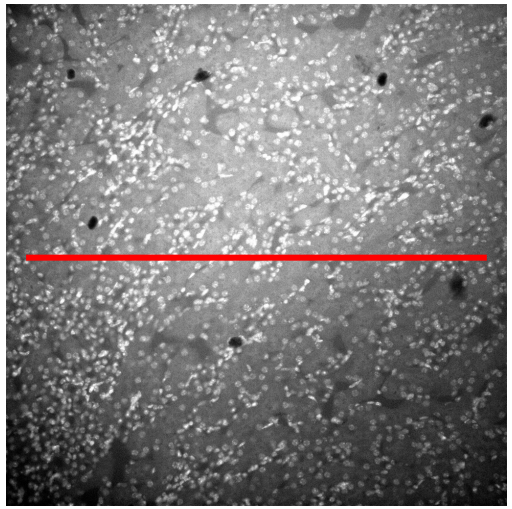


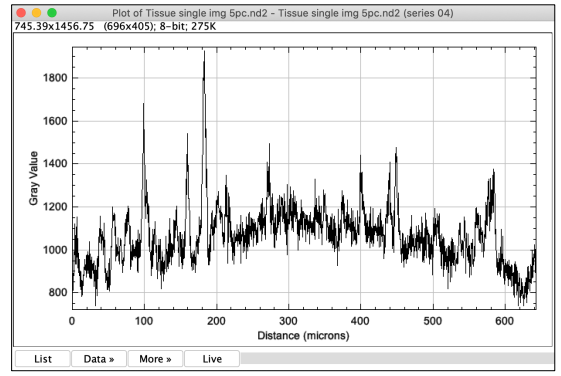
https://imagejdocu.list.lu/gui/process/subtract_background

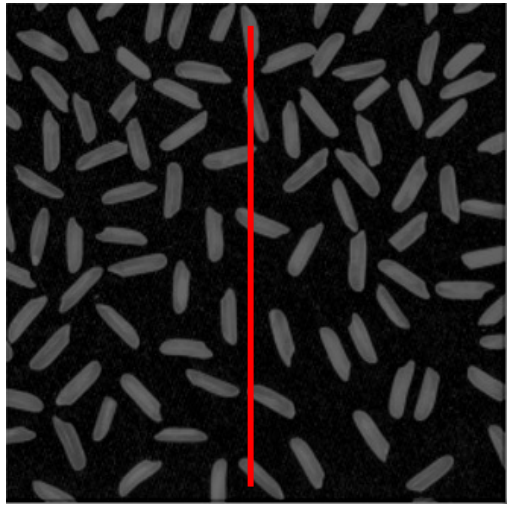
Background correction

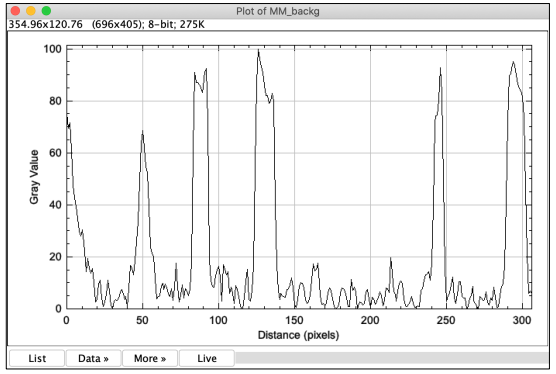


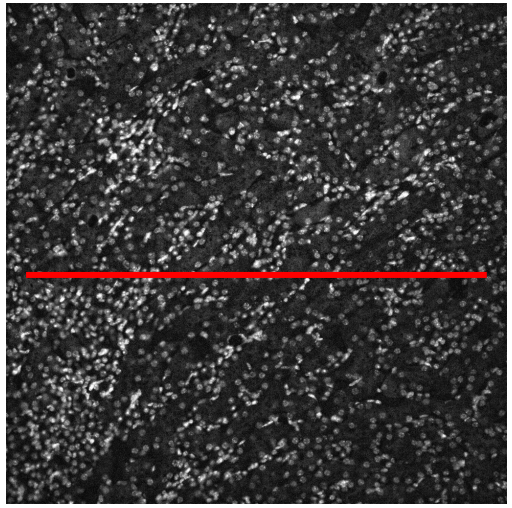


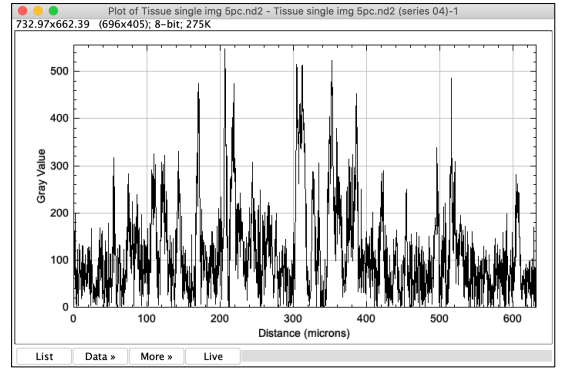












Practical Exercises

- 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/

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)

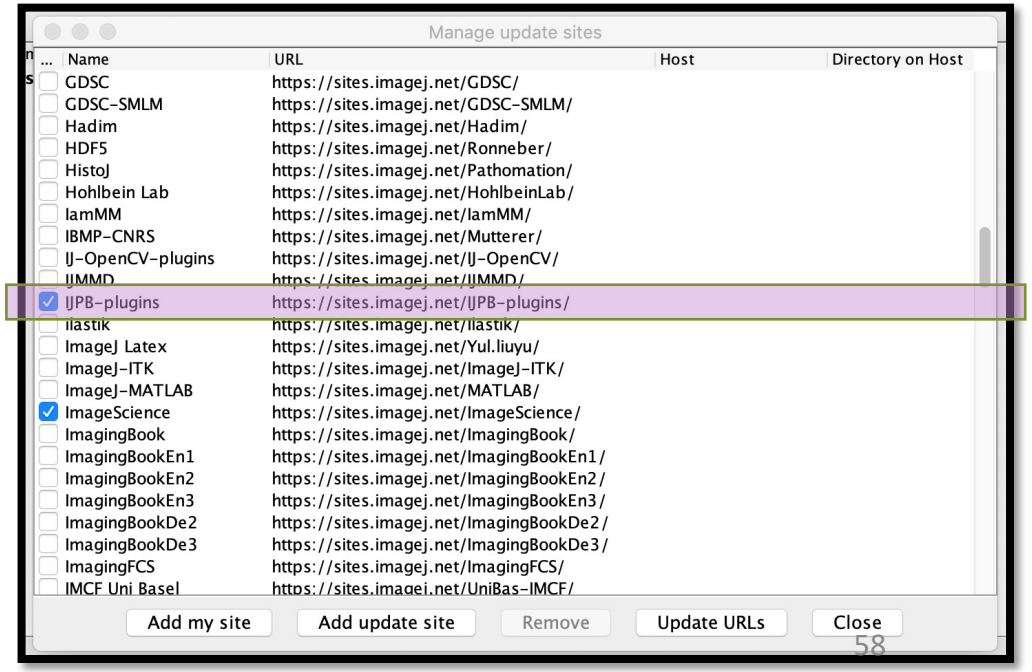
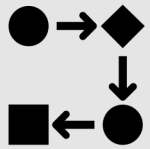


Image Analysis Workflow



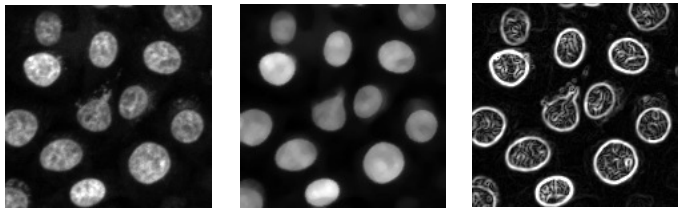
The pathway towards image quantification

