DIGS-BB Dresden International Graduate School for Biomedicine and Bioengineering
"From Nanobiotechnology to Regenerative Medicine"

MPI-CBG LMF / IPF Basics of Quantitative Imaging and Image Processing

Using ImageJ / Fiji

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Nov 2008

CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics

IPF

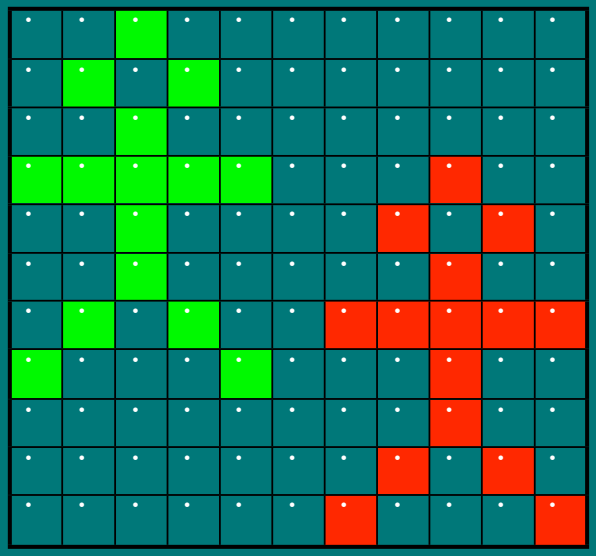
Before you start writing...

Presentations soon available at:

http://tu-dresden.de/med/ifn

Session 1:
Quantitative Imaging?
…what does that mean?

- Art or Science? Photography or Spectroscopy?
- Science = measure something!
  - Numerical Results
  - Statistics!
  - Computers become useful!



What is an Image?

- An image NOT reality - it is an artifact!
  - Image of a point is not a point (Point Spread Function)
    - Deconvolution?
  - Digital - a series of pixels / voxels with a value,
  - NOT Analogue art!

Digitised image of "something"

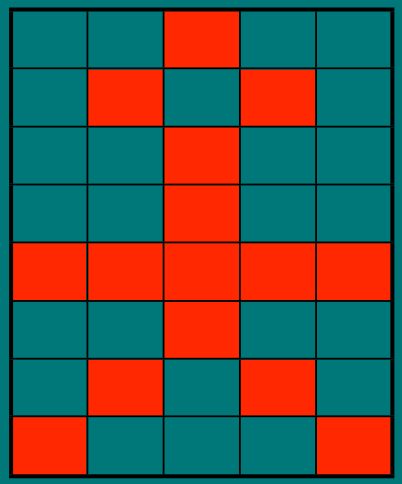


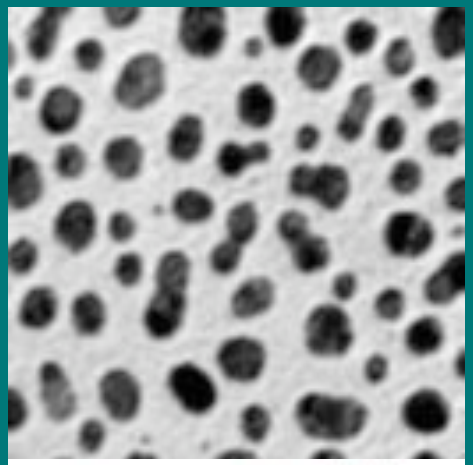
Image Analysis

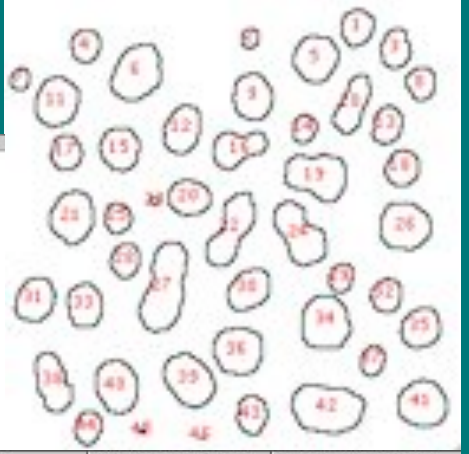
A stick man?
How do I know?
How can computer know?

Image = Information

Images contain information!

- Quantify / Measure / Analyse
- Manipulate Image = Changed Info (Danger)
- Lost Info = Lost Forever!
- Meta data (What, Where, When, How)
- Noise / Background



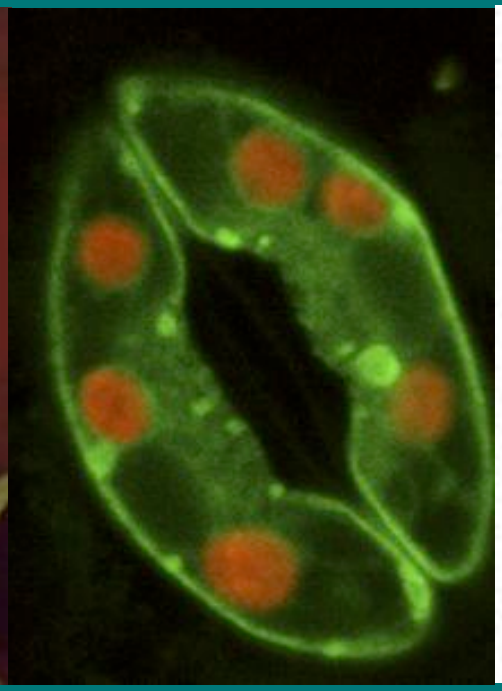


	Area	Mean	StdDev	Min	Max	IntDen	Median	XStart	YStart
1	285	255	0	255	255	72675	255	197	6
2	81	255	0	255	255	20655	255	136	17
3	278	255	0	255	255	70890	255	218	17
4	231	255	0	255	255	58905	255	42	18
5	501	255	0	255	255	127755	255	170	21
6	660	255	0	255	255	168300	255	75	26
7	99	255	0	255	255	25245	255	7	39
8	228	255	0	255	255	58140	255	231	39
9	448	255	0	255	255	114240	255	137	42
10	401	255	0	255	255	102255	255	198	43
11	520	255	0	255	255	132600	255	27	44
12	425	255	0	255	255	108375	255	99	60
13	271	255	0	255	255	69105	255	215	60
14	159	255	0	255	255	40545	255	168	65
15	412	255	0	255	255	105060	255	60	73
16	426	255	0	255	255	108630	255	123	75
17	260	255	0	255	255	66300	255	31	77
18	289	255	0	255	255	73695	255	222	85
19	676	255	0	255	255	172380	255	178	87

Slice	Count	Total Area	Average Size	Area Fraction
blobs.gif	46	17686.000000	384.478261	27.2

Photographer or Spectroscopist?

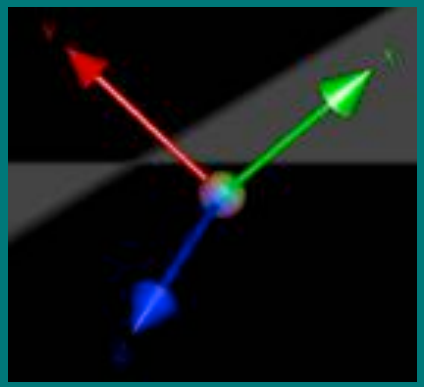
- Art or Science - You Choose
- Morphology can also be quantified!



249	244	240	230	209	233	227	251	255
248	245	210	93	81	120	97	193	254
250	170	133	94	137	120	104	145	253
241	116	118	107	134	138	96	92	163
277	142	121	113	124	115	107	71	179
234	106	84	125	97	108	125	106	204
241	202	102	132	75	73	141	246	252
253	252	244	239	178	199	242	250	245
255	249	244	250	226	231	240	251	253

What can you digitise?

Dimensions!

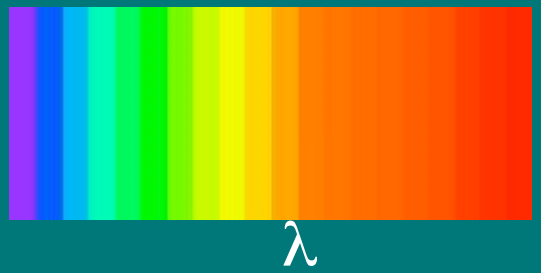


SPACE

INTENSITY

TIME

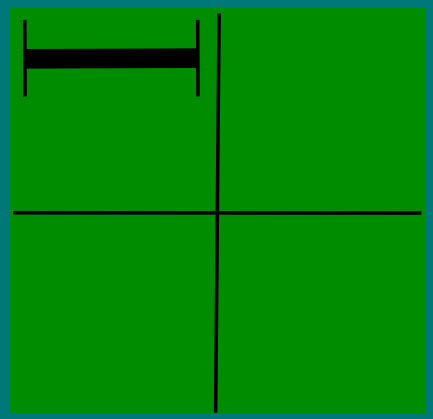
Colour Channels Wavelength



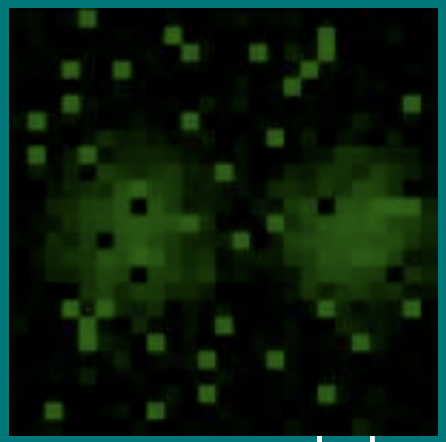
Pixel Size / Resolution

- Remember !!!
  - Nyqvist told us how to do digital sampling: 2.3x smallest feature.

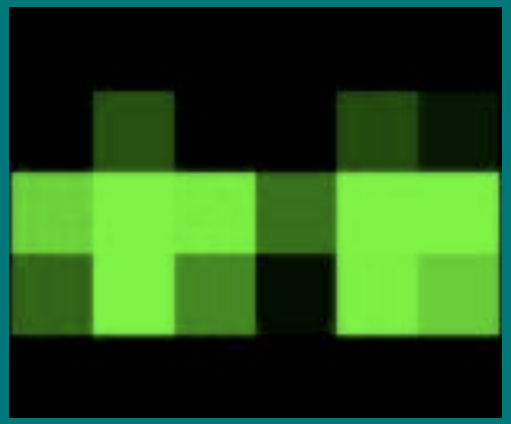
I Airy unit



under sampled



over sampled



correct sampling

Remember - Bit Depth

Measured intensity by detector

digitization

Corresponding level in image

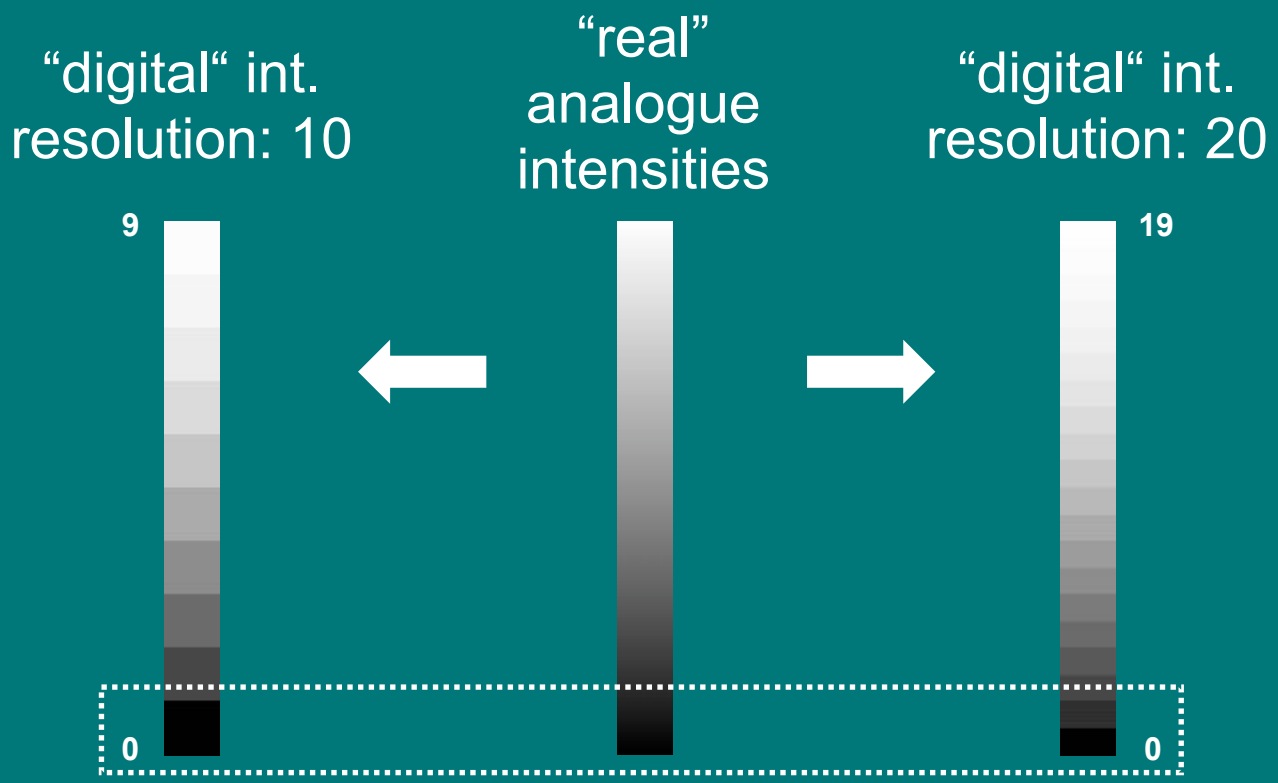
"Bucket" holds 10 electrons

5 electrons counted

Bit depth: 10 levels

Level 5 selected for RAW data "image"

Bit Depth


“digital“ int. resolution: 10
“real” analogue intensities
“digital“ int. resolution: 20
9
19
0
0

Bit Depth

1 bit	2^1	2
8 bit	2^8	256
12 bit	2^12	4096
14 bit	2^14	16384
16 bit	2^16	65536
...		

← segmentation

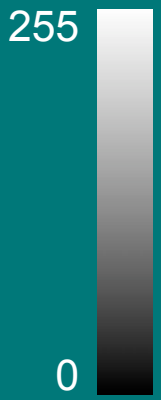
~ limit of human eye, displays...

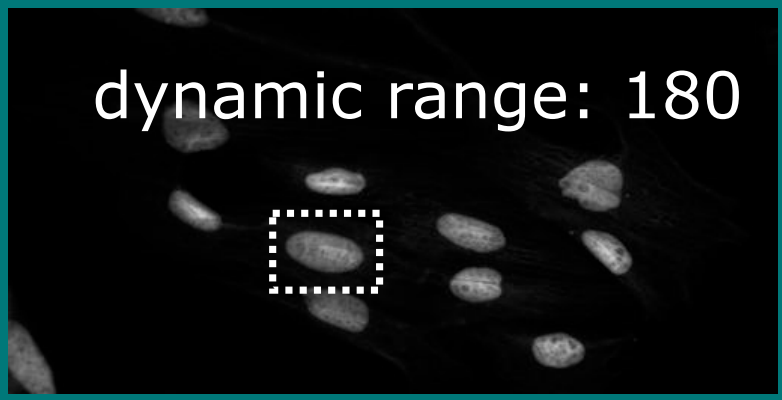
↓ Intensity-related measurements

Bit Depth

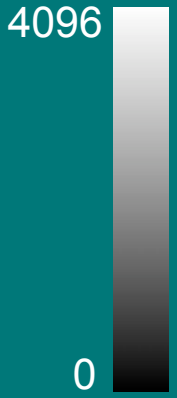
for intensity-related measurements

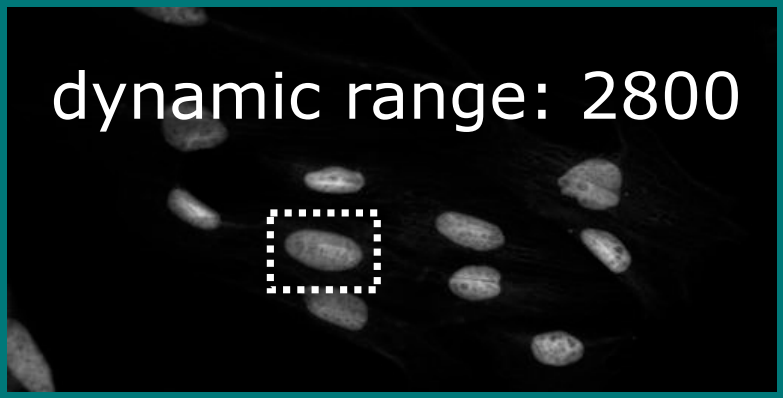
8 bit





12 bit

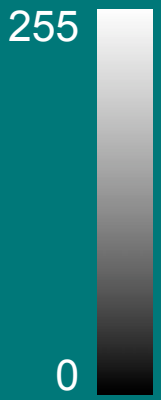


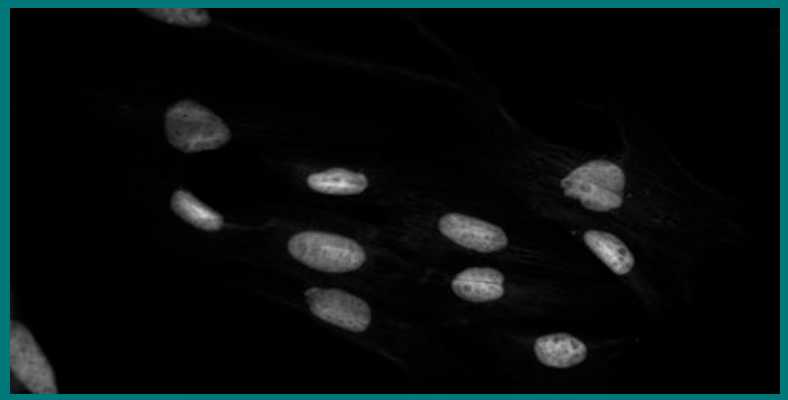


Bit Depth

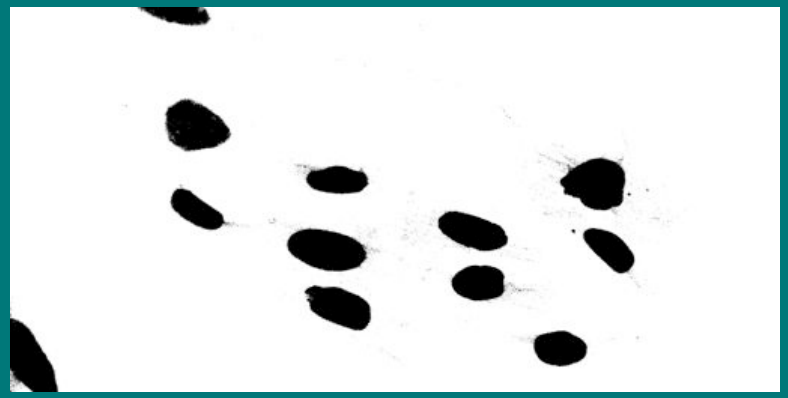
for segmentation

8 bit greyscale





1 bit binary image



Remember:
Intensity / Exposure / Saturation

- Don’t over expose / saturate your image data!
  - Why not? Lost Info!
  - Use look up tables / palettes

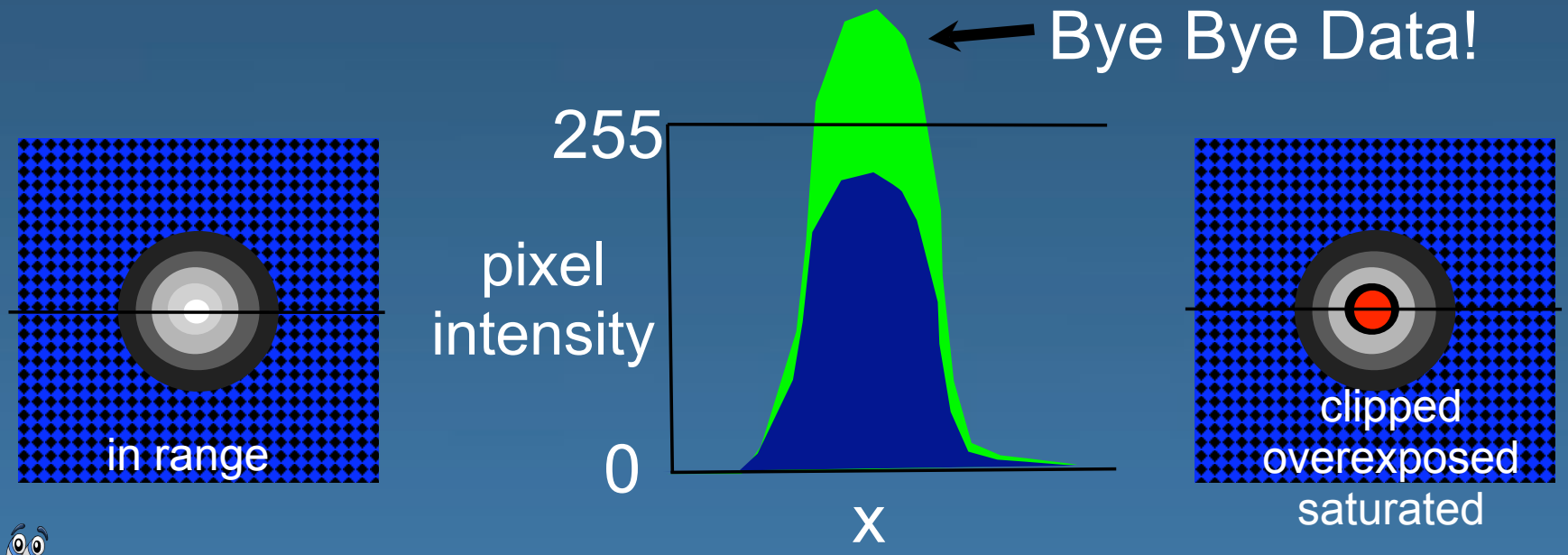
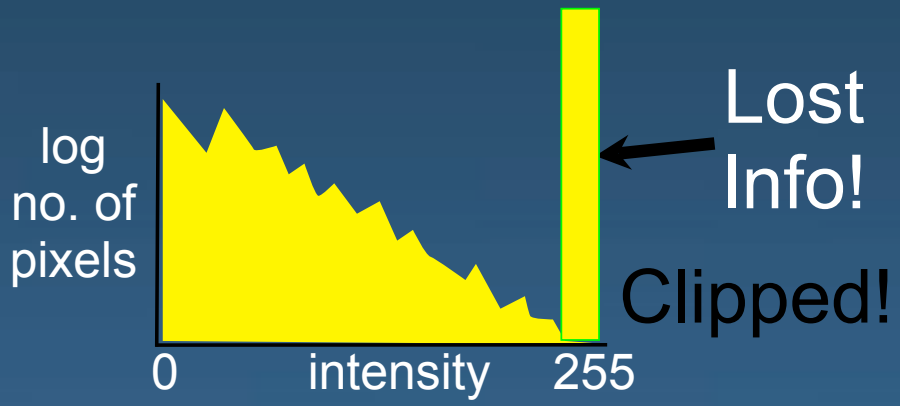
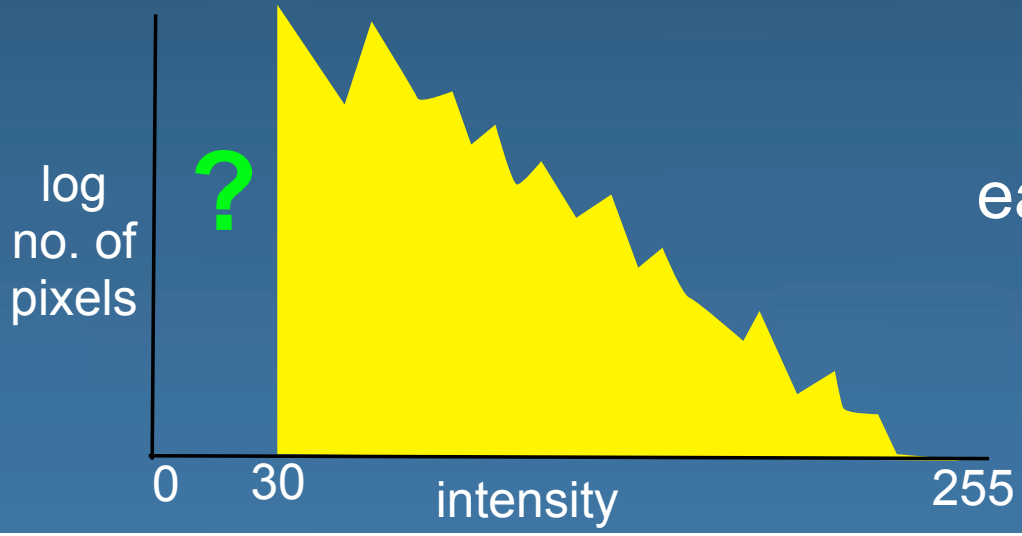


Image Intensity Histograms - Use them!

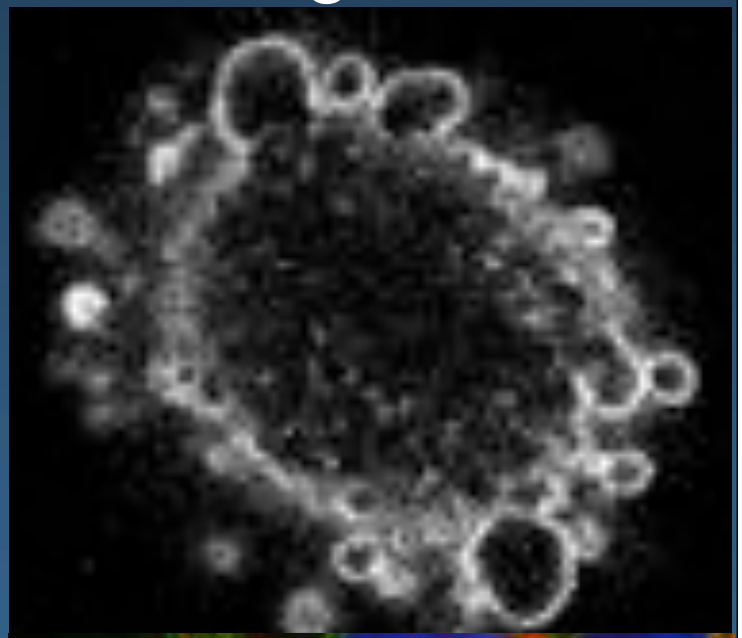


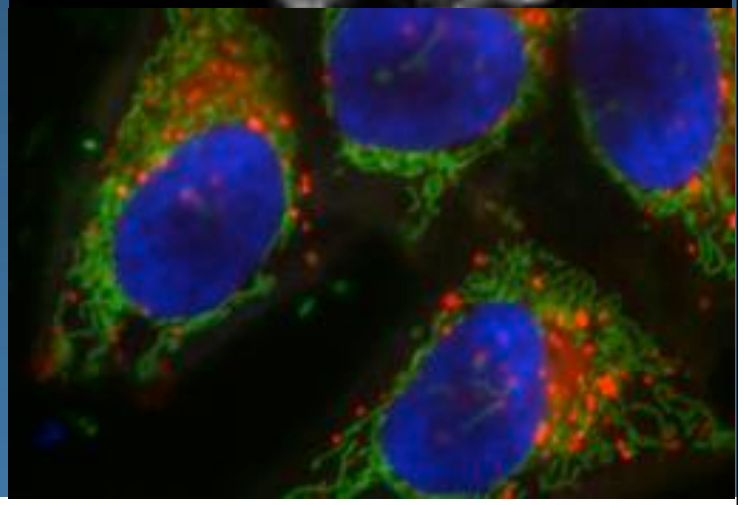


In Histograms:
easily see problems
for image
quantification!

