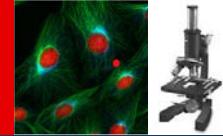


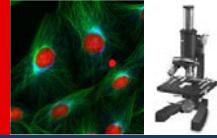
Basic Image Processing (using ImageJ)

Dr. Arne Seitz
Swiss Institute of Technology (EPFL)
Faculty of Life Sciences
Head of BIOIMAGING AND OPTICS – BIOP
arne.seitz@epfl.ch



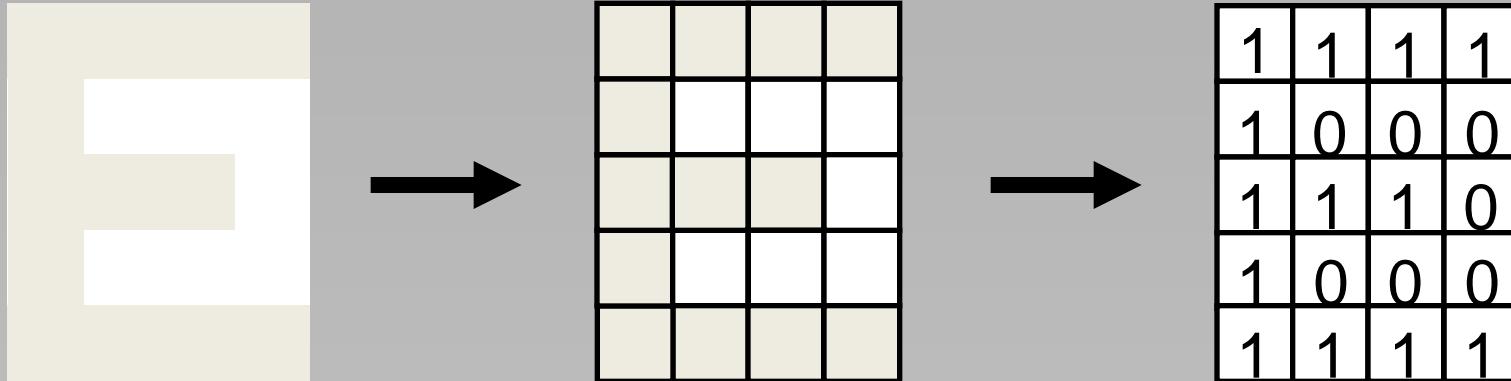
Overview

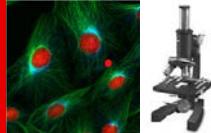
- File formats (data storage)
- Programs for image viewing / processing / representation
- Basic Image Processing (using ImageJ)



Definition Digital image

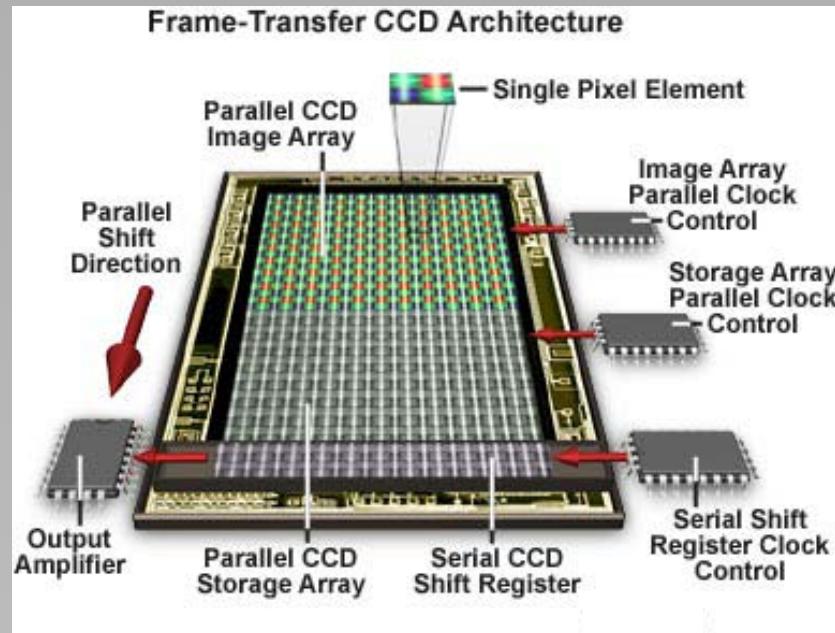
- A digital image is a representation of a two-dimensional image using ones and zeros (binary). (Wikipedia)
- Analog = continuous values
- Digital = discrete steps



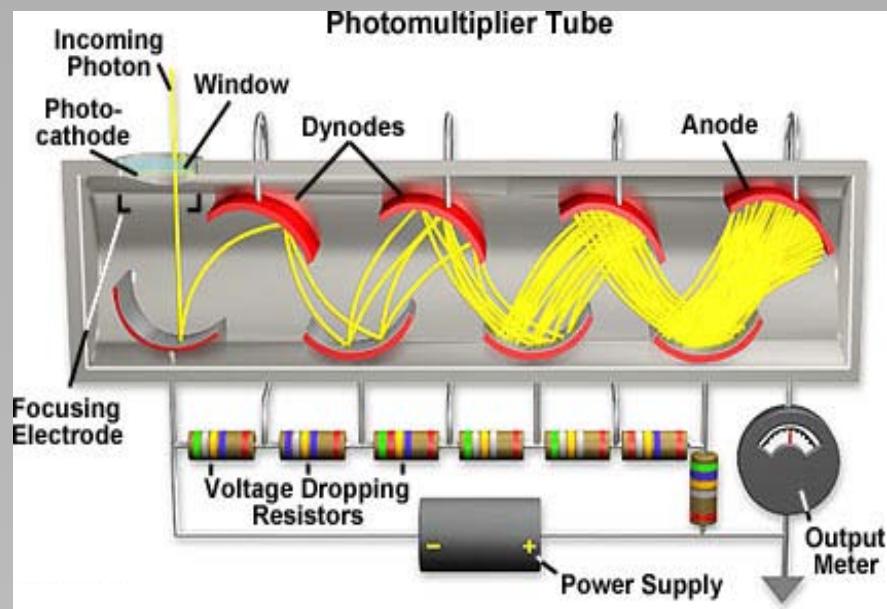


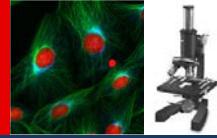
Detection Devices

Array detector



Point detector



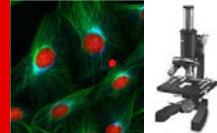


File Formats – data storage

- Lossless image formats
- Lossy compression formats
- Custom formats (microscope companies)
- Sequence vs. single image per file
- 8bit, 12bit, 16bit, 32bit, RGB

Storage:

- Always have at least 1 copy of the data
- Very suitable fileservers (automatic backup)



Lossless Image Formats

TIFF (with or without compression)

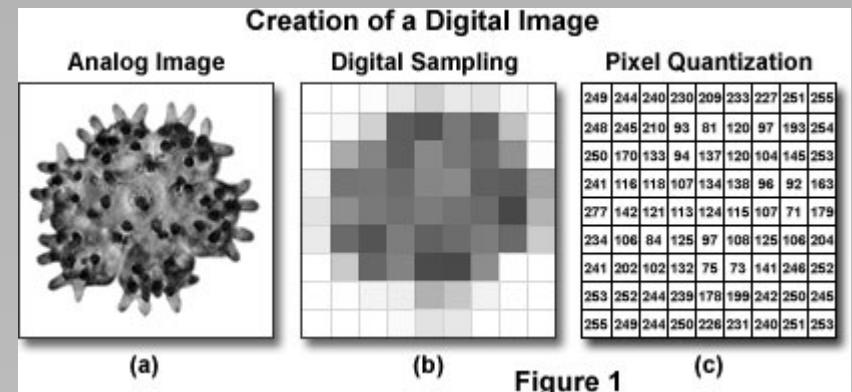
BMP (windows uncompressed)

GIF (graphics interchange format)

PNG (portable network graphics)

Raw data

'text image'



Microscopy Primer

<http://micro.magnet.fsu.edu/primer>

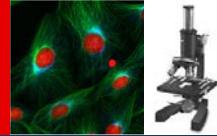


Image Format: TIFF

Tag Image File Format

- Image header with flexible set of ‘tags’ which can be used to store e.g. microscopic settings

Flexible in color space and bit depth

- Microscopy: grayscale 8bit, 16 bit (12bit data)
- Color (e.g. Overlay): RGB (red green blue 8bit each)
- Quantification: 32bit (floating point values)

Always lossless: Uncompressed or compressed

Multiple images possible in one file

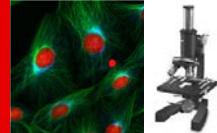
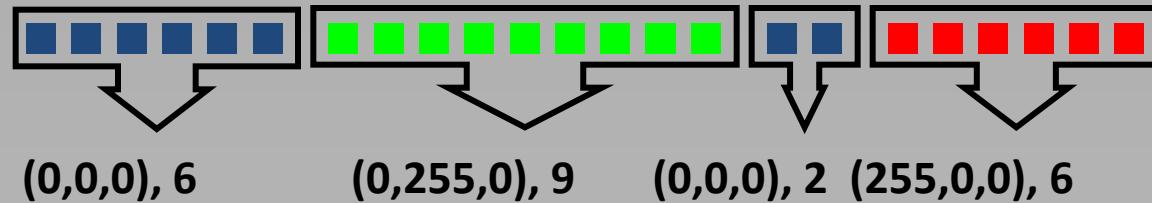
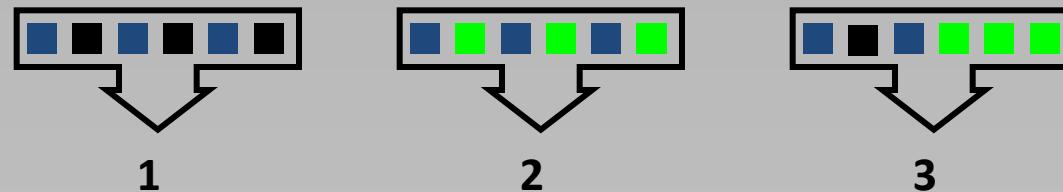


Image Compression: TIFF

Run Length Coding (RLE): first number describes the color, the second the number of following pixels having the same color.



LZW (Lempel-Ziv-Welch): Find repetitive patterns of values and give them a number which is points to an entry of a „dictionary“ (LUT).



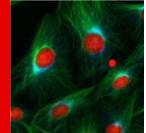


Image Compression: TIFF

Pros:

Extra infos can be written in the ‚tags‘
(e.g. microscope data like objective lens, voxel size)

Everybody can read it

Lossless

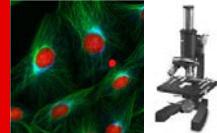
Flexible (8, 16, 32bit grayscale, 8:8:8bit RGB)

256 Floating point values
65536 graylevels

Cons:

Big files

Compressed files can't be loaded by ImageJ



Lossy Image Formats

The lossy compression algorithm takes advantage of the limitations of the human visual senses and discards information that would not be sensed by the eye.
(like mp3 in audio).

Compression level is usually flexible, but the more compressed the more information is lost and artifacts become visible by eye



From: www.wikipedia.org

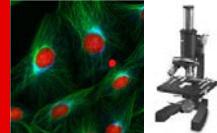
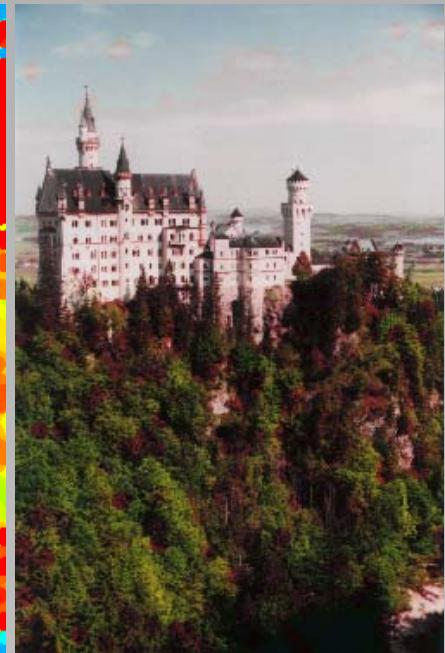
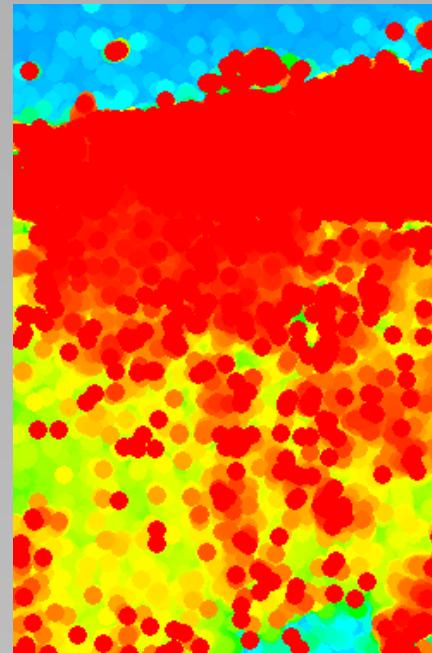
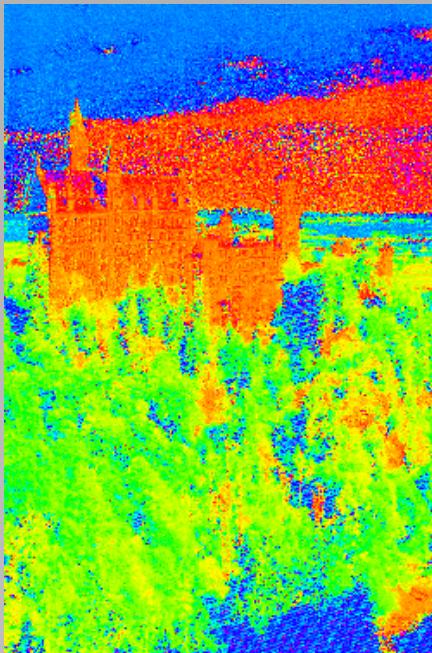


Image Compression: JPG

Split image into color and gray-scale information (color is less important than boundaries)
→ reduce high frequency color information.

Group pixel into 8x8 blocks and transform through discrete cosine transform...



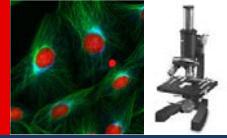


Image Compression: JPG

Pros:

Small Files

True Color

Usable for most photos (real life) and presentations (powerpoint)

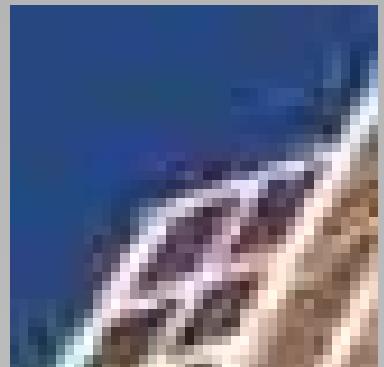


Cons:

Do not use for quantification !

