Light microscopy in Cellular Biology

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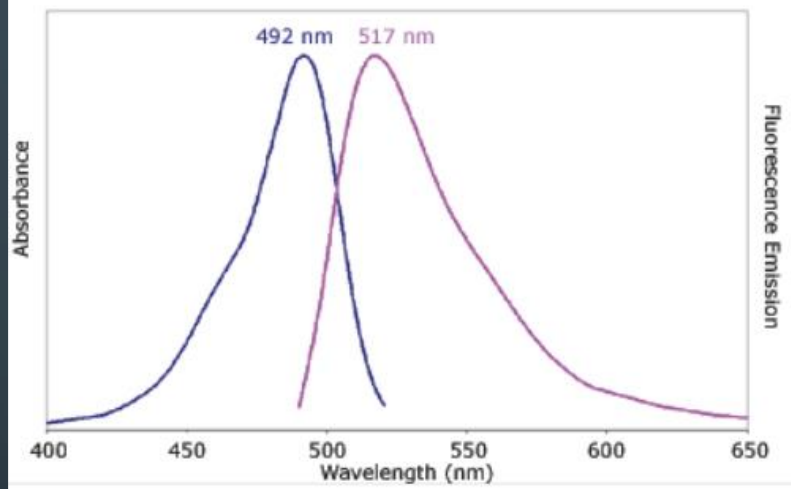
What is fluorescence?

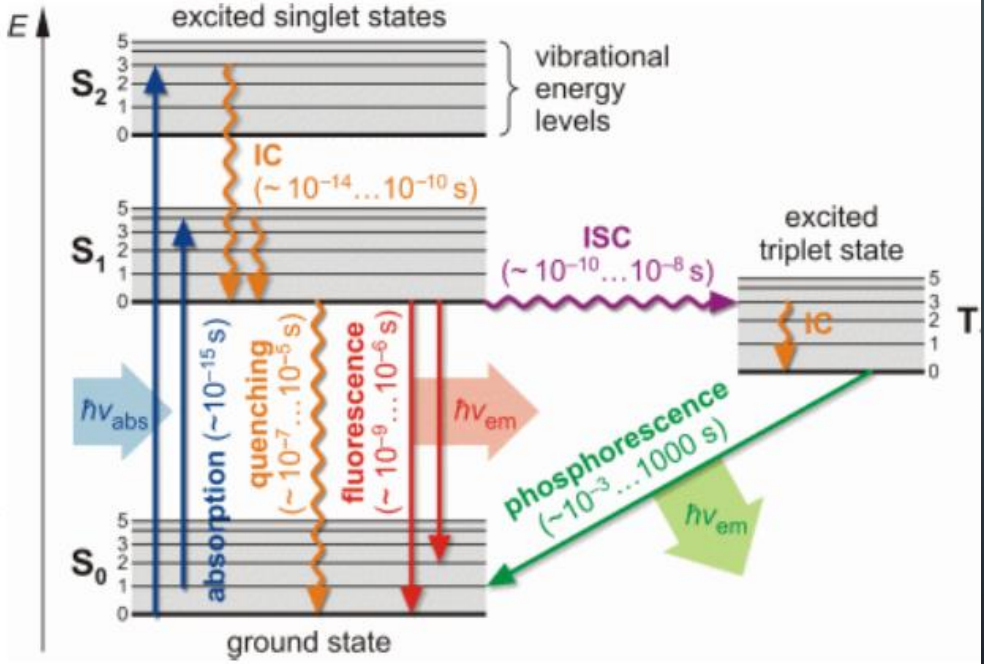
George Gabriel Stokes (1819-1903)

Stokes Shift



Alexa Fluor 488

Jablonski diagram



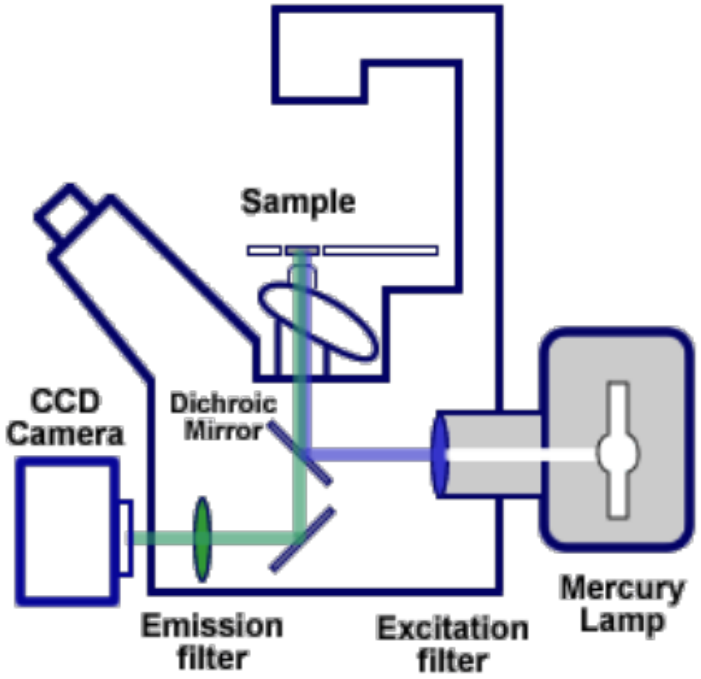
Photoluminescence:

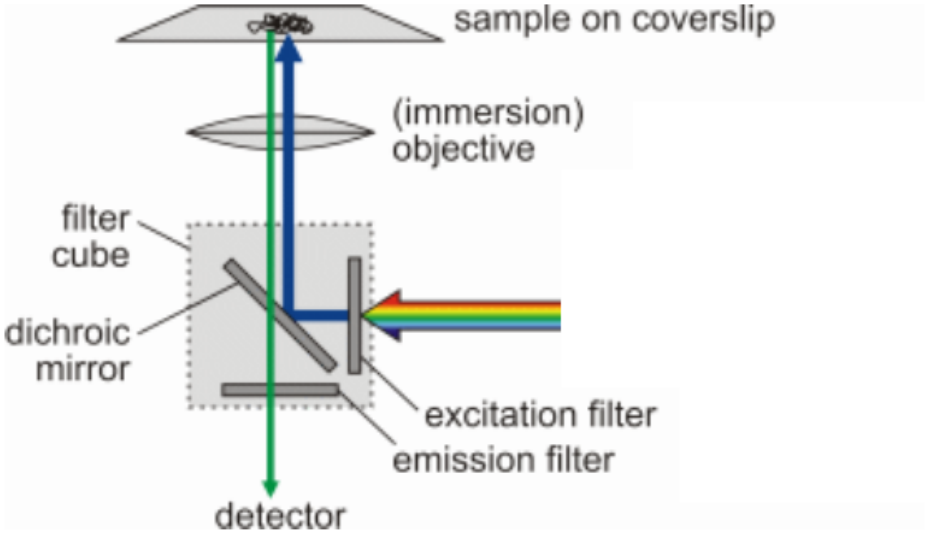
Fluorescence - spontaneous emission of light during transition of the system from its lowest vibrational energy level of an excited singlet state S_1 back to the ground state S_0 (10^{-9} to 10^{-6} s)

Phosphorescence – a non-radiative transition into an isoenergetic vibrational level of a triplet state T_1 , which lasts for 10^{-3} to 1000 s before it decays to the ground state

IC: internal conversion
ISC: intersystem crossing

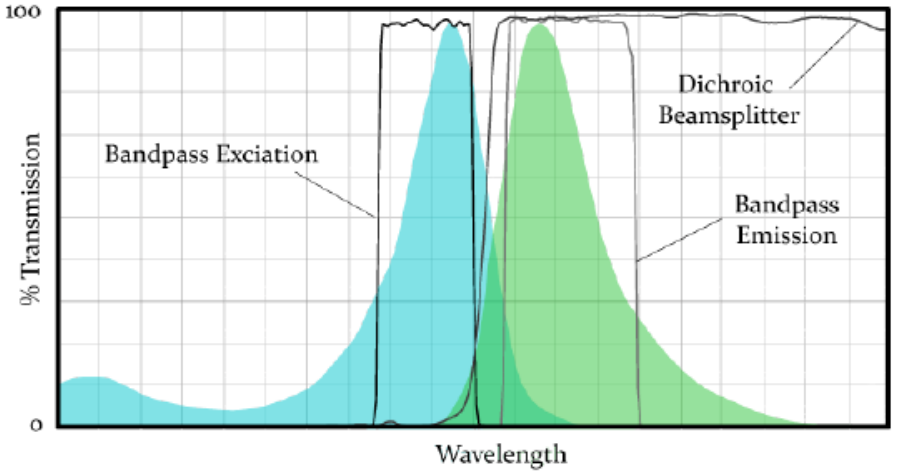
Basic principle of an (Inverted) Fluorescence Microscope





Fluorescence microscope:
Fluorescence light source
Excitation filter
Dichroic mirror
Objective
Emission filter
Camera/eye pieces

Ideal filter cube properties



Excitation
Dichroic
Emission

Upright Fluorescence Microscope

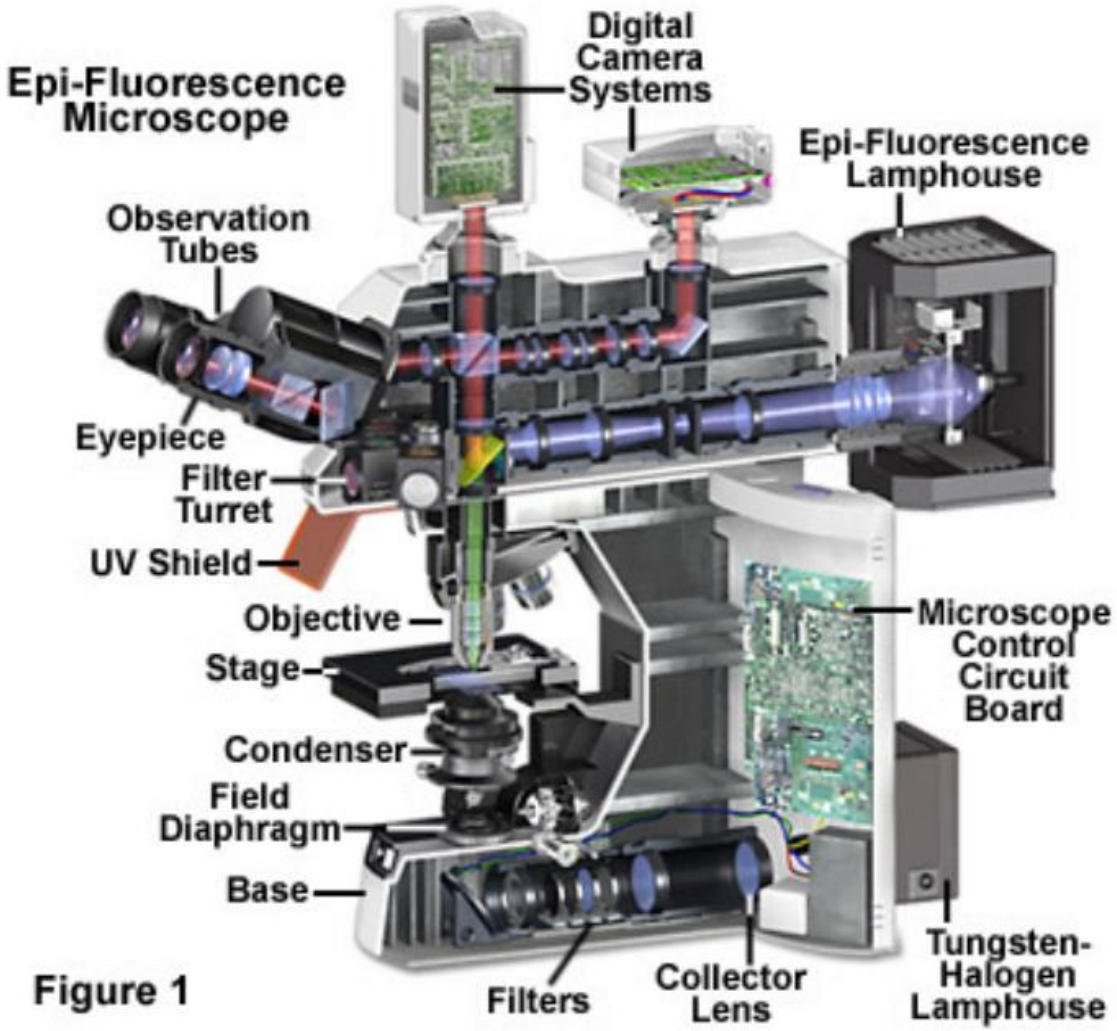


Figure 1

Used for fixed samples on slides and for live imaging where the objective is immersed in the medium

Inverted Fluorescence Microscope

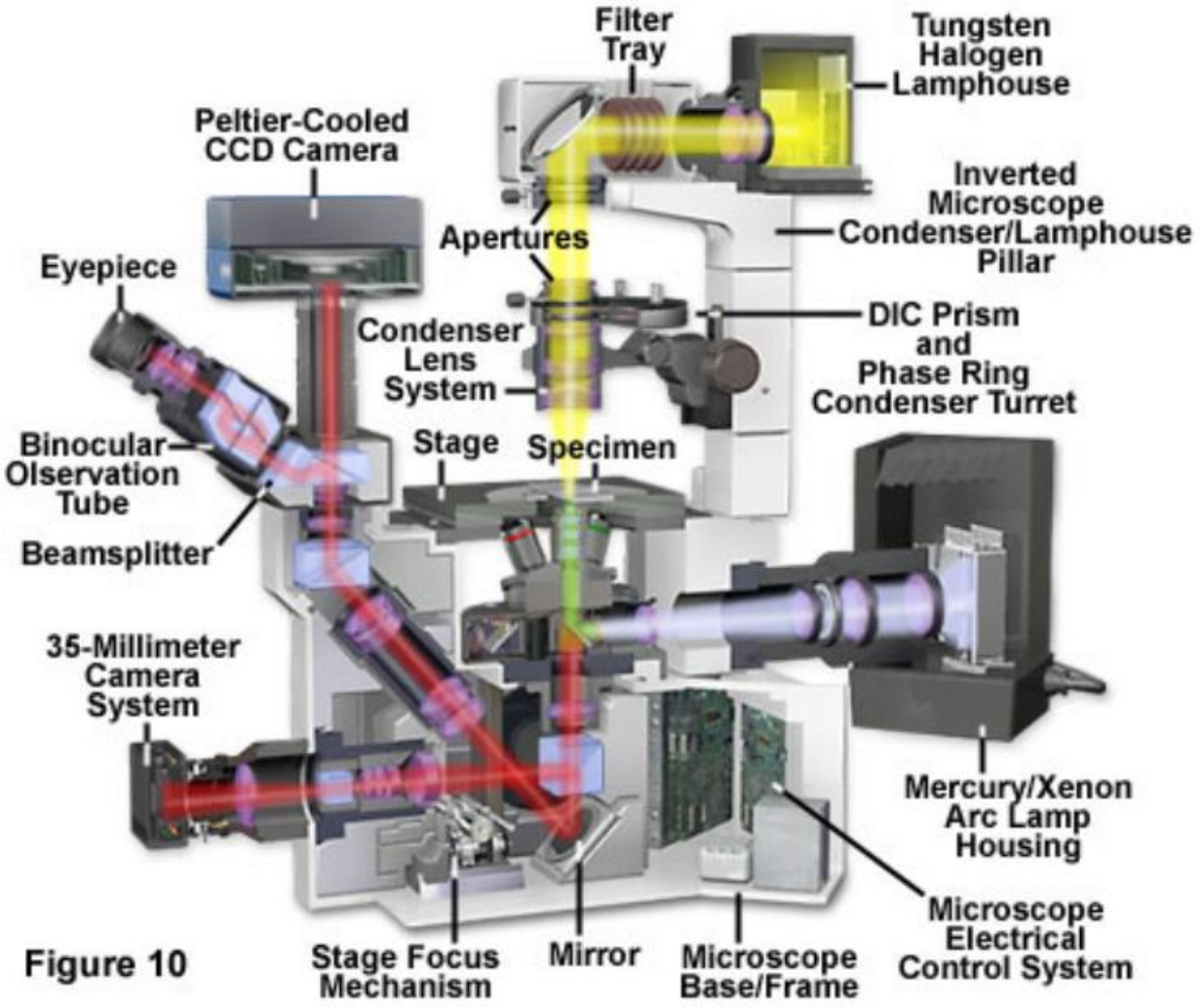
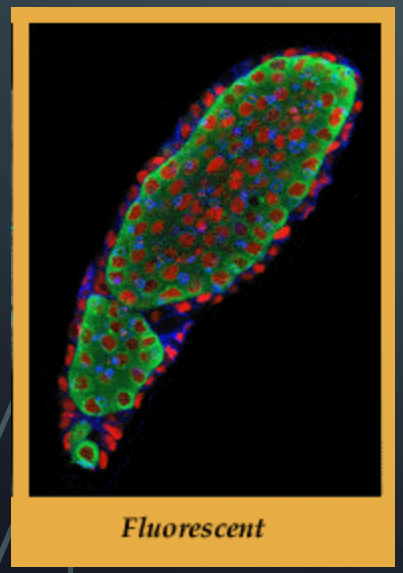
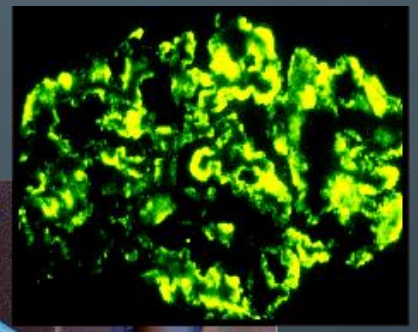


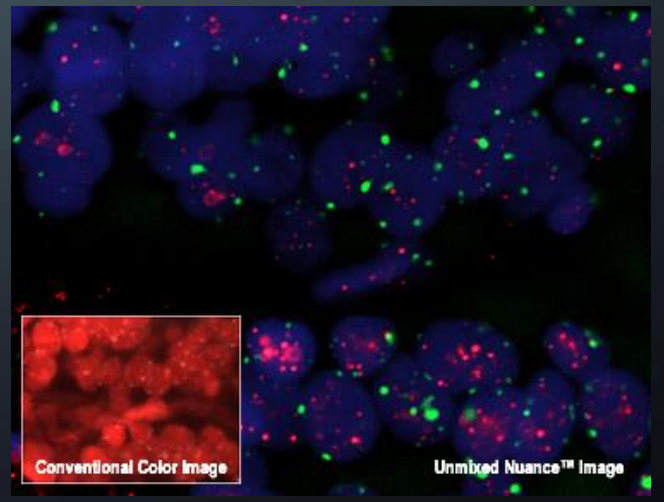
Figure 10

Used for live imaging through a coverslip and for fixed samples on slides
More versatile but danger of oil running down the objective

IMMUNOFLUORESCENCE MICROSCOPY







PREPARATION AND STAINING OF SPECIMENS

- increases visibility of specimen
- accentuates specific morphological features
- preserves specimens

FIXATION

- process by which internal and external structures are preserved and fixed in position
- process by which organism is killed and firmly attached to microscope slide
  - heat fixing
    - preserves overall morphology but not internal structures
  - chemical fixing
    - protects fine cellular substructure and morphology of larger, more delicate organisms

Preparation of Fixed Samples

Why use fixed samples at all?