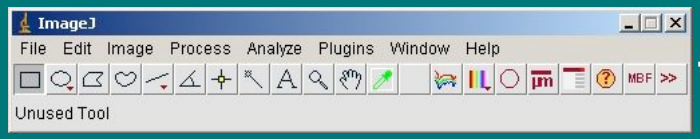
Imaging Experiment Planning:

- What **BIOLOGY** am I trying to measure?
- Do I need 3D, 4D, xD information?
- Resolution? Sampling space and time
- Choose appropriate microscope
  - Don't use confocal LSM just because it is the most expensive microscope.
- Optimise microscope system!
- Statistics!
  - How many images / data points / etc?
- **Controls!!!**





Practical Session 1



toolbar

Fiji is just ImageJ

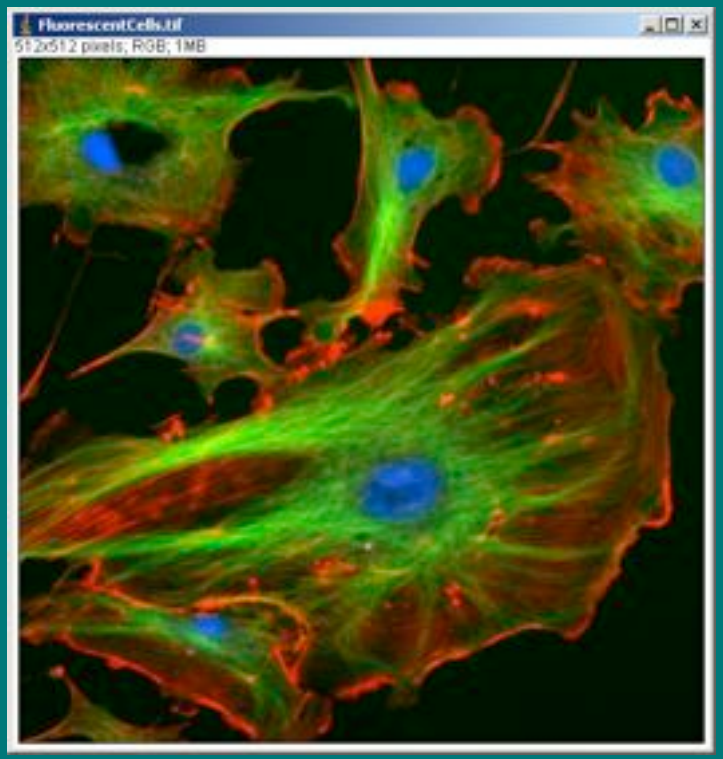
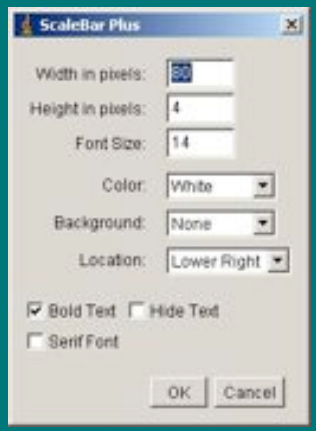


Image window



Settings window

Practical Session 1

- Getting to know “FIJI”
  - (Fiji is just ImageJ)
  - http://pacific.mpi-cbg.de
- Open Sample images - Neuron
- Image - Adjust - Brightness / Contrast (manual - auto)
- Simple measurements ctrl/apple M or Analyse - Measure
  - Cross hairs show position and value of pixels
  - Length
  - Area
  - Intensities etc
    - use: Analyse - Set Measurements

Image Processing

Session 2

- Images Contain “Information”
- Non image information = Meta Data
- Different ways to visualise / display info in images

Session 3

- Filtering images in the spatial, frequency and time domains
- Segmentation - finding and measuring objects in images

Session 2

- RGB Color Space
- Lookup Tables
- Line Profile
- Histogram
- Scatterplot
- Scaling

Practical Session 2

Break

Image Processing?!

255	255	255	255	255	255	255	255	255	255
255	255	255	255	50	50	50	50	255	255
255	255	255	50	50	50	50	50	255	255
255	255	255	50	50	50	50	50	255	255
255	255	255	72	50	50	50	50	255	255
255	255	255	255	50	50	50	255	255	255
255	50	50	50	50	50	50	50	50	255
255	255	255	255	255	50	255	255	255	255
255	255	255	255	50	255	255	255	255	255
255	255	255	255	50	50	50	50	51	168
255	255	255	255	50	255	255	255	255	255
255	255	255	50	255	255	255	255	255	255
255	255	255	50	255	255	255	255	255	255
255	255	50	255	255	255	255	255	255	255

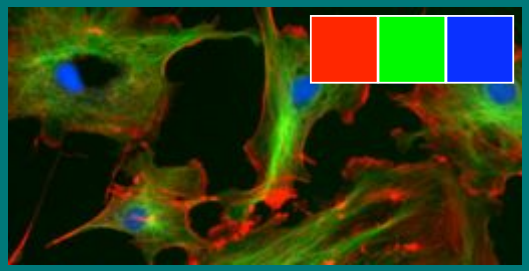
min	50
max	255
mean	194.5
stddev	93.2
area	10x14
pix	140
pix <255	42

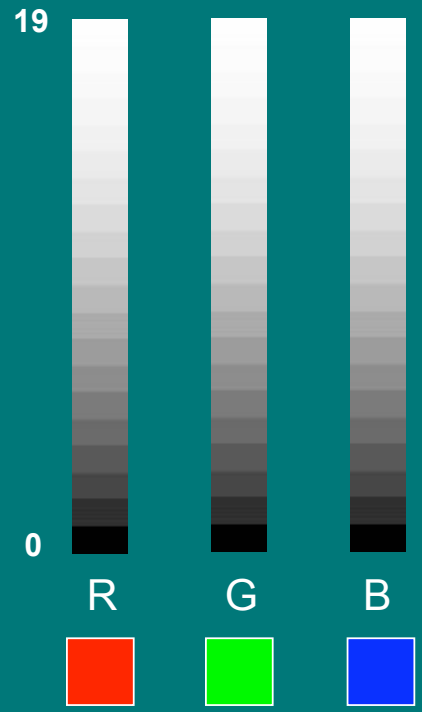
> Image Processing

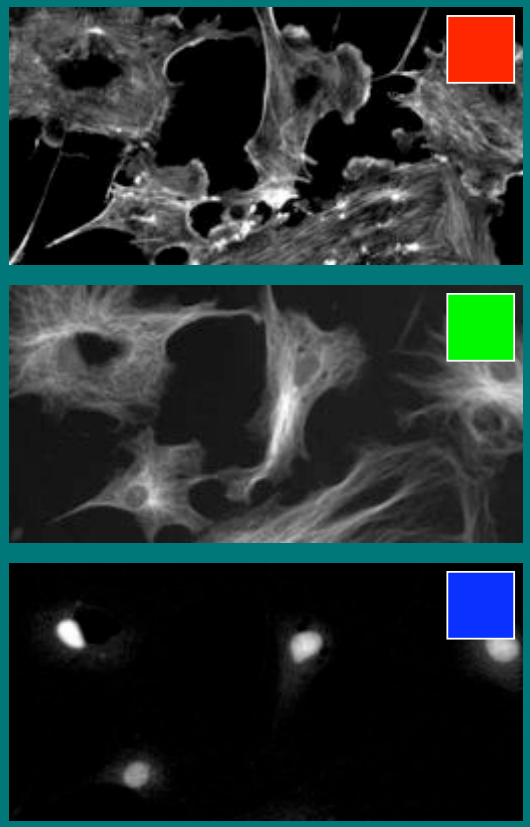
Object: Stick man
Body: 1
Head: 1
Legs: 2 (1 lifted)
Arms: 2 (2 lifted)
Walking left to right
...

> Image Analysis

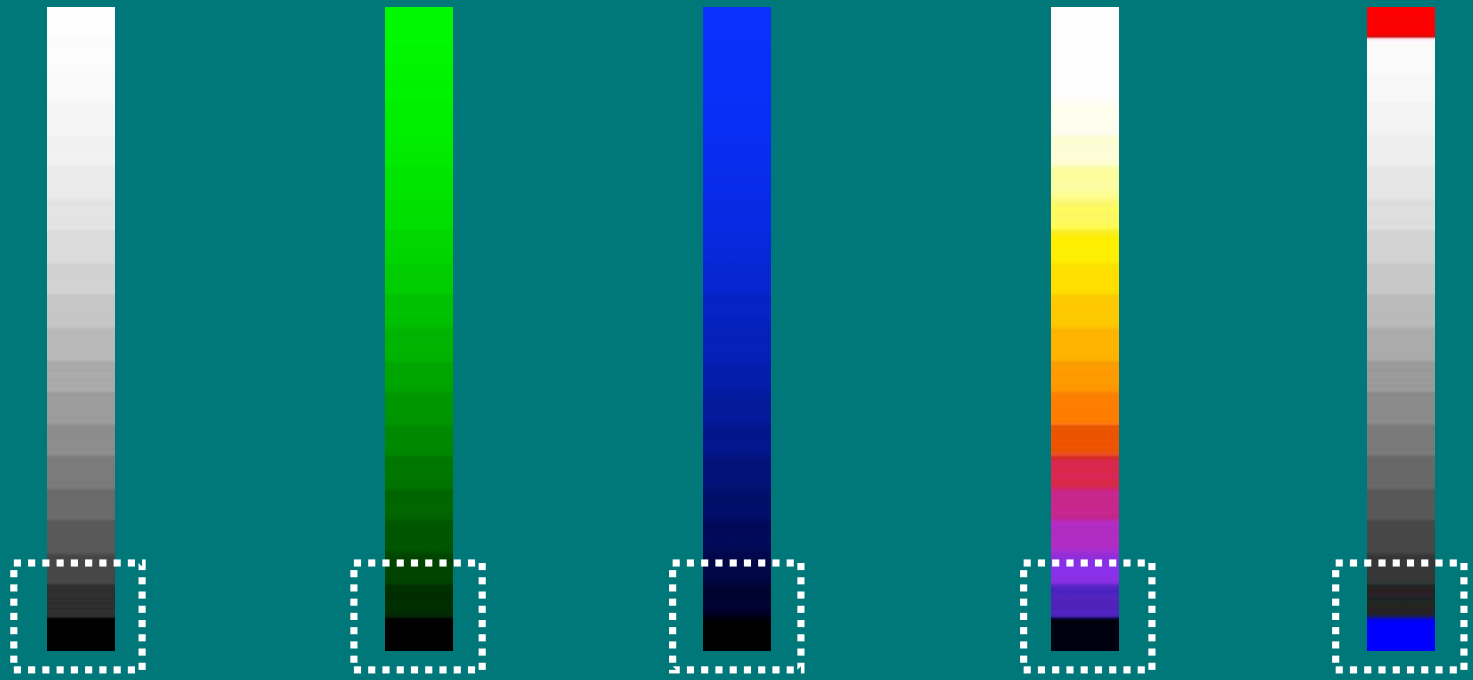
RGB Color Space



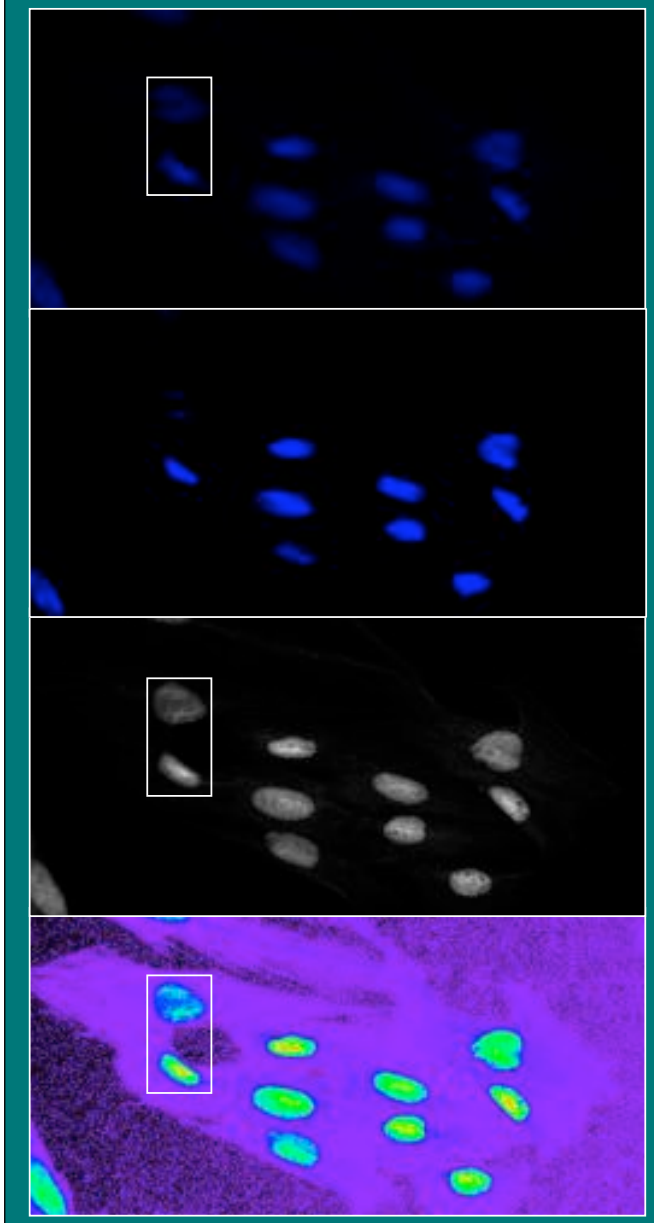




Lookup Tables



"grey" "green" "blue" "fire" "HiLo"



"original"

linear blue

brightness + contrast

data changed/lost!

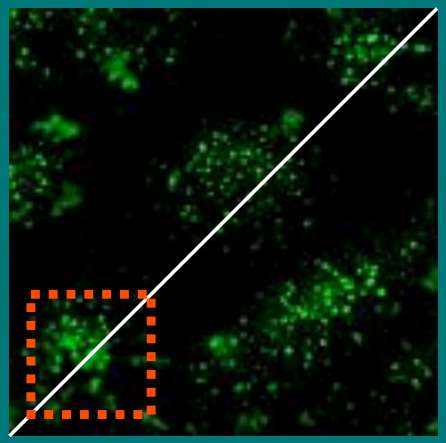
grayscale

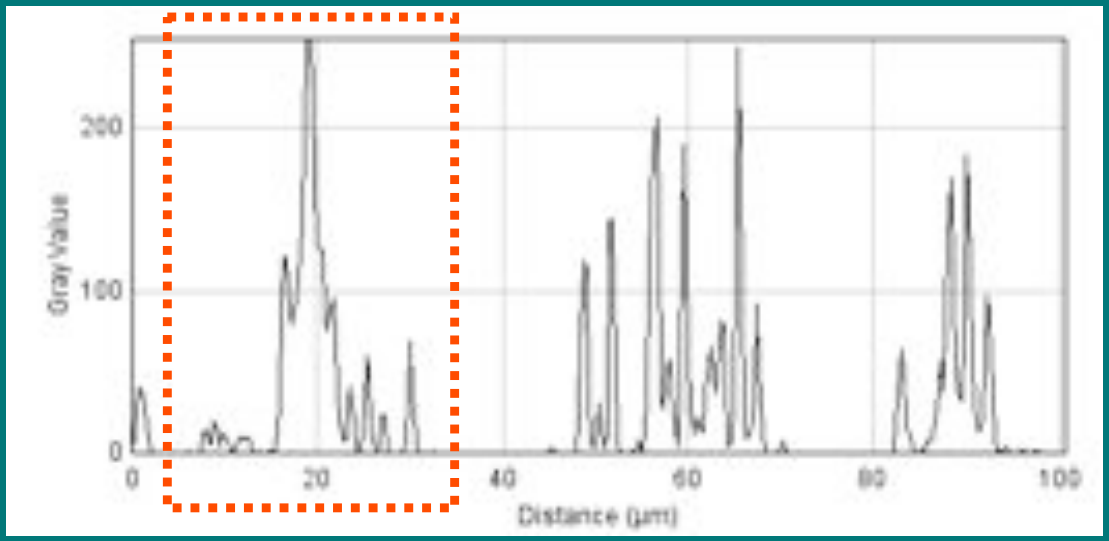
linear

rainbow lookup table

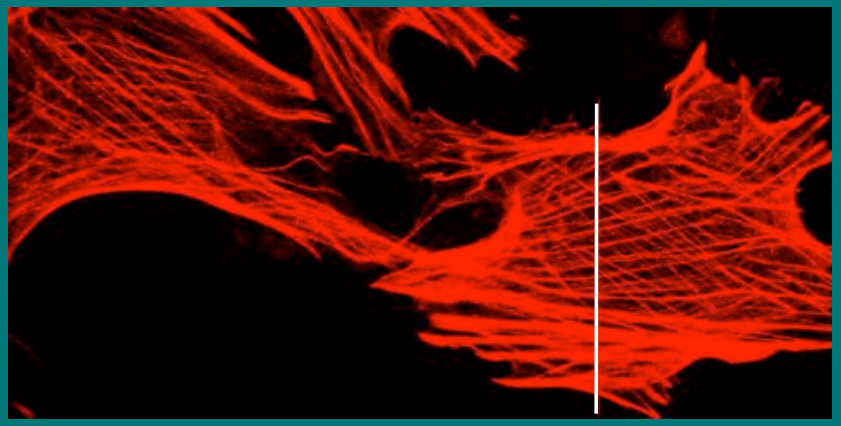
better see different intensity levels

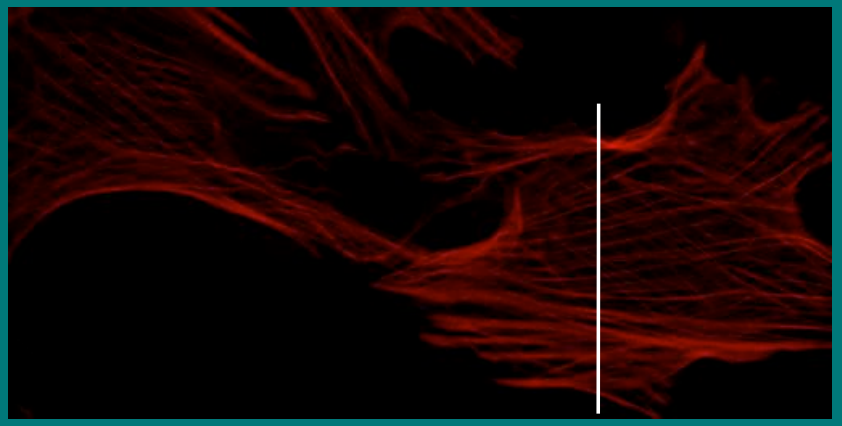
Line Profile

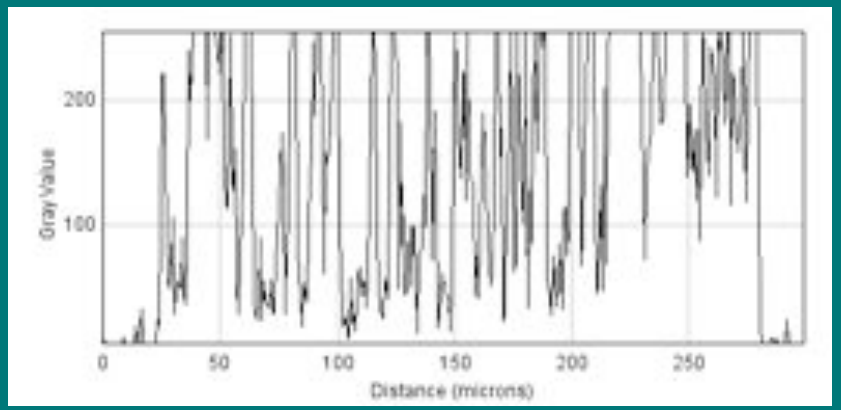


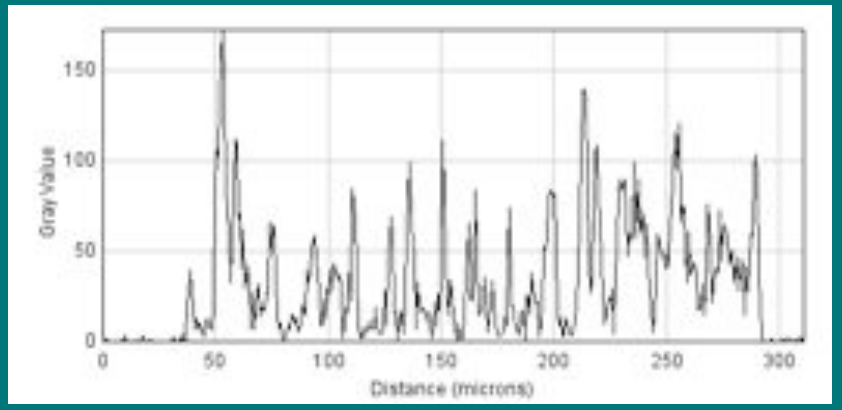


Line Profile



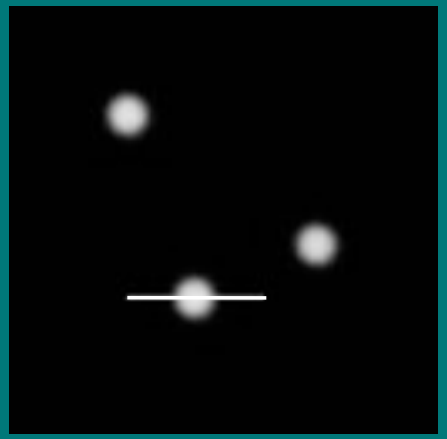


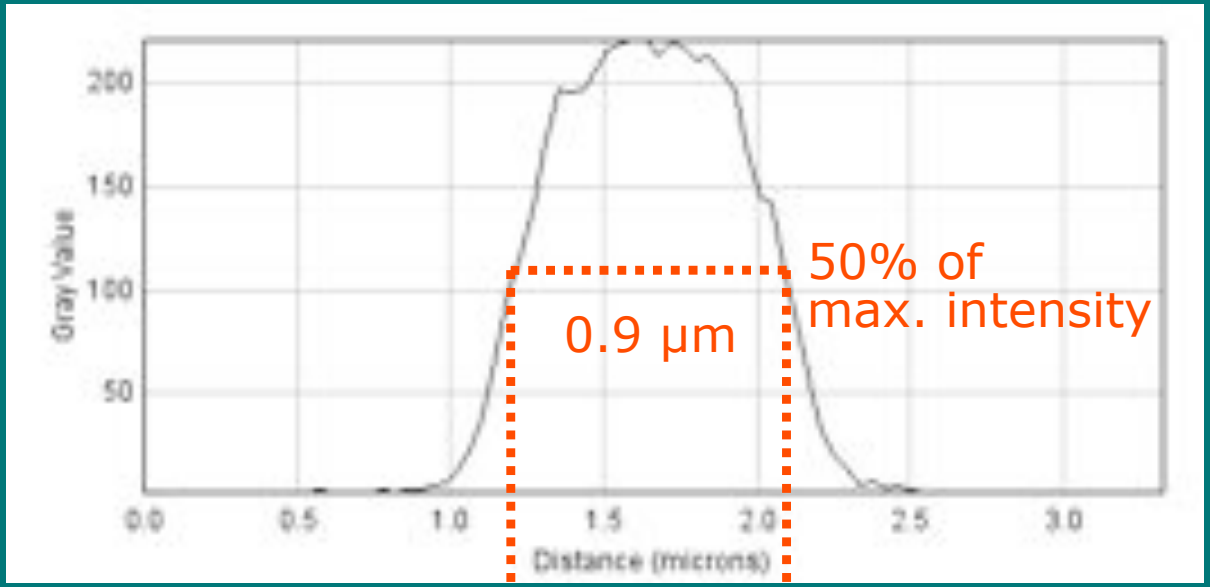




Line Profile

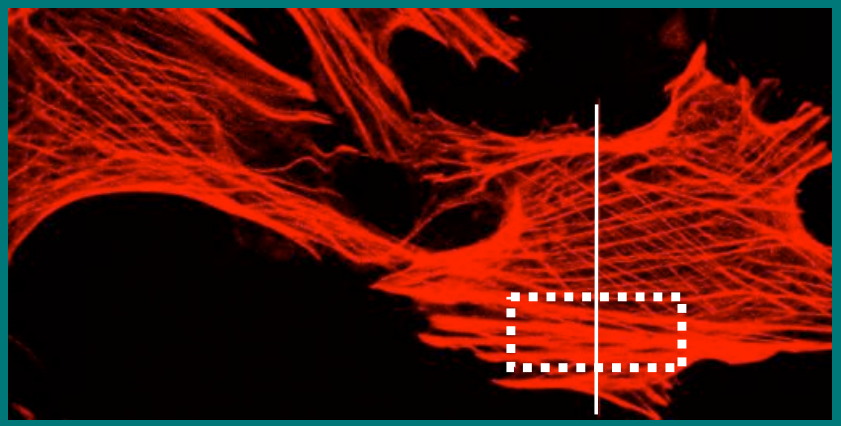
for measurements

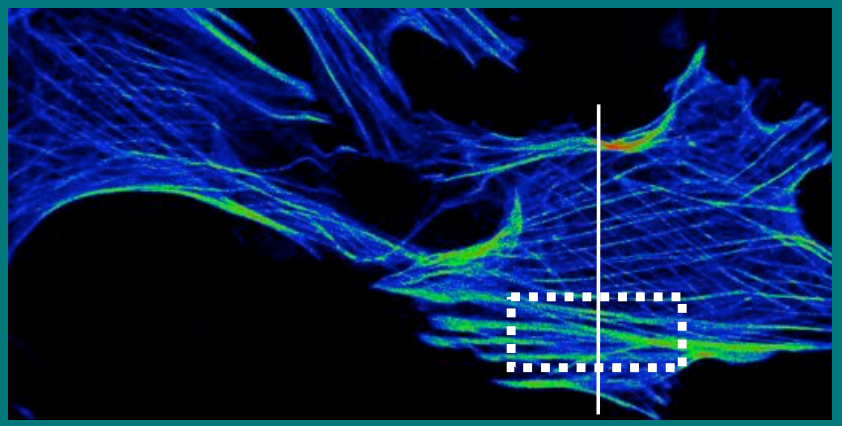


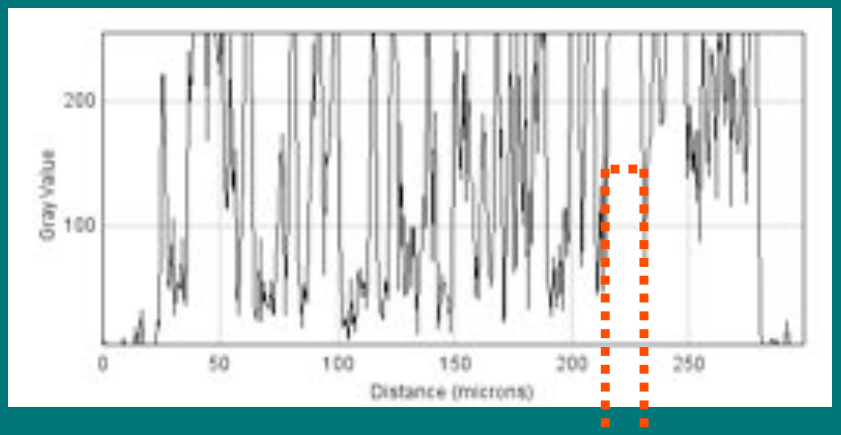


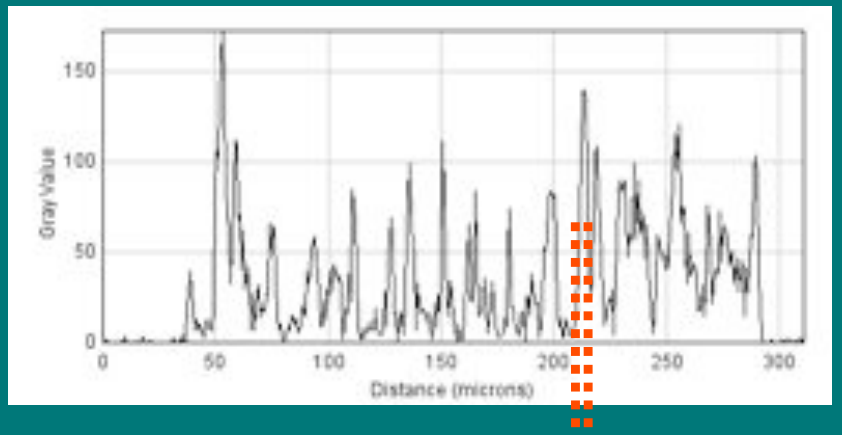
= “Full Width at Half Maximum”

