MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

MICROSCOPY IN CELL BIOLOGY –

aa 2022/2023, 2nd semester

Aula 5A, edificio H2bis ~~14-17~~ 15-18

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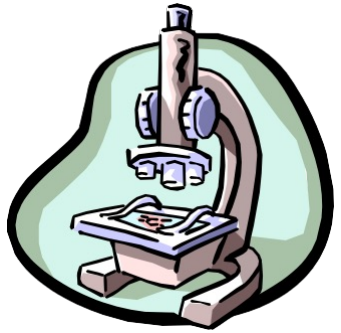
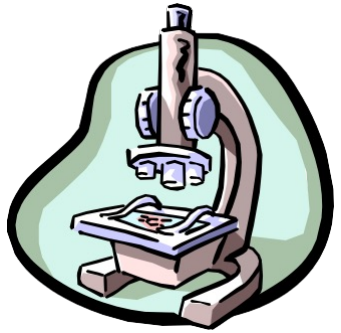
Dipartimento di Scienze della Vita

date	lesson/lab	aula	time
01/03/23	intro	aula5A, H2bis	14-15 (GF and BT), 16-17 (NS)
08/03/23	lesson1	aula5A, H2bis	15-18
15/03/23	lab1	sala microscopia F2, C1	15-18
22/03/23	lesson2	aula5A, H2bis	15-18
29/03/23	lab2	sala microscopia F2, C1	15-18
05/04/23	lesson3	aula5A, H2bis	15-18
19/04/23	lesson4	aula5A, H2bis	15-18
26/4/23	lesson5	aula5A, H2bis	15-18
3/5/23	lab3	CIMA center	15-18
10/5/23	lab4	aula5A, H2bis	15-18



Live imaging microscopy

LIVE IMAGING



- Allows study of dynamic processes in real time in stead of snap-shot of one timepoint

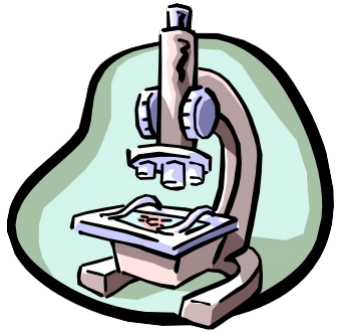
- Results in movie instead of single image

- Dynamic processes of subcellular compartments, cells, cellular networks, organs, entire animals

Examples for dynamic processes of interest

- Cell migration
- Morphological changes
- Physiological parameters (ion concentrations, vesicle dynamics)

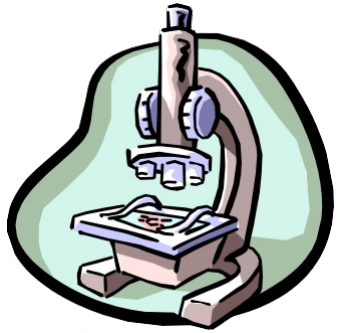
LIVE IMAGING - CHALLENGES



Keeping cells alive

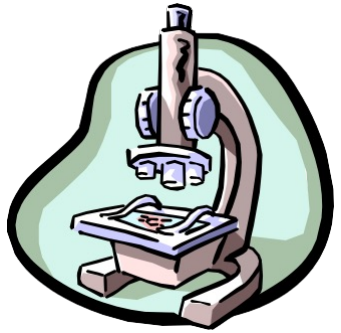
- physiological environment
    - Temperature
    - Ionic and osmotic environment
    - Oxigenation
    - pH (ev. CO_2 concentration for carbonate buffer)
- Mechanical stress
- Phototoxicity
- Chemical toxicity of dyes

LIVE IMAGING - INCUBATION

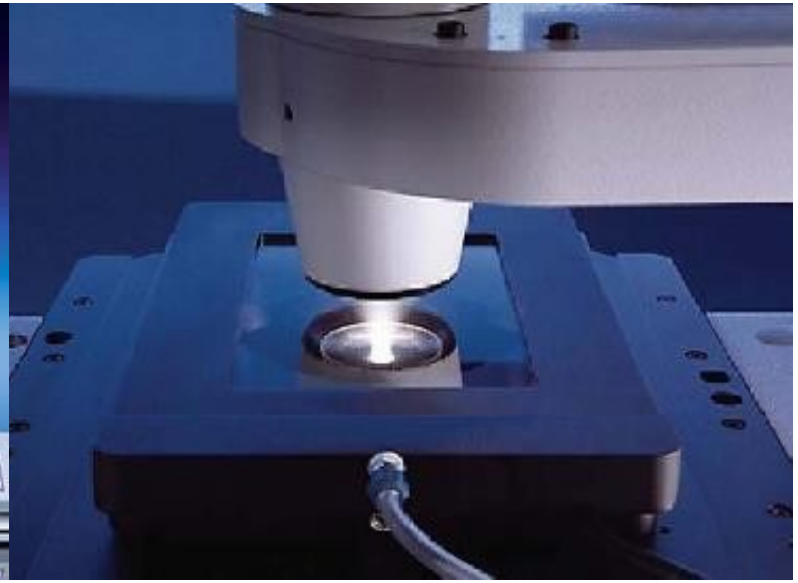


Cell Health Parameter	Short-Term Study	Longer-Term Study
pH	HEPES Buffer	CO_2 Incubator
Temperature	Stage Warmers	Objective Lens Warmers Cell chambers with integrated heating elements Enclose microscope within a heated box
Humidity	Open cell chambers	Tightly sealed cell chambers
Oxygenation	Large volume of media	Media changes during study Large media volume
Osmolarity	Sealed chamber	Enclose system within a humidified chamber

LIVE IMAGING - INCUBATION



Incubation chambers can surround the microscope stand or just the cell dish. Both can maintain cells at the correct temperature for long periods of time.



CONTRASTING TECHNIQUES

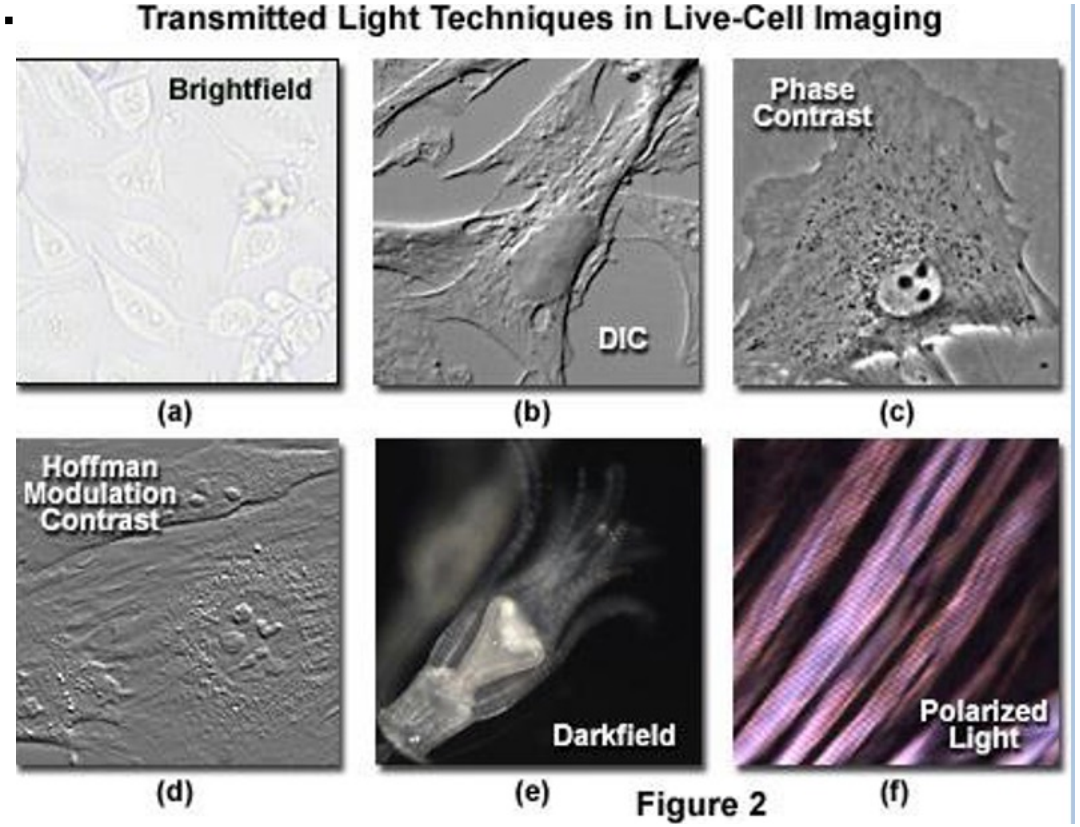
Optical Techniques to Enhance contrast:

Differential Interference Contrast (DIC)
Phase Contrast

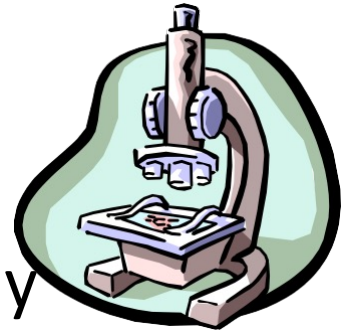
Darkfield

Polarized Light

Fluorescence



PHOTOTOXICITY

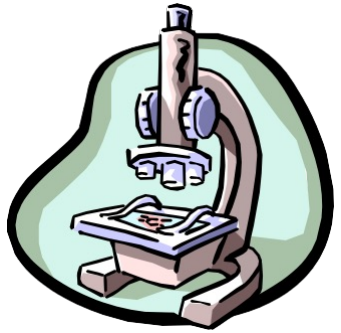


The **central** challenge in live cell imaging – Cells are inherently photosensitive!

- Formation of free radicals by fluorophores only exacerbates the problem
- Free radical formation cannot be prevented, only managed by careful tuning of imaging parameters to reduce light exposure

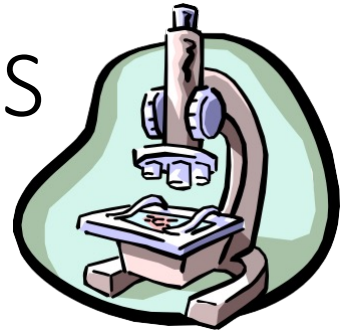
Live cell imaging requires “gentle imaging” - short exposures of low intensity Illumination

FLOURESCENCE LABELLING OF LIVE CELLS



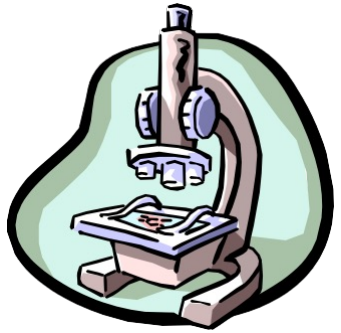
- Detect specific molecules (immunocytochemistry, fluorescent proteins)
- Must keep the specimen transparent
- Targeting live cell (membrane permeability)
- Eventually detect multiple molecules at the same time

MINIMIZING DAMAGE WHILE GETTING GOOG IMAGES

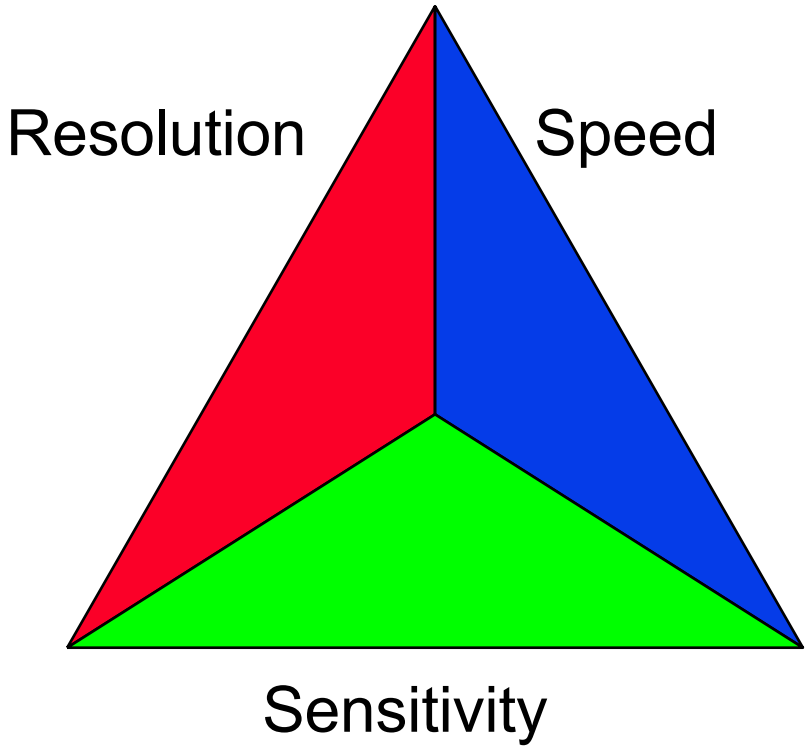


- Resolution vs Speed vs Sensitivity
- Light Gathering Power and Focus Maintenance
- Which immersion media
- Correct coverslip thickness
- 3D Imaging

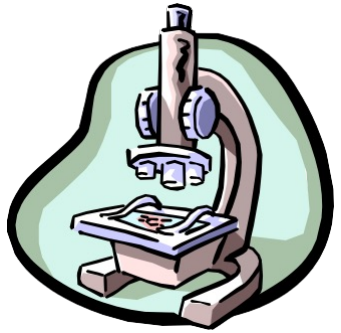
RESOLUTION-SPEED-SENSITIVITY



- Spatial Resolution
  - Depends on application optical magnification and lens apperture
  - Smaller and more pixels = higher resolution
- Sensitivity
  - Larger pixels = higher sensitivity
  - Higher sensitivity = shorter exposure time
  - Shorter exposure time = higher frame rate
  - Larger pixels = lower resolution
- Speed
  - Less pixels = faster readout
  - Faster Readout = higher framerate
  - Less pixels = lower resolution
  - requires fast readout and high sensitivity



CORRECTION IMMERSION MEDIA

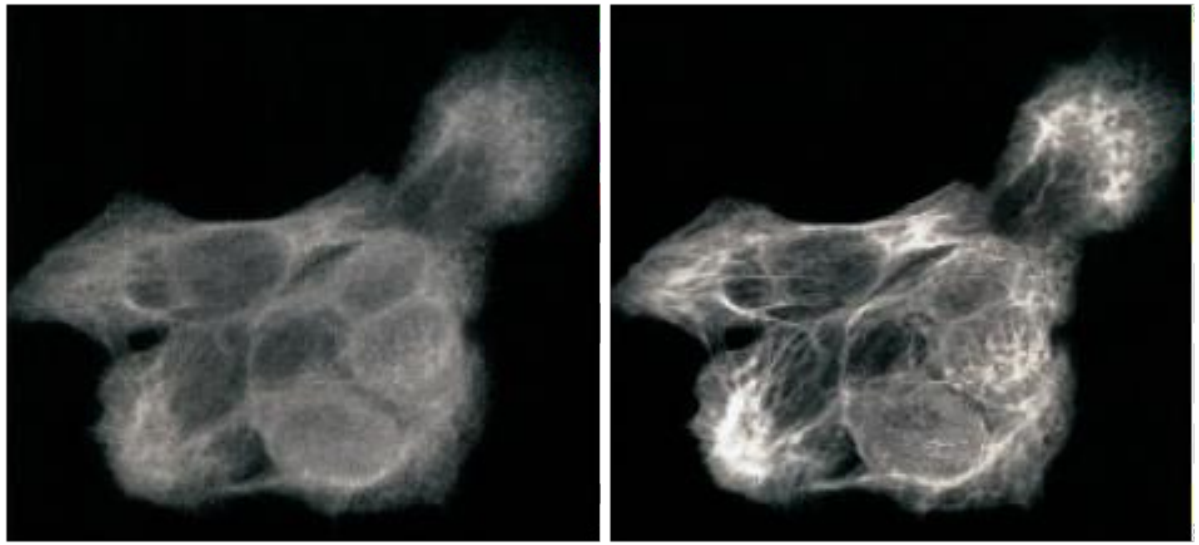


oil immersion objective

NA=1.4

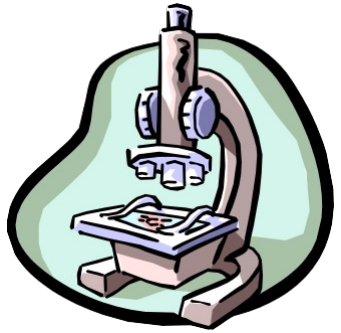
water immersion objective

NA=1.2

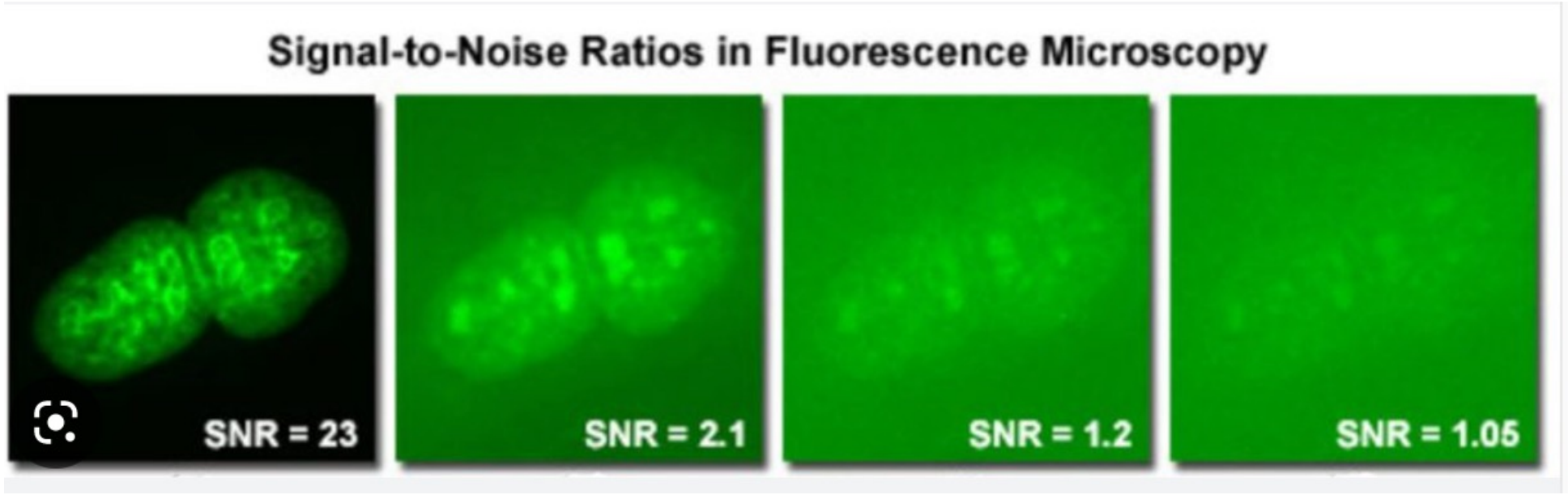


Using water immersion objectives for samples in liquid media captures more signal and preserves resolution better than refractive index mismatched oil immersion objectives.

SIGNAL-TO-NOISE

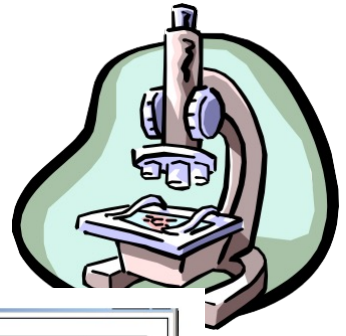


- Camera Sensitivity -
  - Signal needs to be 2-3x brighter than background noise to be detected.

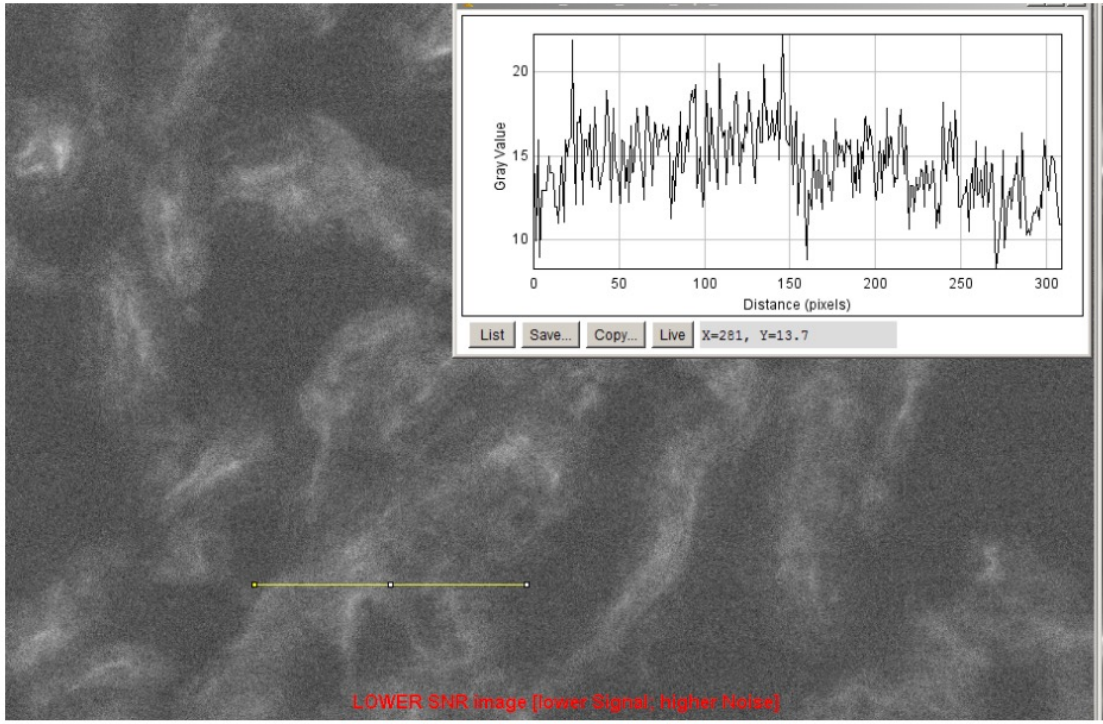


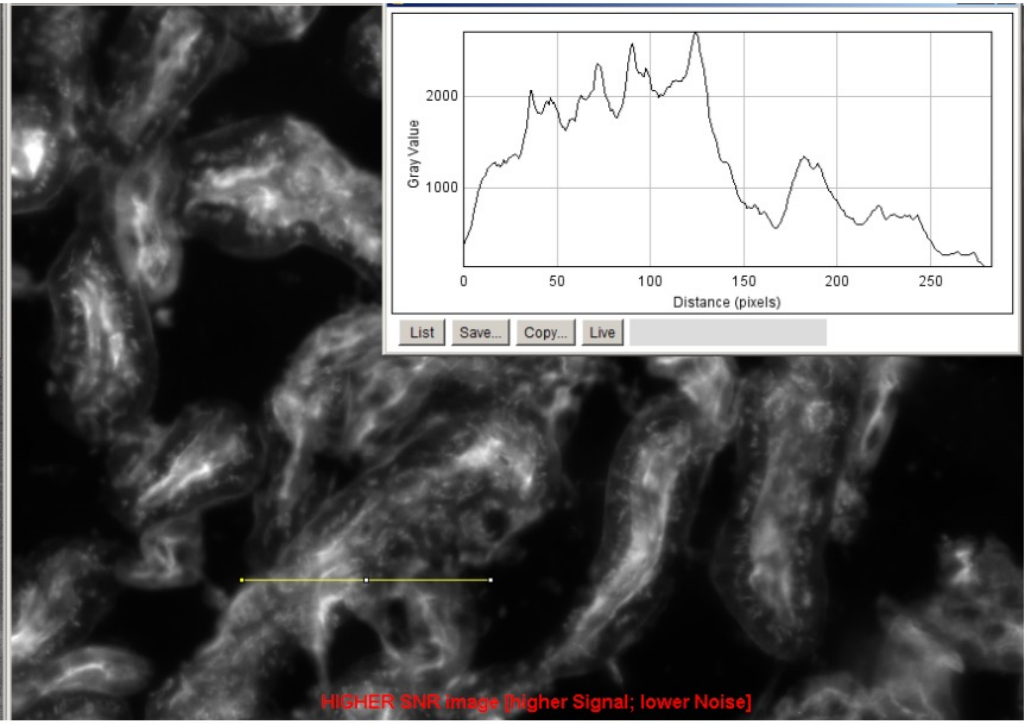
  - Increasing the exposure time (integration time) or increasing the illumination intensity produces more signal.

SIGNAL-TO-NOISE



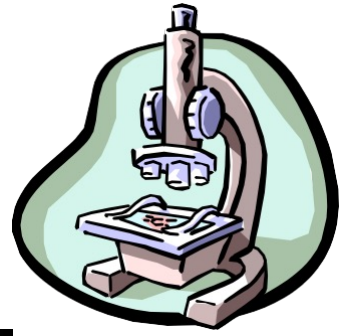
https://scientificimaging.com/knowledge-base/signal-and-noise-quantitative-explanation/

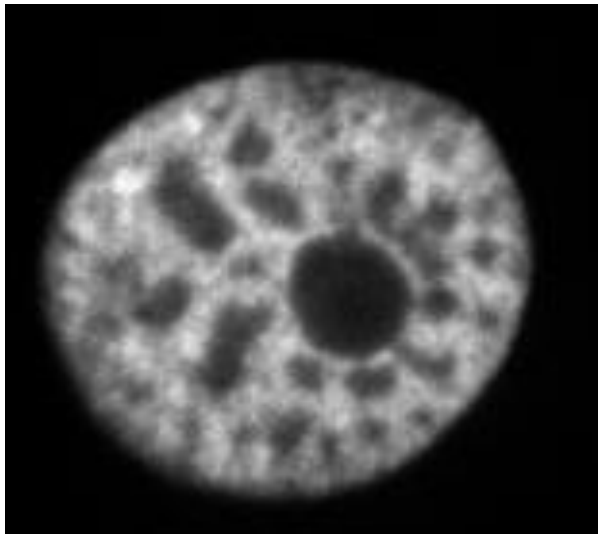




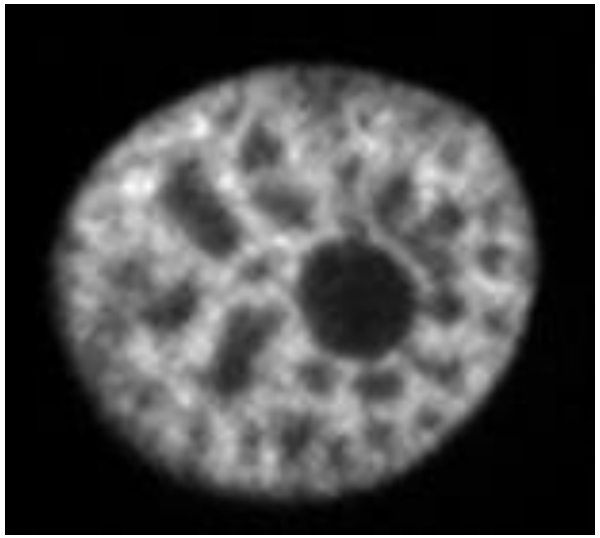
A fluorescence slide was imaged with the same excitation & filters, but with different camera exposures. The LOWER SNR image was obtained with a short=2.5ms exposure and the HIGHER SNR image was obtained with a long=1sec exposure.

RESOLUTION VS SPEED

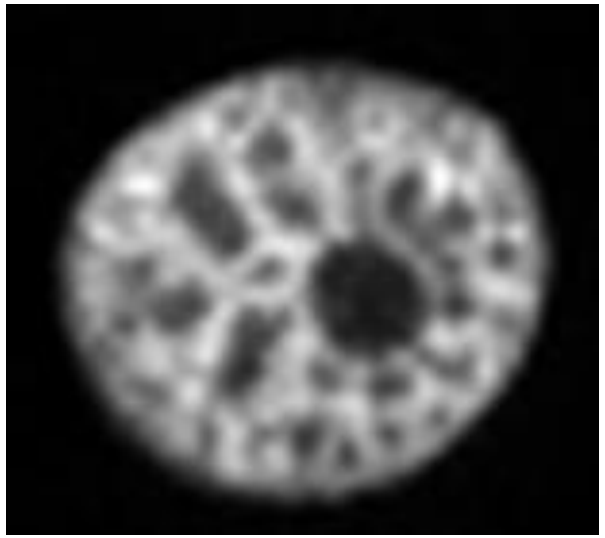




512ms
1x1 binning



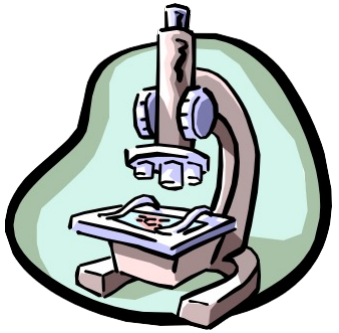
117ms
2x2 binning



28ms
4x4 binning

Using binning shortens exposure times reducing cell phototoxicity and fluorophore photobleaching

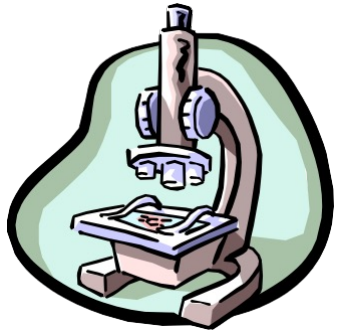
CHANGES IN FLOURESCENCE



- Changes of flourescence intesity
  - Examples: calcium imaging, vesicle release

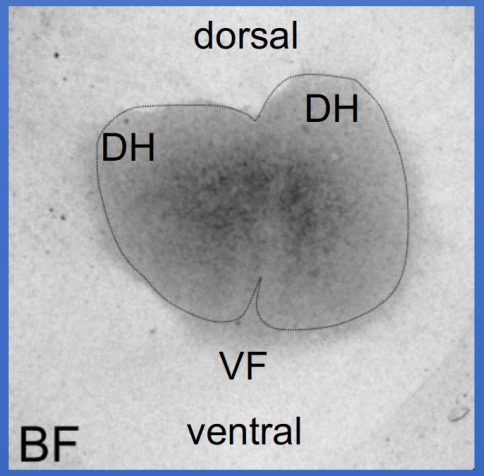
- Changes of flouresence localisation
  - Examples: Mitochondrial dynamics

IMAGING CHANGES IN ION CONCENTRATIONS

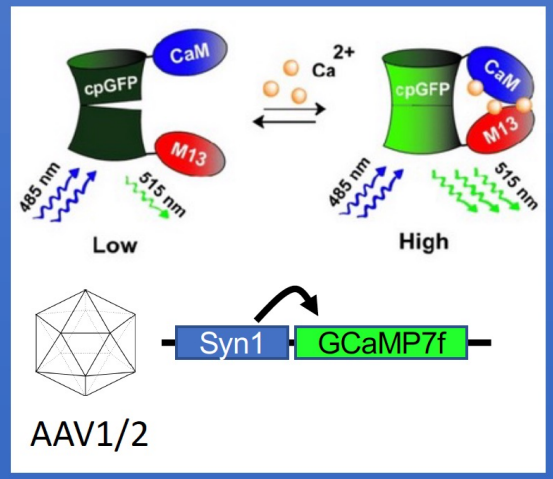


A commonly performed live flourescence imaging method is ion imaging (calcium, chloride, magnesium, etc) using either fluorescent dyes or proteins especially designed to change their emission behavior upon binding of the respective ion.

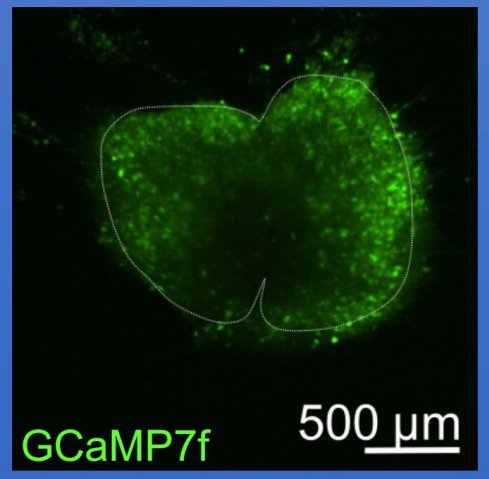
IMAGING CHANGES IN ION CONCENTRATIONS

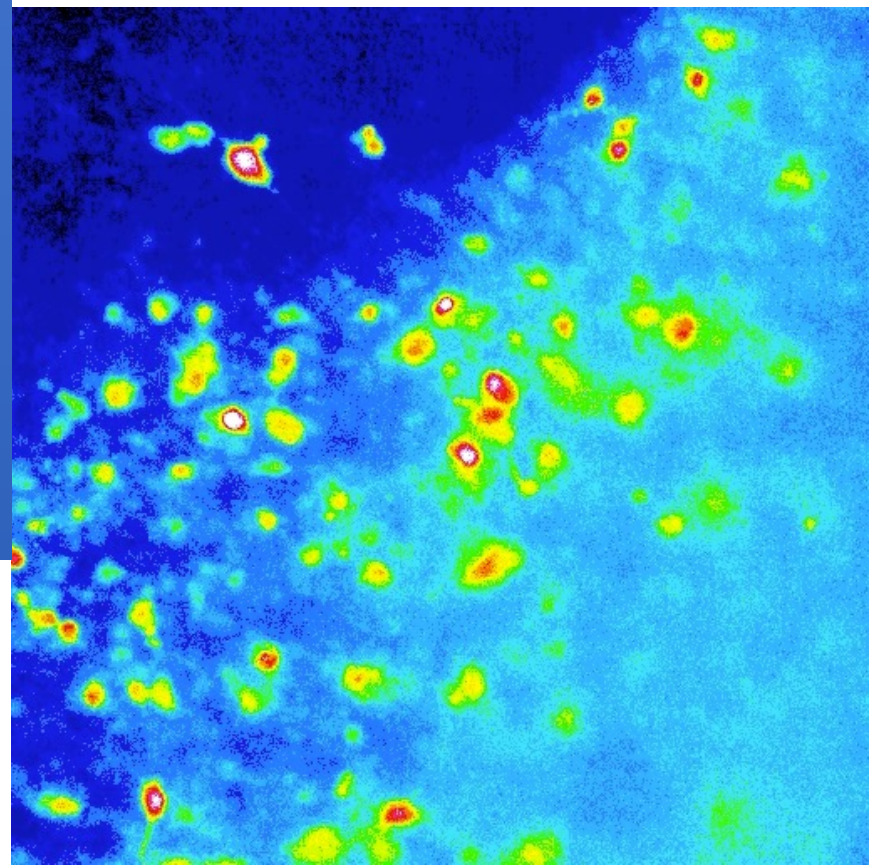


Organotypic spinal cord culture at DIV 18. DH, dorsal horn; VF, ventral fissure.



Organotypic spinal cord cultures were infected with AAV1/2 at DIV13, expressed calcium indicator GCaMP7f under control of the neuron-specific promotor Syn1 for 4-6 days. Image of GCaMP7f fluorescence.