- Primary cells cannot easily be transfected and transgenic animals are time-consuming to produce and not always possible

- Brighter than fluorescent fusion proteins

- Injection of antibodies only possible with big cells (e.g. oocytes)

- Can detect four different labels or even more at the same time

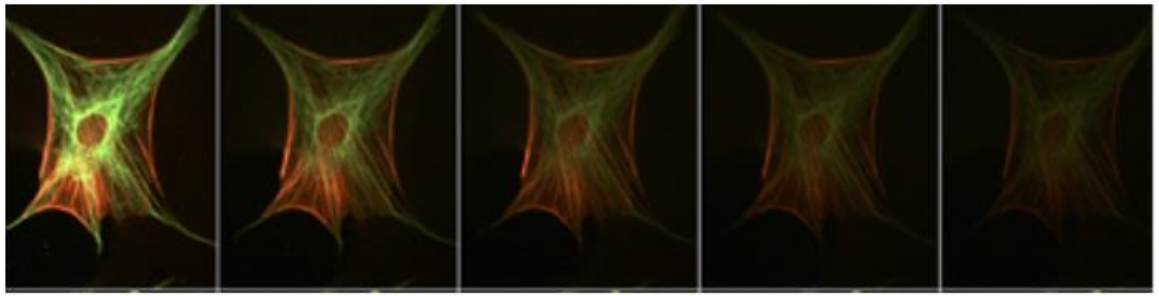
- High-throughput screening

DYES AND SIMPLE STAINING

- dyes
  - make internal and external structures of cell more visible by increasing contrast with background
  - have two common features
    - chromophore groups
      - chemical groups with conjugated double bonds
      - give dye its color
    - ability to bind cells

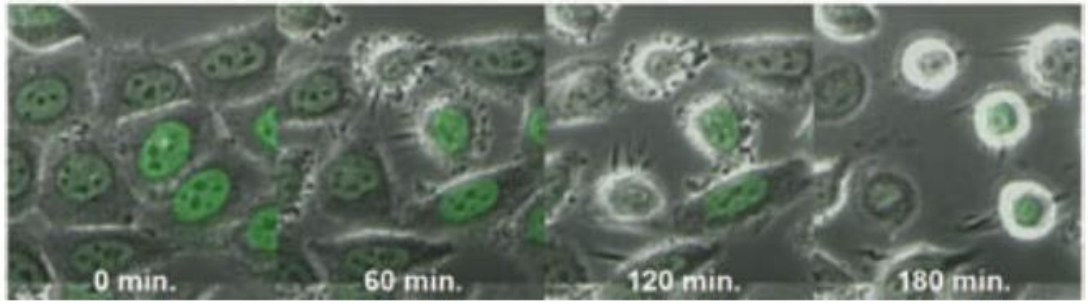
Photobleaching and Phototoxicity

Photobleaching: photochemical destruction of the fluorophore
In an excited triplet state, fluorophores may interact with another molecule to produce irreversible covalent modifications



Phototoxicity: illumination of a fluorophore causes damage to the cell expressing it, eventually leading to cell death
Common situation: the excited dye molecule passes its excess energy on to O_2, creating reactive oxygen species (ROS):

- ROS reacts with dye ⟶ dye bleaches
- ROS diffuses away and reacts with other dyes or cell components

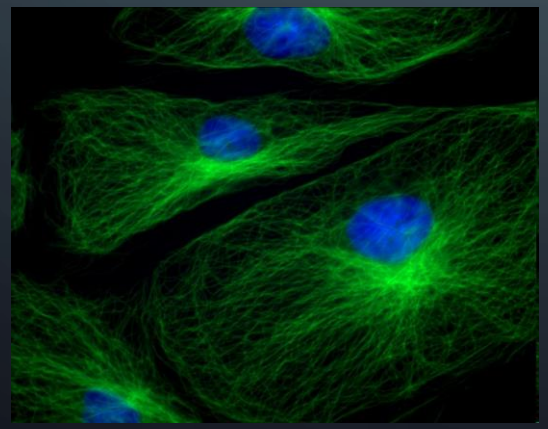


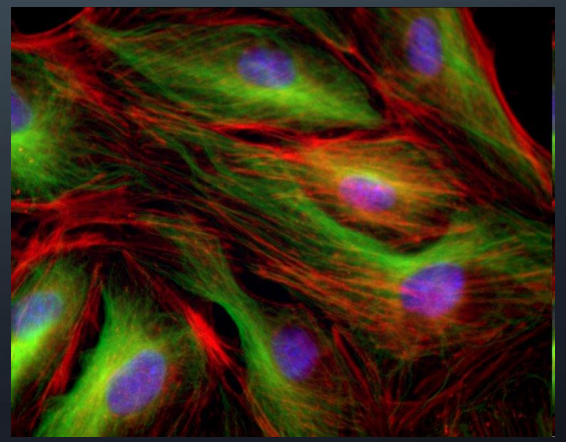
Solution:

- Reduce the intensity of the excitation light and frequency of illumination
- Close down the field aperture in order to restrict the illuminated area

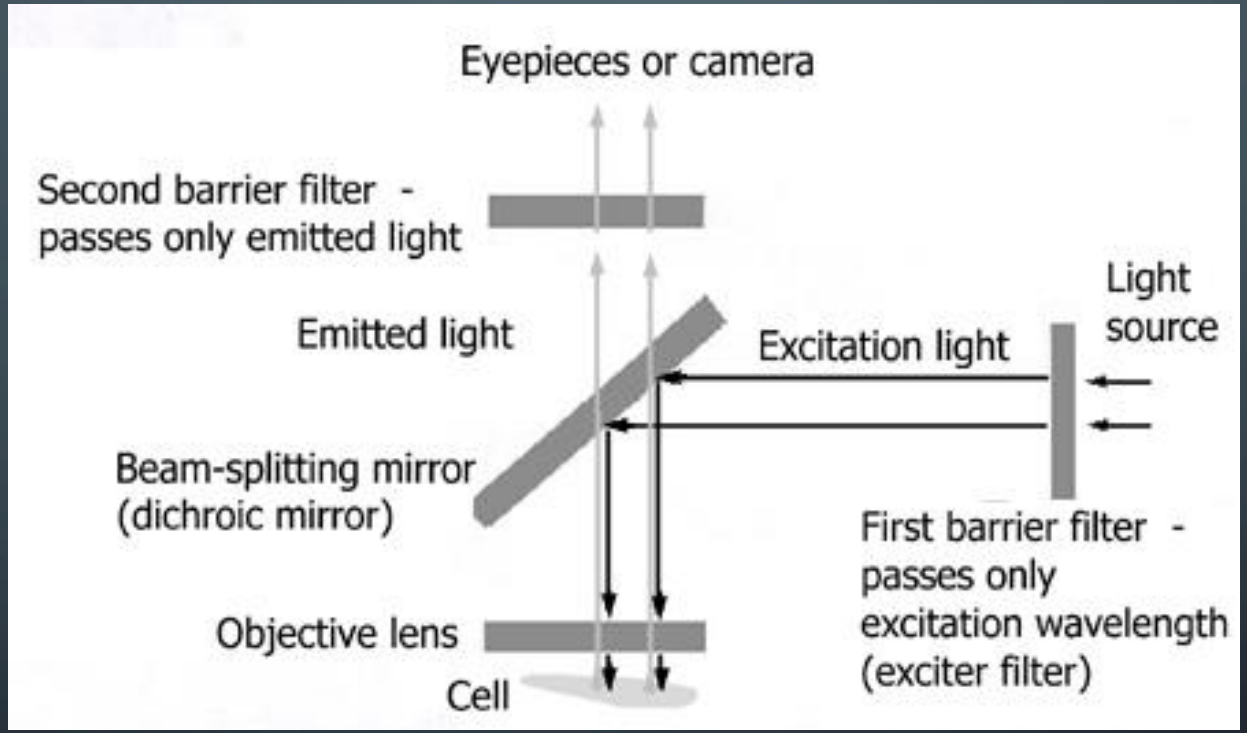
IMMUNOFLUORESCENCE MICROSCOPY:

- When an antibody, or the antiimmunoglobulin antibody used to detect the antibody is labeled with a fluorescent dye
- This method is used when looking at the subcellular location of a protein of interest



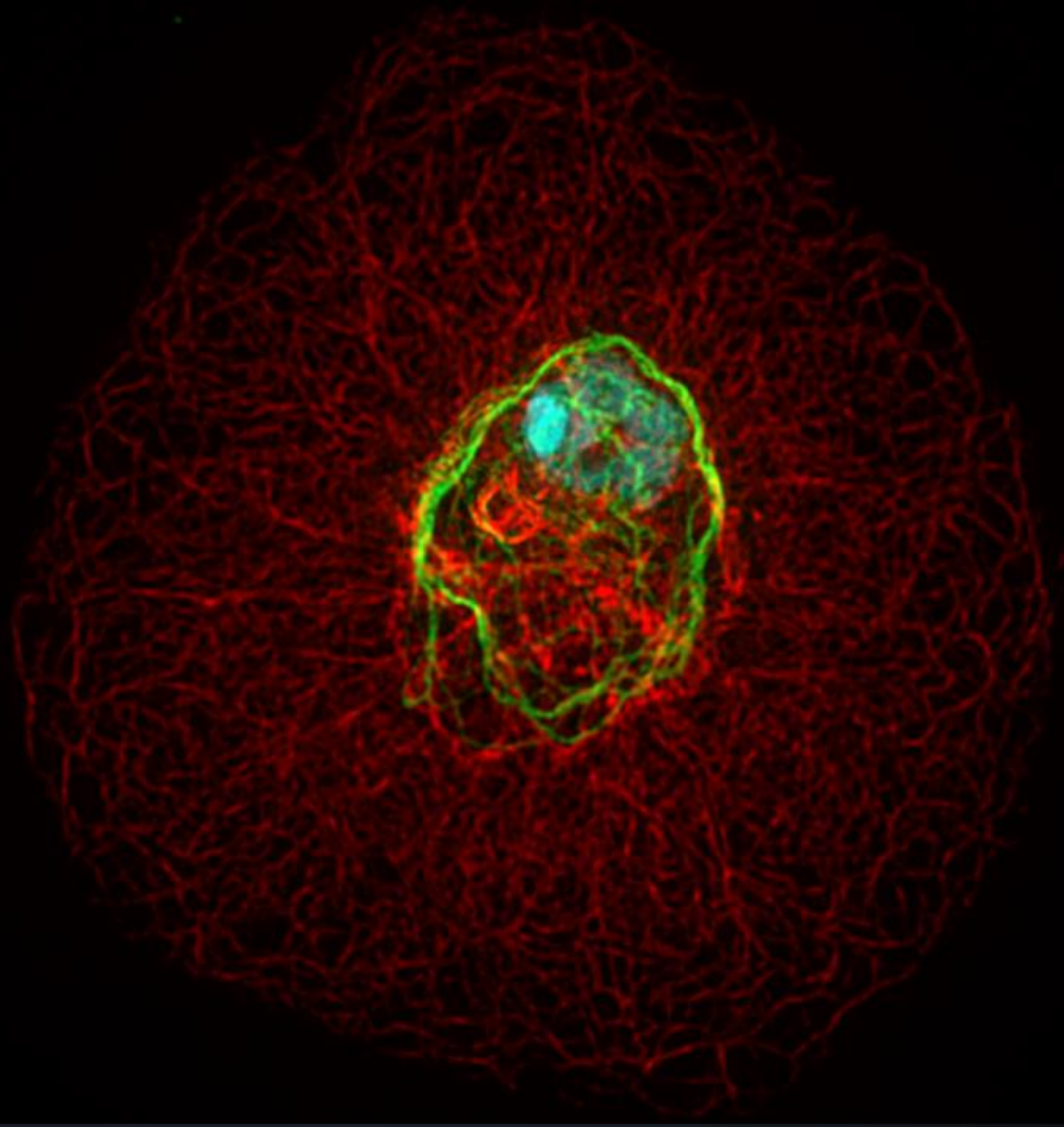


IMMUNOFLUORESCENCE MICROSCOPY:



Typical Immunocytochemistry Protocol

Fixation
Permeabilisation
Washes
Blocking
1° antibody
Washes
2° antibody
Washes
Mounting



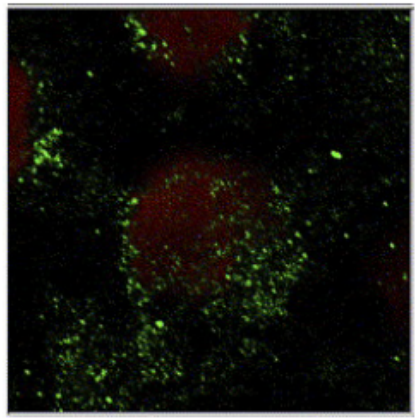
Two Types of Fixation

Denaturing fixation:

Cold methanol or cold acetone stored at -20 °C, samples submerged at -20 °C for 10 to 20 min

- destroys 3D protein structure
- dissolves lipids into micelles
- poor morphological preservation and poor protein retention
- makes some epitopes accessible
- best used after cross-linking fixation

MeOH

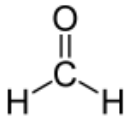


Cross-linking fixation:

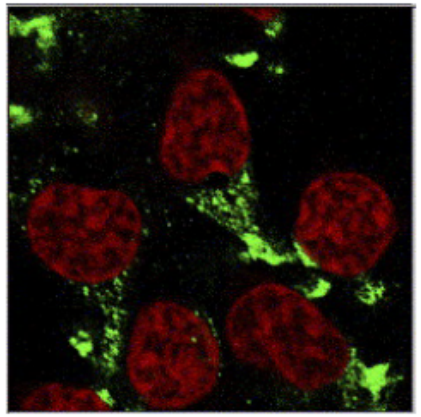
- aldehyde groups cross-link molecules in cells and tissues
- extensive cross-linking prevents antibody penetration

Formaldehyde used for immunocytochemistry in light microscopy

- cross-links 1° amines of Lys and Arg, sulfhydryl groups of Cys, OH groups, double bonds
- binds to amino acids, peptides, proteins and some lipids, but not RNA, DNA or most sugars
- retention of DNA and RNA due to protein cross-linking
- for cultured cells fixation usually for 20 min in 2 - 4% formaldehyde



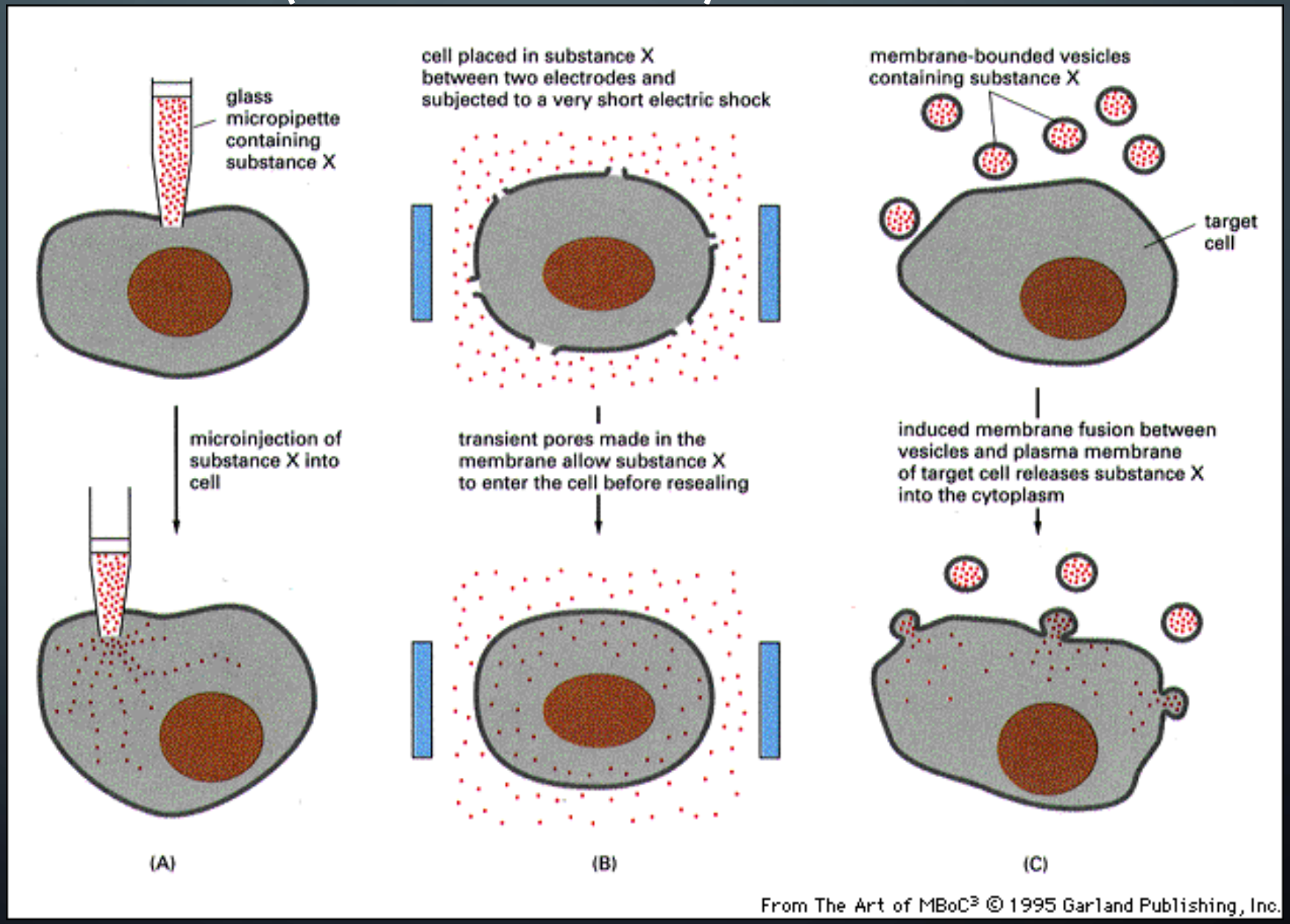
PFA



HOW DO WE GET FLUORESCENT PROBES INTO CELLS

- Kill the cell and make the membrane permeable
- Live cells
  - Diffusion: some can cross membrane
  - Microinjection- stick and tiny needle through membrane
  - Trauma: rip transient holes in membrane by mechanical shear (scrape loading) or electrical pulse (electroporation)
  - Lipid vesicles that can fuse with membrane
  - Transfect with fluorescent protein vector