Line Profile





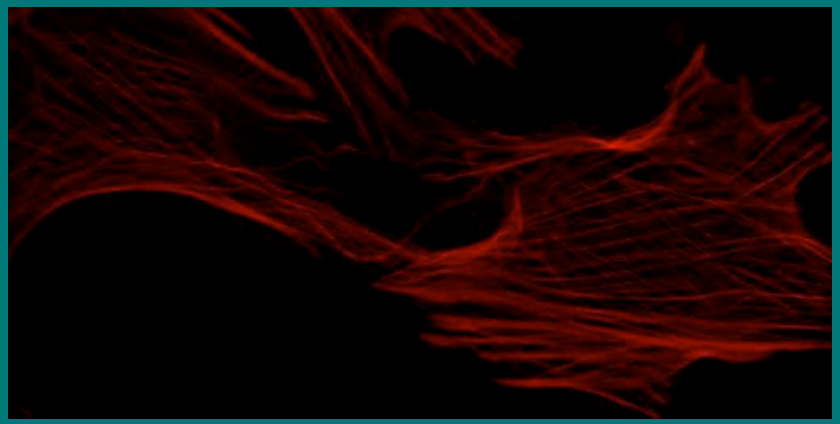


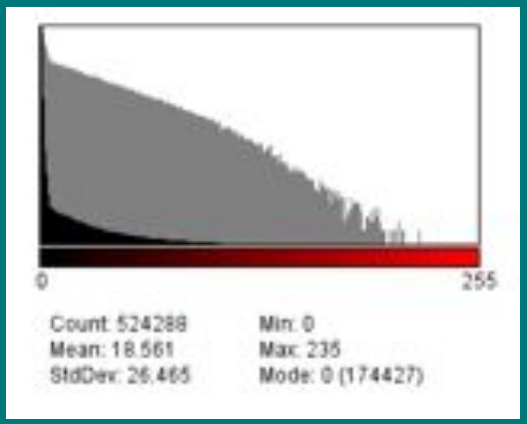


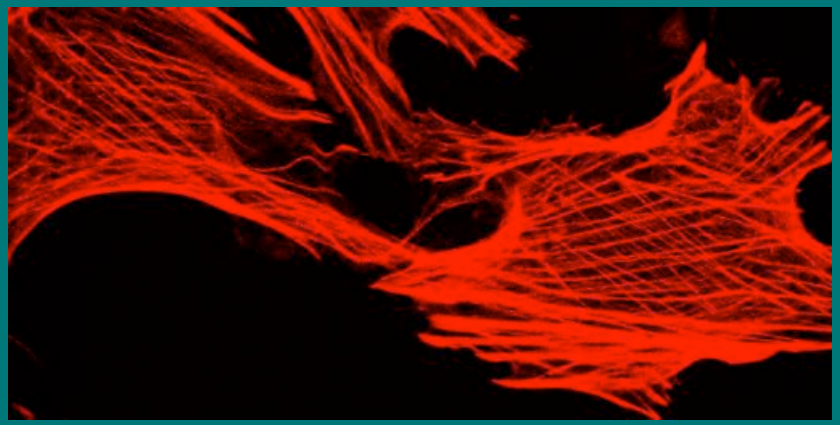
correct ?

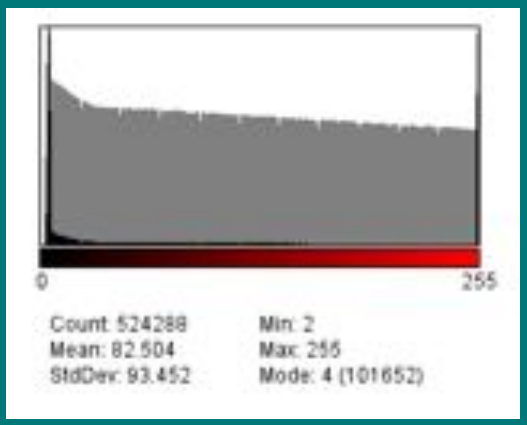
correct !

Histogram



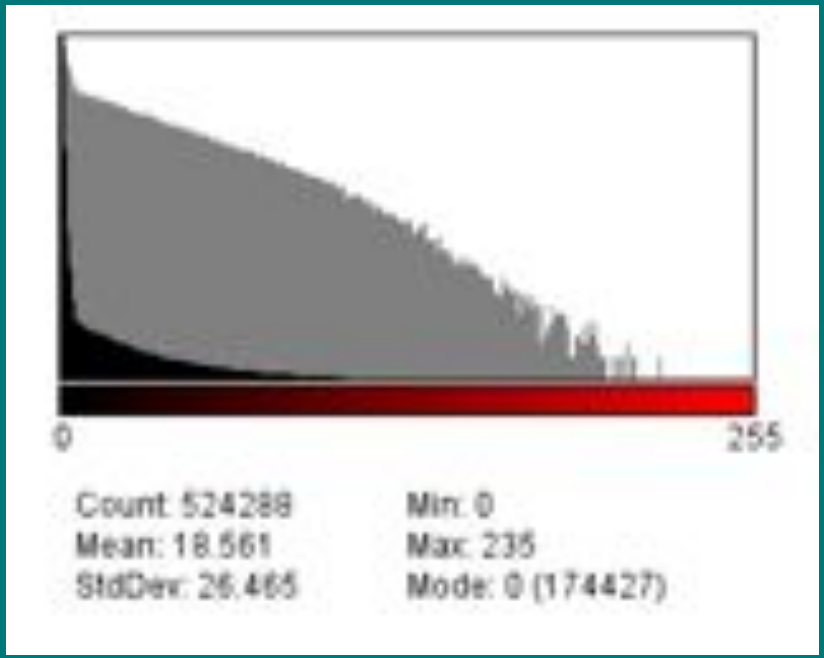

0
255
Count: 524288
Mean: 18.561
StdDev: 26.465
Min: 0
Max: 235
Mode: 0 (174427)

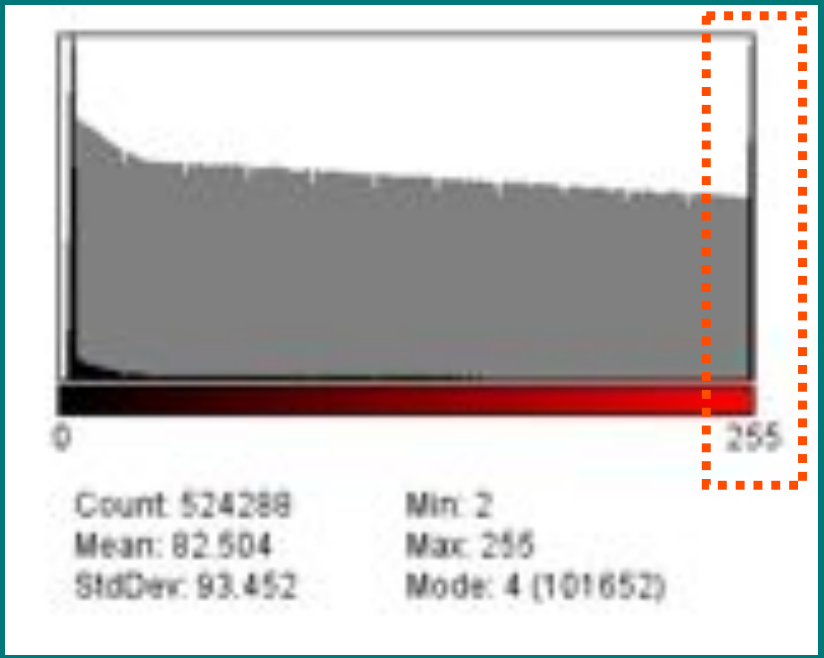



0
255
Count: 524288
Mean: 82.504
StdDev: 93.452
Min: 2
Max: 255
Mode: 4 (101652)

Histogram

fluorescence microscopy



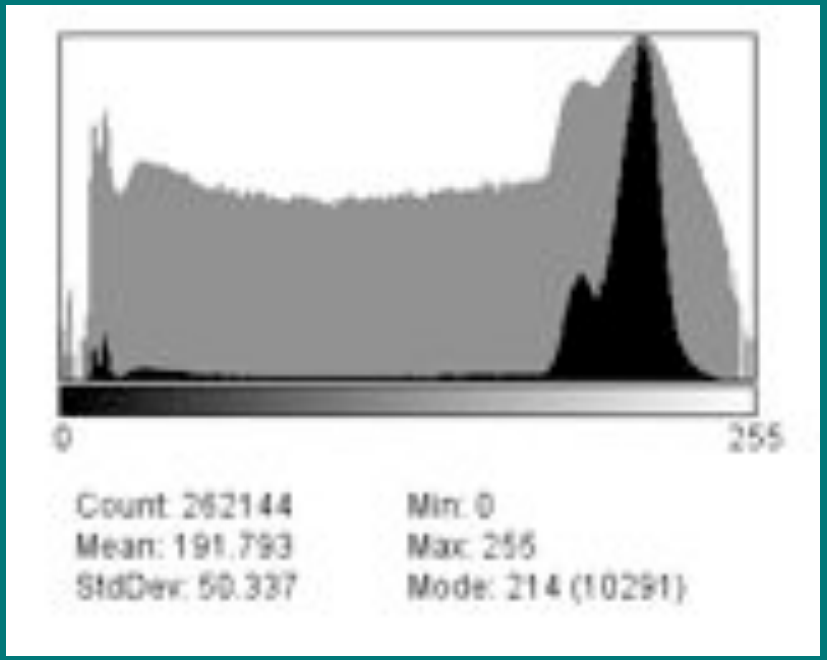


OK

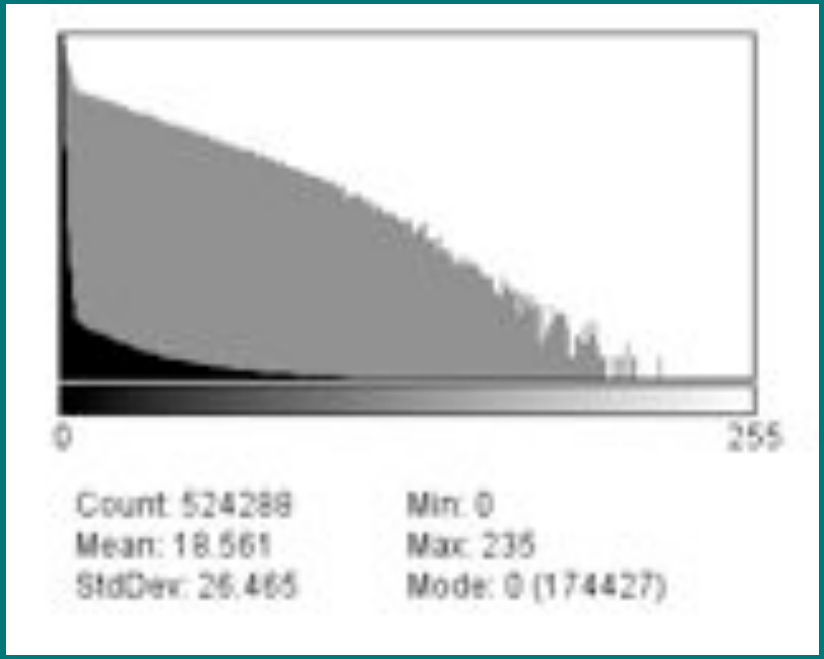
not OK - why?

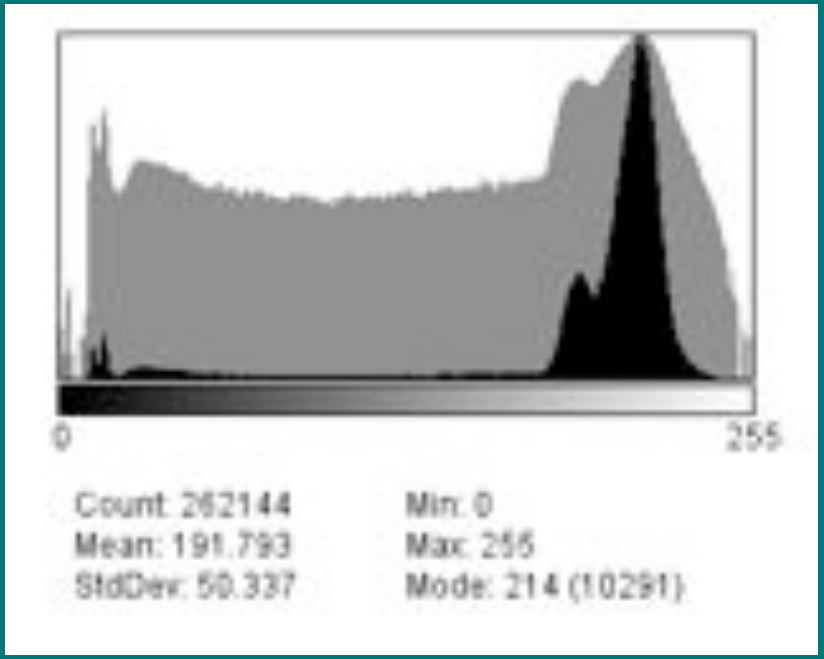
Histogram

brightfield microscopy



Histogram

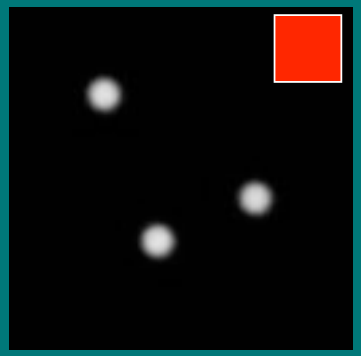


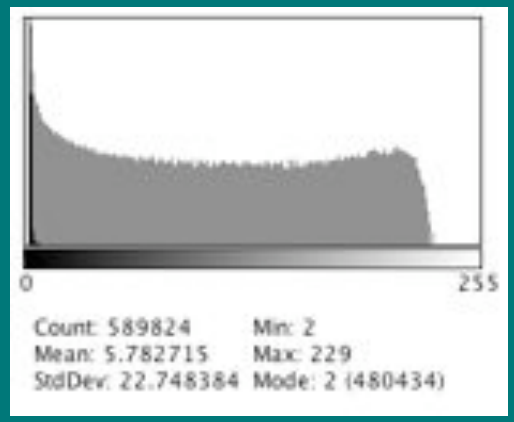


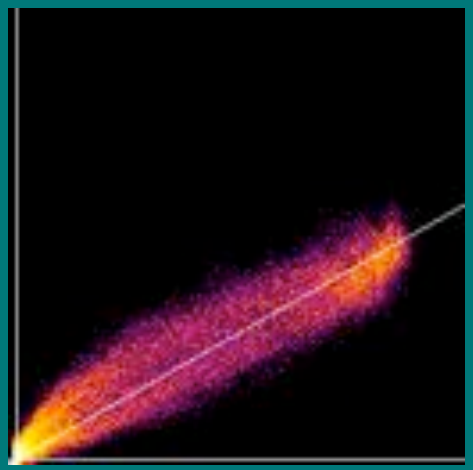
fluorescence

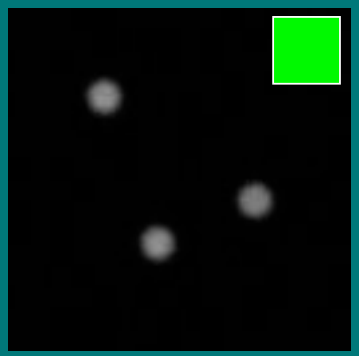
brightfield

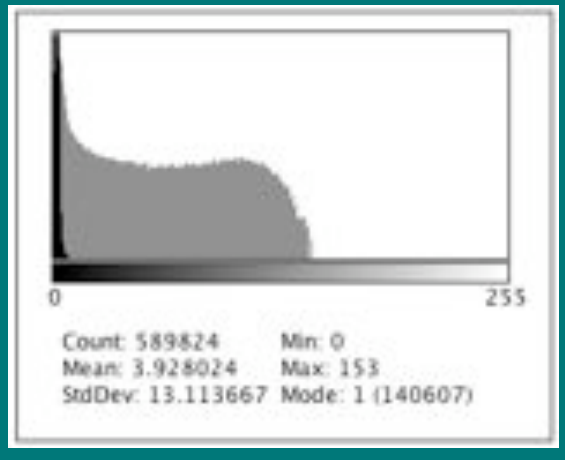
2 Histograms > Scatterplot
2 Histograms > 2D Histogram



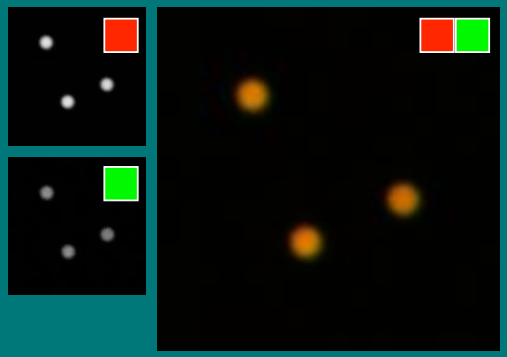




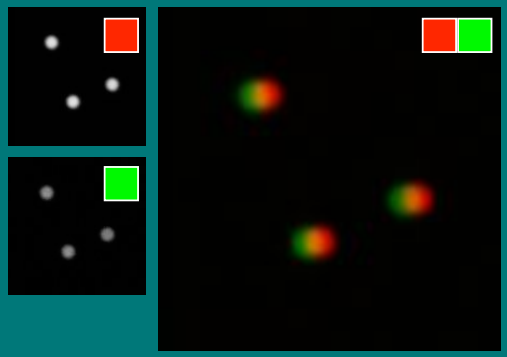




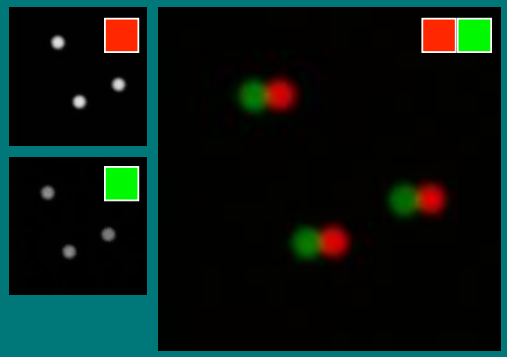
Scatterplot / 2D Histogram



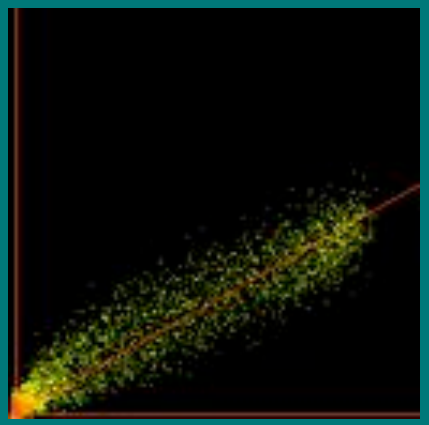
original R+G

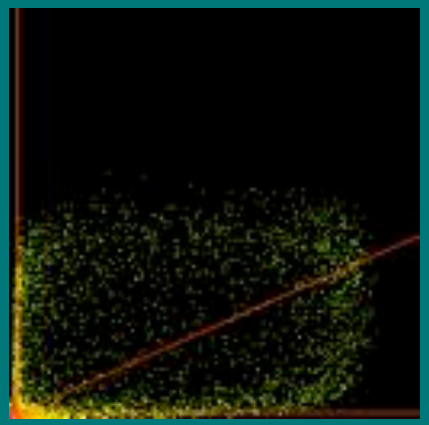


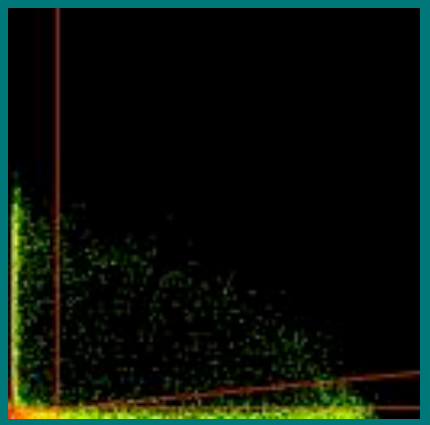
R shifted +10 pix



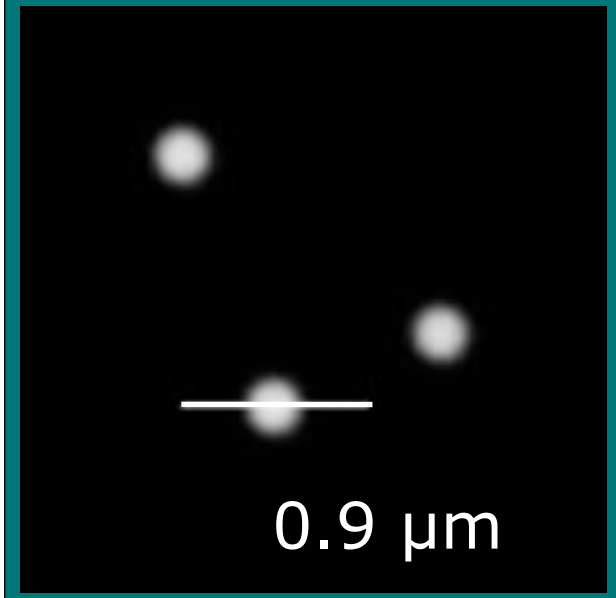
R shifted +20 pix

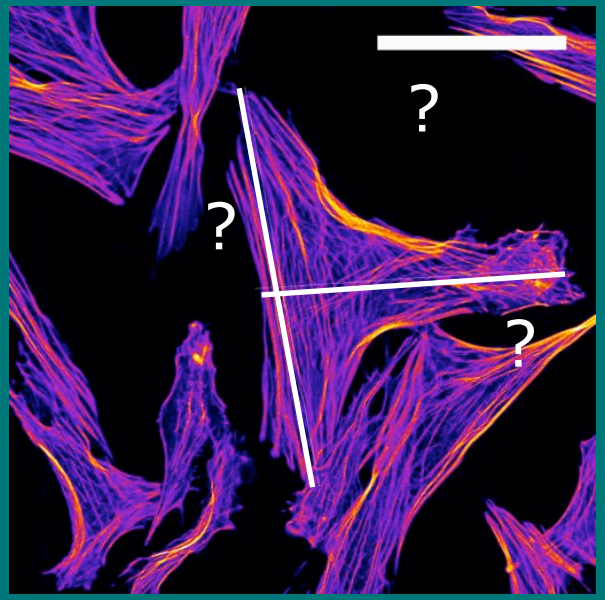






Pixelsize / Scaling





?

?