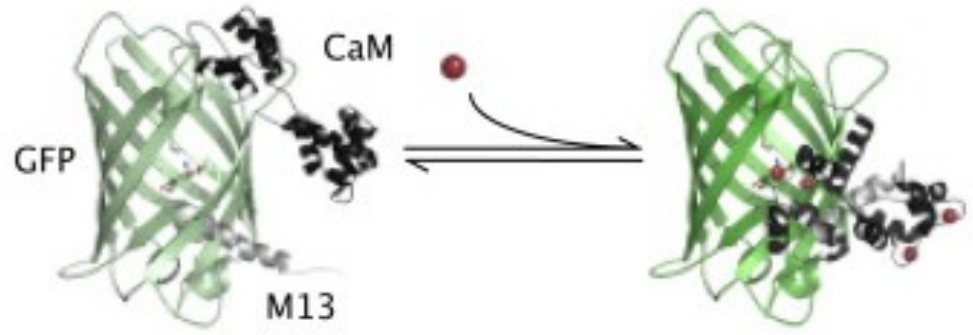


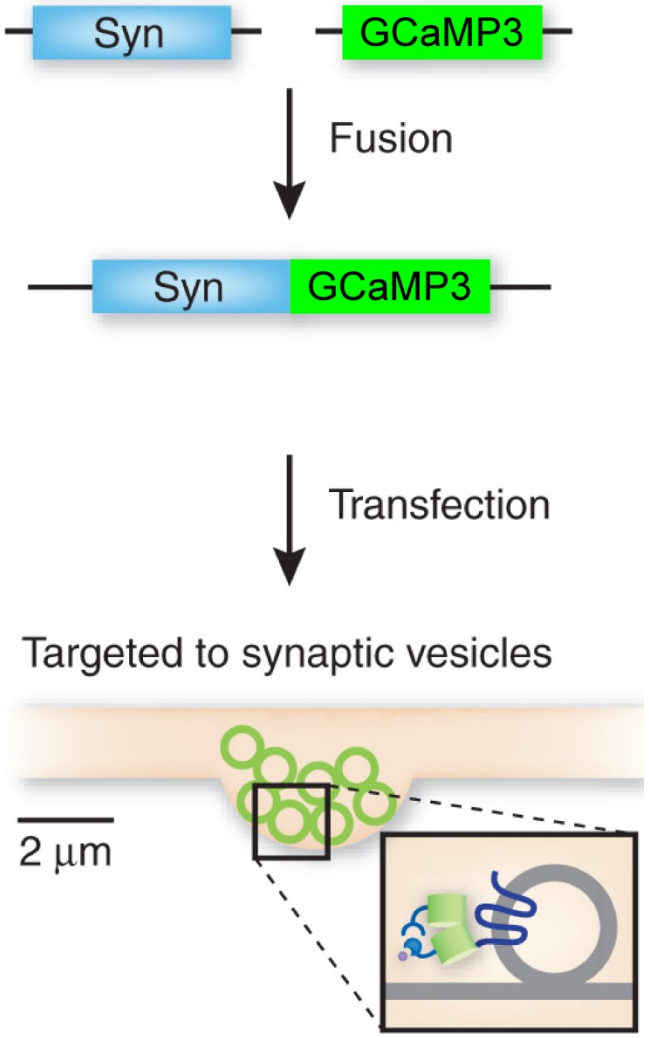


Thalhammer et al, 2022

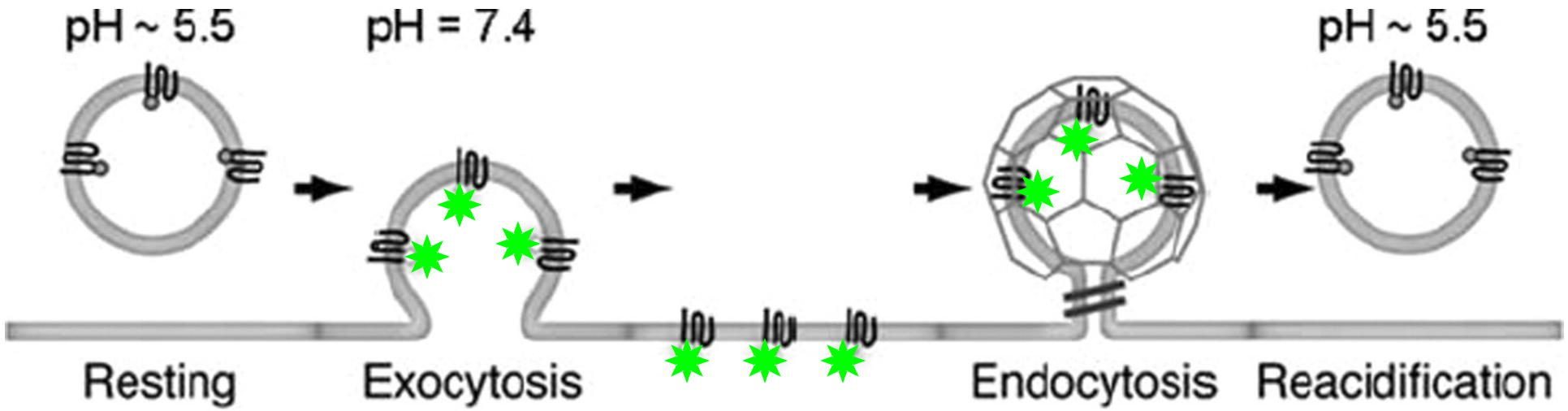
SYNAPTIC TARGETING OF GCaMPs

GCaMP

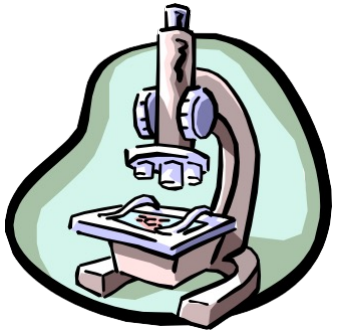




SYNAPTOPHYSIN-PHLUORIN (SYPHY) – IMAGING OF VESICLE RELEASE



CHANGES IN FLOURESCENCE



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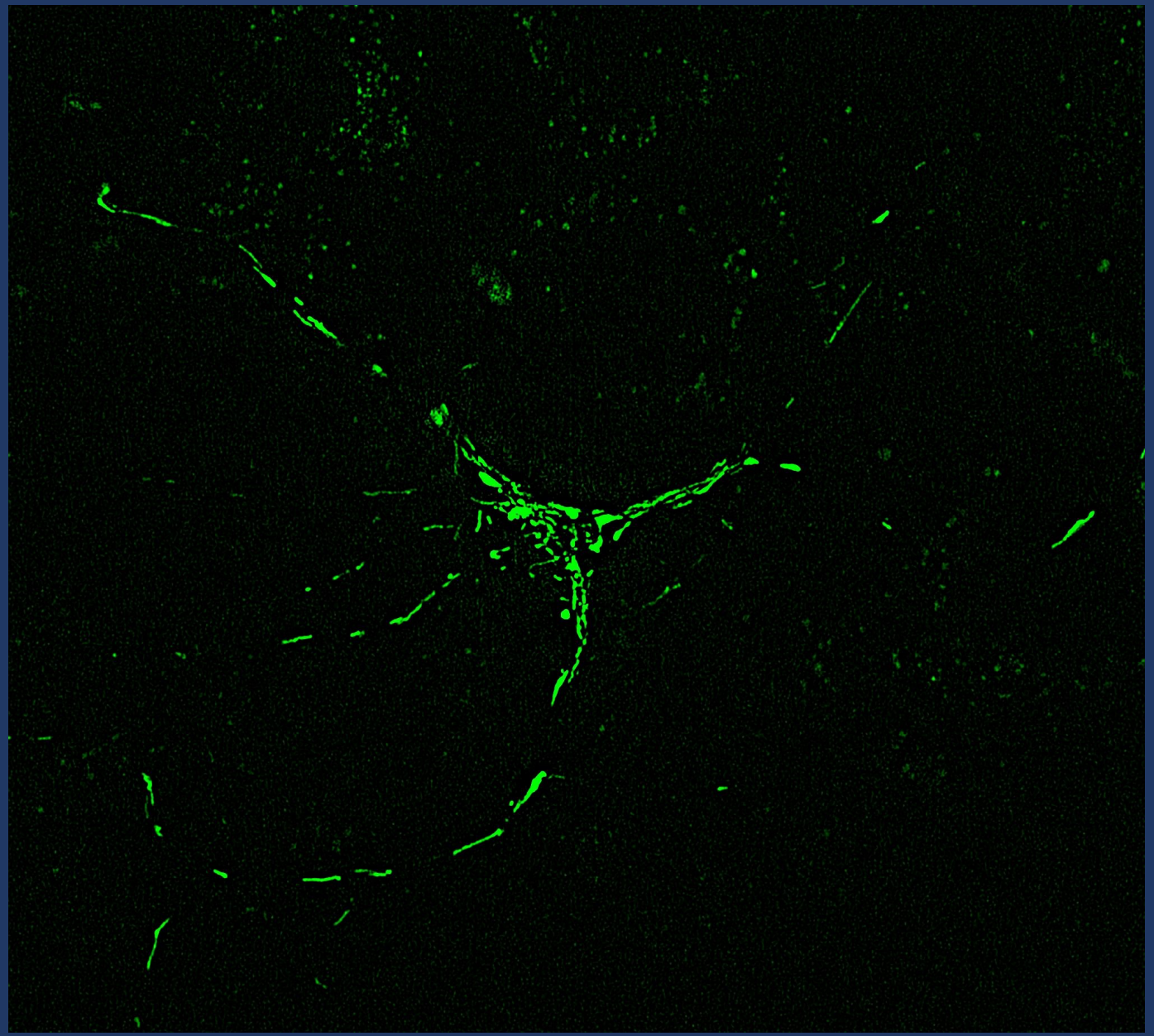
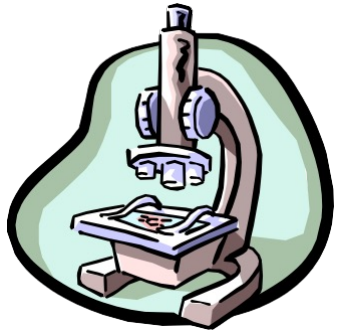
  - Examples: calcium imaging, vesicle release

- Changes of flouresence localisation
  - Examples: Mitochondrial dynamics

MITOCHONDRIAL PARTICLE DYNAMICS

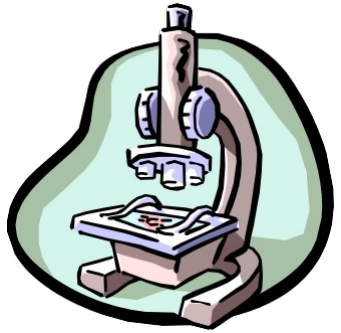


OTHER LIVE IMAGING TECHNIQUES



- Vesicle fusion/release (TIRF or pH-sensitive GFP)
- FRAP
- FRET
- Photoactivation/Photoconversion

RESOLUTION-SPEED-SENSITIVITY



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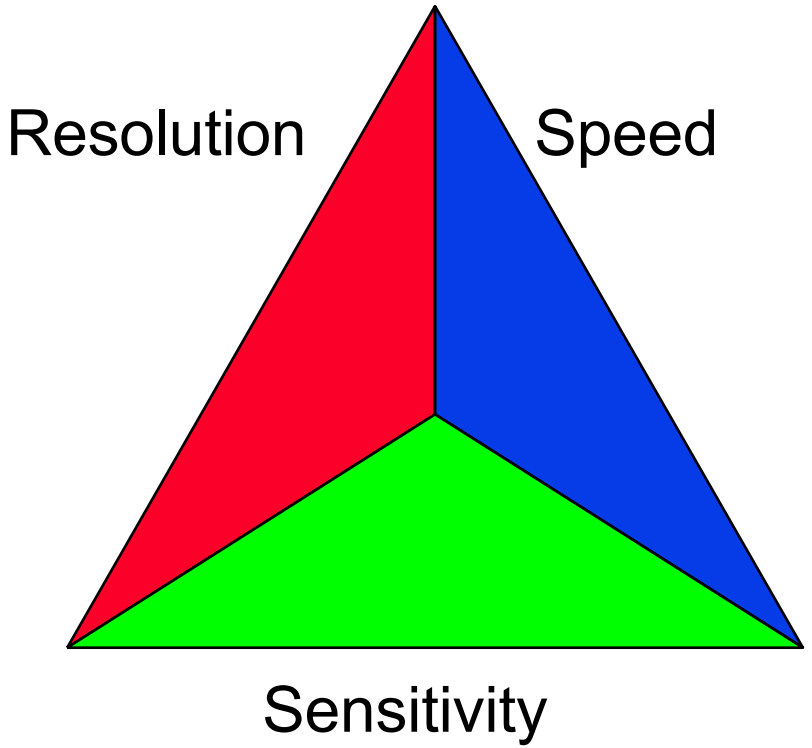


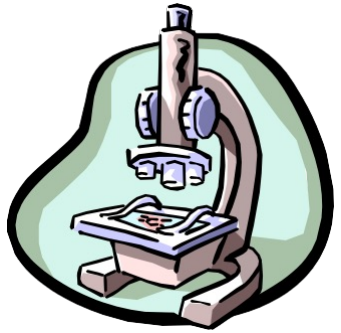
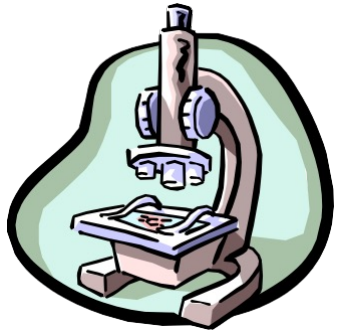


Image processing and analysis

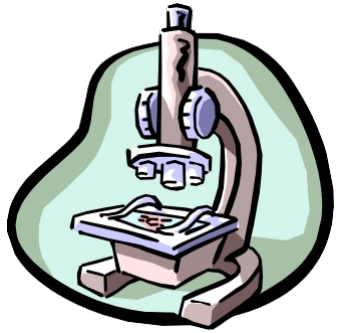
DIGITAL IMAGES



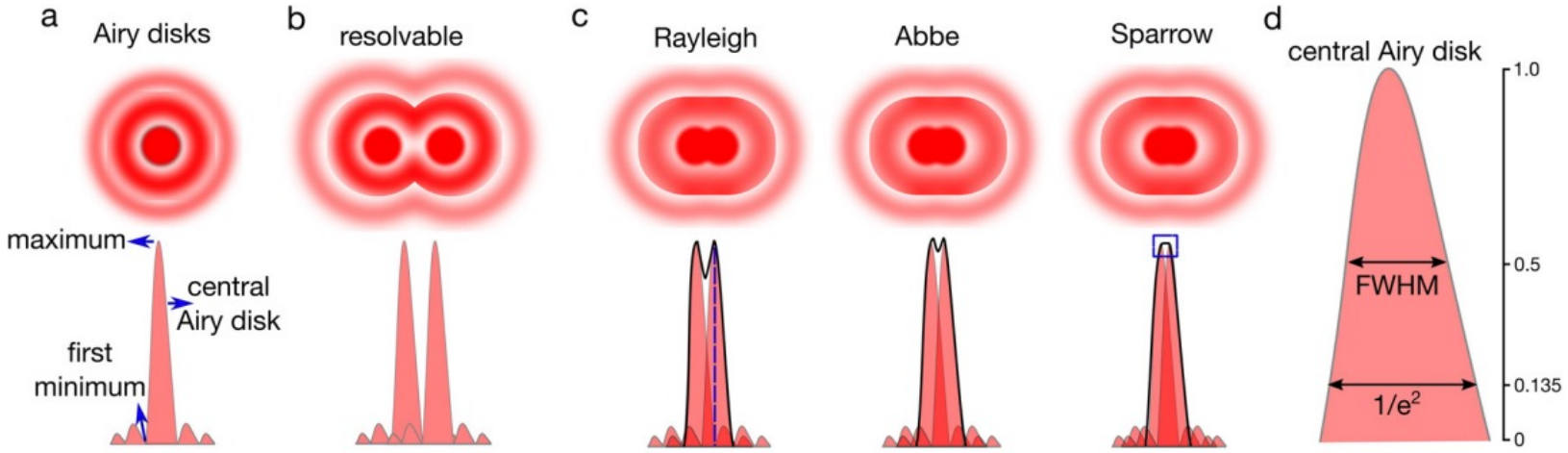
- 2D structure biult from pixels
- Can be seen as a matrix comprised of *m* columns and *n* rows, pixel location denoted by indes (i,j)
- for: $0 \leq I < n$ and $0 \leq j < m$

0,0	0,1	⋯	0,*m*
1,0	1,1	⋯	1,*m*
⋮	⋮	⋱	⋮
n,0	*n*,1	⋯	*n*,*m*

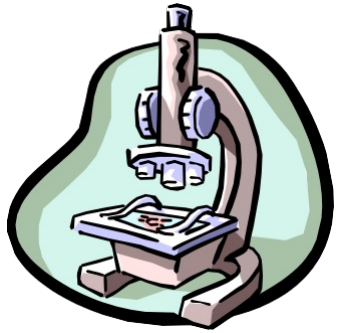
RESOLUTION

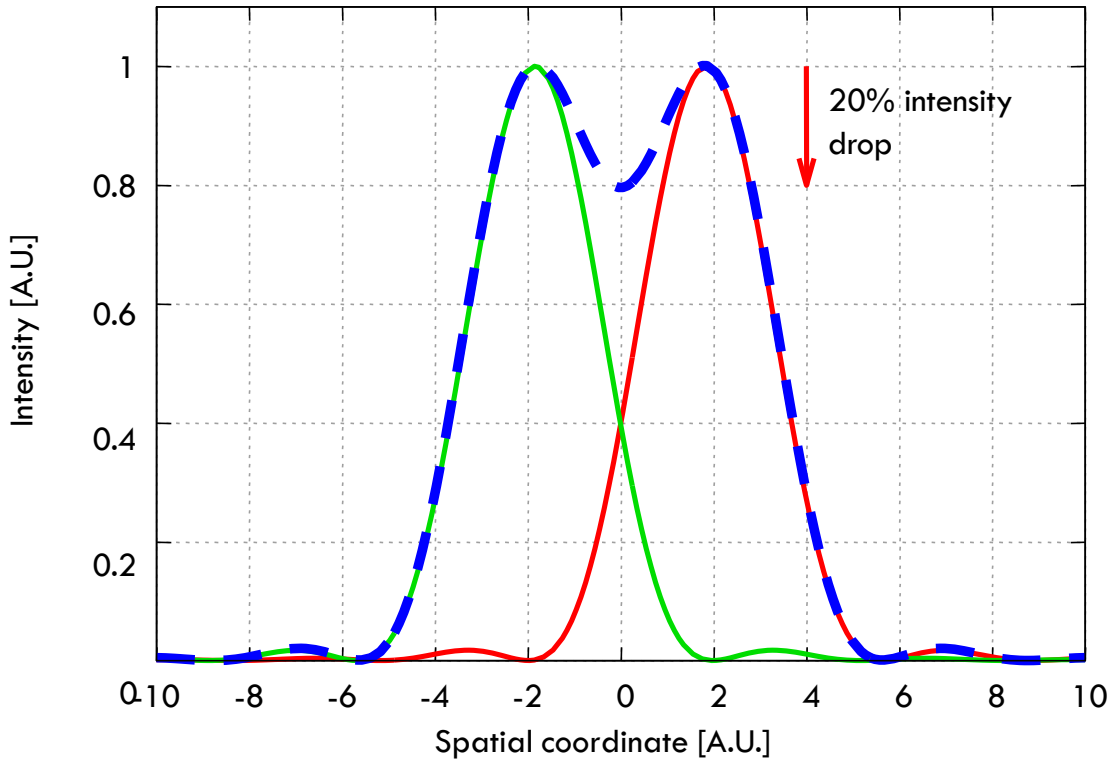


Microscopy	Digital image processing
Rayleigh criterion	The spatial resolution of an image is the physical size of a pixel in an image
Sparrow criterion	
full width at half maximum (FWHM)	



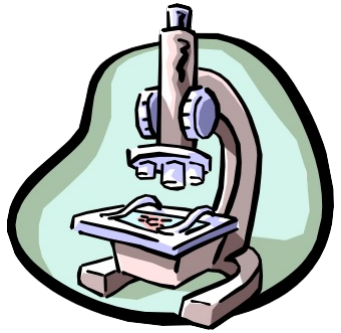
RAYLEIGH CRITERION





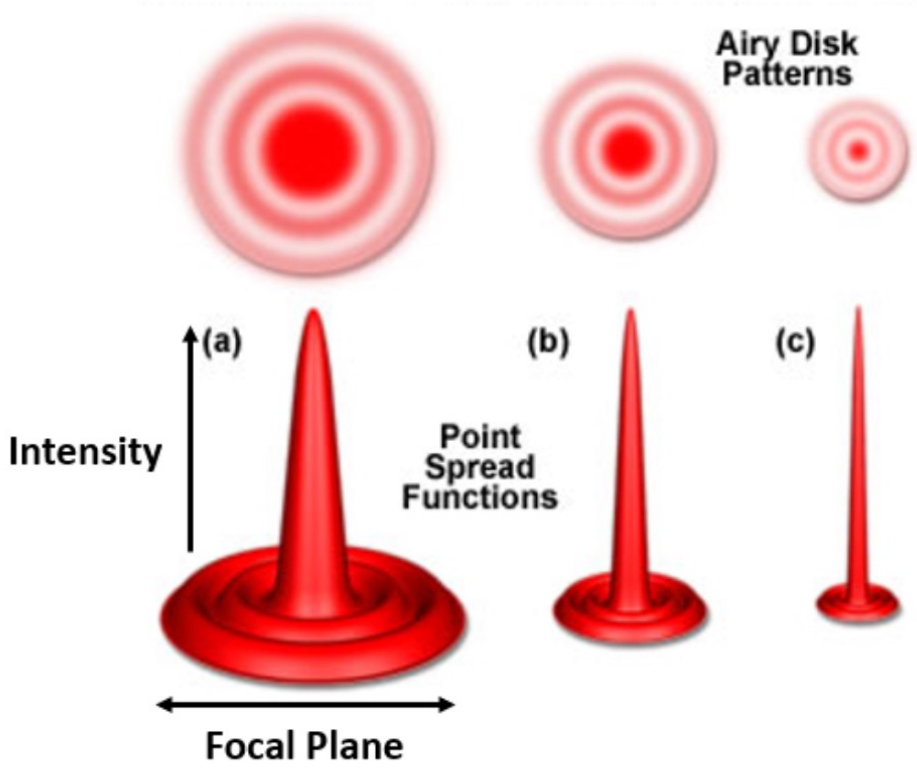
The **Rayleigh criterion** is the generally accepted, although arbitrary, criterion for the minimum resolvable detail – the imaging process is said to be diffraction-limited when the first diffraction minimum of the image of one source point coincides with the maximum of another.

POINT-SPREAD FUNCTION

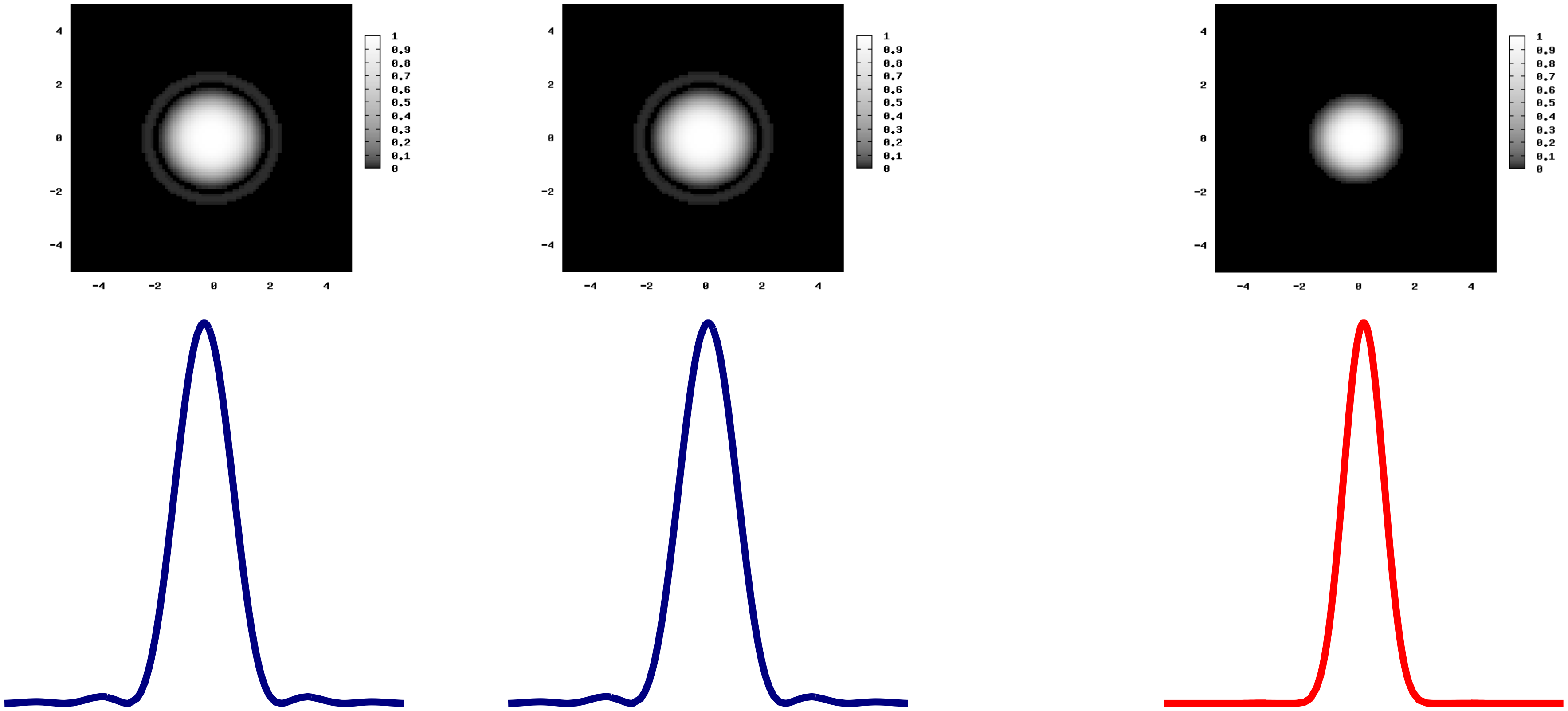


- The point spread function (PSF) describes 3D light distribution in an image of a point source (for a given lens)

- An x-y slice through the center of the wide-field point spread function reveals a set of concentric rings: the so-called Airy disk that is commonly referenced in texts on classical optical microscopy

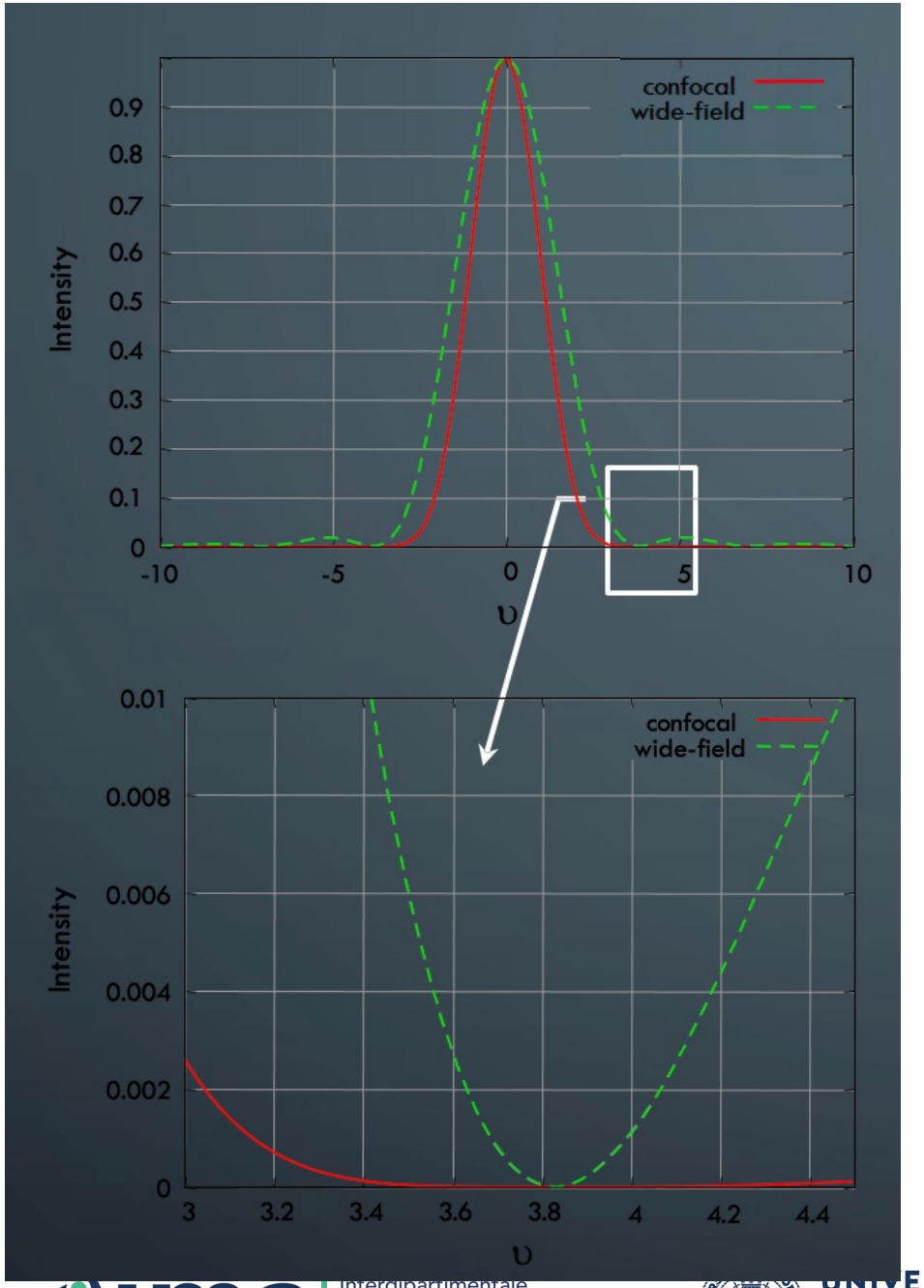


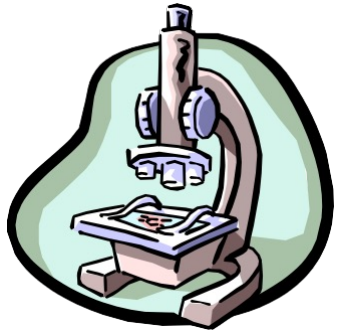
POINT-SPREAD FUNCTION - CONFOCAL



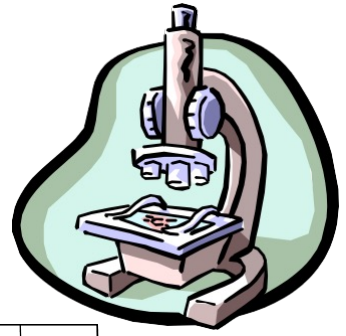
$$PSF_{exc} \bullet PSF_{det} = PSF_{conf}$$

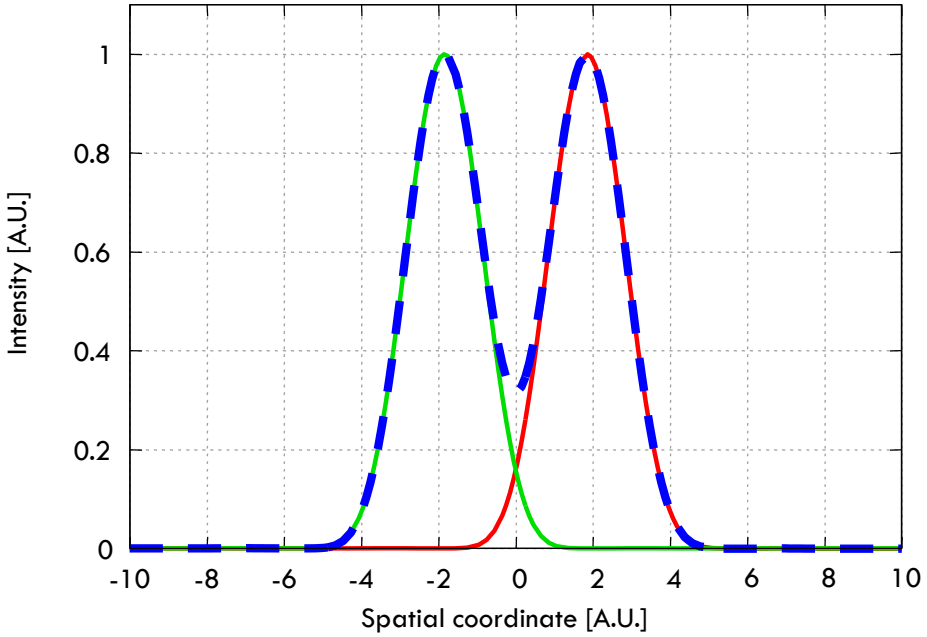
RESOLUTION - CONFOCAL

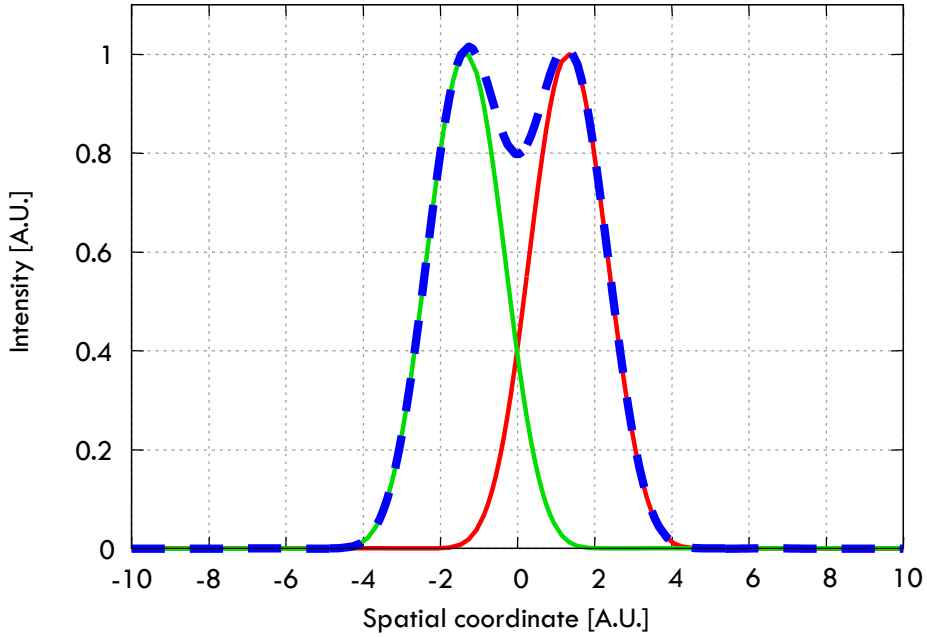




RESOLUTION - CONFOCAL

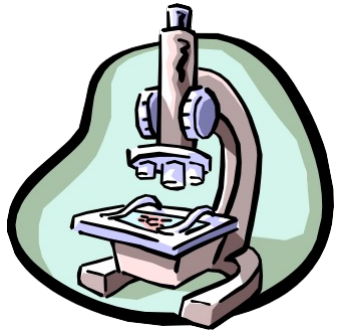


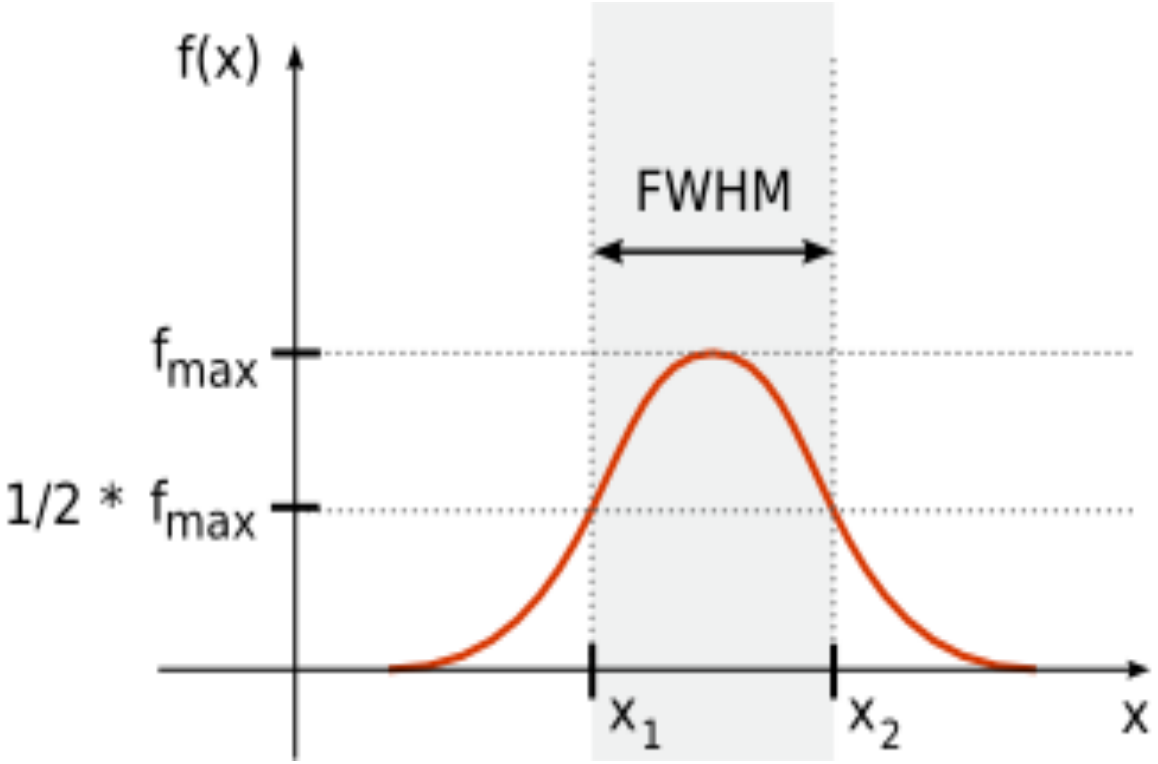




Rayleigh criterion cannot be used directly to define the improvement of resolution in a confocal microscope. The position of the first minimum does not change. The drop in intensity is much larger, though. In fact, we can move two intensity distributions a little bit closer (1.4 times), and still get the required 20% drop in intensity.