LOADING CELLS (ALBERTS 4-59)



Permeabilisation

Aim: to allow fixative to enter the cells/tissue more quickly if necessary
to allow antibodies to penetrate fixed cells/tissue
done by removing lipids with detergents

Detergents:

- polar lipids with a hydrophilic (water soluble) end and a hydrophobic end that binds the hydrophobic moieties of water insoluble compounds and renders them hydrophilic

Nonionic detergents:

- contain methyl groups that participate in hydrogen bonds and are able to solubilise membranes but do not destroy protein-protein interactions

Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.1% in PBS)
Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%)
Nonidet P-40 (Igepal Ca-630 from Sigma-Aldrich): used to permebilise unfixed cells (0.1% in PBS for 5-10s)

Ionic detergents:

- have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures

SDS, deoxycholate, CHAPS
Not used for immunocytochemistry

TYPES OF PROBES

- Some change intensity of fluorescence depending on pH or [Ca^{++}]
- Some bind specific structures
  - ER
  - actin
  - Golgi
  - Plasma membrane
  - Mitochondria
- Fluorescently labeled purified protein
- Antibodies

IMMUNOFLUORESCENCE LOCALIZATION OF PROTEINS IN DEAD/FIXED CELLS

- You can purify almost any protein from the cell (Biochemistry)
- Make an antibody to it by injecting it into a rabbit or mouse (primary antibody)
- Use the antibody to bind to the protein in the fixed cell
- Fixed cells can be made permeable so antibodies can get into interior
- Use a fluorescent “secondary antibody” (anti-rabbit or mouse) to localize the primary antibody

Polyclonal antibodies

Advantage:

- High levels of labelling because they bind several epitopes on the same protein

Disadvantages:

- Can label multiple proteins that share epitopes
- Different batches have different antibodies

Monoclonal antibodies

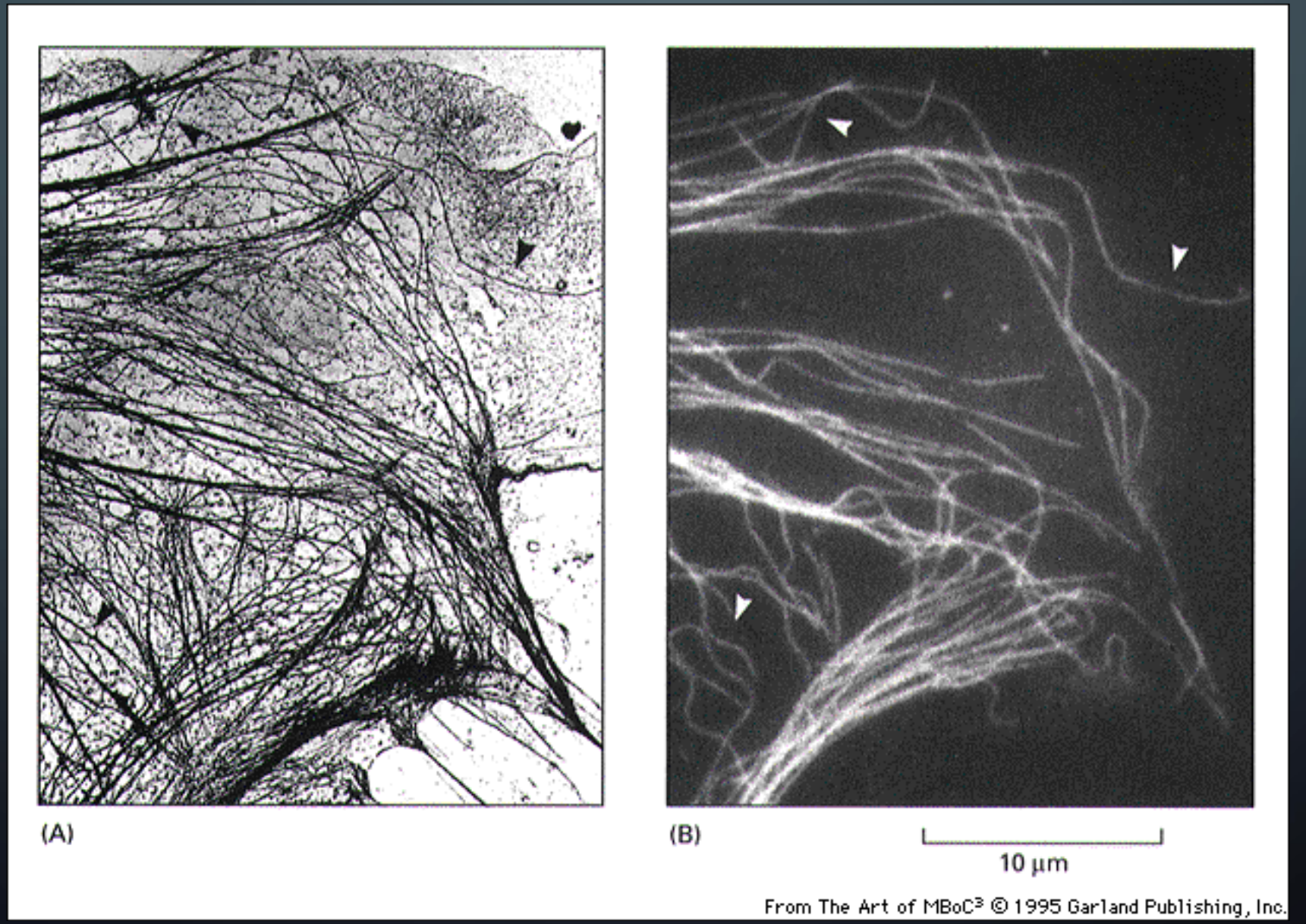
Advantages:

- Single epitope selected for high specificity
- Different clones can be generated to different epitopes on the same antigen
- Single clone can recognise post-transcriptionally modified protein (e.g. phosphorylation)
- Same clone can be generated indefinitely

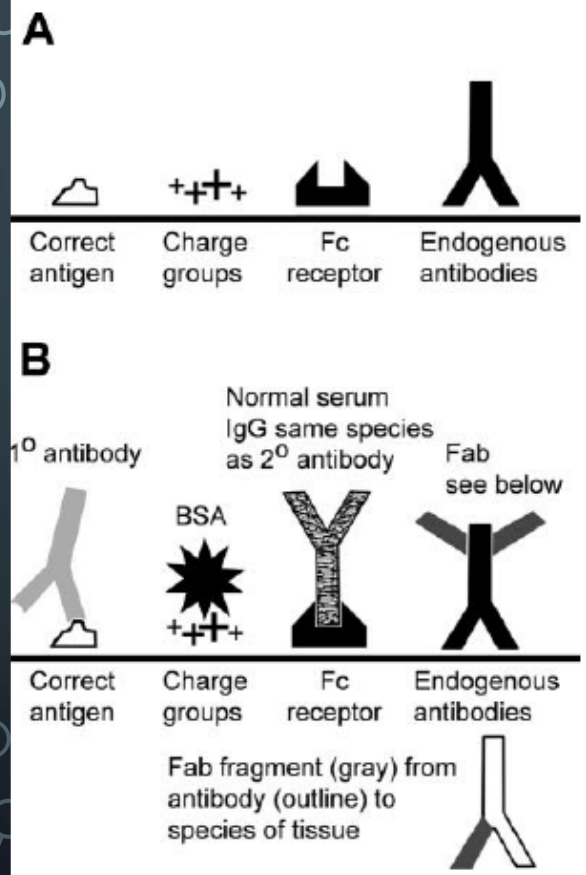
Disadvantages:

- Low levels of labelling possible
- Mostly from mice

ANTI-TUBULIN IMMUNOFLUORESCENT LOCALIZATION OF MICROTUBULES



Blocking



Aim: to allow binding of antibodies only to appropriate sites

Sources of nonspecific binding:

Charged groups

Occur on proteins (esp. histones) or lipids

Also generated by fixation in formalin or glutaraldehyde

To block use bovine serum albumin at 10-30mg/mL (fraction V)

Fc receptors

On macrophages and other immune cells, which bind any antibody

To block whole IgG 1° and 2° antibodies from binding to Fc receptors, incubate cells in buffer containing 5-10% normal serum from the host species of the 2° antibody

Endogenous antibodies

Only a problem for 2° antibodies recognising the same species as your tissue/ cells and only at inflammation sites or in cell cultures of immune system cell types

To block use Fab fragments raised in the same species as the 2° antibody that recognise the species of your tissue/cells as part of the blocking procedure

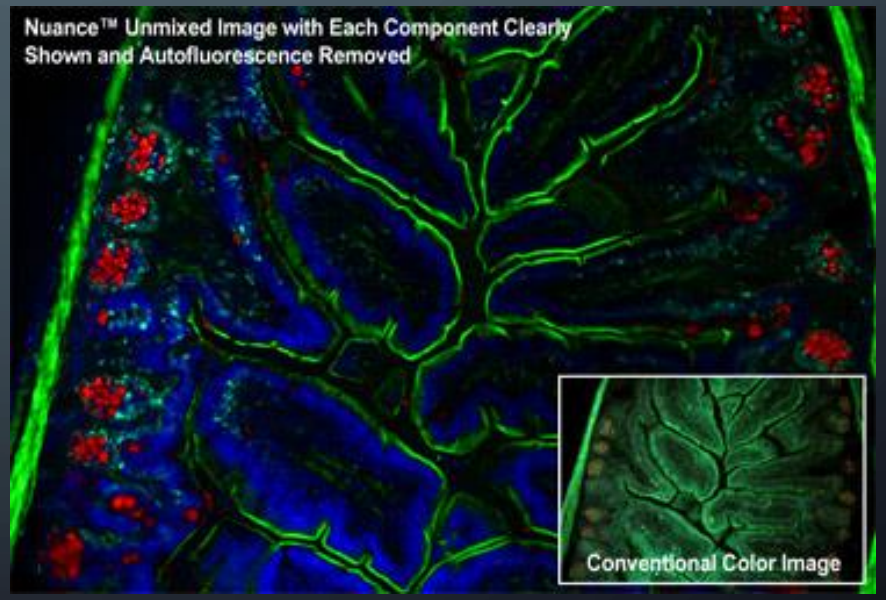
For general blocking can also try MAXblock (Active Motif): protein based, non-mammalian blocking agent, no cross-reactivity with 2° antibodies

TECHNIQUE:

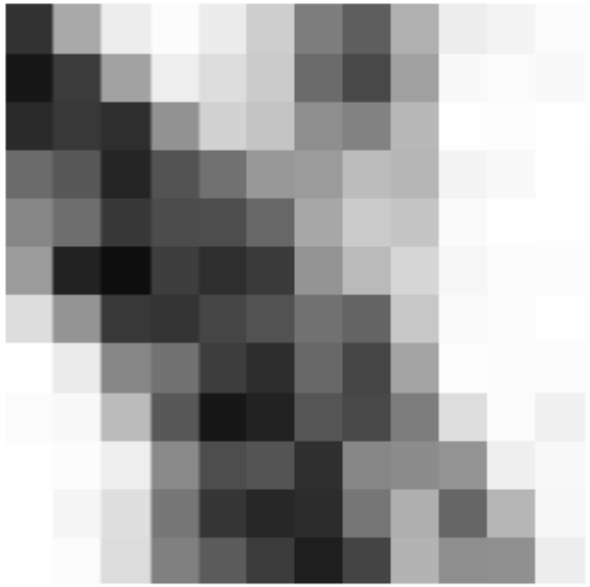
- Common dyes: fluorescein, rhodamine
- Dyes chosen are excited by a certain light wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum
  - Eg. Fluorescein emits green light
  - Eg. Rhodamine emits orange/red light
- By using selective filters in a fluorescence microscope only the light from the dye is detected
- Available fluorescent labels now include red, blue, cyan or yellow fluorescent proteins

USES:

- This can be used to detect the distribution of any protein
- By attaching different dyes to different antibodies the distribution of two or more molecules can be determined in the same cell or tissue sample



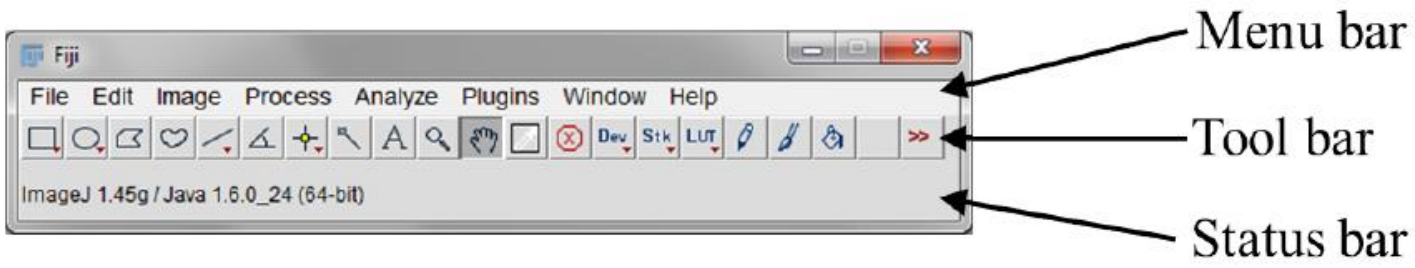
(a) Original image

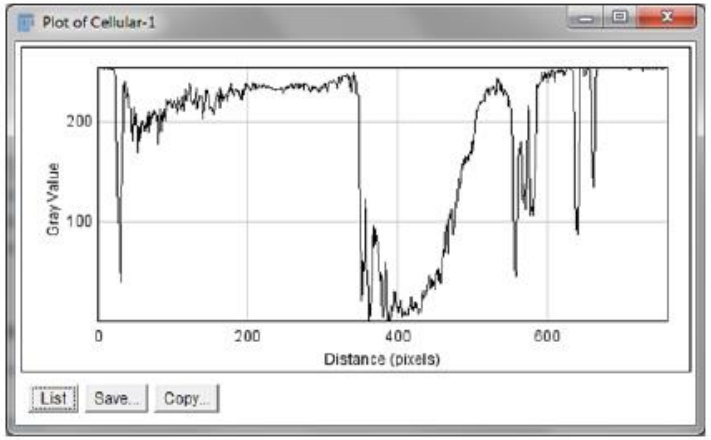


(b) Enlarged view from (a)

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)





Plot window

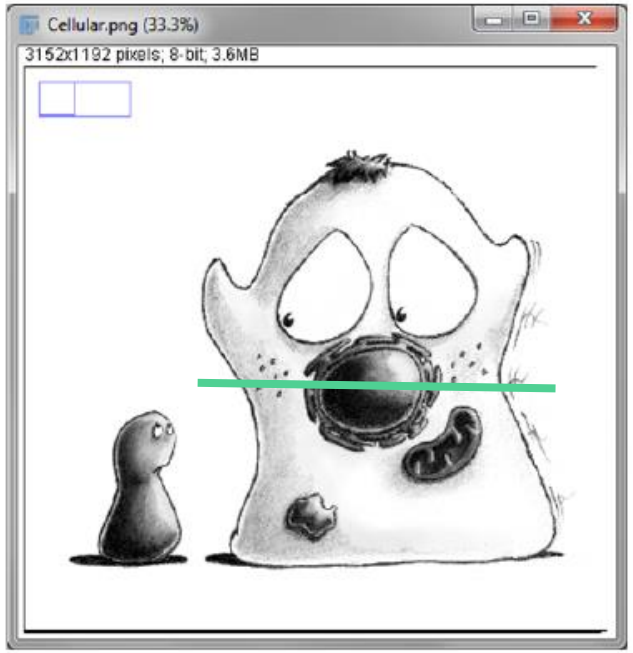
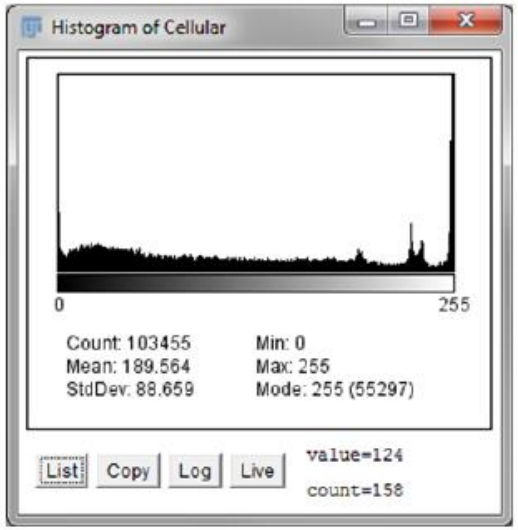
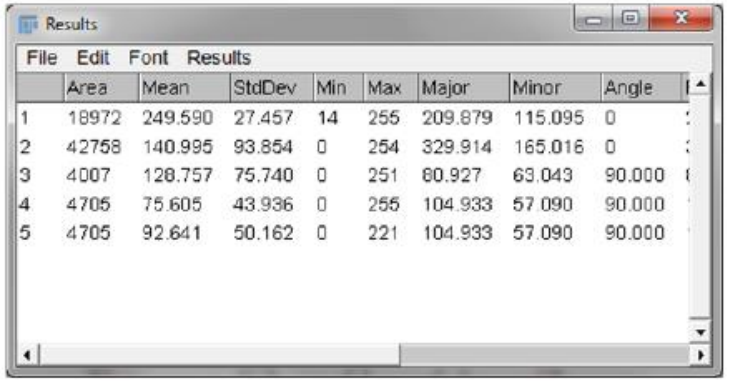