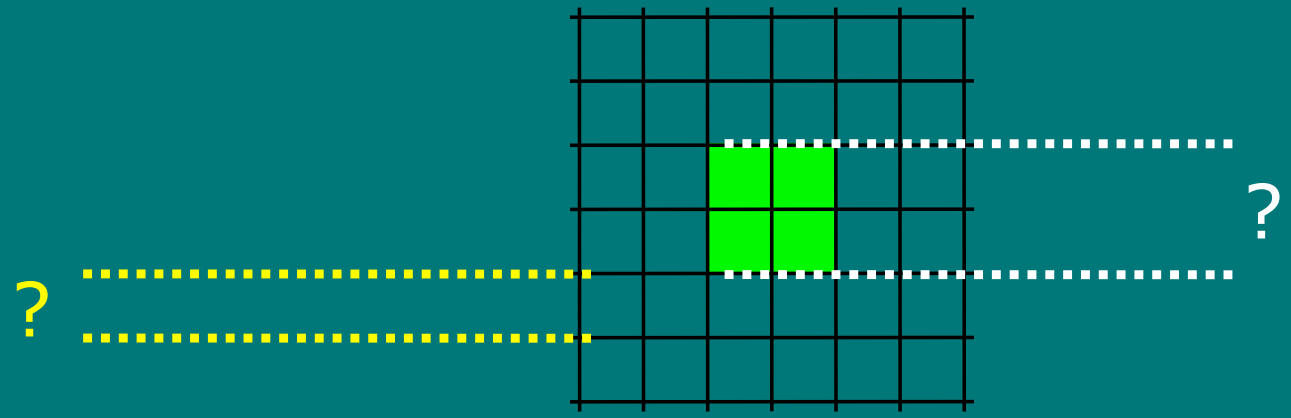
?

Pixelsize / Scaling

- How big is a structure that is represented in my image?

  =

- How big is one pixel?



Pixelsize / Scaling

- Pixelsize given by system
- might be changed / lost while processing
- stored in “metadata”
- dataset for image processing:
  - image data
  - metadata

Practical Session 2

- Bit Depth: Image - Type
- RGB Color Space
  - Colour Channels: Image - Colour - Channels Tool, Split channels etc.
- Lookup Tables: Image - Lookup tables
- Line Profile: Analyse - Plot Profile
- Histogram: Analyse - Histogram (plugins-analyse-2D Histogram)
- Spatial Scaling: Analyse - Set Scale, Analyse-Tools-Scale Bar
- Intensity Scale: Analyse - Tools - Calibration Bar

File - Open Samples - Neuron

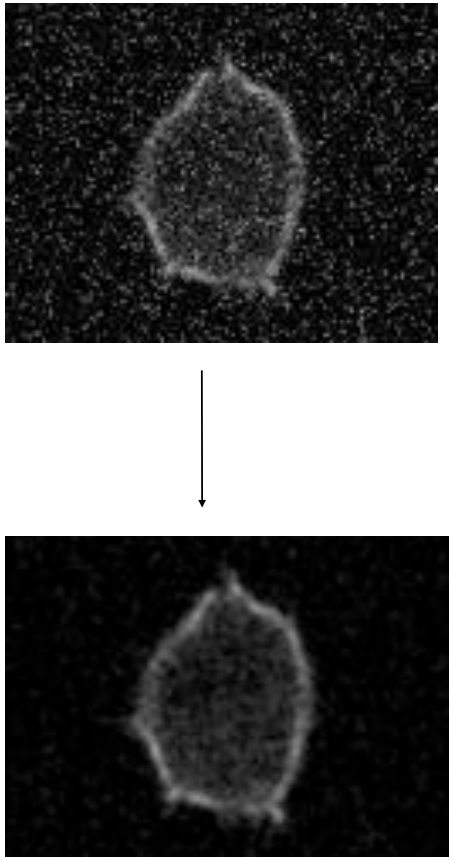
Session 3:

1) Image processing in the spatial / frequency / time domain.
2) Image Segmentation.

1) Filtering - Neighborhood - Fourier space - Time series

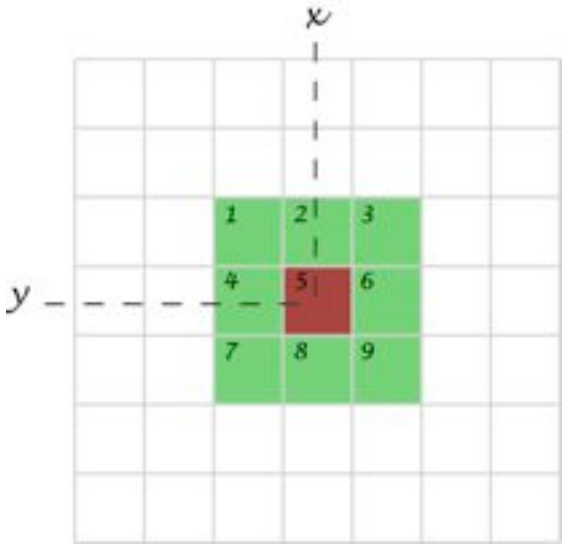
2) Thresholding - Edge Detection - Watershed segmentation

I. Image processing in the spatial domain



- A. Introduction
  - Neighborhood
  - Operation on neighbors
- B. Spatial filters
  - Mean filter
  - Median filter
  - Edge detection

A. Introduction



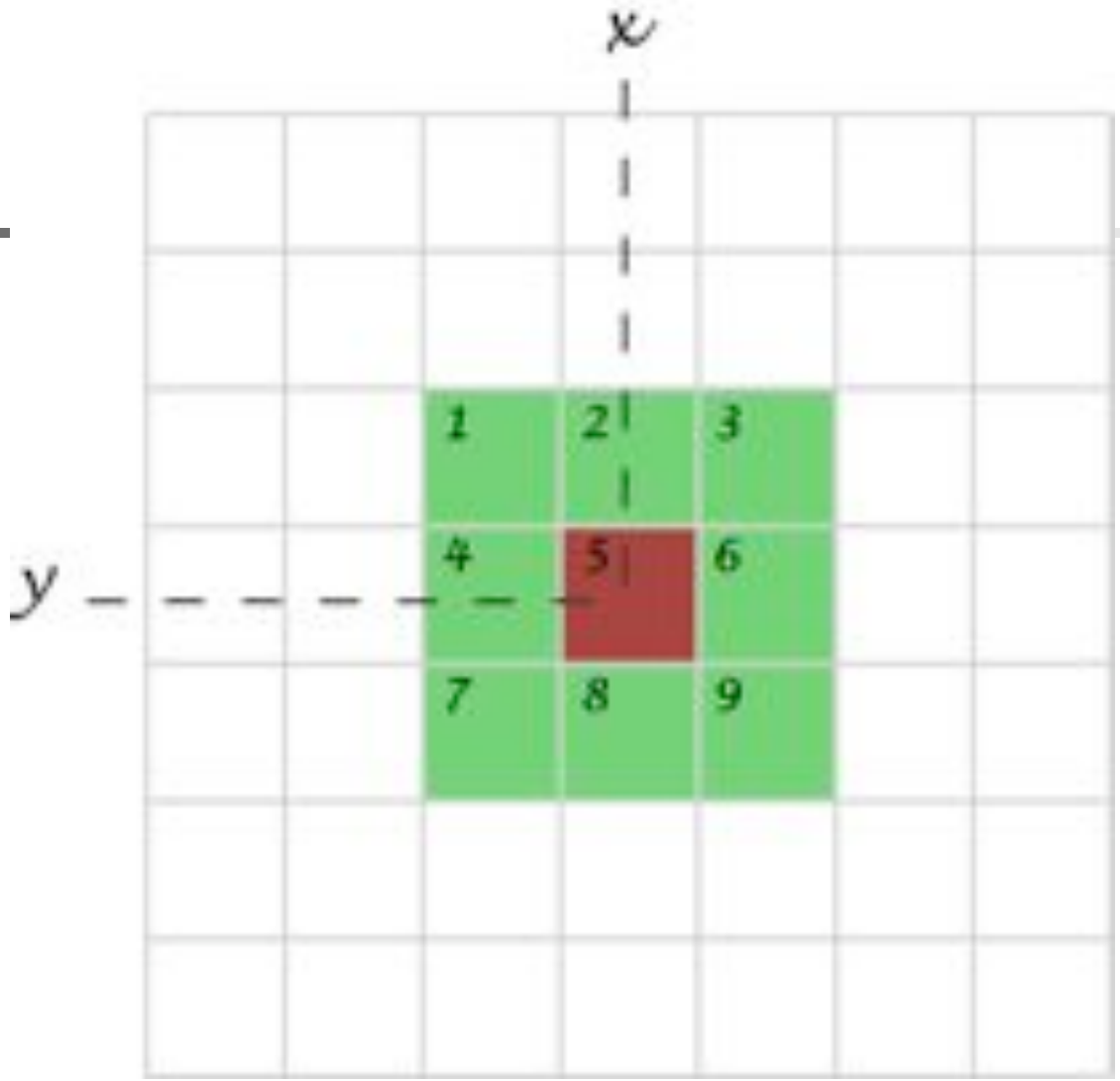
Neighborhood (or kernel): pixels that matters

- Definition

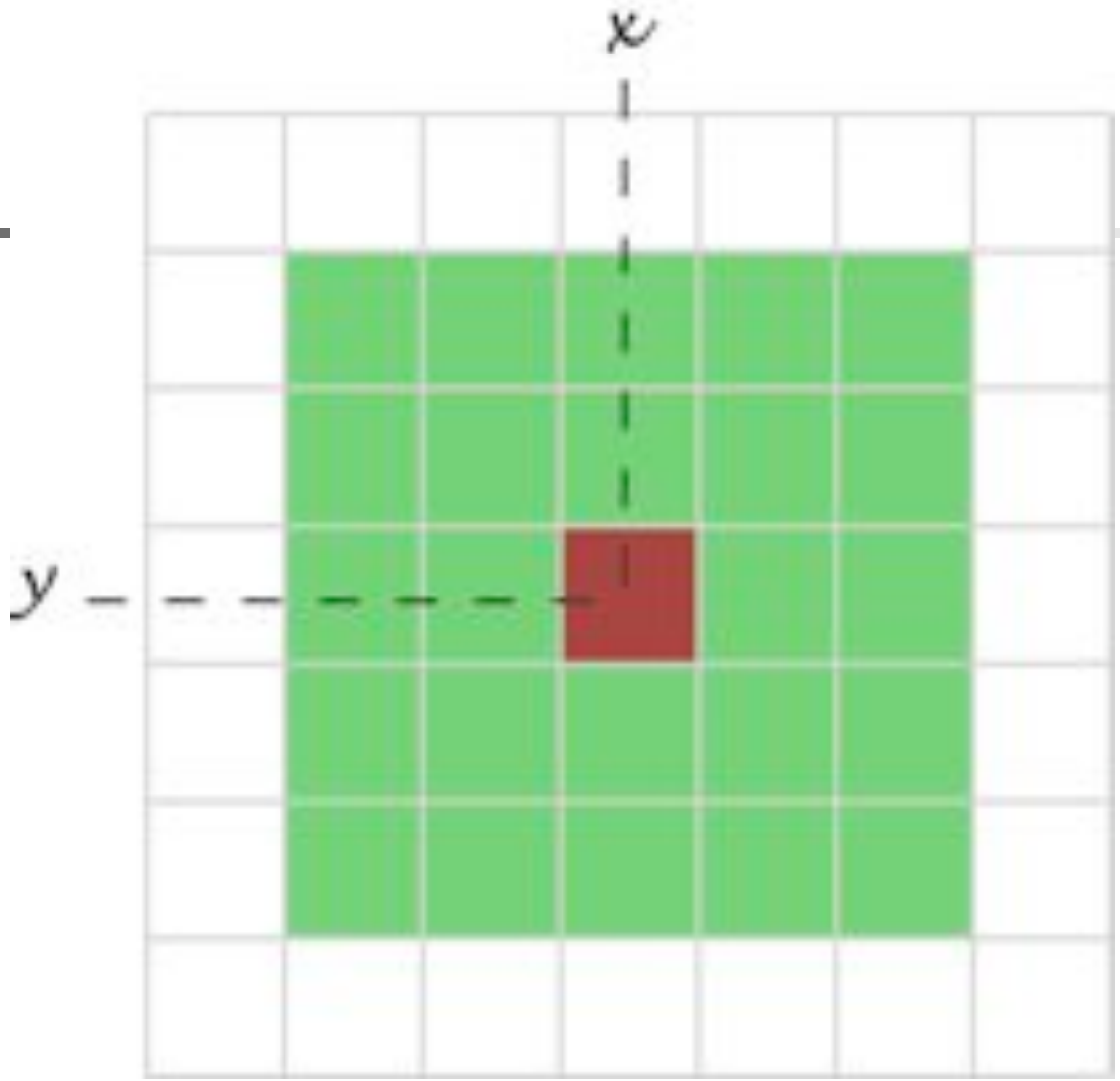
" Transformation or set of transformations where a new image is obtained by *neighborhood operations*."

The intensity of a pixel in the new image depend on the intensity values of "neighbor pixels".

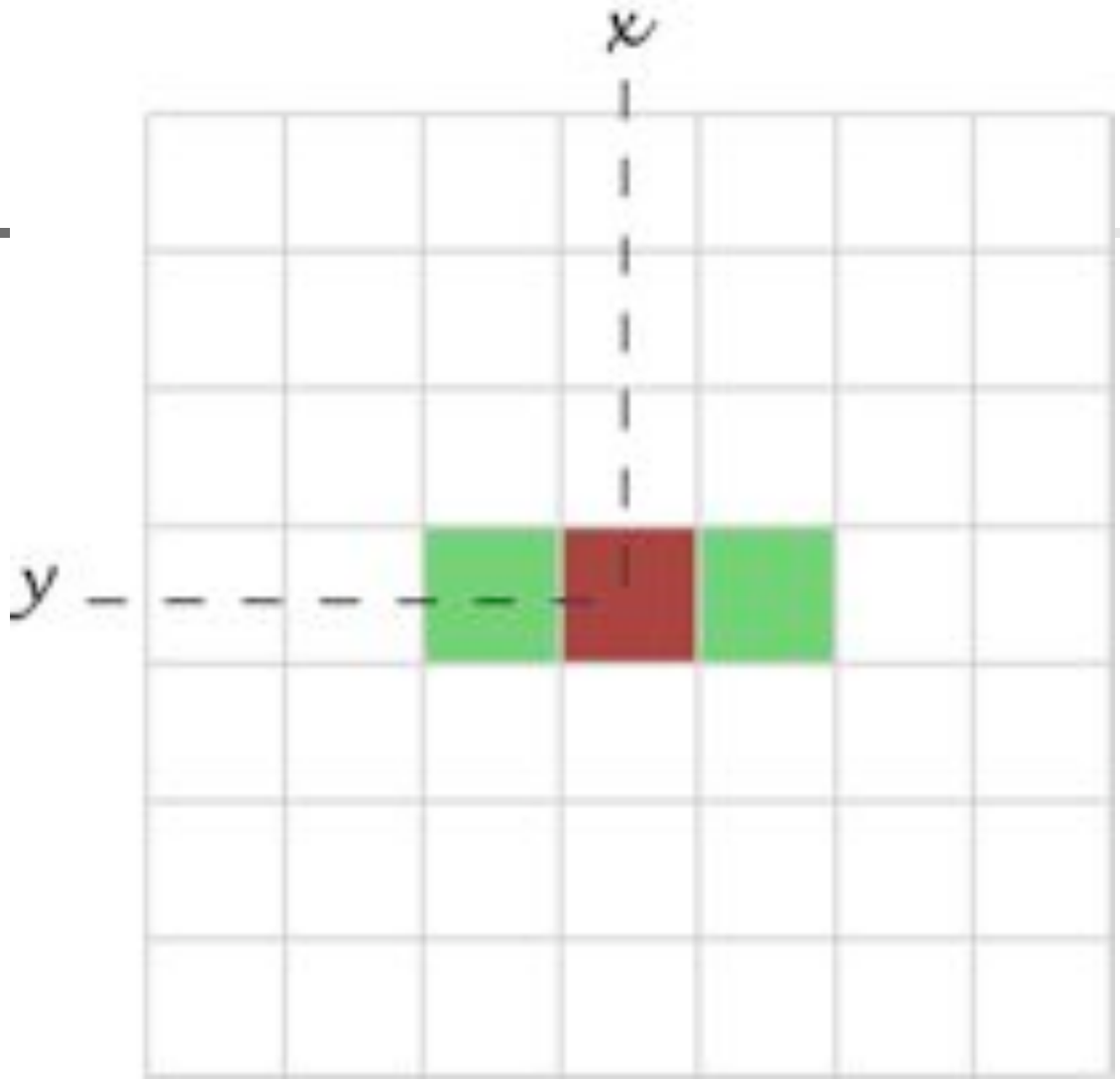
3x3



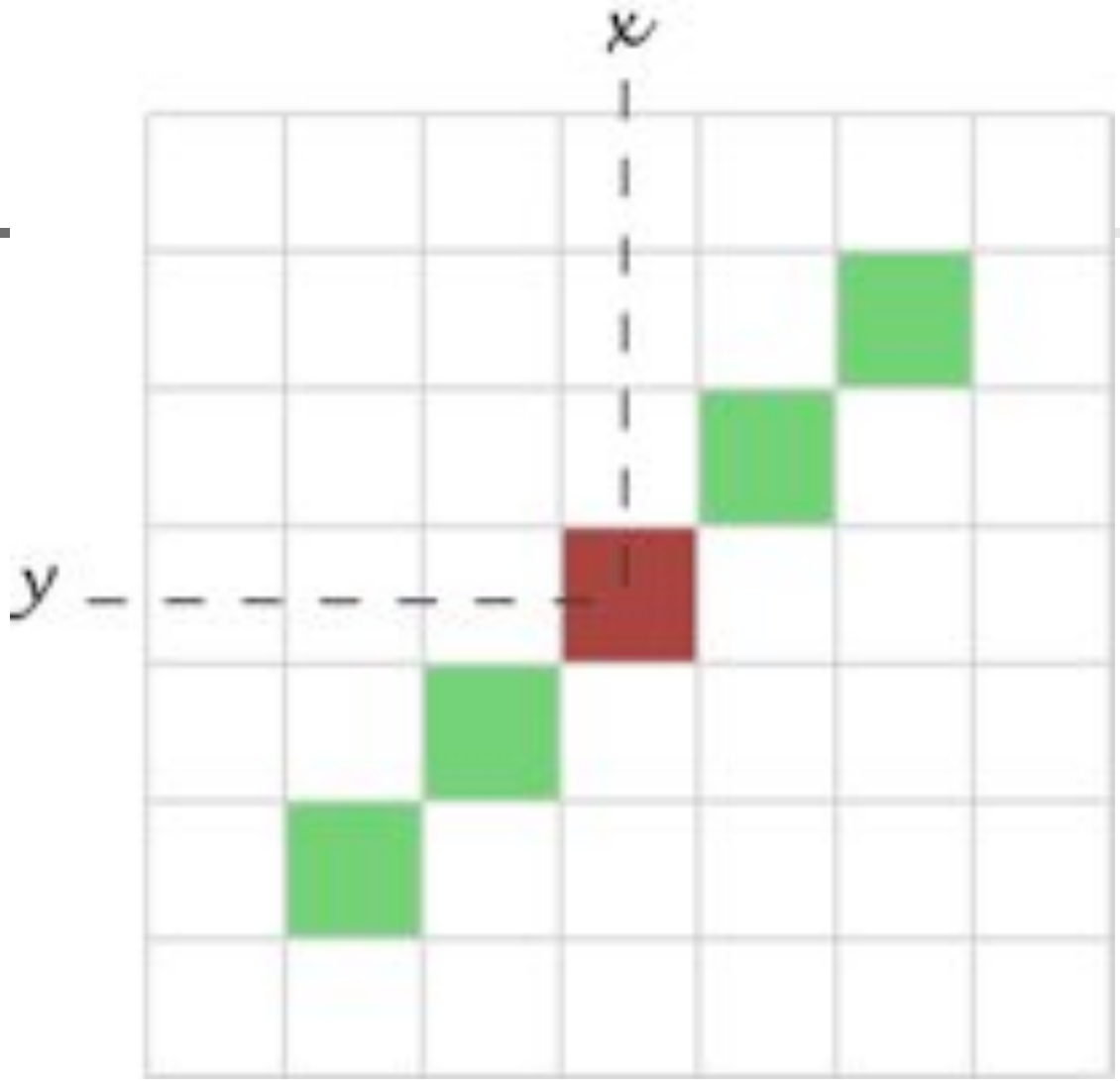
5x5



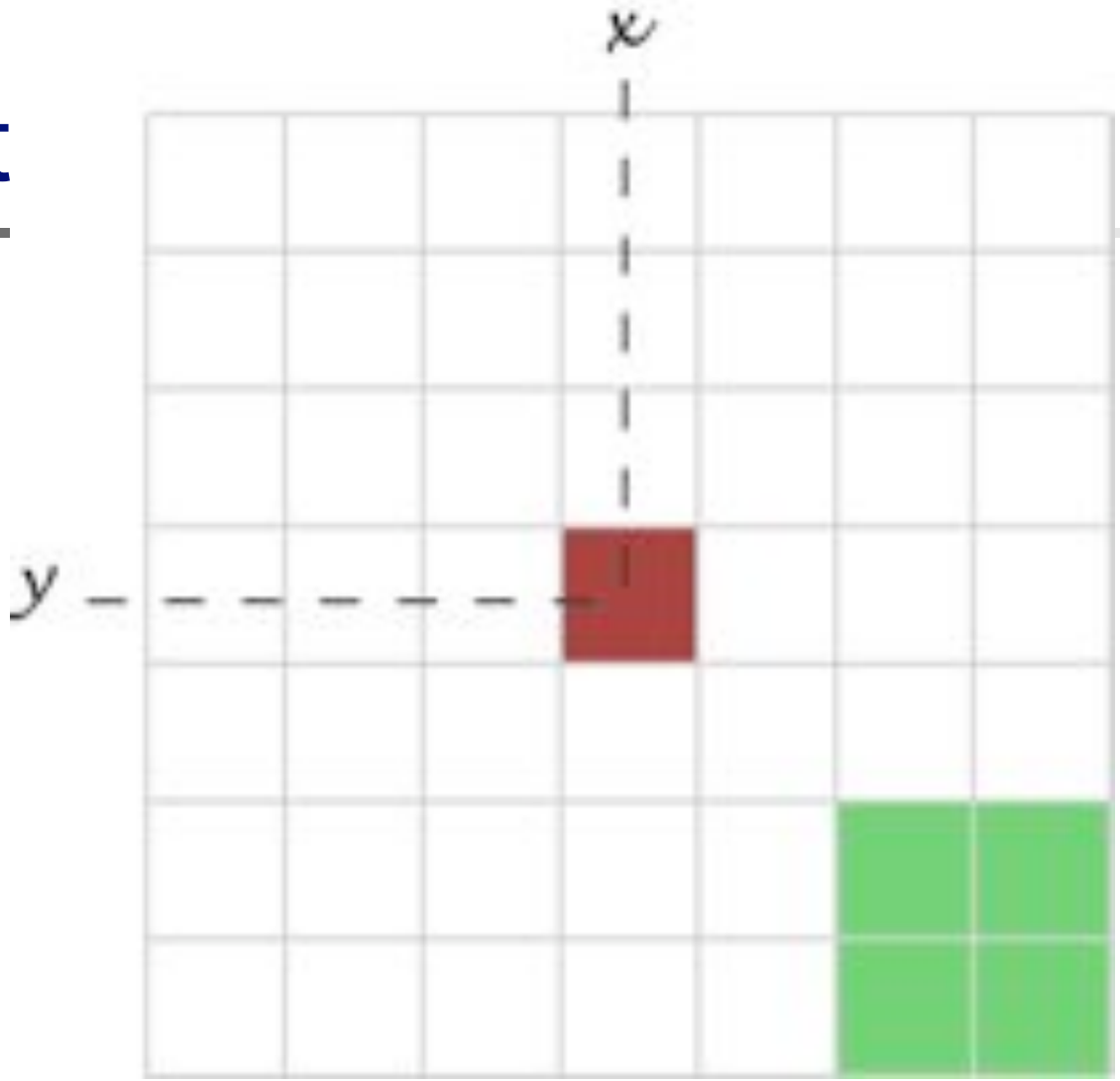
1x3



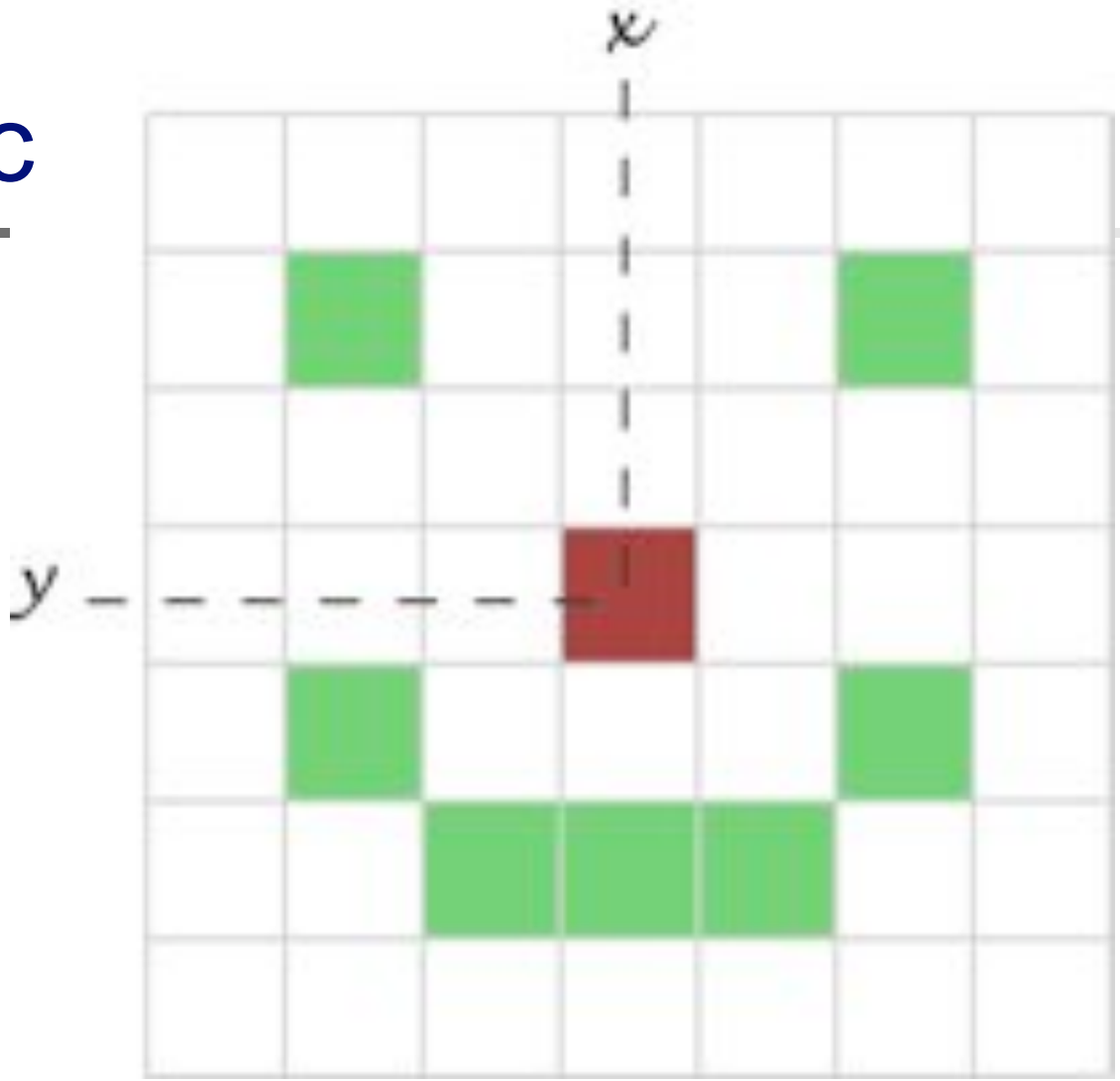
1x5



2x2 shift



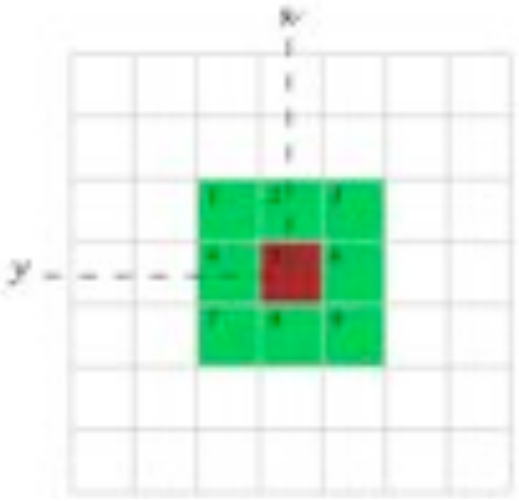
Misc



B. Filtering: The mean filter

Simplest filter: the value of a pixel is replaced by the intensity mean computed over neighbors pixels

$$a_i^* = \frac{1}{N_\Omega} \sum_{j \in \Omega} a_j$$



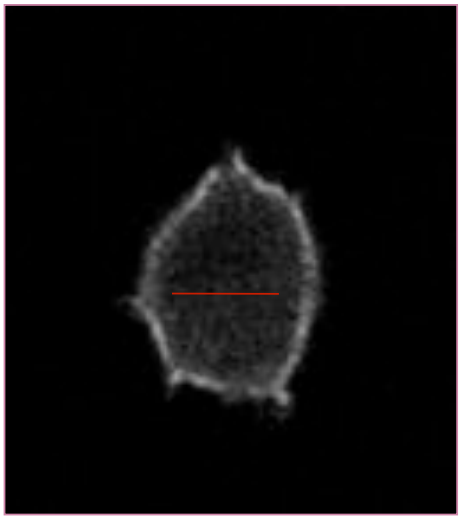
3x3 example:

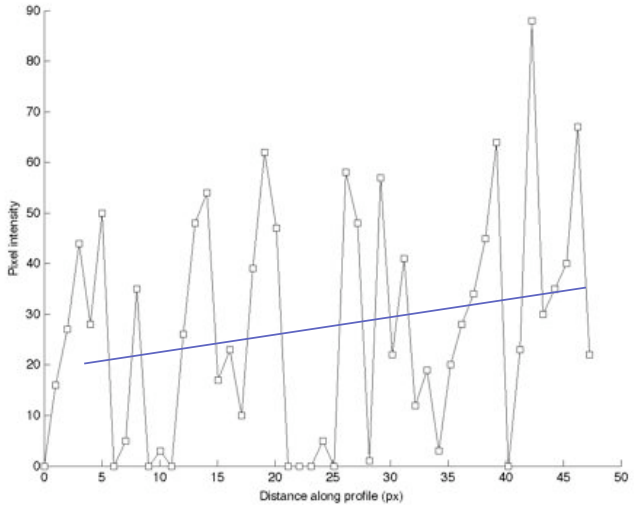
$$a_i^* = \frac{1}{9}\left(a_1 + a_2 + a_3 + a_4 + a_5 + a_6 + a_7 + a_8 + a_9\right)$$

The mean filter

what is it good for?

Noise removal - typically Gaussian / Poisson noise.





(typ. Appears for weak labeling, short exposure time = few photons detected)

The mean filter

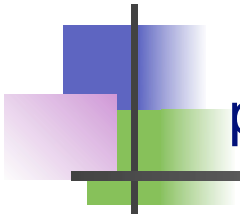
properties - linear filtering

The mean filter is a linear filter:

"*The new pixel value depends on a linear combination of neighbour pixel values*"

(The order of several linear filters in sequence does not matter)

➔ another notation for 3x3 kernel



The mean filter

properties

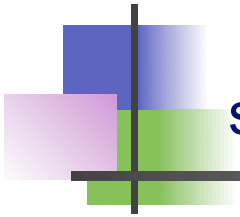
Main property: low-pass filter
(smooths small objects)

- kernel size influence
- number of successive applications

Cases where it fails

- salt & pepper noise

we will do this
in the practical



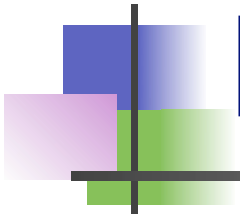
The mean filter

summary

- simplest filter - fast
- is a linear filter
- averages noise, does not eliminate it
- good against Gaussian and Poisson noise

- but
- blurs images - small details are lost
- smoothes edges dramatically

} Low-pass filter



Linear filtering

Properties:

- Applying a linear filter to an image is the same as:
  applying it to all parts, then summing the results.

- When applying a succession of linear filters:
  the order filters are applied in does not matter.

- Mathematical framework underlying it:
  Convolution.

  We can also reverse the process : Deconvolution

Filtering: The median filter

The value of a pixel is replaced by the *median* of the pixel intensity in neighbors pixels

Take neighborhood (*e.g.* 3x3)

5	112	86
235	88	211
137	233	108

Sort it

5
86
88
108
112
137
211
233
235

Take median

112

The median filter

noise elimination

Original:

5	9	6	6	9	5	9
9	5	9	7	8	7	9
8	9	8	6	7	9	9
9	9	7	200	9	6	9
6	5	8	6	9	6	7
9	7	9	9	8	6	7
7	9	5	6	7	6	6

outlier

Median filtered:

0	5	6	6	6	7	0
5	8	7	7	7	9	7
8	9	8	8	7	9	7
6	8	8	8	7	9	6
6	8	8	9	8	7	6
6	7	7	8	6	7	6
0	7	6	6	6	6	0

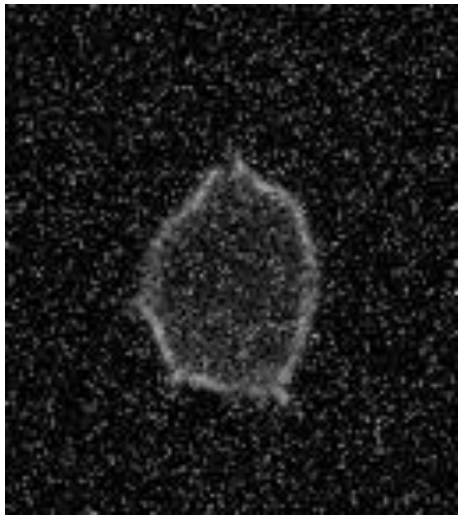
The outlier value has completely been removed from the dataset

The median filter

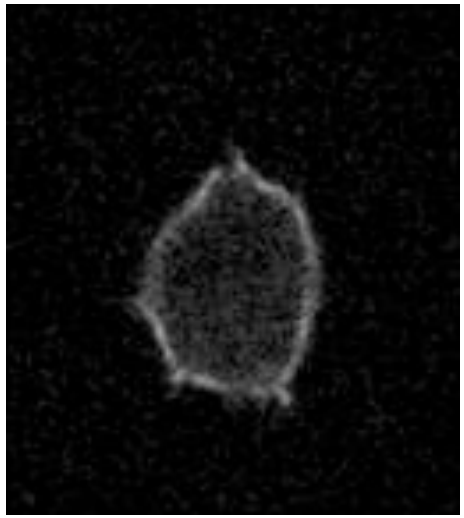
what is it good for?

“Salt & pepper” noise removal

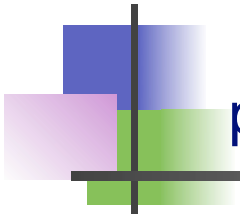
Original:



Median filtered:



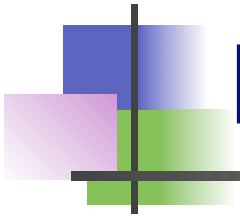
(typ. Appears for very weak labeling - high detector gain etc.)



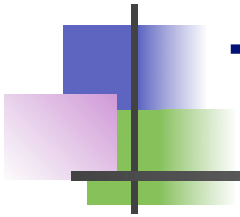
The median filter

properties

- Typically good for “Salt & pepper” noise removal
- *Eliminates* noise
- Slower than mean and similar
  (not such a problem anymore)
- NOT linear
- Edge-preserving



Relax…



The Fourier transform

- The Fourier transform is a way to obtain a new *representation* of the data.
- It is best suited for data with *repetitive patterns* and highlights these patterns.

Don’t worry about the maths for now...

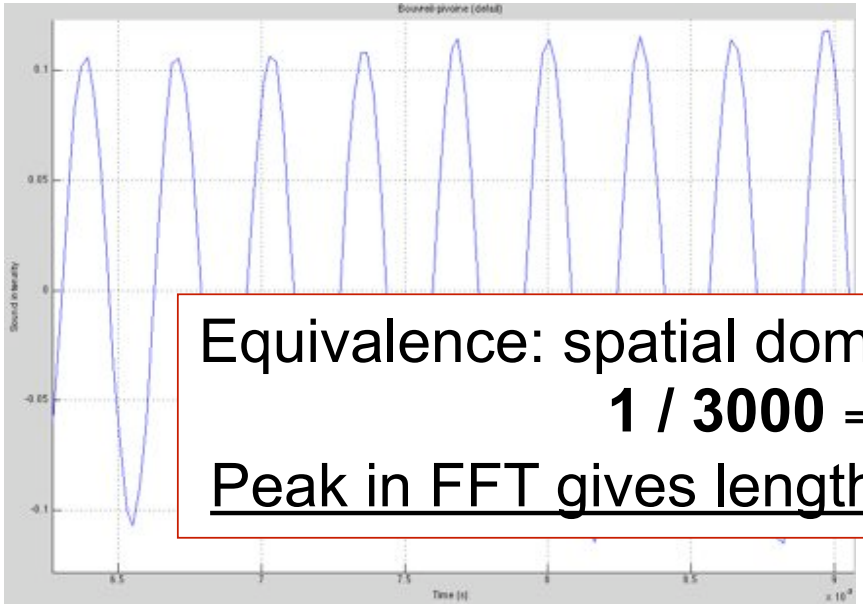
The Fourier transform

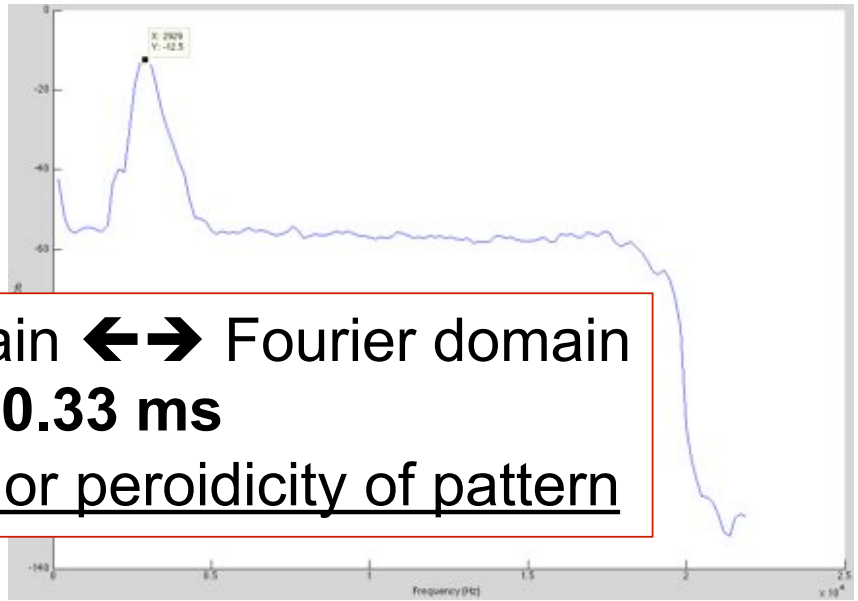
Bird song.

Delay between peaks:
~ 0.35 ms

FFT of this looks like:

Peak in FFT:
~ 3 kHz





Equivalence: spatial domain ⟷ Fourier domain

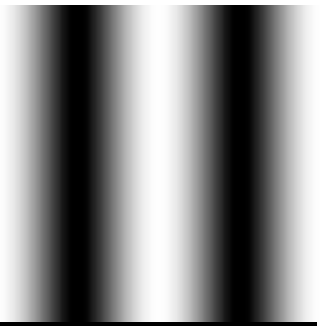
1 / 3000 = 0.33 ms

Peak in FFT gives length or peroidicity of pattern

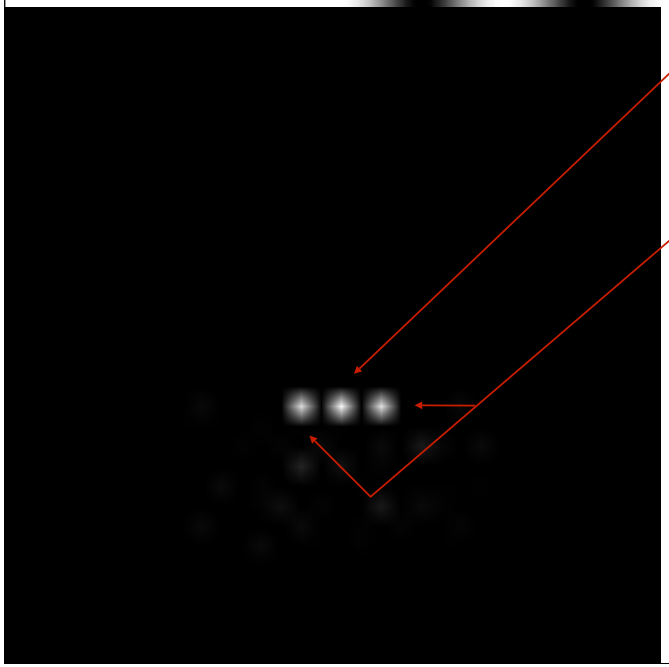
The Fourier transform

in 2D (images)

orig



FFT (zoomed)



Central point: non-varying part of the image (mean)

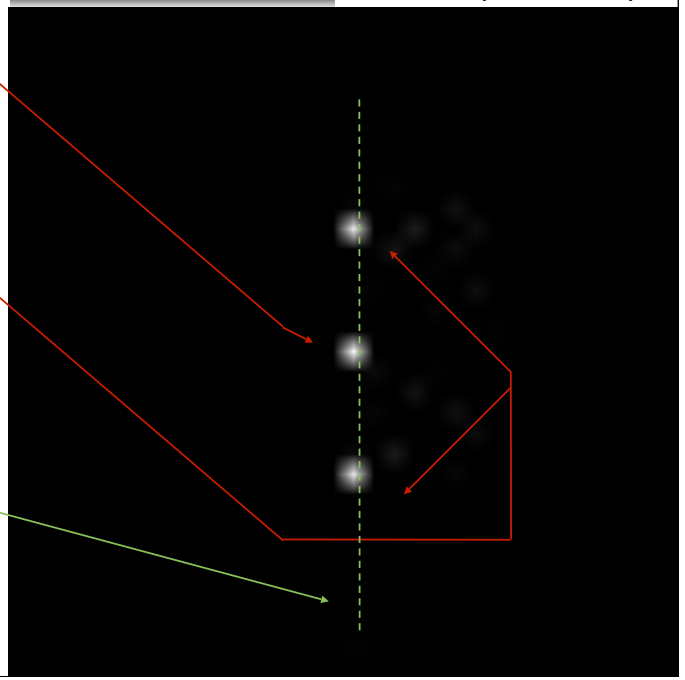
Pattern points: always by pair, the further = the smaller

Angle of pattern point gives pattern orientation

Diffraction pattern?

orig

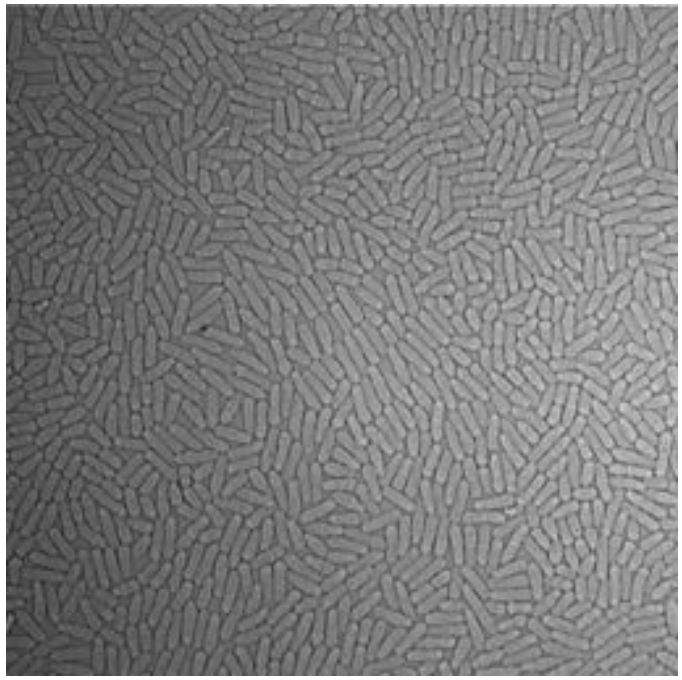
FFT (zoomed)



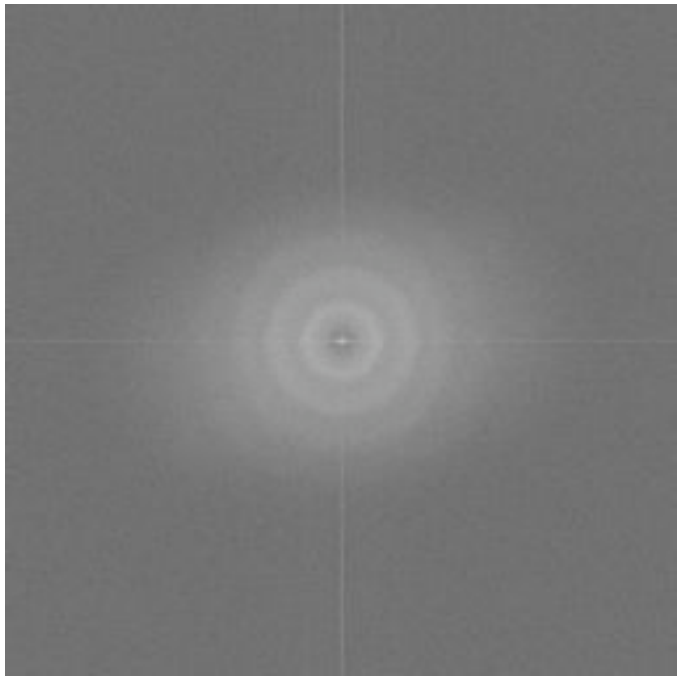
The Fourier transform

real images

… are rarely that clear



S. pombe cells (*Tolic lab*)



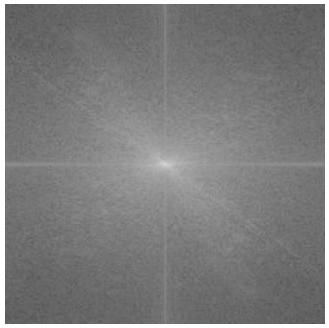
FFT

B. The *inverse* Fourier transform

Because the Fourier image and the real image contain essentially the same information, it is possible to generate a real image from its Fourier representation:

Before:

After:

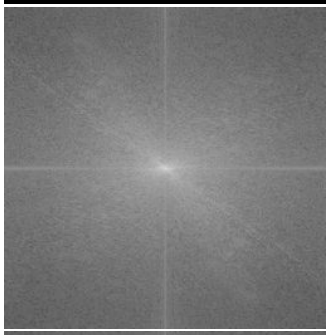


Changed her mind:

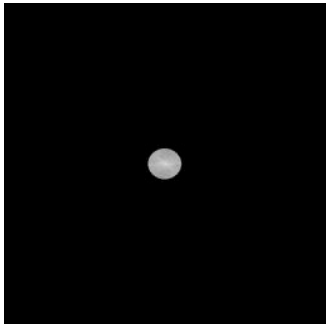
Basically, the same thing happens physically in a microscope. FT image is in the Back Focal Plane of Obj.!

Can use as a filter for detail:

FT

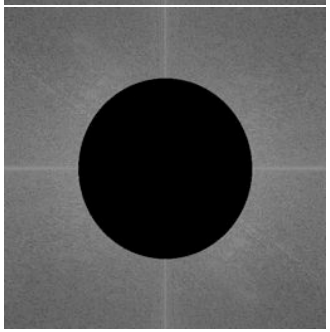


Low freq pass



IFT

High freq pass

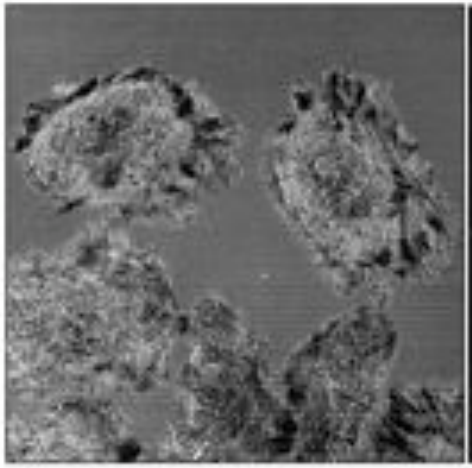


IFT

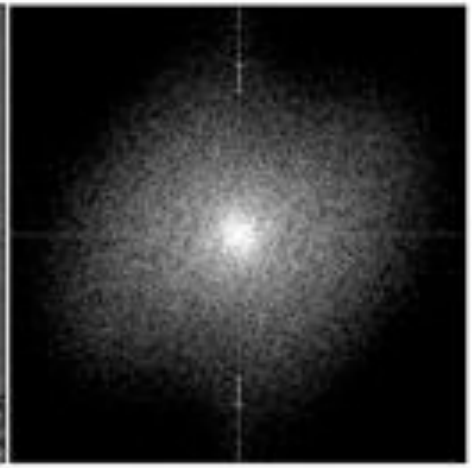
... a filter for periodic noise:

Laser intensity noise from a bad AOTF...

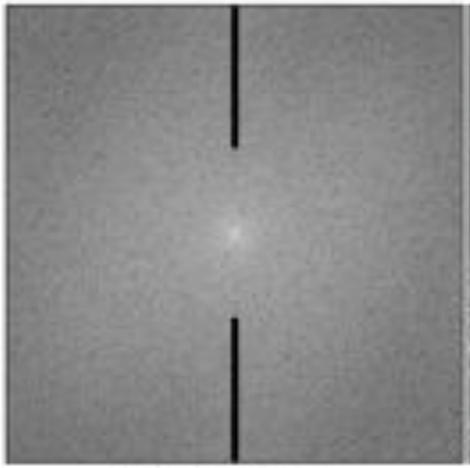
can be removed by frequency filtering in the correct spatial direction.



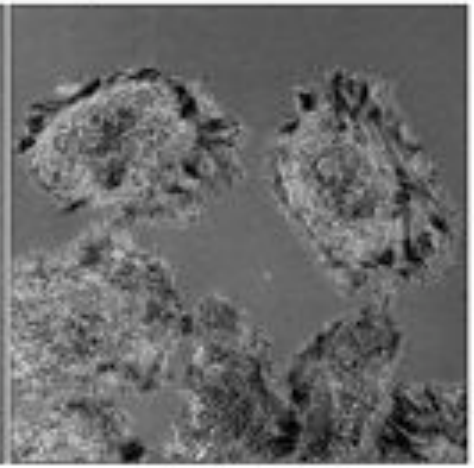
The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.



The power spectrum calculated by ImageJ, contrast enhanced to show the bright spots that represent the X axis fluctuation.



The power spectrum with masks drawn on it.



The inverse transform applying the masks.