Image acquisition

Sample preparation

Pre-processing

- Background subtraction
- Noise reduction
- Filtering, edge detection, etc

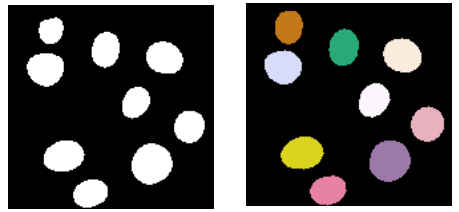


Segmentation

- Separation of foreground/background

Post-processing

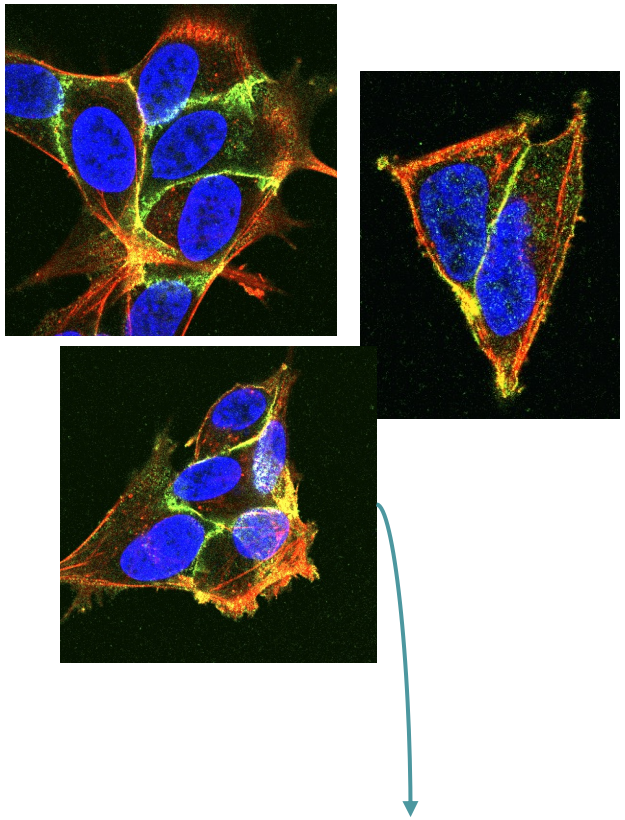
- Refinement of the binary mask
- Labelling of objects



AI tools can be used in different workflow components

- Feature extraction
- Measurements (area, circularity, etc)
- Statistical analysis, classification, interpretation

Quantification

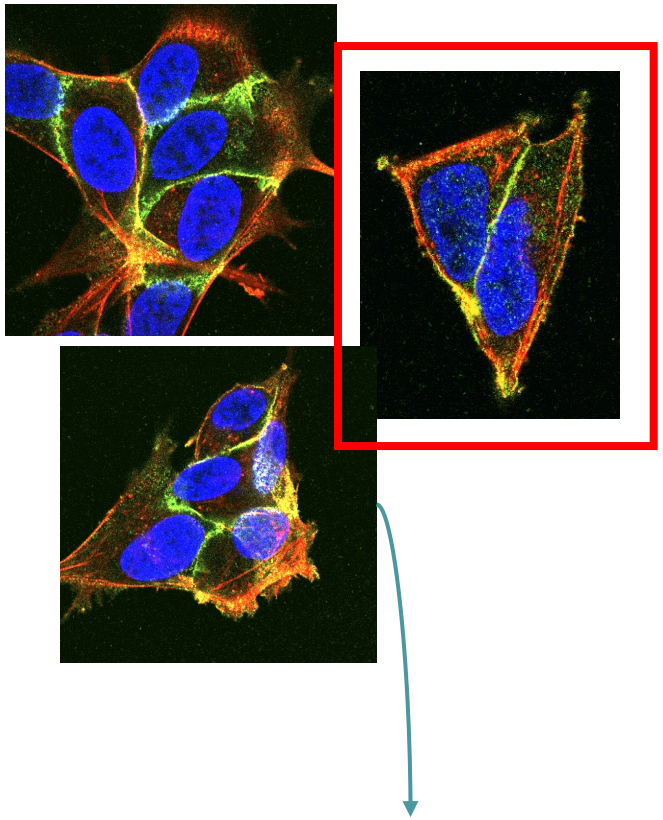


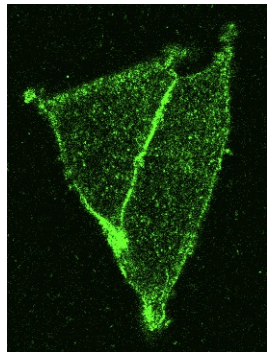
3-channel image dataset

- Nuclei (DAPI - blue)
- β-catenin (GFP - green)
- Actin (Phalloidin - red)

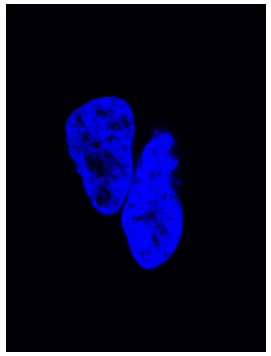
✓ How to quantify the β-catenin translocated from the cell membrane to the cell cytoplasm and nucleus ?

Image Source: LCI / KI Clinical Research School 2021

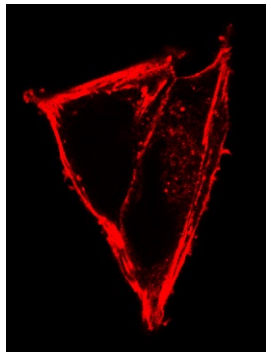




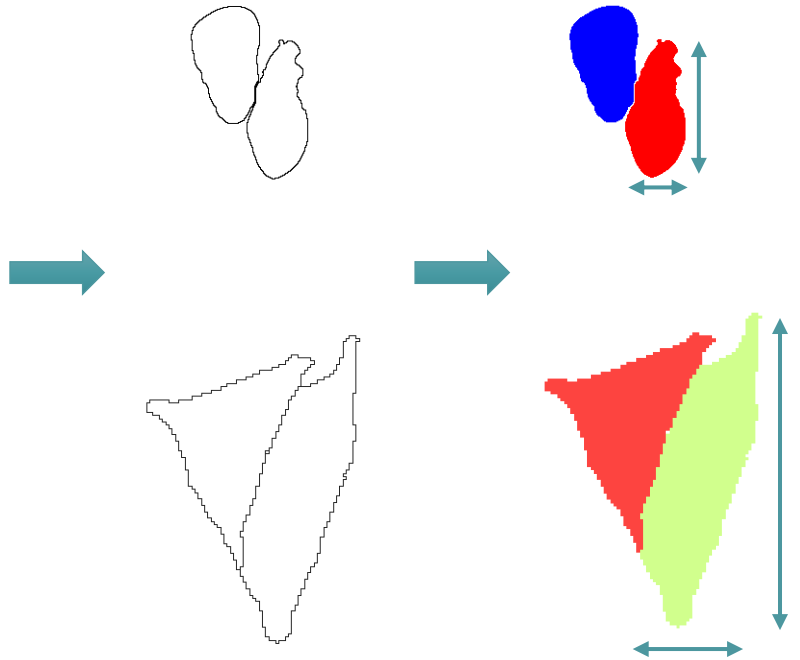
β -catenin



Nuclei (DAPI)

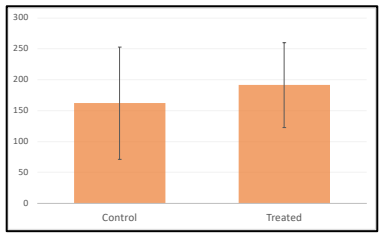


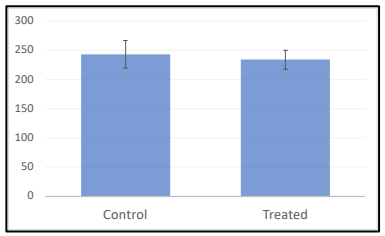
Actin (Phalloidin)



✓ How to quantify the β-catenin translocated from the cell membrane to the cell cytoplasm and nucleus ?

?

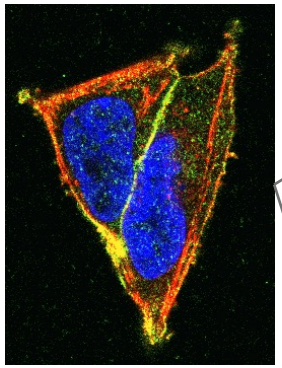




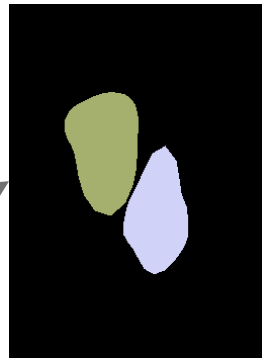
ROI	M_1	M_2	…	…	M_n
	20	102	…	…	0.34
	25	198	…	…	0.29
	58	240	…	…	0.72
	51	211	…	…	0.49

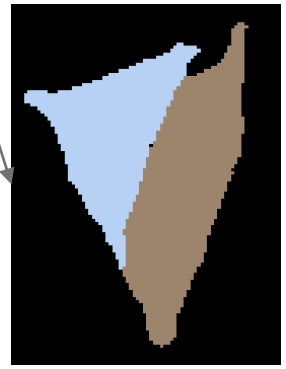
Image Source: LCI / KI Clinical Research School 2021

