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

Basics of Quantitative Image Analysis

or

What you need to know about Image Processing...

but never thought to ask.

MPI-CBG
Image Processing Facility
October 2009

(Practicals Featuring Fiji – is just ImageJ – batteries included)

CBG
Max Planck Institute of Molecular Cell Biology and Genetics

IPF

Before you start writing…

See these slides at:

https://ifn.mpi-cbg.de

under: Teaching

(online also contains practical exercises)

Topics:

- Imaging Experiment Workflow
- Images Contain “Information” - Digital Images.
- What is a pixel?
- ... we also need info “about” the image = Meta Data
- Different ways to visualise / display an image’s information

Also available on the Fiji Wiki

- Fiji is just ImageJ – batteries included http://pacific.mpi-cbg.de
- Fiji tutorials
- DetectInfoLoss, ColocalisationAnalysis and more...
- Whatever you find interesting...

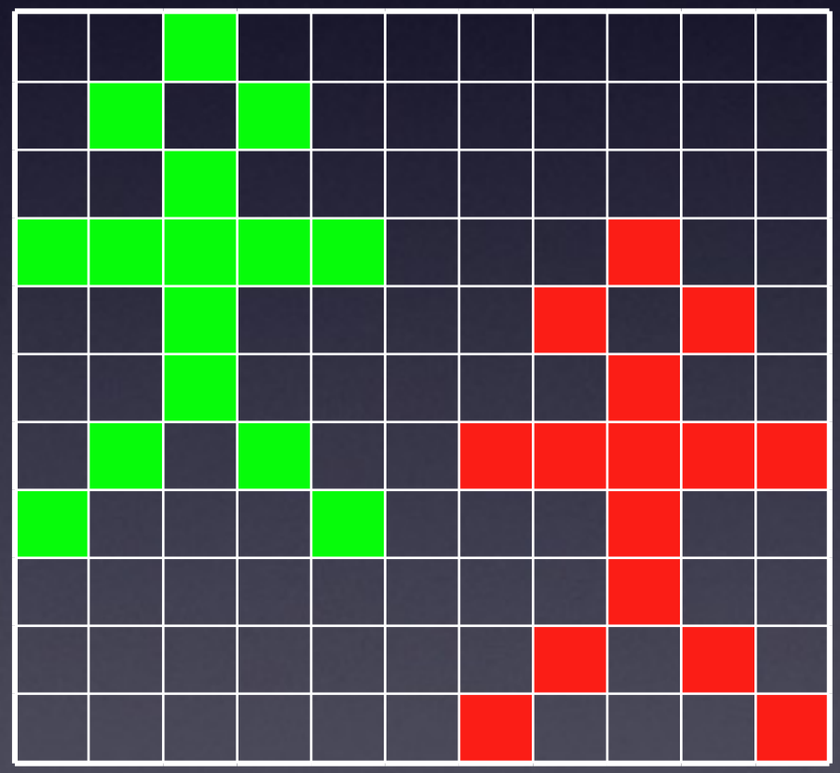
Practicals are included in online version...

Quantitative Imaging?
…what does that mean?

Art or Science? Photography or Spectroscopy?

Science = measure something!

- Numerical Results
- Statistics!
- Computers become useful...



What is Image Analysis / Quantif cation?

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
Std.Dev. 93.2
Area 10x14
Pix 140
Pix <25542

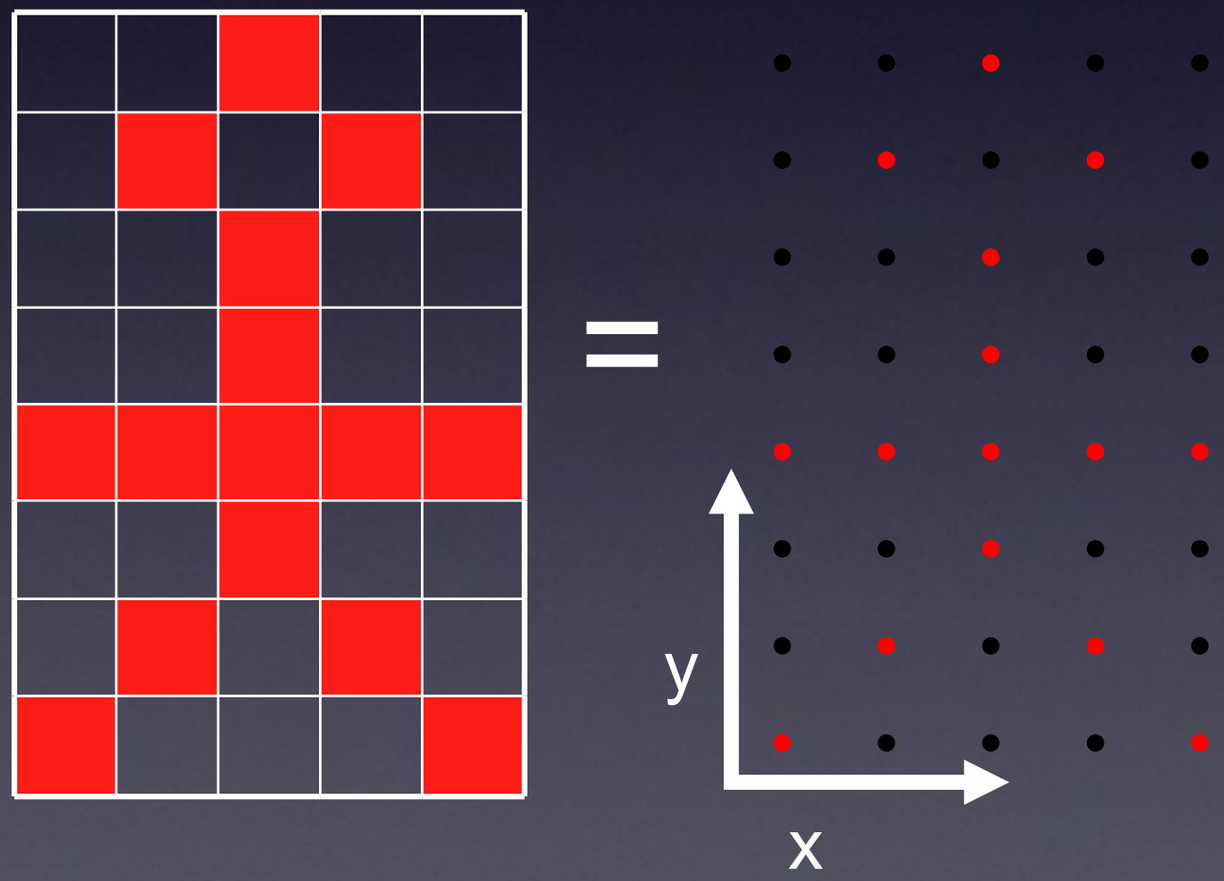
= Image Analysis?
or
= Image Measurement?