Image window

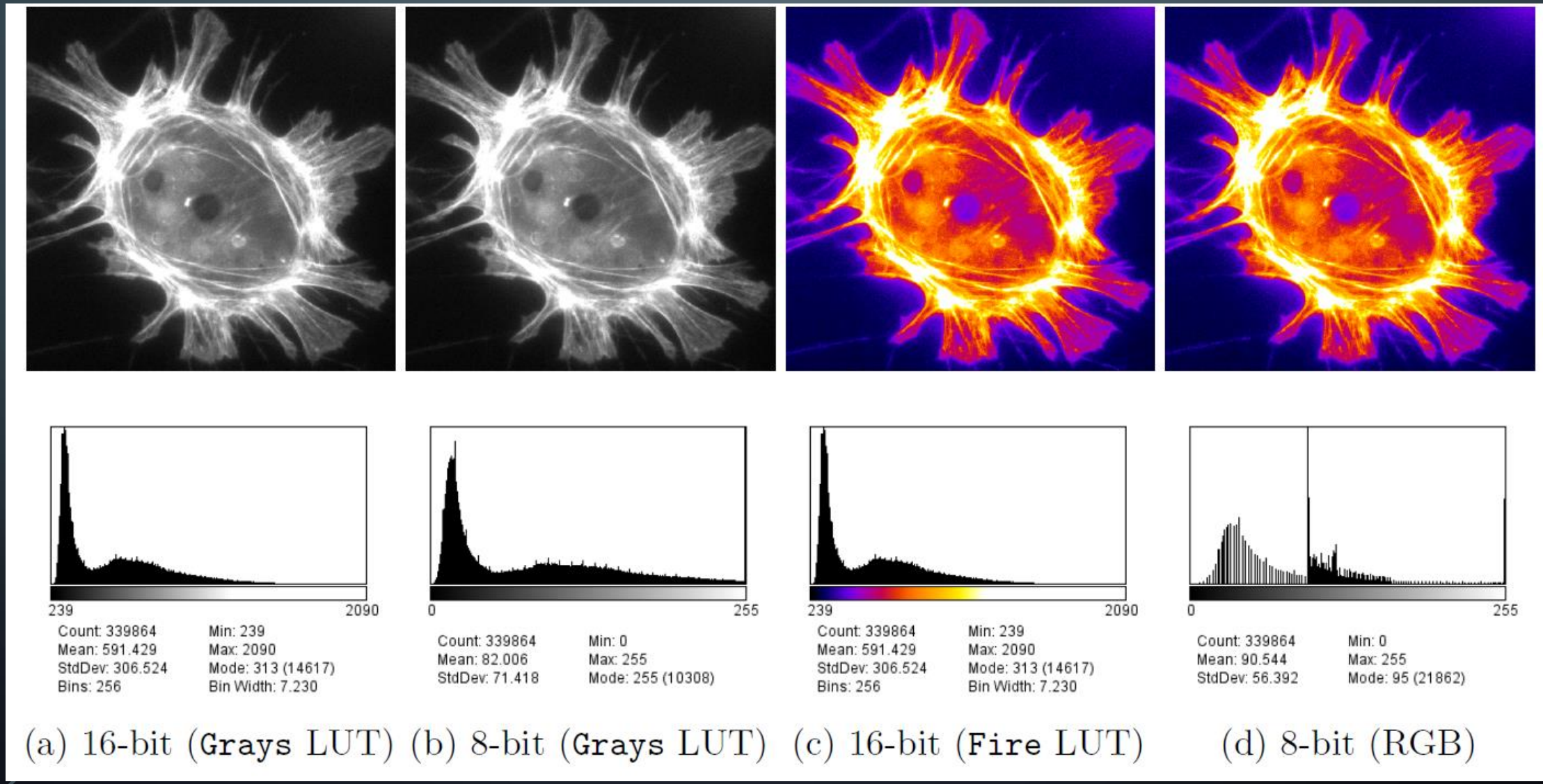


Histogram window



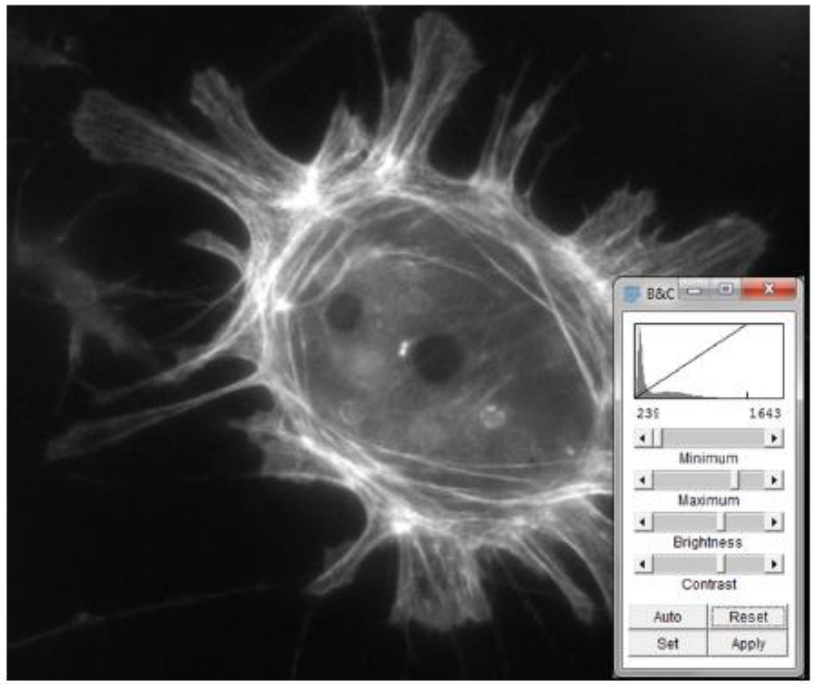
Results table

Do not trust your eyes for image comparisons

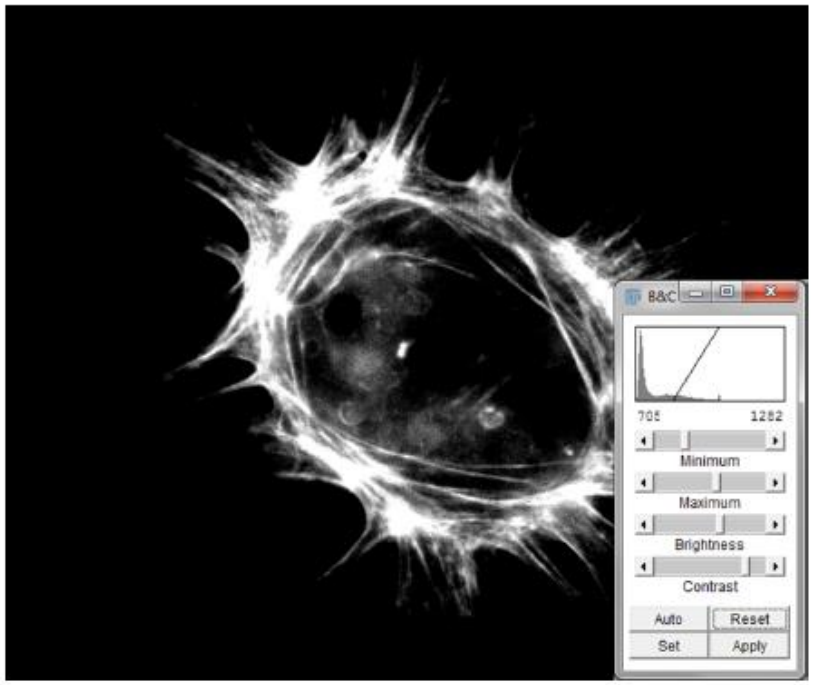


(a) 16-bit (`Grays` LUT) (b) 8-bit (`Grays` LUT) (c) 16-bit (`Fire` LUT) (d) 8-bit (RGB)

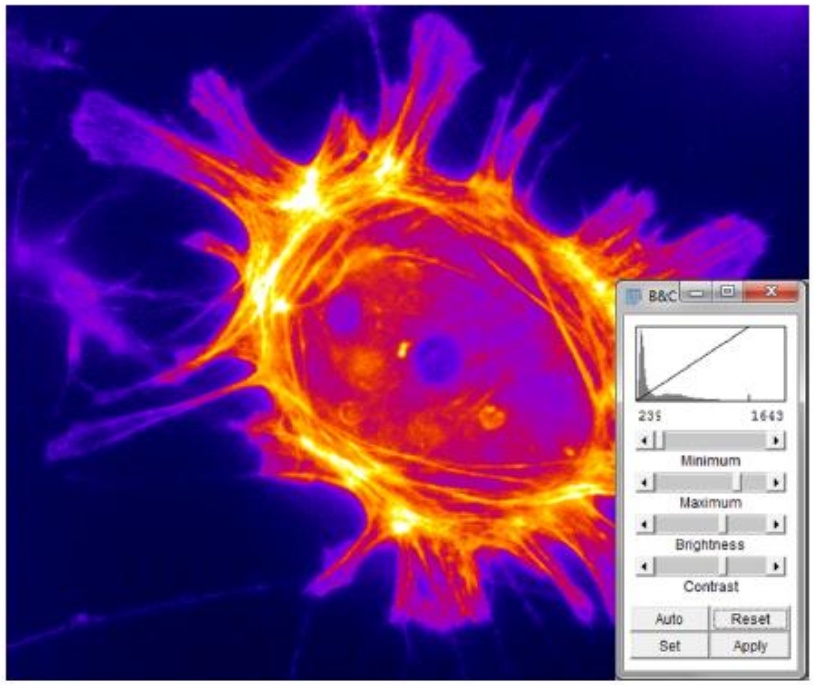
The same image can be displayed in different ways by adjusting the contrast settings or the LUT.
Nevertheless, despite the different appearance, the values of the pixels are the same in all three images.



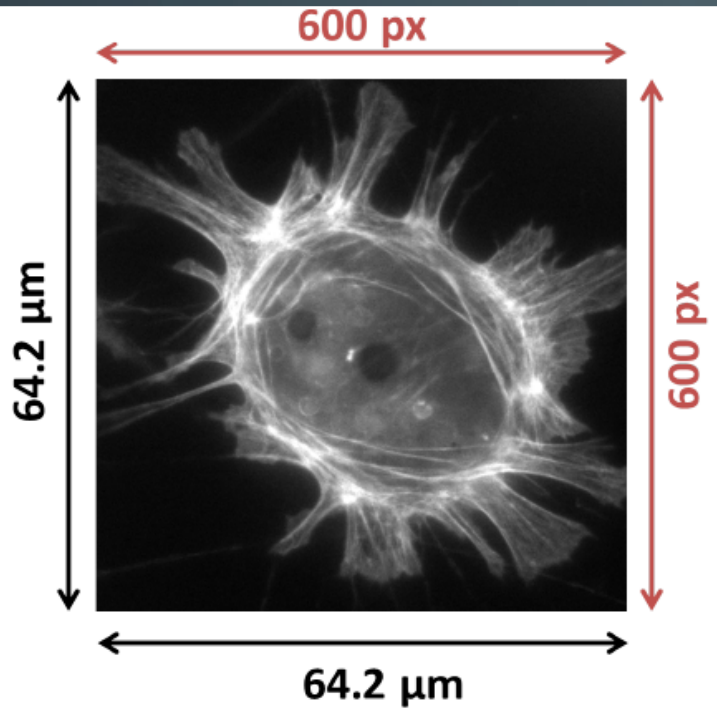
(a) Grayscale

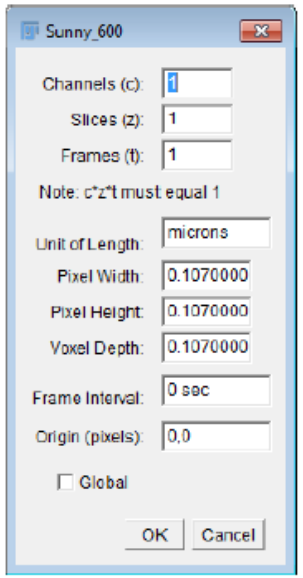


(b) Grayscale (high contrast)

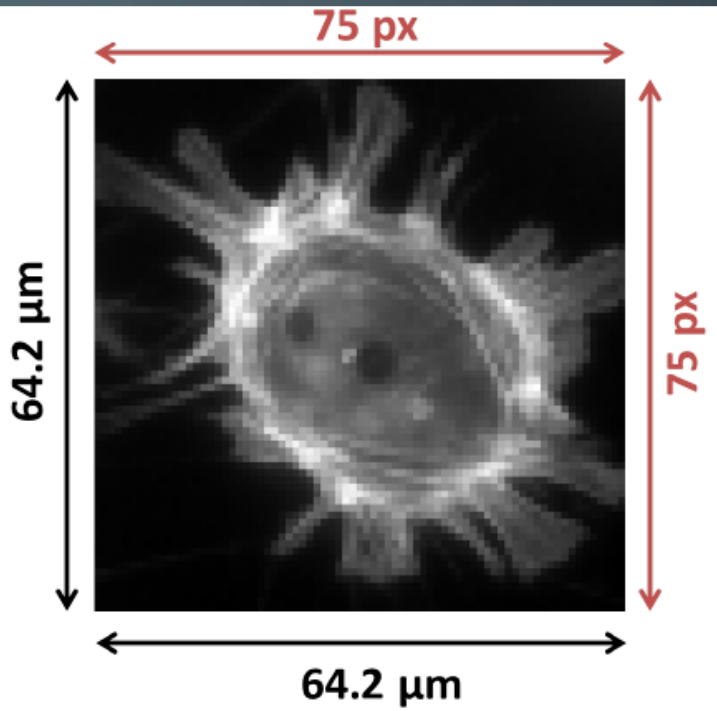


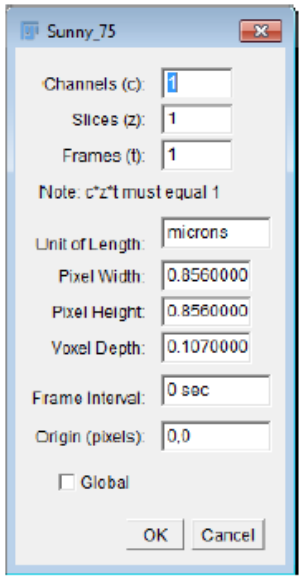
(c) Fire LUT





(a) 600 × 600 pixel image and its properties





(b) 75 × 75 pixel image and its properties

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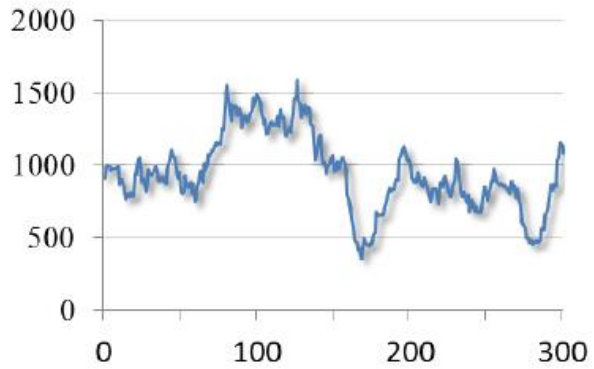
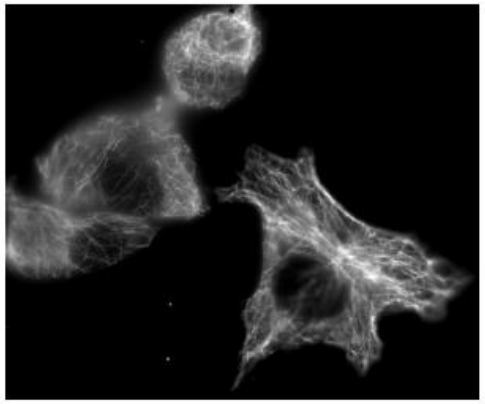
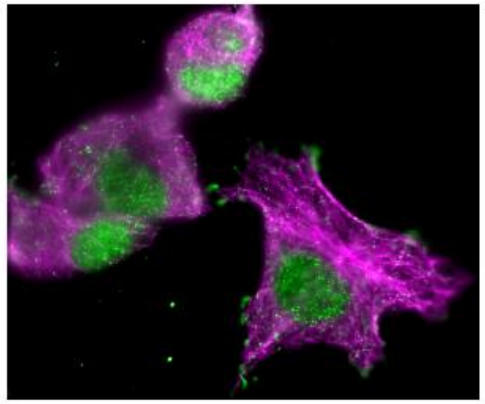
(a) 0 dimensional