„Unrelevant“ photoinfos get lost

Every file-saving reduces the quality



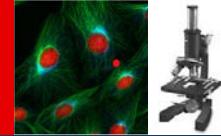


Image Viewers

ImageJ (Java based, freeware, Win/MAC/Linux)

Irfanview (www.irfanview.com/)

- Freeware
- Convert (e.g. tif → jpg)
- Batch processing

ACDSee (ACD Systems)

Microscope companies

- Zeiss Image Browser / Axiovision LE
- Leica LCS Lite
- Olympus Viewer

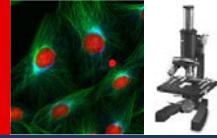


Image Representation

ImageJ

Imaris (Bitplane):

- 4 floating licenses
- installed on image processing workstations

Photoshop, Paintshop, Illustrator, Corel Draw
(, Powerpoint)

Volocity (Improvision):

Custom software of microscopes

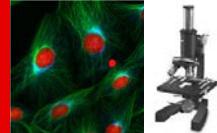
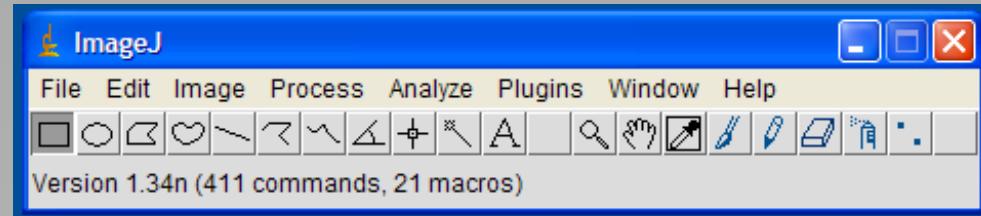


Image Processing



ImageJ

(<http://rsb.info.nih.gov/ij/index.html>)

- installed on all image processing workstations
- Installation: <http://pacific.mpi-cbg.de/wiki/index.php>
(Fiji=ImageJ+plugins+regular update)
- Manual: www.uhnresearch.ca/facilities/wcif/imagej/
(also available as pdf)
- Additional plugins: <http://rsb.info.nih.gov/ij/plugins/index.html>

Metamorph (Universal Imaging),

- installed on 2 image processing workstations

Custom software of microscopes

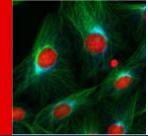


Image Processing Basics

Visual Image Inspection

Lookup tables (LUT) and LUT operations

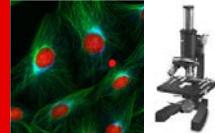
Histogram, brightness, contrast

Filter

Threshold

Measurements

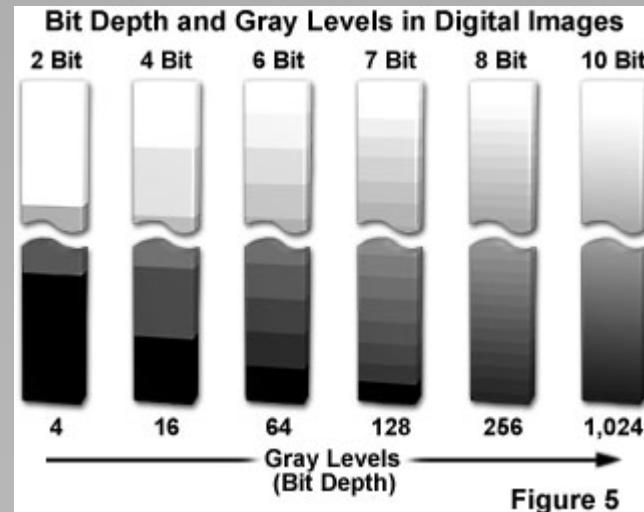
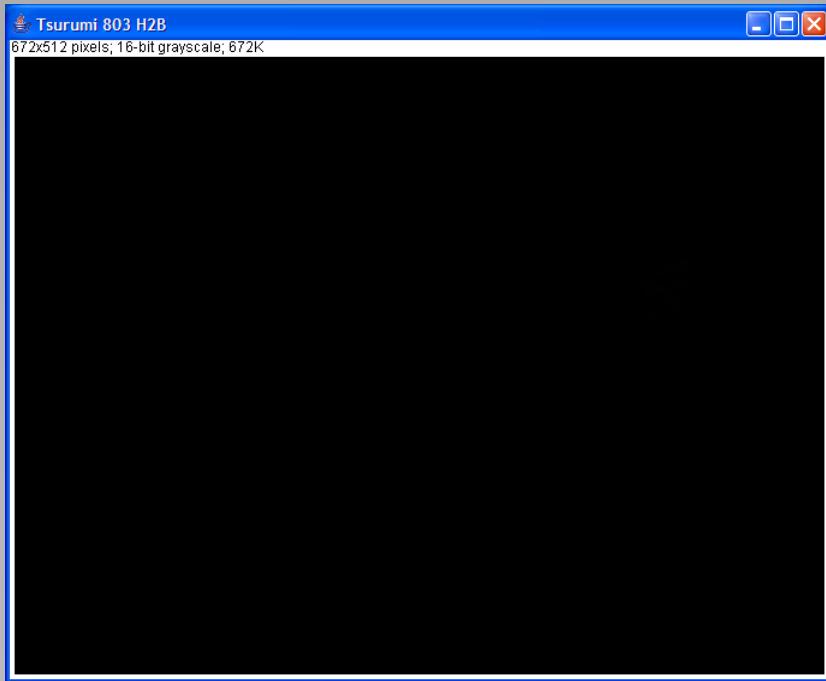
Color functions



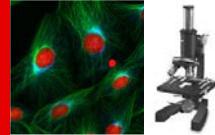
Biolmaging & Optics Platform

Visual Image Inspection

Displaying images, histogram

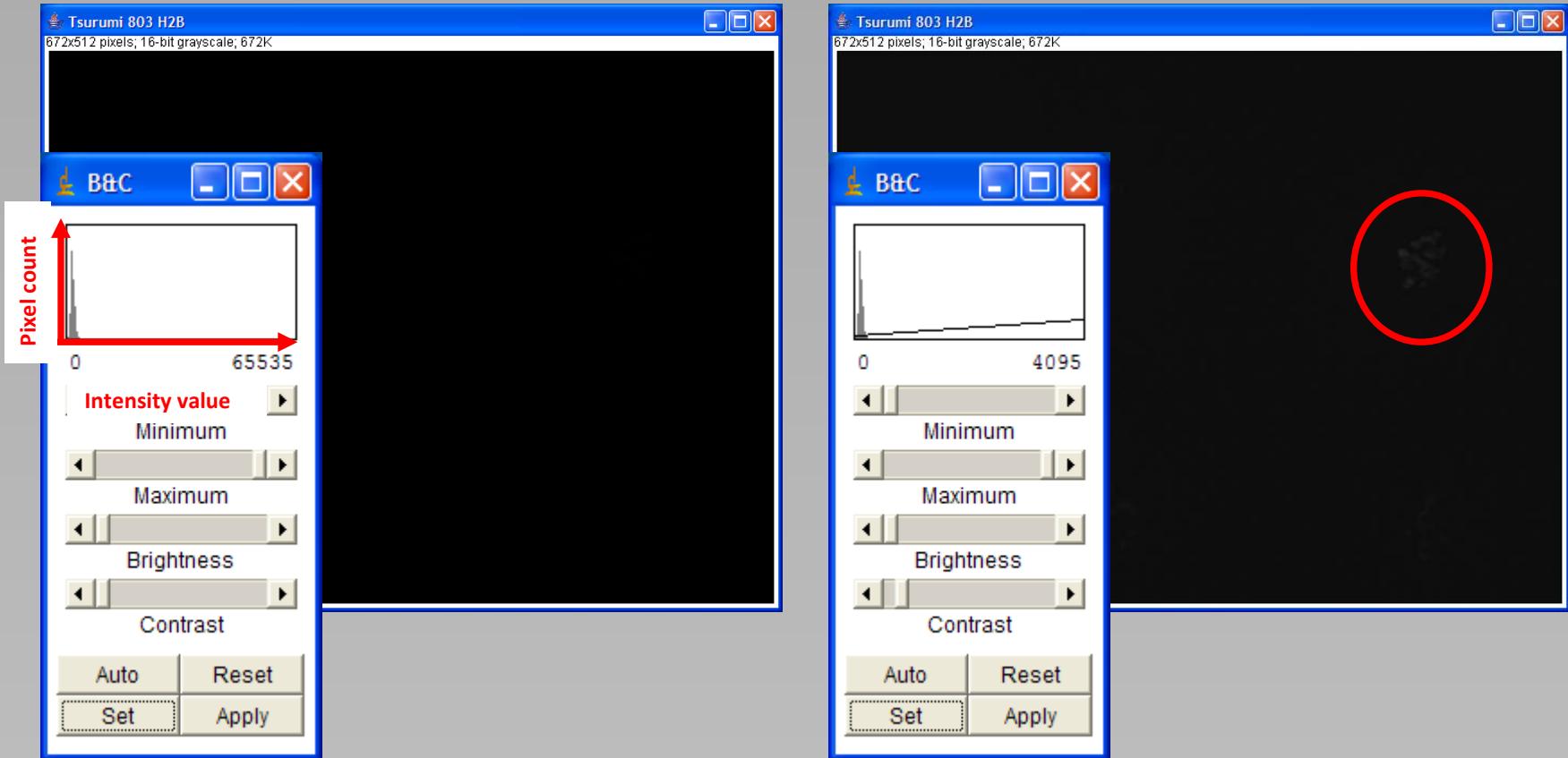


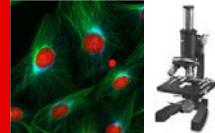
Microscopy Primer
<http://micro.magnet.fsu.edu/primer>



Visual Image Inspection

Displaying images, histogram

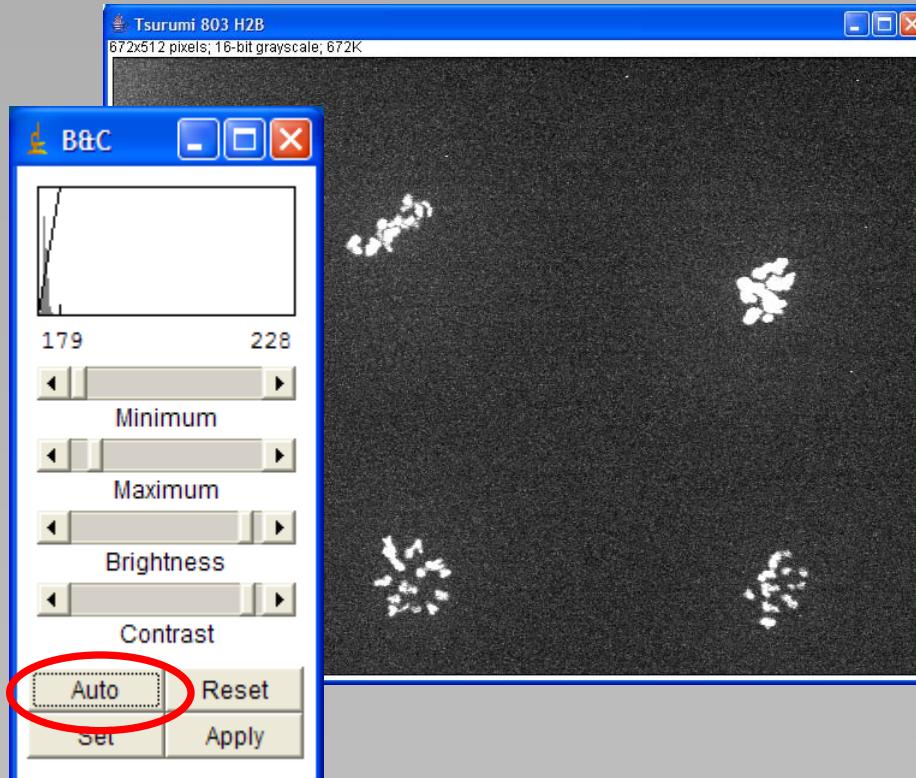




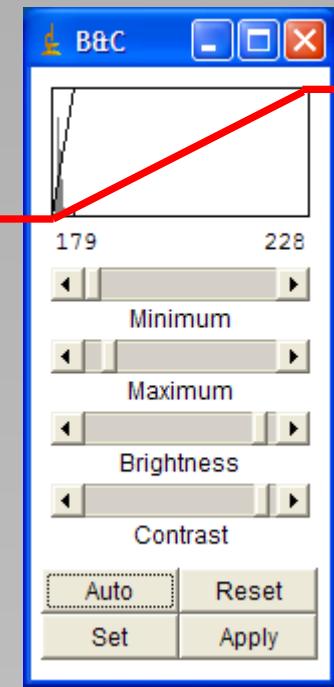
LUT operations

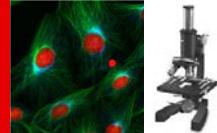
Lookup table (LUT)

- Displays can only show 256 gray values (8bit) per color
- Data is unchanged, it's only “mapped” differently

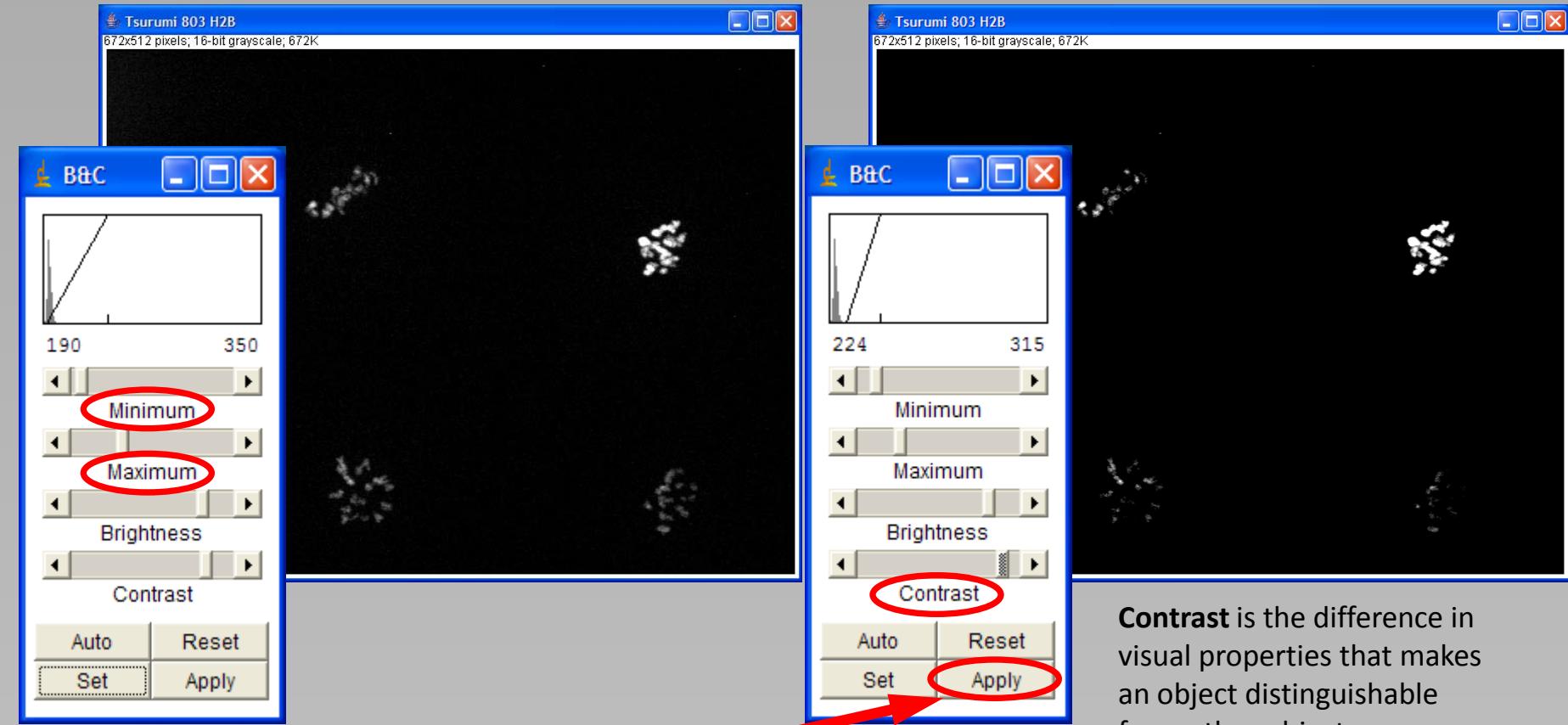


Data Intensity	Displayed Intensity
0	0
...	...
179	0
180	5
181	10
...	...
226	
227	
228	255
229	255
65535	255

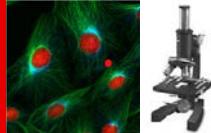




Brightness, Contrast

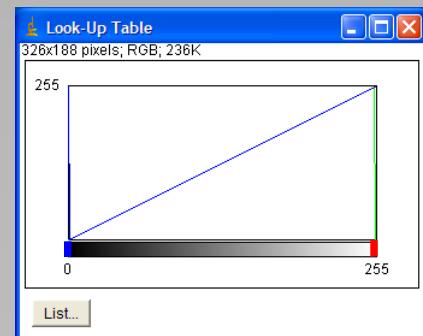


Caution: Apply modifies the data!