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

= Interpretation of Analysis result?

What is a (Digital) Image anyway..?

- An image is only a representation of reality
  - An image is NOT a “real” copy of the object
    - It’s an artifact! Less info in the image than the object.
  - Image of a point is NOT a point (Point Spread Function)
  - Space/Intensity/Time - Digitised by detector, CCD or scanner
- Digital images are NOT analogue art - it’s just numbers!



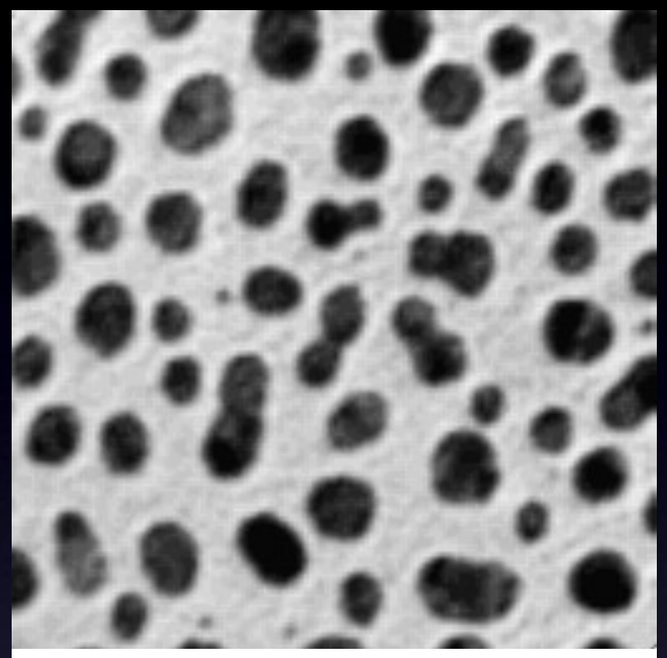
A digital image of ???

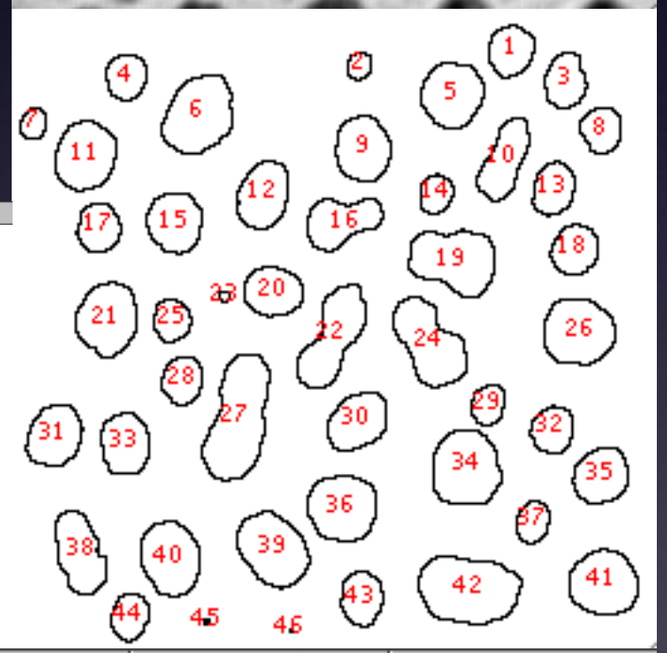
Image Analysis
(Brain or Computer)

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

Image = Information

- Images contain information!
  - Quantify / Measure / Analyze
  - 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