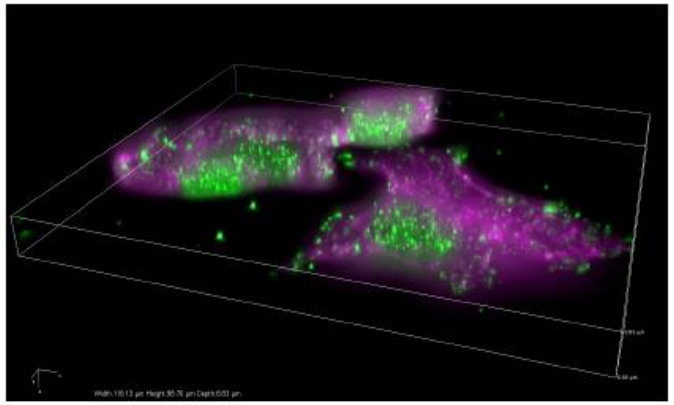
(b) 1 dimensional



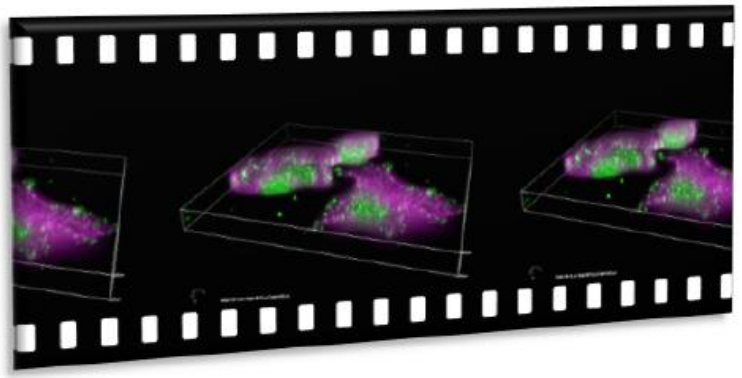
(c) 2 dimensional



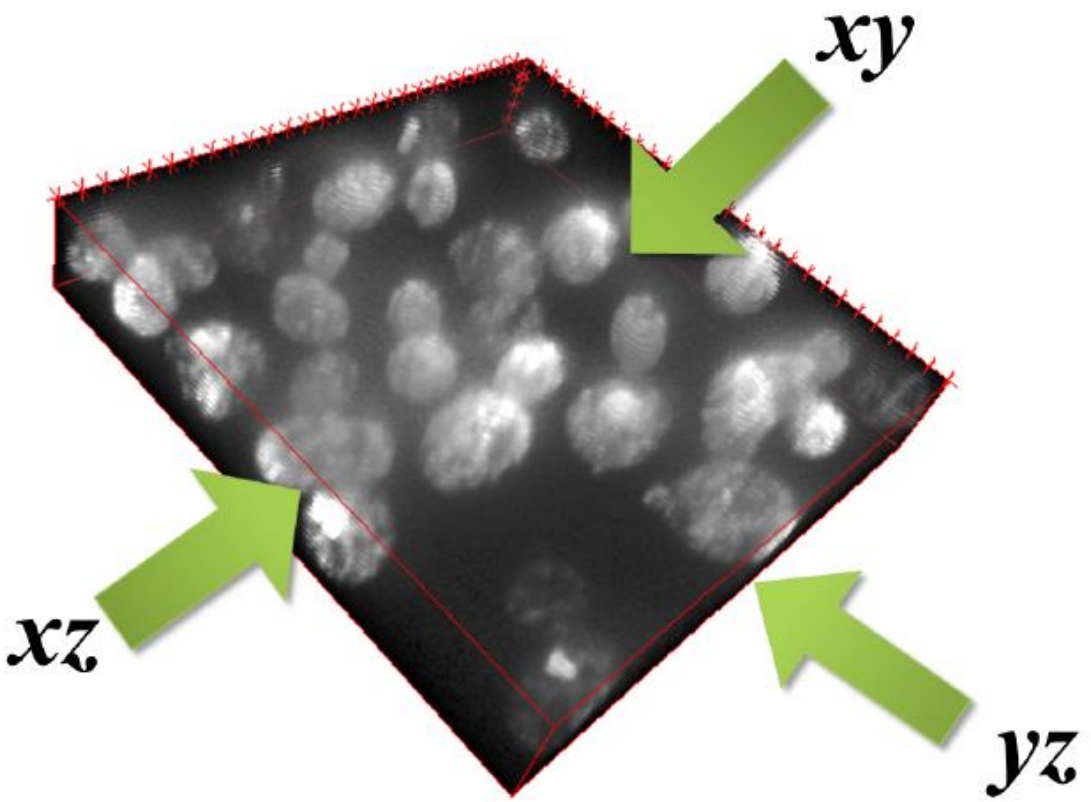
(d) 3 dimensional



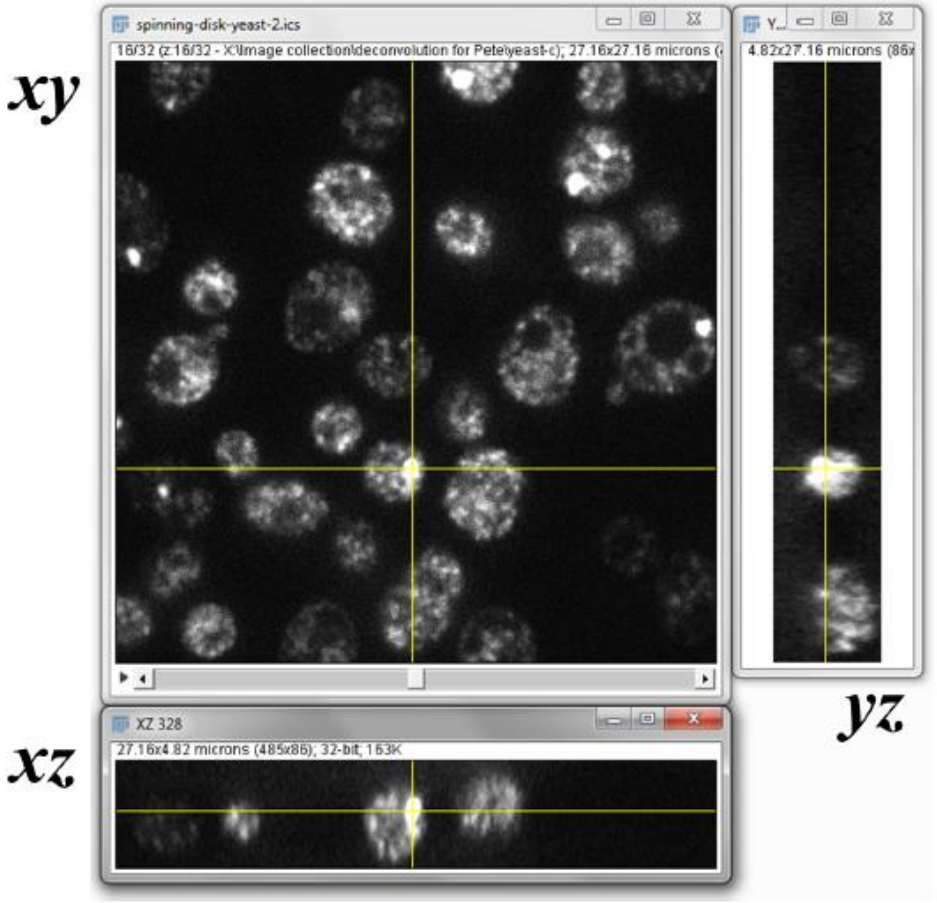
(e) 4 dimensional



(f) 5 dimensional

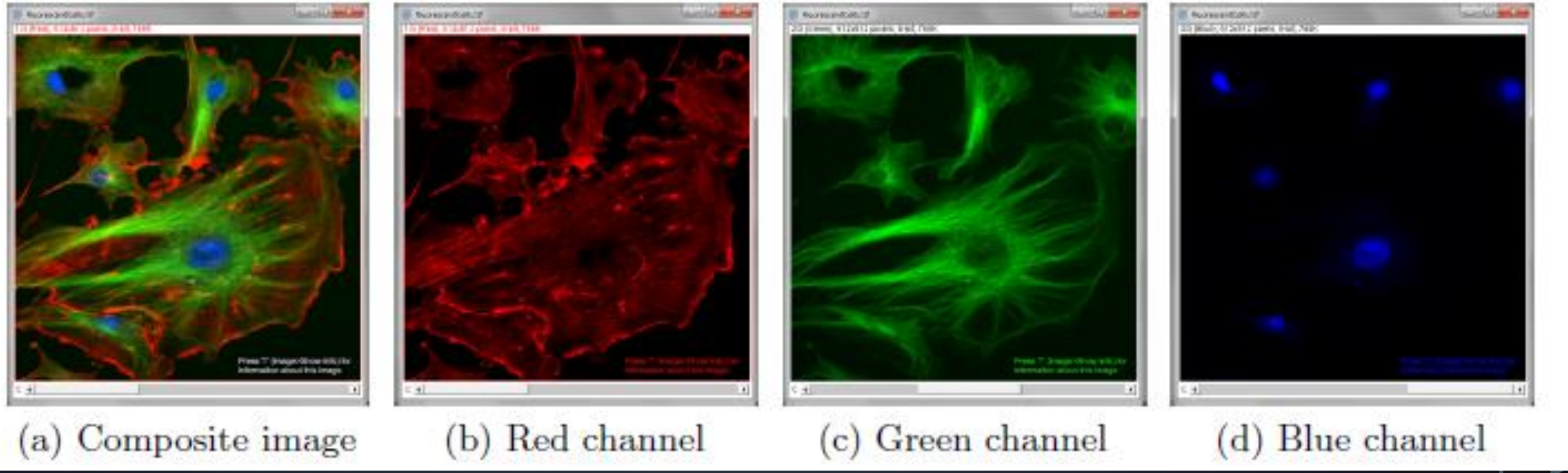


(a) Volume rendering

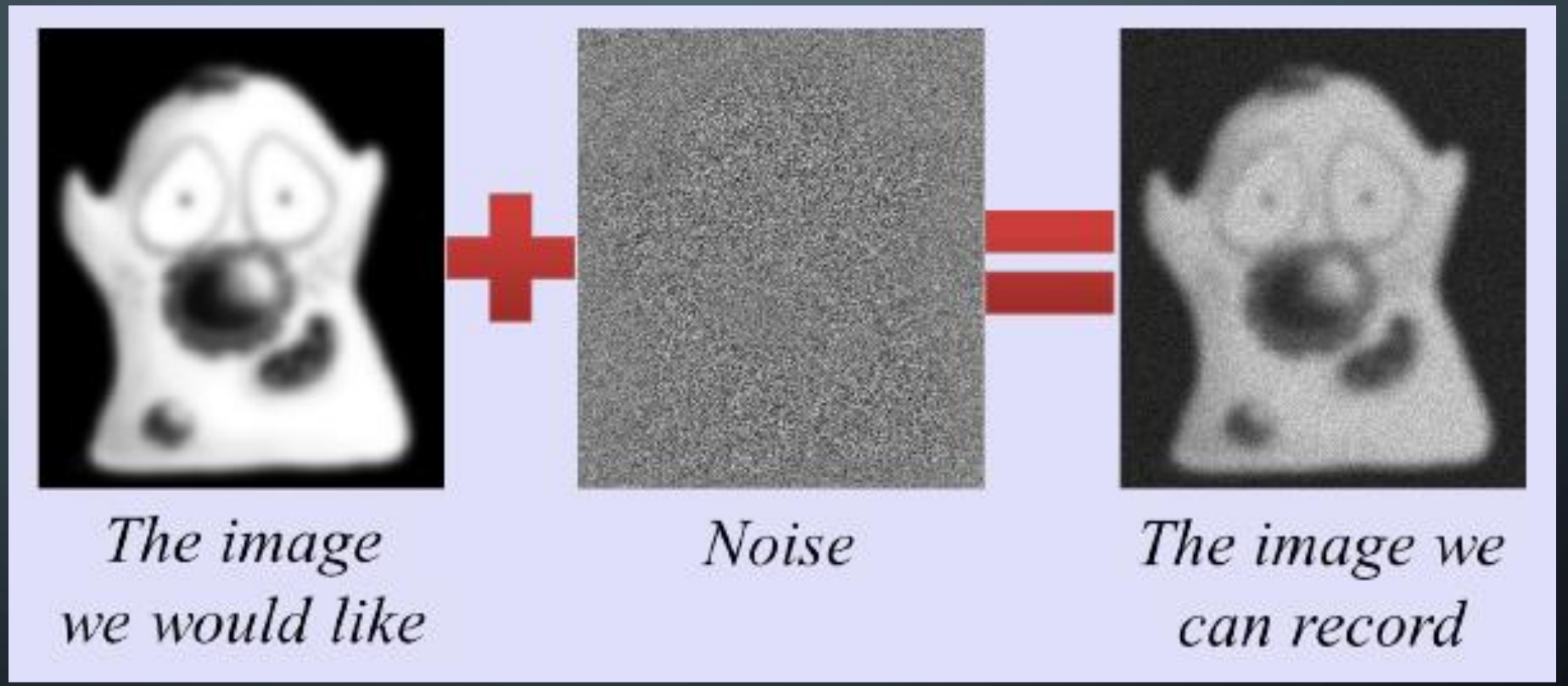


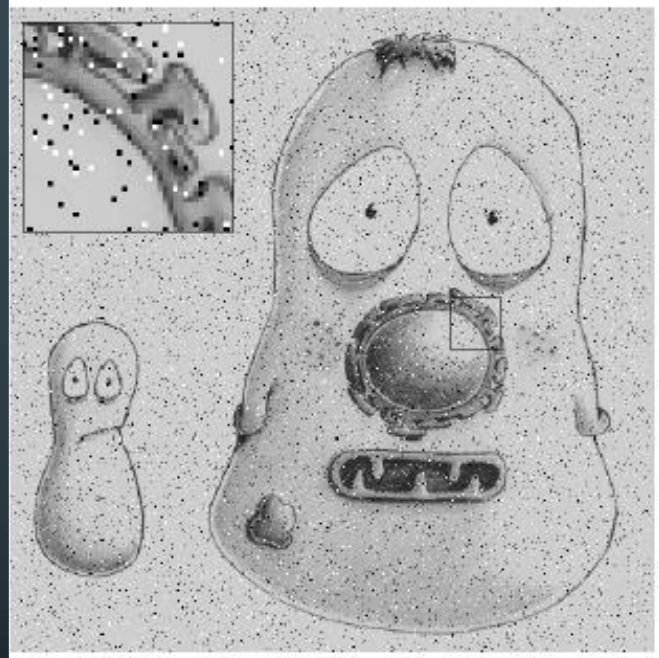
(b) Orthogonal views

ImageJ composite image sample Fluorescent Cells. Using the Channels Tool...
and the slider at the bottom of the window, you can view the channels individually or simultaneously.