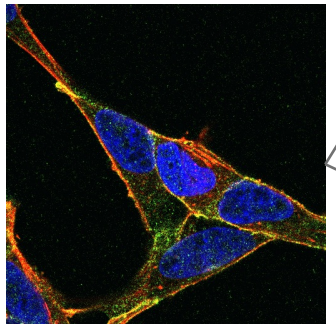
Other images of the dataset



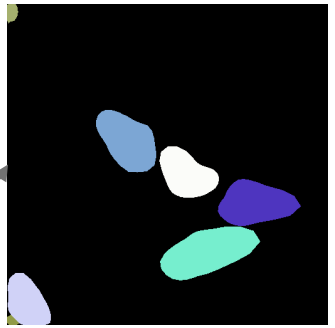
Example #1

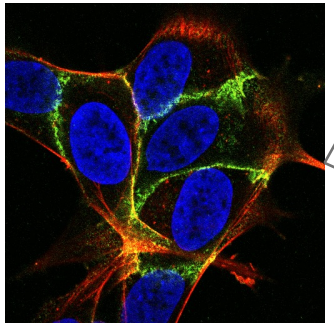






Example #2





Example #3

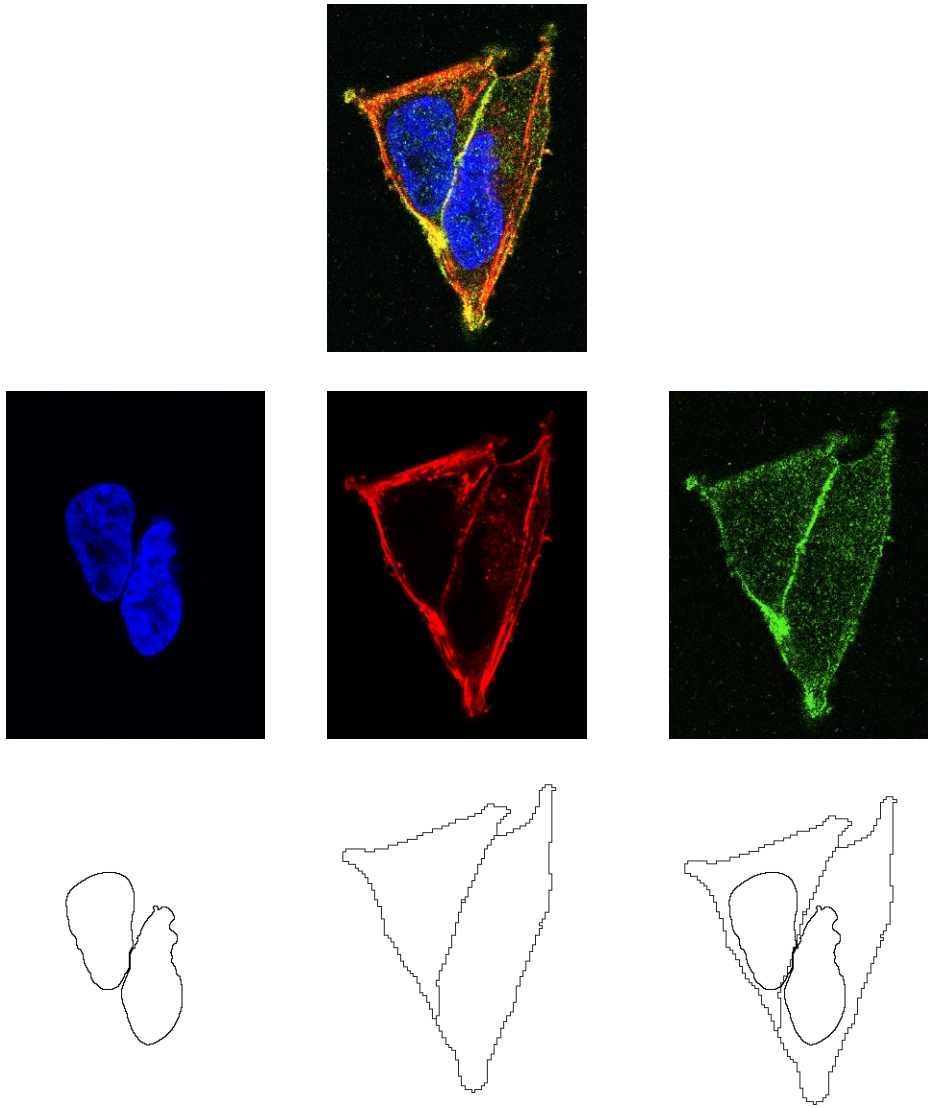
Summarizing the workflow…

Split Images into three separate channels

Get outlines of nuclei from DAPI image

Get outlines of cytoplasm from actin image

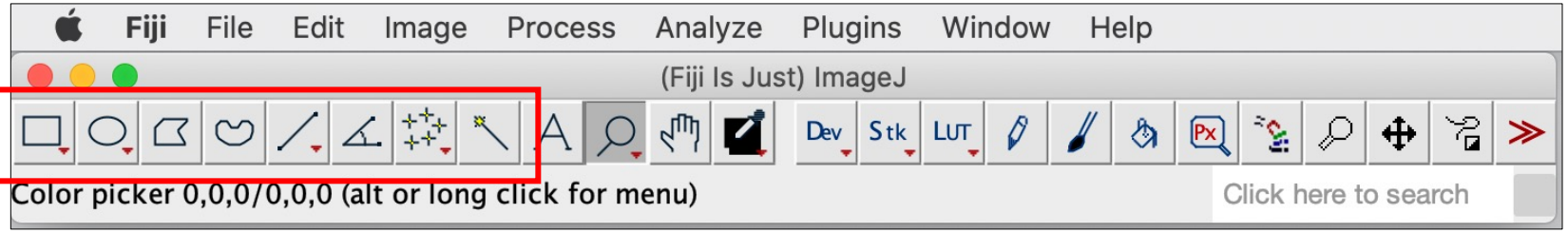
Measure β-catenin in the area defined by the outlines

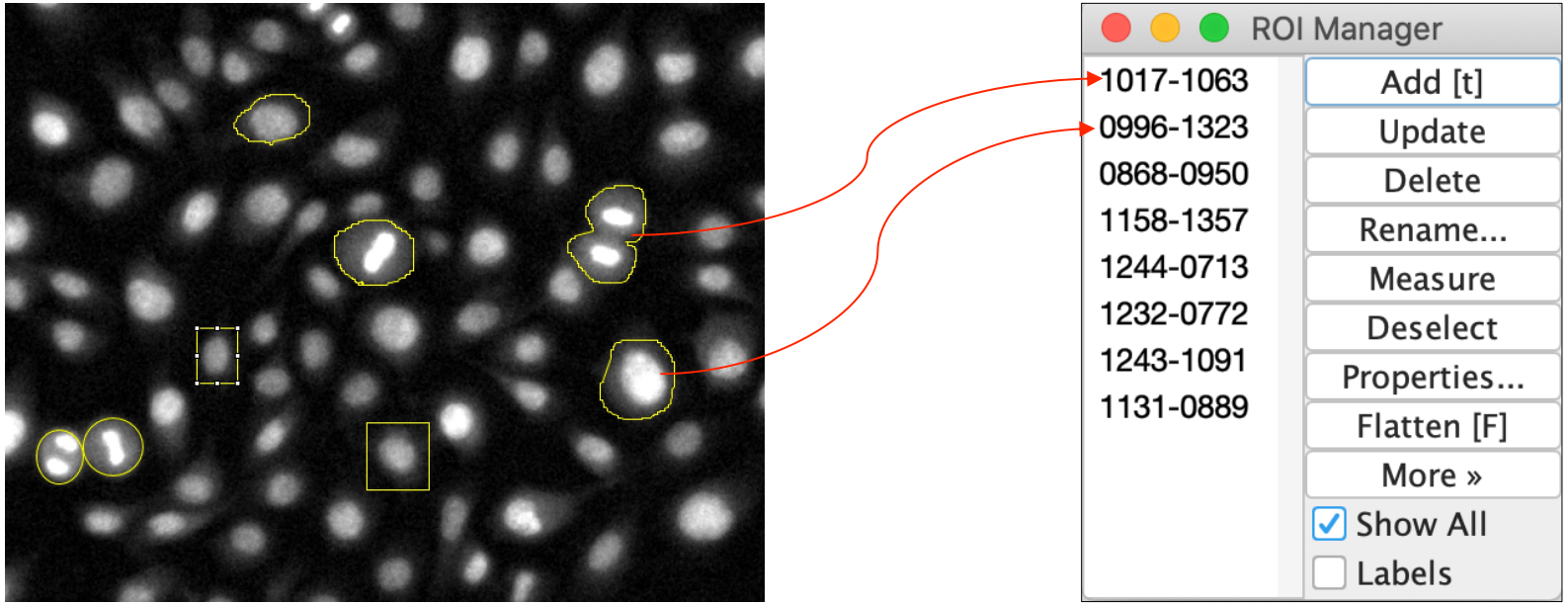


Region of Interest (ROI)