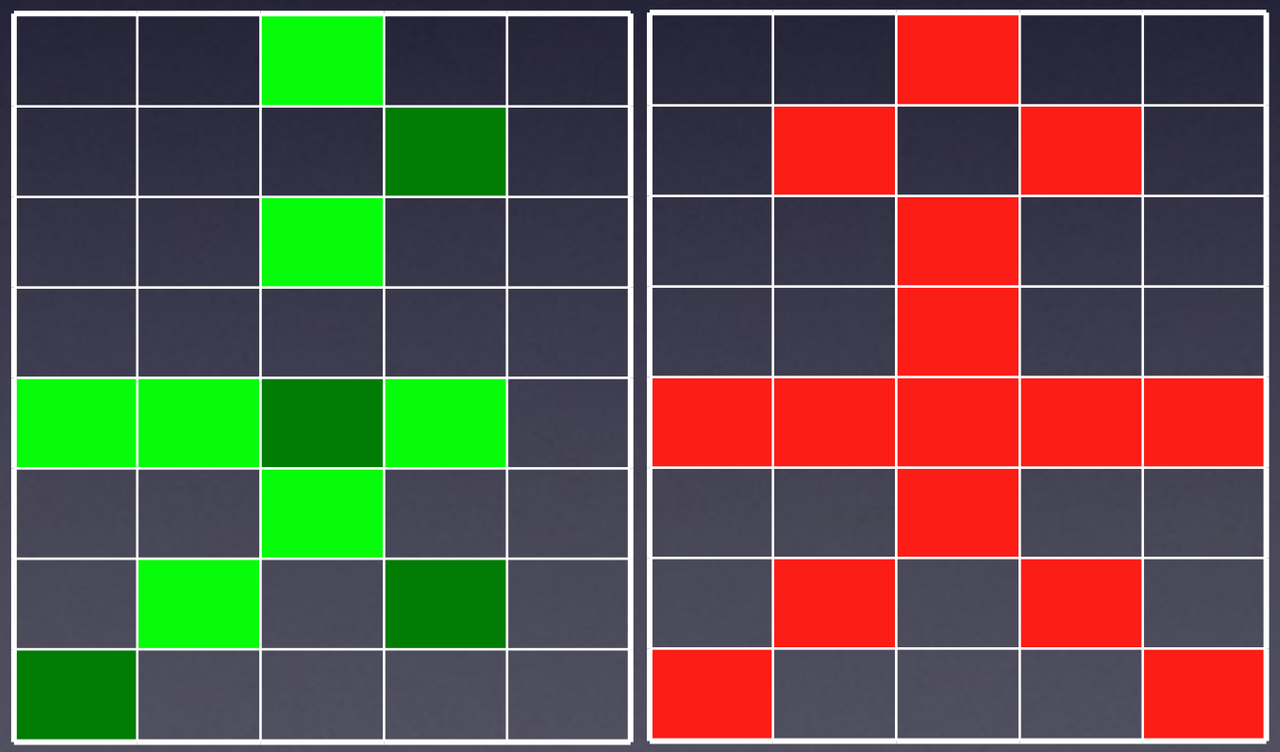
A digital image:
How many objects?
How “bright” are they?
How big are they?
What are they?
etc.

Image Data? What is it?

- Intensity is related to what? Something physical?
  - Dye concentration? Or is it? Why not? Internal Control.
  - Noisy Images? Averaging? Pixel Time?
- Comparison of 2 colours / dyes / proteins - Biology / BioChemistry / Interaction ?
- Shapes, Movement, Structure?

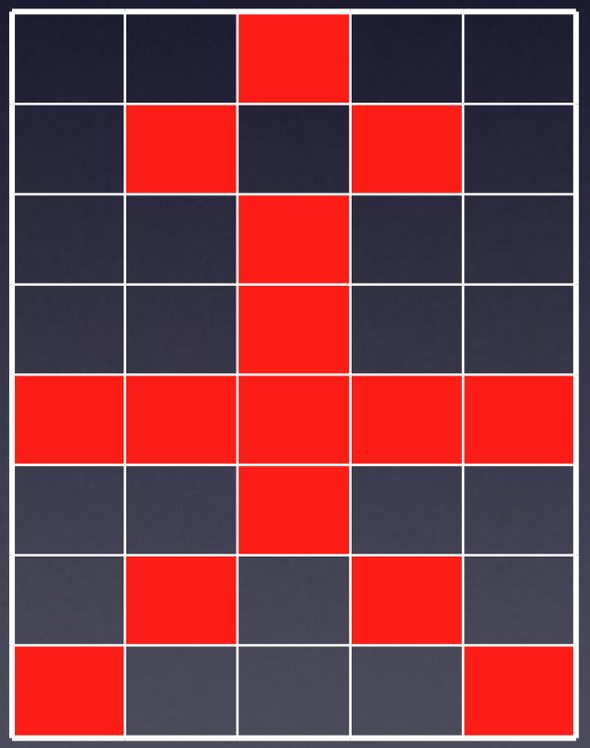
A digital image
With 2 channels / colours

What can you say here?



Photographer or Spectroscopist?

- We can show you how to take pretty pictures (Art)
- We can teach you how to get useful information (Science)
- You have to choose which you want to be!



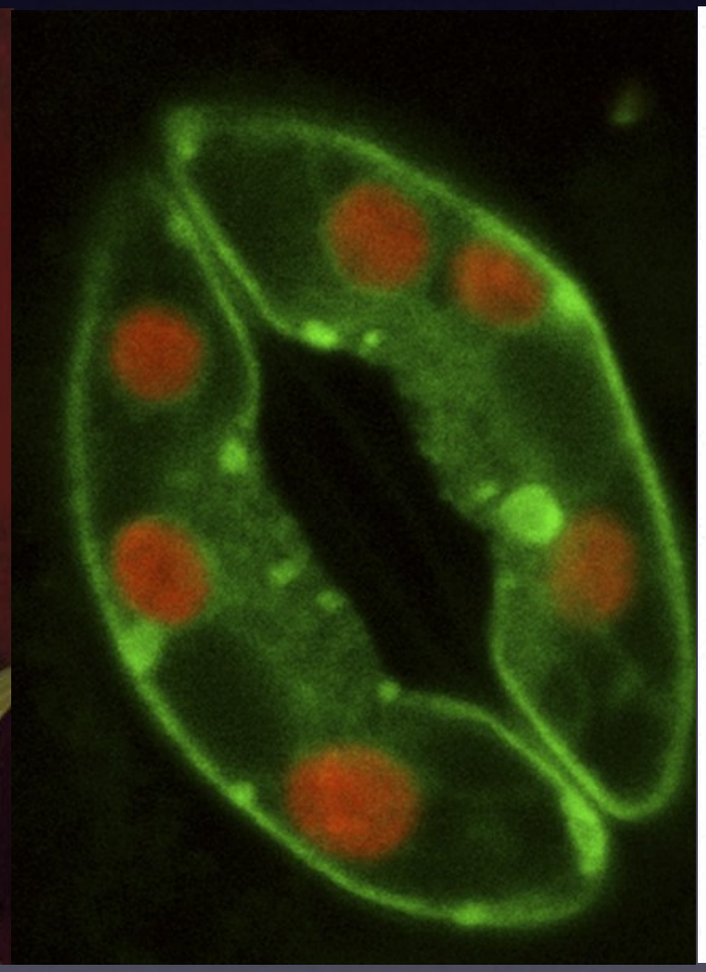
This

Is simply a way to
“Visualise”