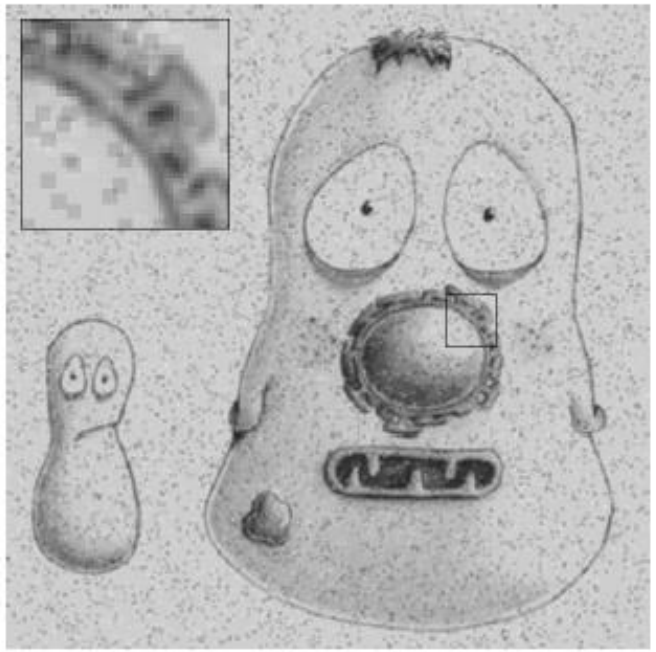


(a) Composite image (b) Red channel (c) Green channel (d) Blue channel

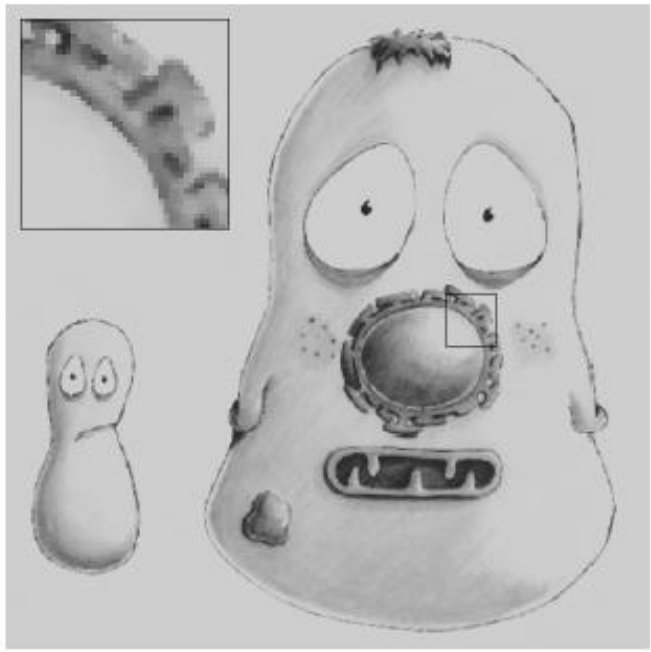

The image we would like
Noise
The image we can record



(a) Speckled image



(b) Mean filter



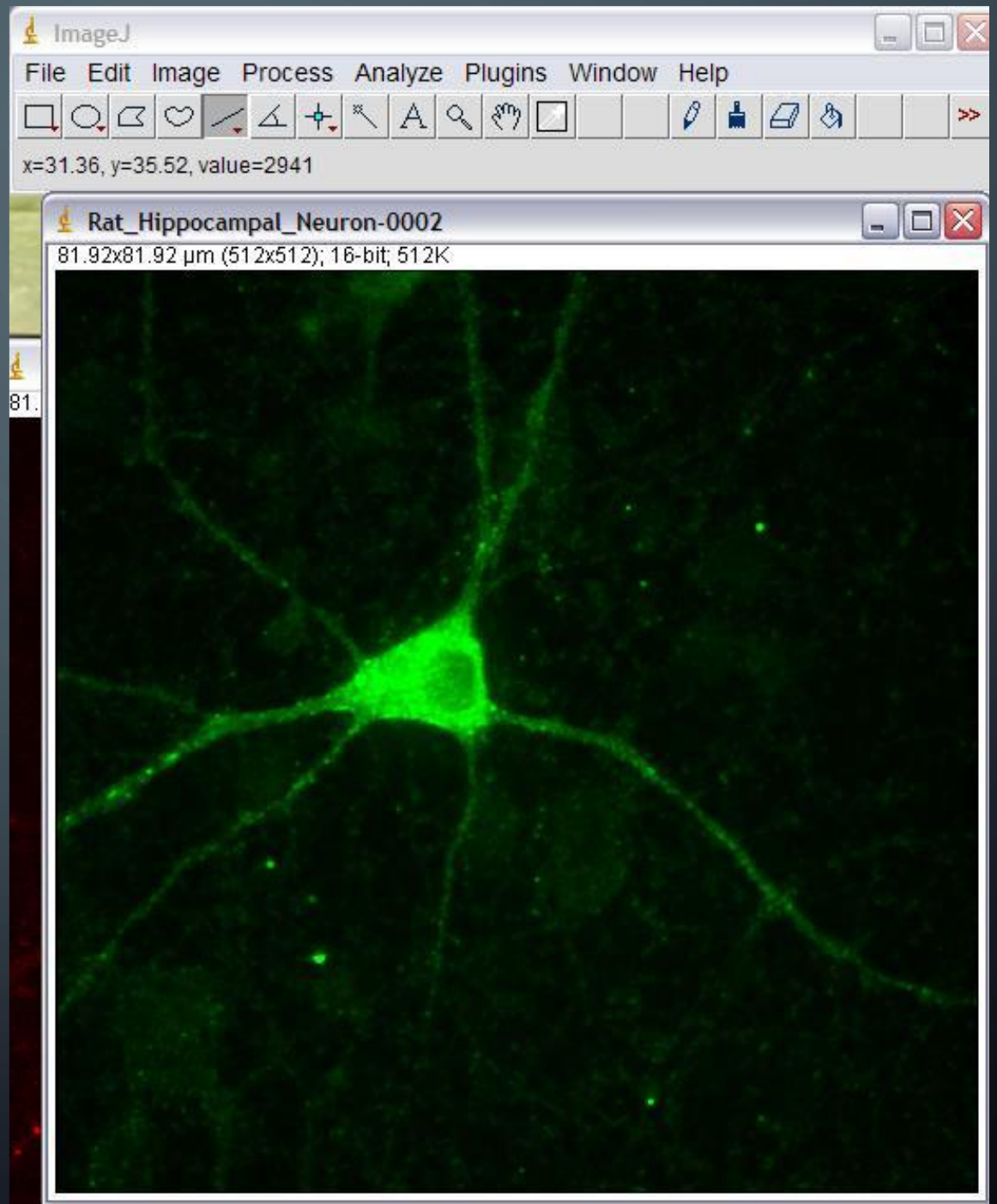
(c) Median filter

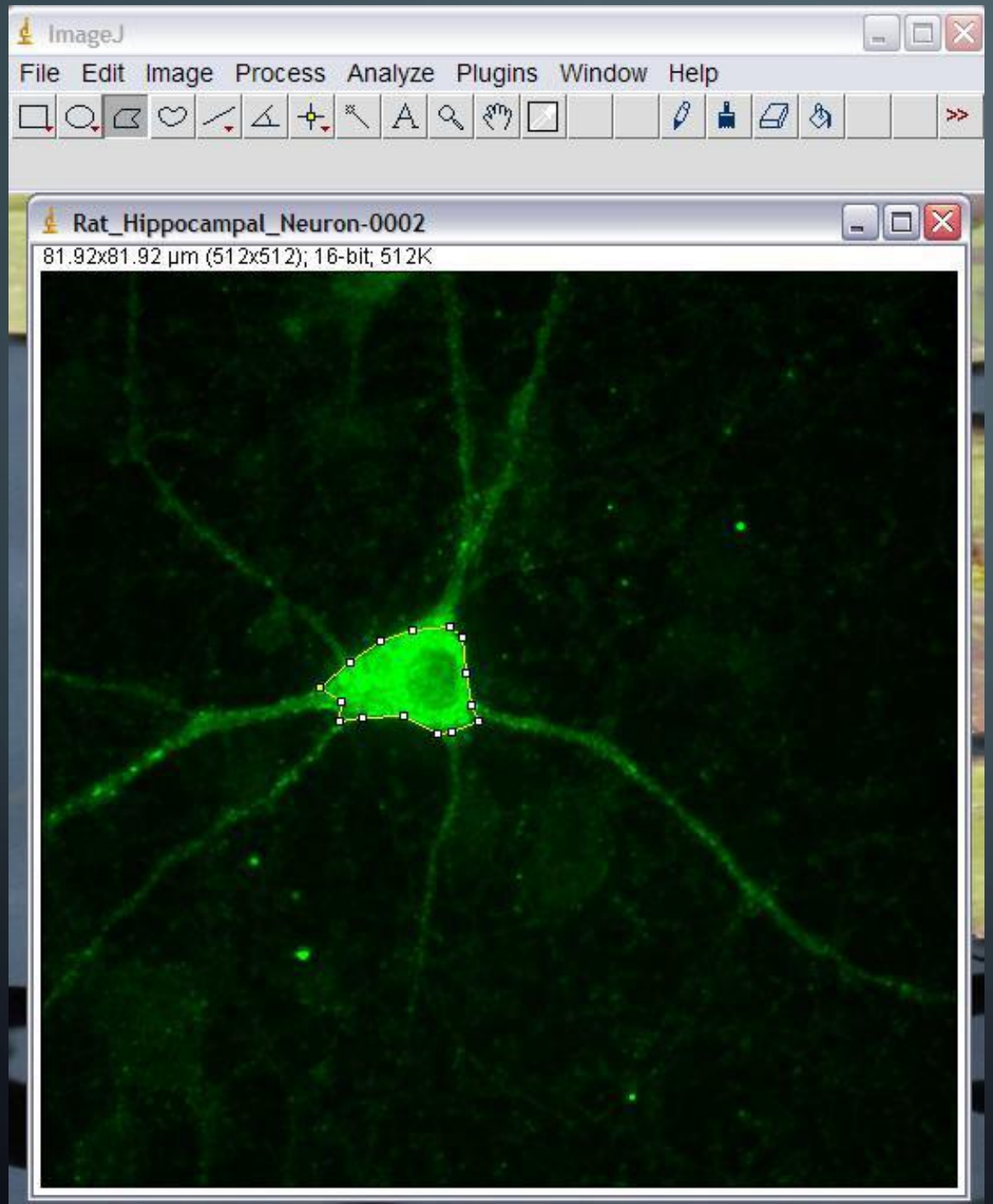
Basic Intensity Quantification with ImageJ

Pretty pictures are nice, but many times we need to turn our images 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

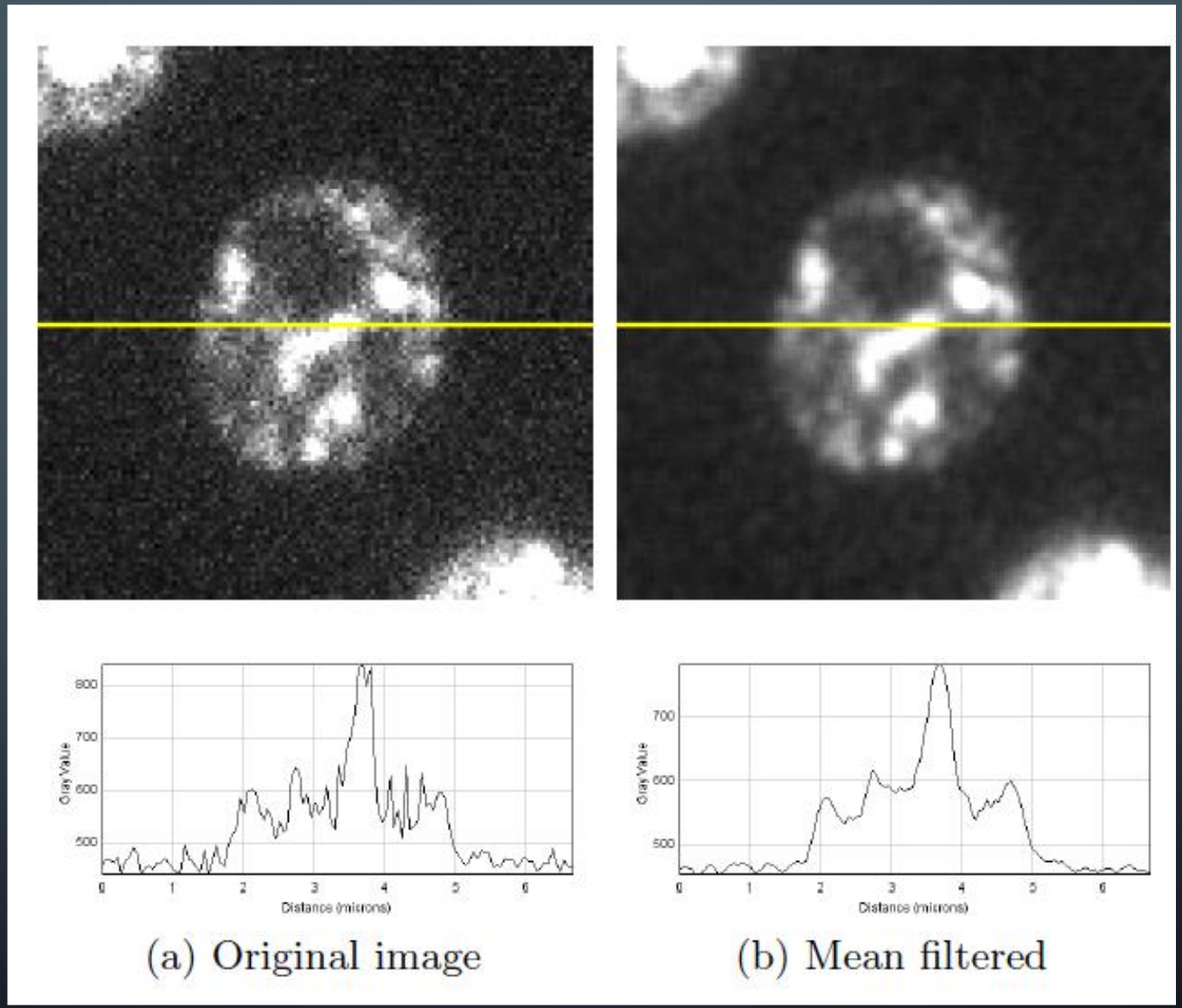




If you want to limit your measured area to just your object you draw a region of interest (ROI) around your object with one of the drawing tools (in the toolbar) and then

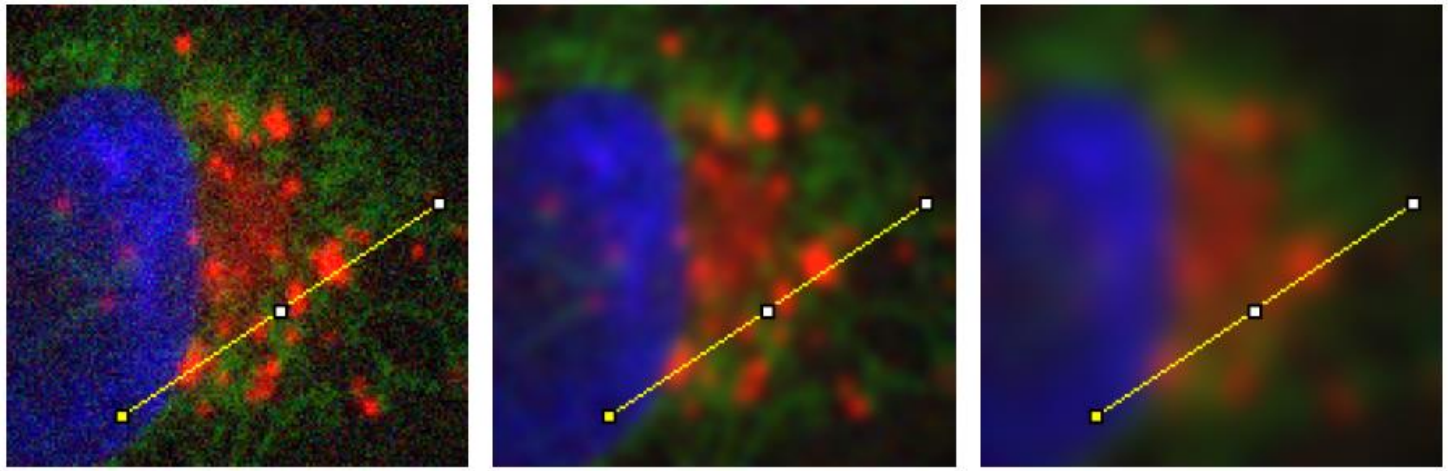
Analyze

Measure

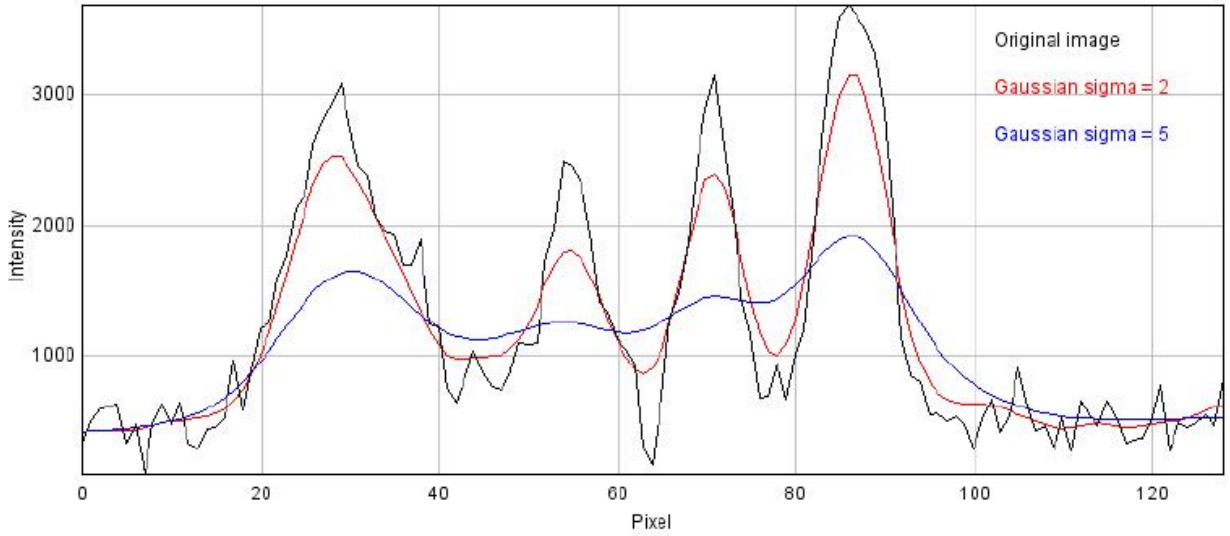


(a) Original image

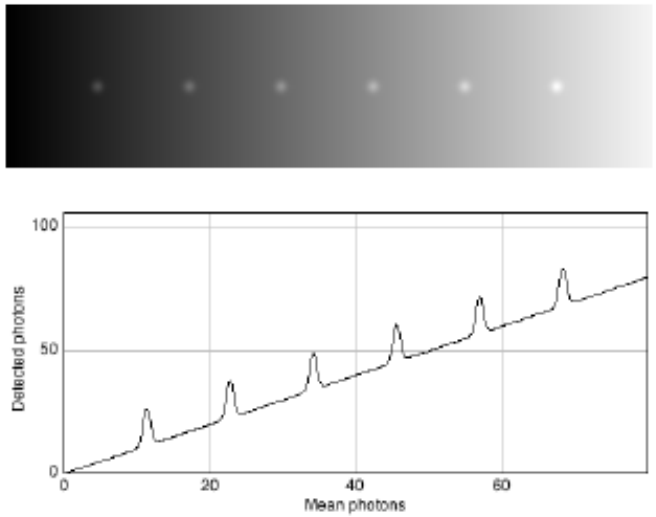
(b) Mean filtered

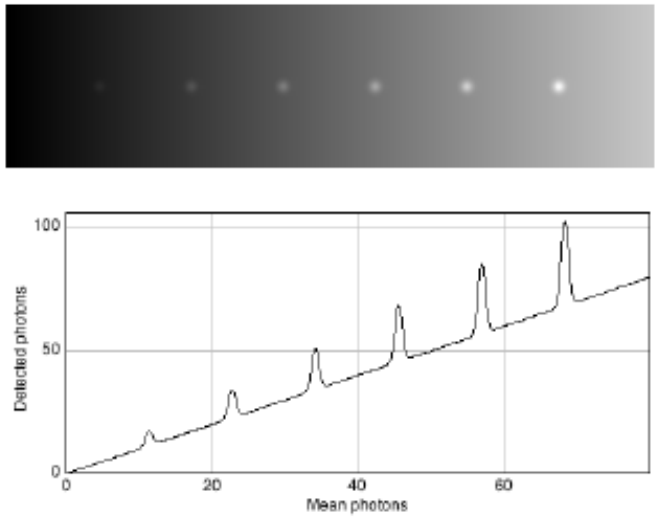


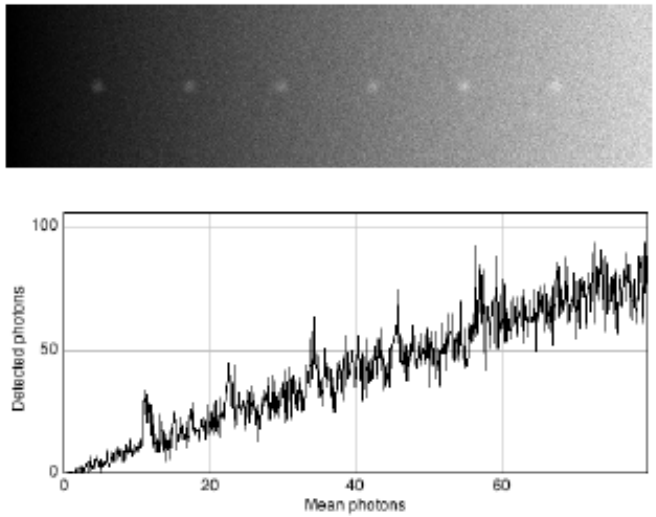
(a) Original image (b) Gaussian $\sigma = 2$ (c) Gaussian $\sigma = 5$



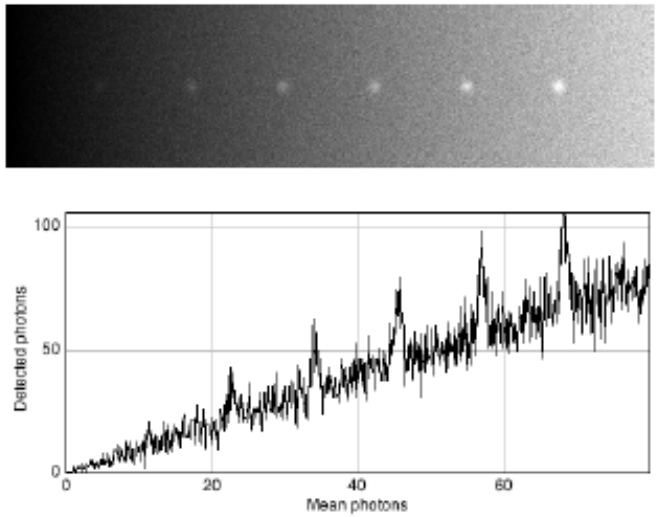
(d) Profile plots of the intensity in the red channel of the image