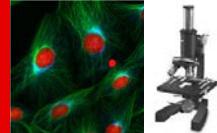


Color LUT

The pixel contains a „pointer“ to an array, where the actual pixel values are stored

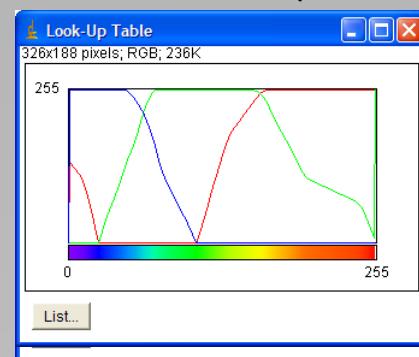
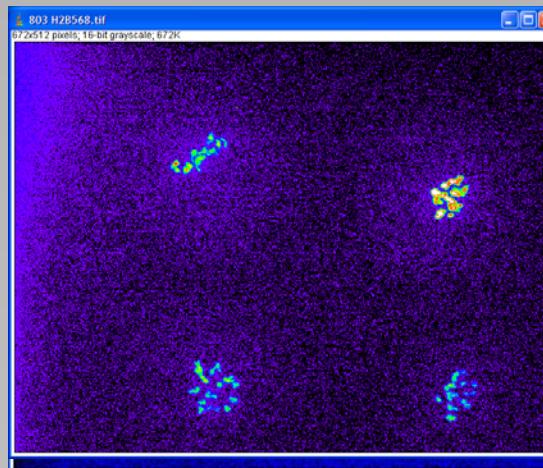


“HiLo” LUT

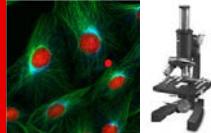


Color LUT

The pixel contains a „pointer“ to an array, where the actual pixel values are stored



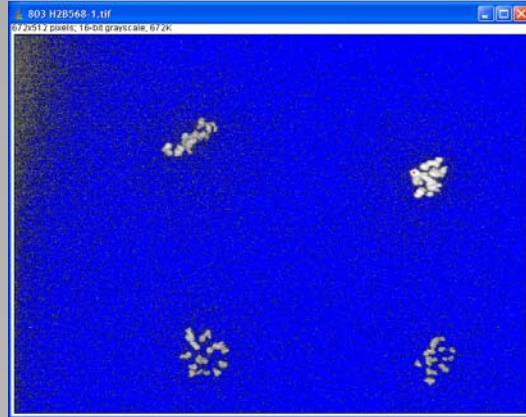
“Rainbow” LUT



Non-linear Histogram Stretch

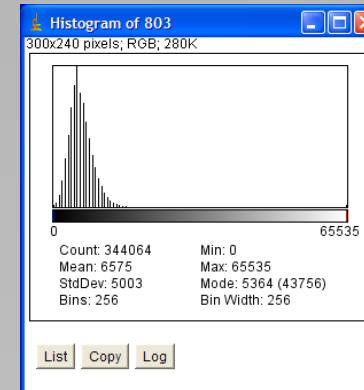
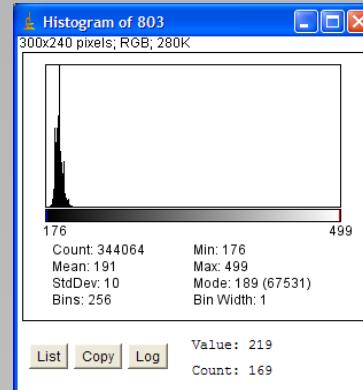
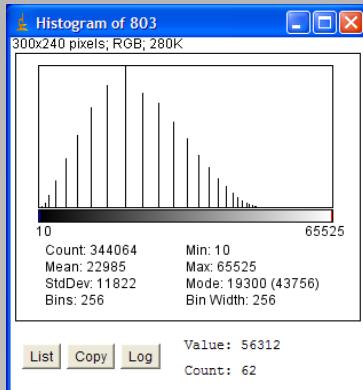
Enhance contrast by (changing data):

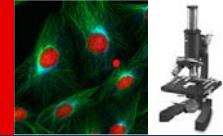
“Equalization” non-linear stretch
based on square root of the intensity



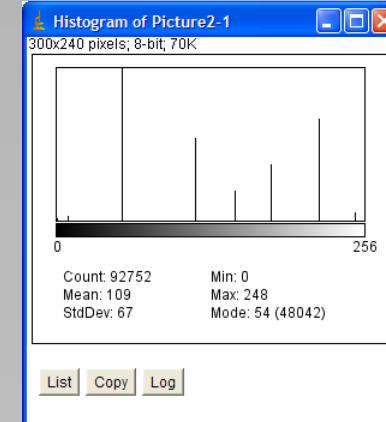
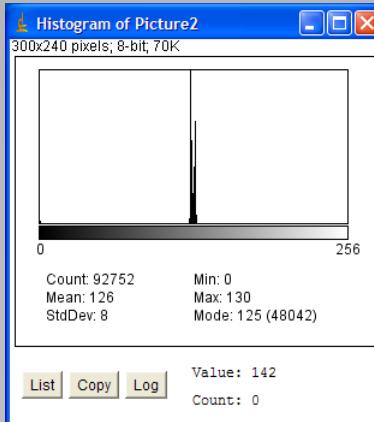
Raw data

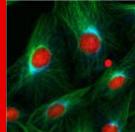
Linear stretch
“Normalization”





Equalization





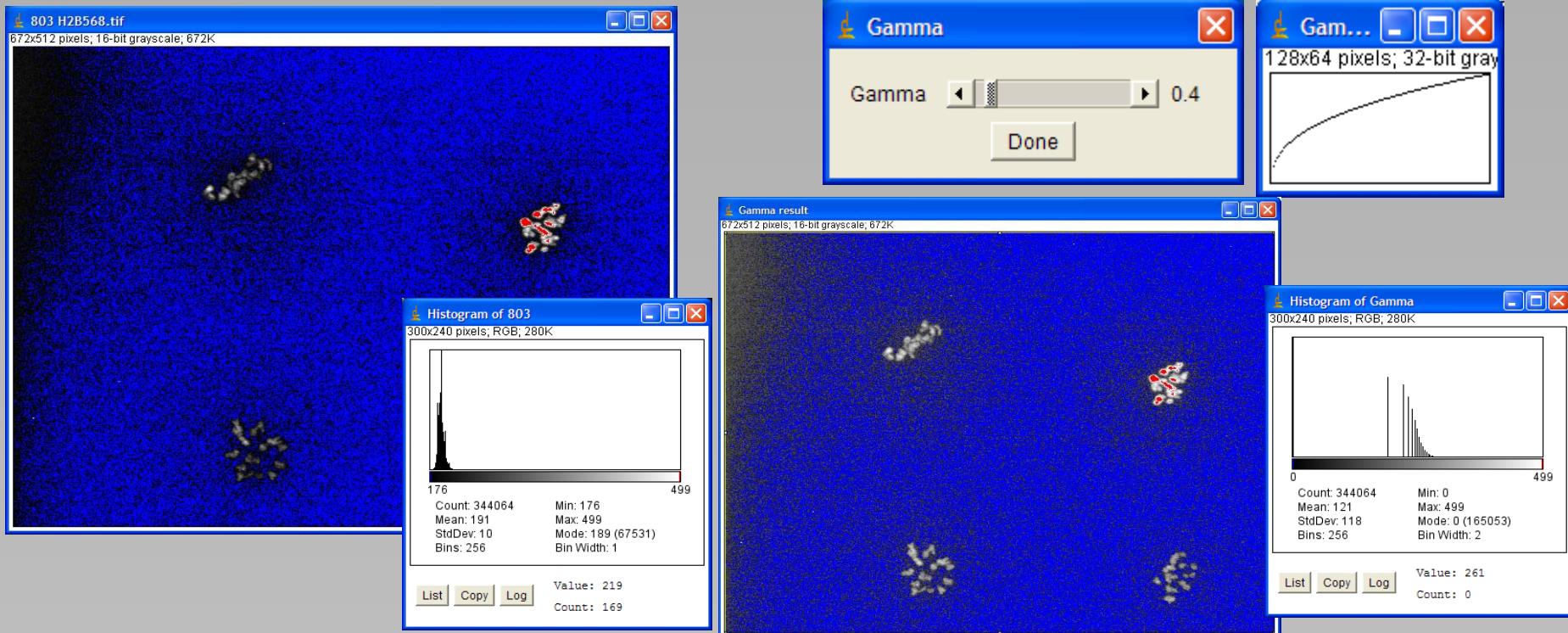
Biolmaging & Optics Platform

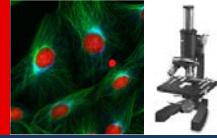
Gamma

Gamma is a non-linear histogram adjustment

8 bit images:

$$\text{New intensity} = 255 \quad [(old\ intensity/255) \gamma]$$





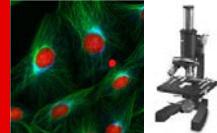
Filtering

Image processing filters are mainly used to:

- suppress the high frequencies in the image, *i.e.* smoothing the image, noise reduction
- or suppress the low frequencies, *i.e.* enhancing or detecting edges in the image

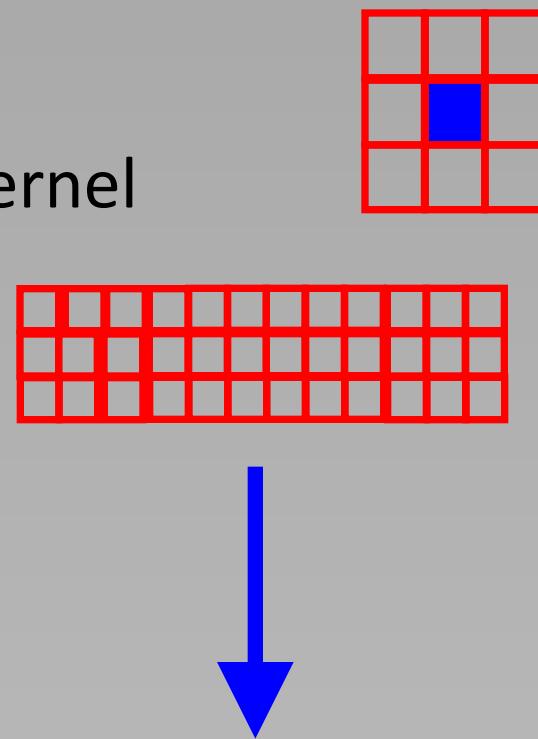
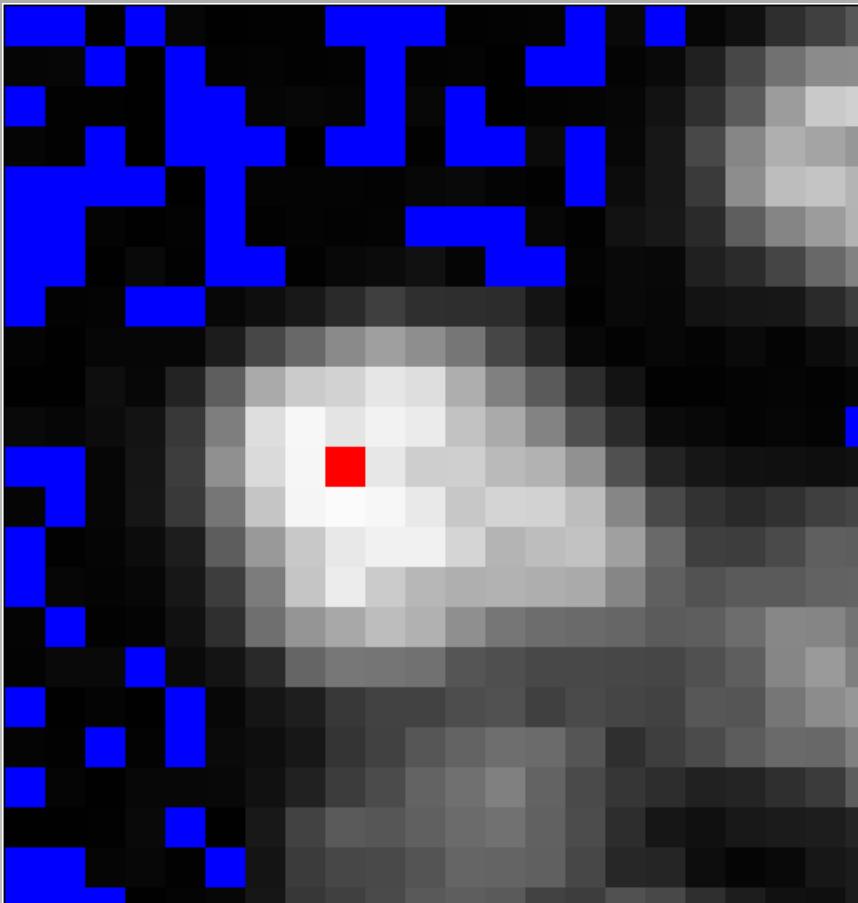
An image can be filtered either in the frequency or in the spatial domain.

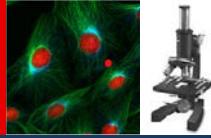
- Filtering in the frequency domain requires Fourier transform first and re-transformation after application of the filter.
- Filtering in the spatial domain is done by convolving the image with the filterfunction.