This

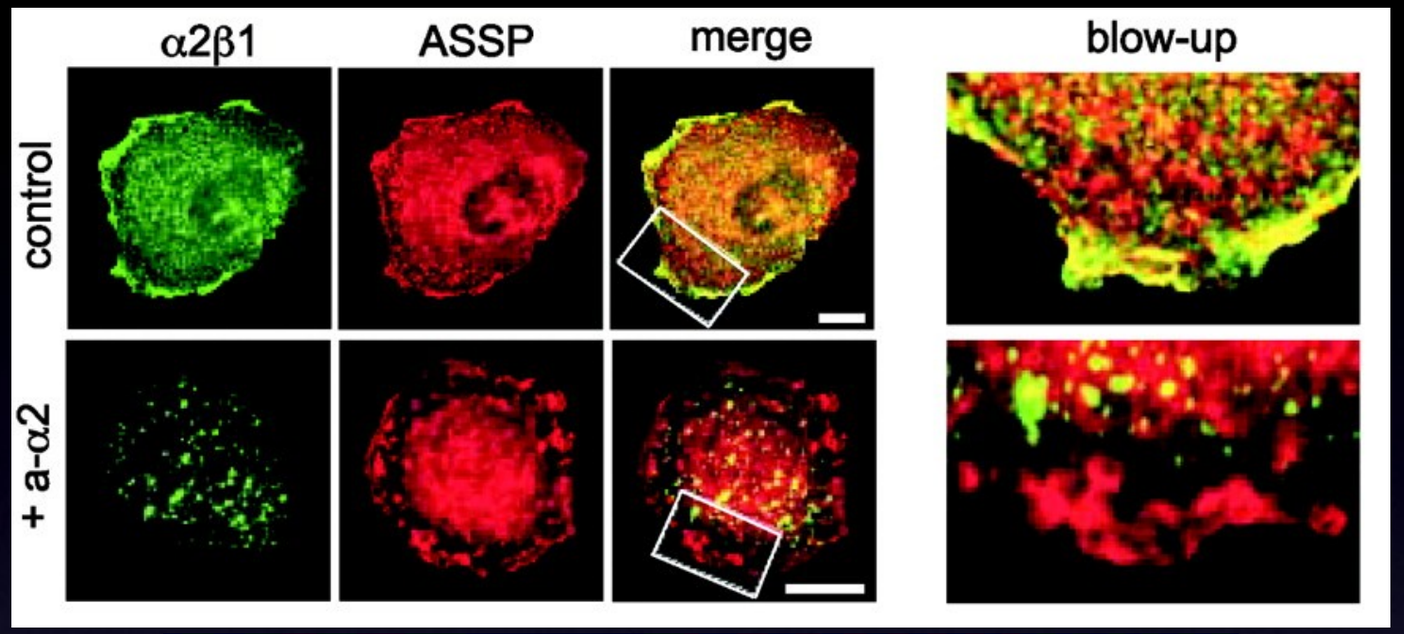
Photographer or Spectroscopist?

- Science or Art - You Choose
- Objectivity vs. Subjectivity
  - What I “think” I see *vs.* What IS there
  - 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	183
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	248	252
253	252	244	239	178	199	242	250	245
255	249	244	250	226	231	240	251	253

“Colour Merge” images could ruin your life



It is not possible to objectively decide about colocalisation by eye in a red-green merge image!

You see embedded spirals, of green, pinkish-orange, and blue?