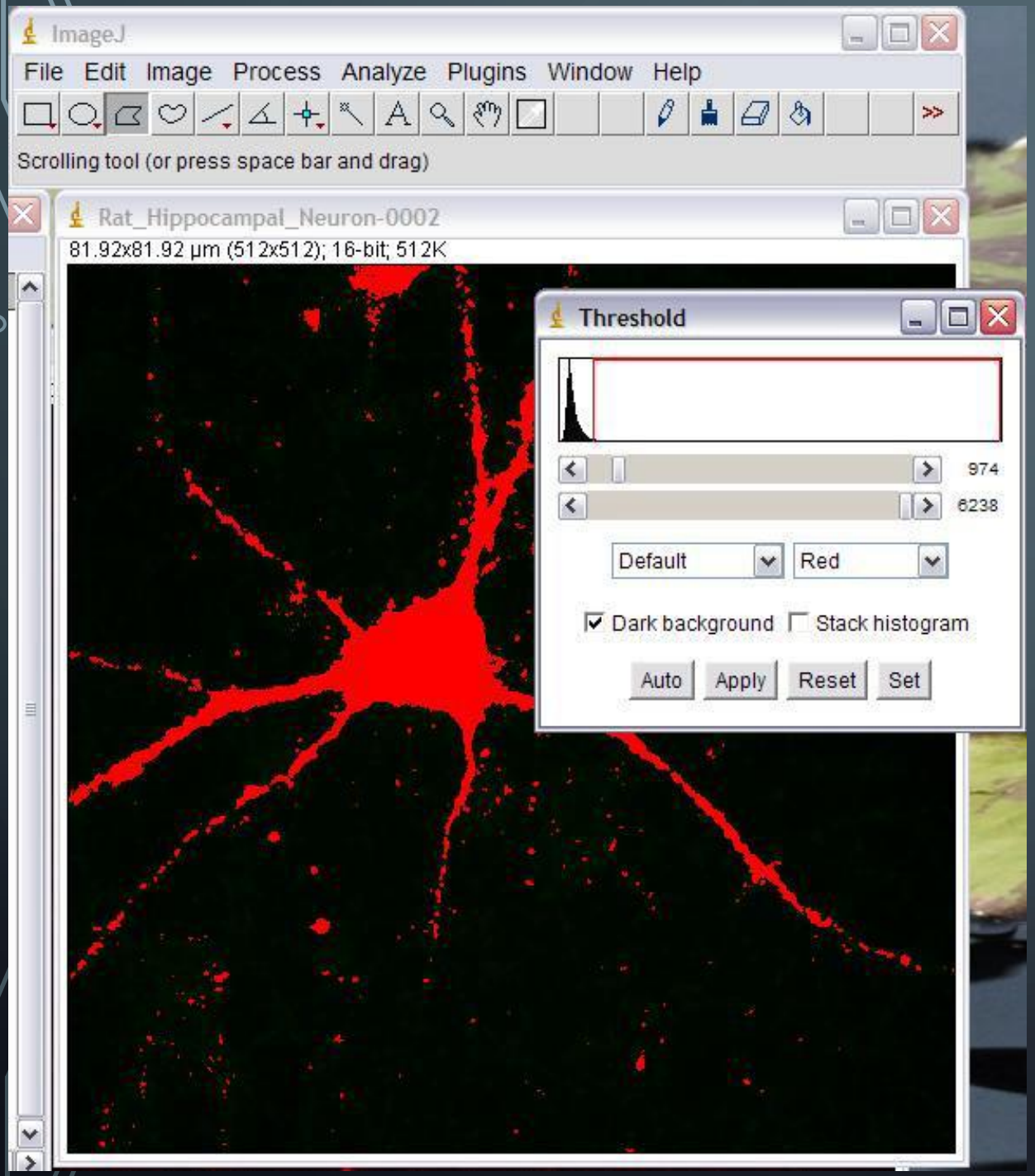




(a) Seeing spots with the same absolute brightness



(b) Seeing spots with the same relative brightness



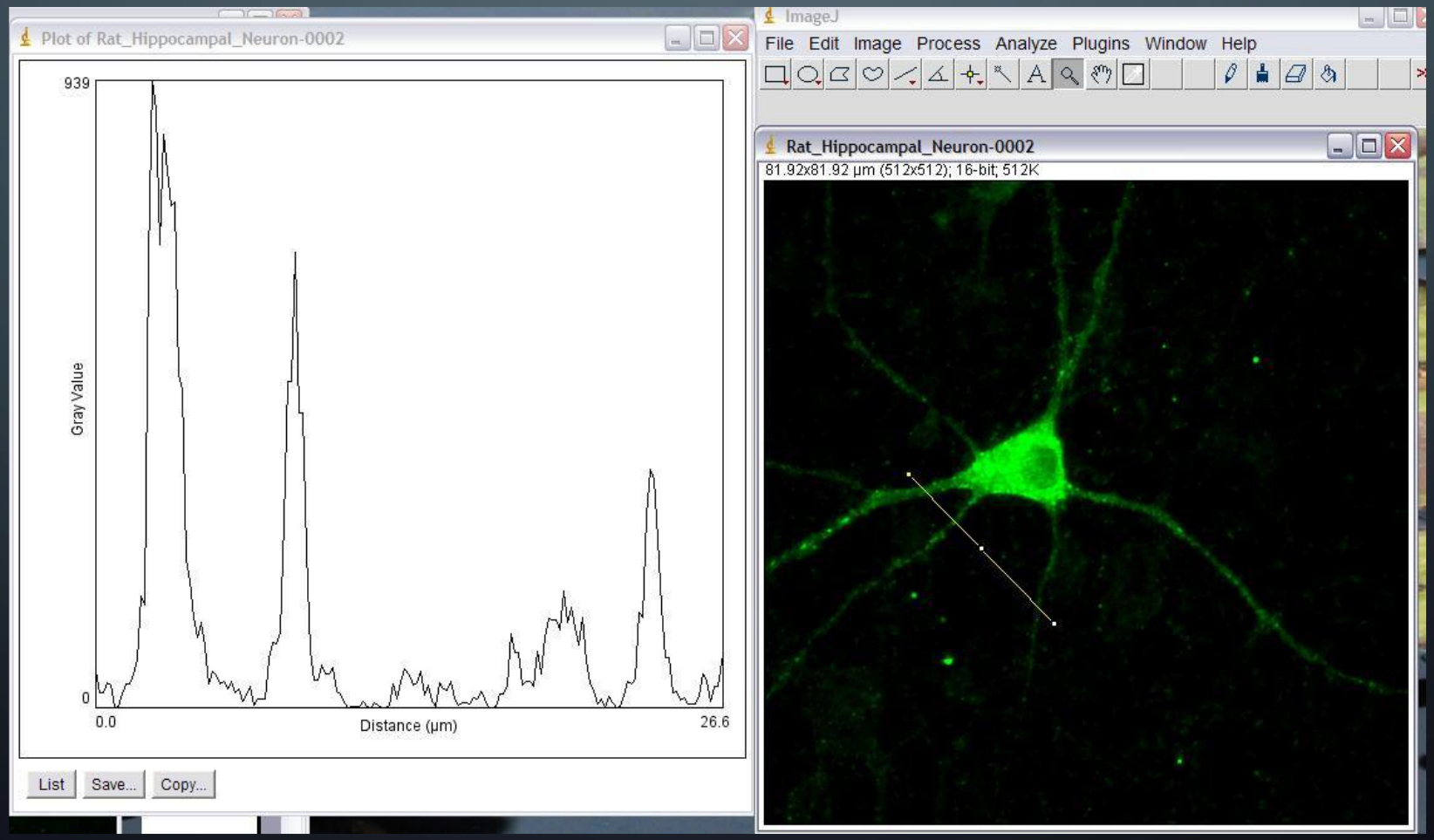
Alternatively, you can go to Analyze > Set Measurements

and check off the box next to “Limit to Threshold.”
Then use
Image > Adjust >Threshold

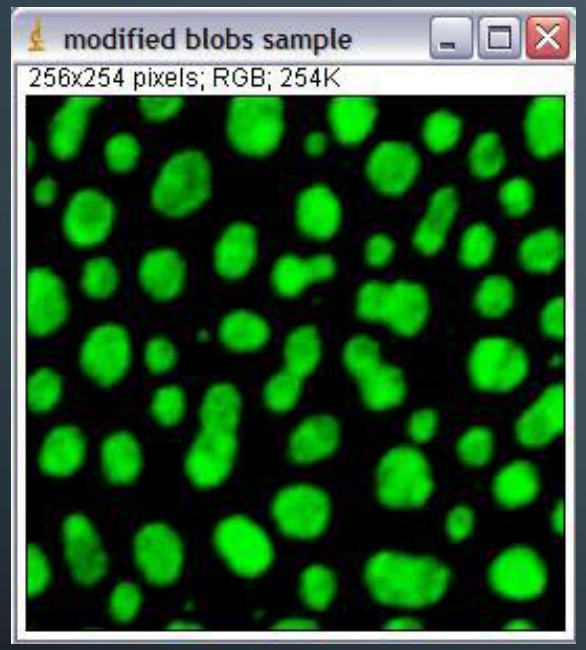
to highlight the area you want to analyze, and then
>
Analyze

You can use Analyze > Plot Profile to create a plot of intensity values across features in your image.

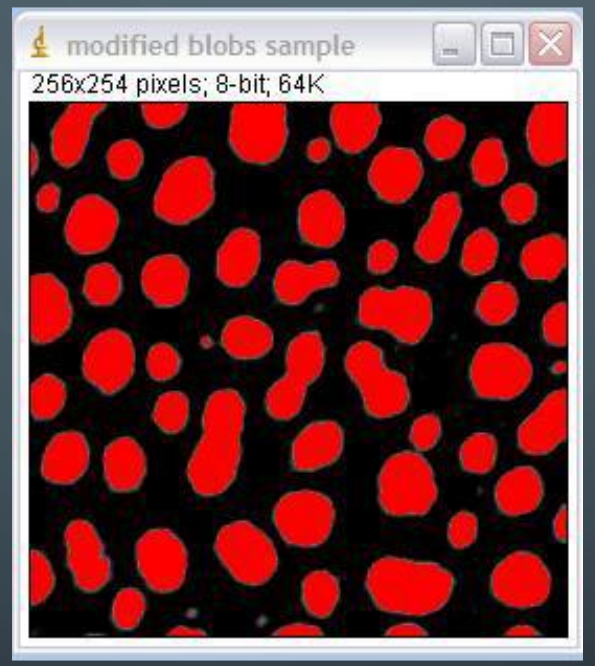
In the example below, the plot gives the intensity values along the line drawn across three cell processes.



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



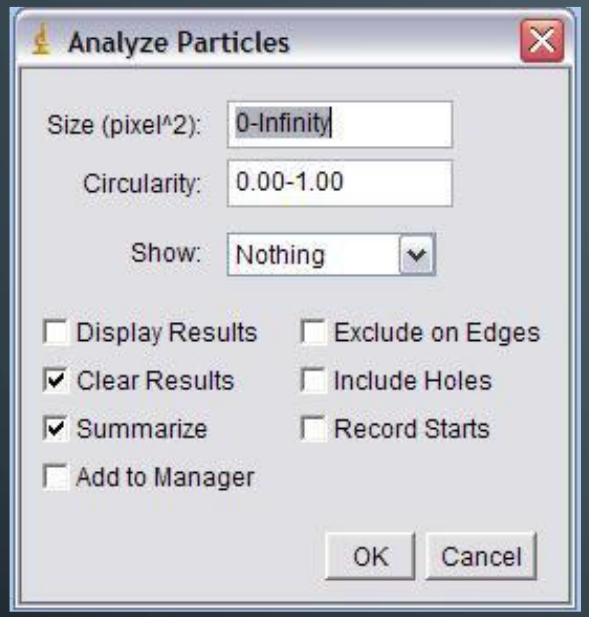
convert to grayscale



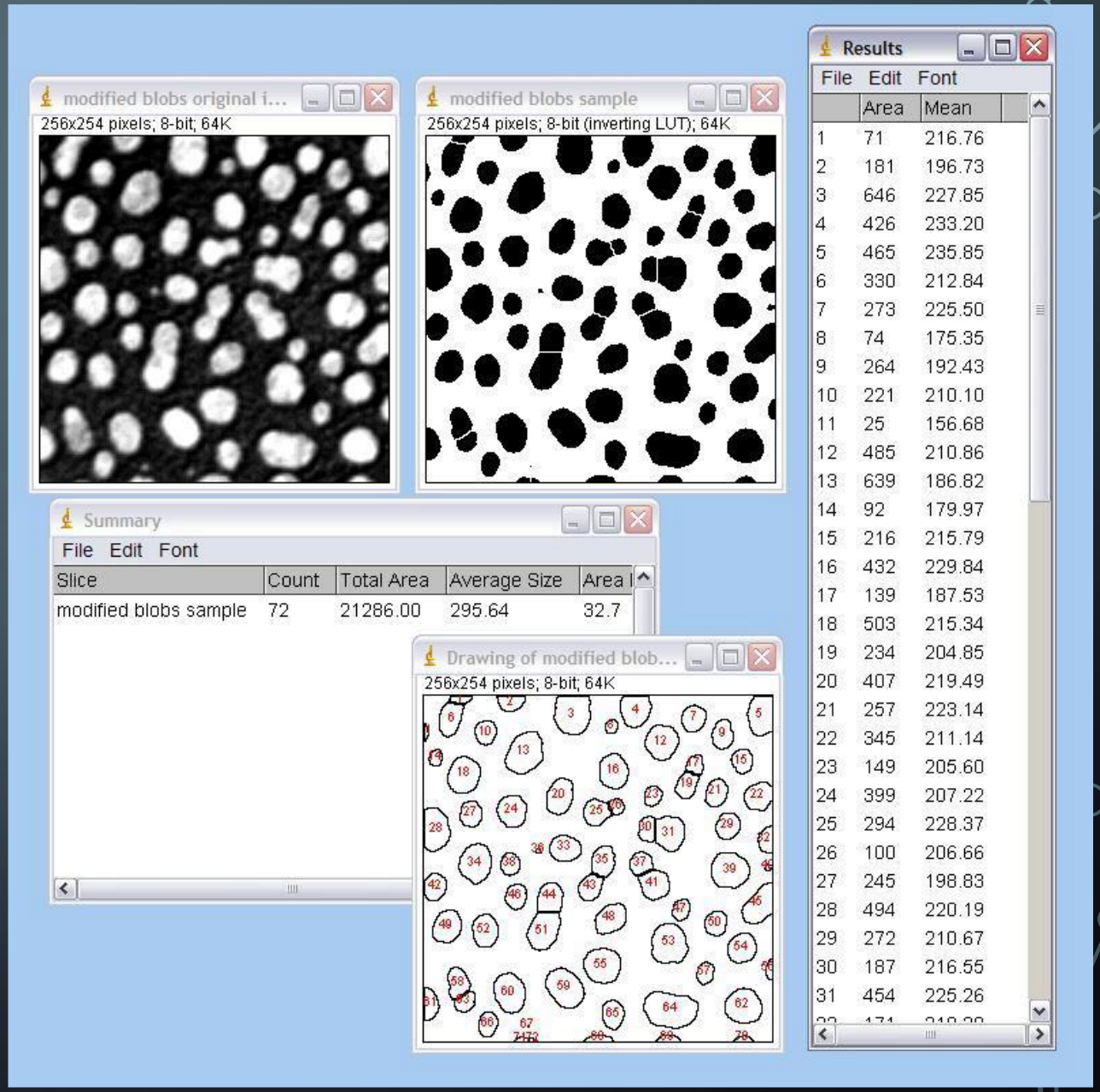
create a binary image,

Process > Binary > Watershed

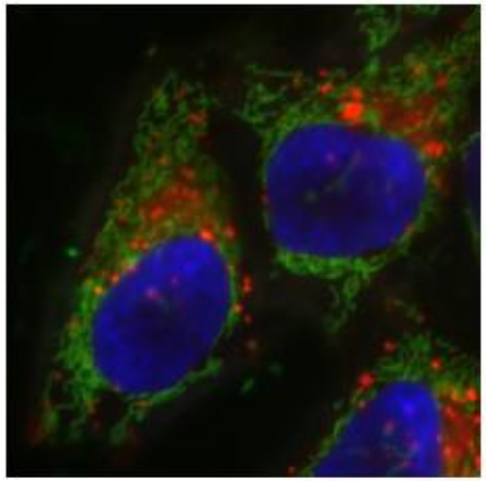
Analyze > Analyze Particles



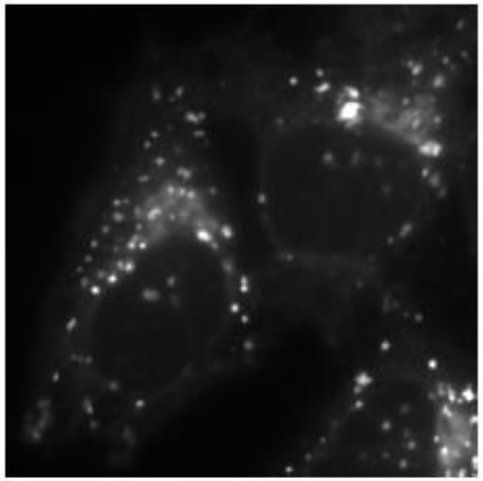
>



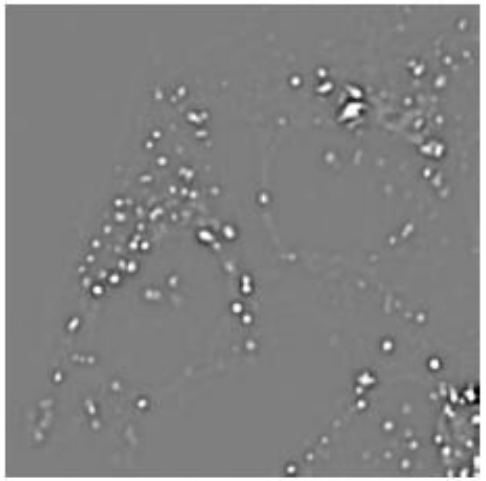
An simple image analysis workflow for detecting and measuring small spots, applied to the red channel of the sample image HeLa Cells.