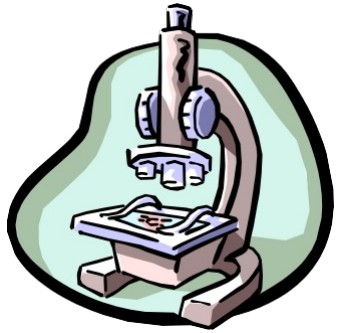
RESOLUTION - CONFOCAL

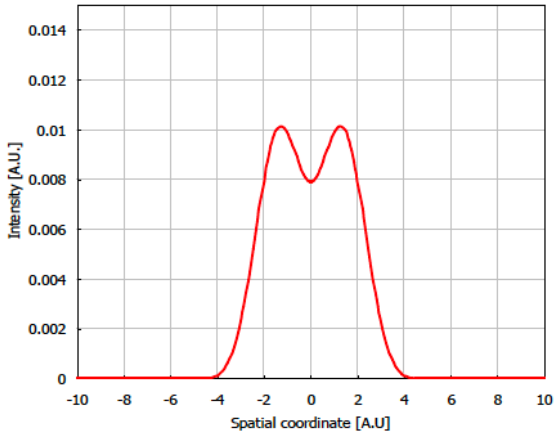


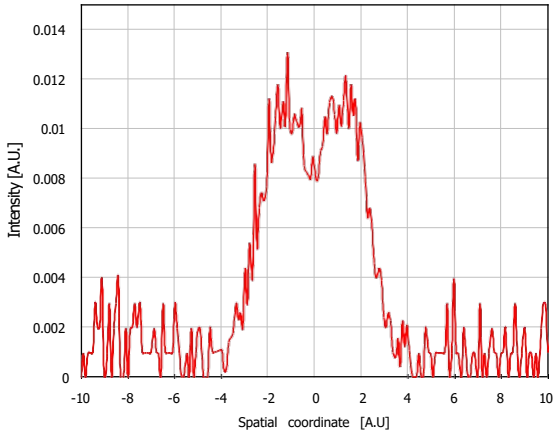


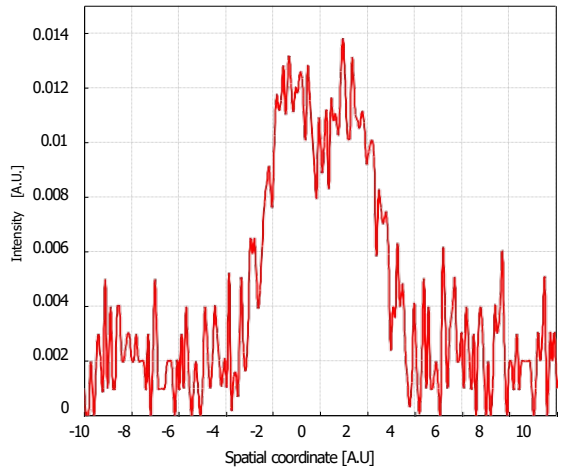
The full width at half maximum (FWHM) is a parameter commonly used to describe the width of a "bump" on a curve or function. It is given by the distance between points on the curve at which the function reaches half its maximum value.

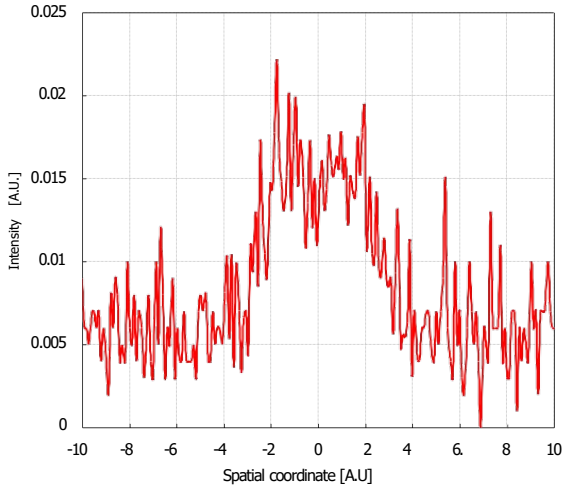
SIGNAL-TO-NOISE RATIO AND RESOLUTION





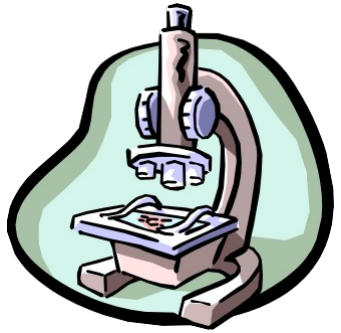






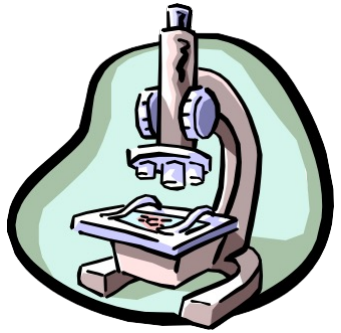
The influence of Poisson noise on two intensity distributions separated spatially according to the Rayleigh criterion.

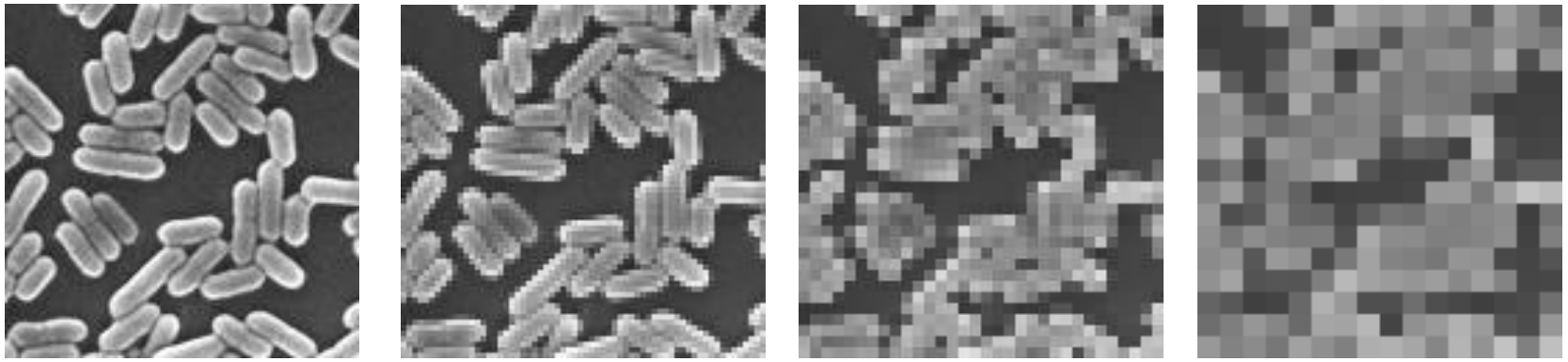
DIGITAL RESOLUTION IN ELECTRONIC PUBLISHING



- **Image resolution** is measured in pixels per inch (ppi).
- An image with a resolution of 100 ppi, contains 10,000 pixels in a Square inch (100 pixels wide x 100 pixels high = 10,000).
- The higher the resolution, the more pixels in the image. A 2-by-2-inch image with a resolution of 100 ppi would have 40000 pixels.
- The same image with a resolution of 300 ppi would have 360,000 Pixels in the same 2-by-2-inch area.

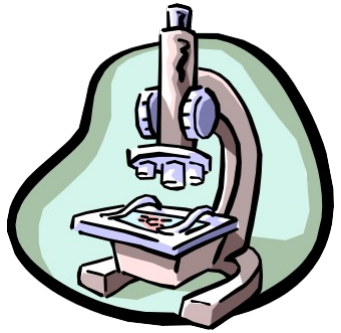
DIGITAL RESOLUTION





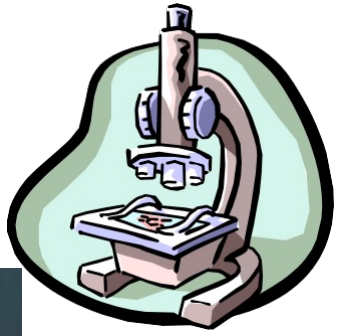
128x128 64x64 32x32 16x16

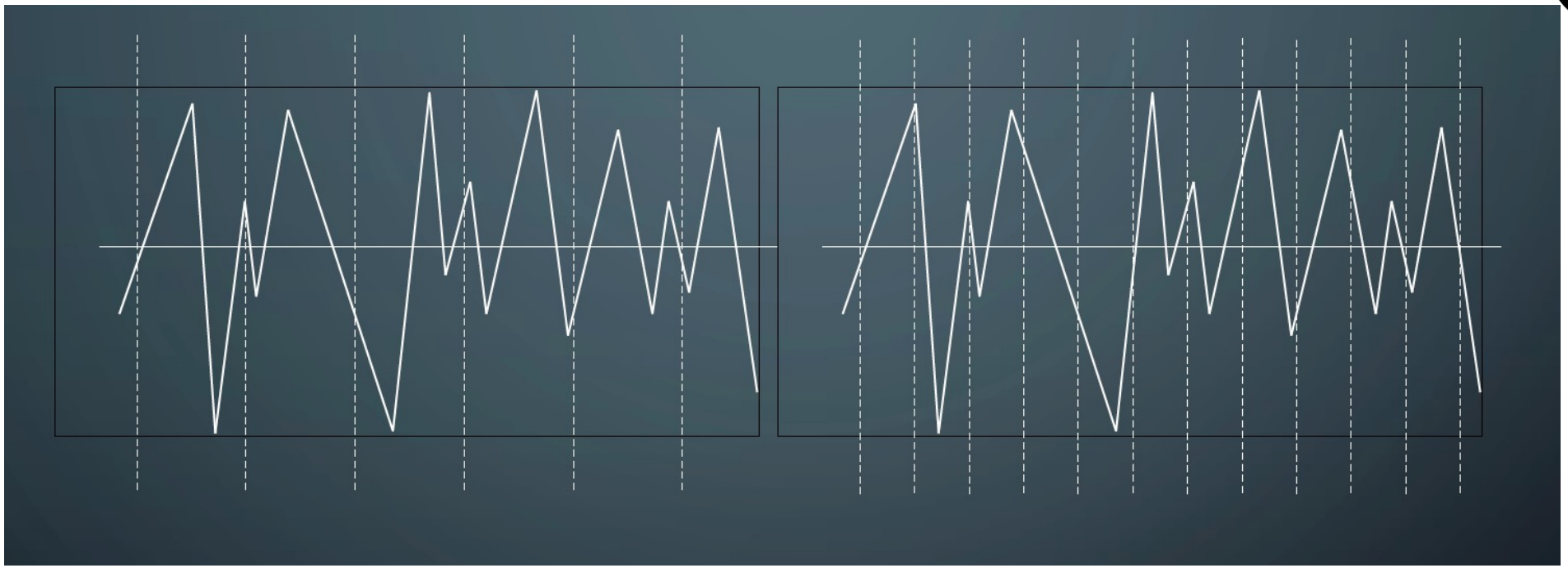
SAMPLING THEORY



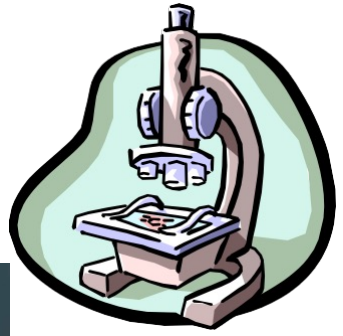
- Nyquist Theorem
  - sampling frequency (*f*) required to represent the true identity of the sample: to capture the periodic components of frequency *f* in a signal we need to sample at least 2*f* times
- Nyquist claimed that the rate was 2*f*; in reality the rate is 2.3*f* (~at least 2 times the highest frequency)

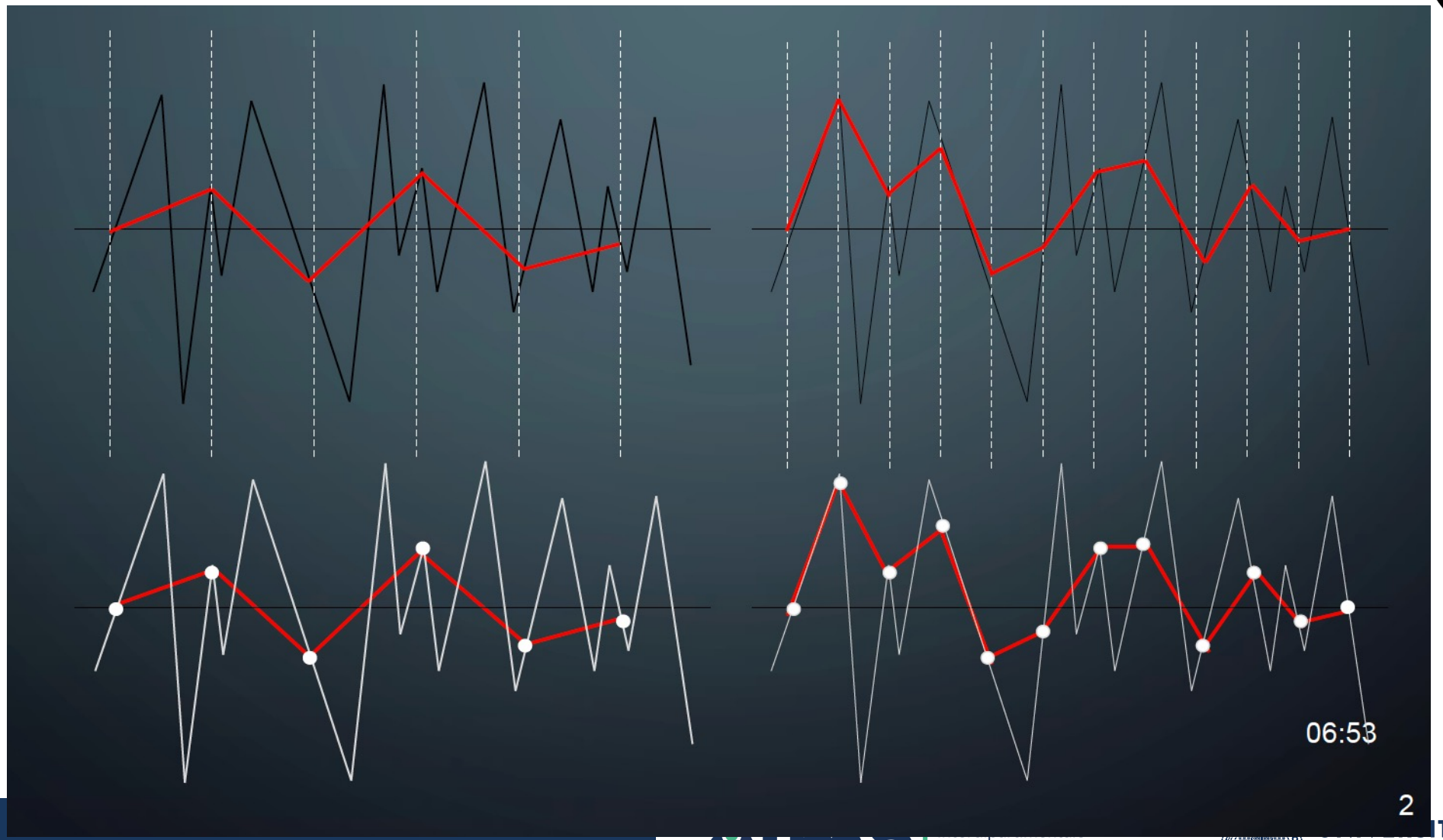
SAMPLING AND NYQUIST



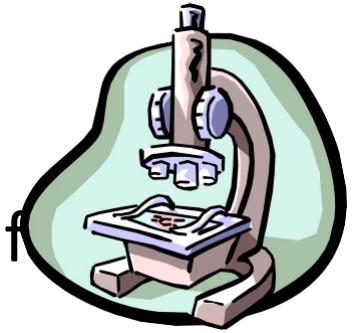


SAMPLING AND NYQUIST





SAMPLING AND NYQUIST



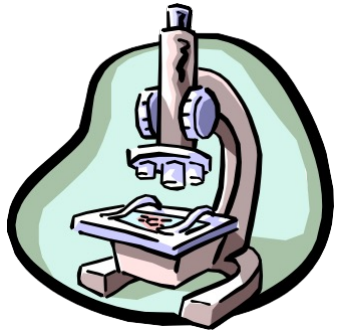
An image that is digitized at a rate of 600 dpi will only be 300 dpi if enlarged 2X and printed

- practical sampling guideline for printed images is that the final image should have at least the resolution of the printing device

For light and electron micrographs of biological tissue, prints with a final resolution of 600 dpi will preserve most detail. However, fine granular structures such as autoradiography silver grains or colloidal gold particles used in immunolabeling will not be as sharp as those in a traditional photograph

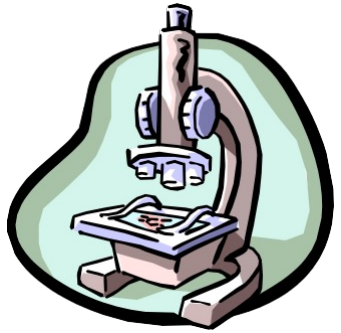
- the final print resolution may need to be 1200 dpi or higher.

BIT RESOLUTION OR PIXEL DEPTH



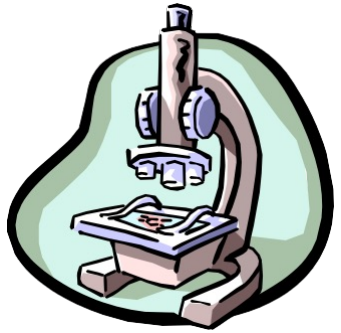
- This is a measurement of the number of **bits of information** per pixel.
- The pixel depth will determine how much color or gray scale Information is available for each pixel.
- Greater pixel depth means more available colors and more accurate color representation.
- Pixels in binary images have a depth of 1 (on or off), and are black and white images
- A pixel with a bit depth of 8 has 2^8, or 256, possible values; and a pixel with a bit depth of 24 has 2^{24}, or 16 million possible values (red=8, green=8, blue=8).
- Common values for pixel depth range from 1 to 24 bits per pixel.

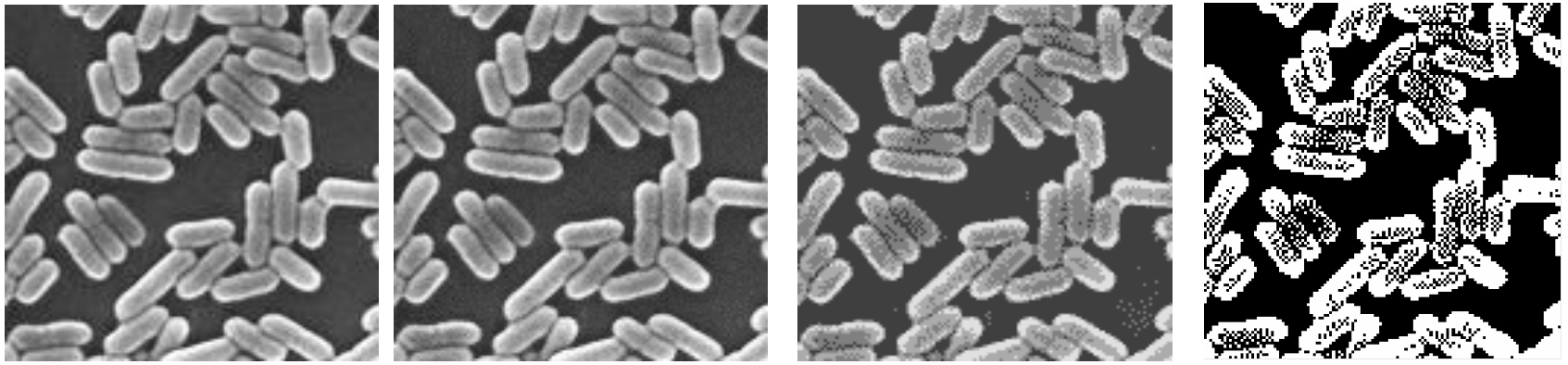
QUANTIZATION



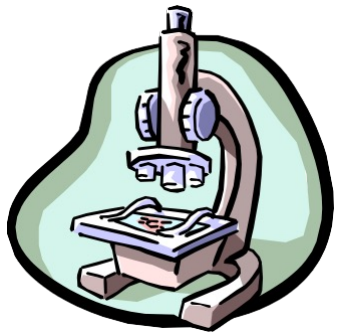
- A set of *n* quantization levels comprises the integers: *0, 1, 2, …, n-1*
- *0* and *n-1* are usually black and white respectively, with intermediate levels rendered in various shades of gray.
- Quantization levels are commonly referred to as **gray levels**.
- *n* is usually an integral power of two: *n=2b*, where *b* is the number of bits used for quantization.
- If *b=1* then the image is **binary**

QUANTIZATION

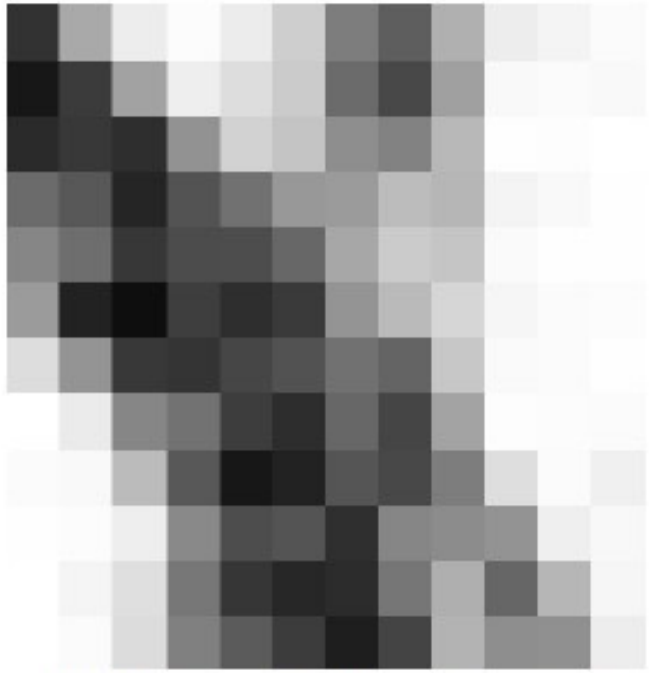




256 16 4 2



(a) Original image

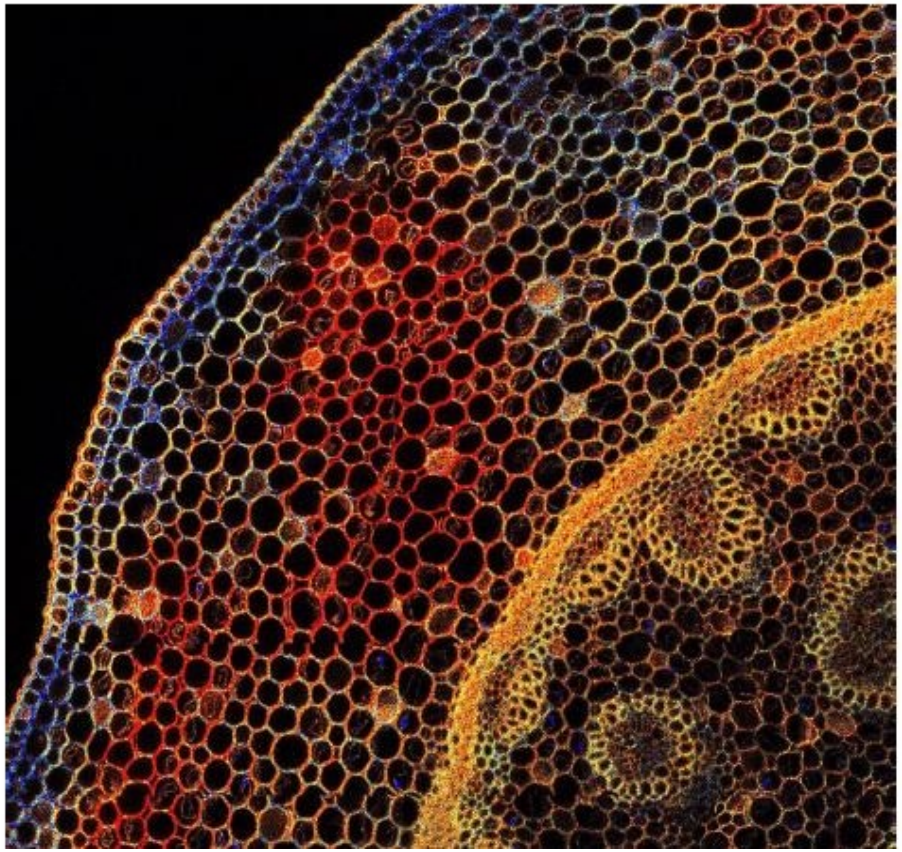


(b) Enlarged view from (a)

50	169	237	252	236	206	125	94	176	237	244	251
23	59	162	238	221	203	107	72	160	249	251	249
42	56	46	146	210	196	142	130	184	254	253	255
107	88	37	83	113	152	155	188	182	244	249	254
134	110	55	76	77	103	167	203	197	250	254	254
155	34	14	62	46	58	148	186	214	246	251	252
221	148	56	52	70	82	113	100	199	250	251	254
255	235	134	114	61	45	103	69	163	253	252	251
251	249	187	88	23	34	85	72	125	222	251	240
254	252	238	137	77	84	47	134	140	147	239	248
255	245	223	119	54	39	44	118	175	102	182	246
255	251	220	128	91	60	30	68	179	142	144	237

(c) Pixel values of (b)

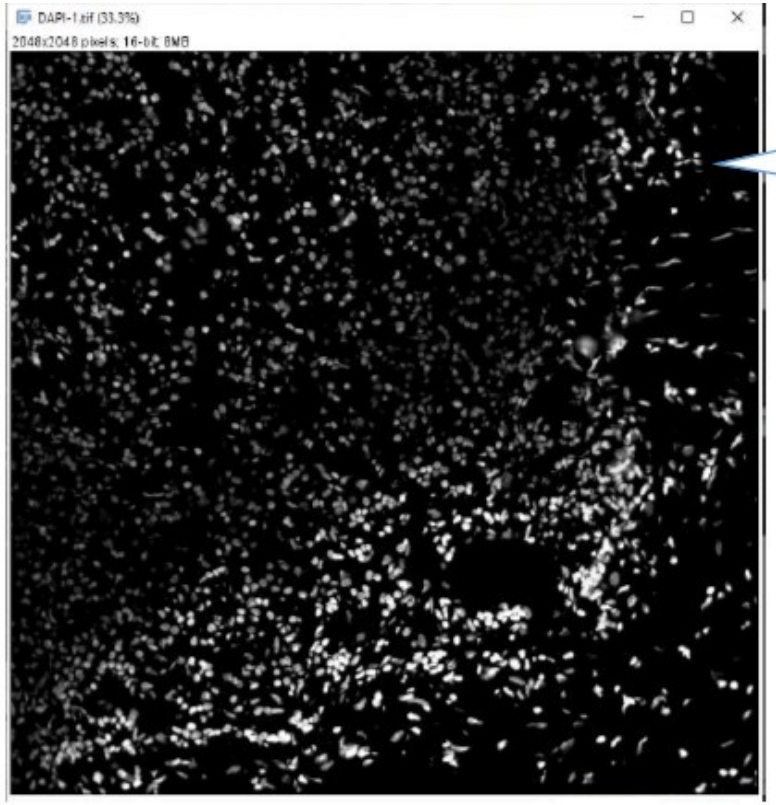
Deriving quantitative information from images of biological samples



How many cells are there in the outer ring?

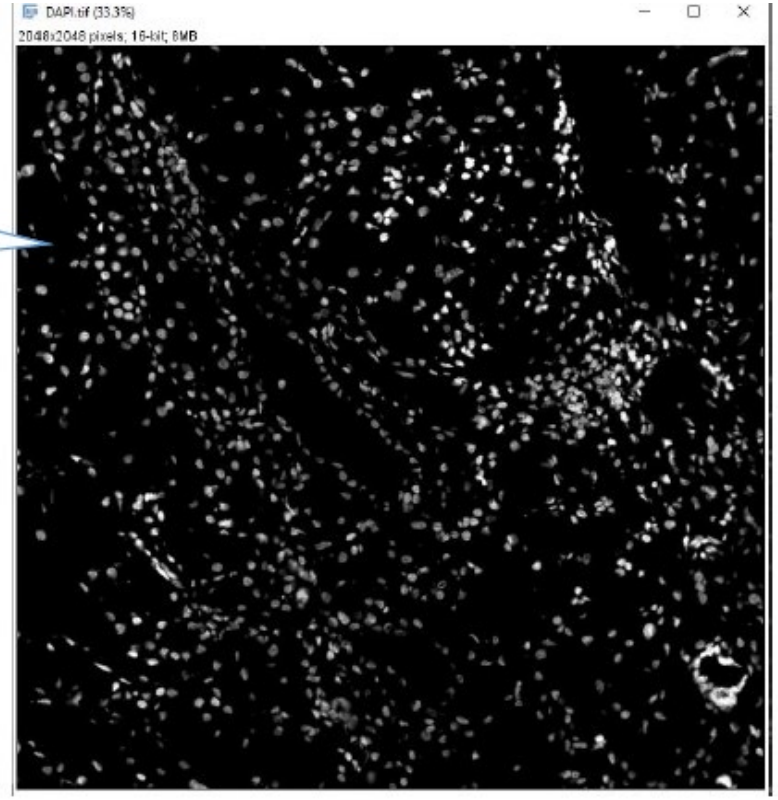
How high is the intensity in the inner ring?

How is the signal in the blue channel distributed?



Nuclei in this image are ... _

... more dense than in this image.

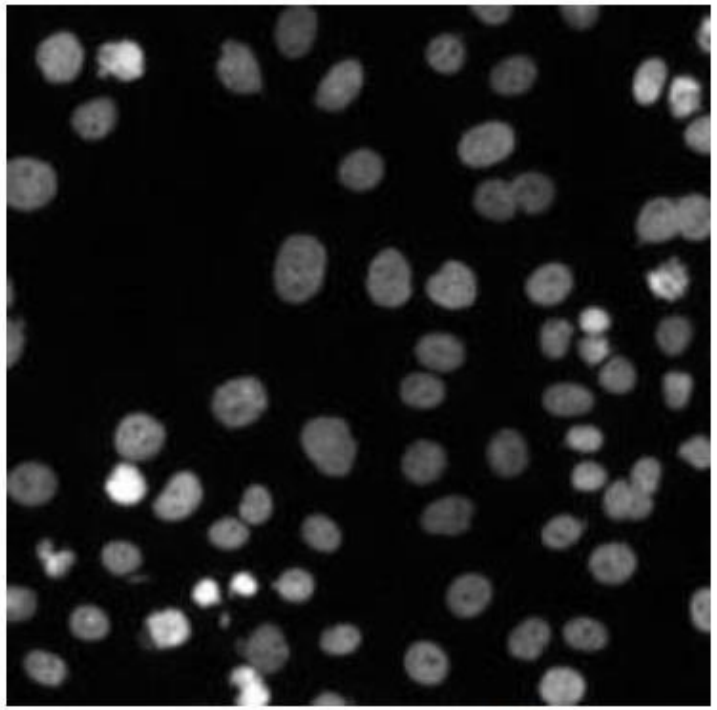


Use automation for less subjective analysis.