Incredibly, the green and the blue spirals…

<u>are the same color!</u>

The “green” has orange,

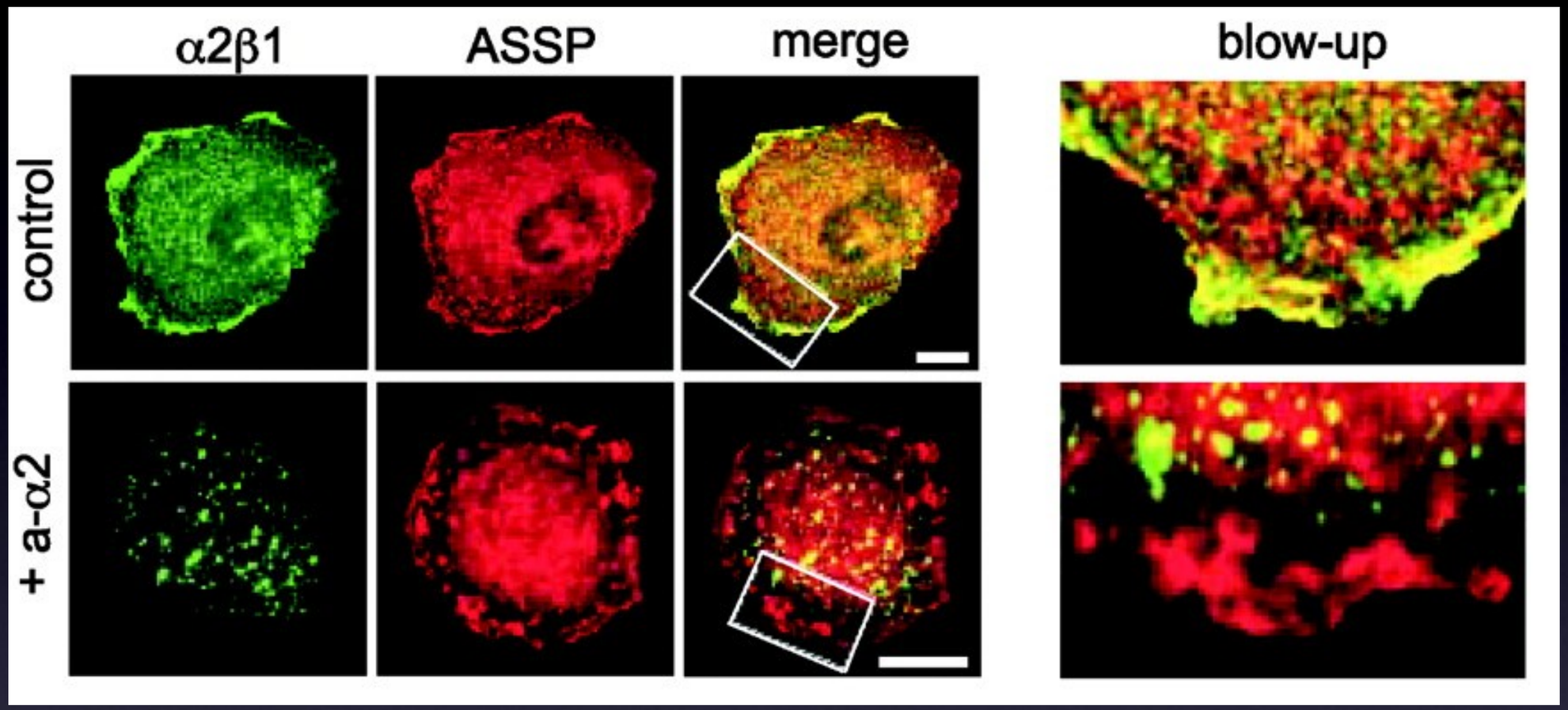
and the “blue” has magenta

lines which confuse the brain

<u>Moral of the story:</u>

Don’t Trust Your Eyes!

Colocalisation/Correlation

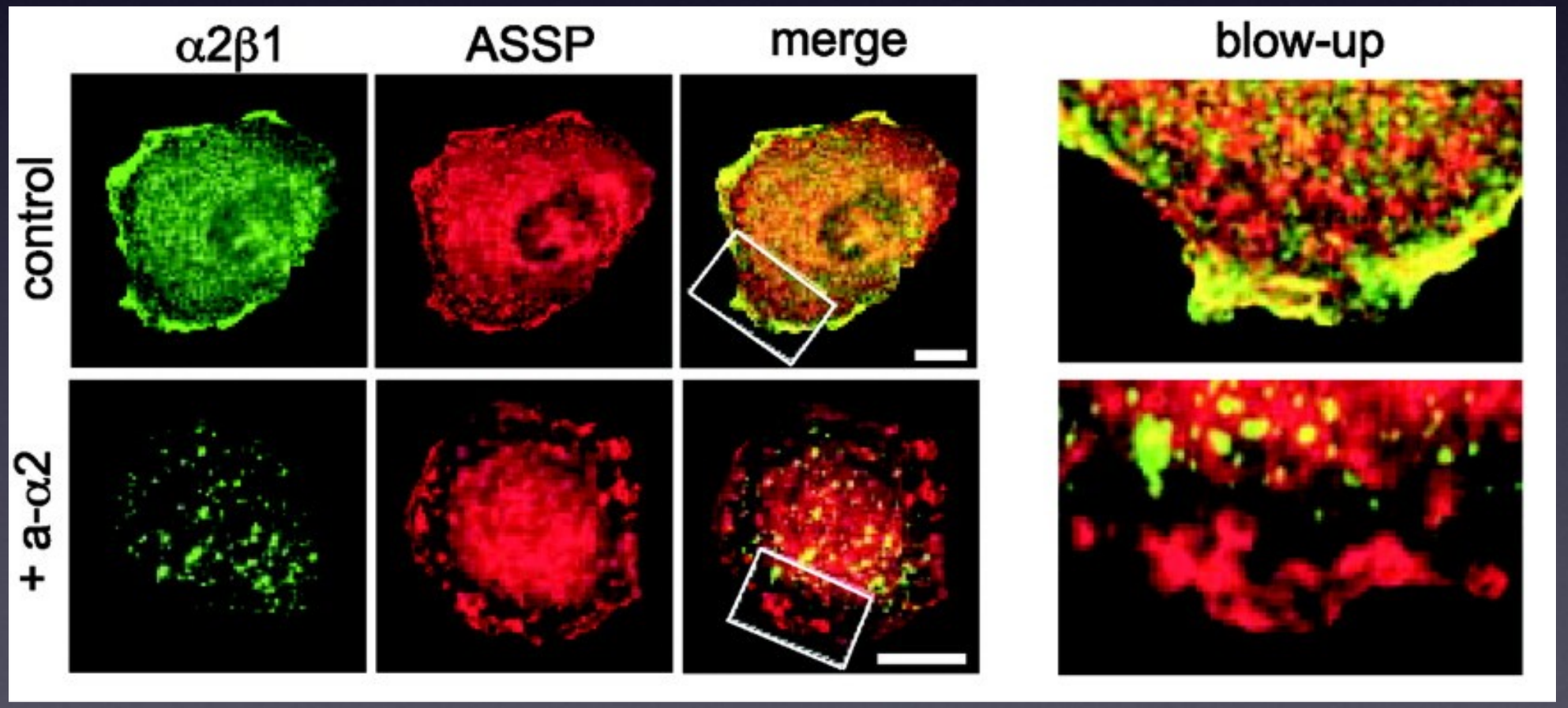


The past:
“I see yellow - therefore there is colocalisation”
but published images “look” over exposed.
No colocalisation definition + No stats = No Science.