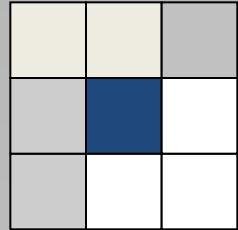
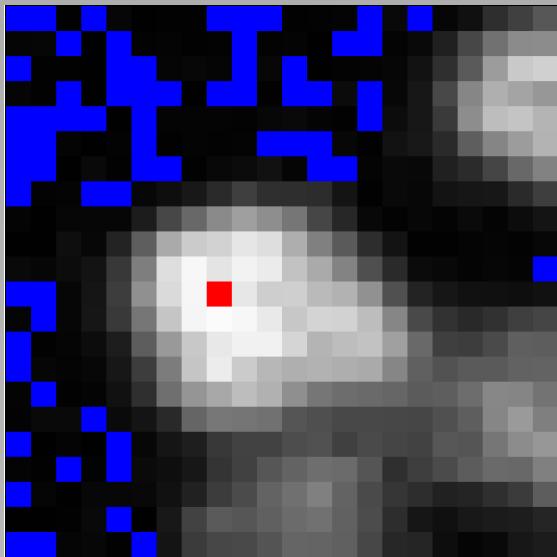
Filtering

Shifting and multiplying a filter kernel



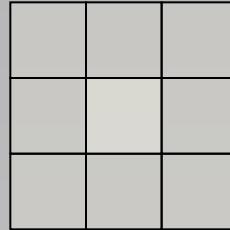
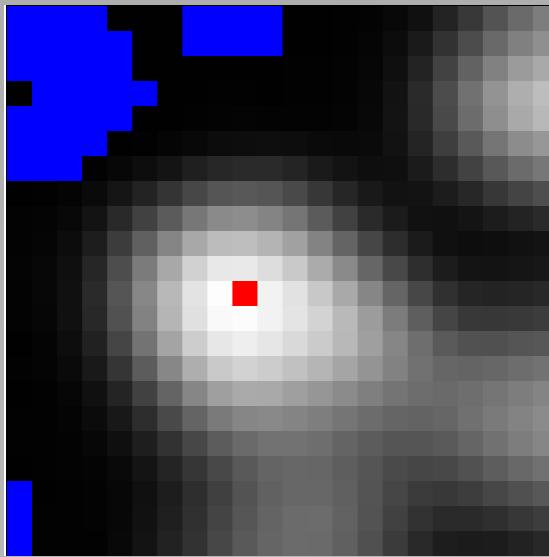


Noise Reduction: Mean

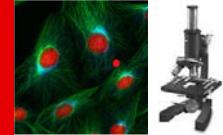


$$\begin{array}{|c|c|c|} \hline \frac{1}{9} & \frac{1}{9} & \frac{1}{9} \\ \hline \frac{1}{9} & \frac{1}{9} & \frac{1}{9} \\ \hline \frac{1}{9} & \frac{1}{9} & \frac{1}{9} \\ \hline \end{array}$$

mean



Mean 1pt



Noise Reduction: Gaussian

Filtering with a gaussian
bell-shaped kernel:

10	25	3
9	33	5
4	6	8

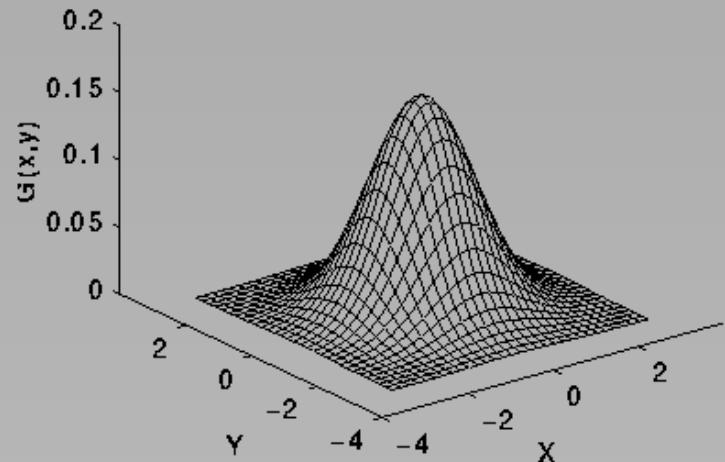
$$\frac{1}{16}$$

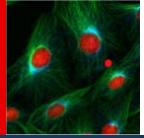
1	2	1
2	4	2
1	2	1



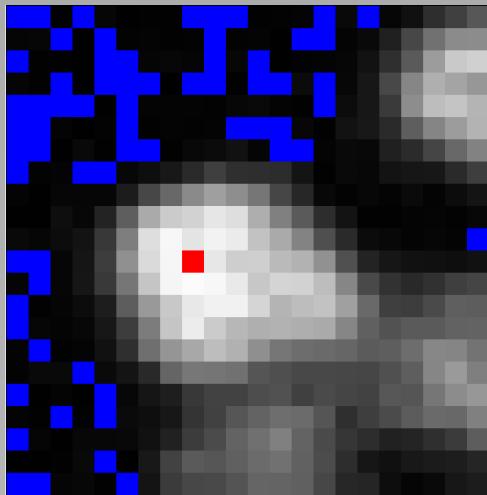
$$\frac{1}{16}$$

10	50	3
18	132	10
4	12	8

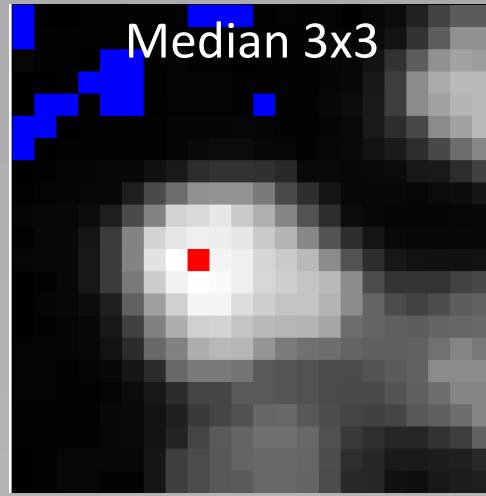




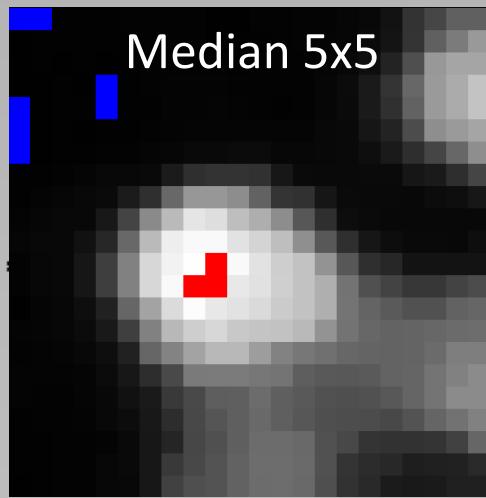
Noise Reduction: Median



median



Median 3x3



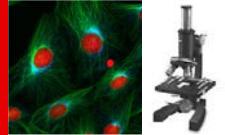
Median 5x5

123	125	126	130	140
122	124	126	127	135
118	120	150	125	134
119	115	119	123	133
111	116	110	120	130

Neighbourhood values:

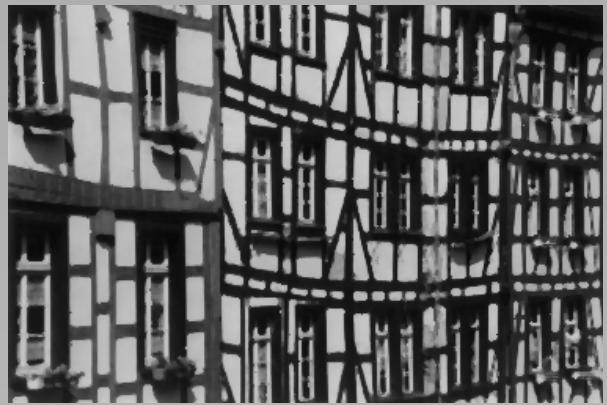
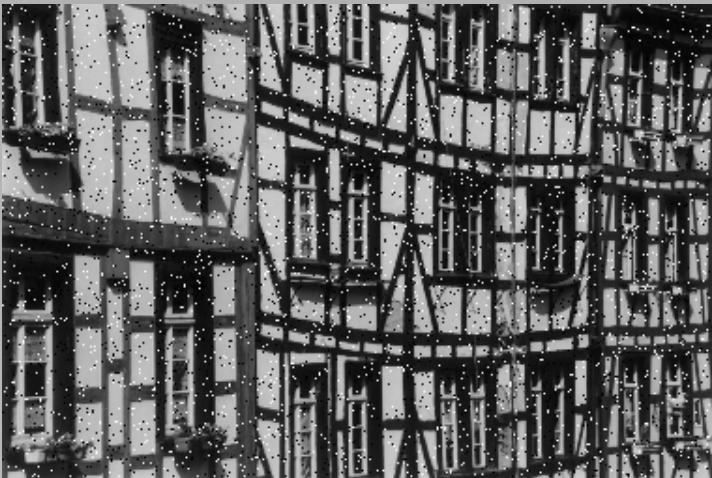
115, 119, 120, 123, 124,
125, 126, 127, 150