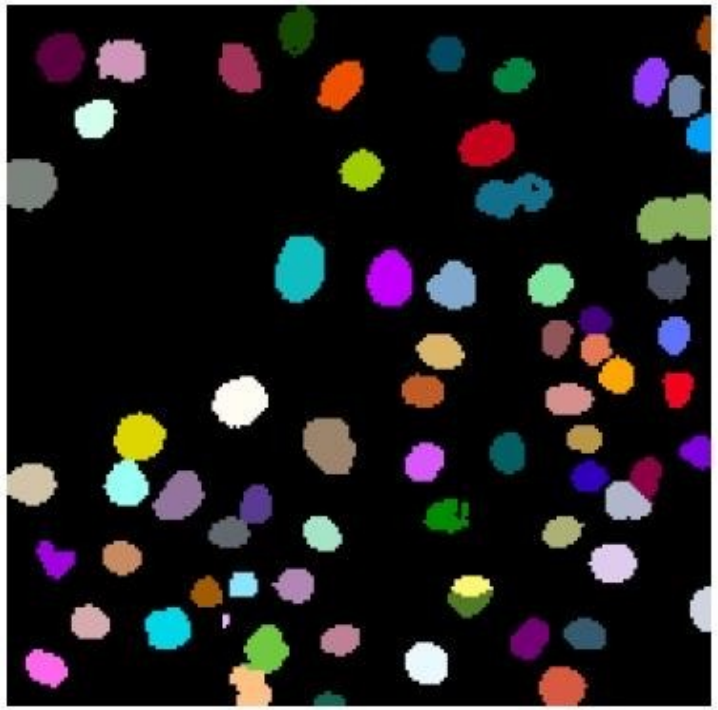
Image data source: Pascual-Reguant, Anna. (2021). Immunofluoresce
staining of a human kidney (#2, peri-tumor area) obtained by MELC [I

Algorithms must be reliable. Visualization helps gaining trust in methods

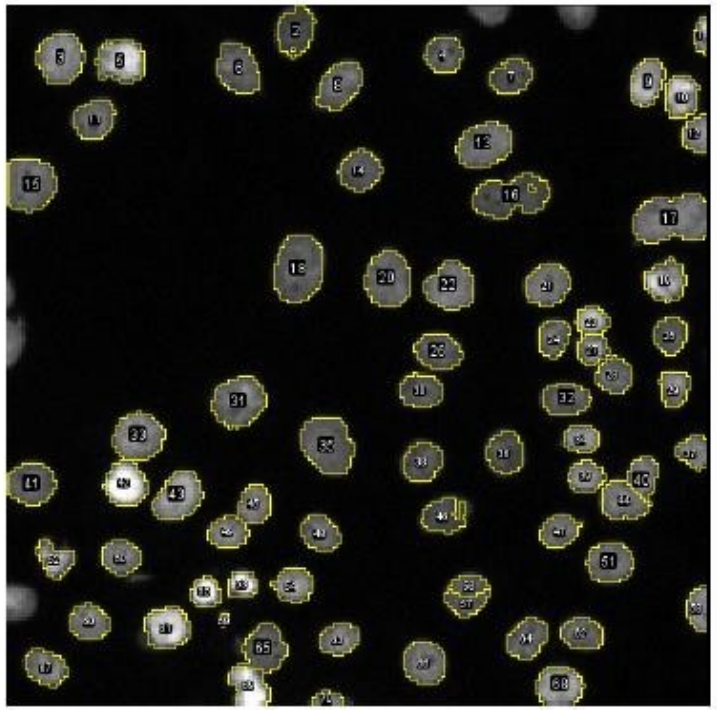
Original image



Label image



Overlay



There are 70 nuclei

QUANTITATIVE MICROSCOPY IS SUPPOSED TO BE:

- **Quantitative**
  - We derive numbers from images which describe physical properties of the observed sample.
- **Objective**
  - The derived measurement does not depend on who did the measurement. The measurement is free of interpretation.
- **Reliable (trustworthy / validated)**
  - We are confident that the measurement is describing what it is supposed to describe.
- **Reproducible**
  - Somebody else can do the experiment under *different conditions* and gets similar measurements. For this, documentation is decisive!
- **Repeatable**
  - We can do the same experiment twice under the *same conditions* and get similar measurements.

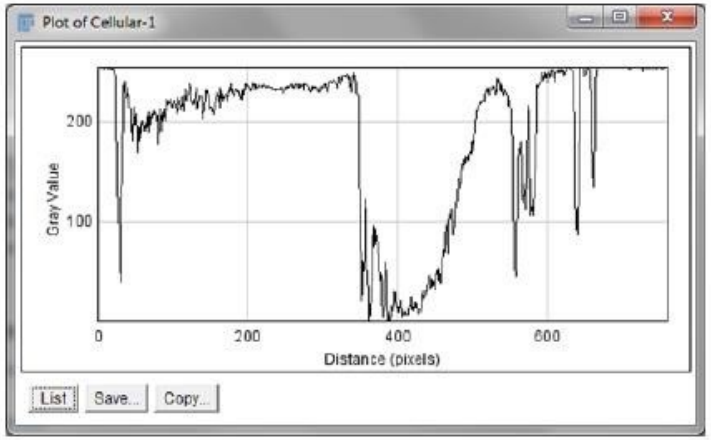
IMAGE ANALYSIS IS PART OF THE EXPERIMENT

- Think about how to analyze your images before starting the experiment.
- Consider adapting your experiment so that quantitative image analysis can be performed easily.
- Think about controls, counter-proves, an easy to falsify null-hypothesis
- Do simple experiments.
- How can you exclude yourself from the experiment?
- Think of blinding yourself or fully automate analysis.
- One experiment usually answers just one or less questions.

COMMON QUESTIONS IN QUANTITATIVE MICROSCOPY

1. Numbers (particles, spots, nuclei. etc)
2. Morphometry (length, Feret diameters, roundness, etc)
3. Densitometry (intensity base analysis on fluorophores)
4. Colocalization
5. Live imaging & tracking

FIJI – freely available image analysis program



Plot window

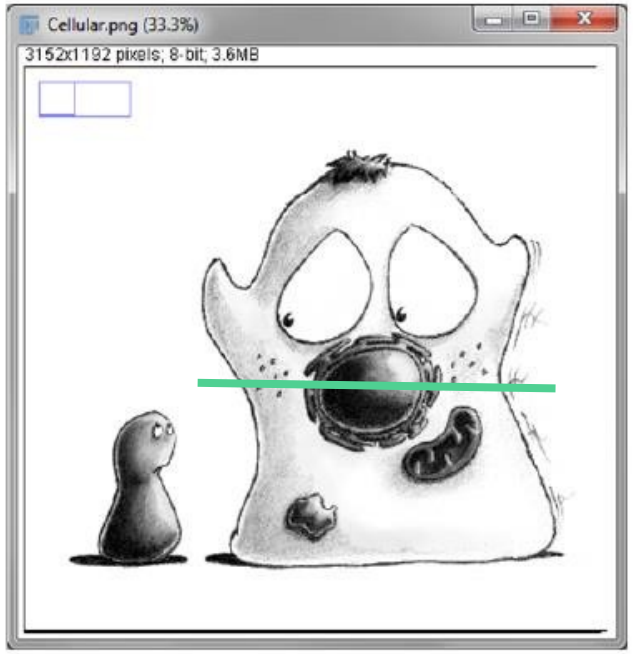
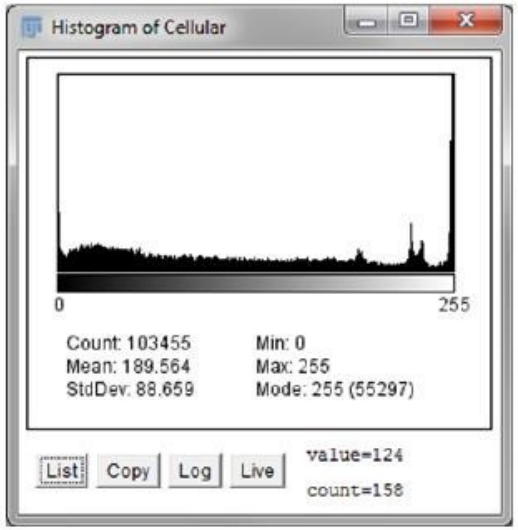
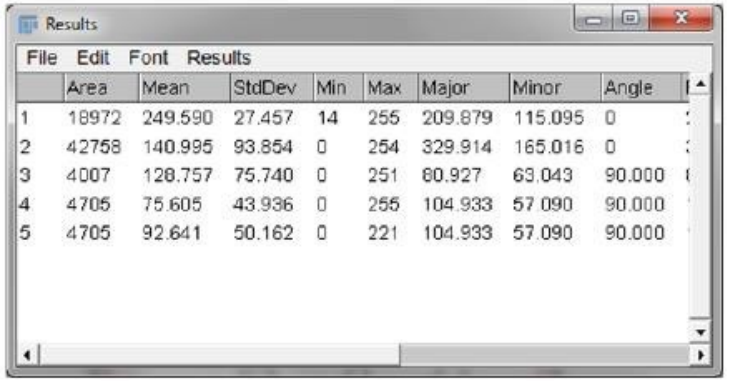


Image window

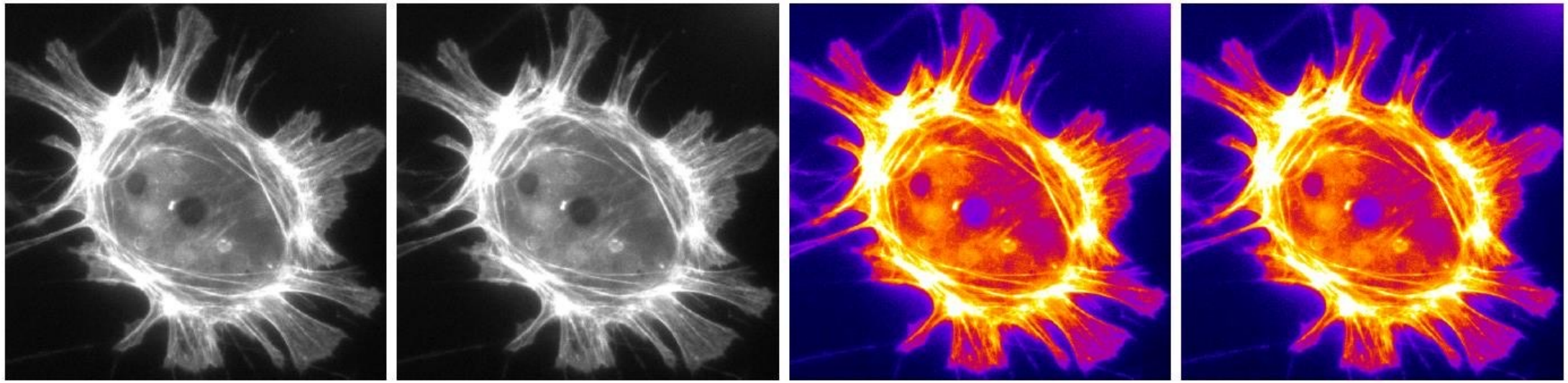


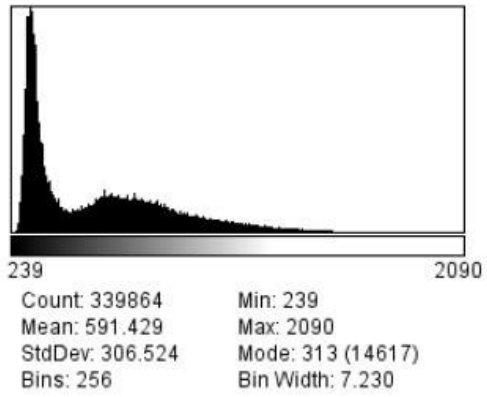
Histogram window



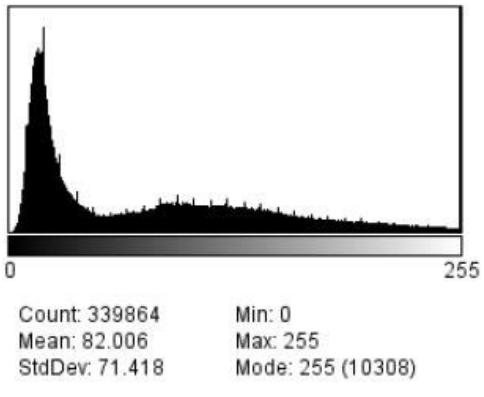
	Area	Mean	StdDev	Min	Max	Major	Minor	Angle
1	18972	249.590	27.457	14	255	209.879	115.095	0
2	42758	140.995	93.854	0	254	329.914	165.016	0
3	4007	128.757	75.740	0	251	80.927	63.043	90.000
4	4705	75.605	43.936	0	255	104.933	57.090	90.000
5	4705	92.641	50.162	0	221	104.933	57.090	90.000

Results table

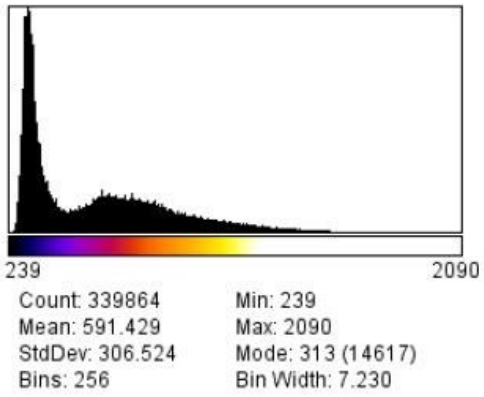




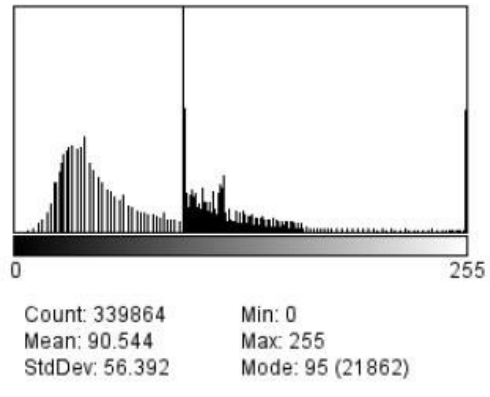
(a) 16-bit (`Grays` LUT)



(b) 8-bit (`Grays` LUT)

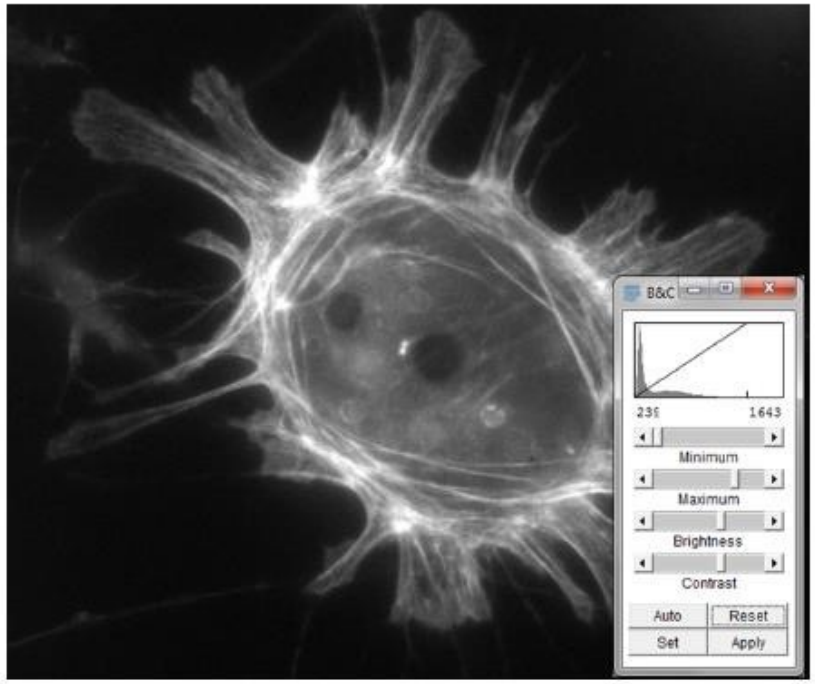


(c) 16-bit (`Fire` LUT)

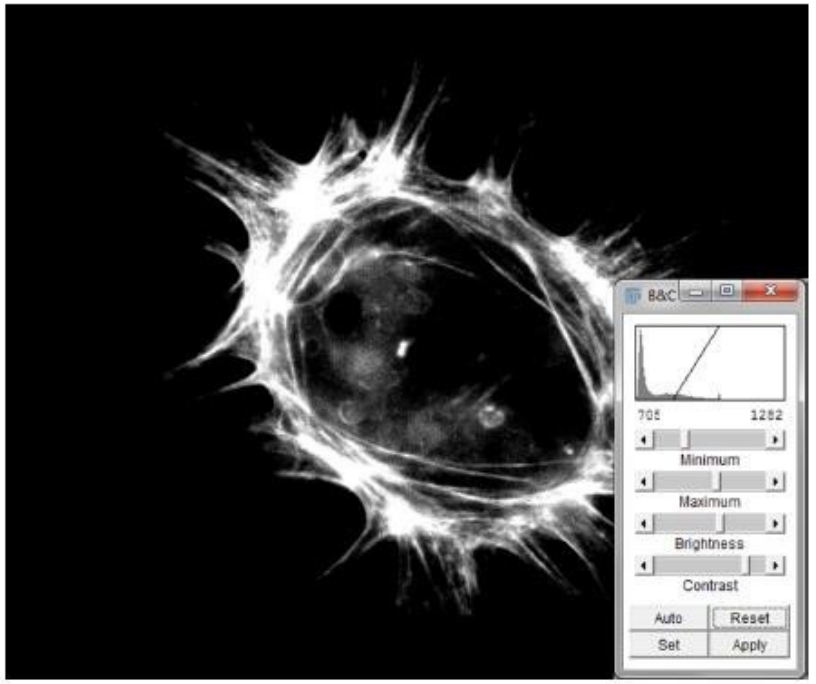


(d) 8-bit (RGB)

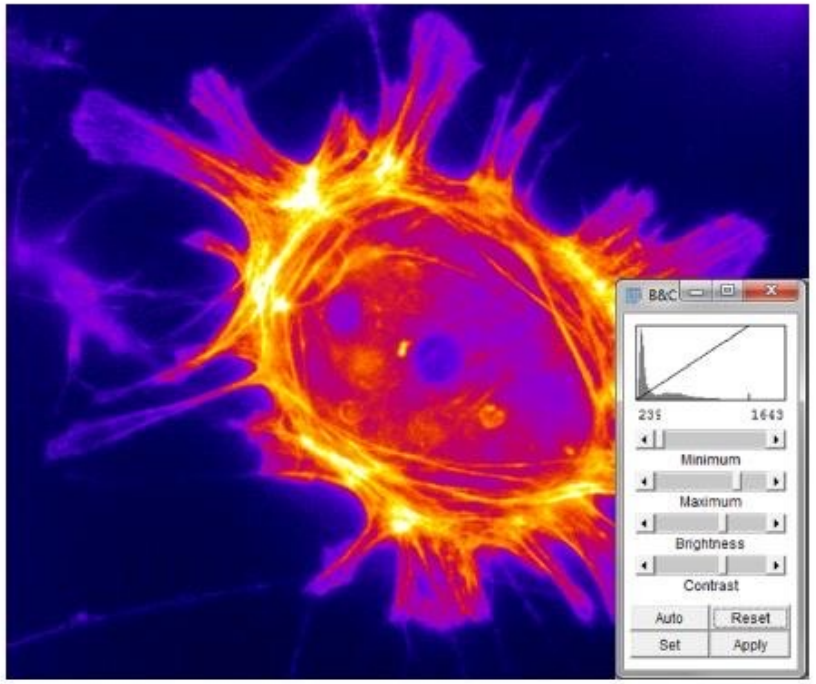
- The same image can be displayed in different ways by adjusting the contrast settings or the LUT.
- Nevertheless, despite the different appearance, the values of the pixels are the same in all three images.



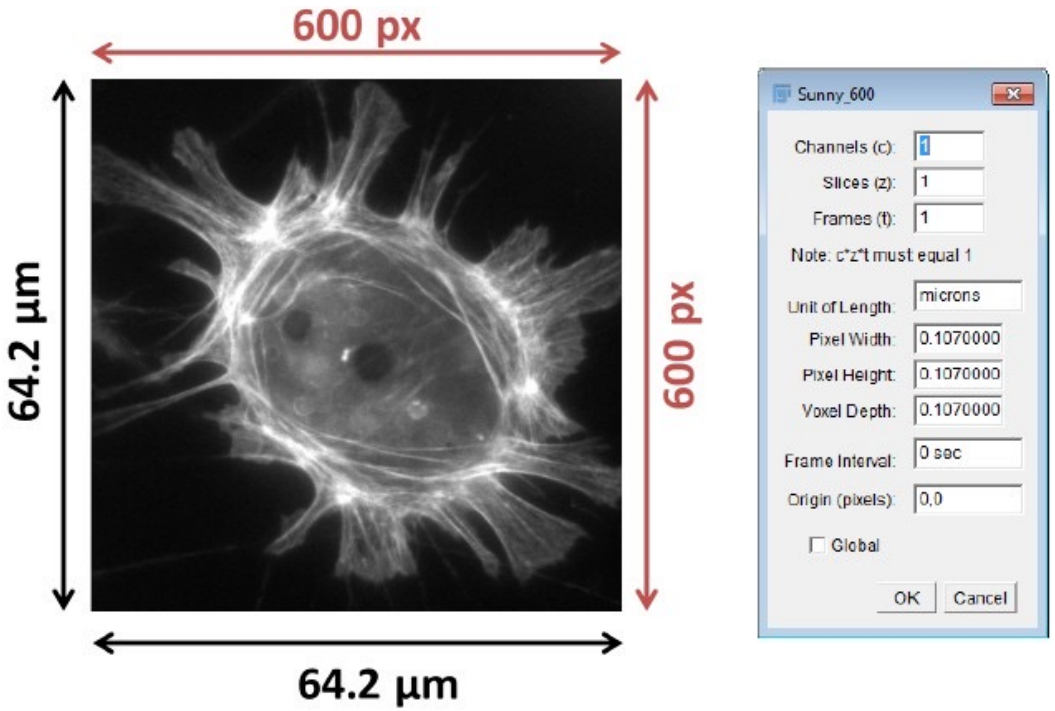
(a) Grayscale



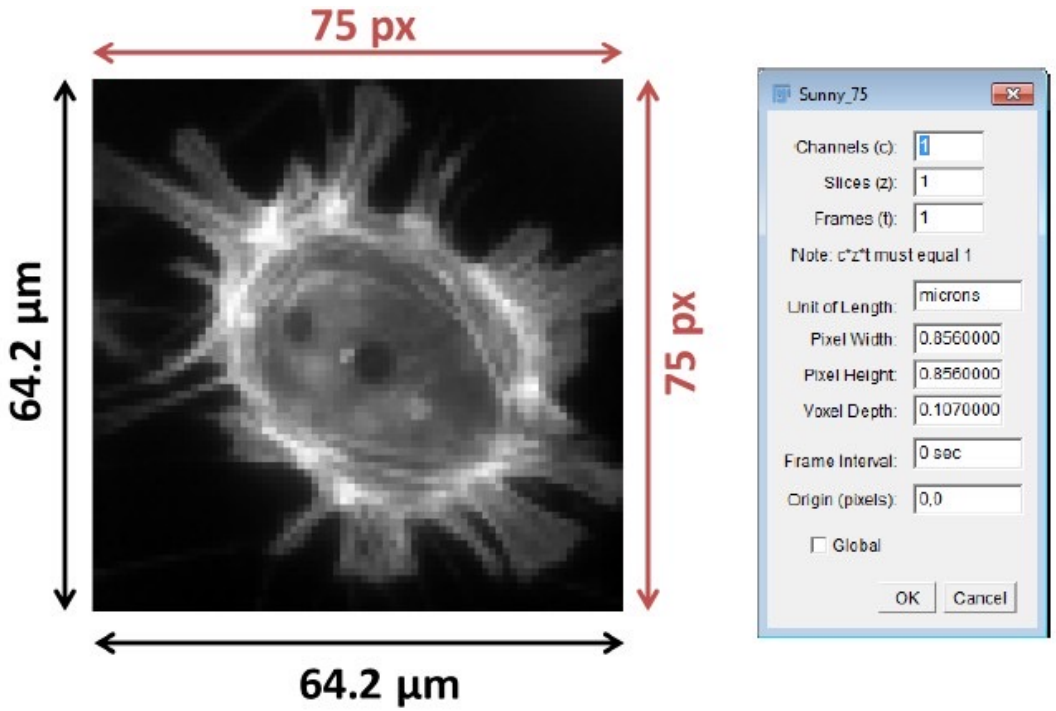
(b) Grayscale (high contrast)



(c) Fire LUT

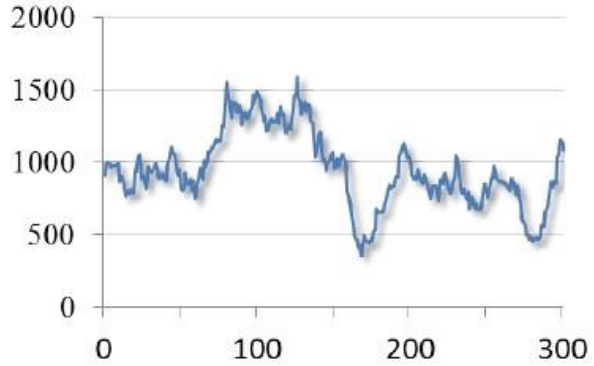


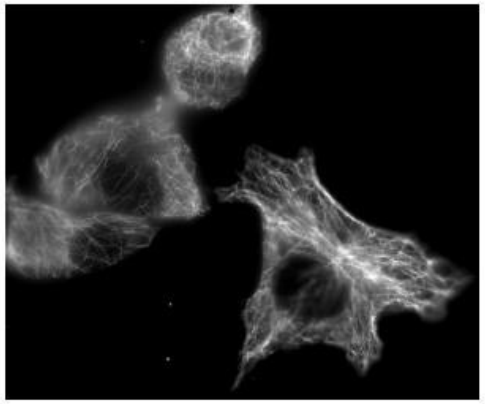
(a) 600×600 pixel image and its properties