From Now On: 3D. Quantification. Correlation. Statistics.
Complementary methods: BioChemical, Optical (FRET, FLIM)

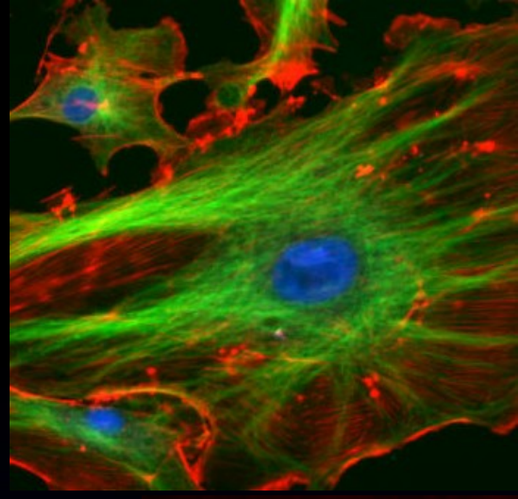
Colour Merge Images? Only for Art!

- Channel Merge Images? What are they good for?
  - Apart from looking pretty... not much.
  - Scientific conclusions from the image below?
  - Colour blind people - see green and red the same!
    - So use Magenta and Green!

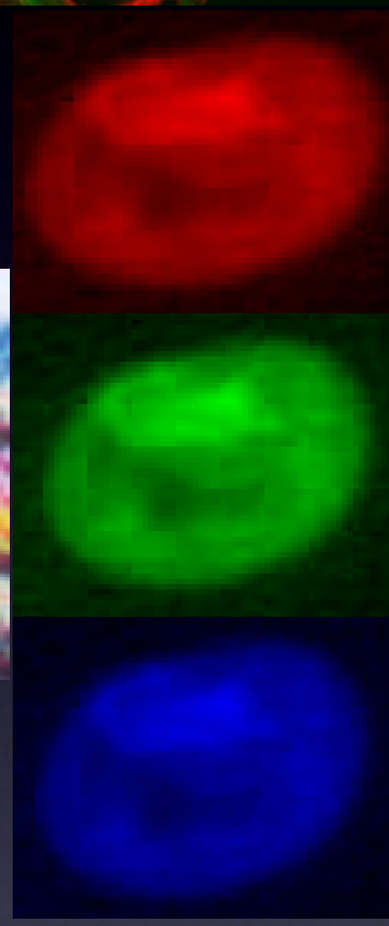


Publishing Images

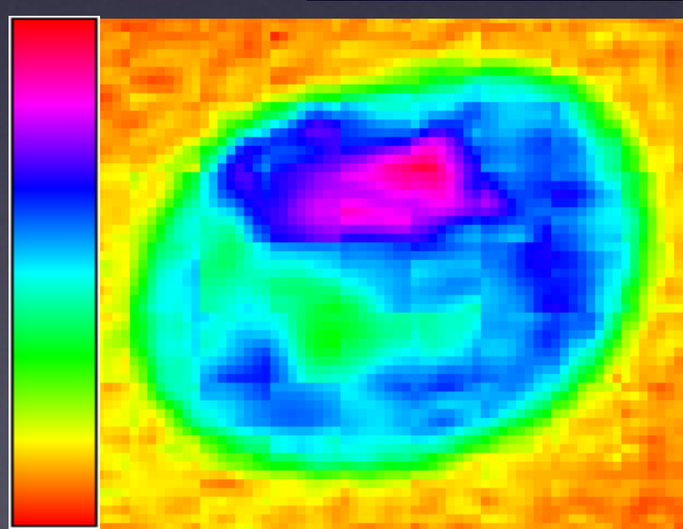
or “how Photoshop can ruin your career”



- Which Image? How Many? Prettiest? Representative?
  - Why is there no online database for images, like PDB or Genbank?
- CCD/PMT sees intensities differently than your eye/brain
  - LUT? Gamma correction? Calibrate Monitor - we have the tools!
- RBG colour space is not what we print!
  - RGB = Visualise (LCD and CRT computer screen)
  - CMYK = Print: Inks used are NOT RGB.
  - Journal Image Screen Image

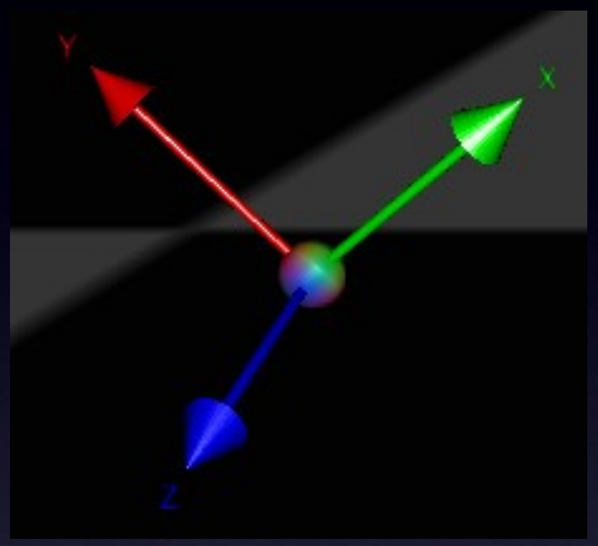


- Author instructions - image format? TIFF CMYK
- Materials and Methods - exact image processing done
- Image = data Don’t corrupt information!
  - PDF - Reviewer CAN check image processing results!
  - Compression - Lossless OK - Lossy (JPEG) very bad.
  - You wouldn’t do it to any other kind of data.

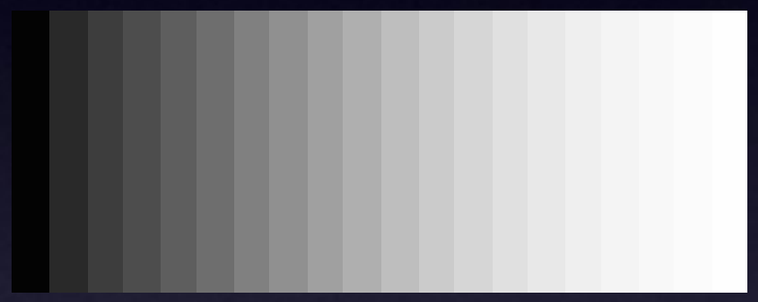


What can you digitise?