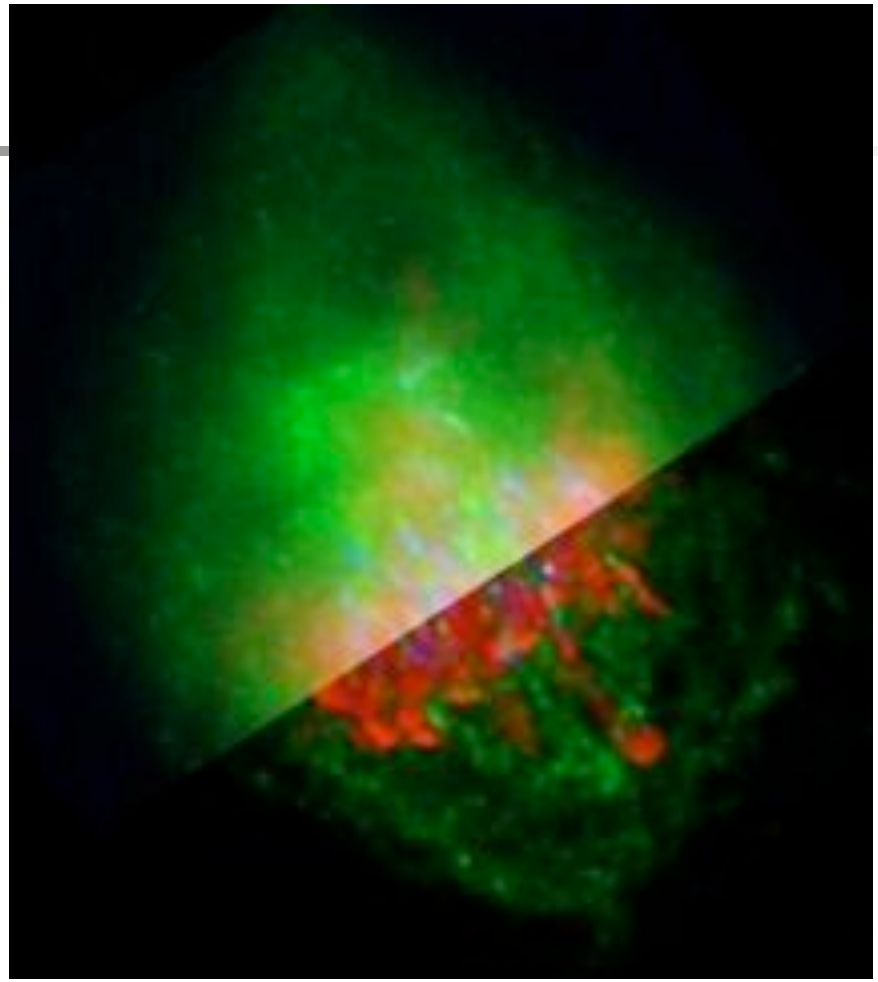
... during “Deconvolution”:

Take Image and PSF image

Do Fourier transforms

Image FT / PSF FT

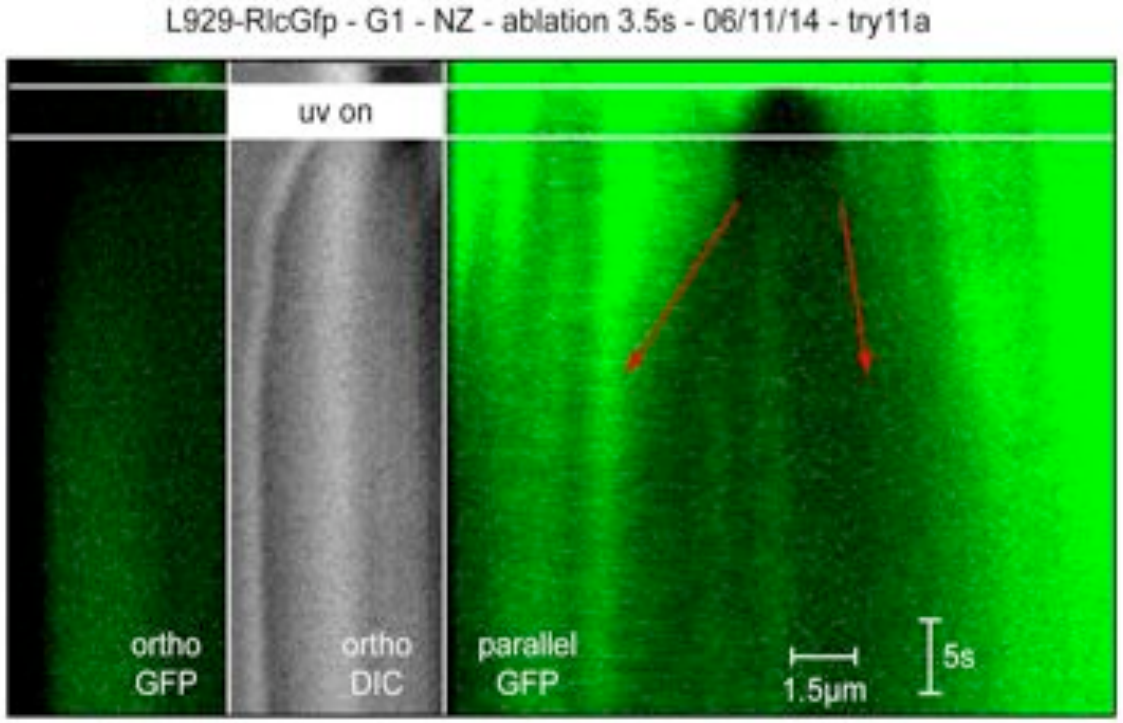
Reverse FT of result
=
Deconvolved image with much improved contrast and less out of focus signal.



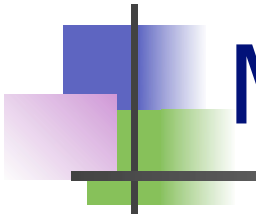
A metaphase human cell stained for DNA (red), centromeres (blue) and the anaphase promoting complex/cyclosome (green). Upper part: original data, Lower part: deconvolved with Huygens Professional. Recorded by Claire Acquaviva, Pines Lab.

Time? Just another dimension

Dealing with multiple images files (a.k.a. *stacks*): timelapse movies, 3D stacks, …



- Intensity over time
- Kymographs

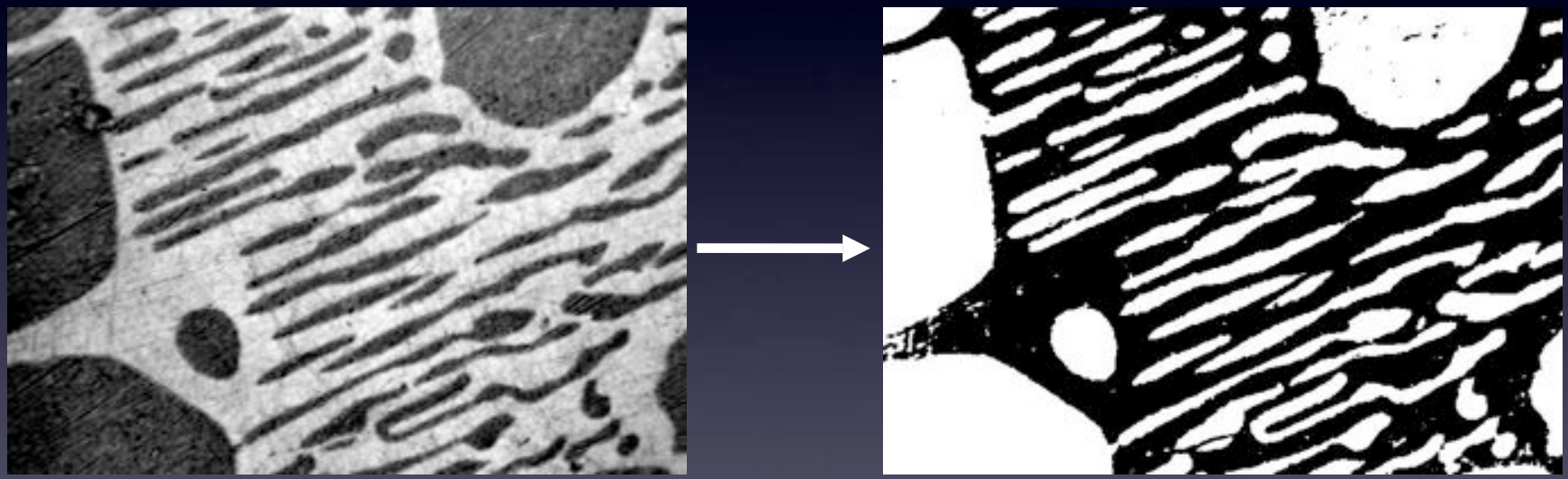


Motion blur

Motion blur = average over time

Does this happen in your sample? Frame Rate?

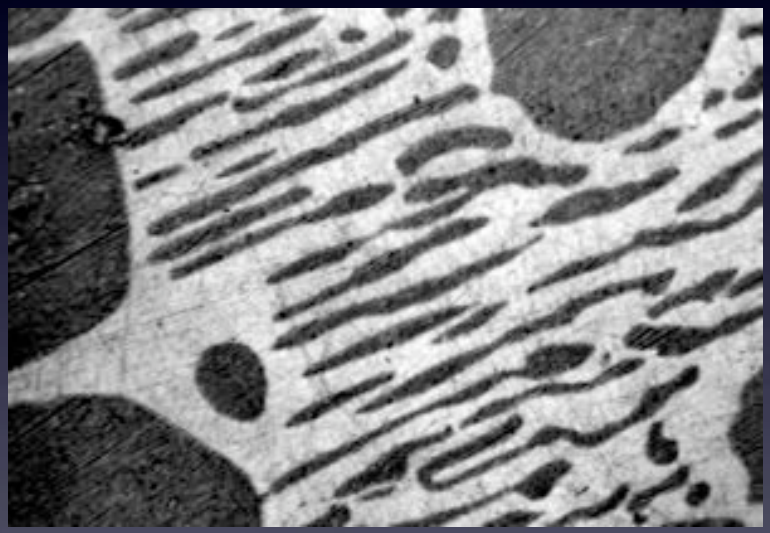
What is "Image Segmentation"?



"Greyscale" image

foreground background

What is "Image Segmentation"?



"Scalar Intensity" image

"Binary" image

What is “Image Segmentation”?

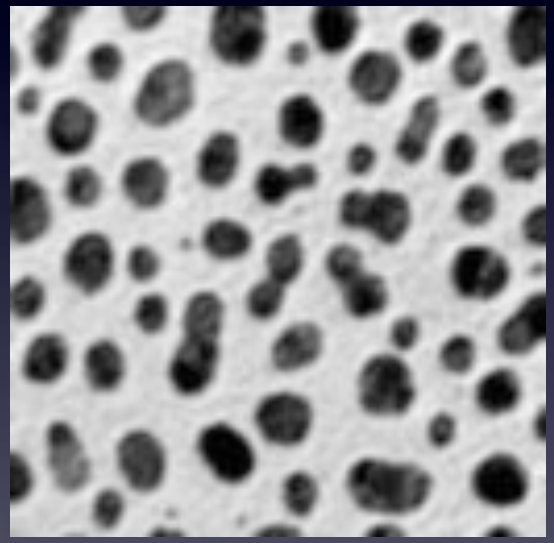
1	65	13	55	2
2	3	34	2	1
4	0	31	1	2
1	33	3	54	3
56	3	2	1	34

0	1	1	1	0
0	0	1	0	0
0	0	1	0	0
0	1	0	1	0
1	0	0	0	1

“Scalar Intensity” image

“Binary” image

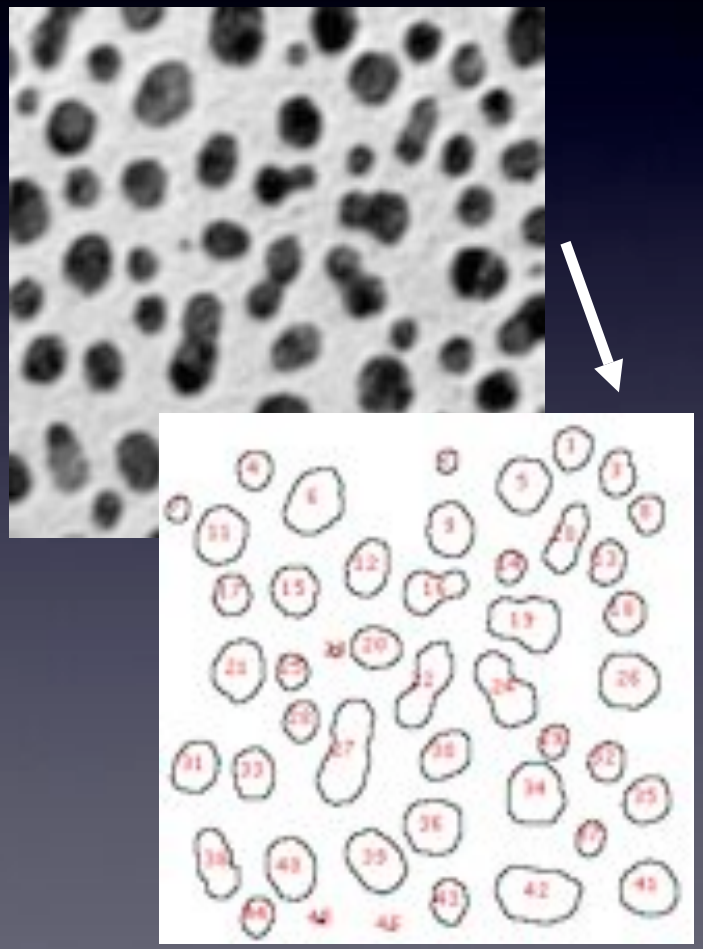
What is "Image Segmentation"?



"Scalar Intensity" image

"Labelled Objects"

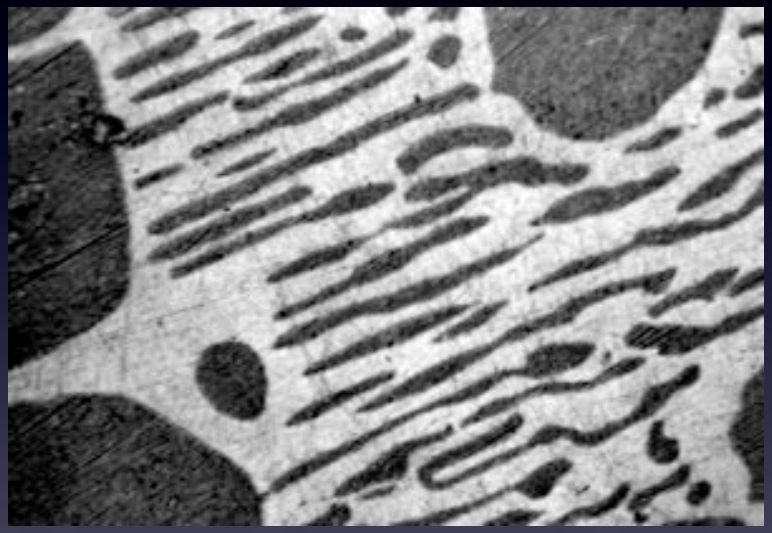
What is "Image Segmentation"?



High Information Content
65536 pixels, 0-255 value

Lower Information Content
But easier to interpret biological meaning:
45 "objects" with properties: size, shape, intensity etc.

“Thresholding” (Intensity Histogram Split)

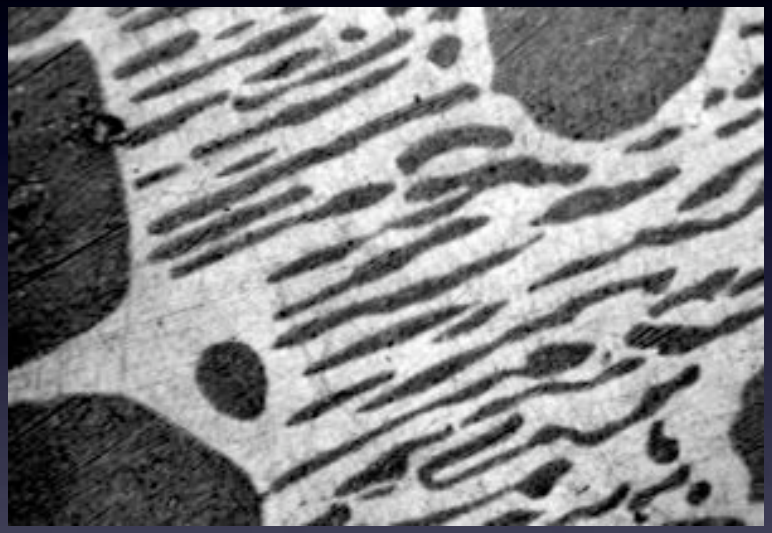


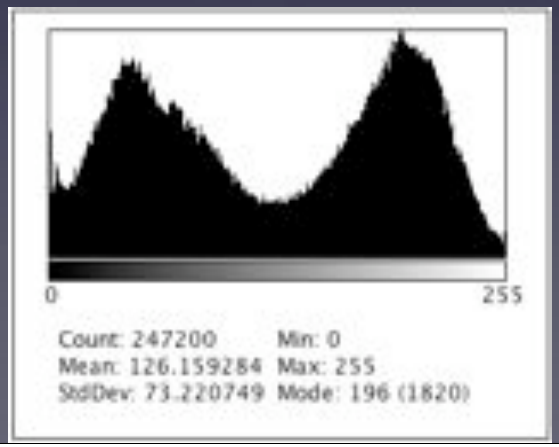
Clear difference between foreground and background?
Image not very noisy?

Choose an intermediate grey value = “threshold”

Determines foreground and background.

“Thresholding” (Intensity Histogram Split)



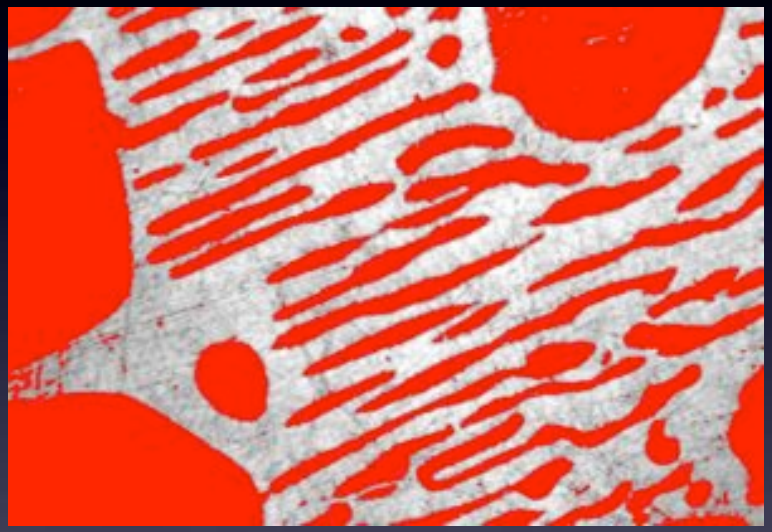


How to choose the grey level for thresholding?

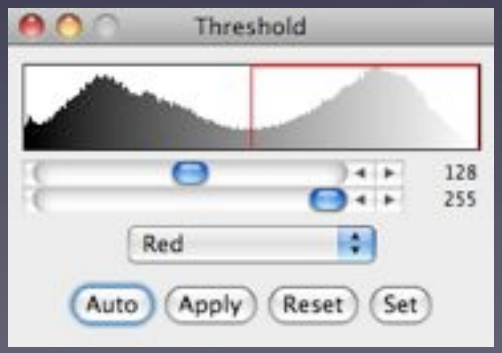
Look at pixel intensity histogram of whole image…

Is there an obvious place?

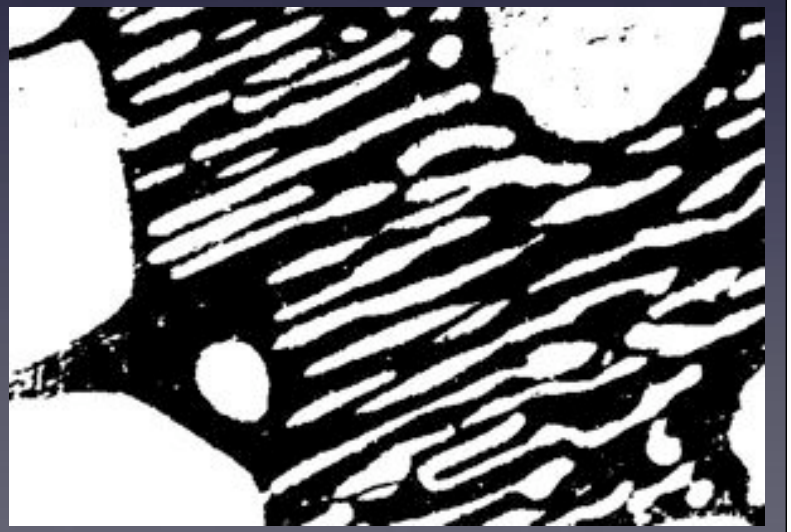
“Thresholding” (Intensity Histogram Split)



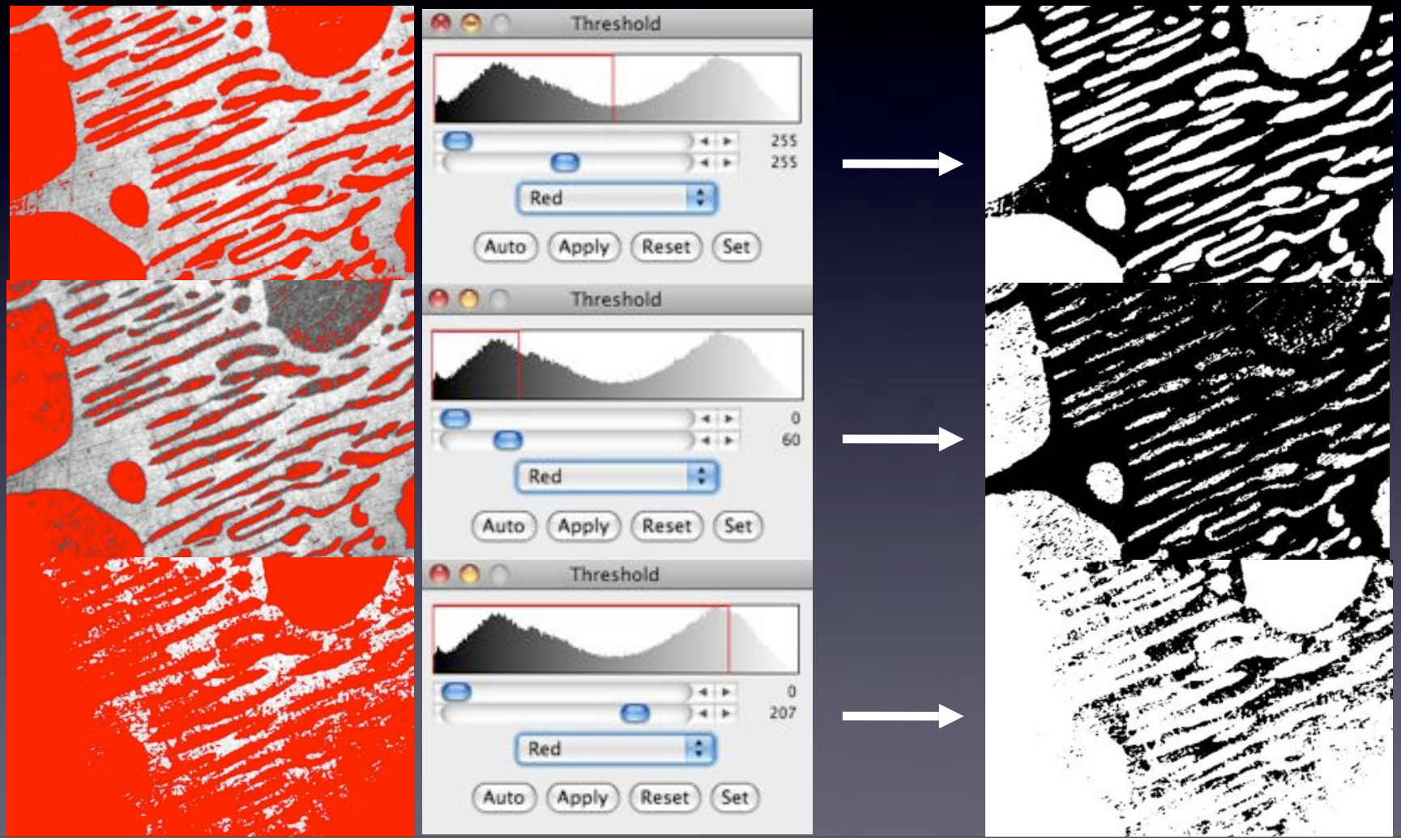
Histogram is bimodal, so put threshold in the trough between the peaks!



Note, in this case:
Foreground = “dim” objects
Background = “bright” objects



“Dumb Global Threshold”
(Subjective - User Biased)



Computed Global Threshold
Objective - Reproducible

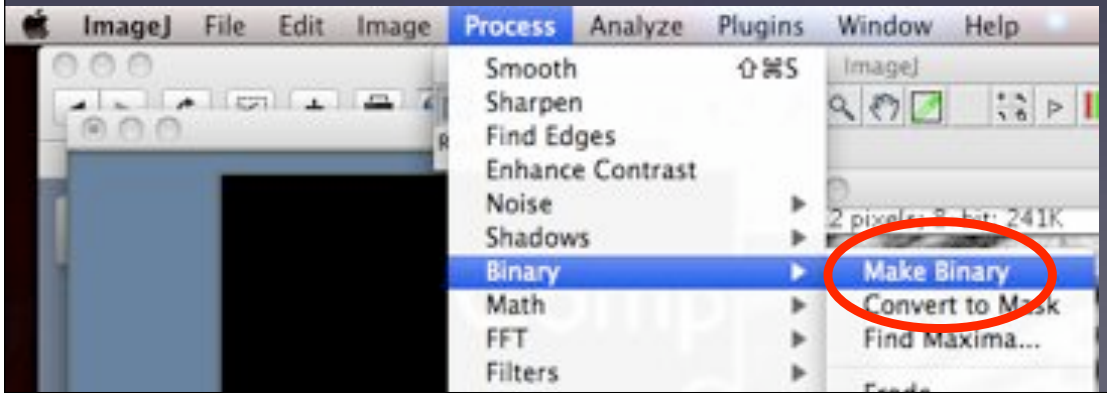
ImageJ - Auto Threshold (and Make Binary):

Initial guess of Threshold, T

Compute mean pixel intensity of background and foreground

Tnew =
0.5 x (mean of foregrnd + mean of bkgrnd)

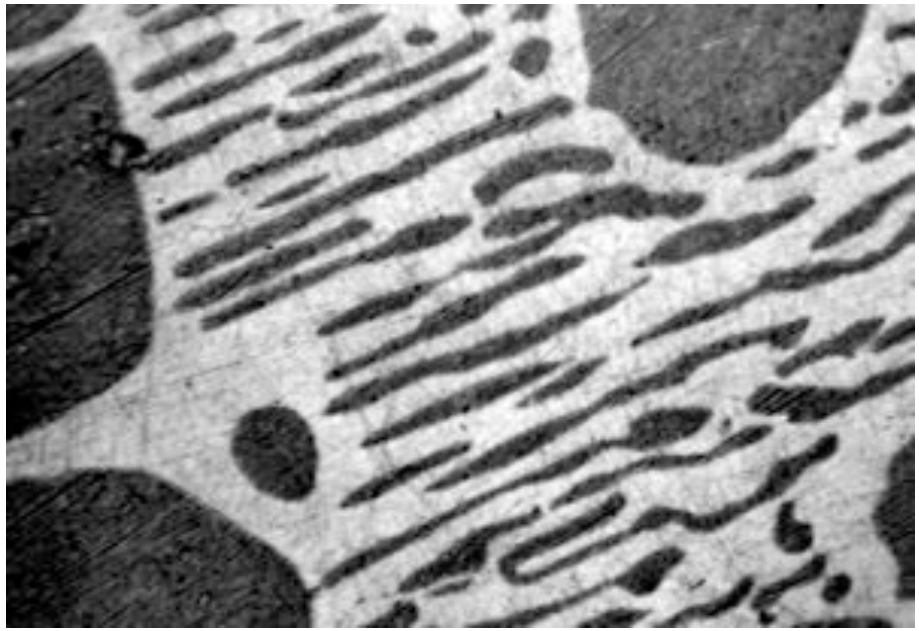
Iterate until Tnew no longer changes.