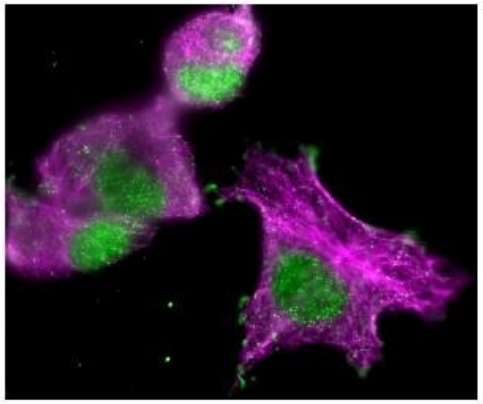


(b) 75×75 pixel image and its properties

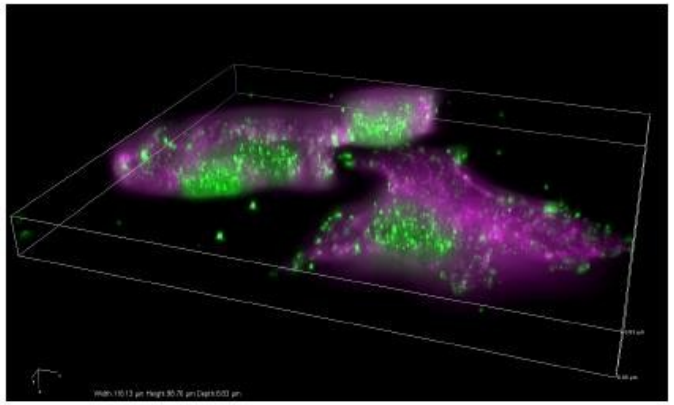
102

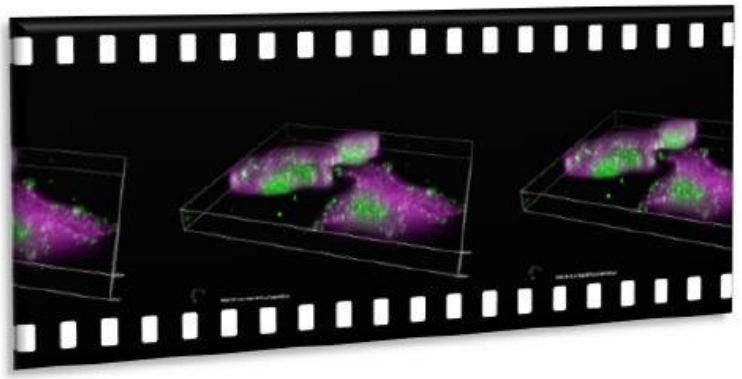




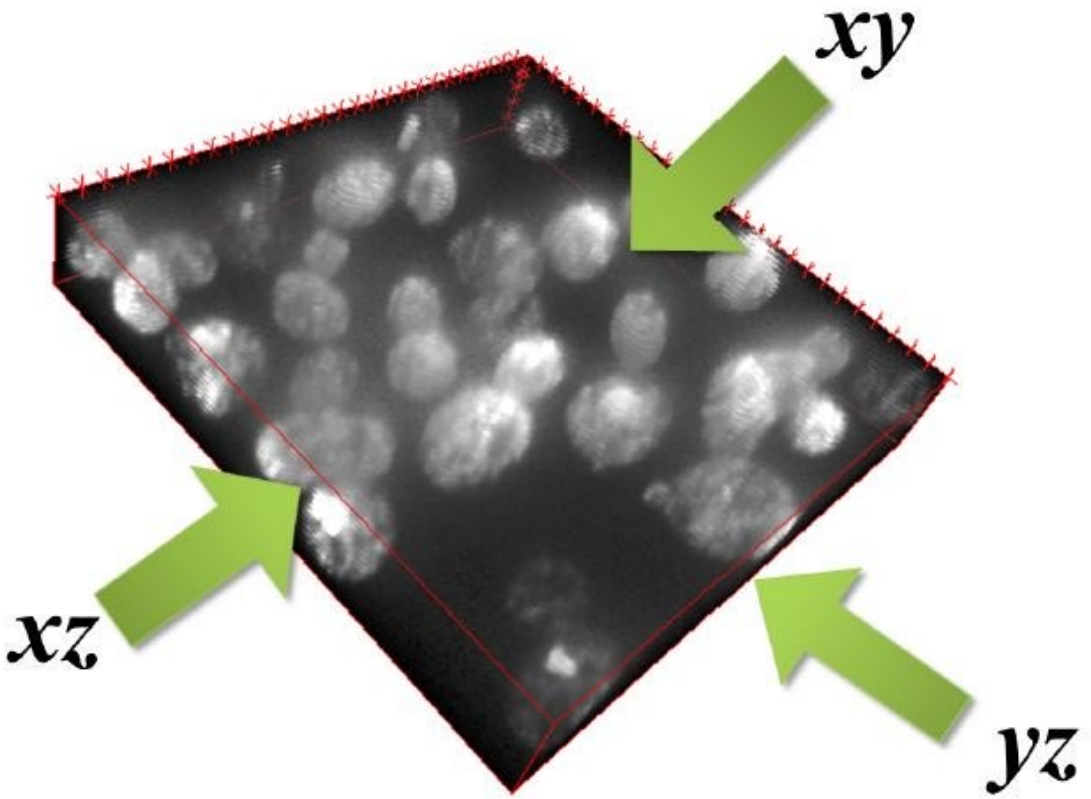


(a) 0 dimensional (b) 1 dimensional (c) 2 dimensional (d) 3 dimensional

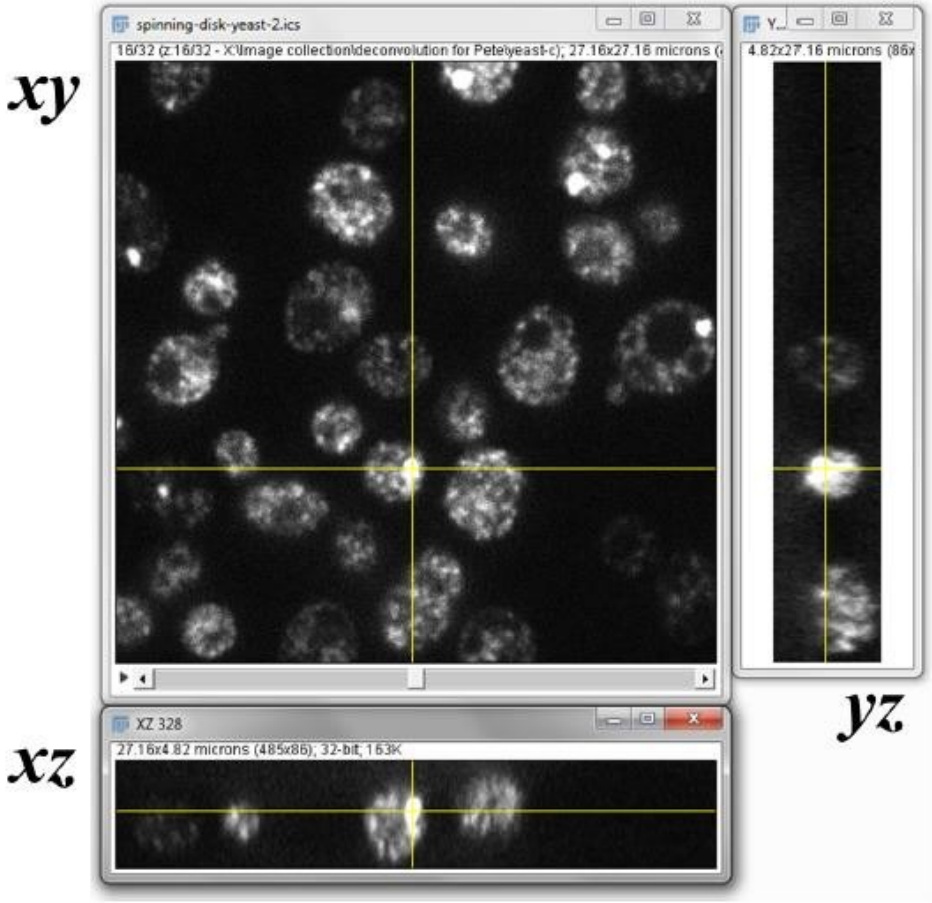




(e) 4 dimensional (f) 5 dimensional

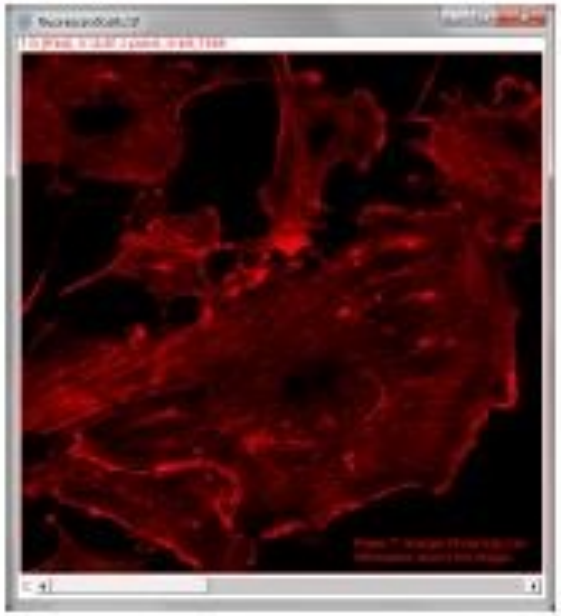


(a) Volume rendering



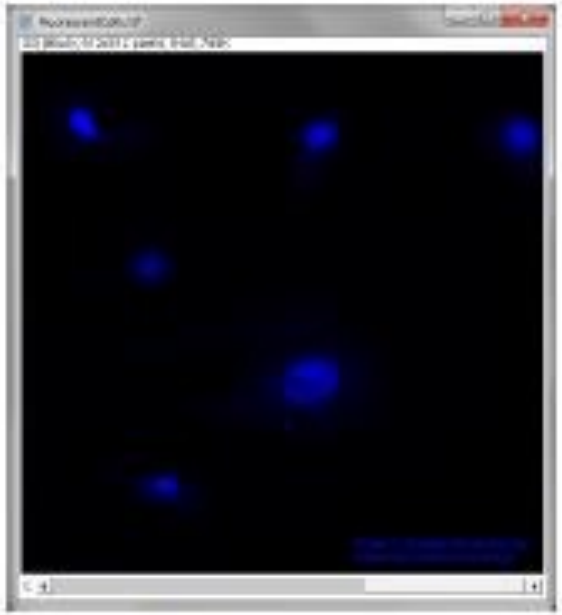
(b) Orthogonal views

(a) Composite image

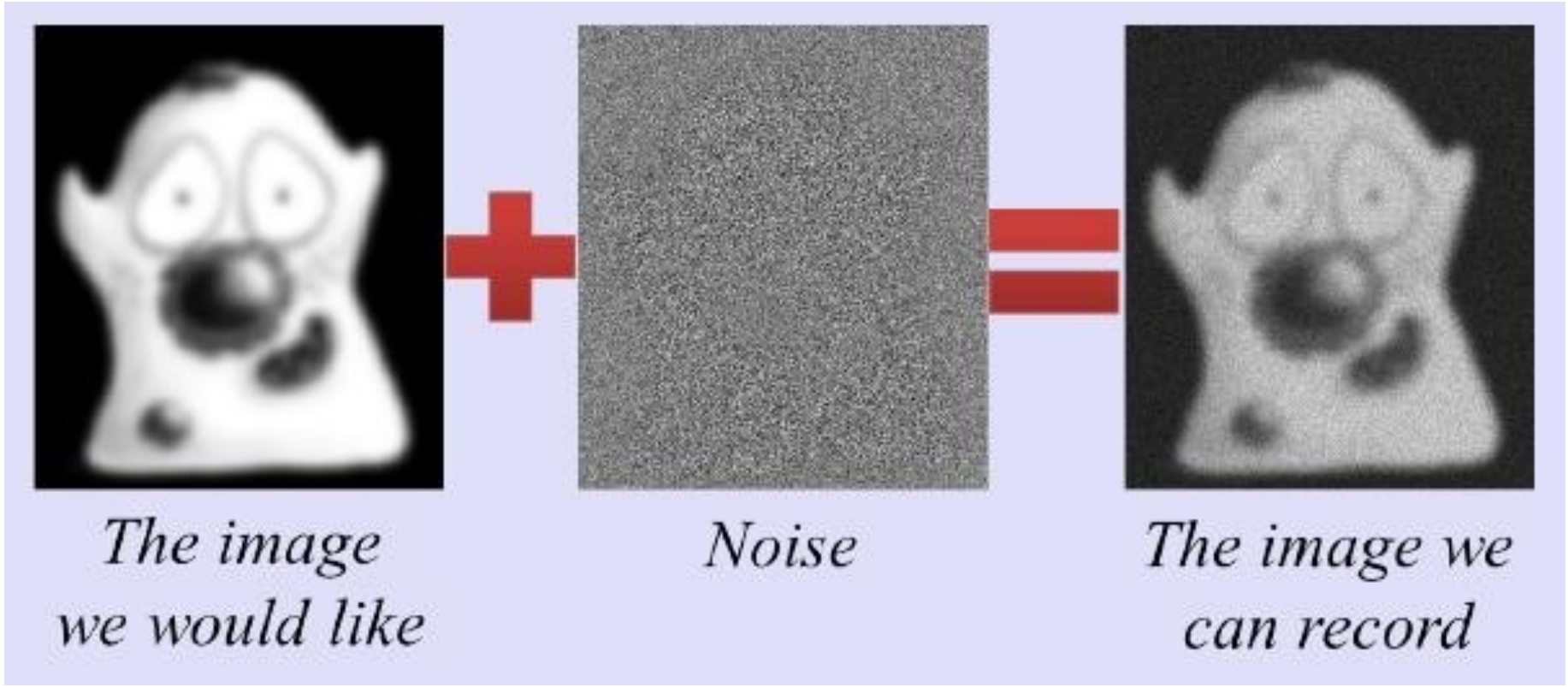


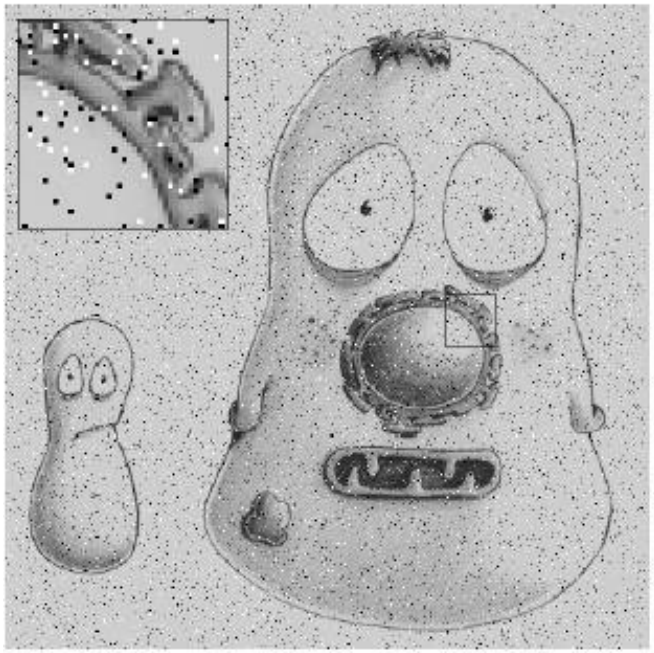
(b) Red channel

(c) Green channel

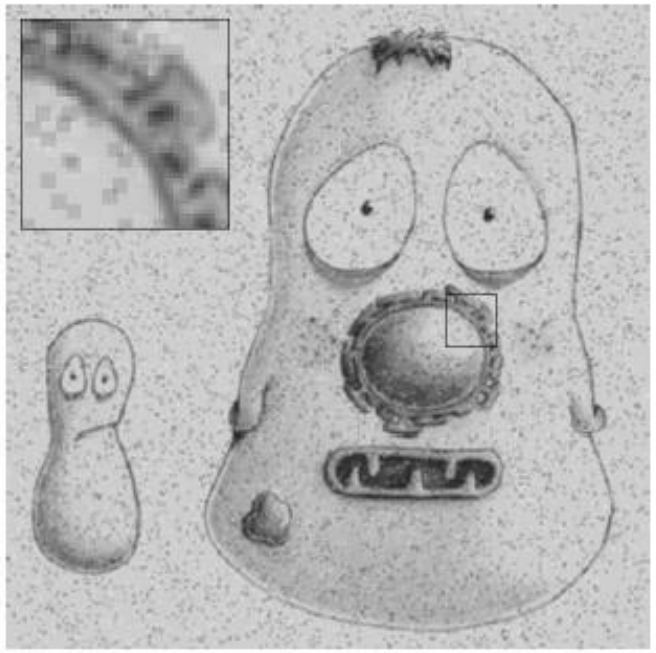


(d) Blue channel

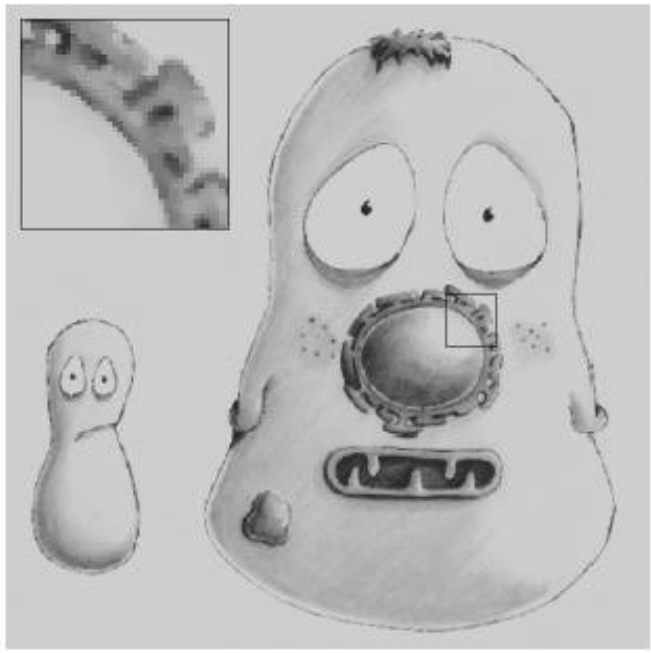

The image we would like
Noise
The image we can record



(a) Speckled image



(b) Mean filter



(c) Median filter

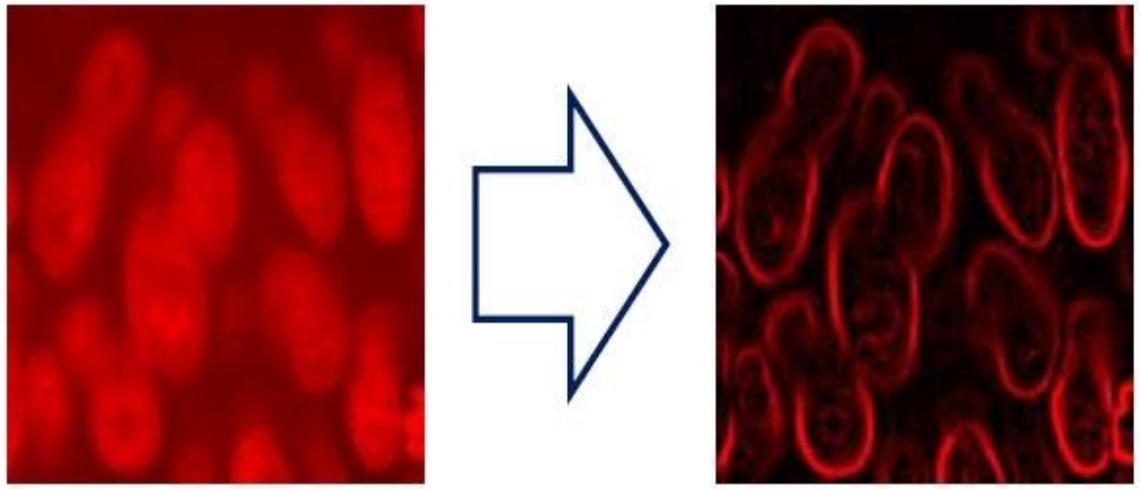
Basic Intensity Quantification with ImageJ

- Images mostly needed to be transformed into quantifiable data
- ImageJ is useful for getting information from images, including pixel intensity.
- There are a number of different ways to get intensity information from images using the base package of ImageJ (no plugins required).

IMAGE FILTERING

Today

- Filters
- Image math

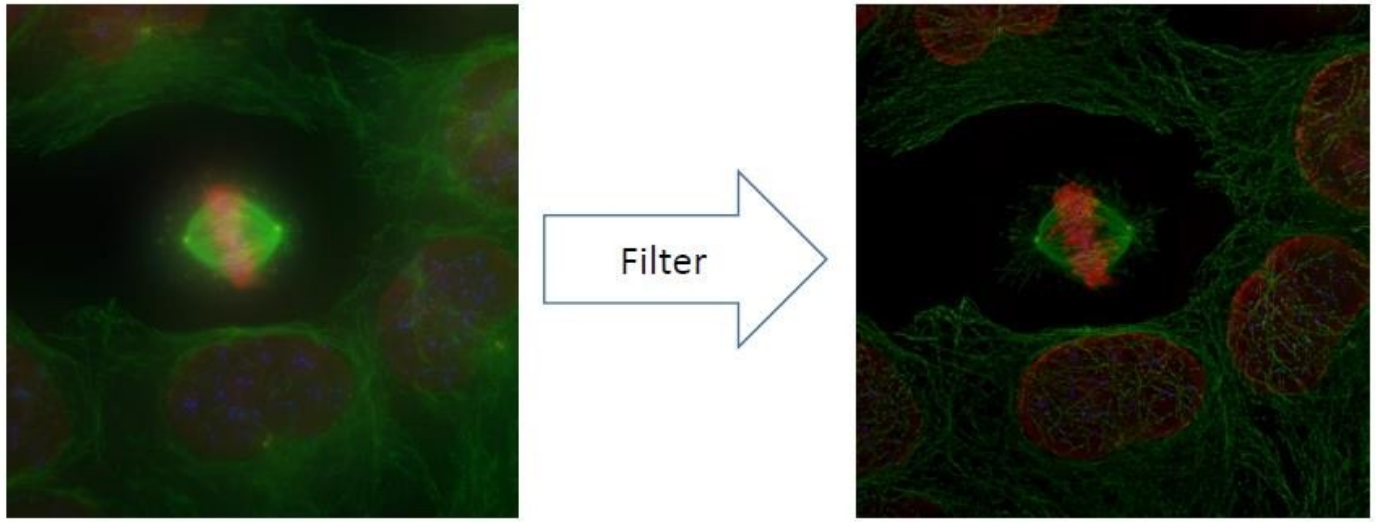


FILTERS

- An image processing filter is an operation on an image.
- It takes an image and produces a new image out of it.
- Filters change pixel values.

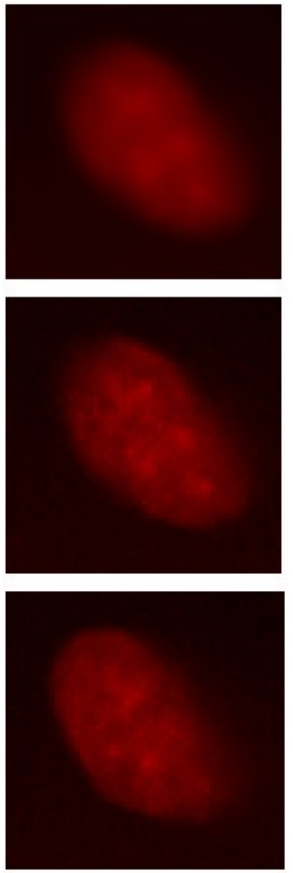
- There is no “best” filter. Which filter fits your needs, depends on the context.
- Filters do not do magic. They can not make things visible which are not in the image.

- Application examples
  - Noise-reduction
  - Artefact-removal
  - Contrast enhancement
  - Correct uneven illumination



EFFECTS HARMING IMAGE QUALITY

- Noise is a general term for unwanted modifications that a signal may suffer during capture, storage, transmission, processing, or conversion.
- In microscopy, image quality suffers from
  - shot noise: Statistical variation of the photons arriving at the camera
  - dark noise: Statistical variation of how many electrons are generated if a photon arrives in a camera pixel (temperature dependent).
  - read out noise: introduced by the electronics, especially the pixel and the analog-digital-converter
  - Physical/optical effects: aberrations, defocus
  - Biological/physiological/structural effects: motion, diffusion



NOISE

- When dealing with noise in microscopic image processing, we mostly mean the noise visible in the images.

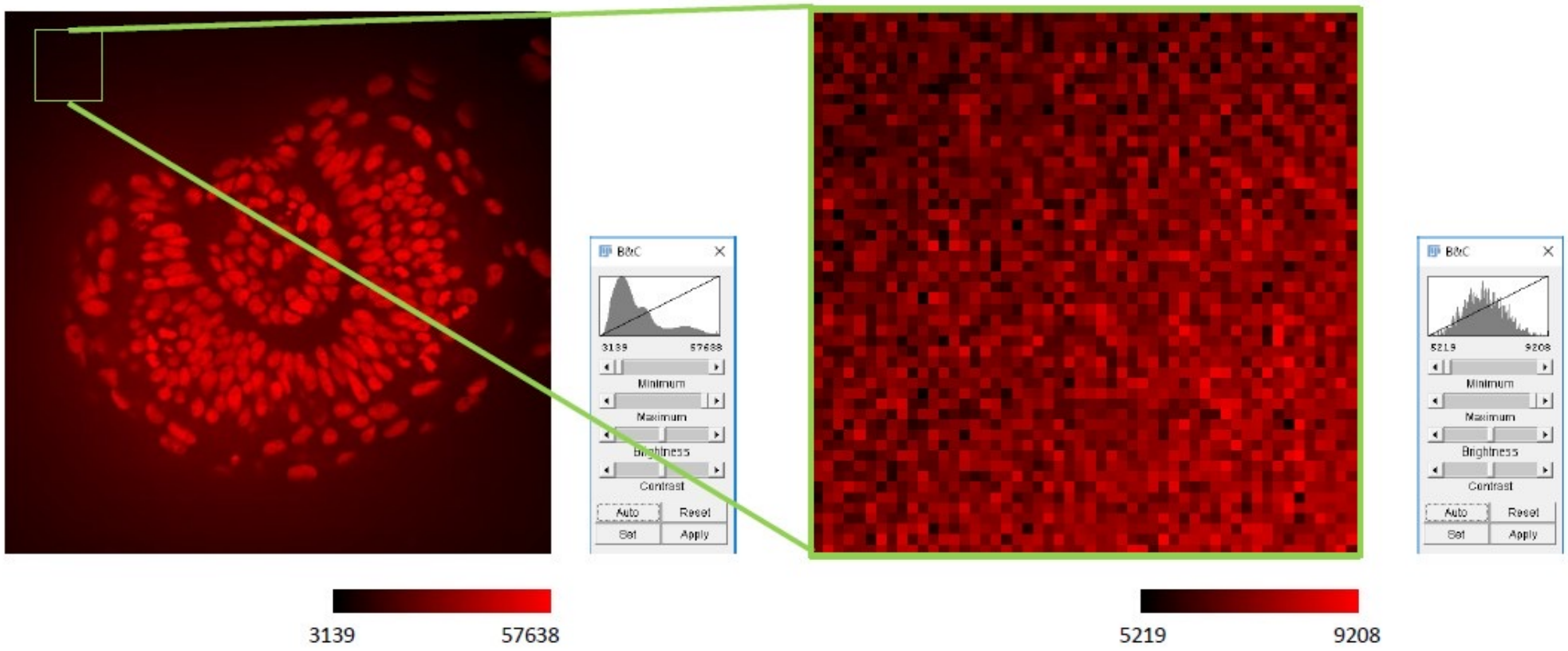


IMAGE CORRECTION/NOISE REMOVAL

- We need to remove the noise to help the computer *interpreting* the image

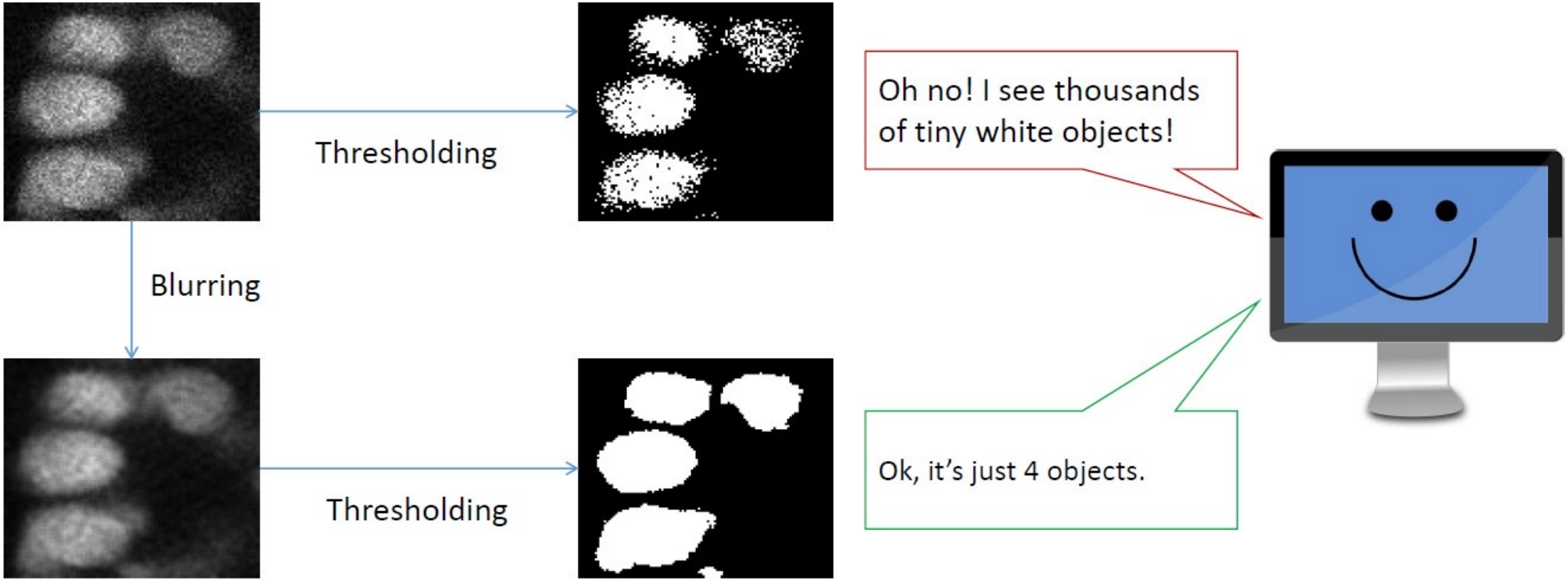
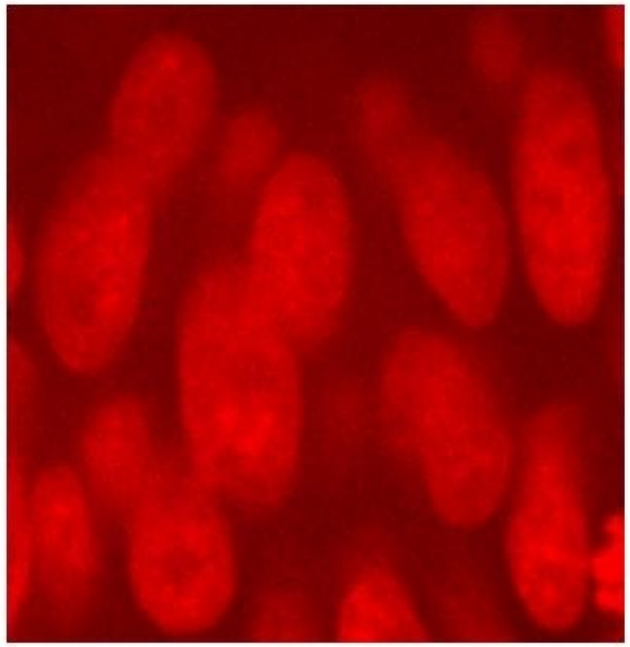
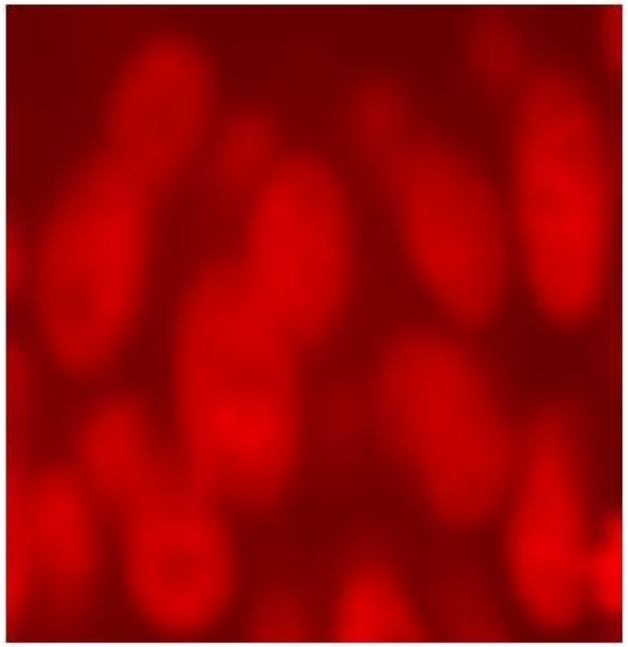


IMAGE CORRECTION/NOISE REMOVAL

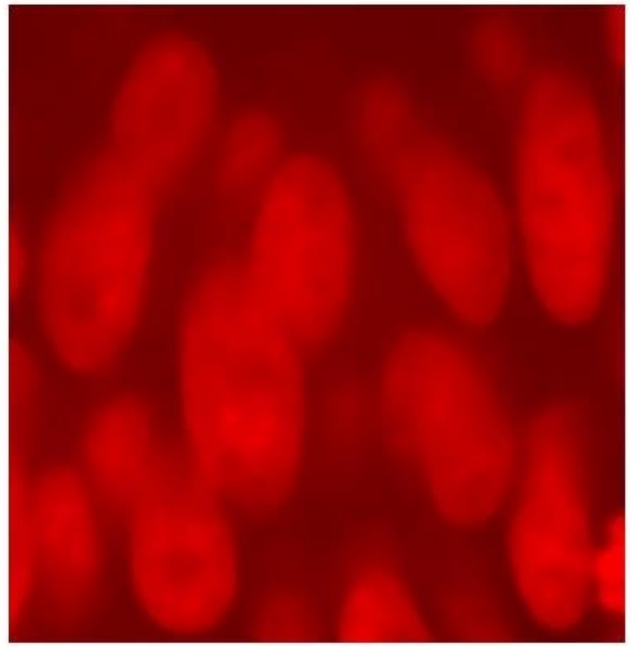
- Noise removal using *filters*



Original image



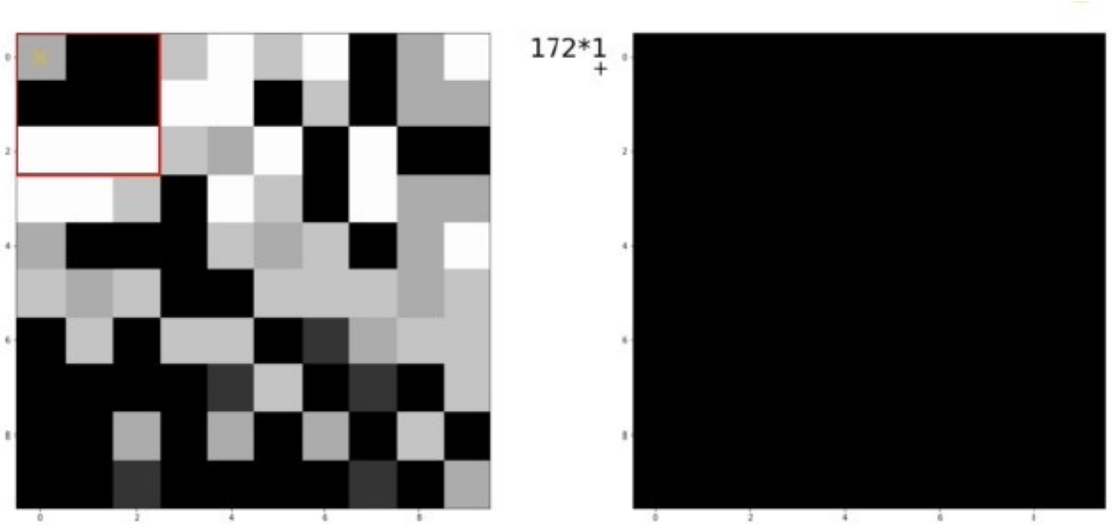
Gaussian blur filtered



Median filtered

LINEAR FILTERS

- Linear filters replace each pixel value with a linear combination of surrounding pixels
  - Basically, linear filtering is a Convolution
  - It needs a kernel (weight template)
  - Result: new image where each pixel is replaced by the weighted sum of pixel values in the neighbourhood.



- Kernels are matrices describing a linear filter

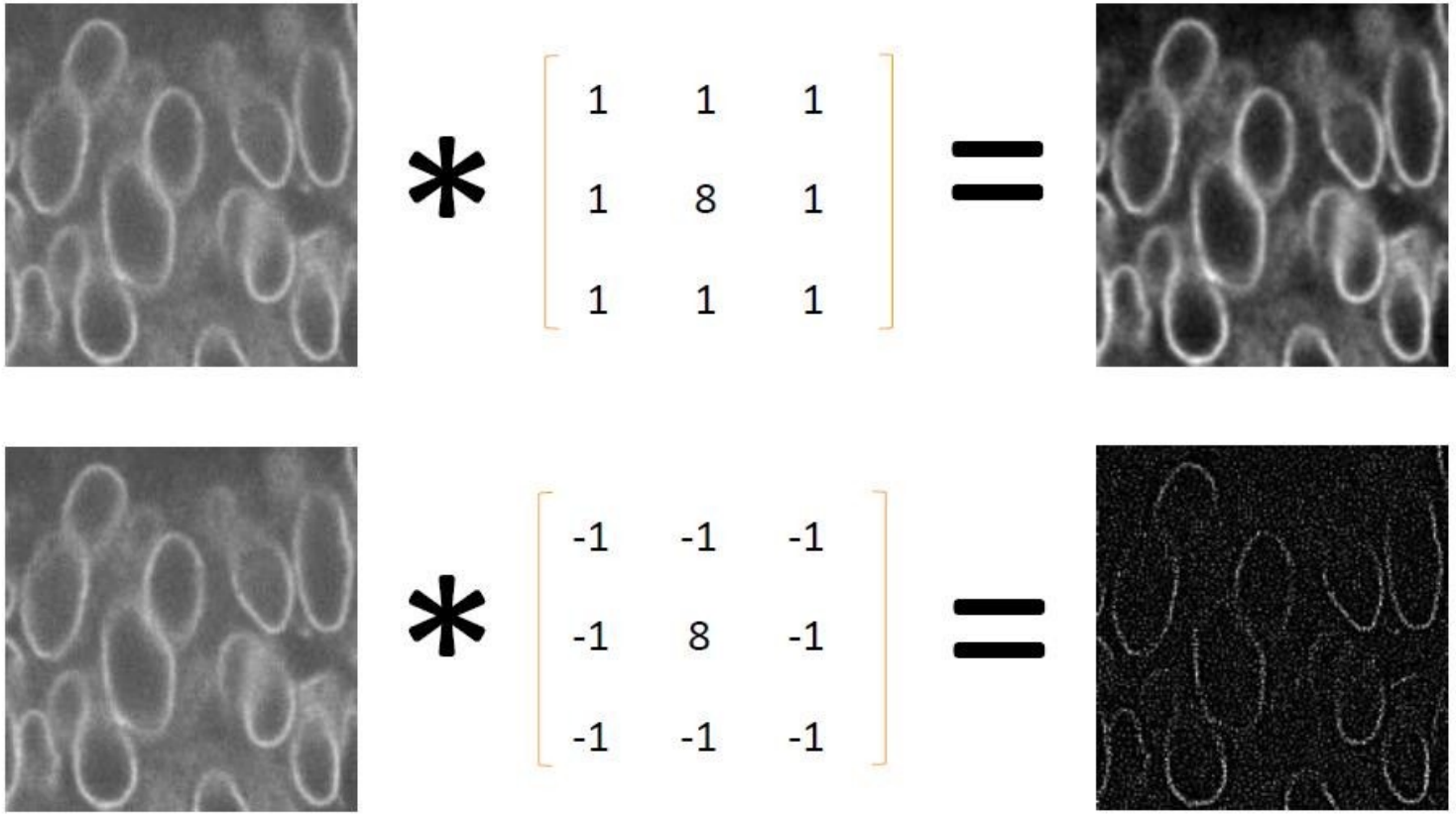
Mean filter, 3x3 kernel

$$\begin{bmatrix} 1/9 & 1/9 & 1/9 \\ 1/9 & 1/9 & 1/9 \\ 1/9 & 1/9 & 1/9 \end{bmatrix}$$

LINEAR FILTERS

- Terminology:
  - "We convolve an image with a kernel."
  - Convolution operator: *

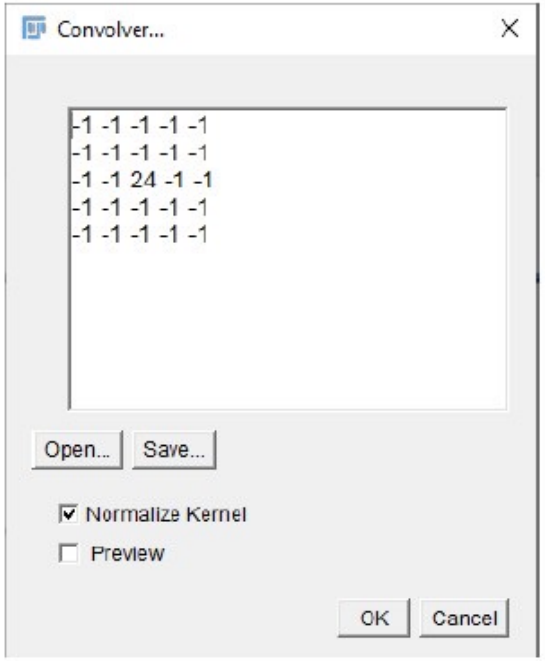
- Examples
  - Mean
  - Gaussian blur
  - Sobel-operator
  - Laplace-filter

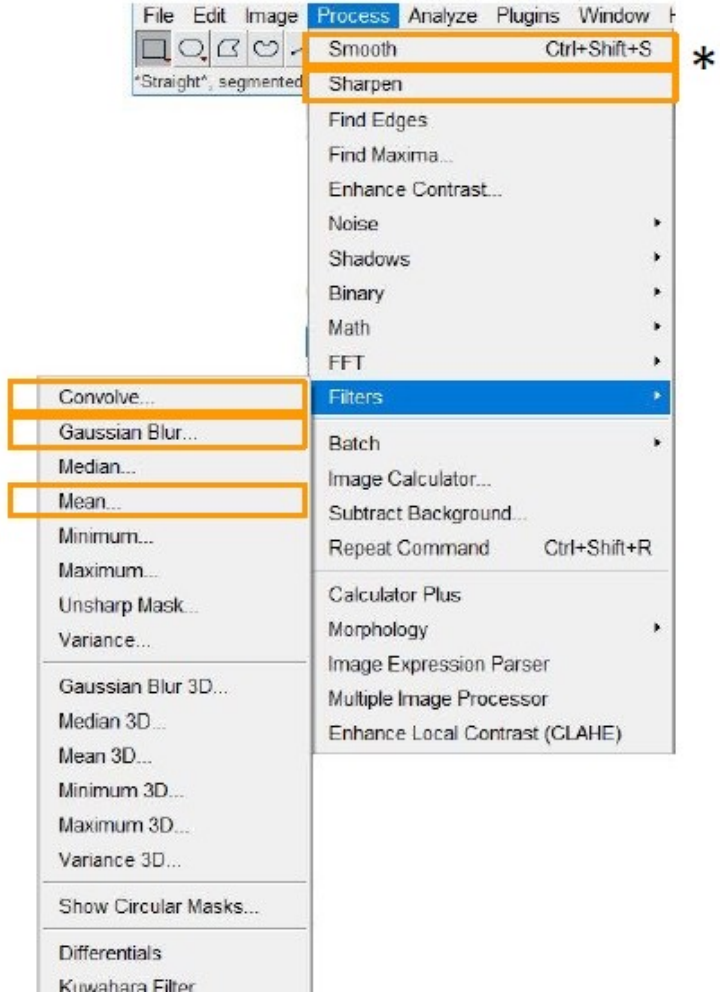


LINEAR FILTERS

Linear filters:

- Convolve…
  - Allows us to specify our kernel





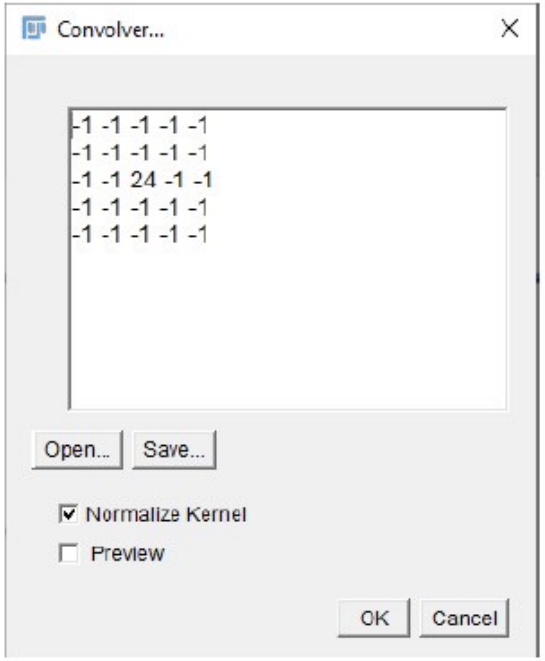
*

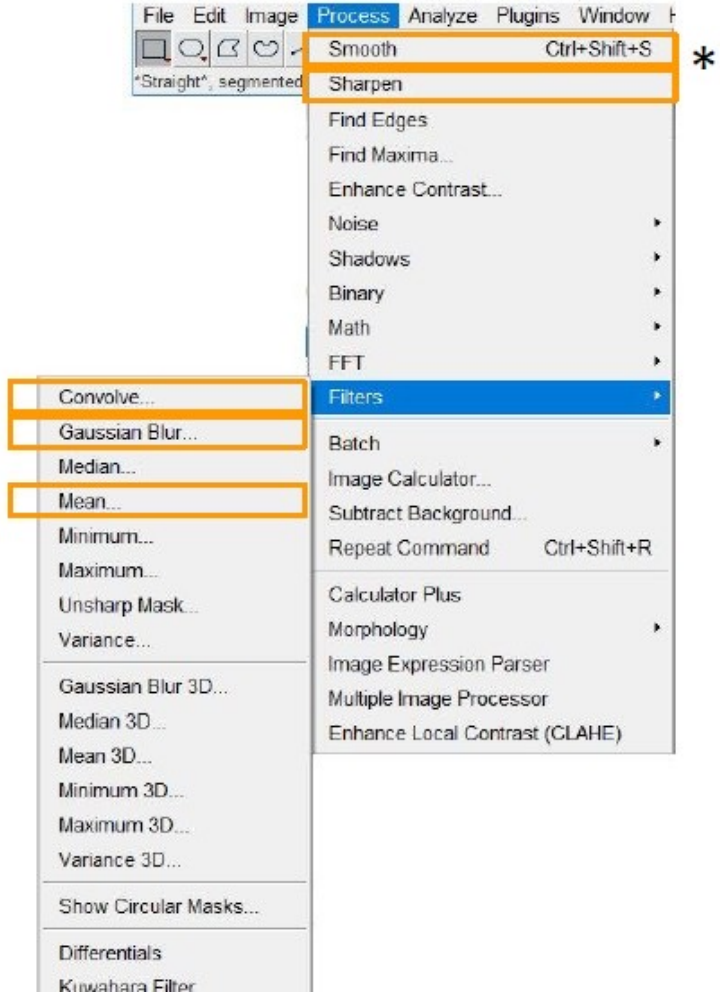
- Gaussian blur
- Mean
  - * Smooth is a 3x3 Mean filter

LINEAR FILTERS

Linear filters:

- Convolve…
  - Allows us to specify our kernel



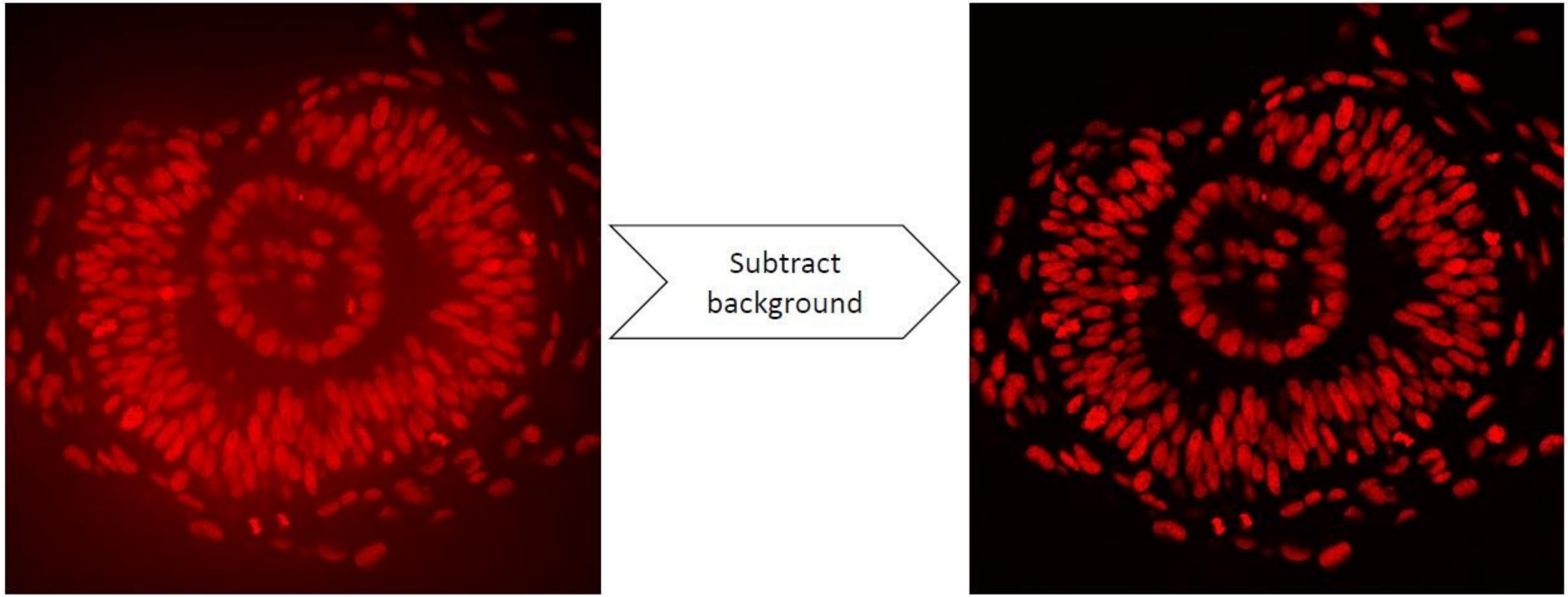


*

- Gaussian blur
- Mean
  - * Smooth is a 3x3 Mean filter

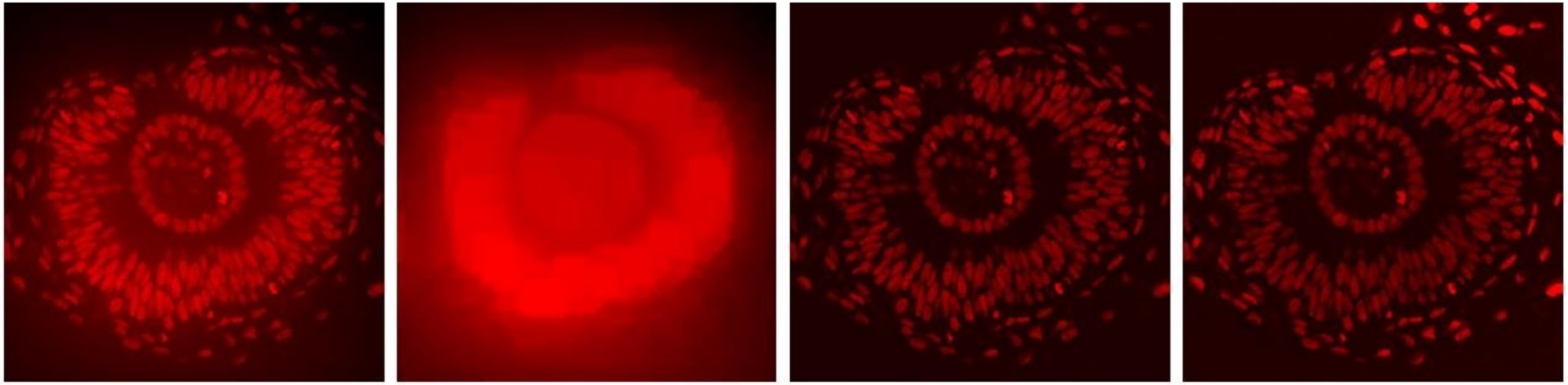
BACKGROUND REMOVAL

- Differentiating objects is easier if their background intensity is equal.



BACKGROUND REMOVAL

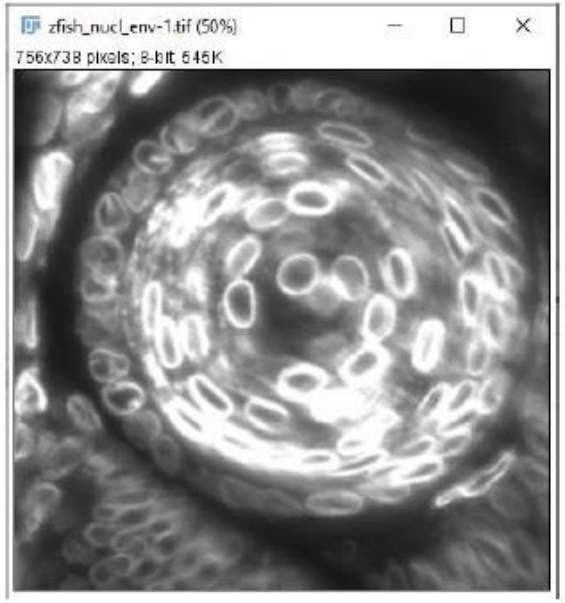
- Depending on the effect we want to correct for, it might make sense to divide an image by its background.

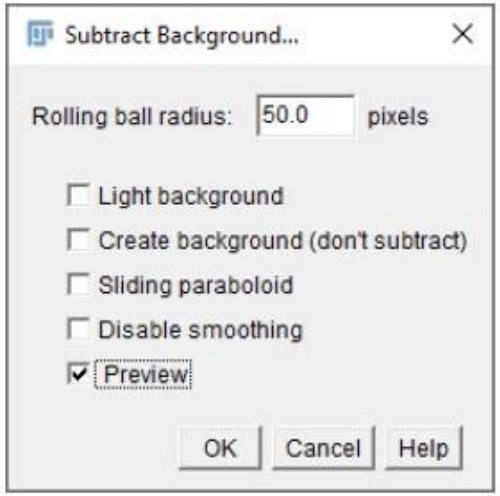


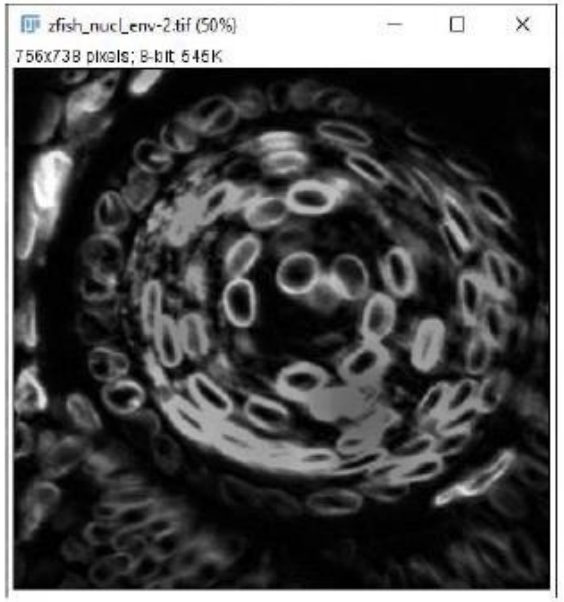
Original image | Extracted background | Subtracted background | Image divided by background

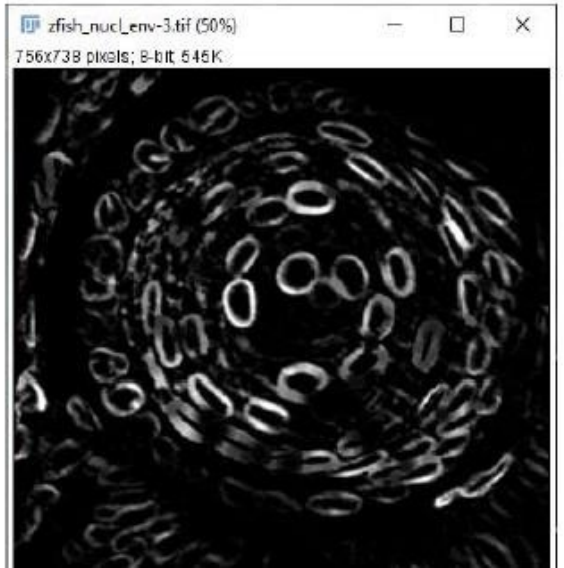
BACKGROUND REMOVAL

- Process > Subtract background…
  - The rolling ball radius should be a bit larger than the objects you are analyzing.
  - Try the preview checkbox while changing parameters!



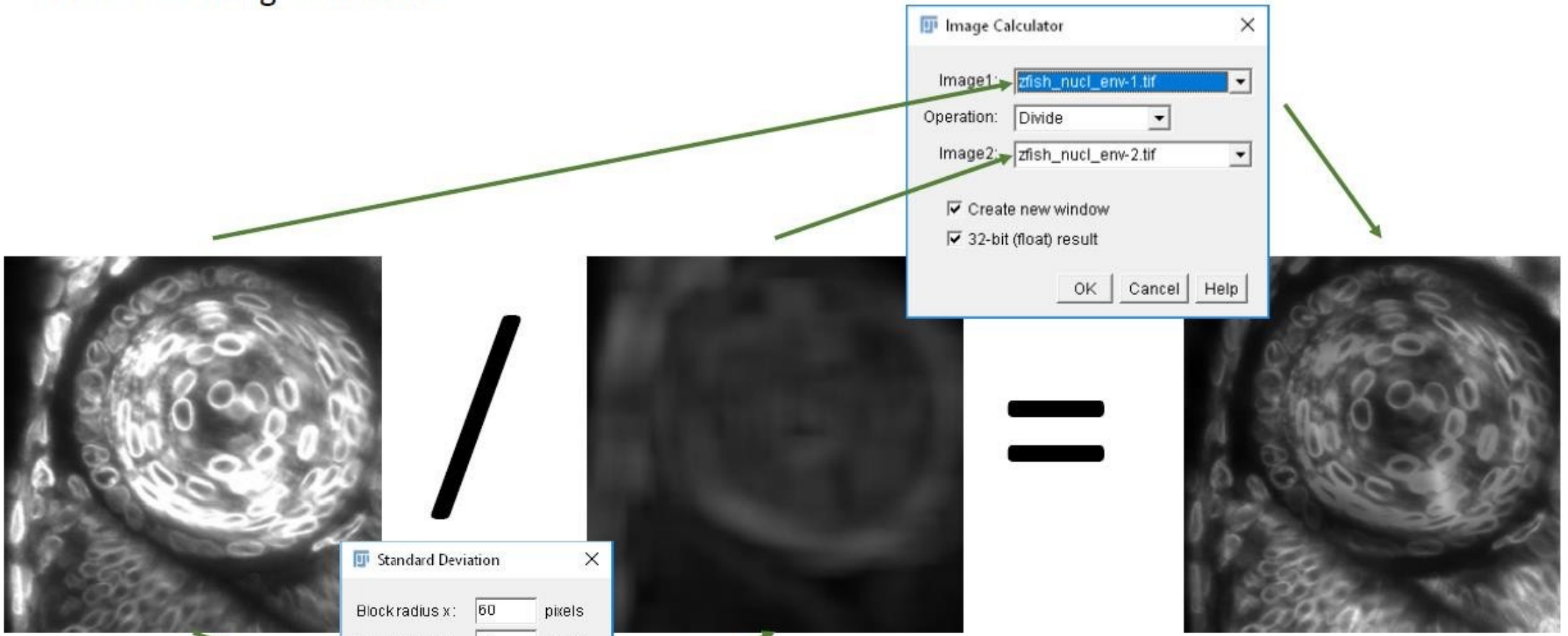






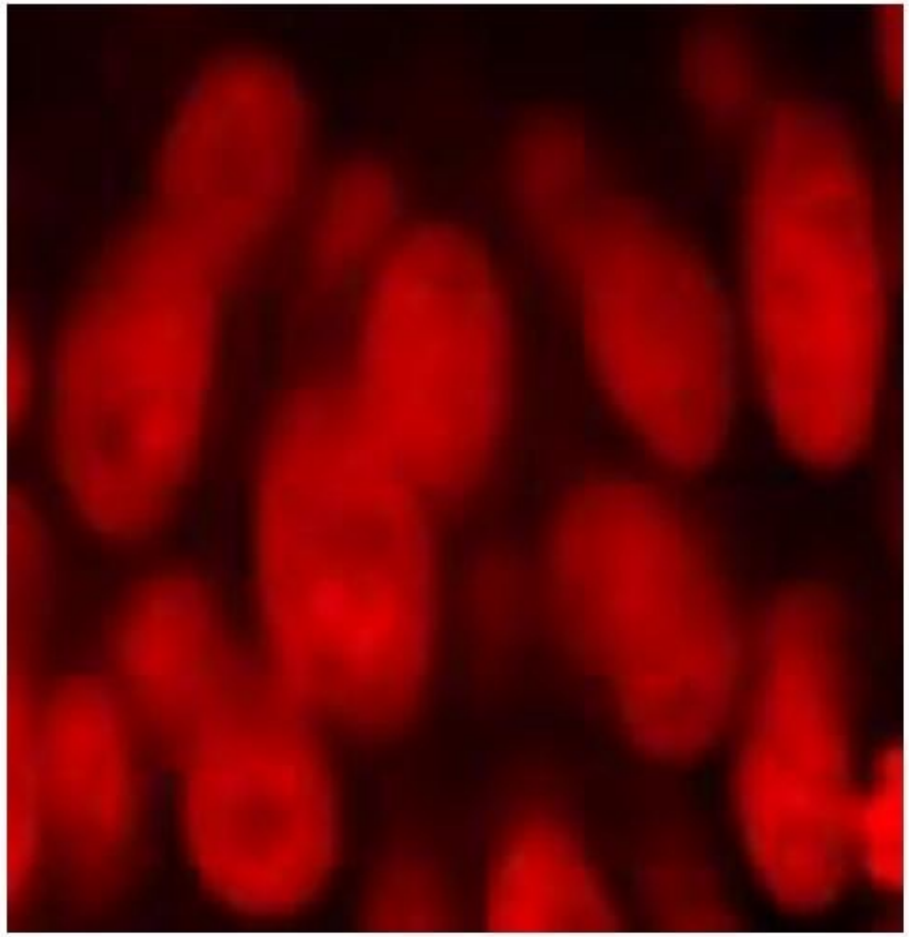
BACKGROUND REMOVAL

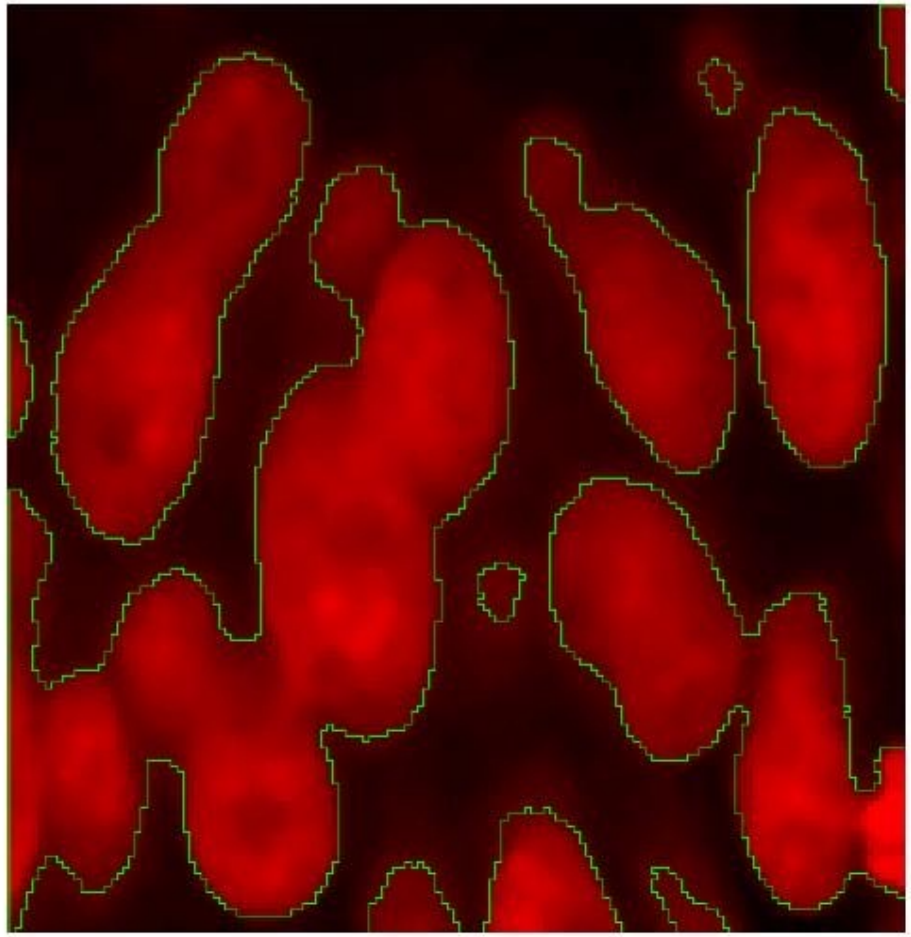
- Plugins > Integral image filters > Standard deviation
- Process > Image calculator



MATHEMATICAL OPERATIONS ON IMAGES

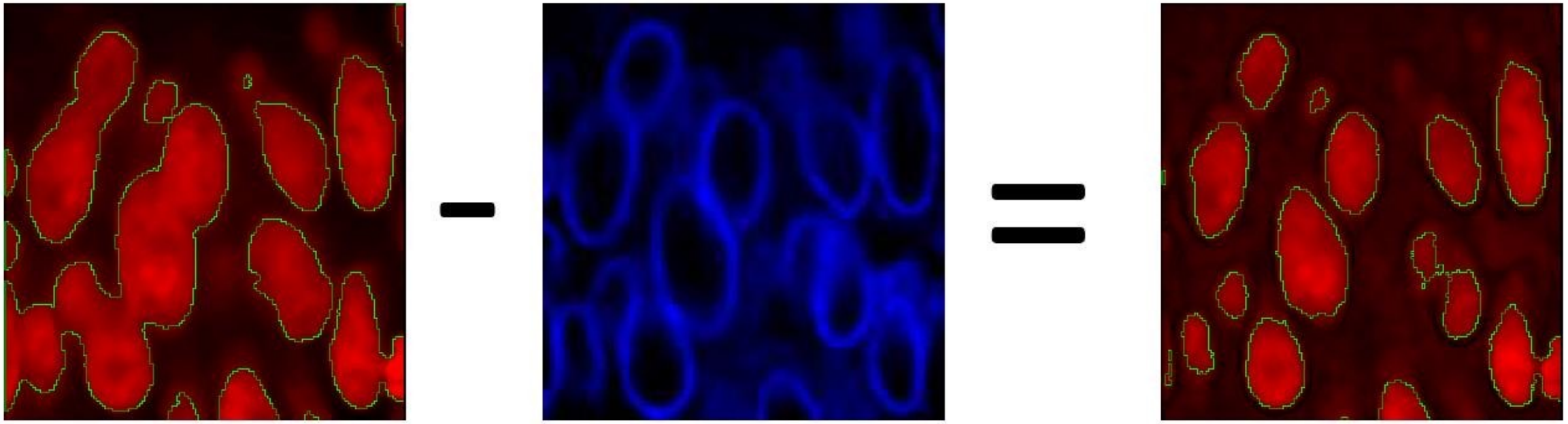
- It might be hard to differentiate objects from single images…





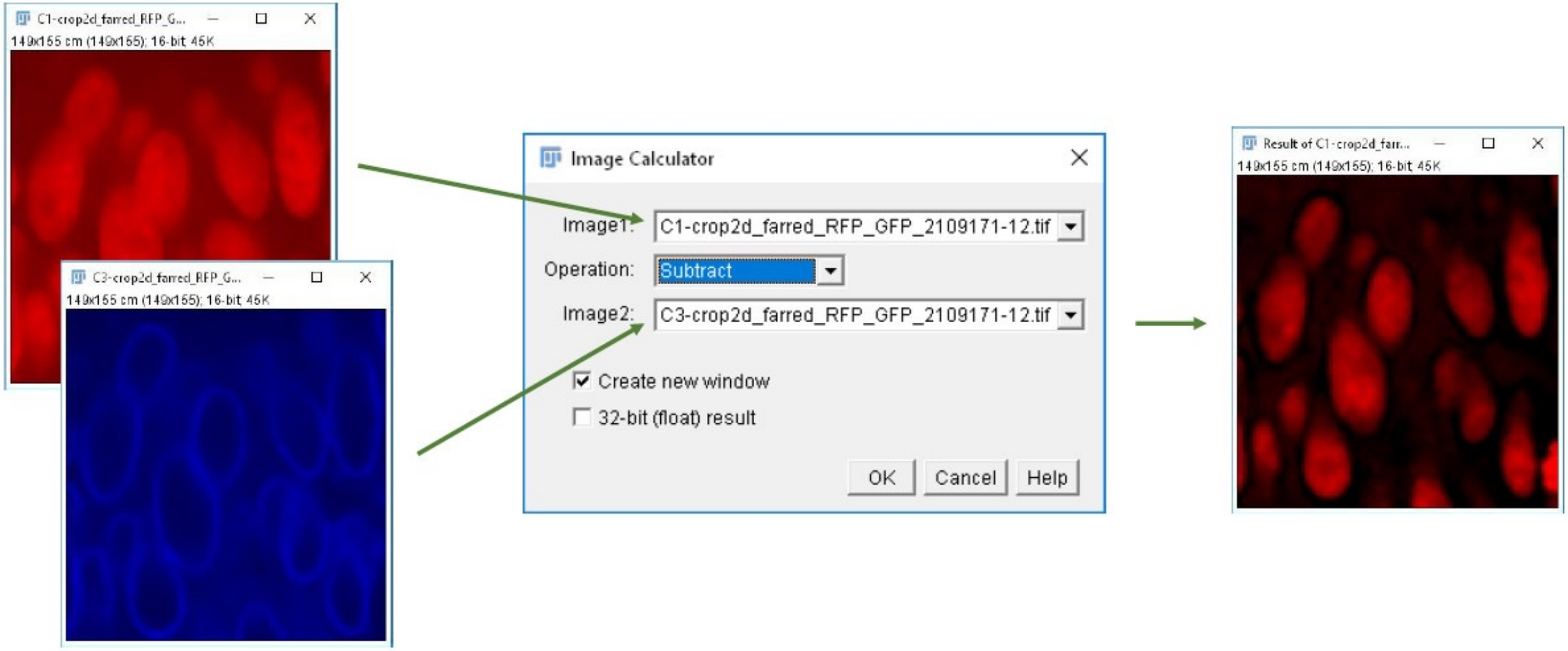
MATHEMATICAL OPERATIONS ON IMAGES

- Segmentation of nuclei can be improved by subtracting a channel visualizing nuclei envelope from the nuclei channel.



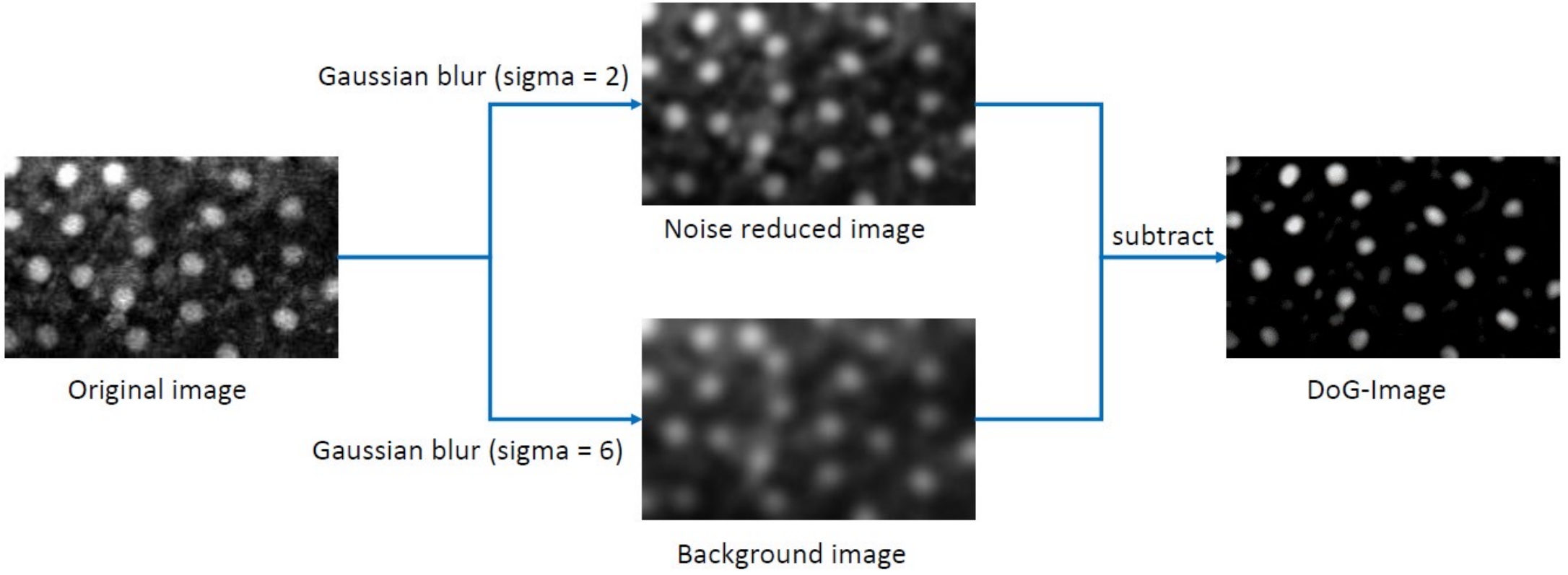
MATHEMATICAL OPERATIONS ON IMAGES

- Use the *Image Calculator* to subtract, add, multiply or divide images.



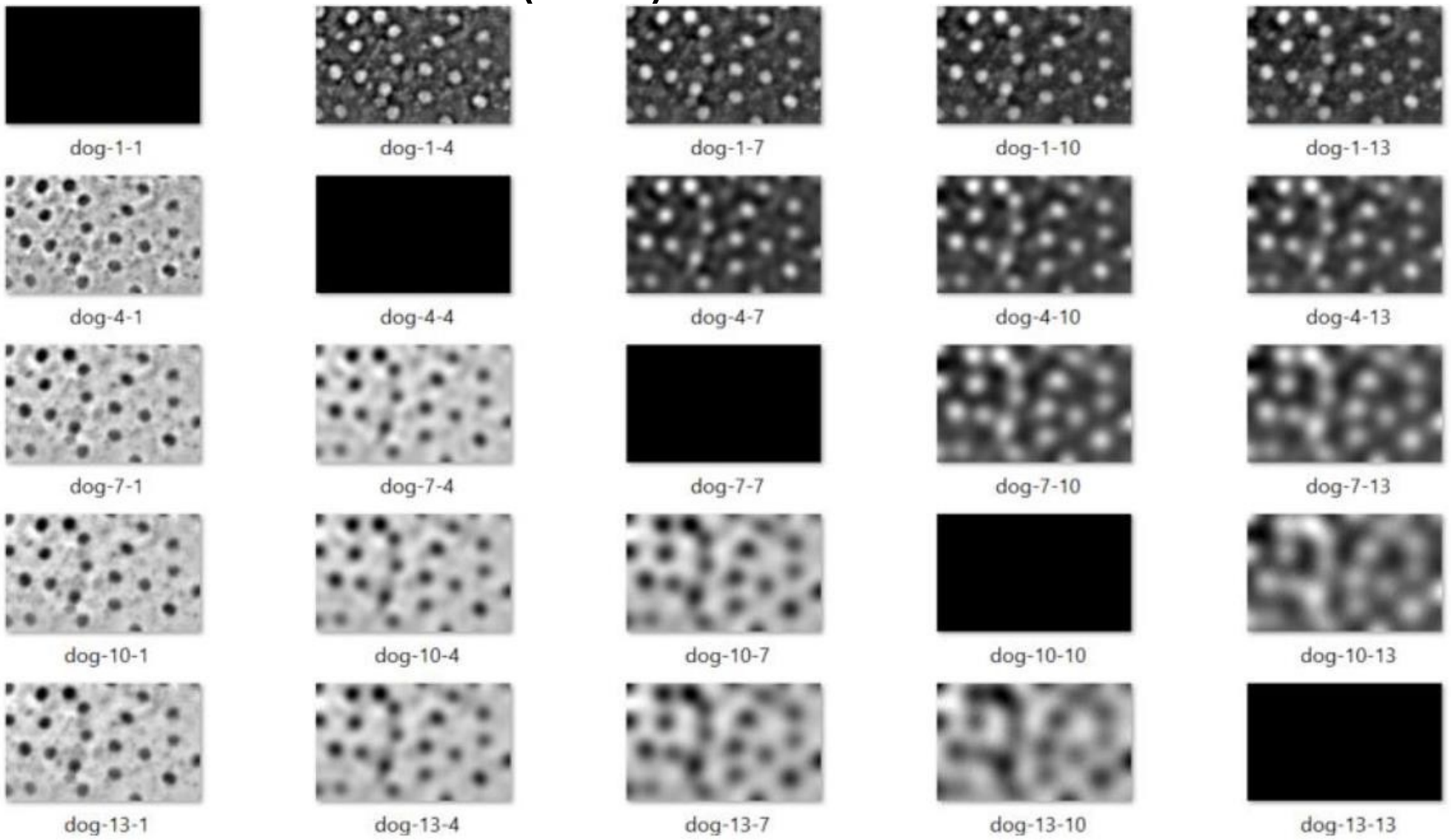
DIFFERENCE OF GAUSSIAN (DoG)

- Improve image in order to detect bright objects.