Dimensions!



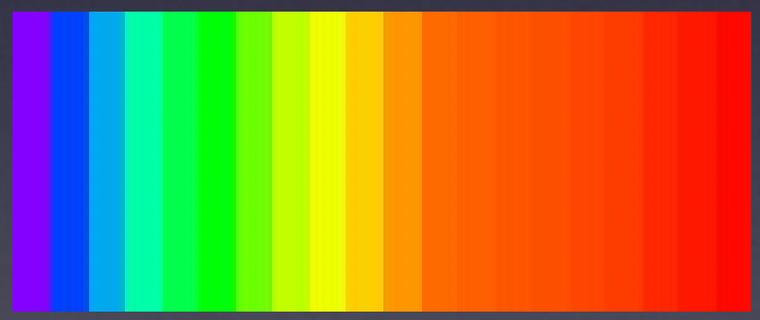
SPACE



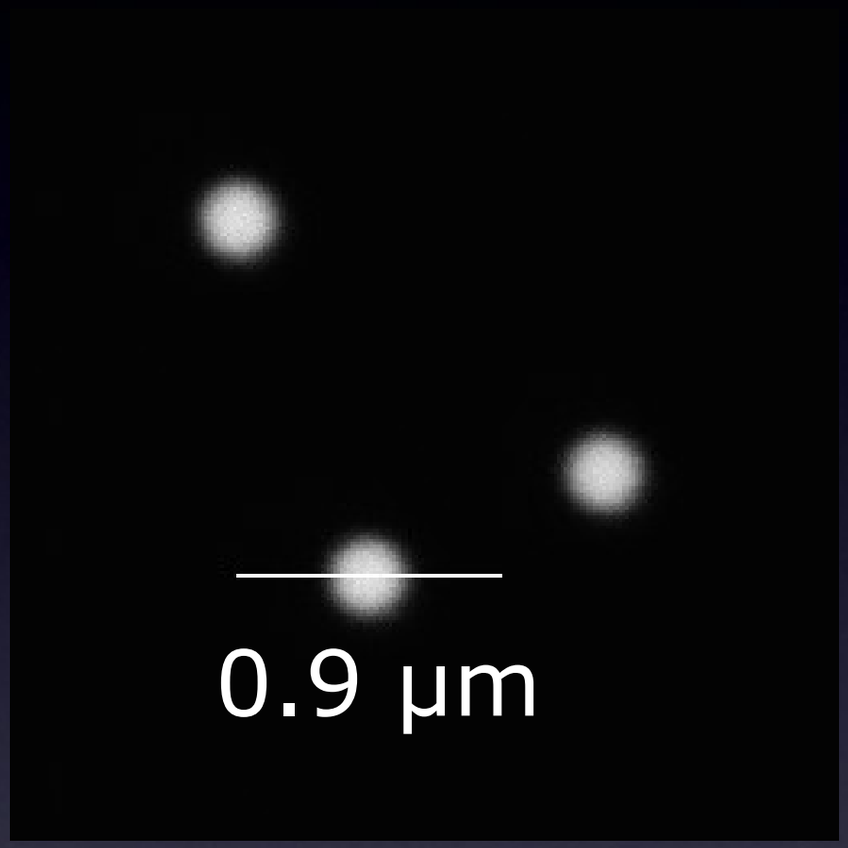
INTENSITY

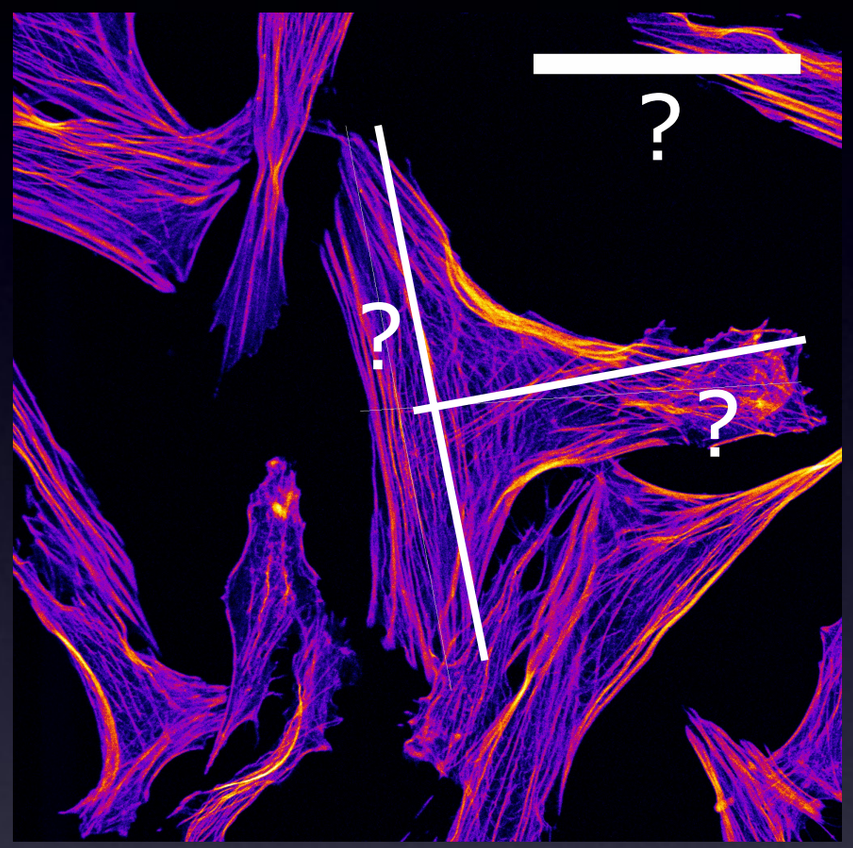
TIME

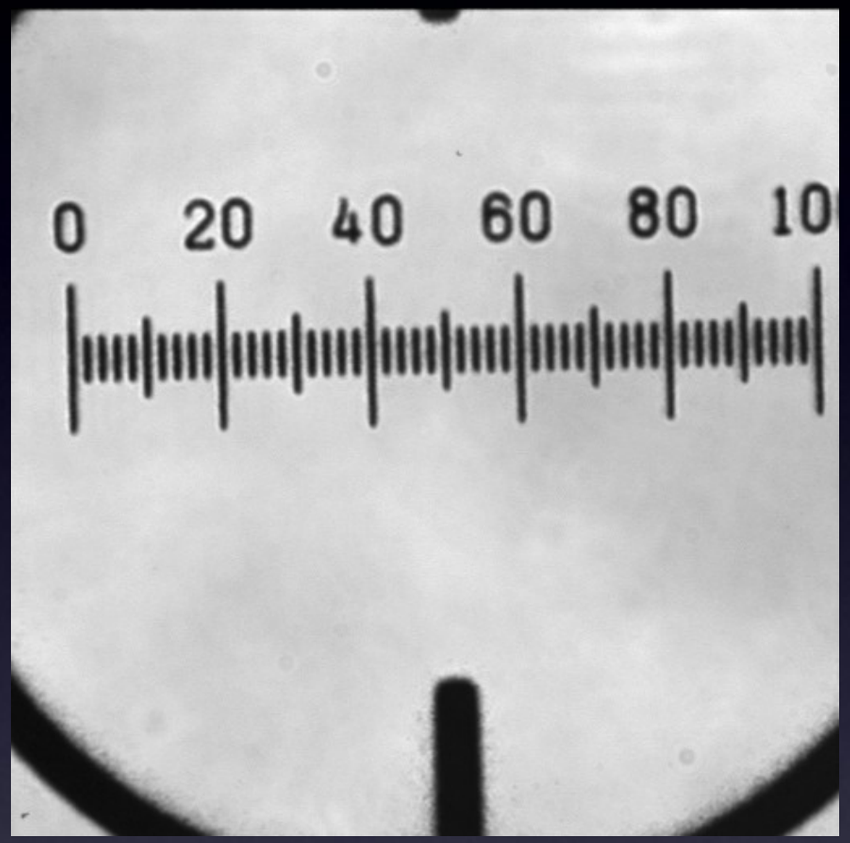