- Can be annotated manually

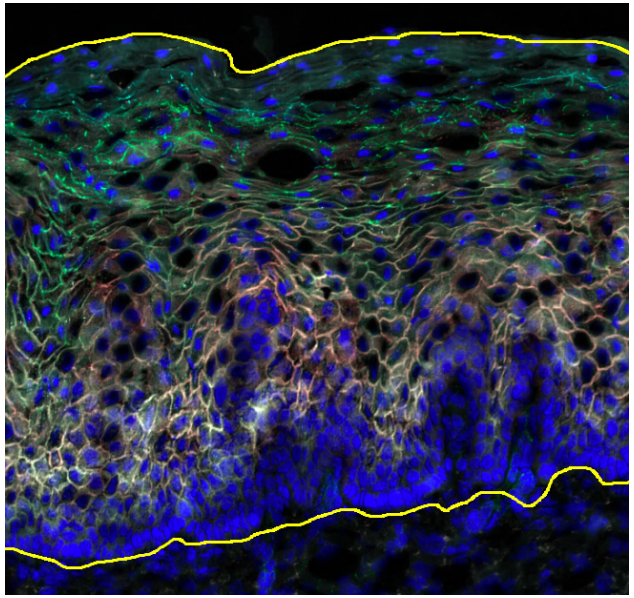
annotations tools



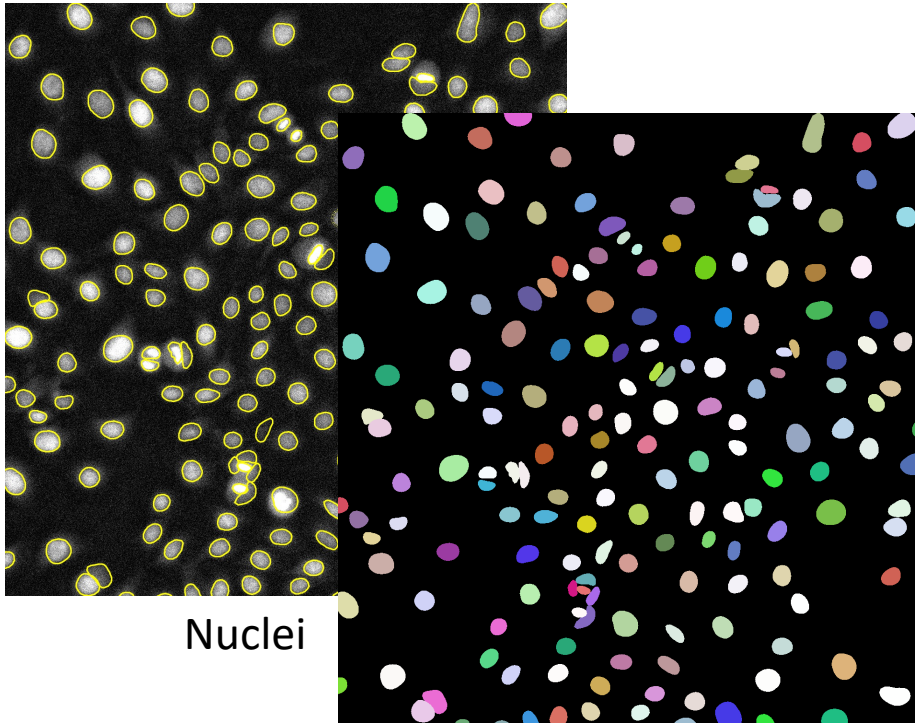


Region of Interest (ROI)

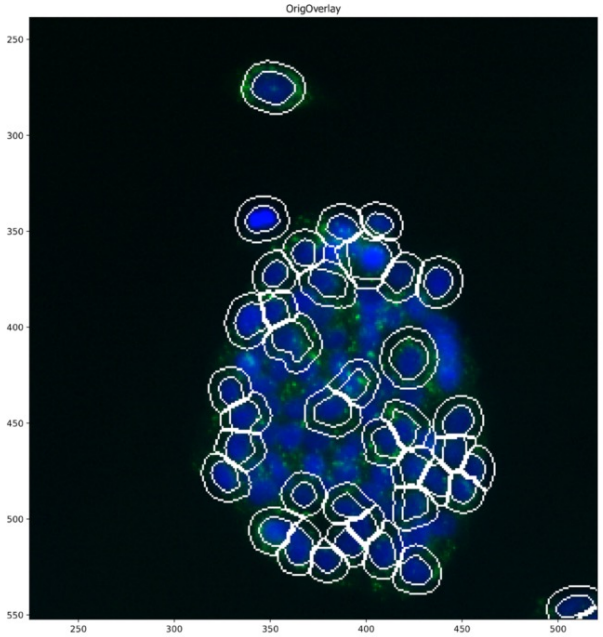
- Can be obtained via segmentation
  - ROIs may correspond to cells, nuclei, filamentous structures, parts of a tissue, etc



ROI: epithelial tissue



Nuclei

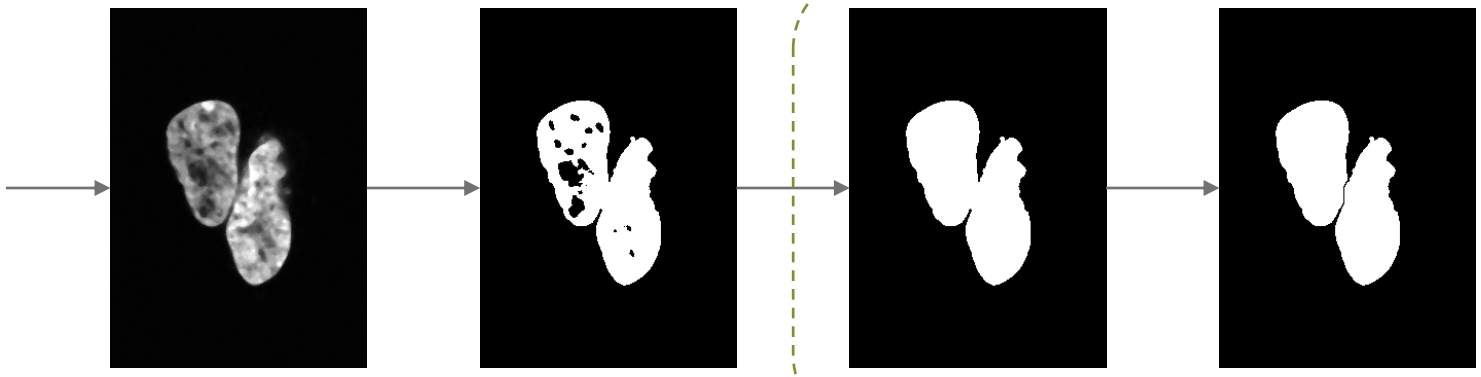


Cell membrane

https://biifsweden.github.io/projects/2018/12/05/CasimiroCastillejo/
https://biifsweden.github.io/projects/2019/10/25/AnnelieTjernlund2019-1/