Median value: 124



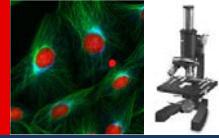
Noise Reduction: Median, Mean

Median, 1pt



Mean, 1pt

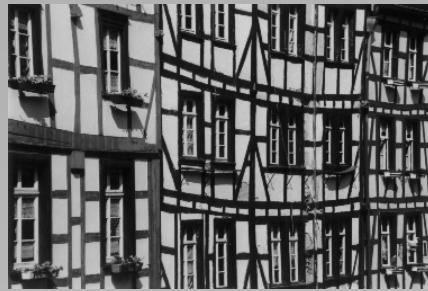




Median-, Mean-, Max-, Min-Filter



Median, 5pt



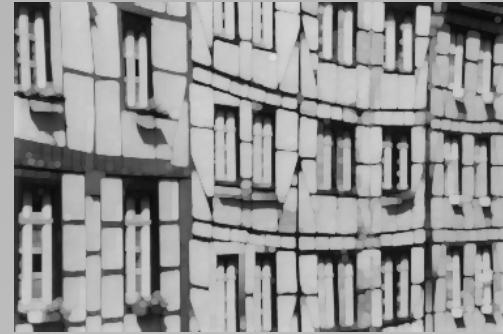
Mean, 5pt

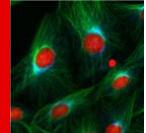


Min, 2pt



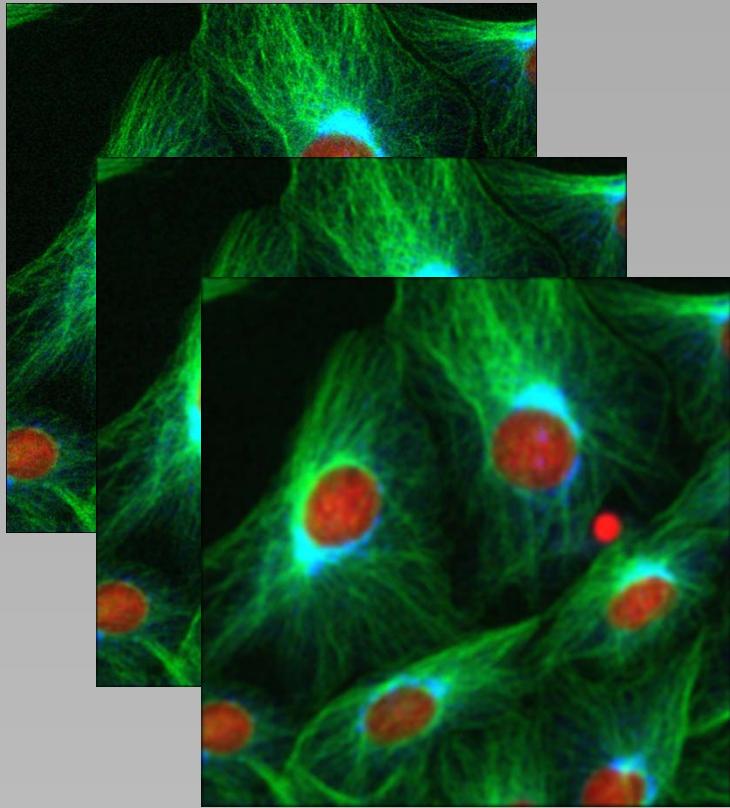
Max, 2pt



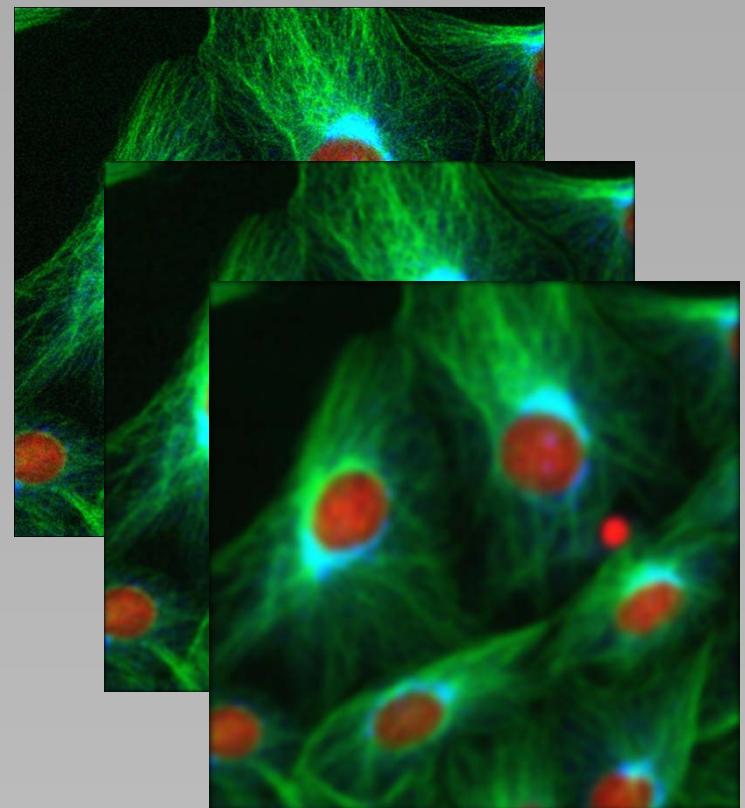


Biolmaging & Optics Platform

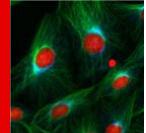
Mean-, Gauss-Filter



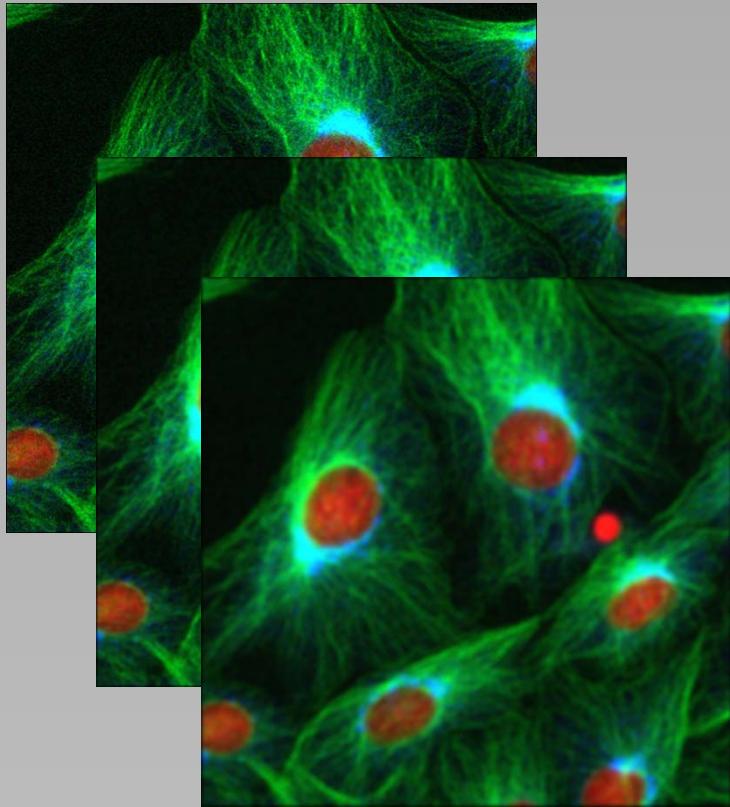
Mean, 2pt, 4 pt



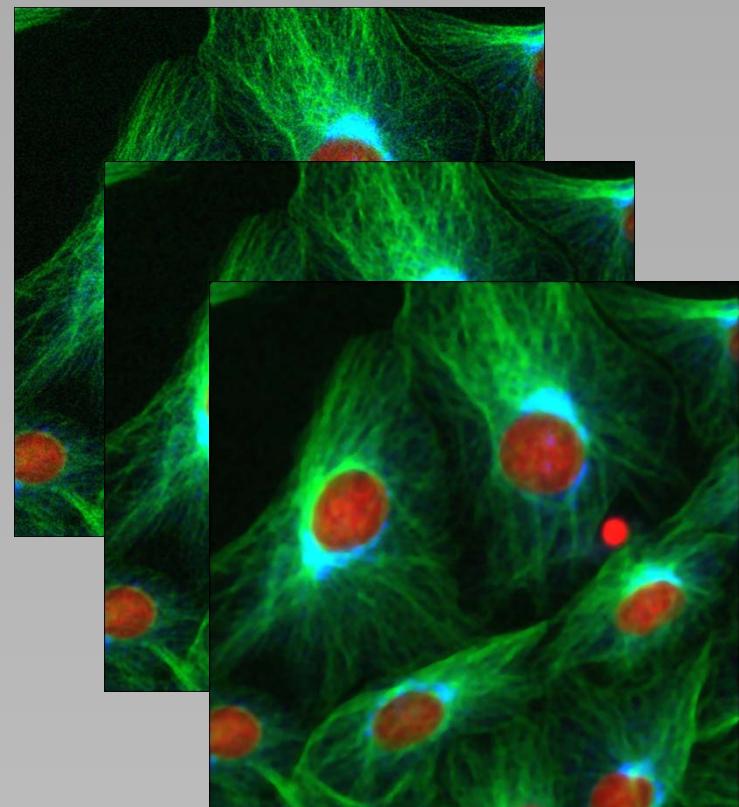
Gauss, 2pt, 4 pt



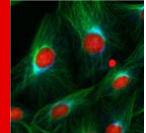
Mean-, Median-Filter



Mean, 2pt, 4 pt

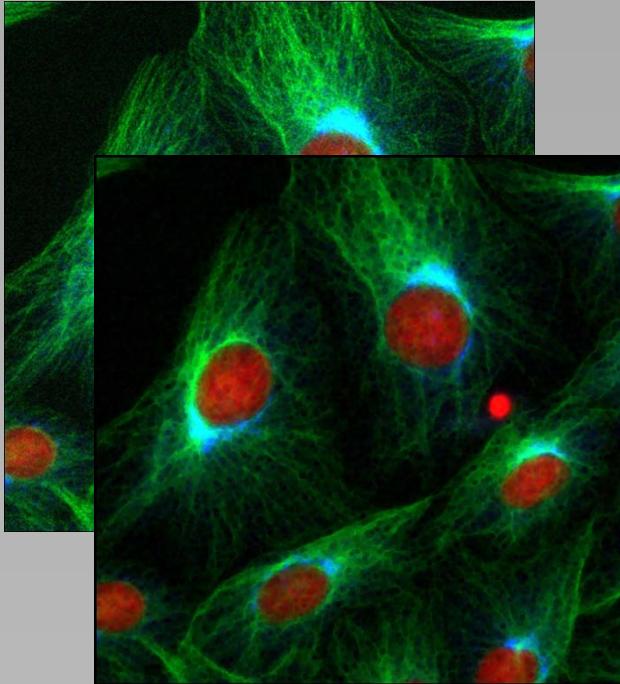


Median, 2pt, 4 pt

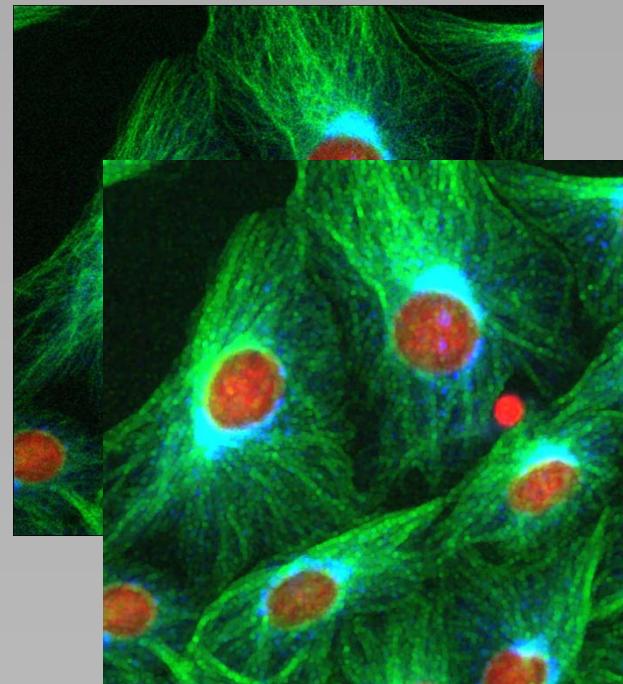


Biolmaging & Optics Platform

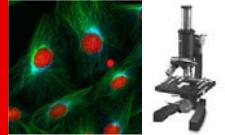
Min-, Max-Filter



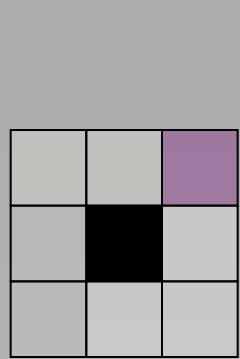
Min, 2pt



Max, 2pt

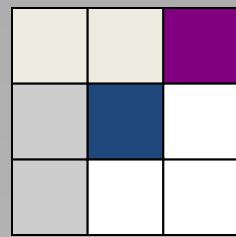


Sharpen / Blur



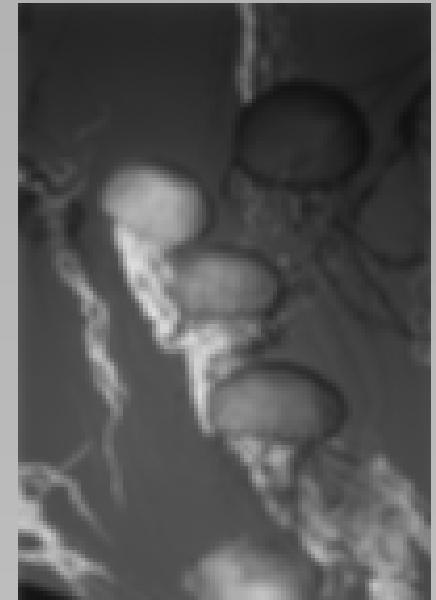
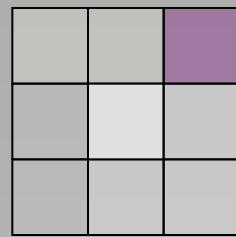
$$\begin{matrix} -1 & -1 & -1 \\ -1 & 9 & -1 \\ -1 & -1 & -1 \end{matrix}$$

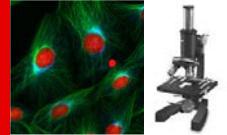
← sharpen



$$\begin{matrix} 1 & 1 & 1 \\ 1 & 2 & 1 \\ 1 & 1 & 1 \end{matrix}$$

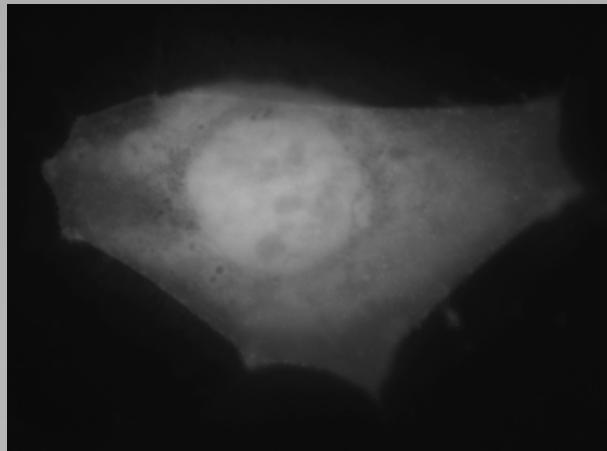
→ blurring





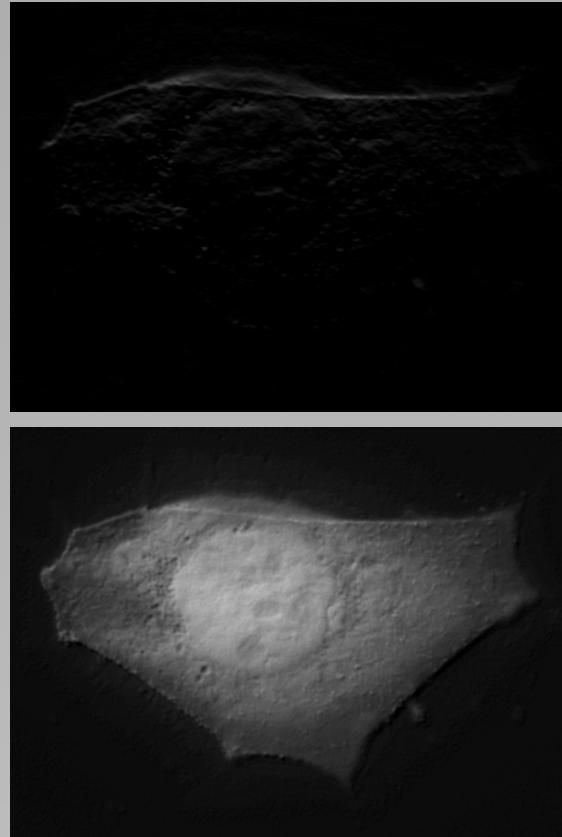
Biomaging & Optics Platform

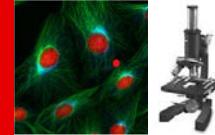
Example: Edge-Finding with derivatives



$$\begin{array}{rrr} -1 & -1 & -1 \\ 0 & 0 & 0 \\ 1 & 1 & 1 \end{array}$$

$$\begin{array}{rrr} -1 & -1 & -1 \\ 0 & 1 & 0 \\ 1 & 1 & 1 \end{array}$$





Background Subtraction

Even background:

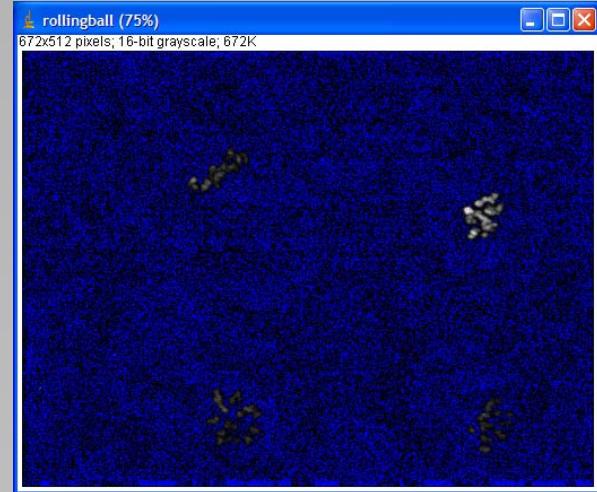
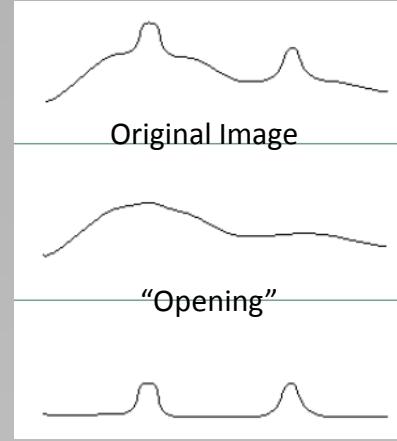
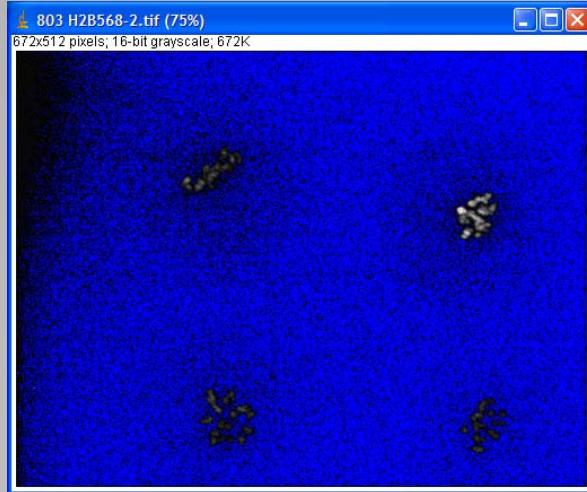
- subtract average background from image

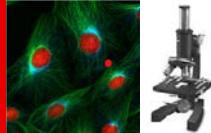
Subtract “background image”

(same exposure time without illumination)

Uneven background: Rolling ball filter

- Use kernel larger than diameter of largest object

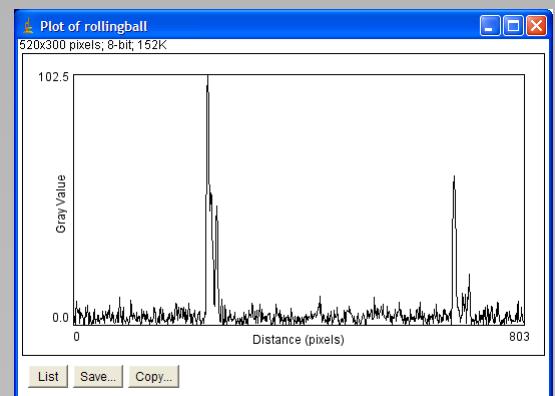
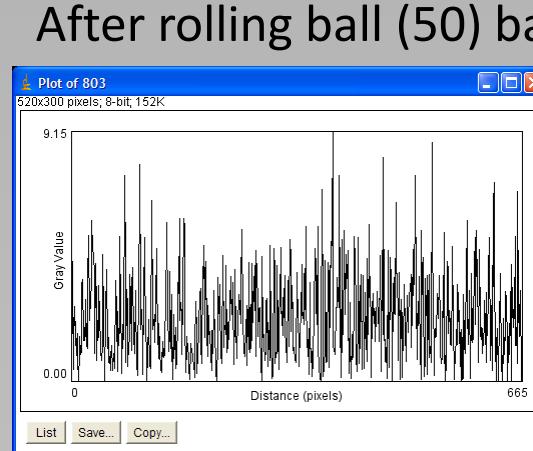
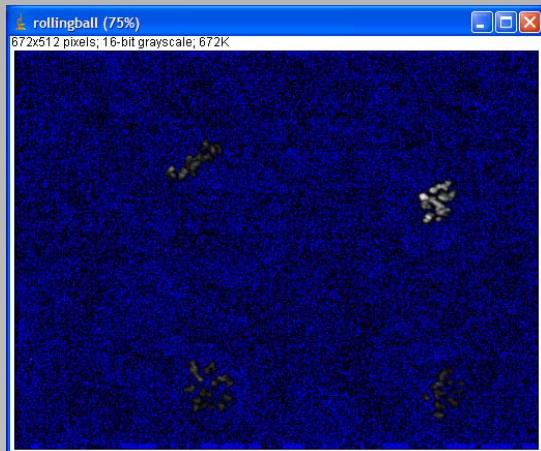
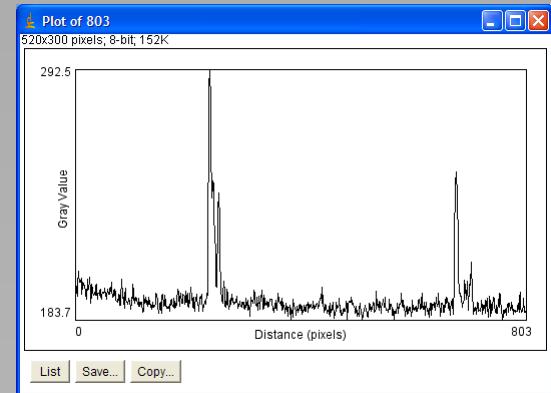
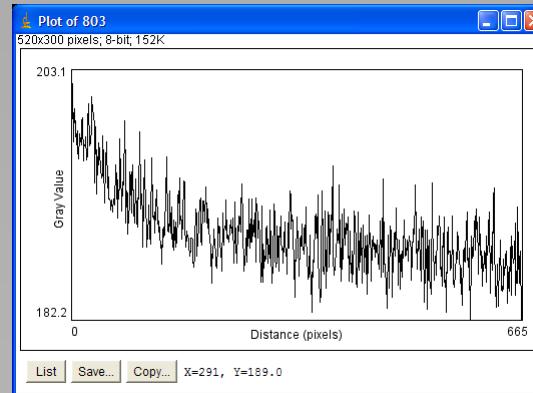
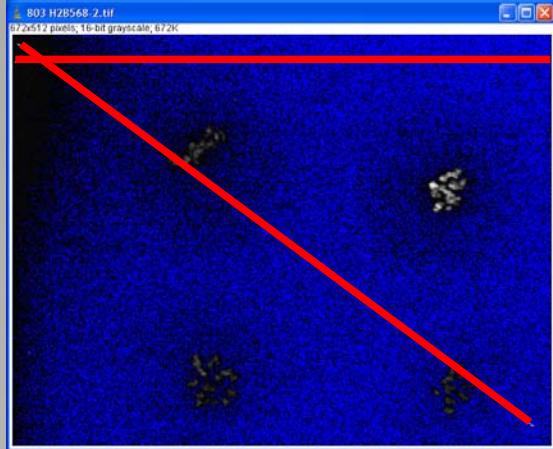