Note:
Manual threshold set?
Make Binary uses
that dumb threshold!

Also see “Otsu”, “K-means Clustering”, “Maximum Entropy”, “Mixture Modelling” and others.

Edge Detection: The Sobel filter



- Images may contain objects
- The objects have edges
- How can we find the edges?

Edge Detection

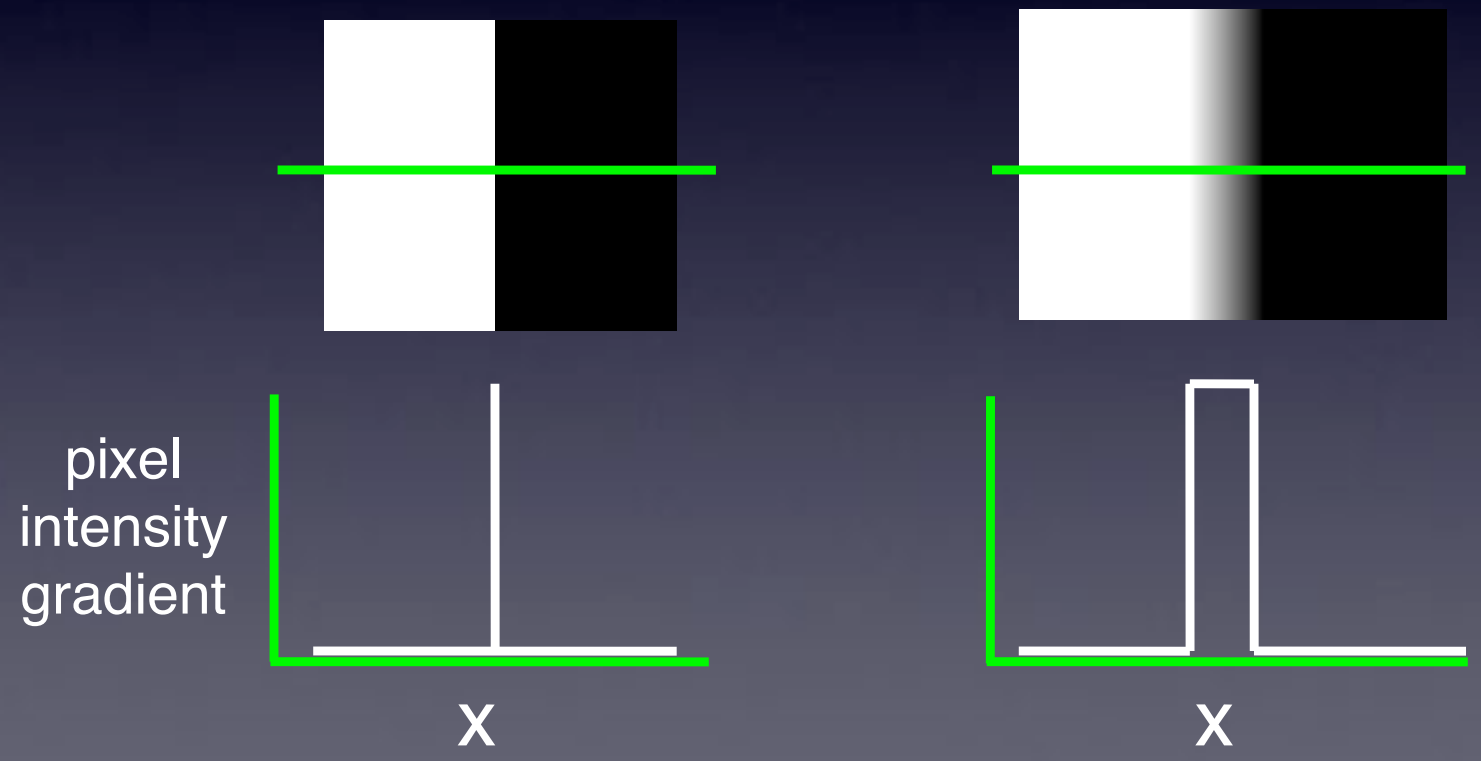
What is an “edge” ?

- “Hard Edge” - Adjacent black - white pixels
- “Soft / Fuzzy Edge” - common in images
  - Especially for small diffraction limited objects (vesicles / membranes)
  - Noise makes edges look softer

Edge Detection
”Image Gradient”

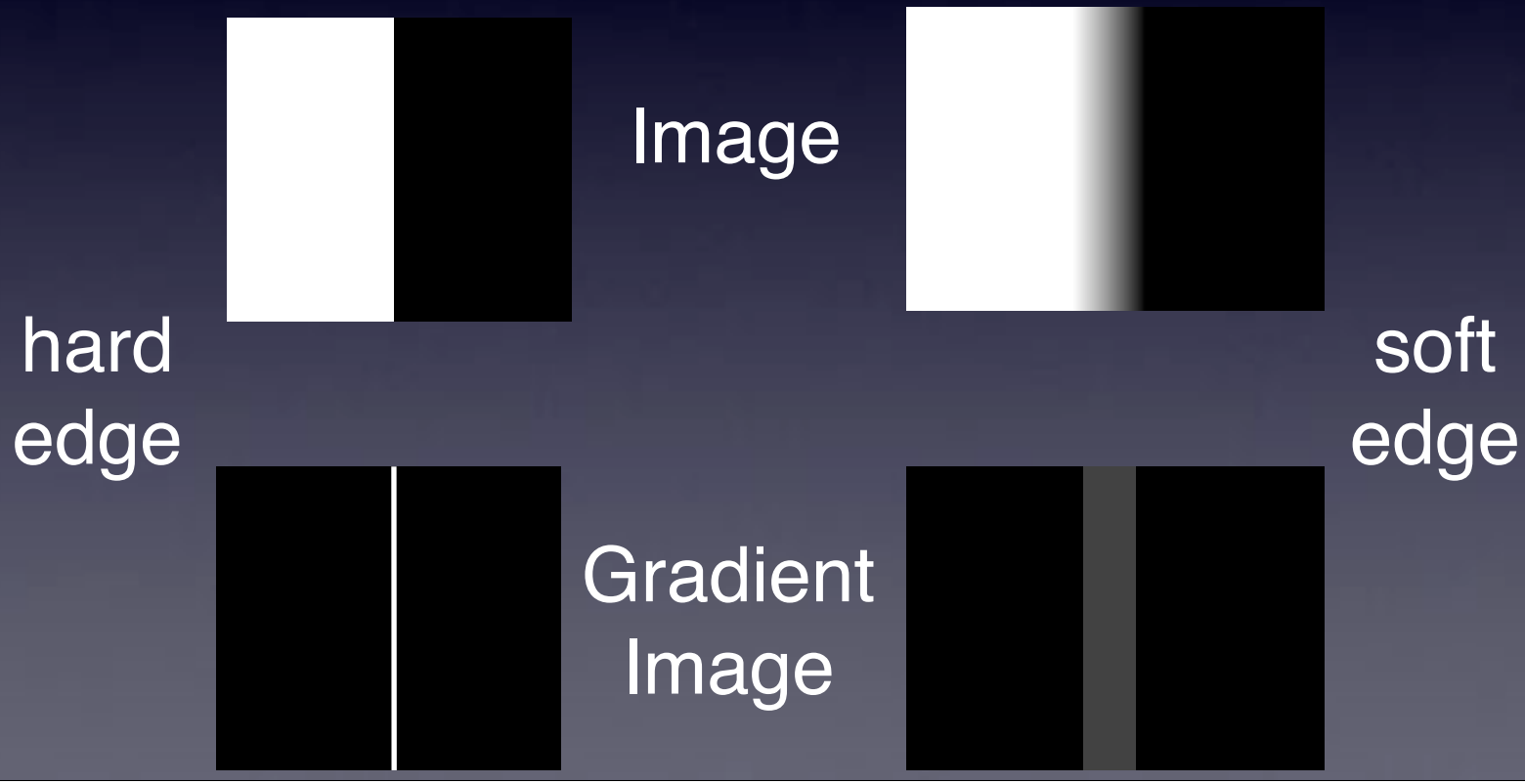
What is a “Gradient Image” ?

Rate of change of pixel intensity (1st derivative)



Edge Detection
”Image Gradient”

What is a “Gradient Image” ?
Rate of change of pixel intensity (1st derivative)



”Image Gradient” - How?

Sobel filter - 3x3 convolution filters in x AND y

- find edges with x and y components
- compute total gradient magnitude
- approximates 1st derivative of image

-1	0	+1
-2	0	+2
-1	0	+1

+1	+2	+1
0	0	0
-1	-2	-1

$$\text{output} = \sqrt{g_x^{\,2} + g_y^{\,2}}$$

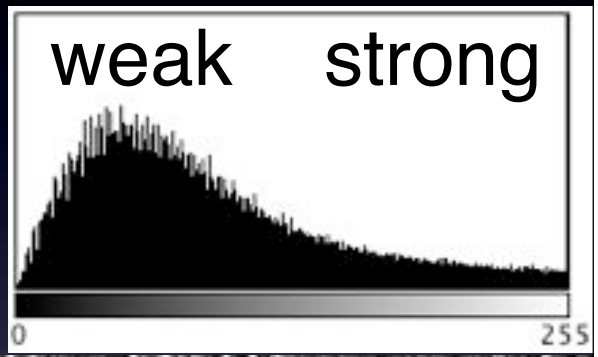
$$|\, g_x \,| \quad + \quad |\, g_y \,| \quad = \quad |\, g \,|$$

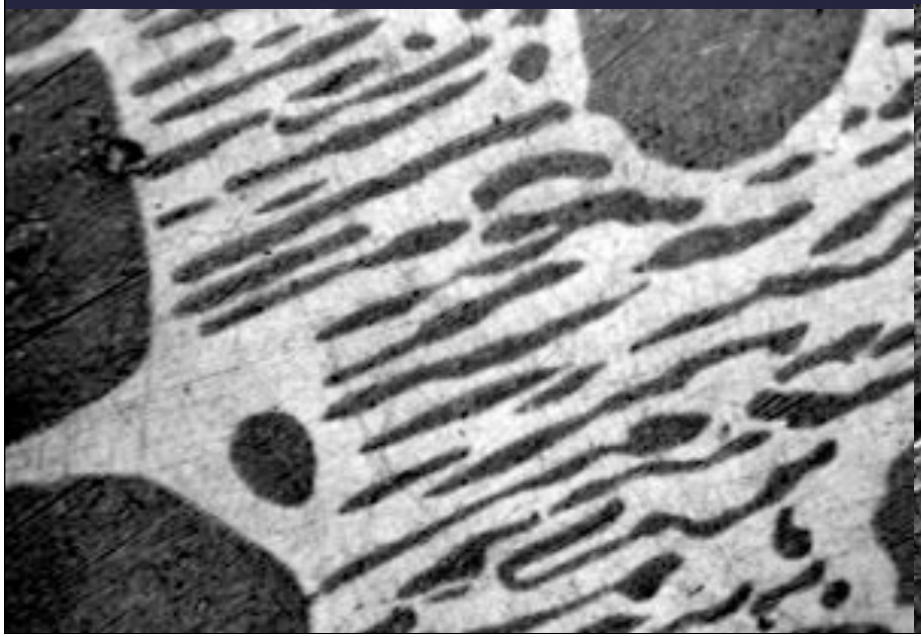
Gradient Image - Real Sample:

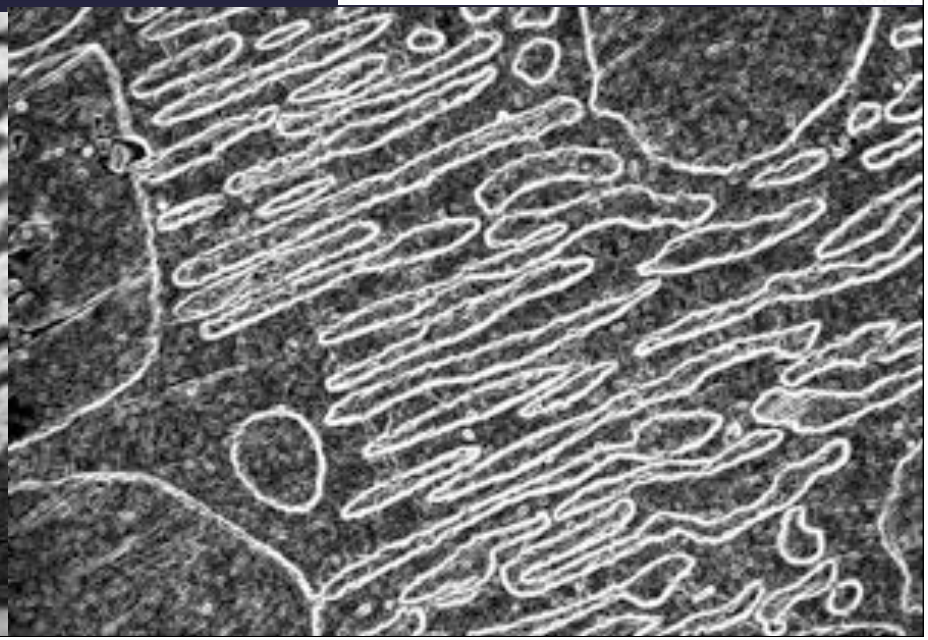
Real / Biological images:

- Sobel filter
- many edges
- many weak edges from noise

gradient image histogram



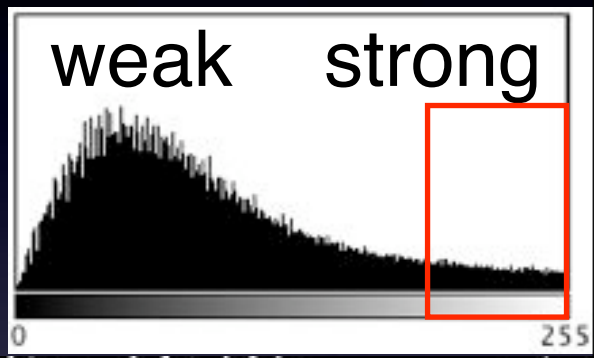


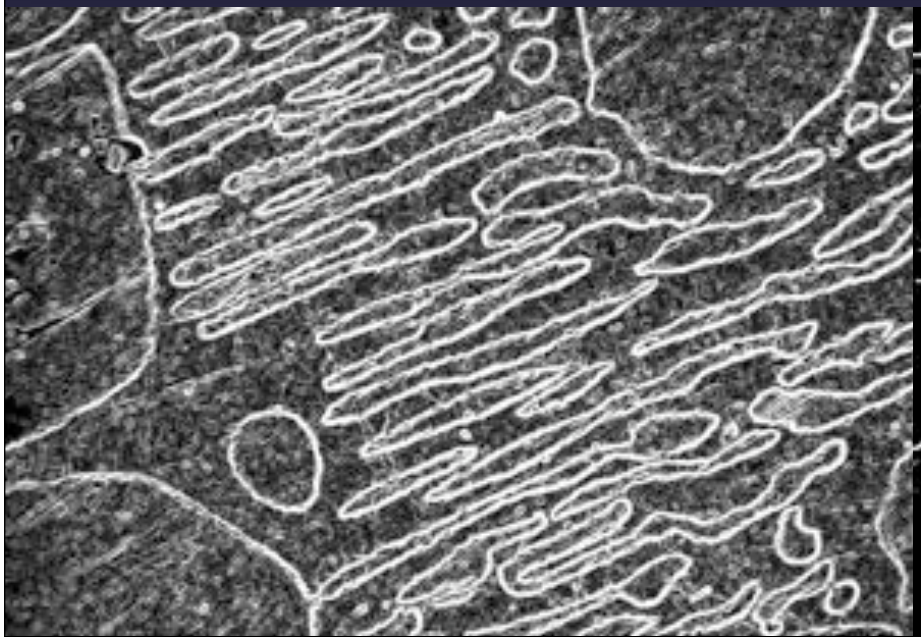


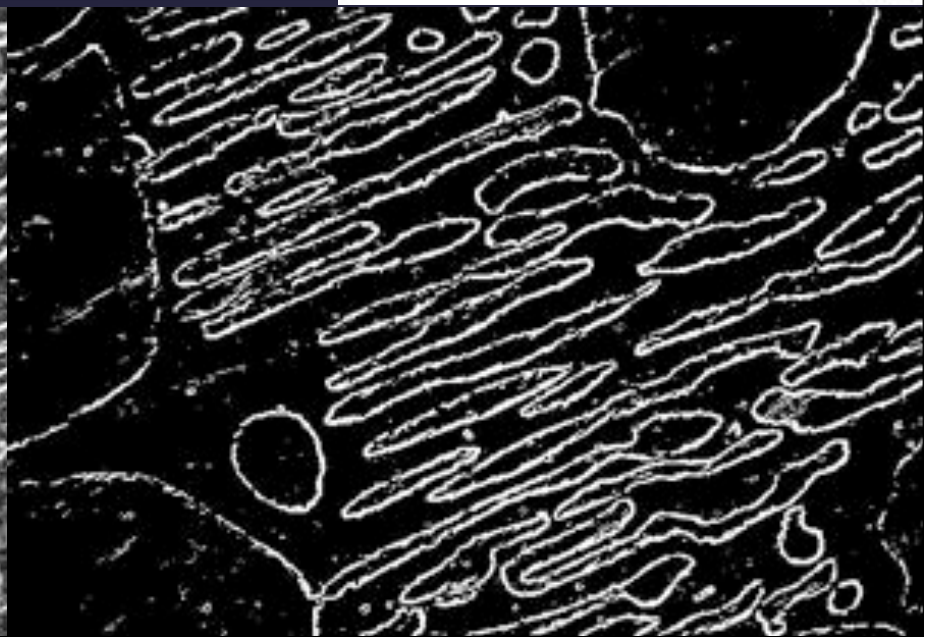
Gradient Image - Strong Edges?

Remove weak edges?

- Threshold the gradient image
- Smoothing filter beforehand

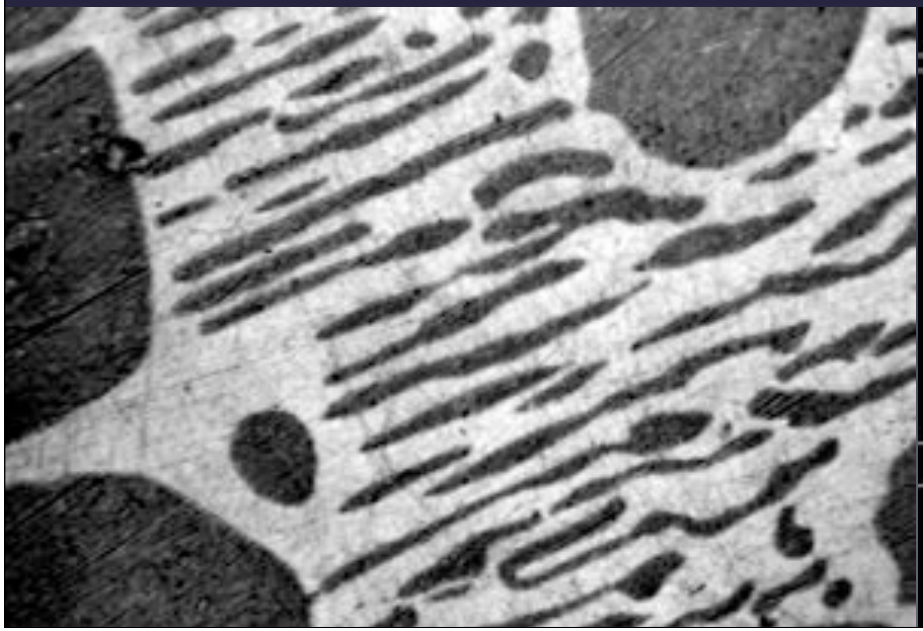


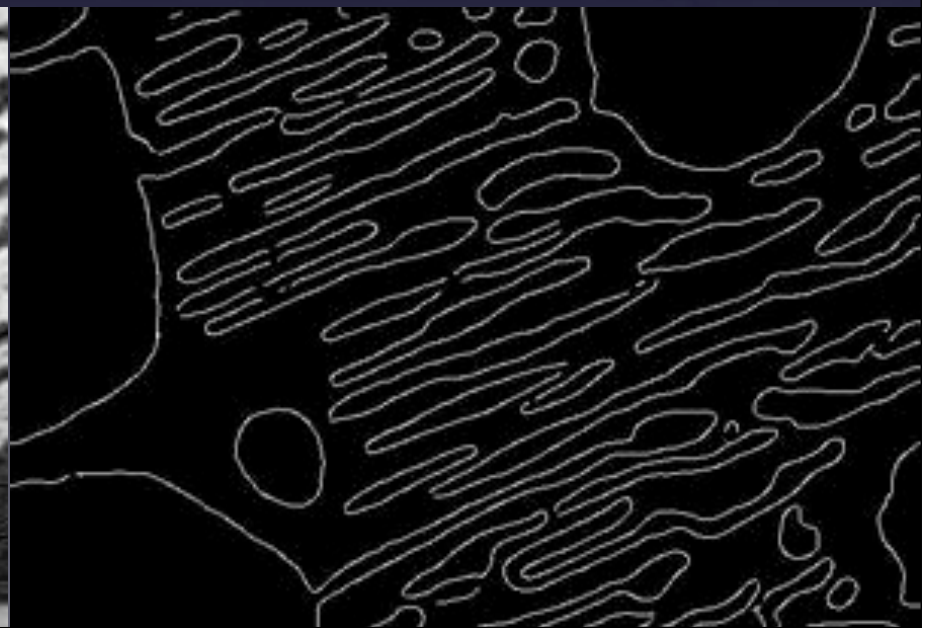




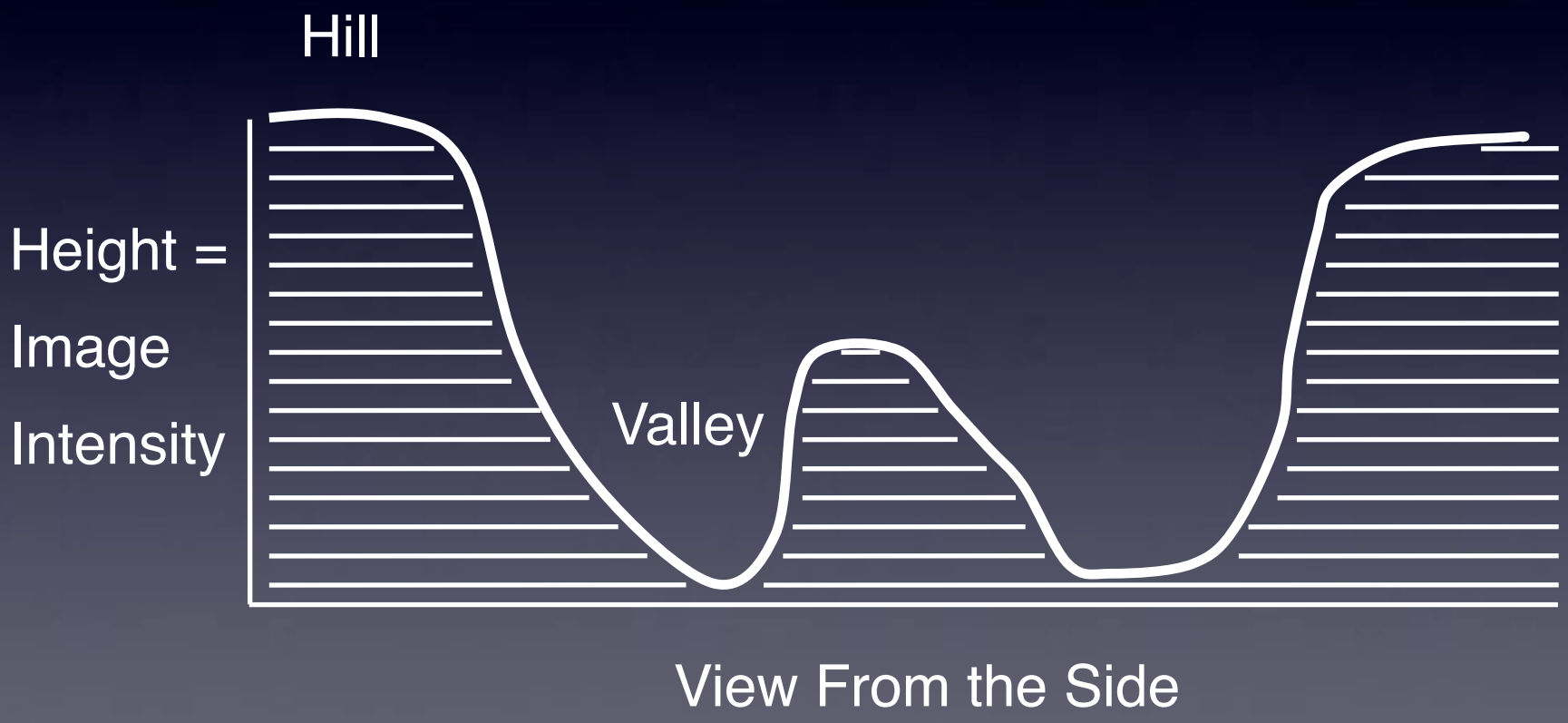
“Canny” Edge Detection

- Remove weak/noisy edges - keep strong
  - Gaussian smooth image + hysteresis threshold gradient image
- Make edges sharp - 1 pixel wide
  - Non maximal suppression of gradient image