Get outlines of nuclei from DAPI image

Nuclei



Pre-processing / filtering

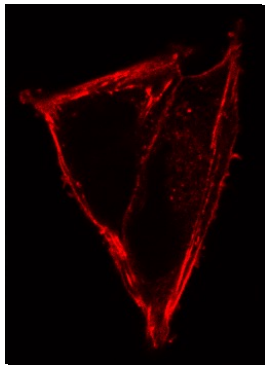
Segmentation by thresholding

Fill holes

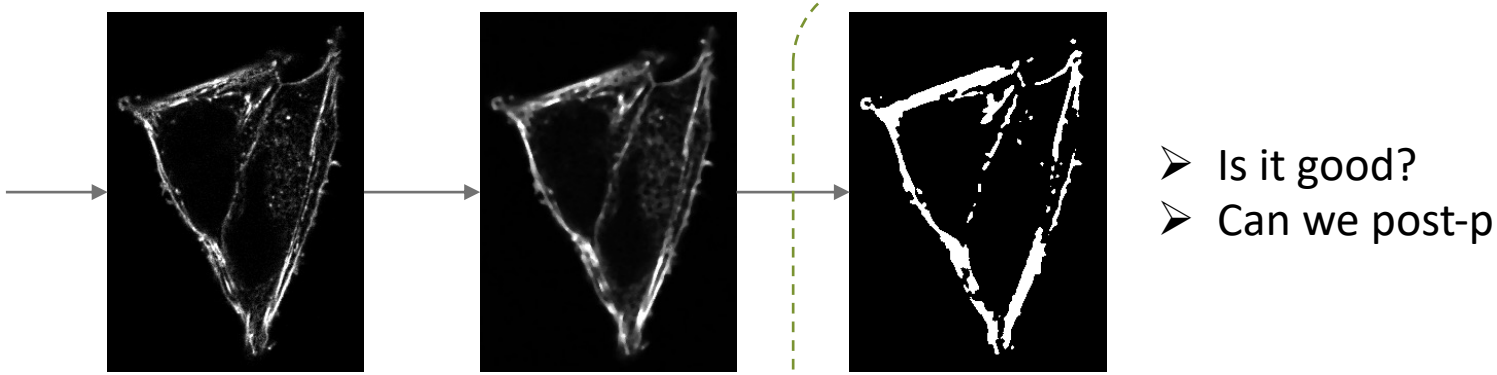
Watershed

Post-processing

Get outlines of cytoplasm from actin image



Actin



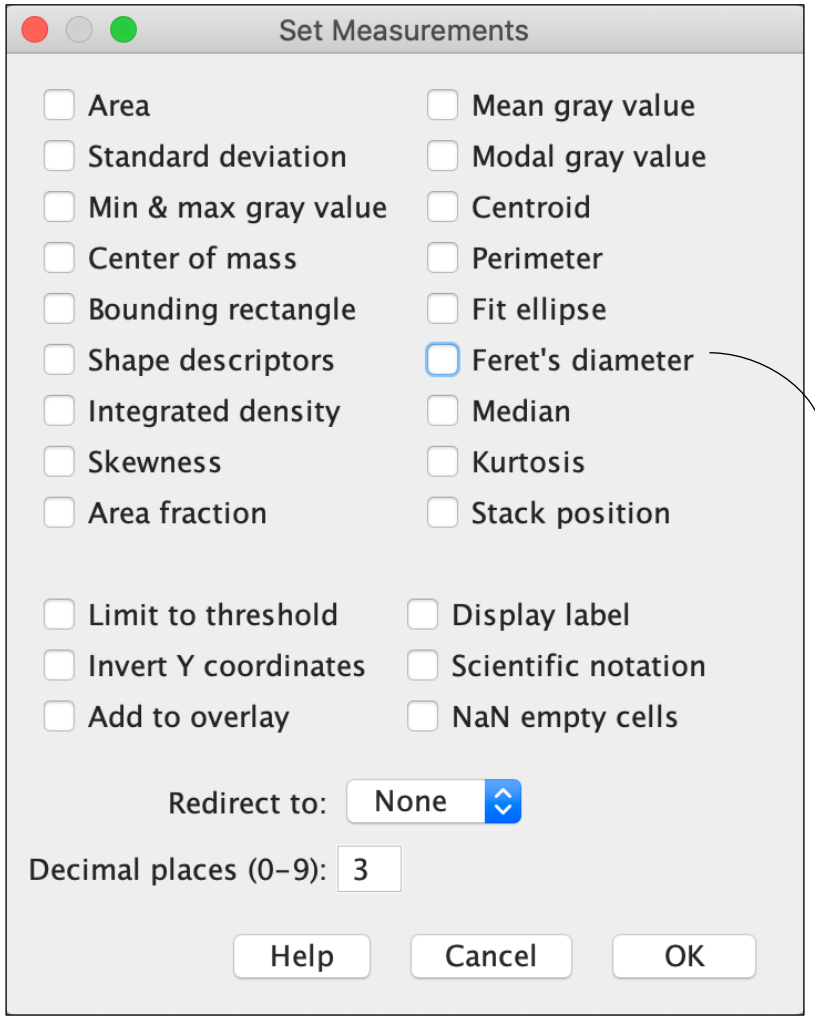
Pre-processing / edge enhancement

Pre-processing / filtering

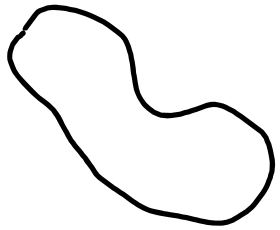
Segmentation by thresholding

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

Image quantification: 2D measurements

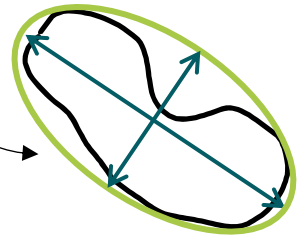


✓ Shape-related measurements



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

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



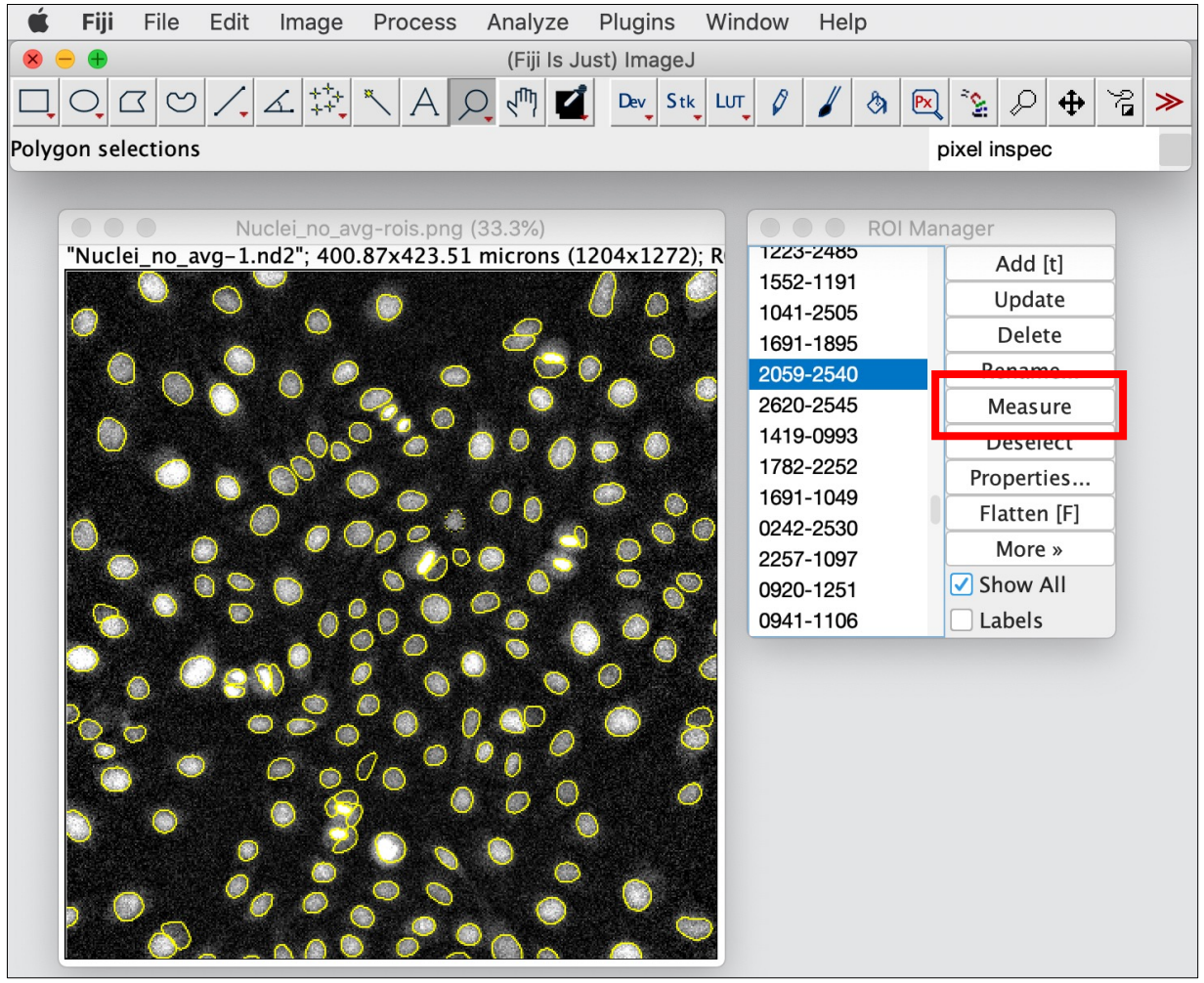
$$AR\ (aspect\ ratio) = \frac{major\ axis}{minor\ axis}$$