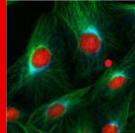
Biolimaging & Optics Platform

Line Profile

Without background subtraction



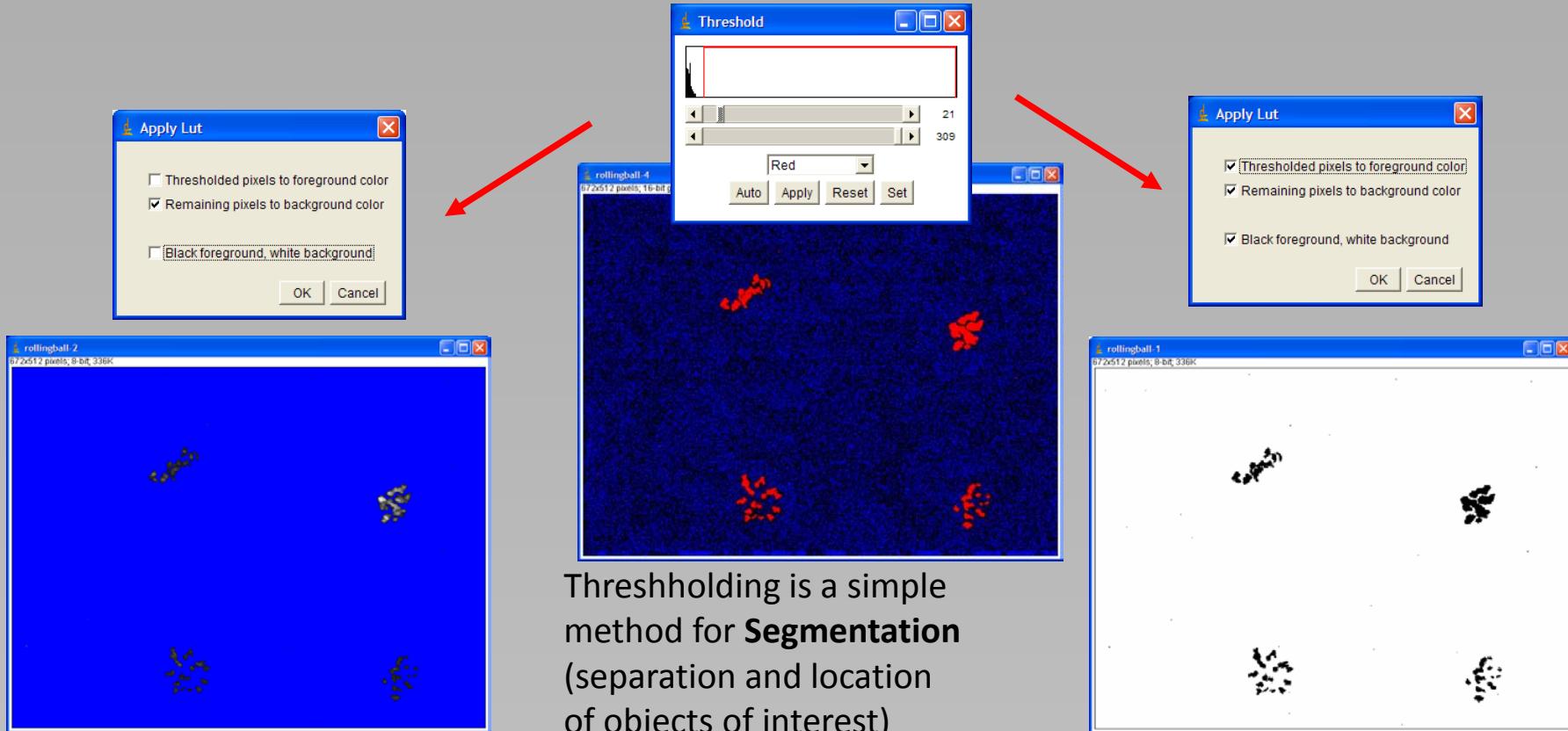
After rolling ball (50) background subtraction



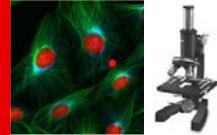
Biolmaging & Optics Platform

Thresholding

Thresholding is used to change pixel values above or below a certain intensity value (threshold):

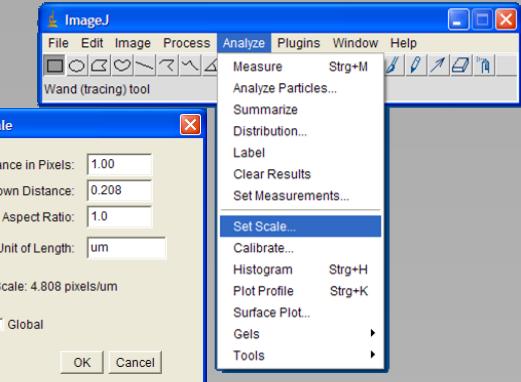
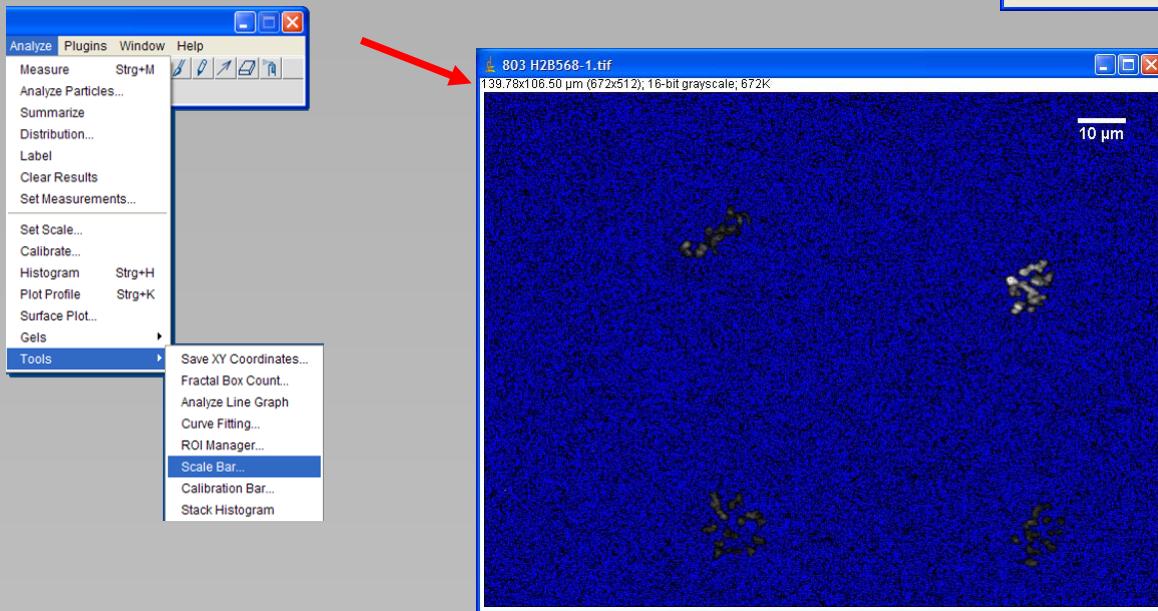


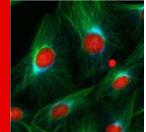
Thresholding is a simple method for **Segmentation** (separation and location of objects of interest)



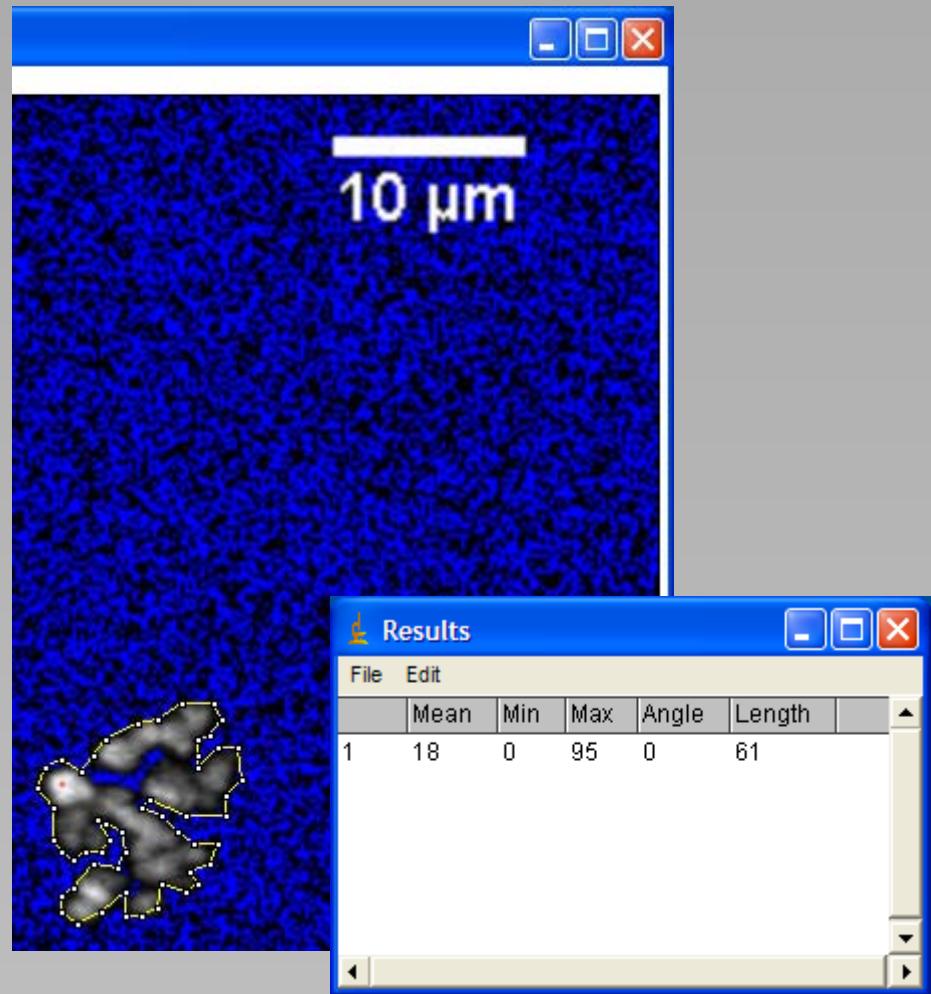
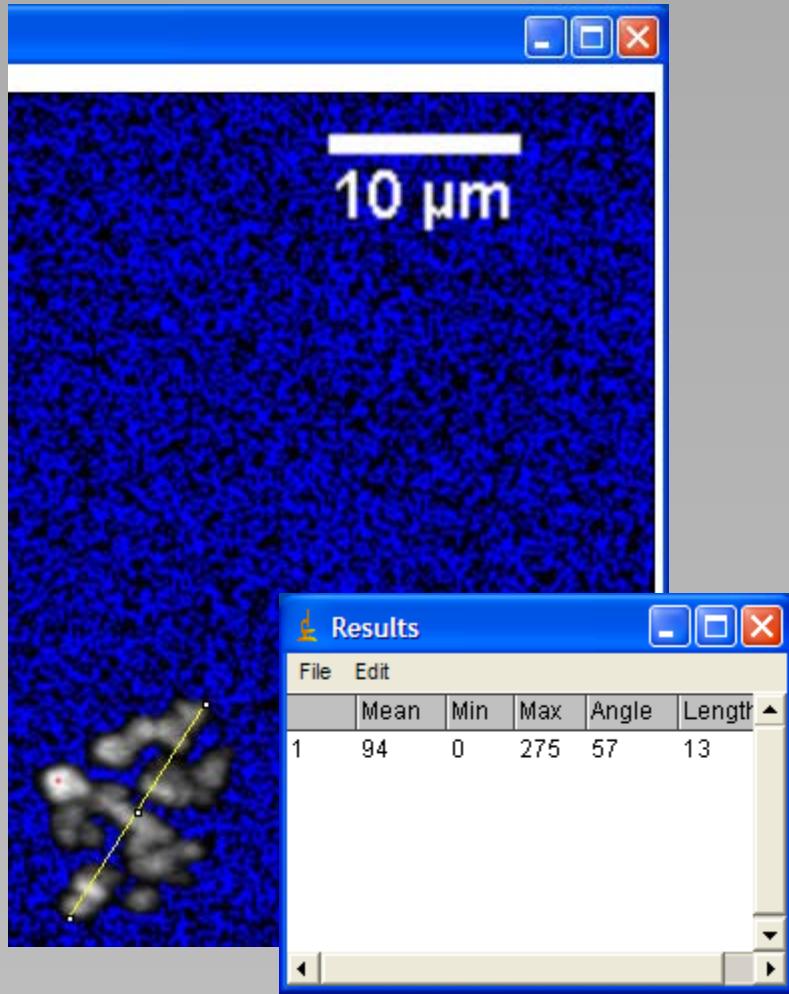
Measuring Sizes

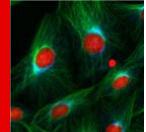
Set Scale with pixel (voxel) size
Include Scalebar



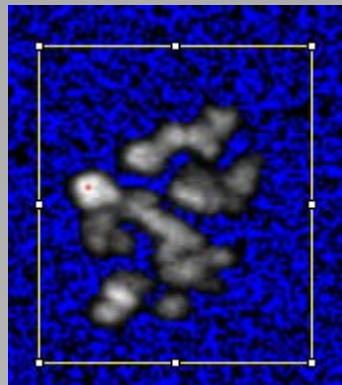


Measuring Length

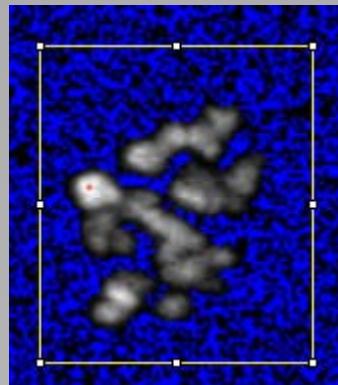




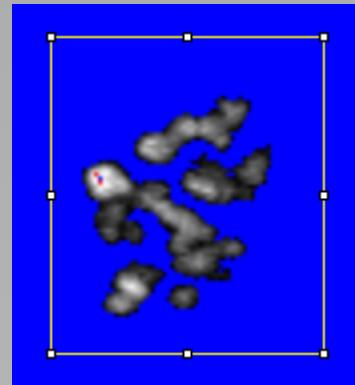
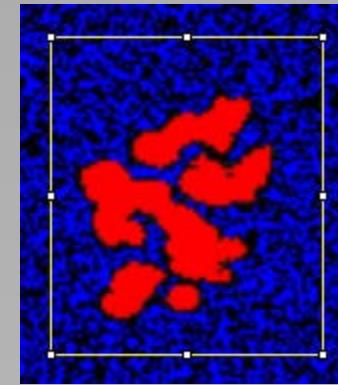
Area Measurement