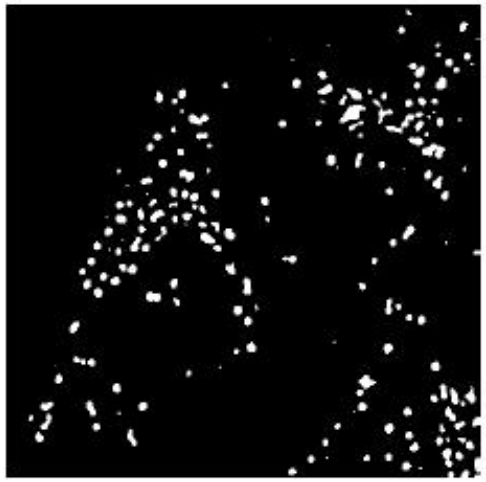
(a) Original image



(b) Extract channel

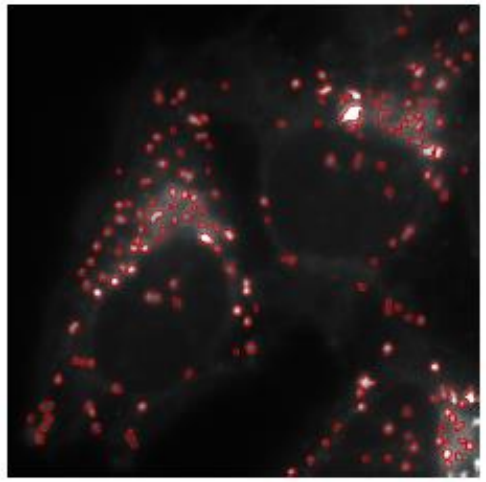


(c) Apply filters

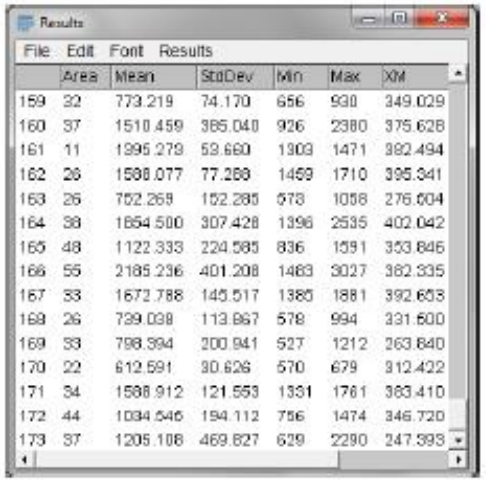


(d) Apply threshold

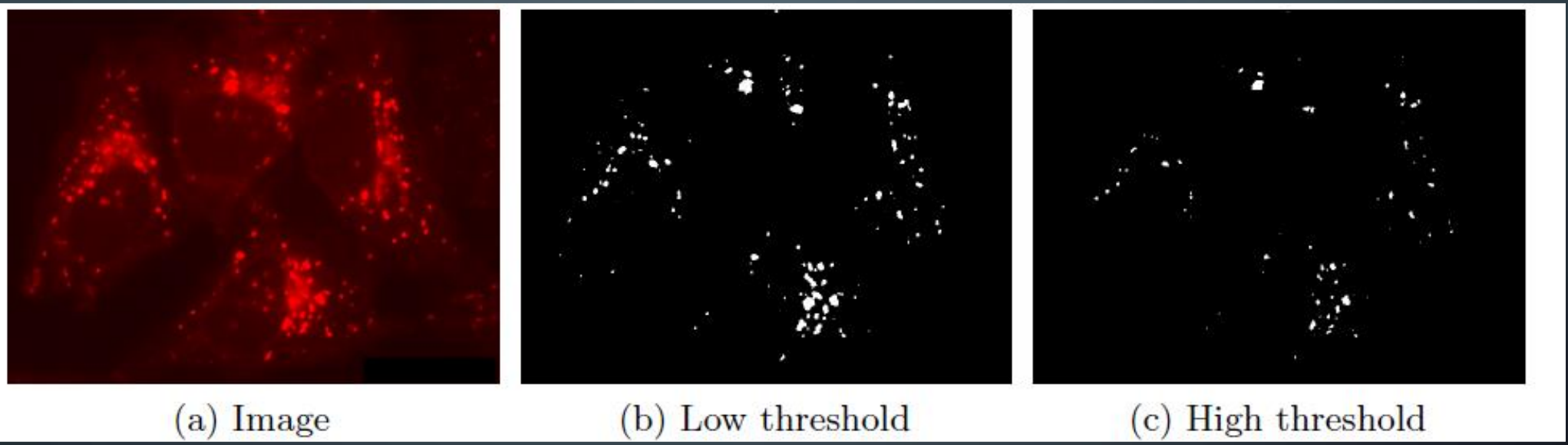
(e) Refine detection



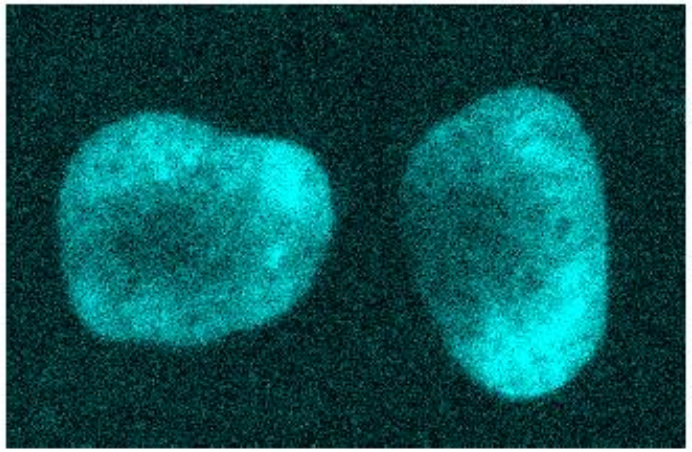
(f) Relate (e) to (b)



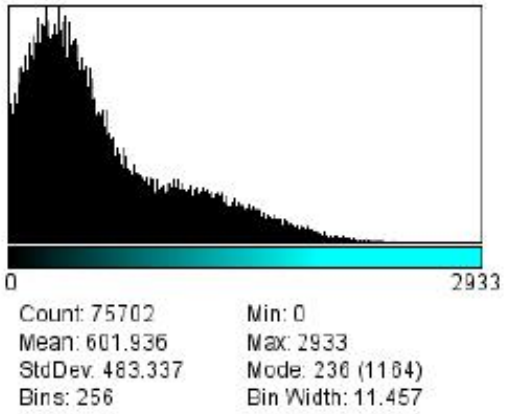
(g) Measure



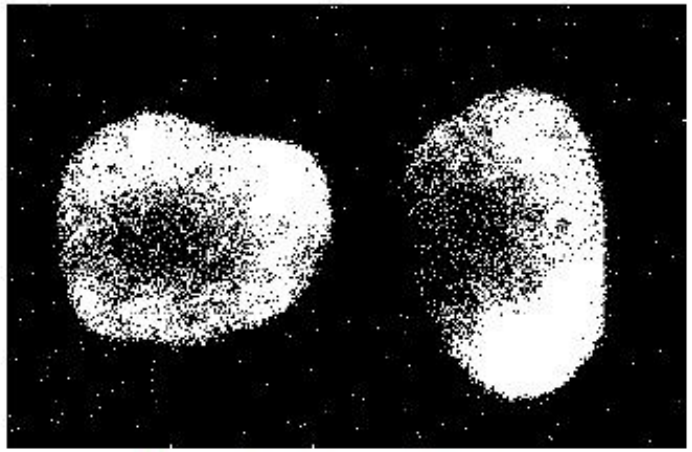
(a) Image (b) Low threshold (c) High threshold



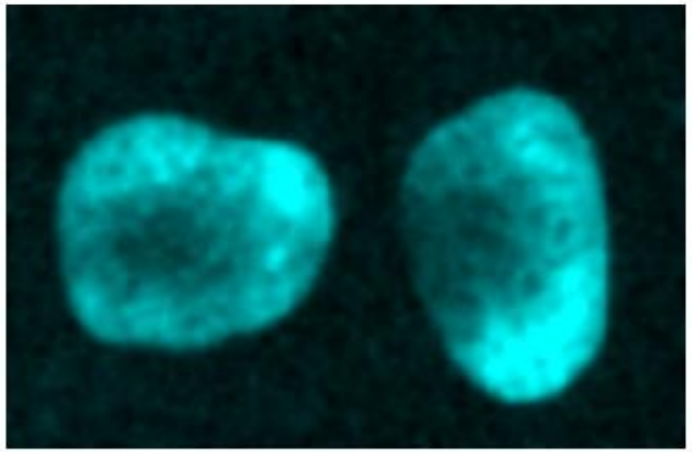
(a) Noisy image



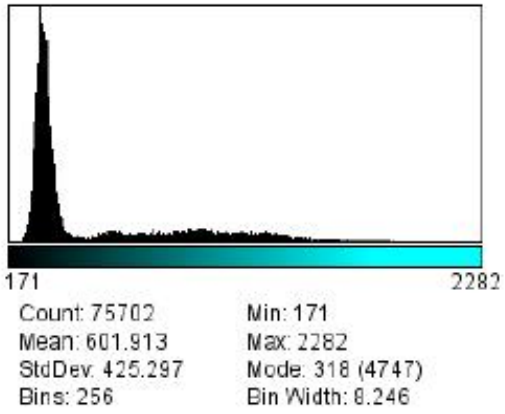
(b) Histogram of (a)



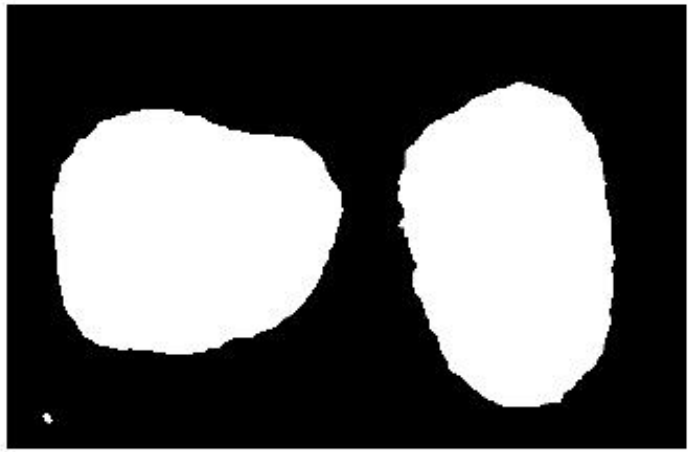
(c) Threshold applied to (a)



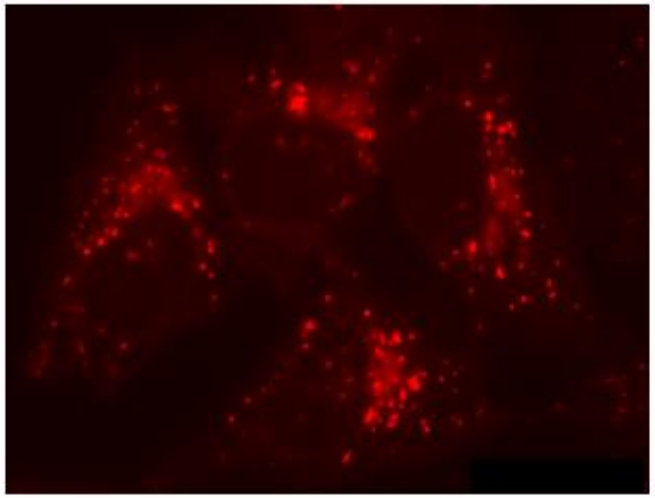
(d) Gaussian filtered image



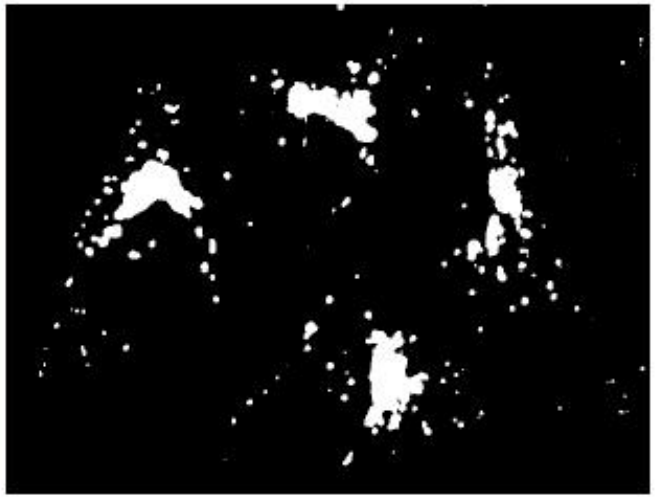
(e) Histogram of (d)



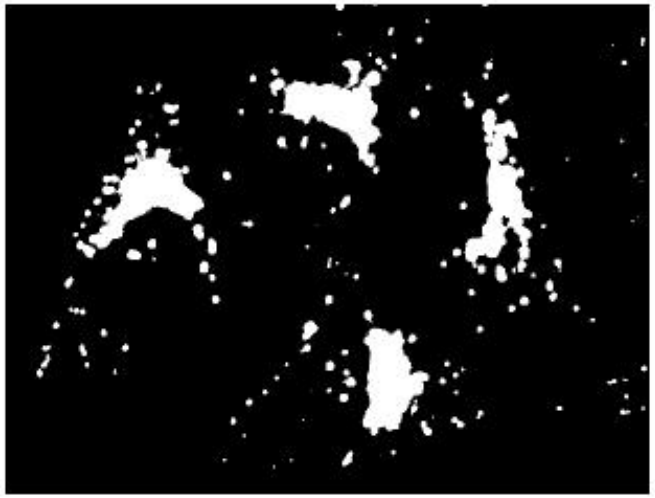
(f) Threshold applied to (d)



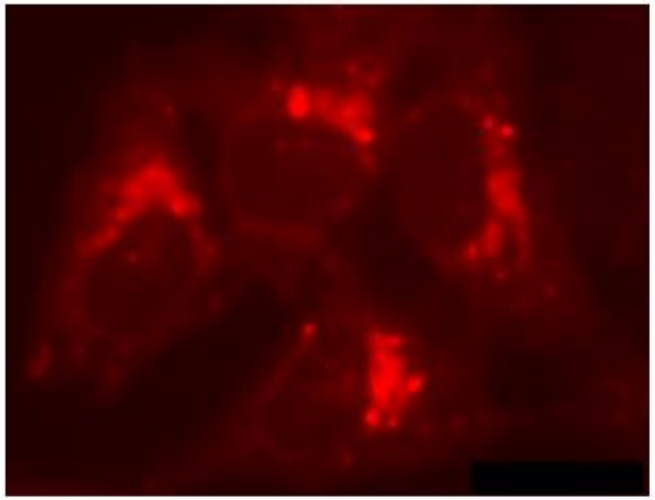
(a) Original image



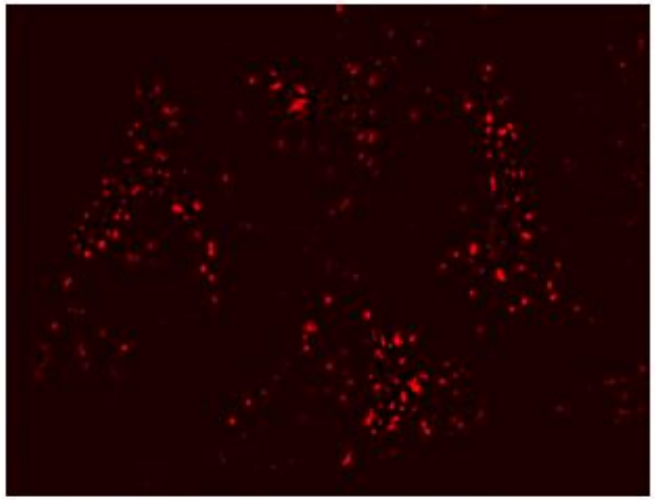
(b) Otsu's threshold



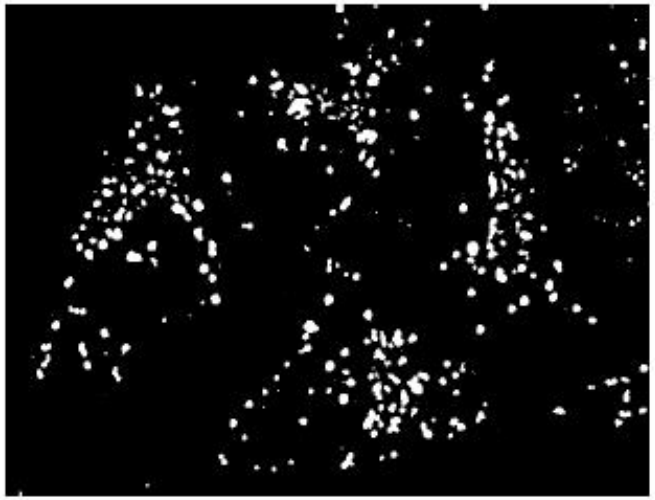
(c) Triangle threshold



(d) Median filtered image



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



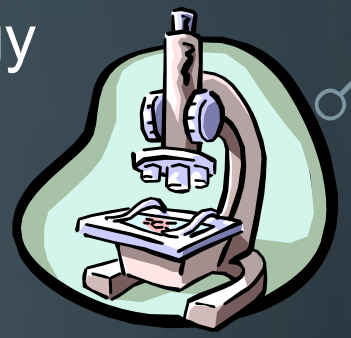
(f) Triangle threshold of (e)

ImageJ How to Measure Mean Fluorescence Intensity Over Timelapse Image Stack

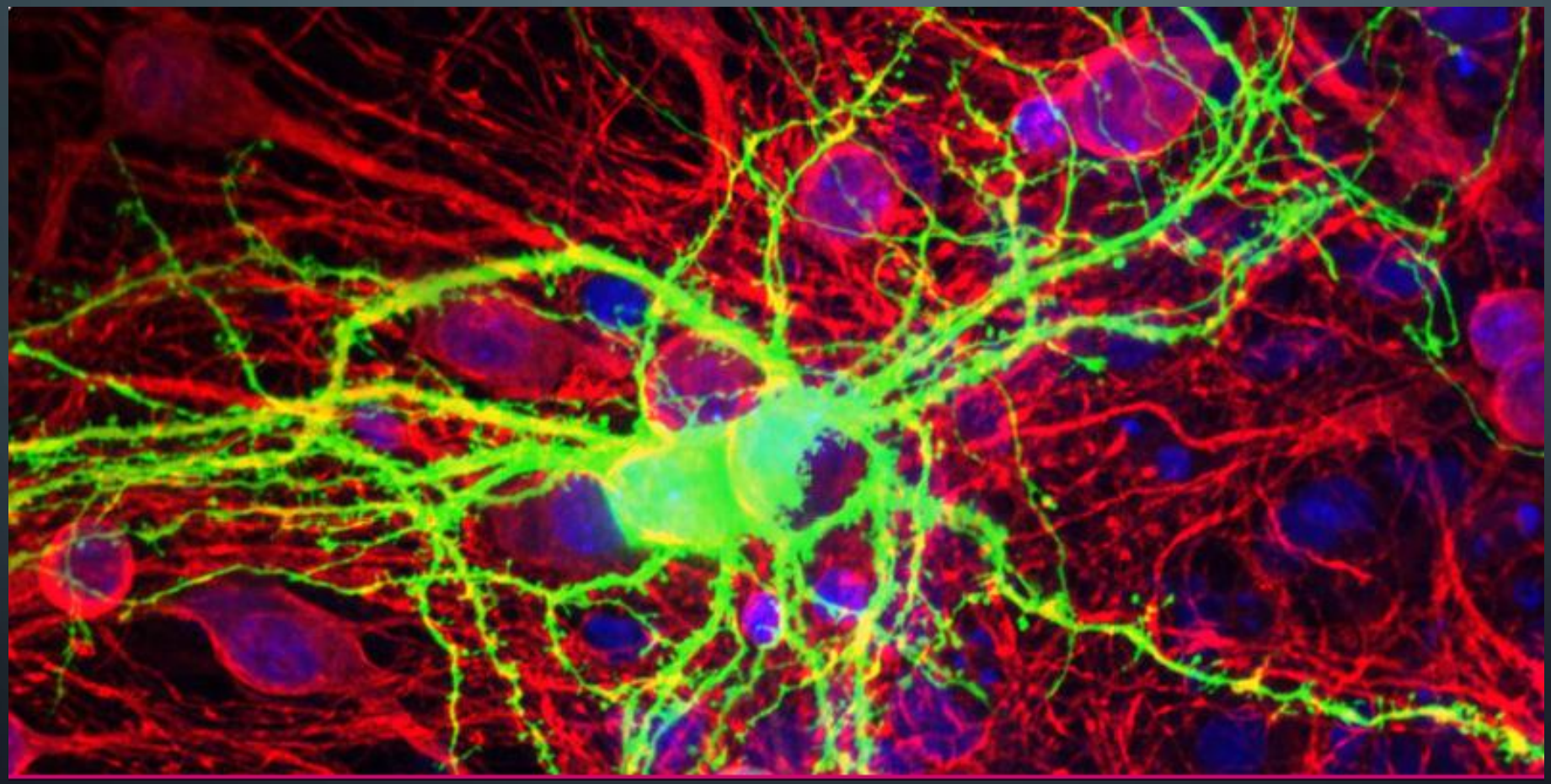
https://youtu.be/GHvndpGQKe4

Short introduction to histogram processing

https://youtu.be/nlRhHb04u_k



Question?



Thanks for your attention!

Gabriele Baj
gbaj@units.it