✓ 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

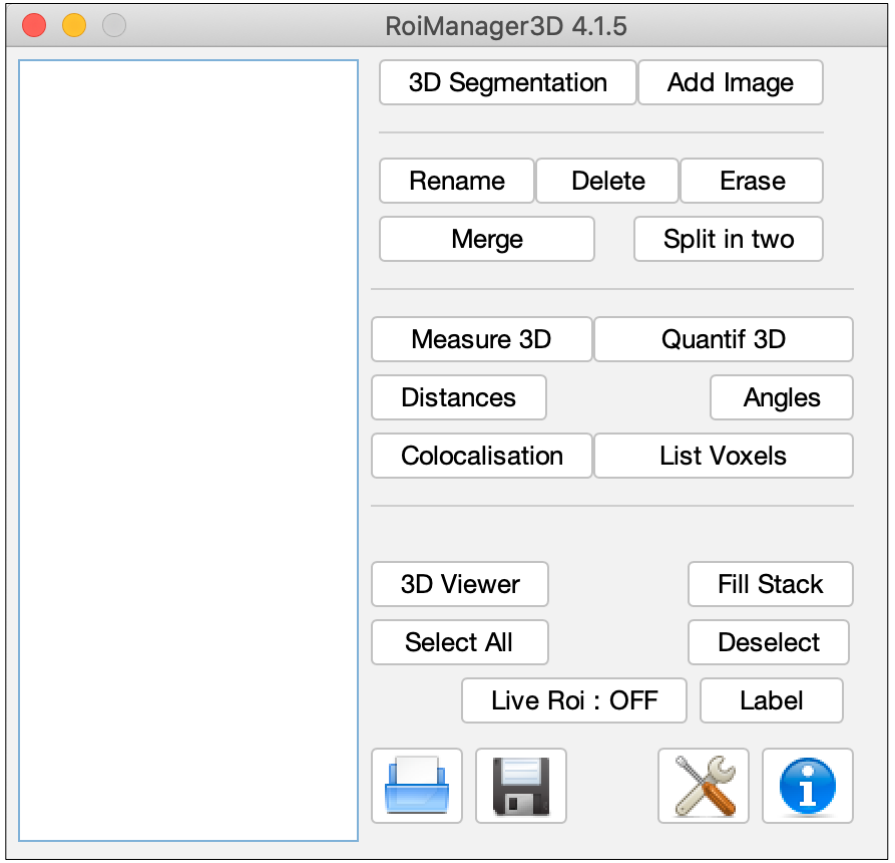
Results

	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



https://imagej.net/plugins/3d-imagej-suite/

Let's practice in Fiji

- Image files to be used in this session are located in ../data_day1/part3/
- Mannualy 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

- 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 Couting
  - Open “Cell_Colony” from Fiji samples
  - Run “Edit / Invert”
  - Subtract Background
  - Run “Find Maxima”

Ensuring image quality for image analysis

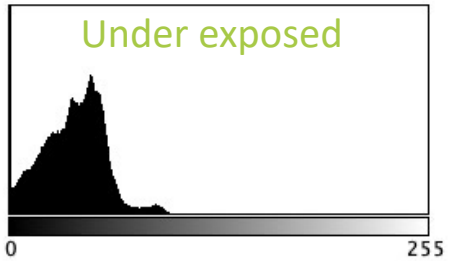
Image artifacts

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

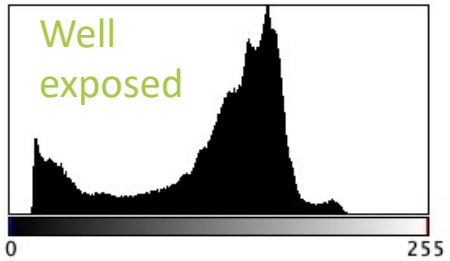
Let's have a look at some examples…

Saturation

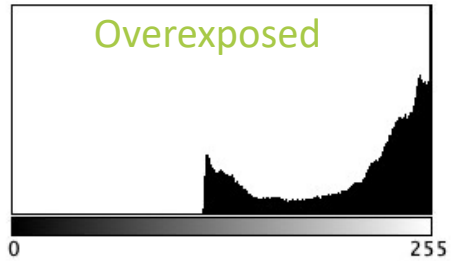
- Histograms help to identify saturation



Count: 397987 Min: 0
Mean: 29.183 Max: 112
StdDev: 23.387 Mode: 0 (104833)



Count: 397987 Min: 9
Mean: 122.848 Max: 220
StdDev: 46.984 Mode: 157 (7435)



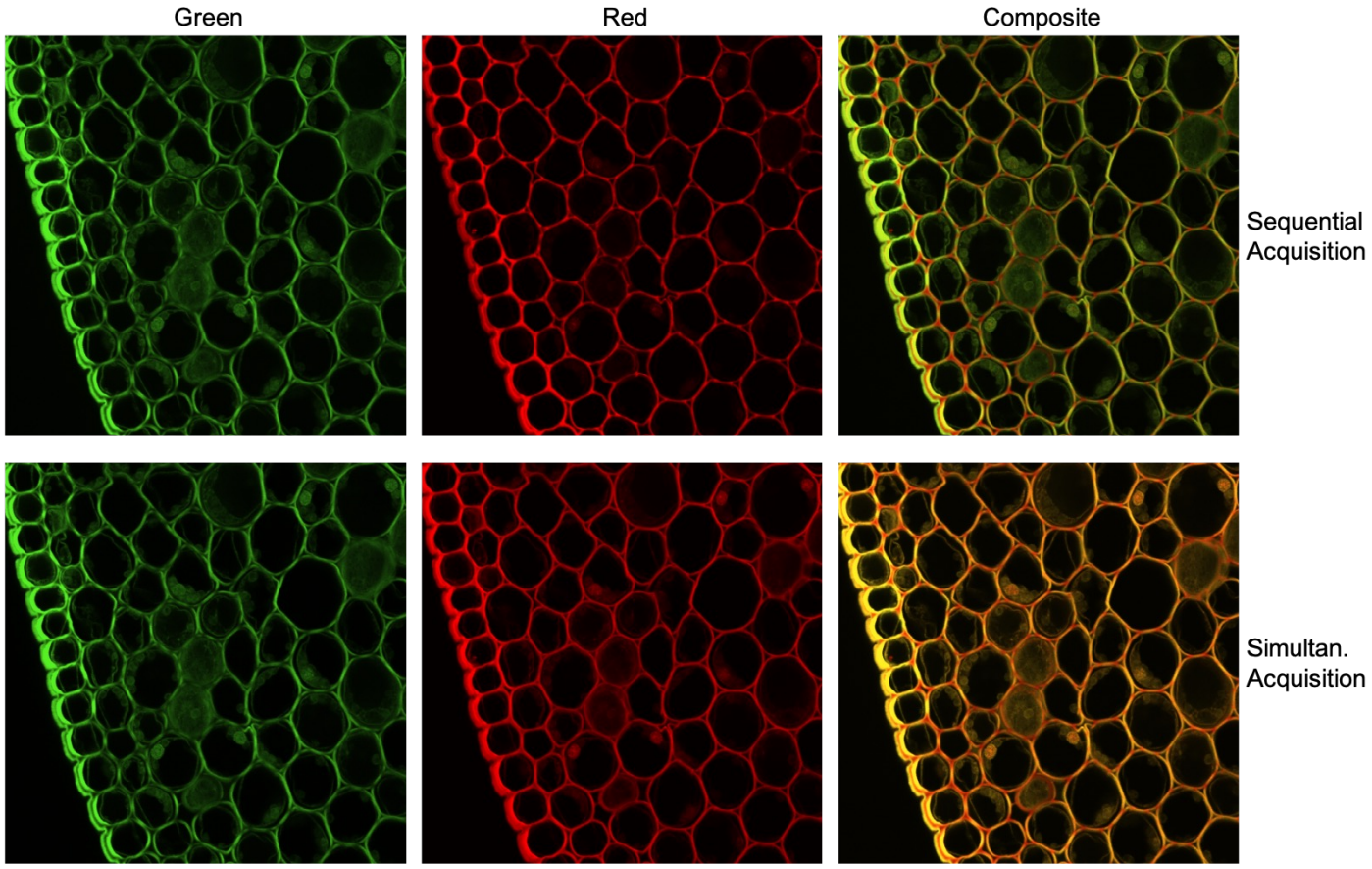
Count: 397987 Min: 112
Mean: 222.028 Max: 255
StdDev: 43.466 Mode: 255 (130741)