16bit image

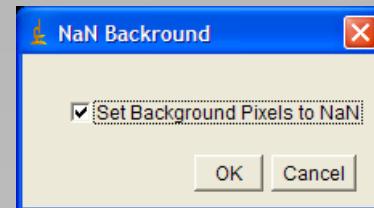


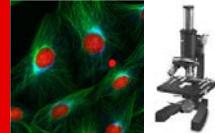
32bit image

32bit image,
background thresholded
to “Not a Number”16bit image,
same threshold
as in 32bit image
but not applied

Results

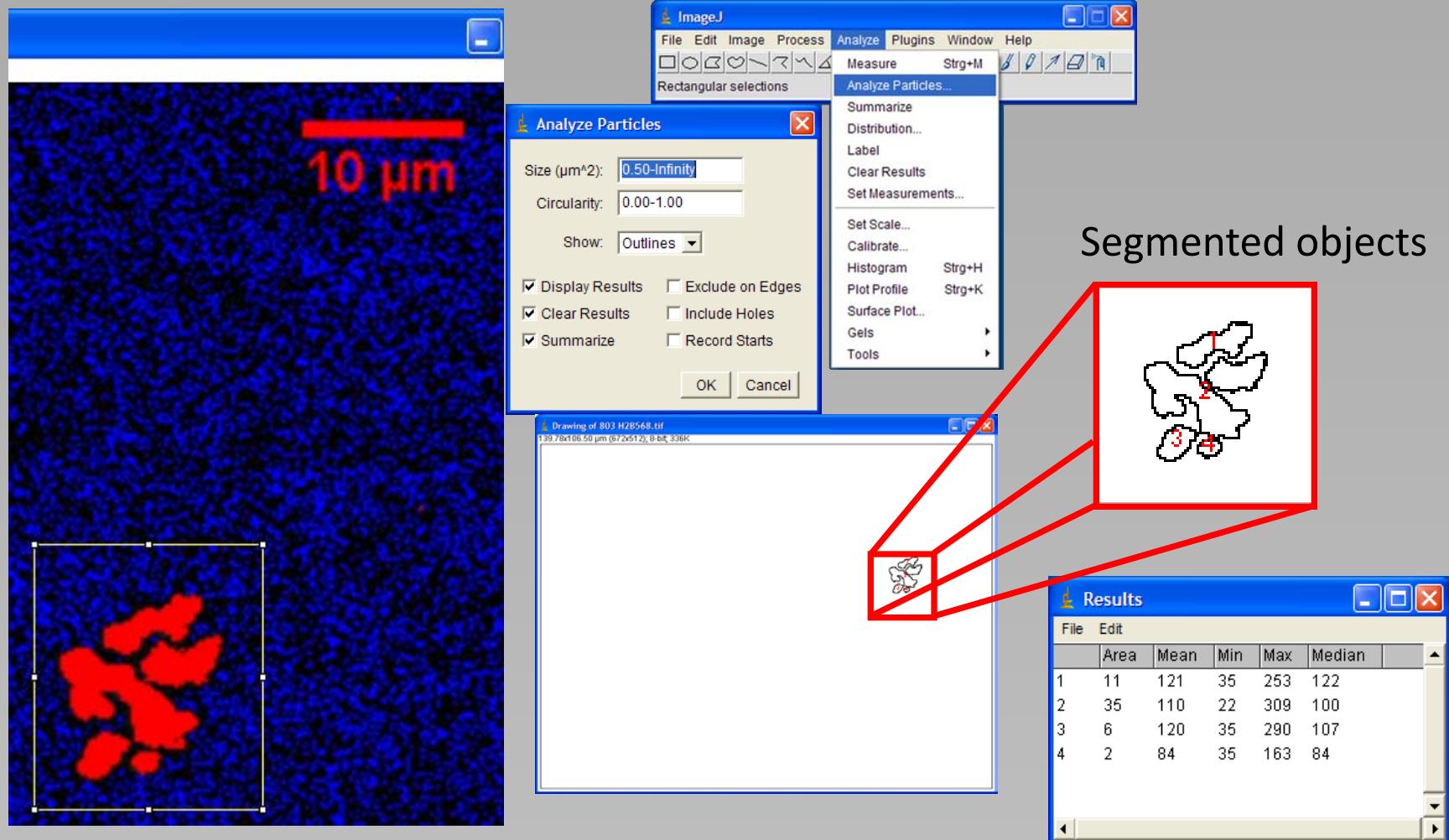
	Area	Mean	Min	Max	Median
1	232	26	0	298	3
2	232	26	0	298	3
3	50	109	35	292	101
4	50	109	35	292	101

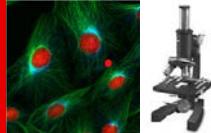




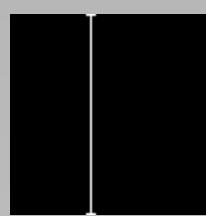
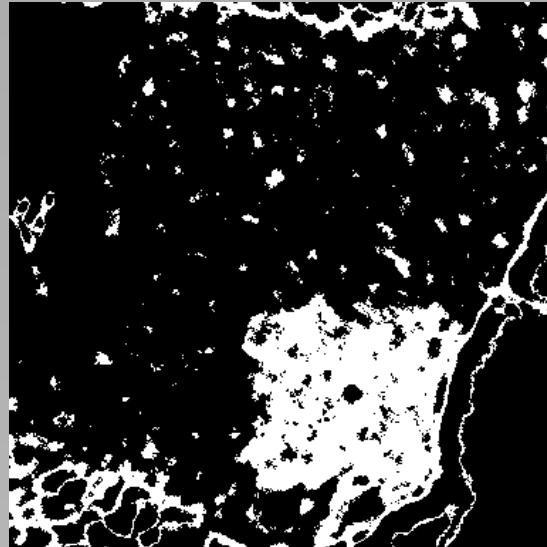
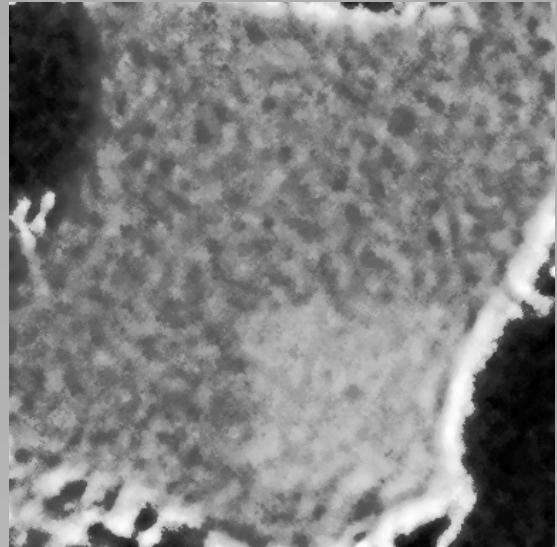
Biomaging & Optics Platform

Analyze Particles

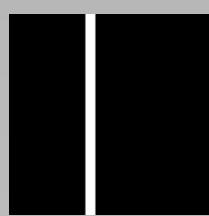




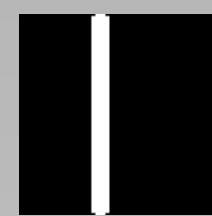
Threshold and Opening/Closing



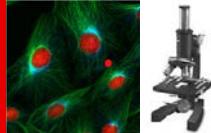
dilate



erode

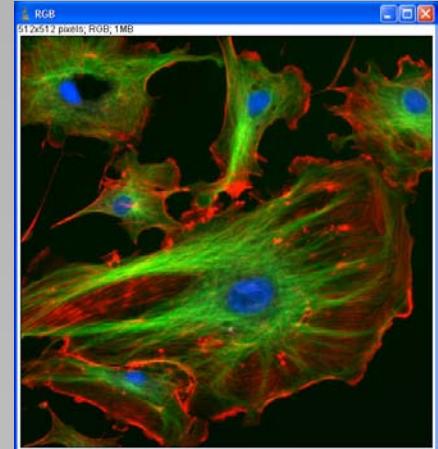
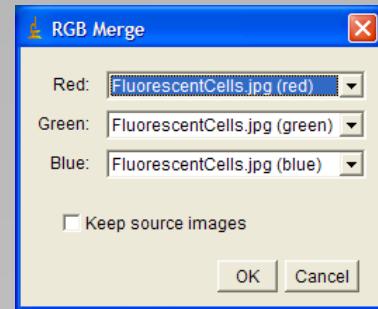
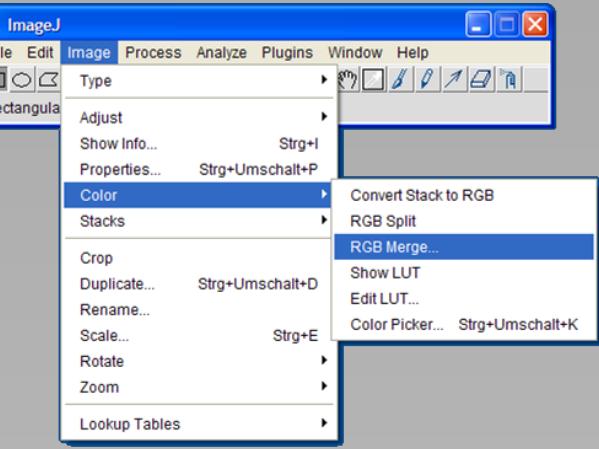
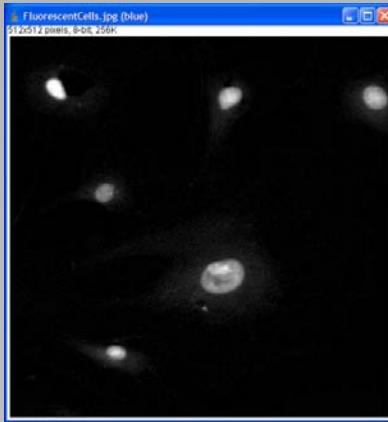
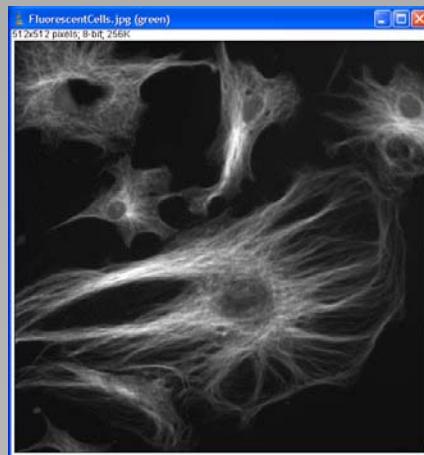
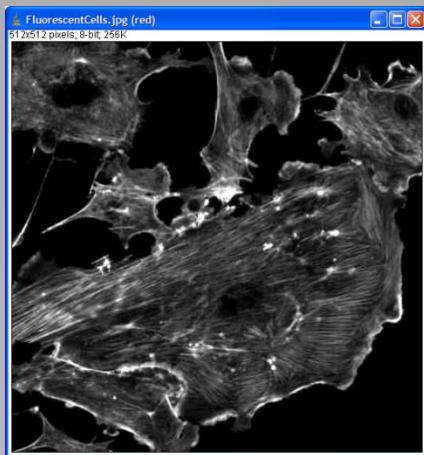


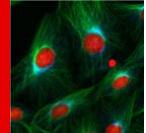
Closing: Dilate/Erode
Opening: Erode/Dilate



Color Functions

RGB Merge /RGB Split





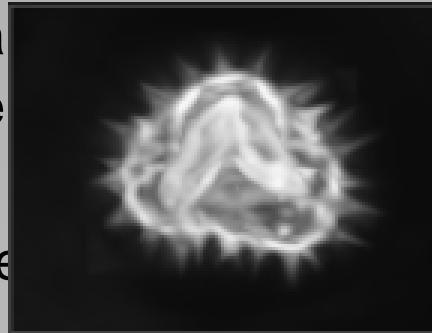
Deconvolution

From Object to Image

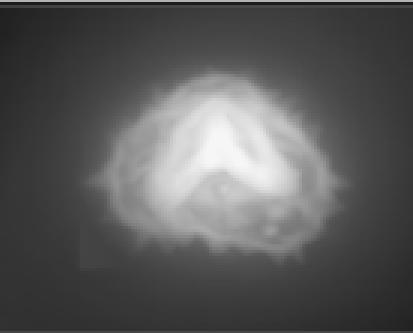
Effects causing Image degradation:

Noise

- Signal
- Noise



Image



Scatter

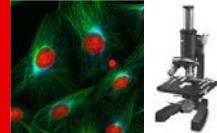
- Causes

digital
refra

Glare

- Random disturbance of light in the system

Blur

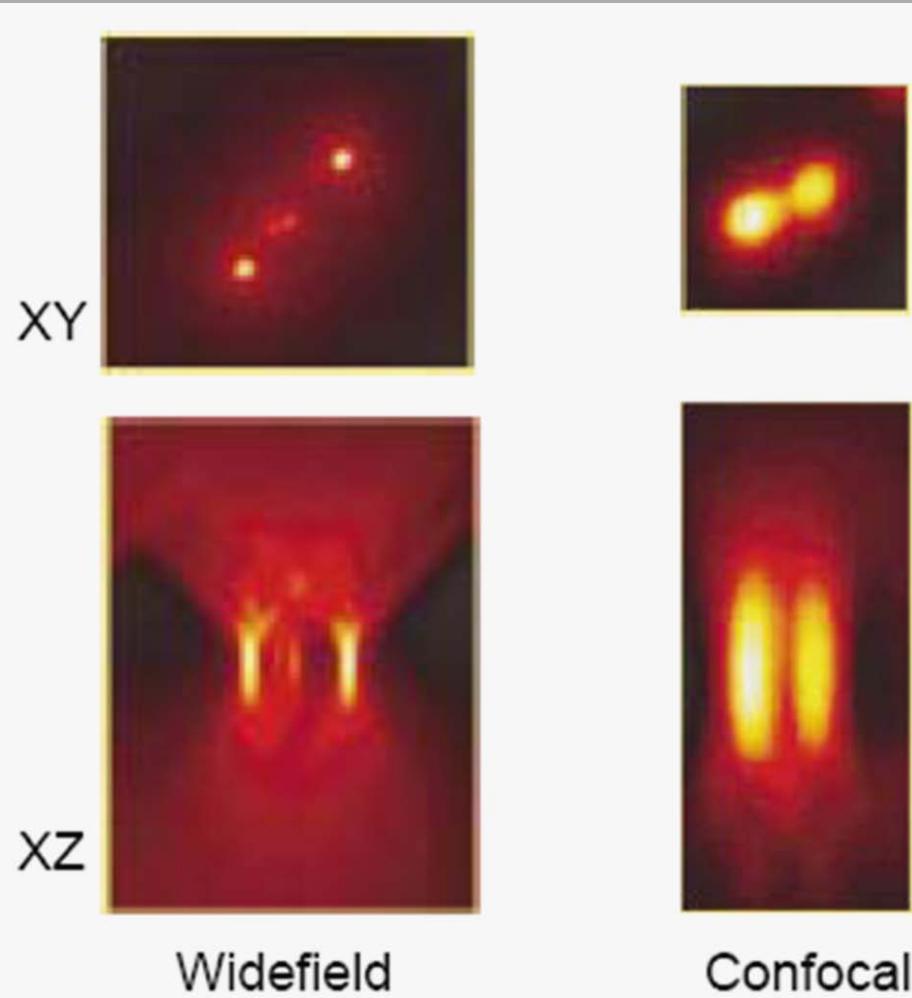


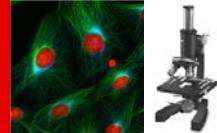
Point Spread Function (PSF)

A Point Spread Function is
the **3D diffraction pattern**
of a “point” source of light.