DIFFERENCE OF GAUSSIAN (DoG)

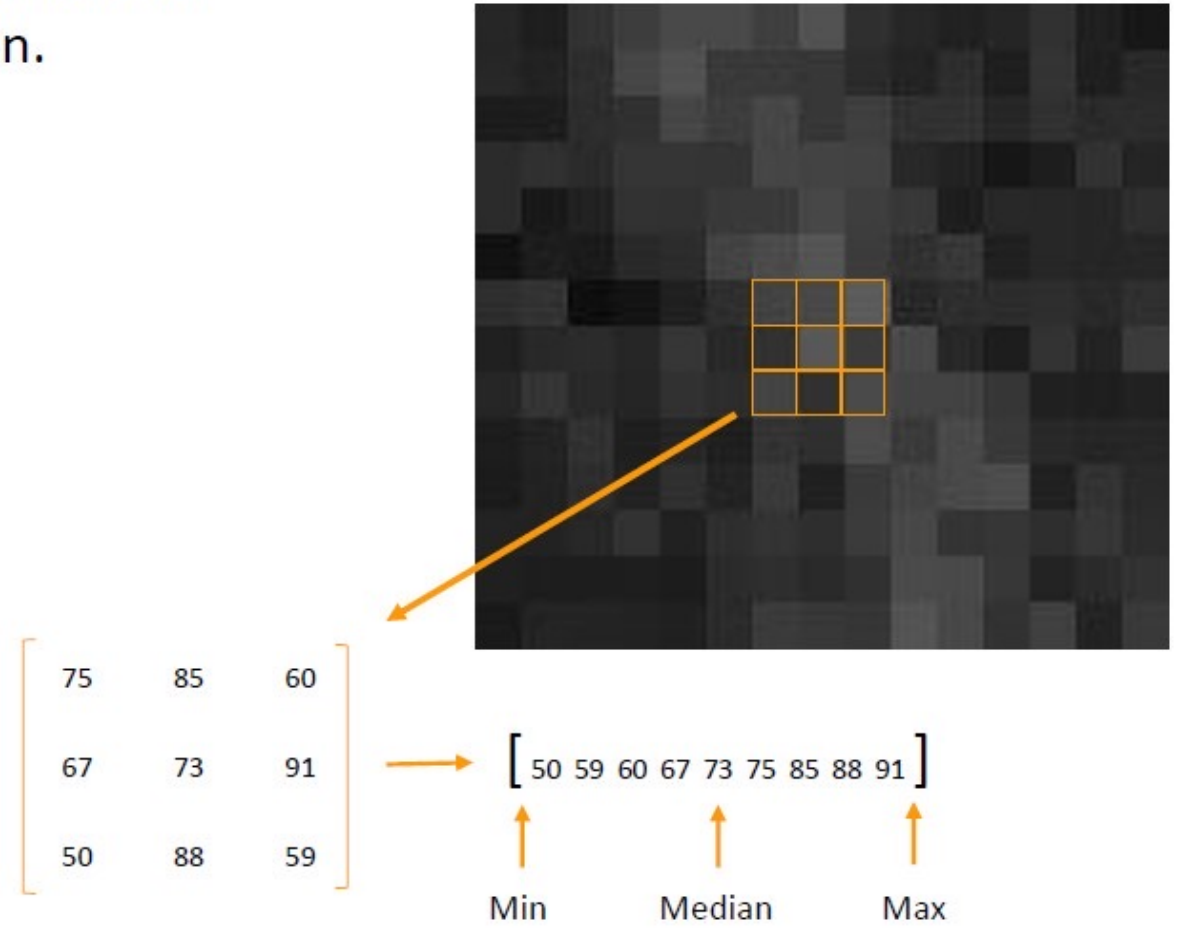
- Example DoG images



NON-LINEAR FILTERS

- Non linear filters also replace pixel value inside as rolling window but in a non linear function.

- Examples: order statistics filters
  - Min
  - Median
  - Max
  - Variance
  - Standard deviation



NON-LINEAR FILTERS

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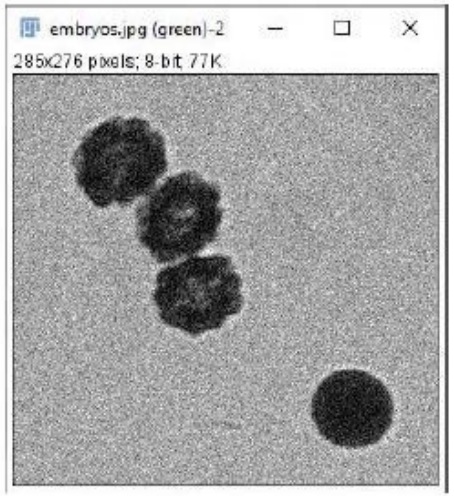
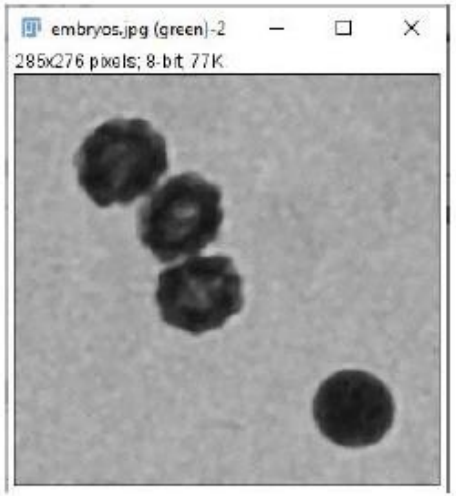
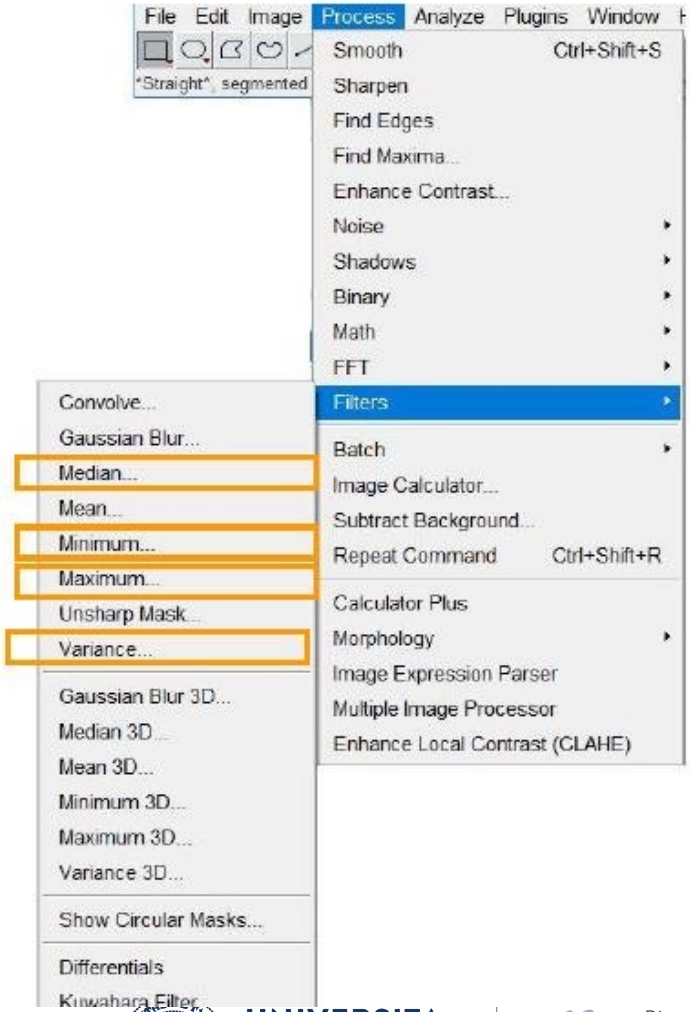
- Examples:
  - Min
  - Median
  - Max
  - Variance

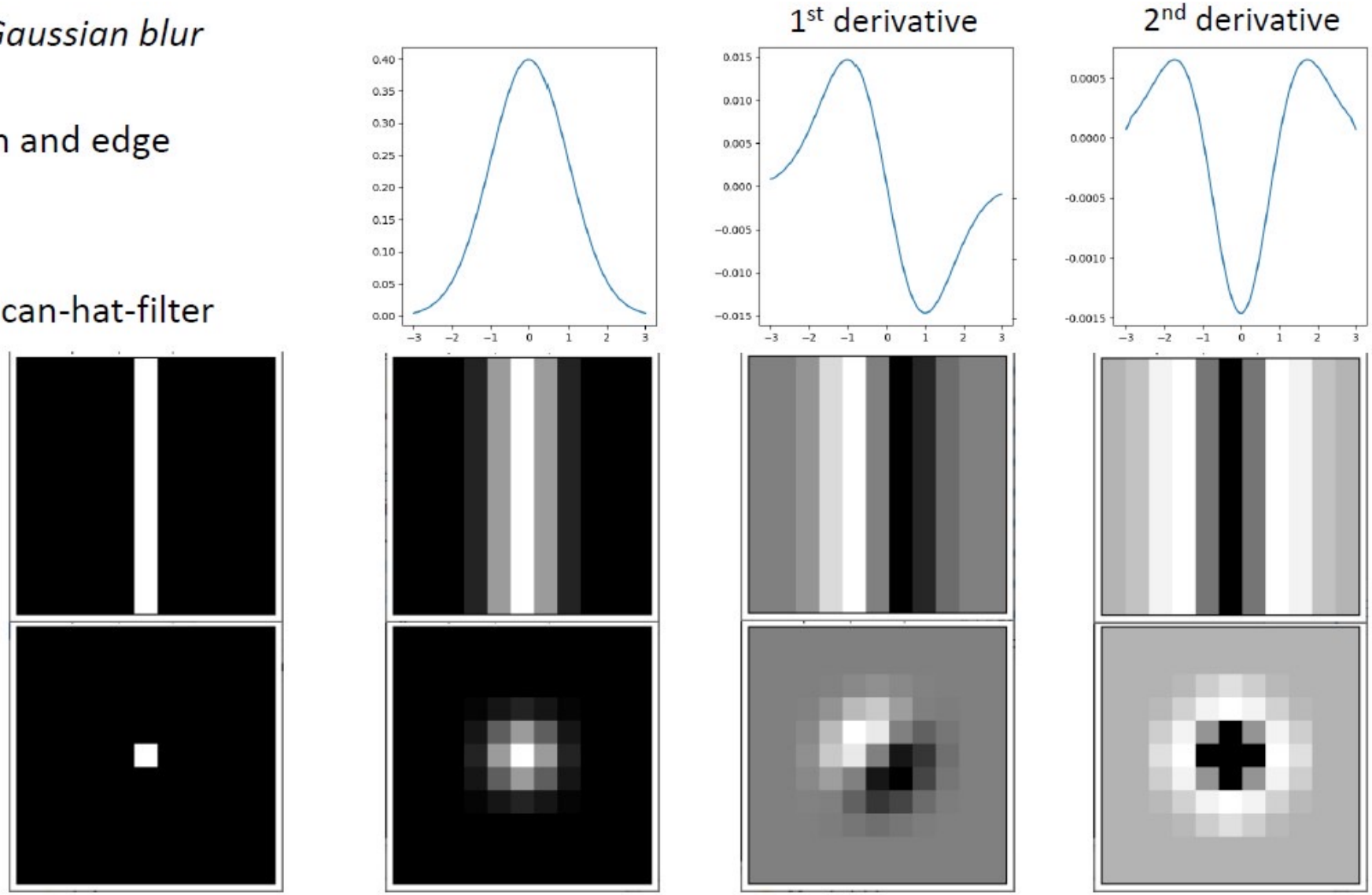






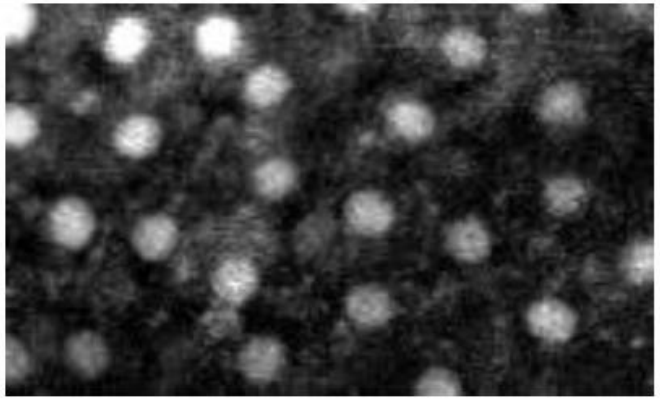
LAPLACE FILTERS

- *Second derivative of a Gaussian blur filter*
- Used for edge-detection and edge enhancement
- Also known as the Mexican-hat-filter



LAPLACIAN OF GAUSSIAN (LoG)

Laplace filter

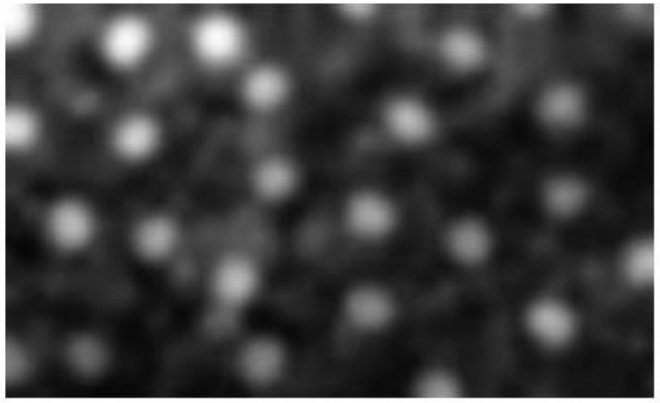


$$* \begin{bmatrix} -1 & -1 & -1 \\ -1 & 8 & -1 \\ -1 & -1 & -1 \end{bmatrix} =$$

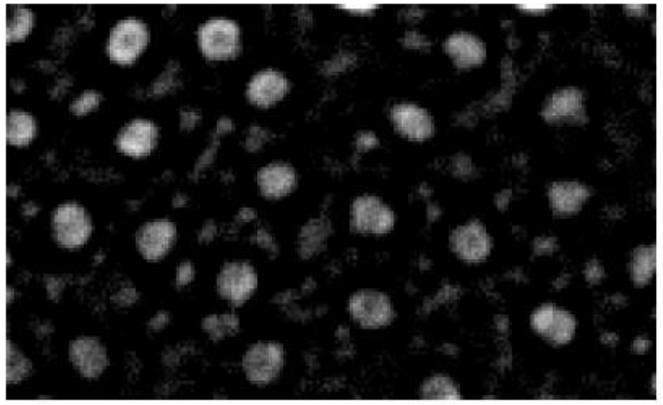
Laplace filtered image

Gaussian blur

Laplacian of Gaussian filter



$$* \begin{bmatrix} -1 & -1 & -1 \\ -1 & 8 & -1 \\ -1 & -1 & -1 \end{bmatrix} =$$

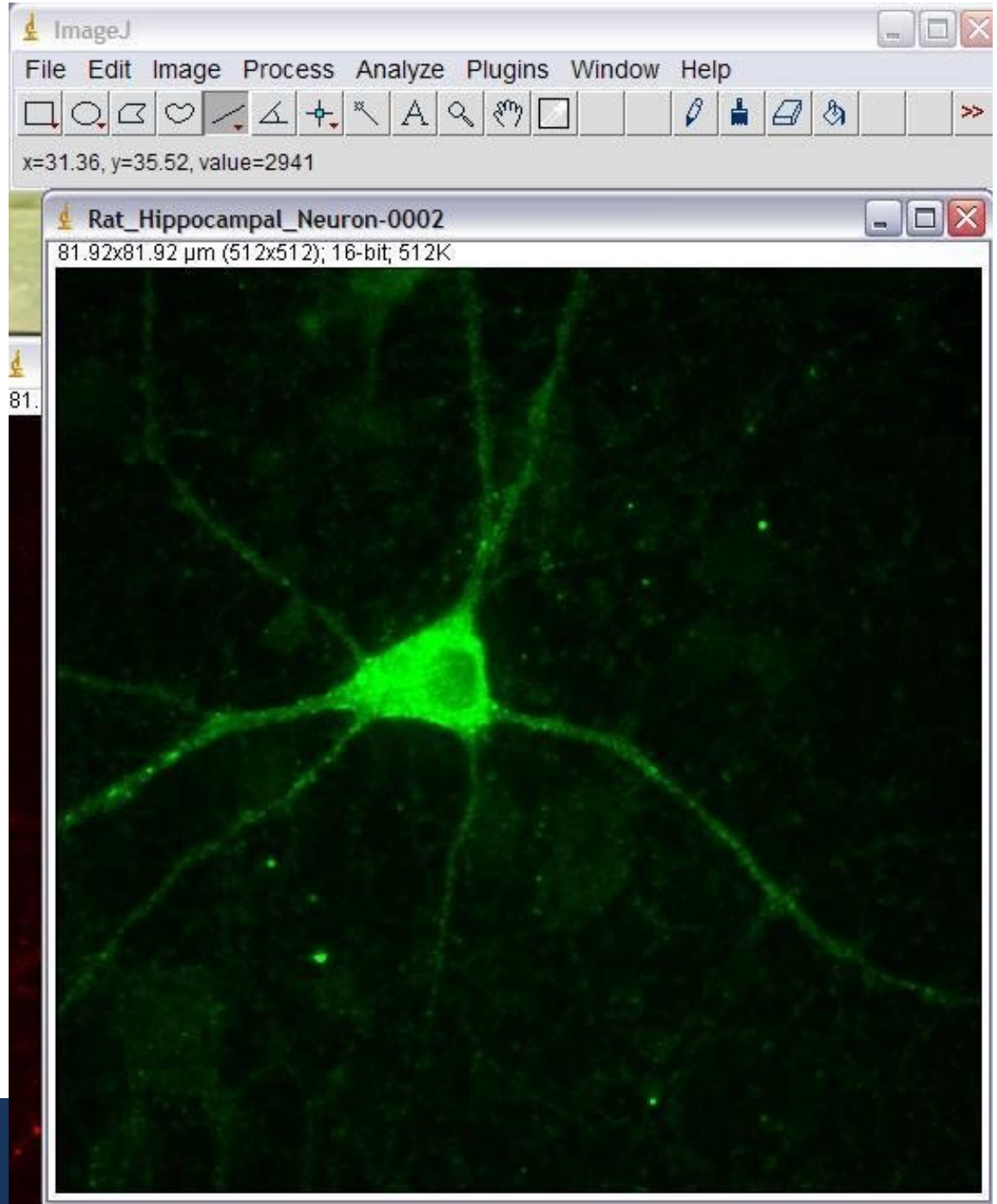


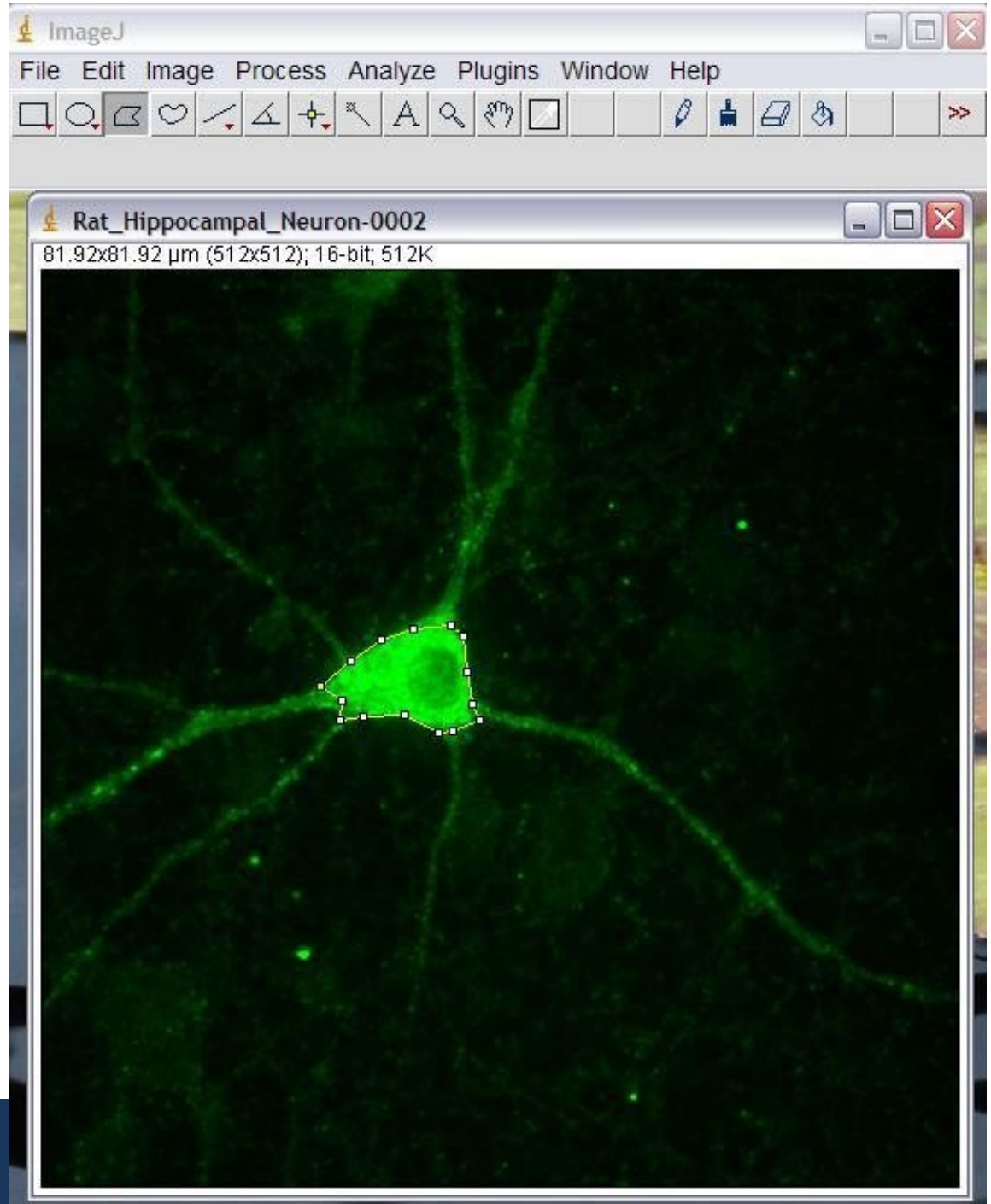
LoG image

Basic Intensity Quantification with ImageJ

- Images mostly needed to be transformed into quantifiable data
- ImageJ is useful for getting information from images, including pixel intensity.
- There are a number of different ways to get intensity information from images using the base package of ImageJ (no plugins required).

Quantify Gray Levels Across an Entire Image or Single Object/Region

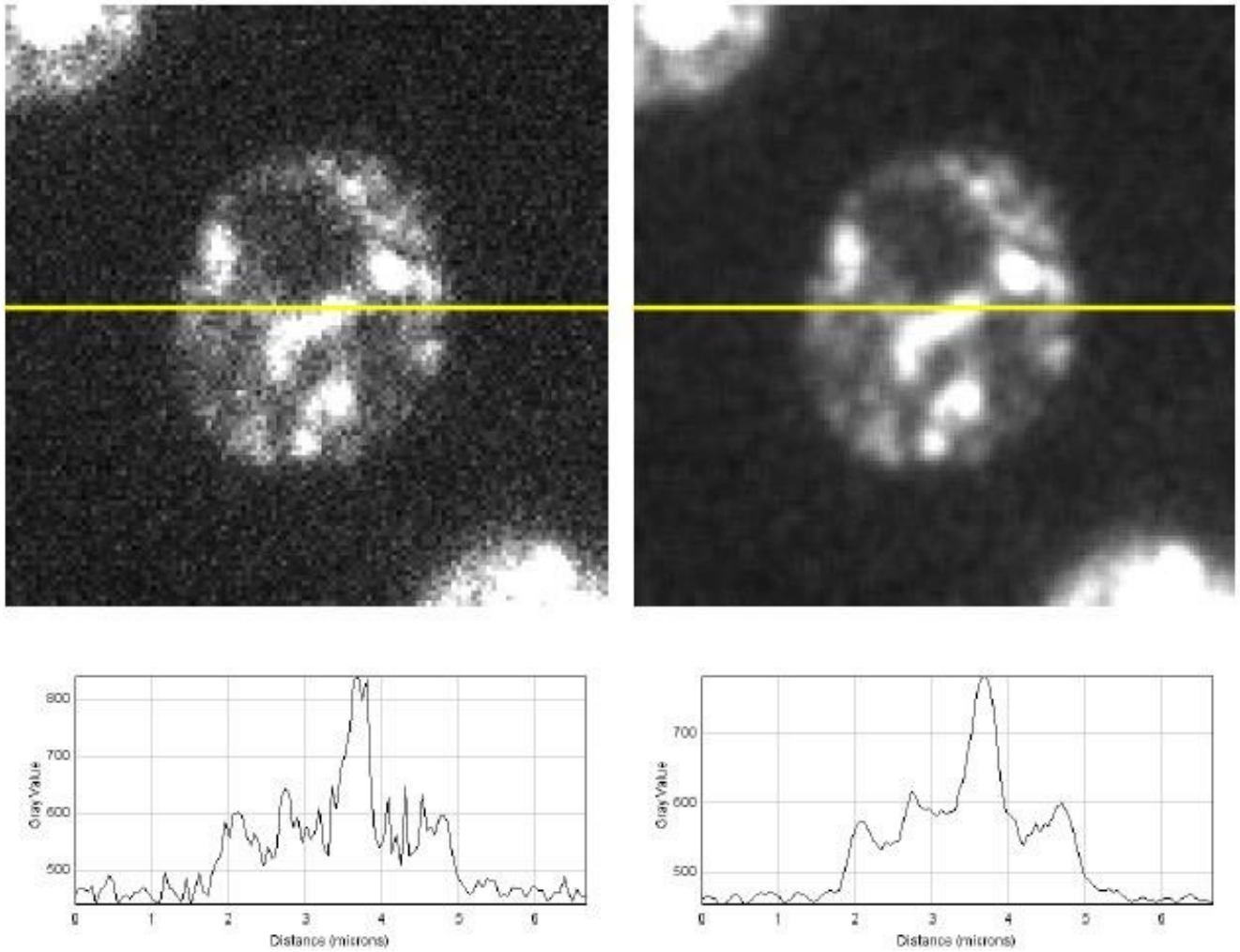




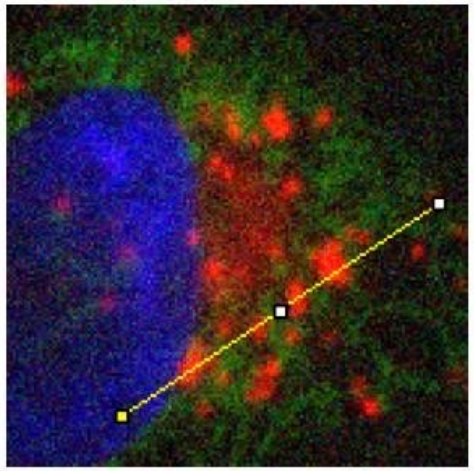
If you want to limit your measured area to just your object you draw a region of interest (ROI) around your object with one of the drawing tools (in the toolbar) and then

Analyze

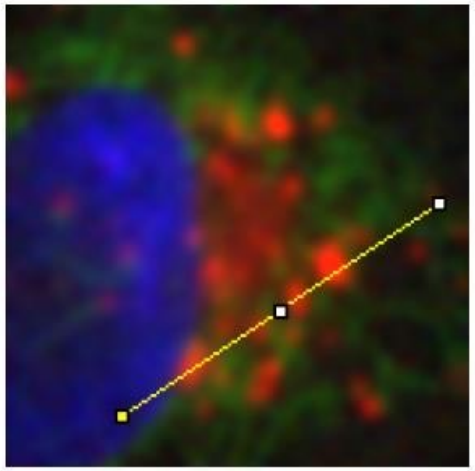
Measure



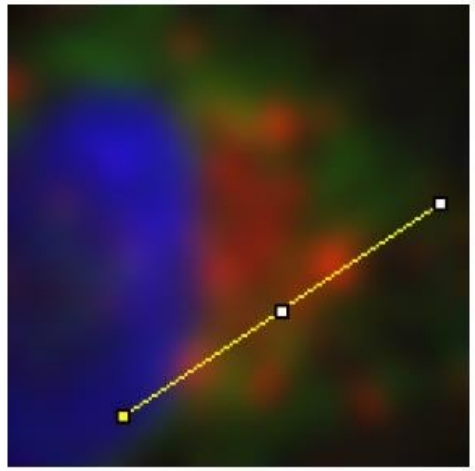
(a) Original image (b) Mean filtered



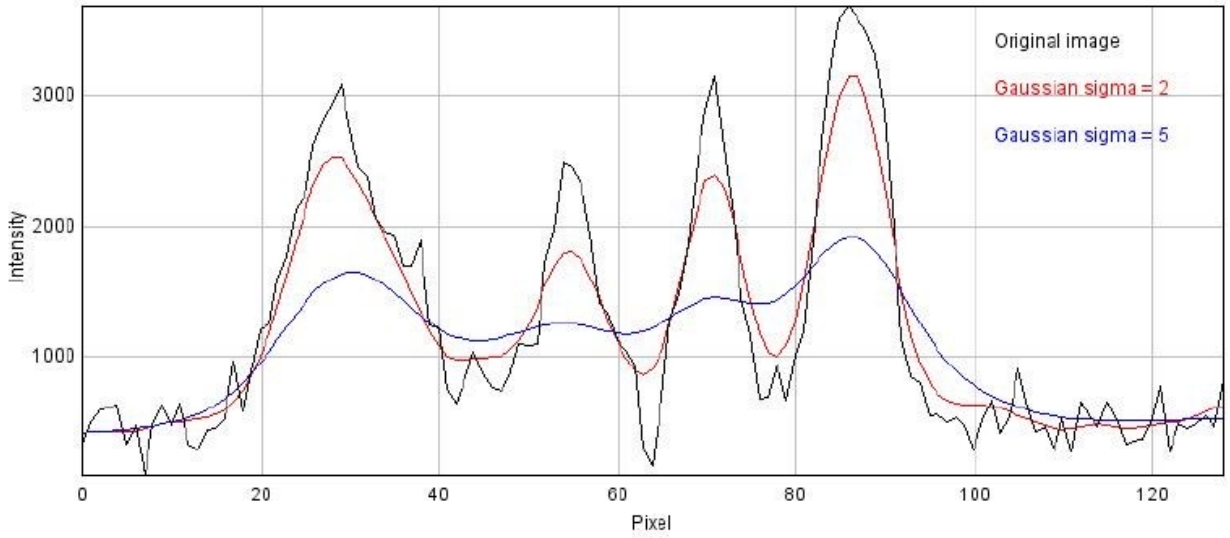
(a) Original image



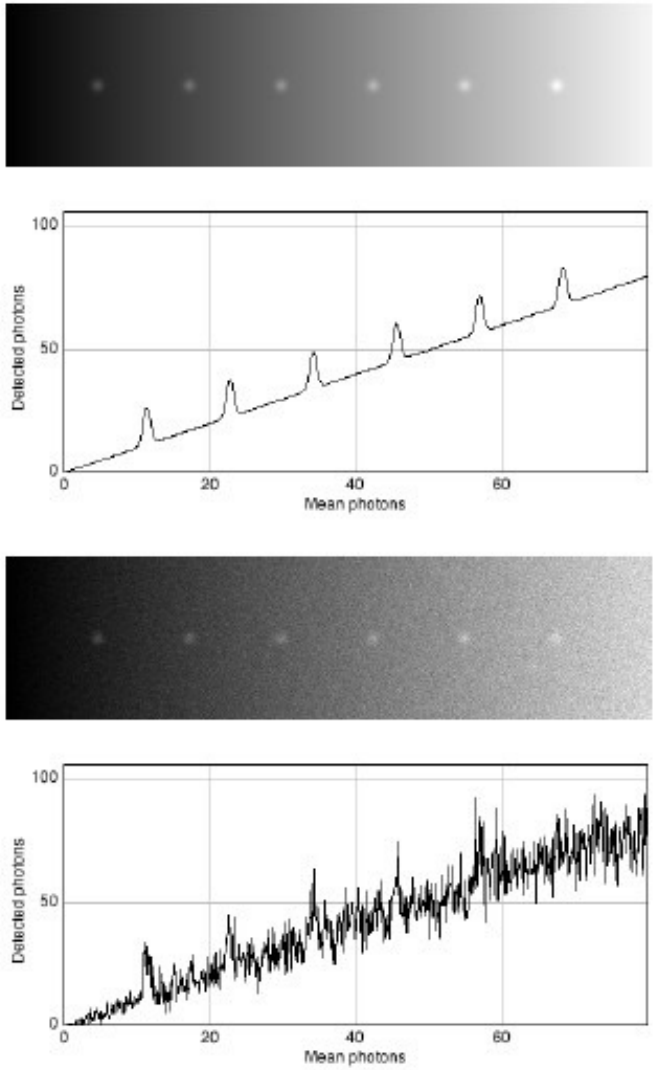
(b) Gaussian $\sigma = 2$



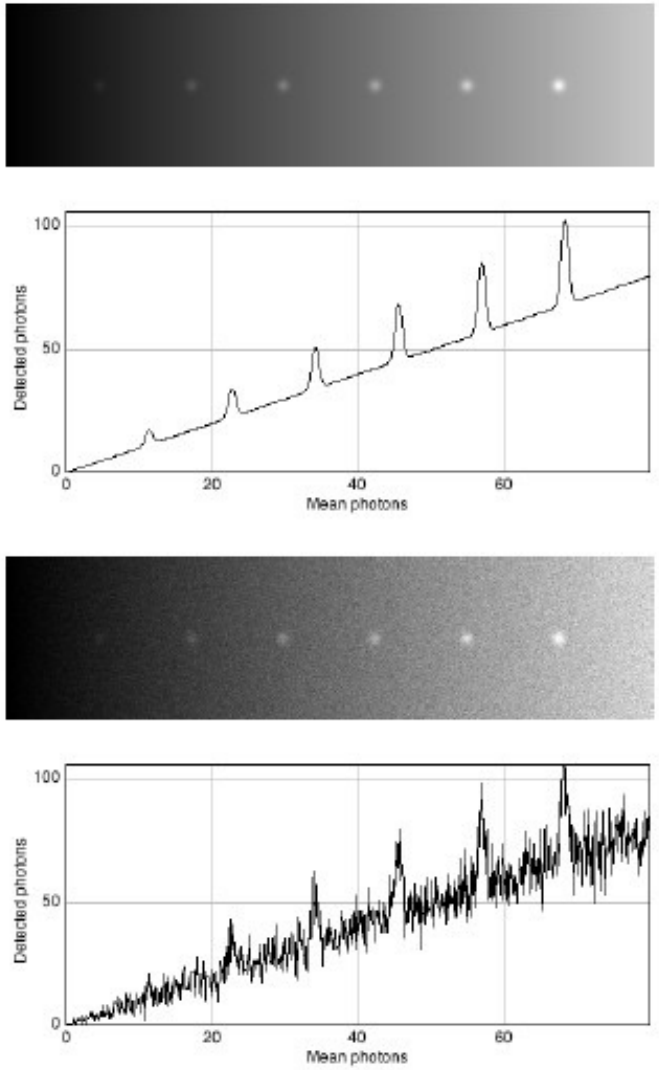
(c) Gaussian $\sigma = 5$



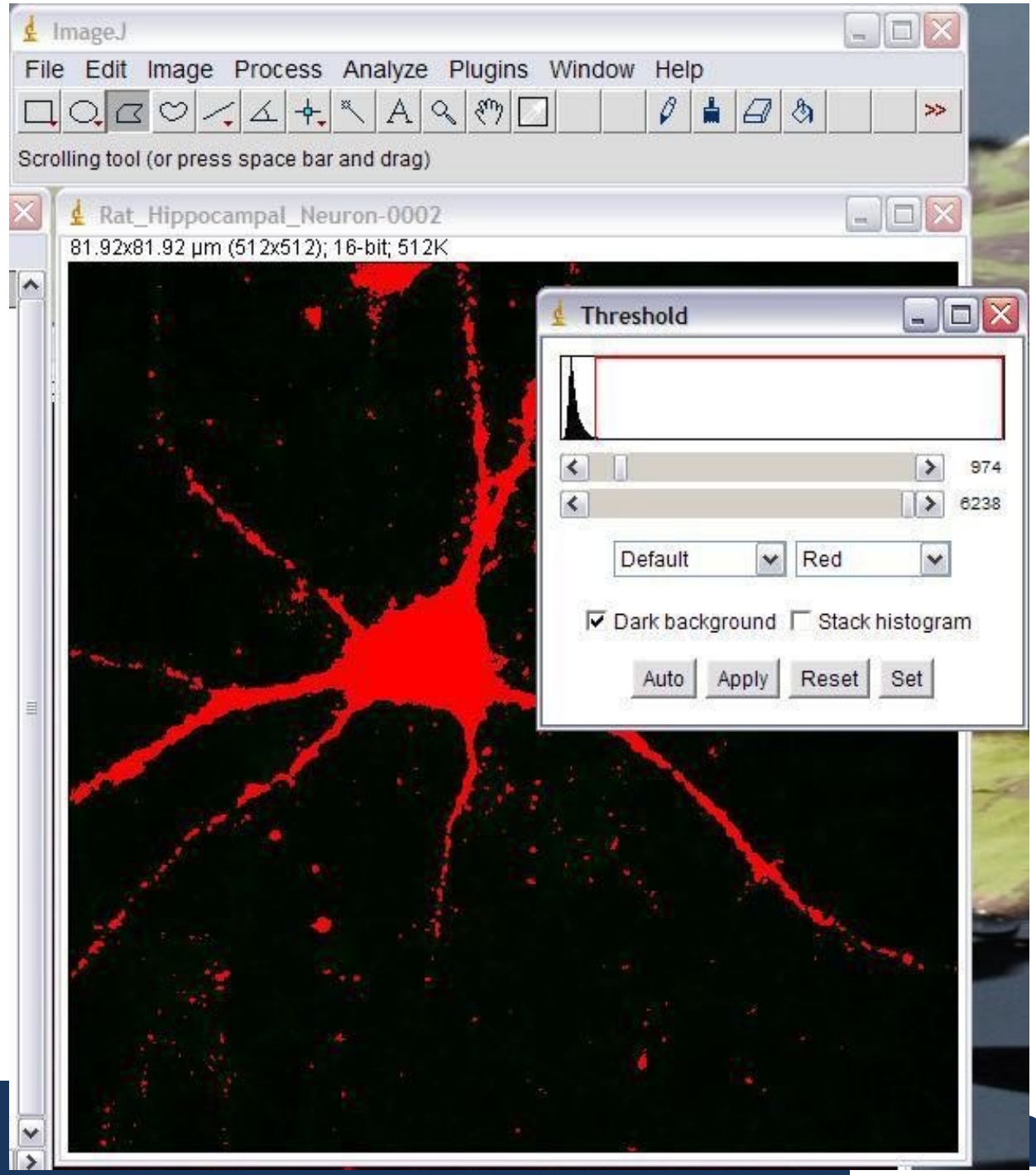
(d) Profile plots of the intensity in the red channel of the image



(a) Seeing spots with the same absolute brightness



(b) Seeing spots with the same relative brightness



Alternatively, you can go to Analyze > Set Measurements

and check off the box next to "Limit to Threshold."