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

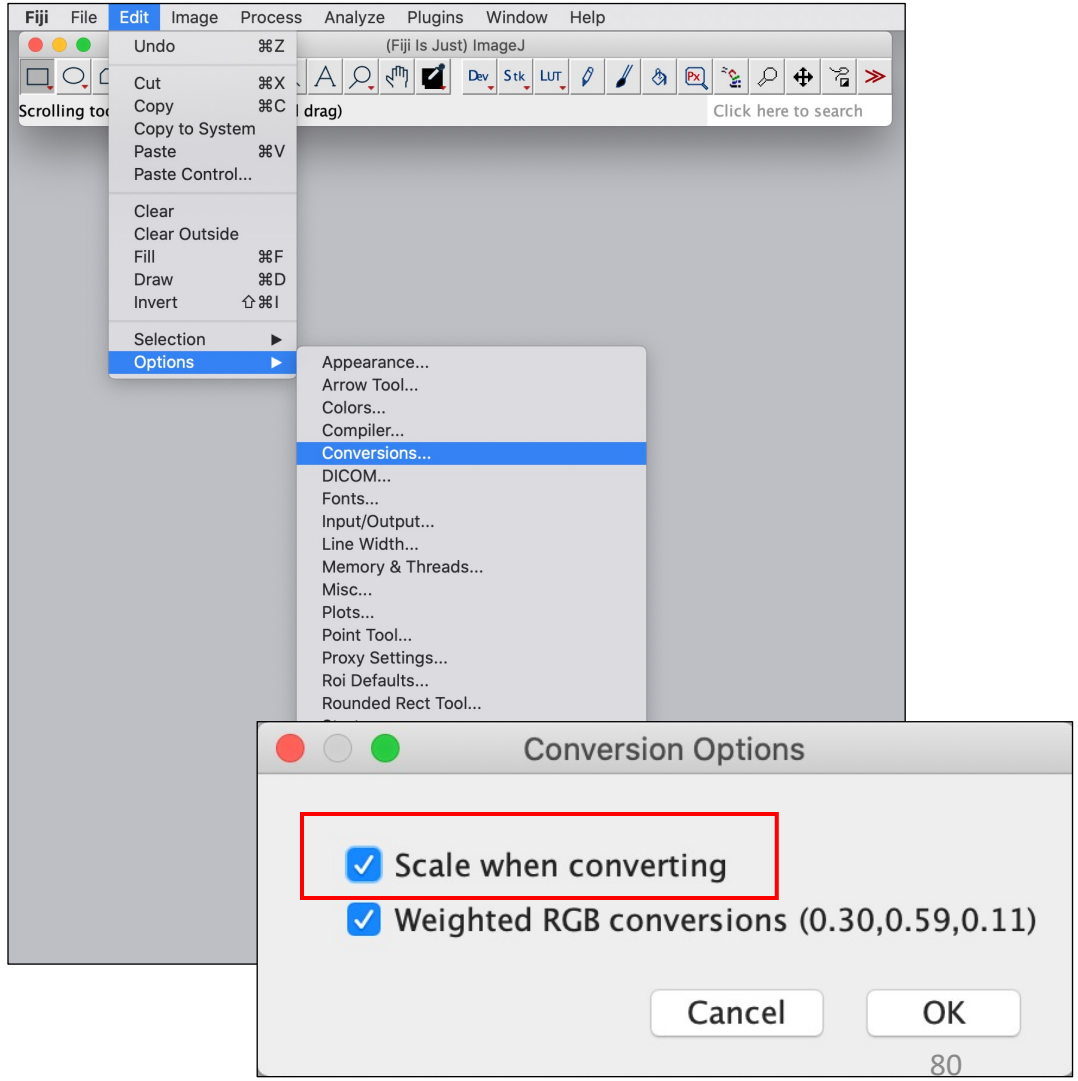
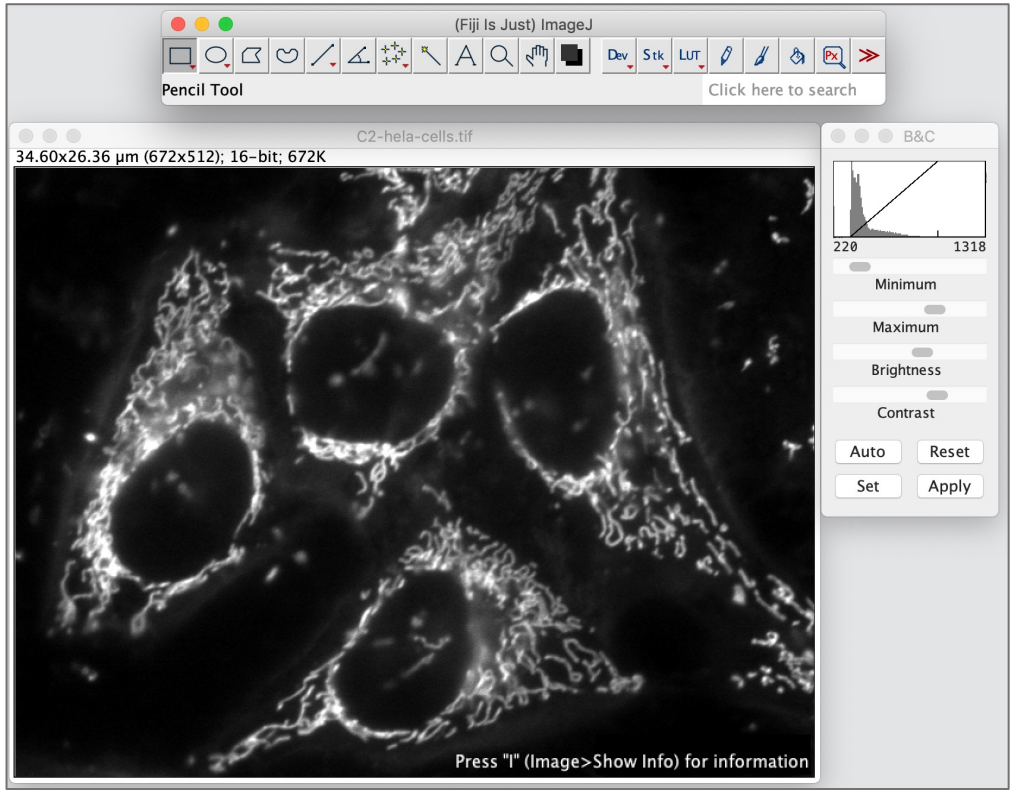


Image operations

- 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

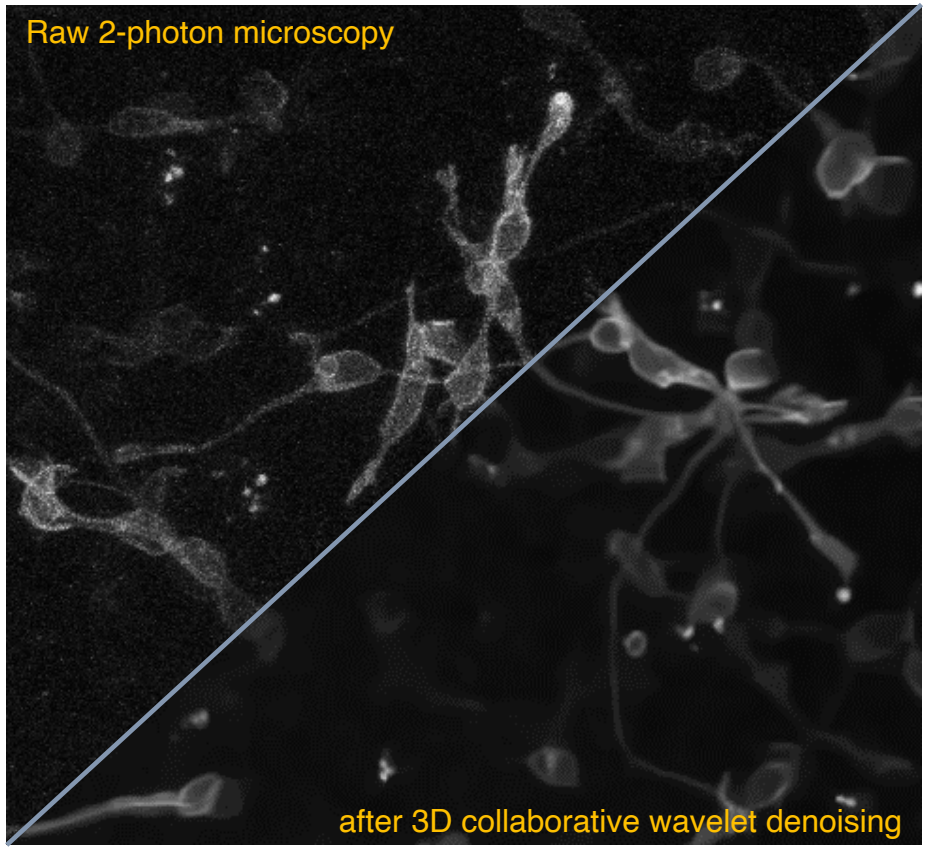
- Some image artifacts cannot be corrected by image analysis
  - Ex: saturation, bleedthrough, etc