Widefield = hourglass shape

Confocal = American Football
shape





Convolution of an Object

Object can be referred as
accumulation of points

Each point is visible as a **PSF**

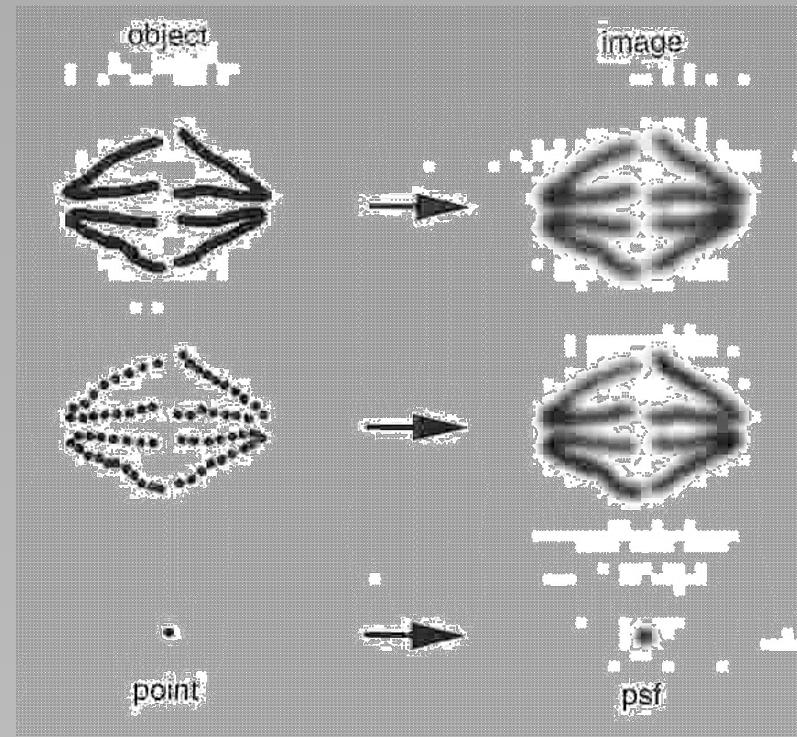
$$\text{Object} \otimes \text{PSF} = \text{Image}$$

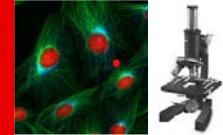
\otimes = convolution

Image process hast to be

- **Linear**
- **Shift invariant**

Convolution is in principle a reversible **mathematical equation**





Constrained Iterative

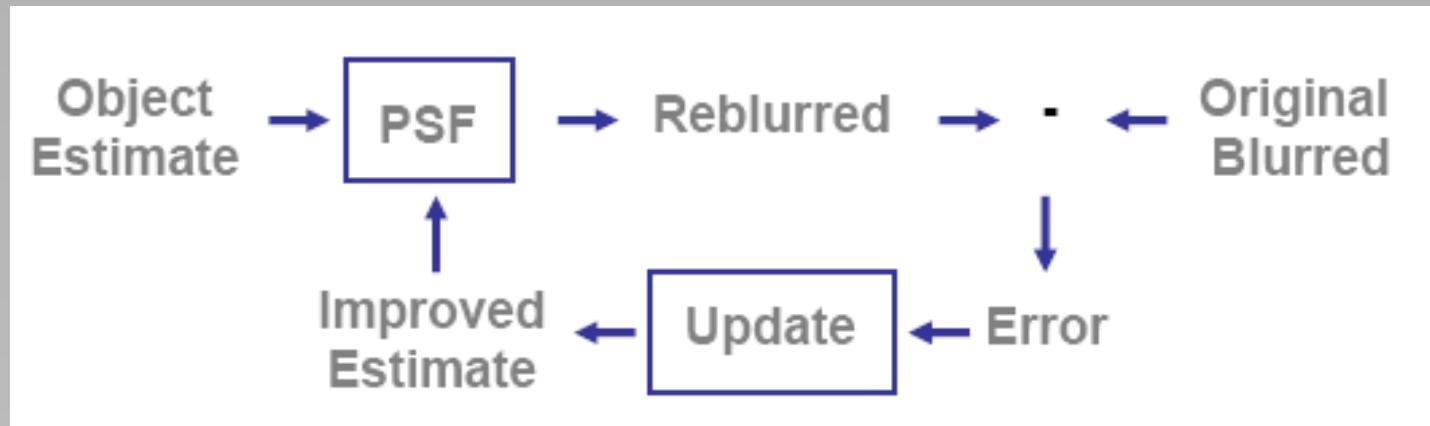
Constrained:

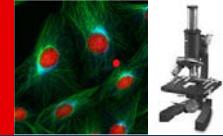
→ “**Nonnegativity**”

→ **Smoothing or regularization** to suppress noise amplification

Iterative:

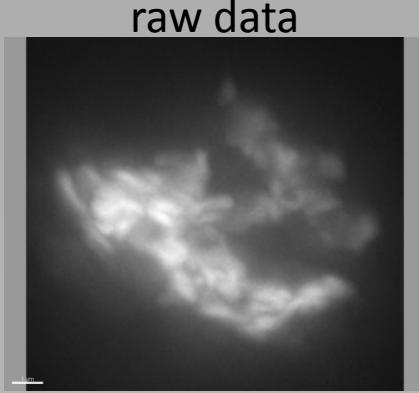
Best estimate is found in a successional serial of calculations.



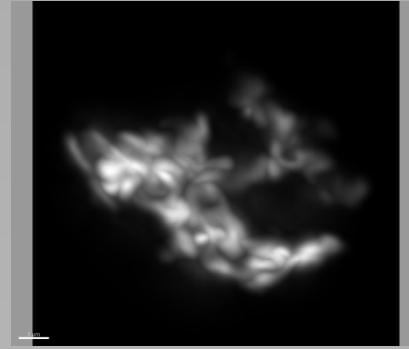


Different Algorithms...

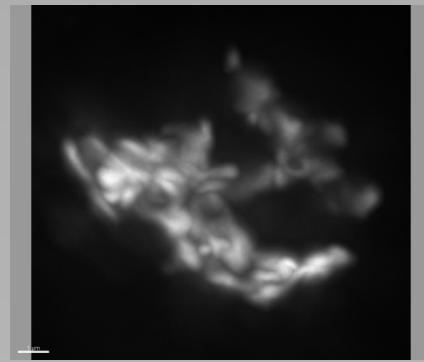
...lead to different Results



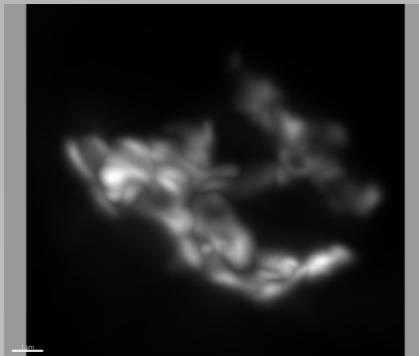
AutoQuant: non blind 15 It



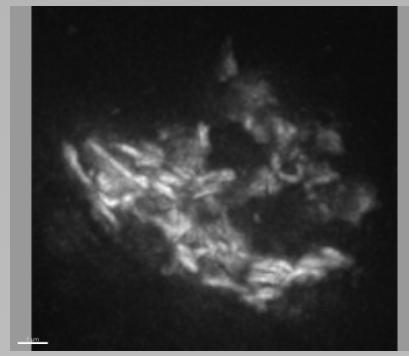
AutoQuant: Blind 15 It



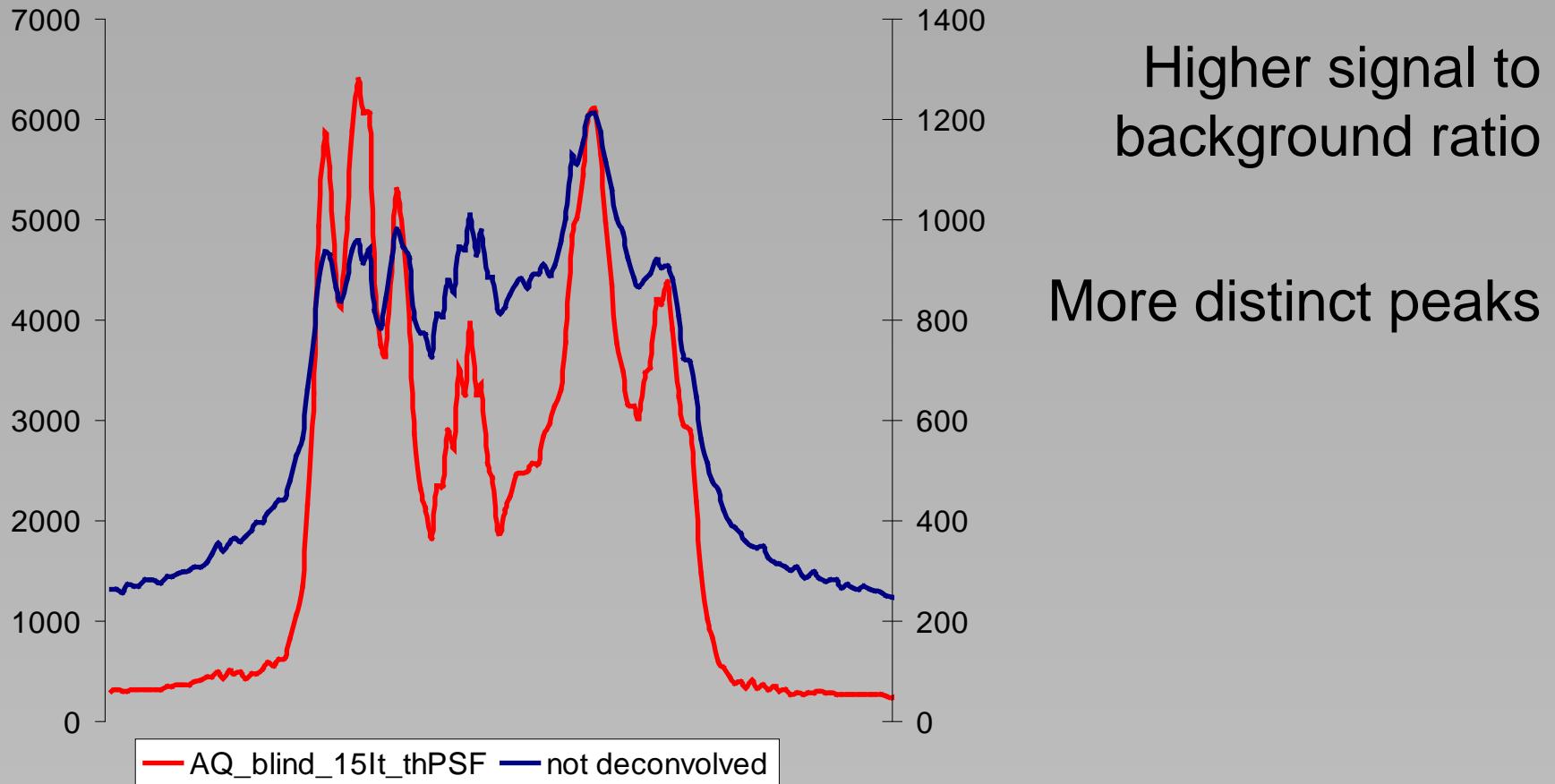
Huygens: CMLE 30 It



SoftWorx: 30 It



Signal improvement





WF Deconvolution

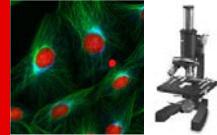
Computational subtraction of blur
or reassignment to the assumed source

Advantages:

- Good **light efficiency** (esp. with reassignment)
- CCD instead of PMT (**high Quantum efficiency**)
- **Fast** stack recording possible → low **bleaching**

Disadvantages:

- Need for **high computational systems**
- **Artefacts** can not be excluded

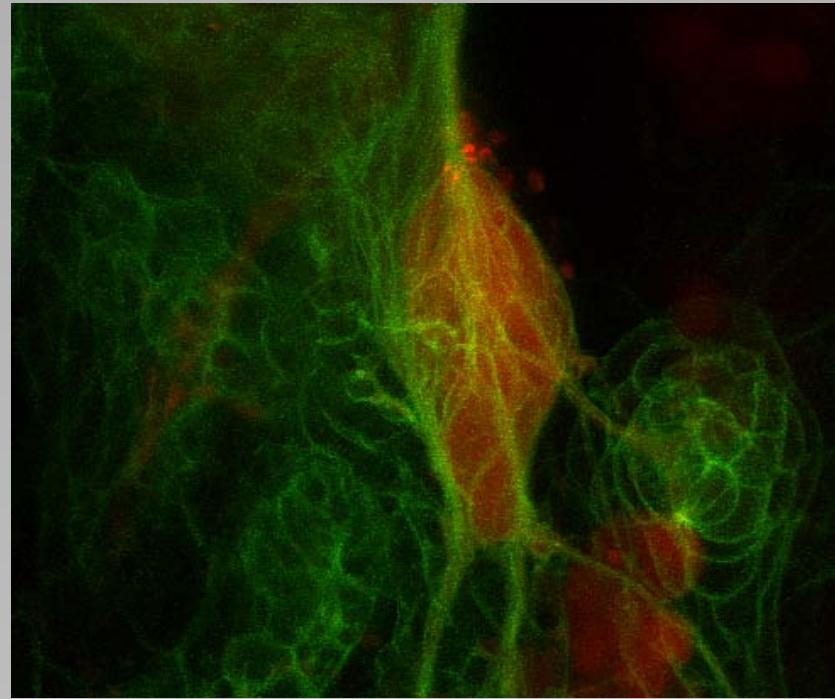


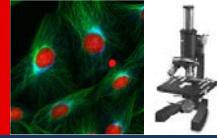
WF Decon vs. Confocal

To deconvolve or not to deconvolve

That is **not** the question:

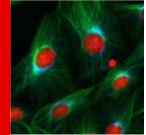
→ WF + Deconvolution is no real alternative to Confocal pictures as they can also be deconvolved





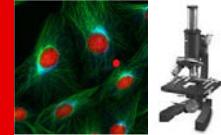
Conclusions

- Keep environment constant and convenient
- Use powerful dyes
- Think about required resolution
(x, y, z, t, brightness, channel number) to minimize photostress
- Use appropriate microscopy method



Summary

- Use lossless file formats for archiving important data
- Image processing is an important step in generating (optimal) results
- Only use documented image processing steps/routines



More about image processing

1. Lecture

M. Unser, EPFL

see also website: <http://bigwww.epfl.ch/>

2. Books

a) W. Burger, M. J. Burge

Digital Image Processing, Springer 2008

b) J. C. Russ

The image processing Handbook, CRC Press 2007

3. PT-BIOP

EPFL, SV-AI 0241, SV-AI 0140

<http://biop.epfl.ch/>