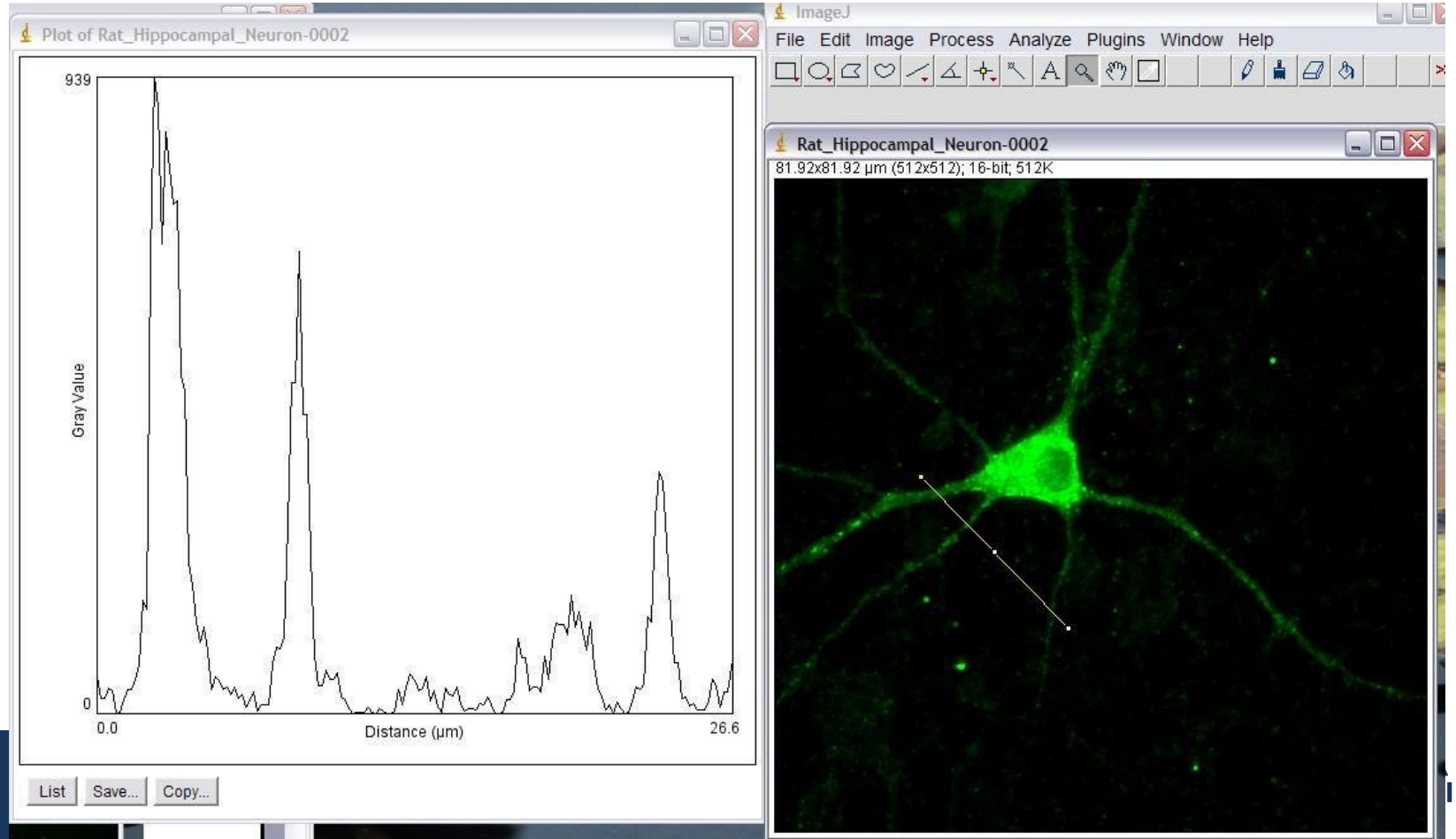
Then use

Image > Adjust >Threshold

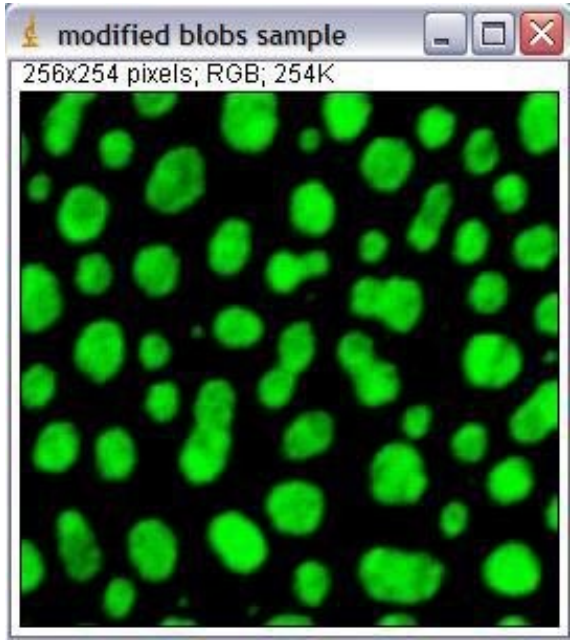
to highlight the area you want to analyze, and then

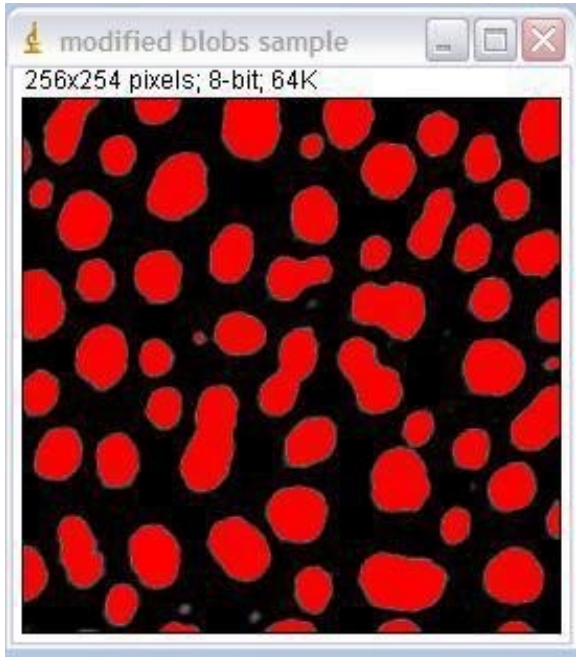
>Analyze

- You can use Analyze > Plot Profile to create a plot of intensity values across features in your image.
- In the example below, the plot gives the intensity values along the line drawn across three cell processes.



To Quantify Gray Levels for Each Object in Images with Multiple Objects



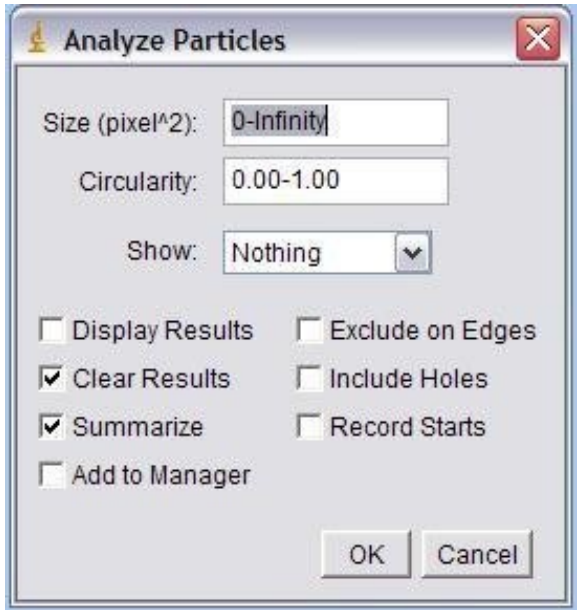


convert to grayscale

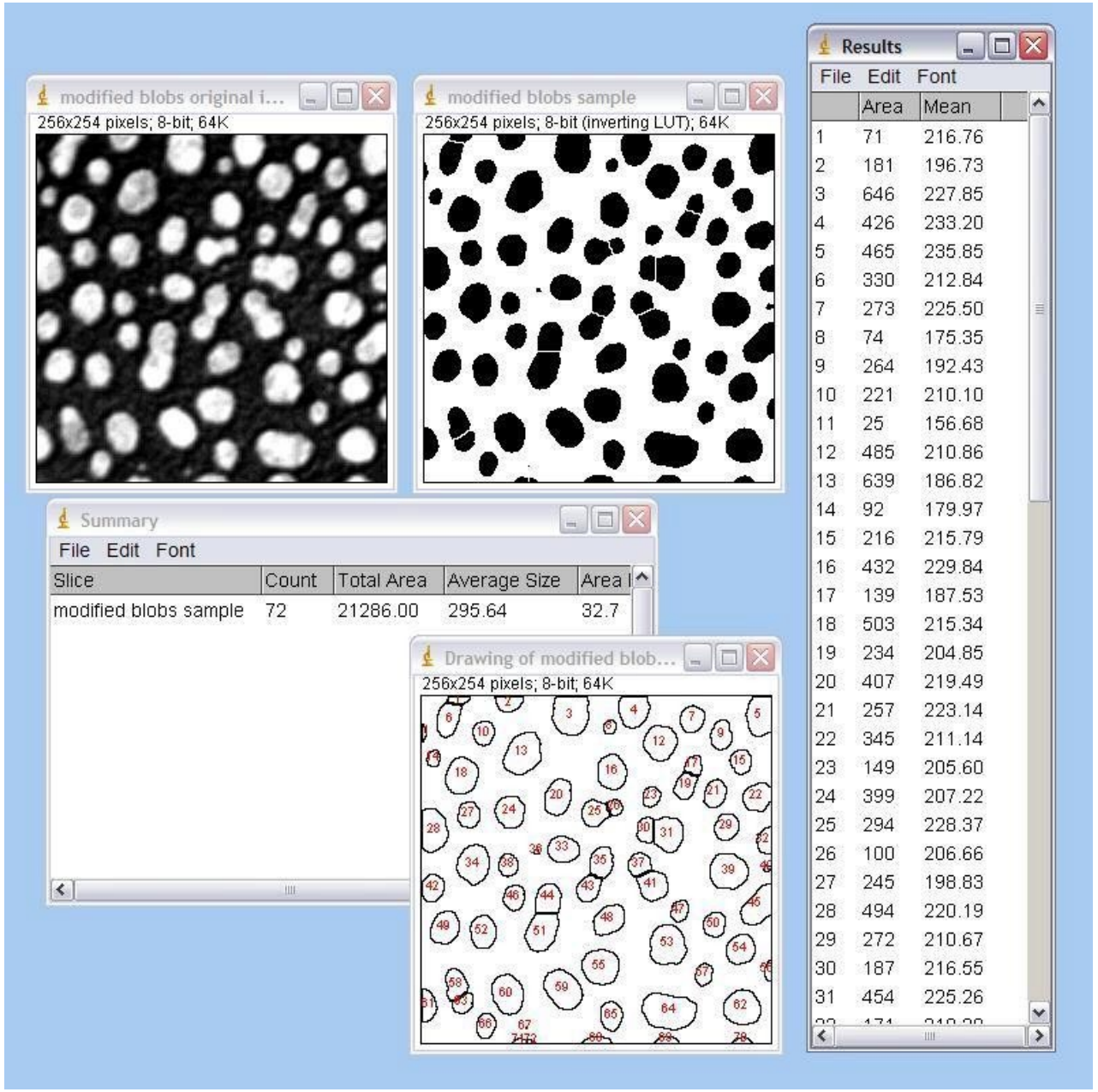
create a binary image,

Process > Binary > Watershed

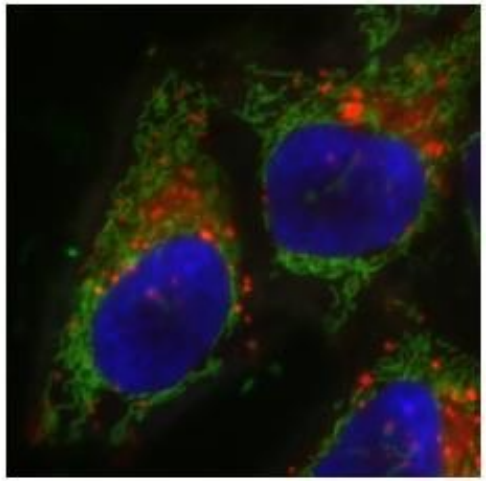
Analyze > Analyze Particles



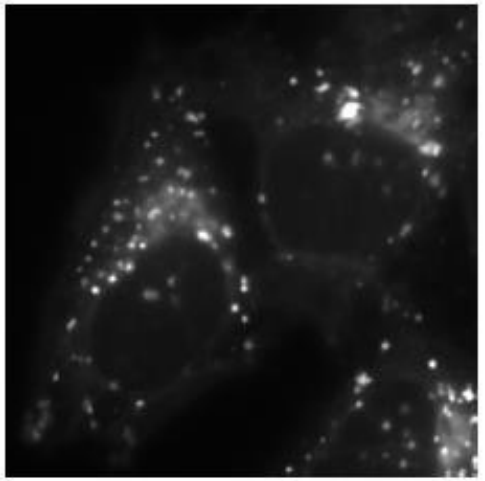
>



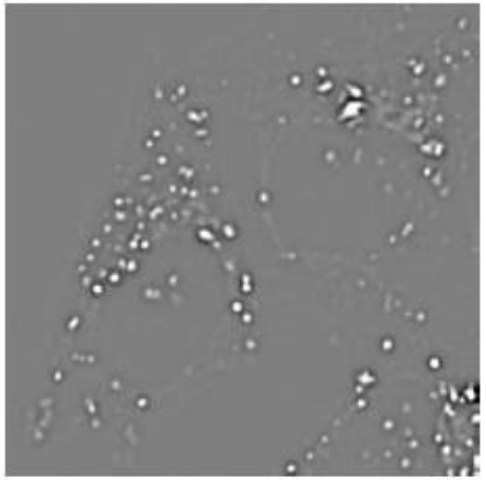
An simple image analysis workflow for detecting and measuring small spots, applied to the red channel of the sample image HeLa Cells.



(a) Original image



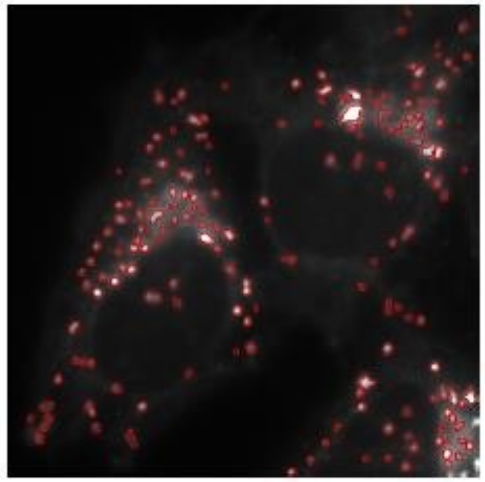
(b) Extract channel



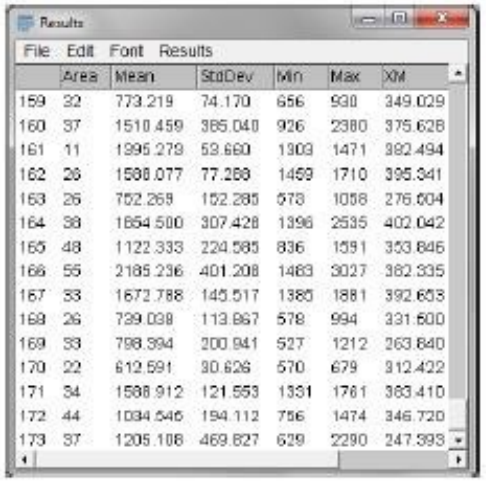
(c) Apply filters

(d) Apply threshold

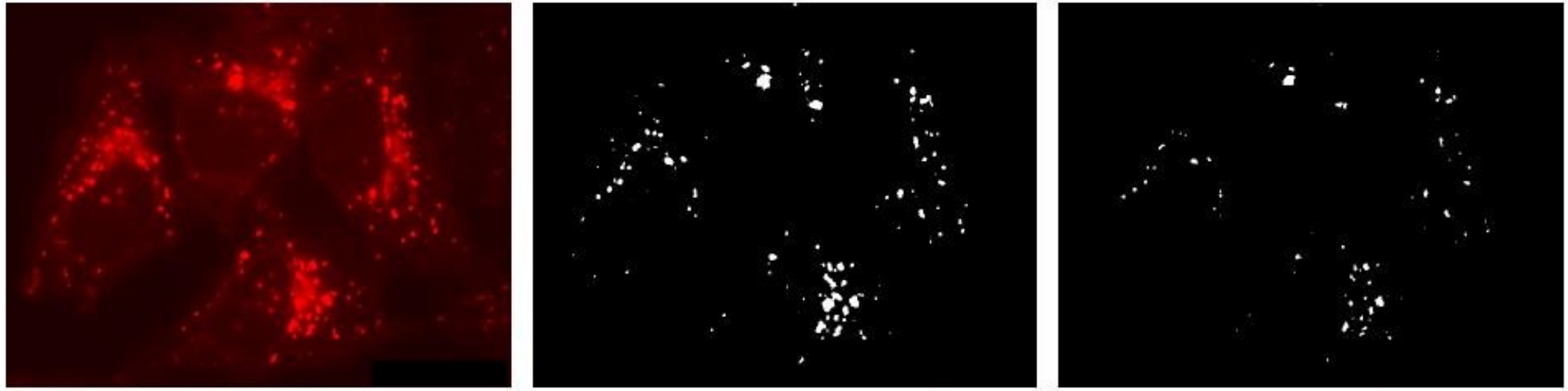
(e) Refine detection



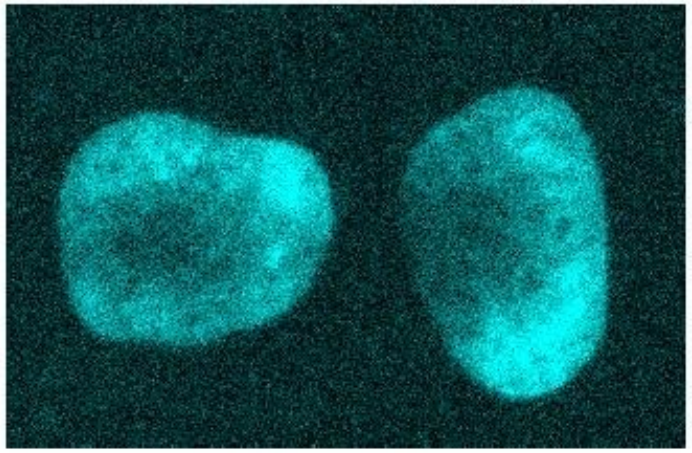
(f) Relate (e) to (b)



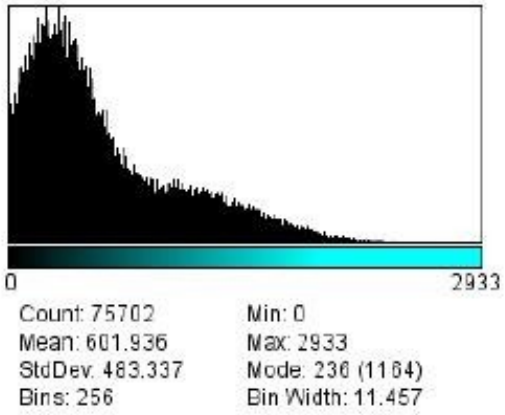
(g) Measure



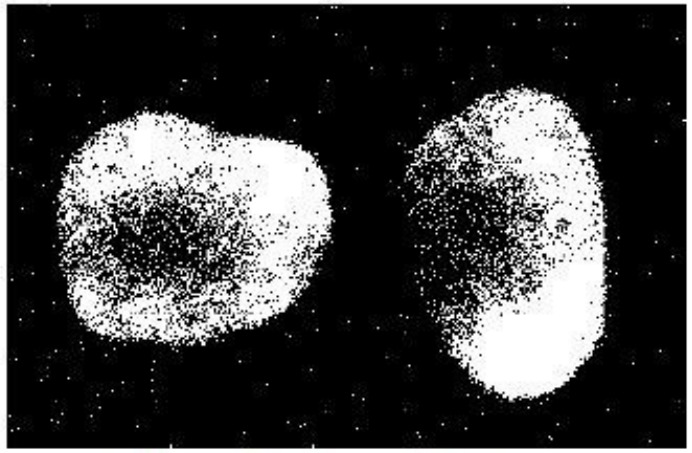
(a) Image (b) Low threshold (c) High threshold



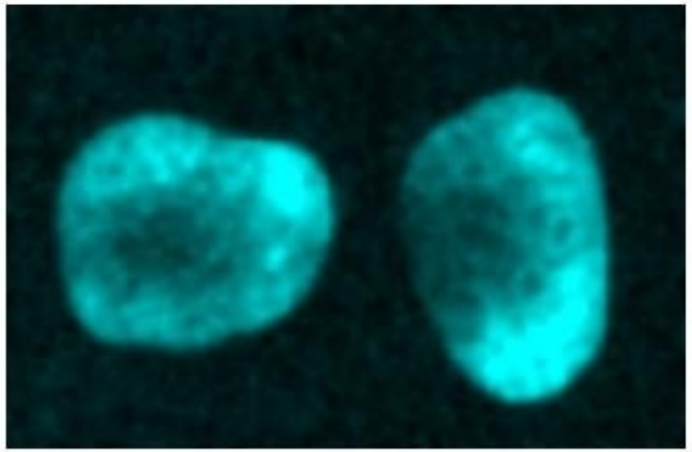
(a) Noisy image



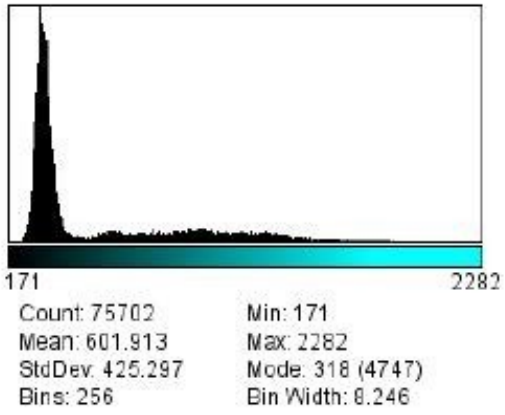
(b) Histogram of (a)



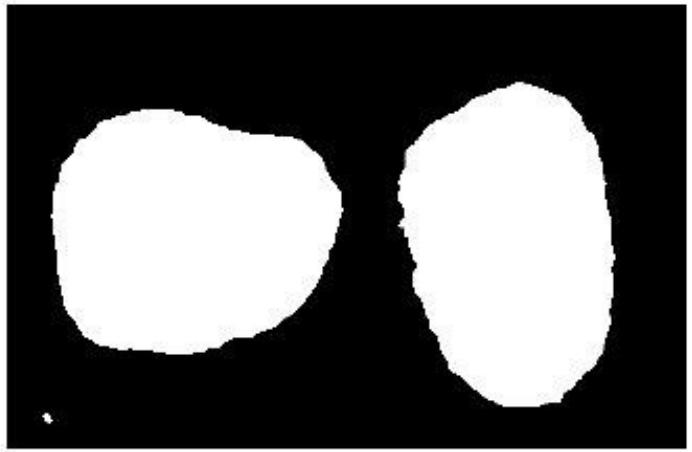
(c) Threshold applied to (a)



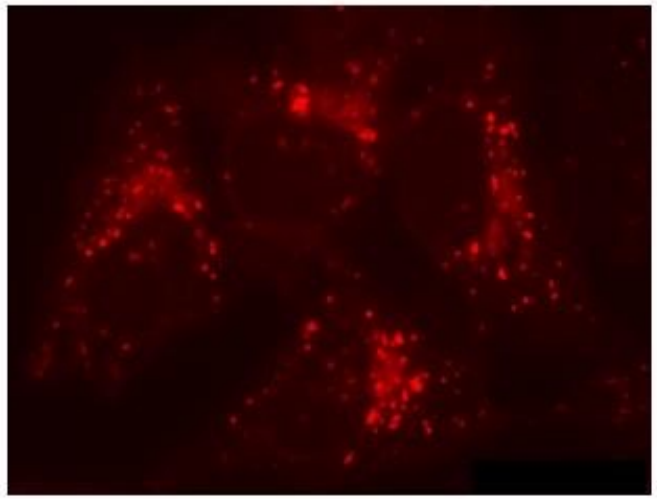
(d) Gaussian filtered image



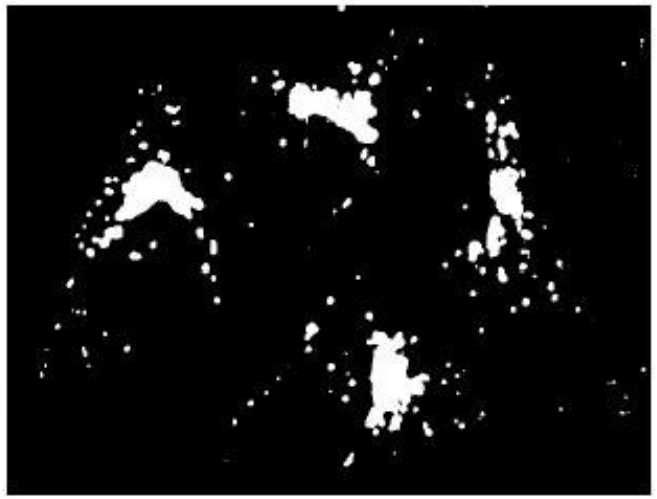
(e) Histogram of (d)



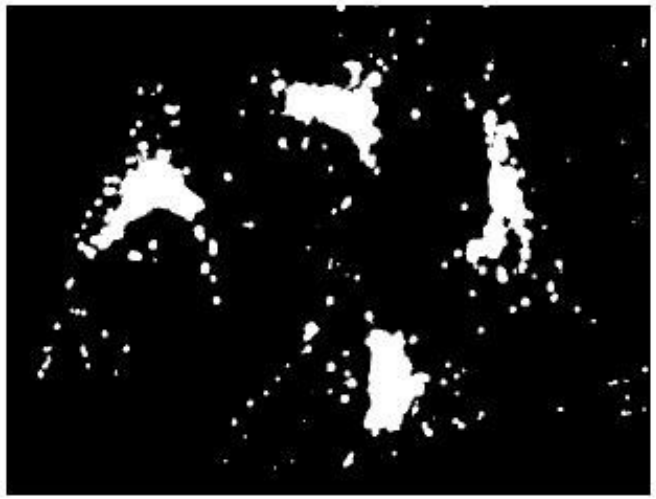
(f) Threshold applied to (d)



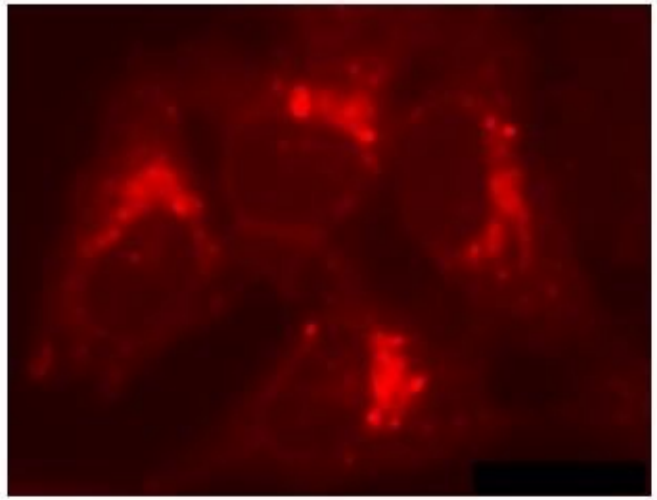
(a) Original image



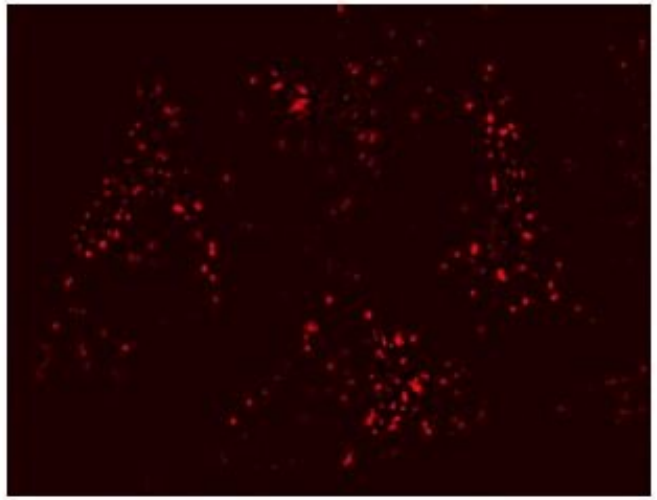
(b) Otsu's threshold



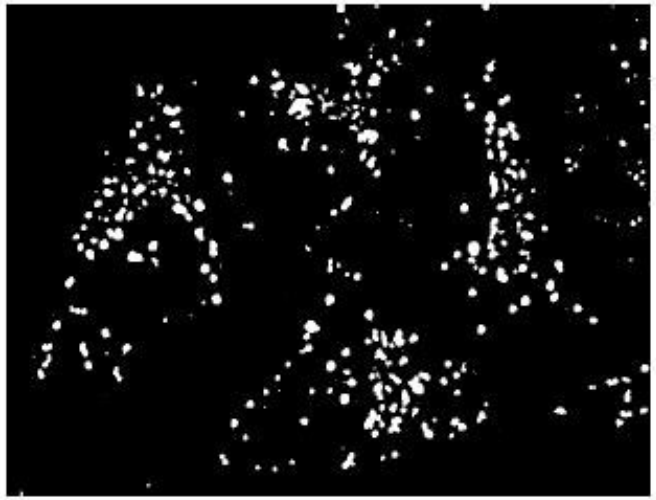
(c) Triangle threshold



(d) Median filtered image



(e) Result of (a)-(d)



(f) Triangle threshold of (e)

ImageJ How to Measure Mean Fluorescence Intensity Over Timelapse Image Stack

https://youtu.be/GHvndpGQKe4

Short introduction to histogram processing

https://youtu.be/nIRhHb04u_k