- Others can be corrected, but sometimes it's better to correct them while acquiring the image

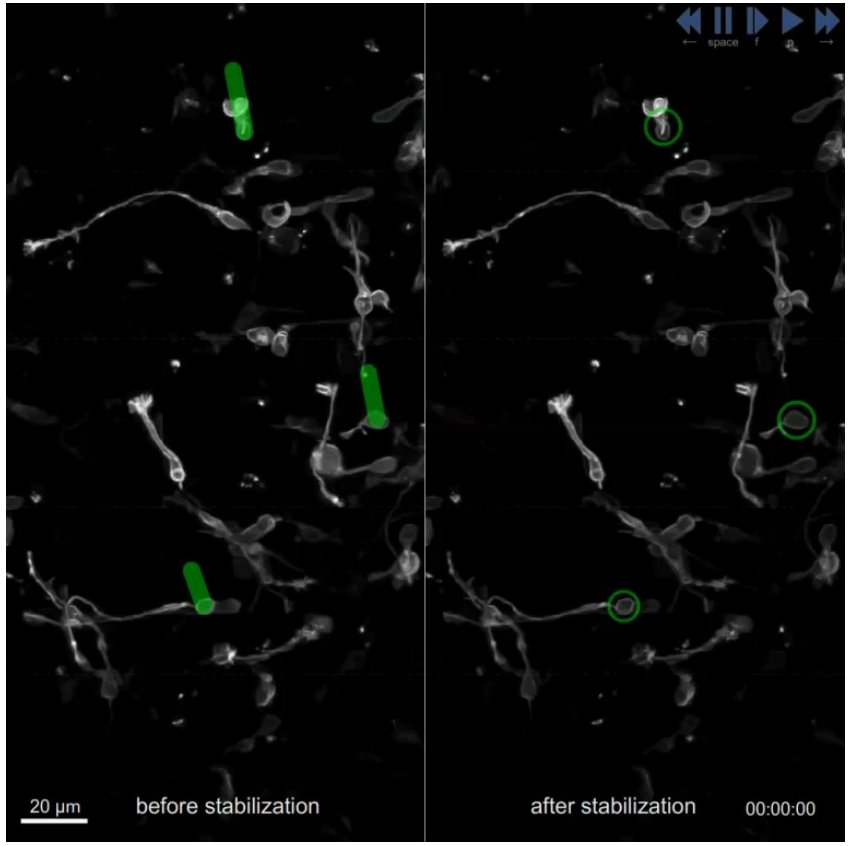
Examples…

Image processing and enhancement

Denoising



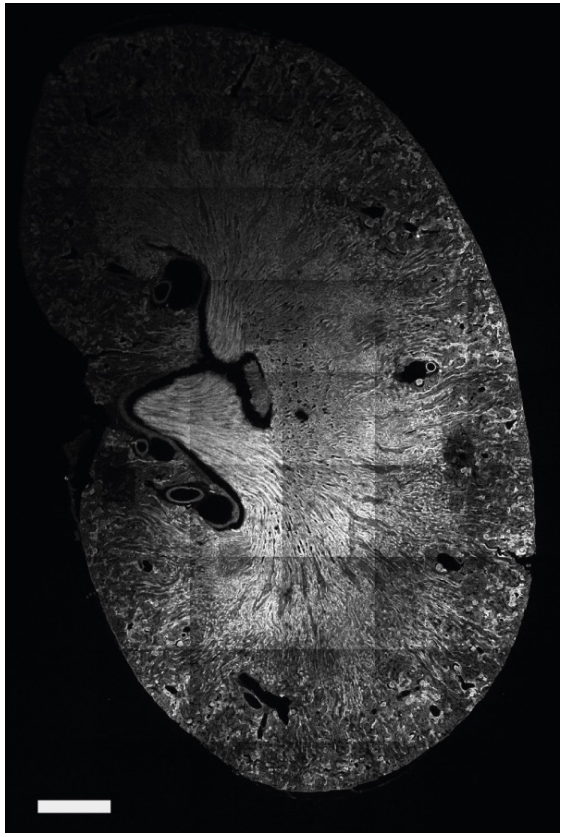
Stabilization



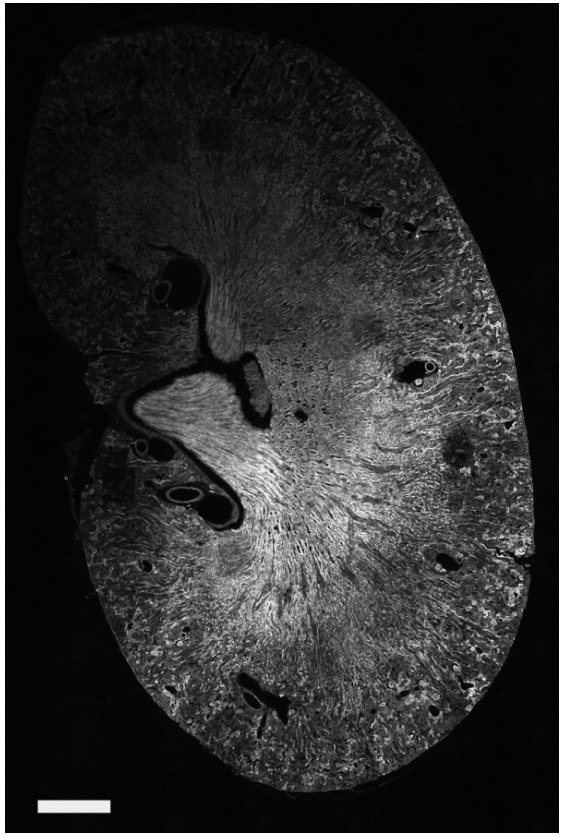
K. Smith *et al.* General Constraints for Batch Multiple-Target Tracking Applied to Large-Scale Videomicroscopy, *CVPR* (2008)

Illumination correction

Original test image mosaic

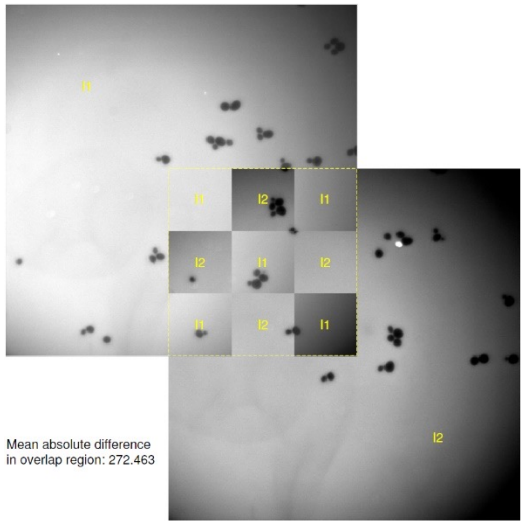


Corrected image

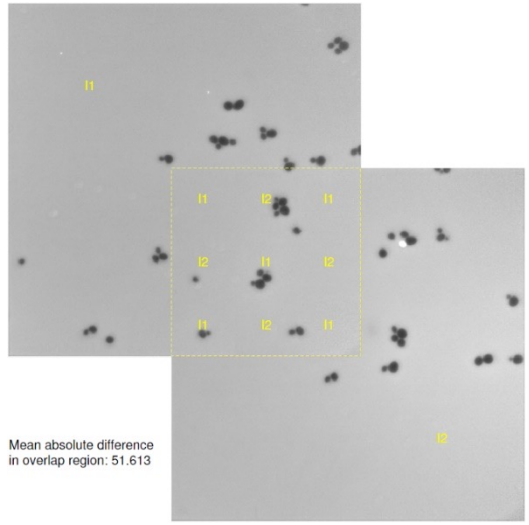


Fluorescent marked mouse kidney section. Confocal microscope with glass slide air interface.

Original test image pair



Corrected pair



K. Smith *et al.* CIDRE: An Illumination Correction Method for Optical Microscopy. *Nature Methods*, 12 (5) 404-406 (2015)

Practical Exercises

- 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

- 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

gmirand@kth.se

@giselehbm

Gisele Miranda

More info

- ✓ https://www.scilifelab.se/facilities/bioimage-informatics/
- ✓ https://biifsweden.github.io/

Thank you!