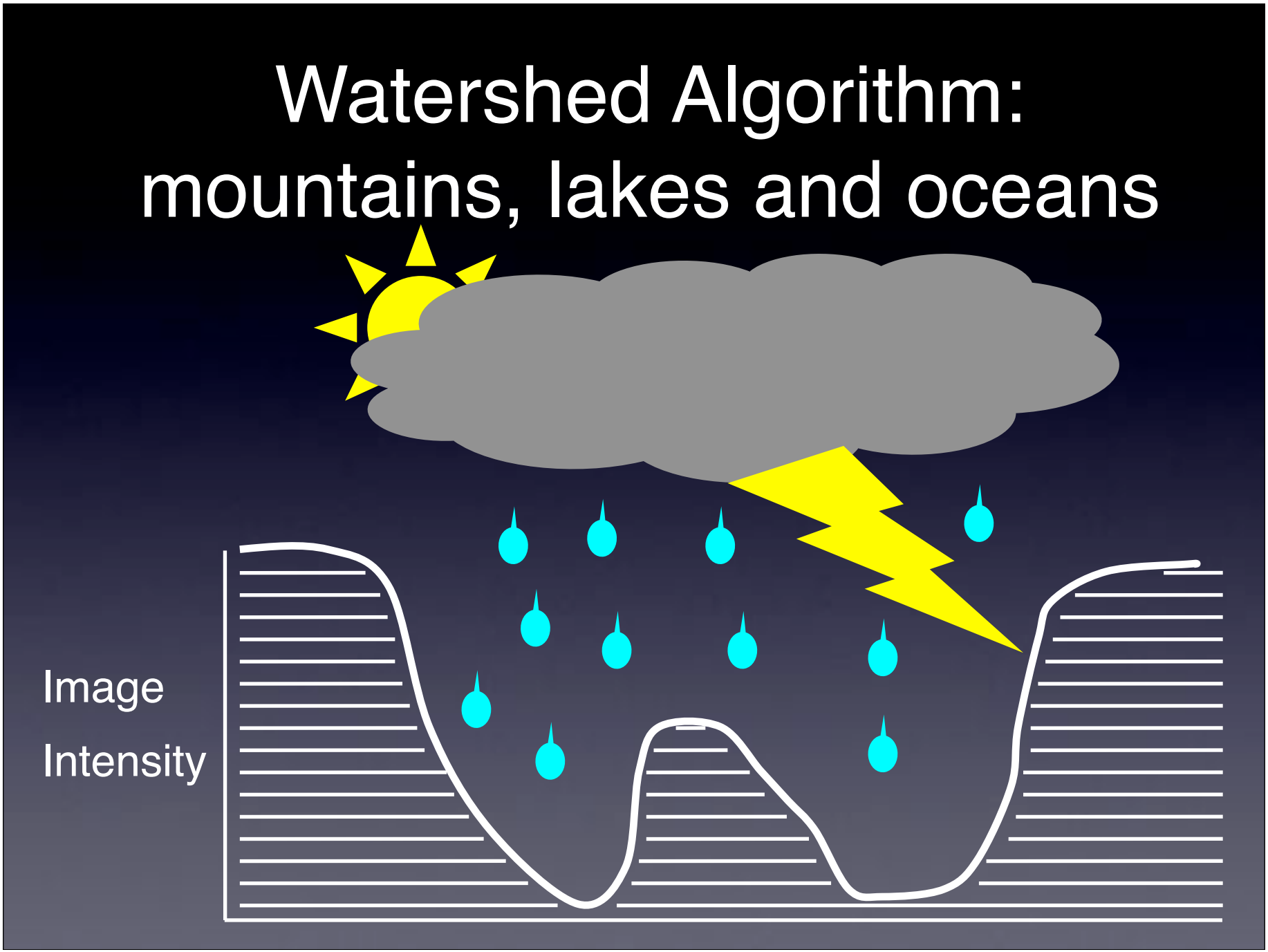




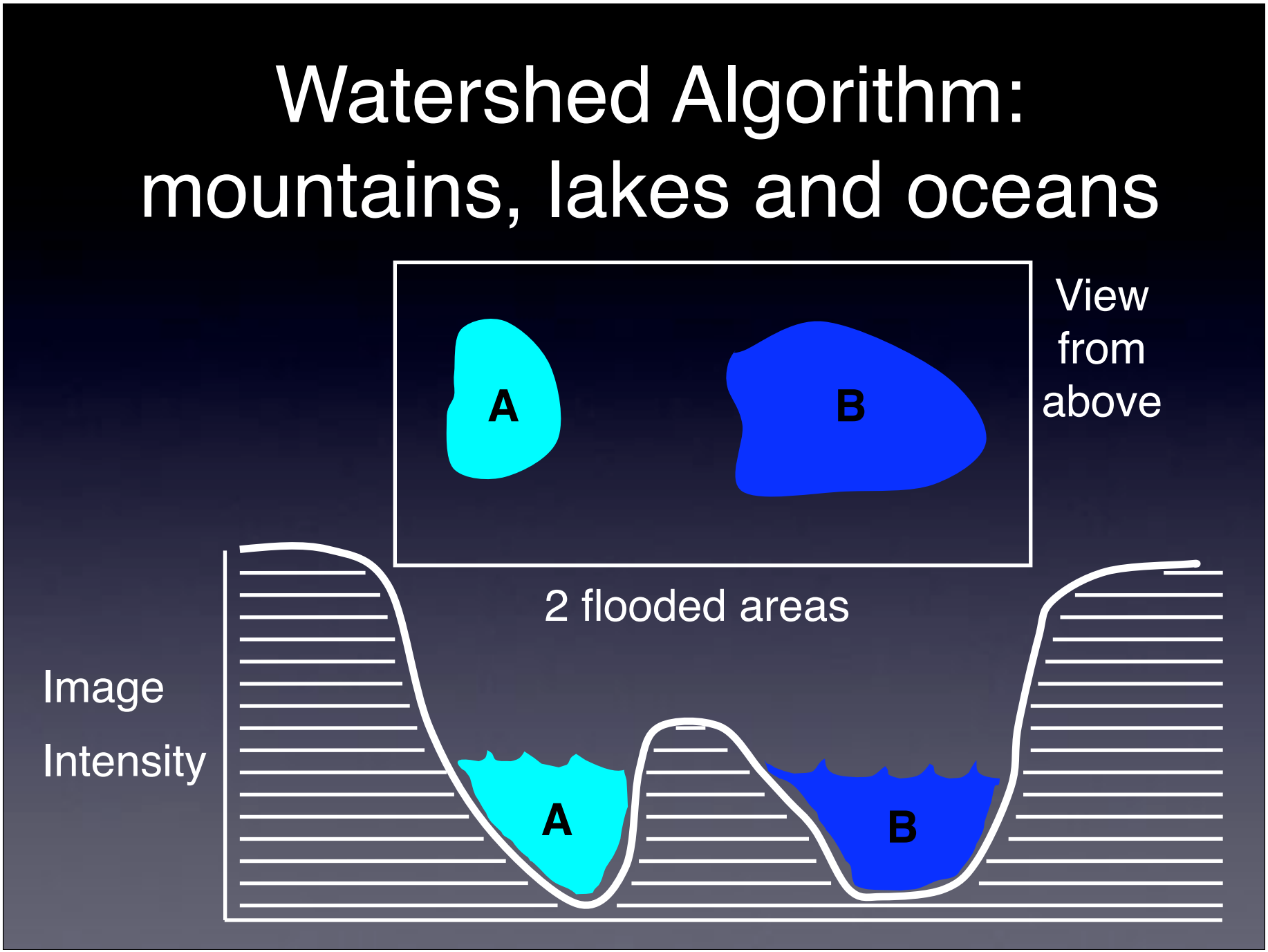
Watershed Algorithm: mountains, lakes and oceans



Watershed Algorithm: mountains, lakes and oceans



Watershed Algorithm: mountains, lakes and oceans

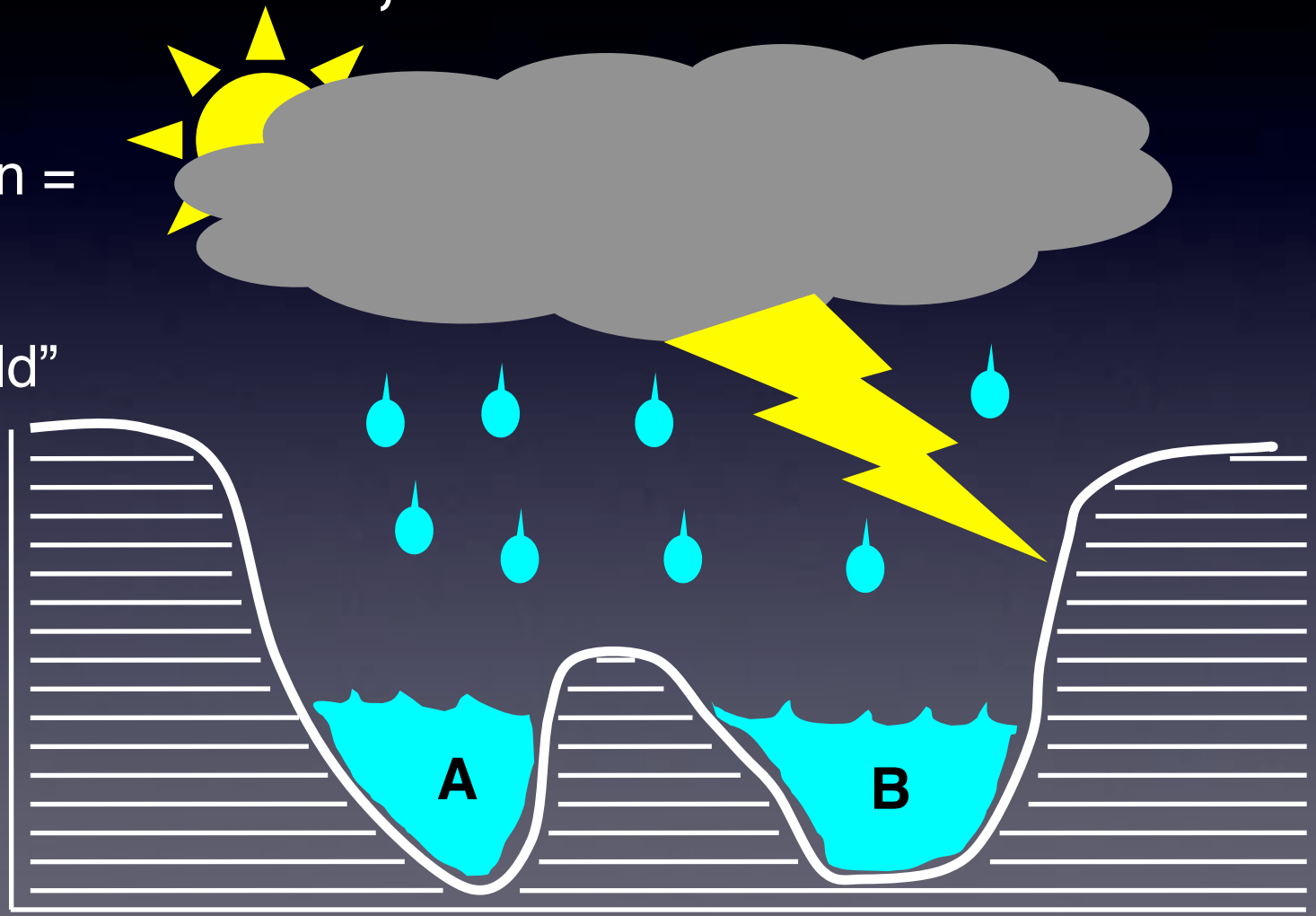


Watershed Algorithm: mountains, lakes and oceans

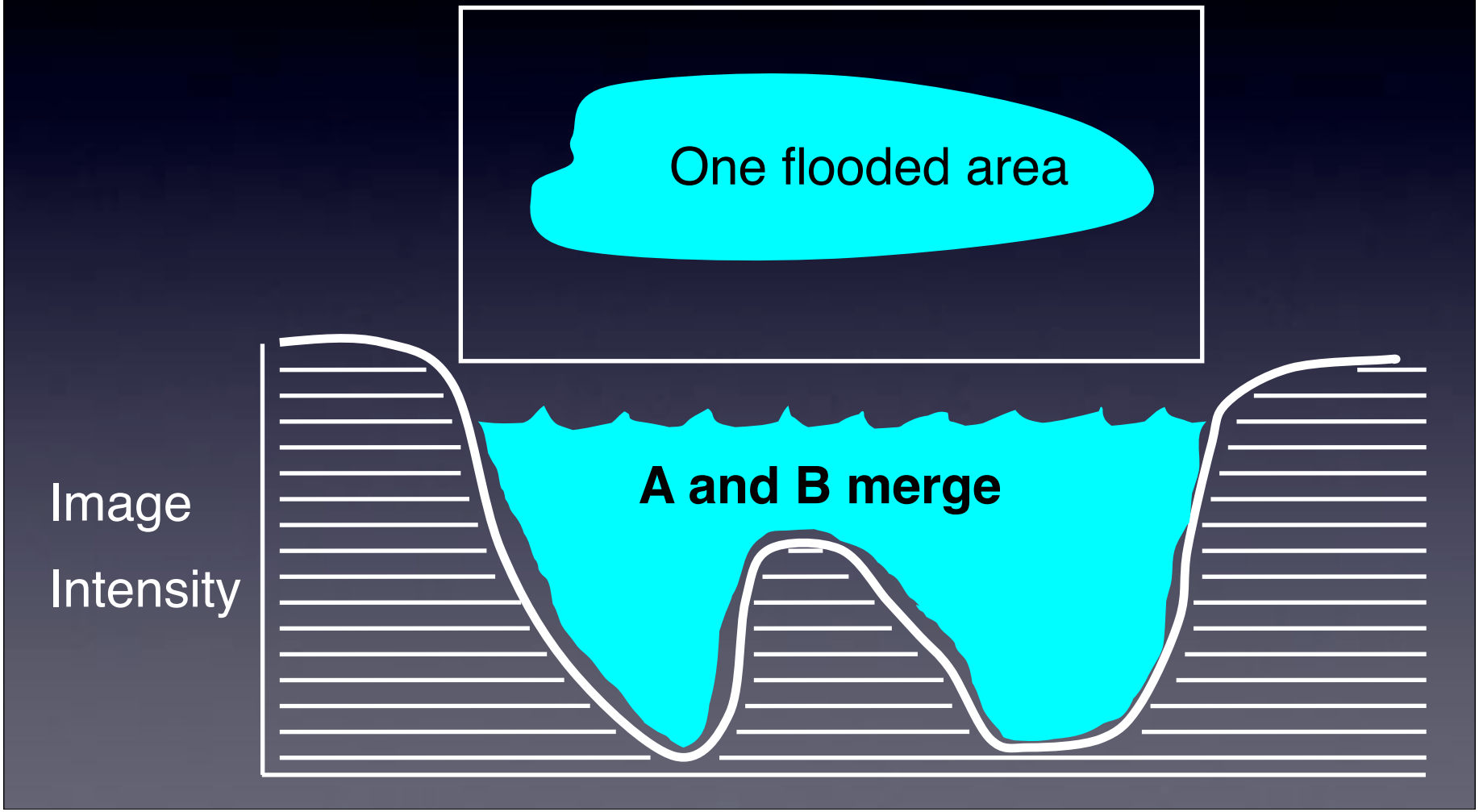
More rain =

increase

“threshold”

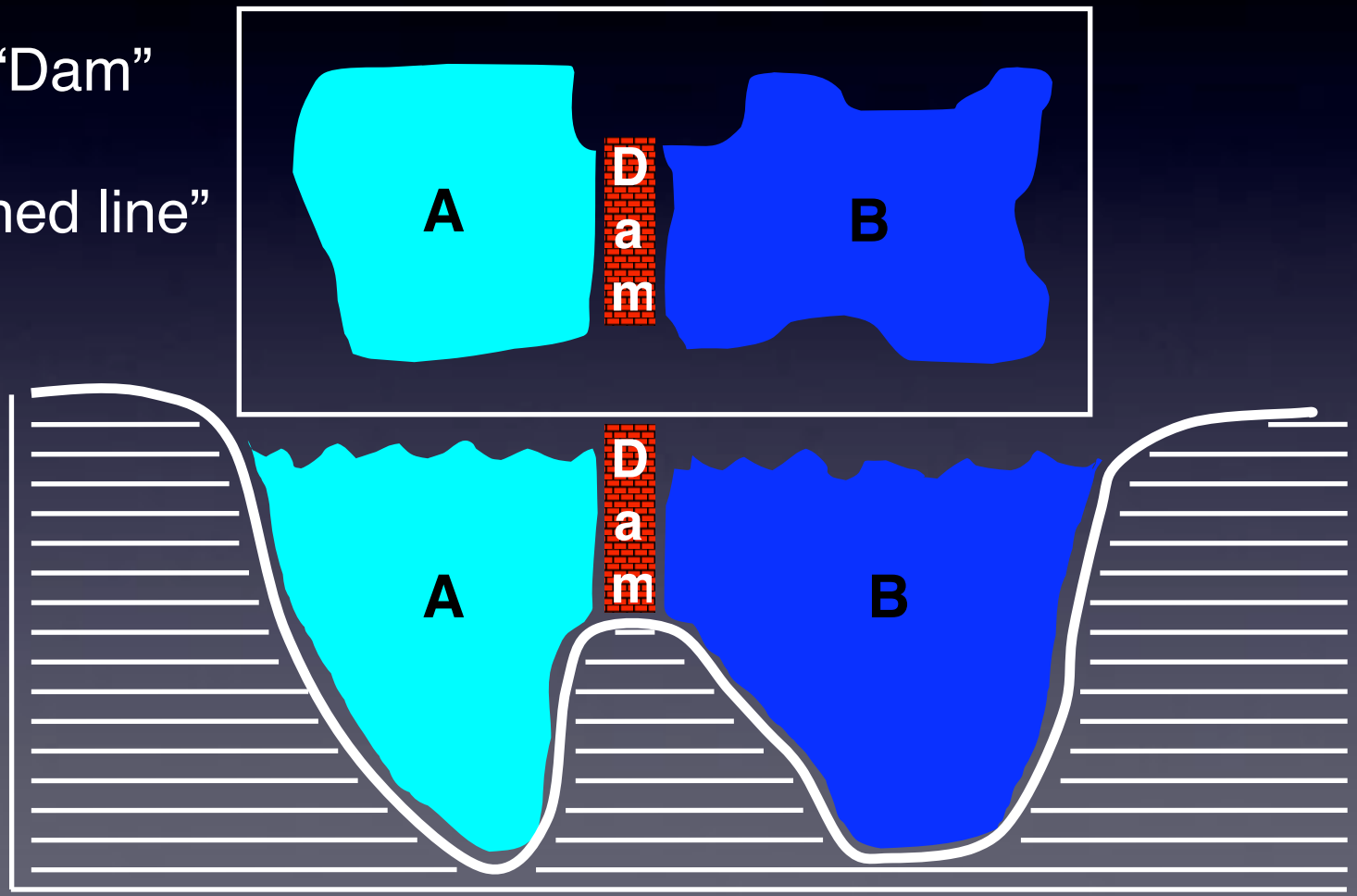


Watershed Algorithm: mountains, lakes and oceans



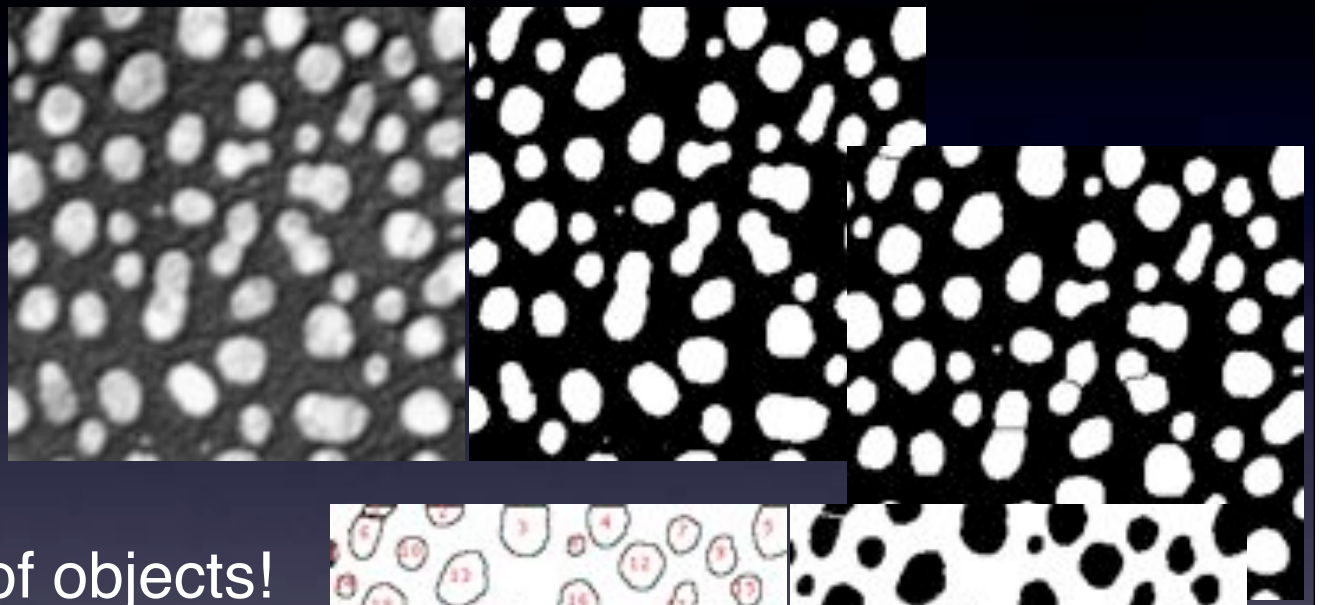
Watershed Algorithm: mountains, lakes and oceans

Make a “Dam” at the “Watershed line”



Watershed to find object number

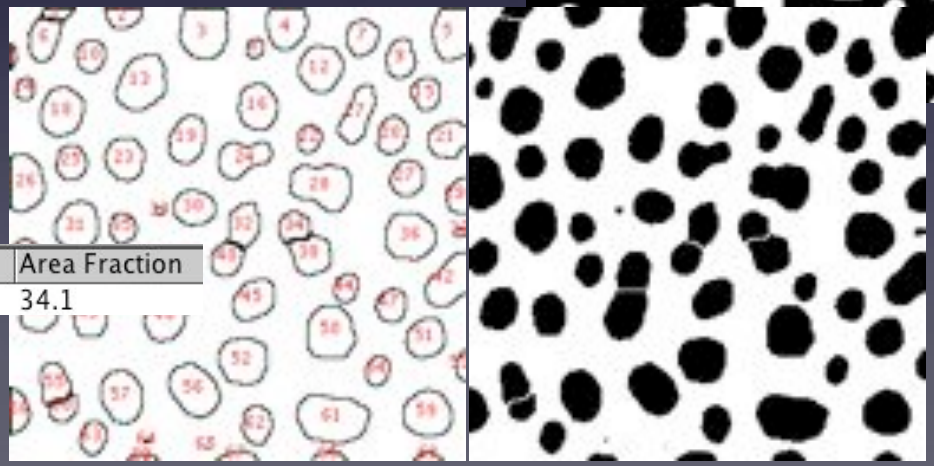
- Blobs.gif
- Make Binary
- Watershed
- Invert
- Analyse Particles



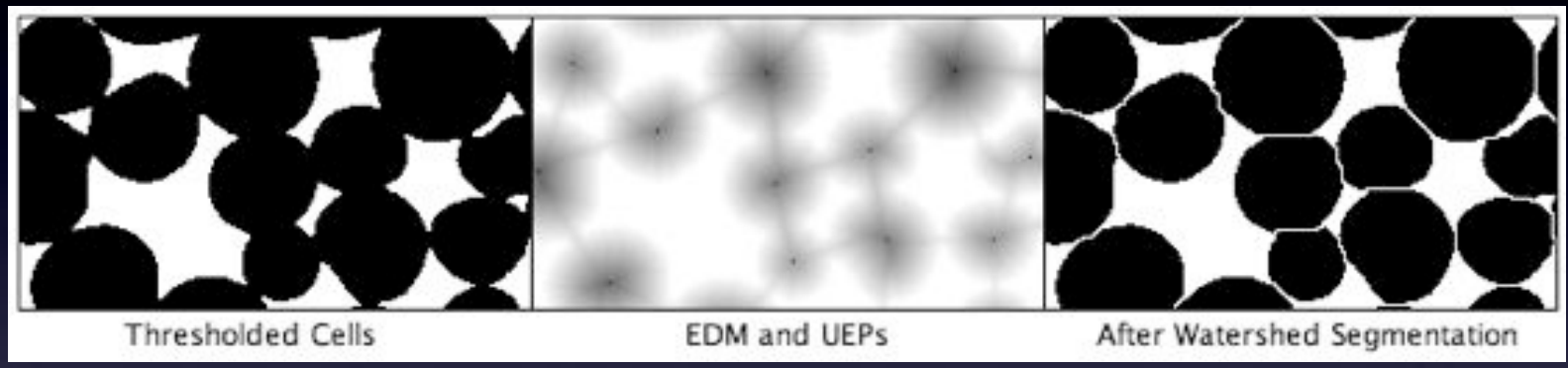
- Gives number of objects!

(imagine there were too many to count by hand, eg Many Cells)

Slice	Count	Total Area	Average Size	Area Fraction
blobs-bin-WShed-inv.tif	69	22159.000000	321.144928	34.1



Watershed to separate touching objects



- Euclidian Distance Map
- Ultimate Eroded Points
- Fill with water from UEP
  - until hits edge of object, or dams between objects

Practical Session 3

- Simple Image Filtering
  - Mean / Median Filter (change no of pixels)
    - Open Samples - Neuron
  - FFT , filter, Inverse FFT
    - mess up Bridge
- Simple Image segmentation - Blobs (inverse LUT)
  - Manual threshold - make bin
  - Auto thresh - make bin
    - Image - Adjust - Threshold
  - Watershed
    - Make bin - Watershed
  - Analyse objects

Links and Further Reading

- Standard Text Book

  Digital Image Processing 2nd Ed.
  Gonzalez and Woods, Prentice Hall
- Image Processing Facility
  - Intranet - Services and Facilities - Image Processing Facility
  - Wiki - info for beginners - tips - software documentation
  - https://zope.mpi-cbg.de/intranet/services/image-processing-facility
- ImageJ
  - Fiji - http://pacific.mpi-cbg.de Fiji Wiki and docs.
  - http://rsb.info.nih.gov/ij/ ImageJ home
  - MacBioPhotonics plugins collection
    - http://www.macbiophotonics.ca/downloads.htm
- Email: ipf@mpi-cbg.de