Colour Channels Wavelength



Pixel Size / Spatial Calibration





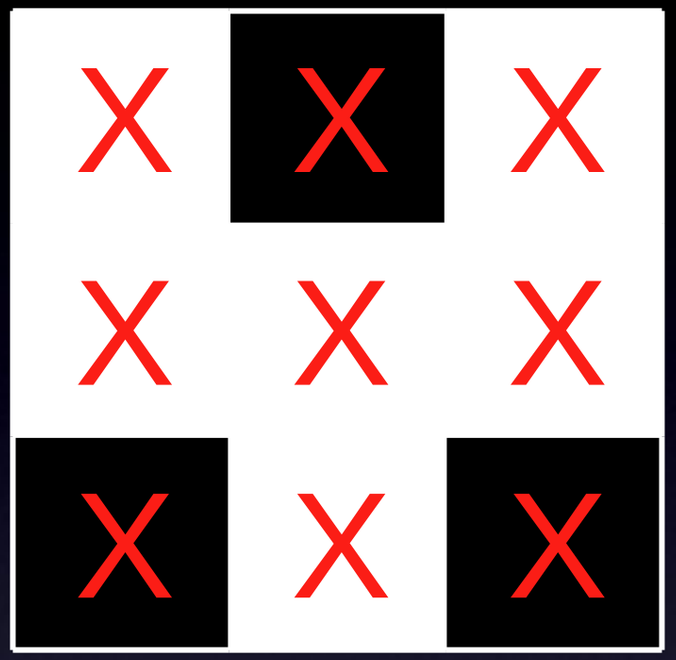


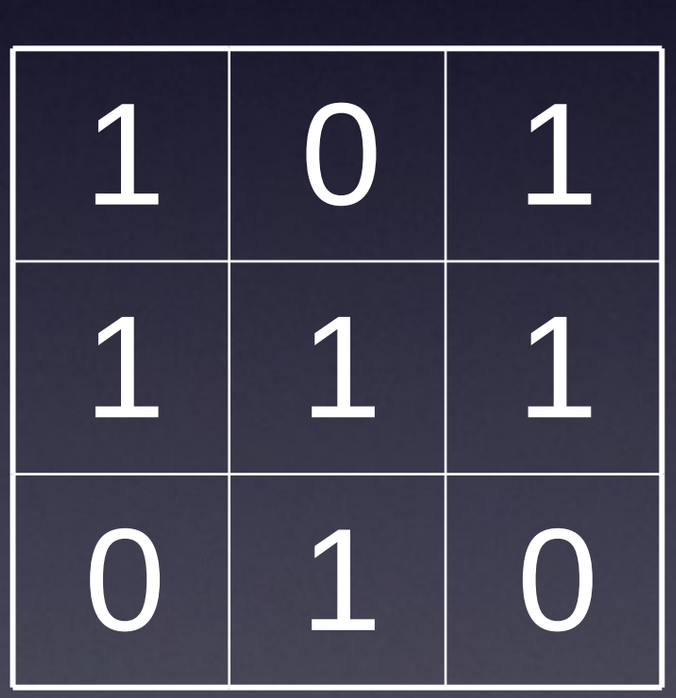
?

?

?

A pixel is NOT a little square!!!





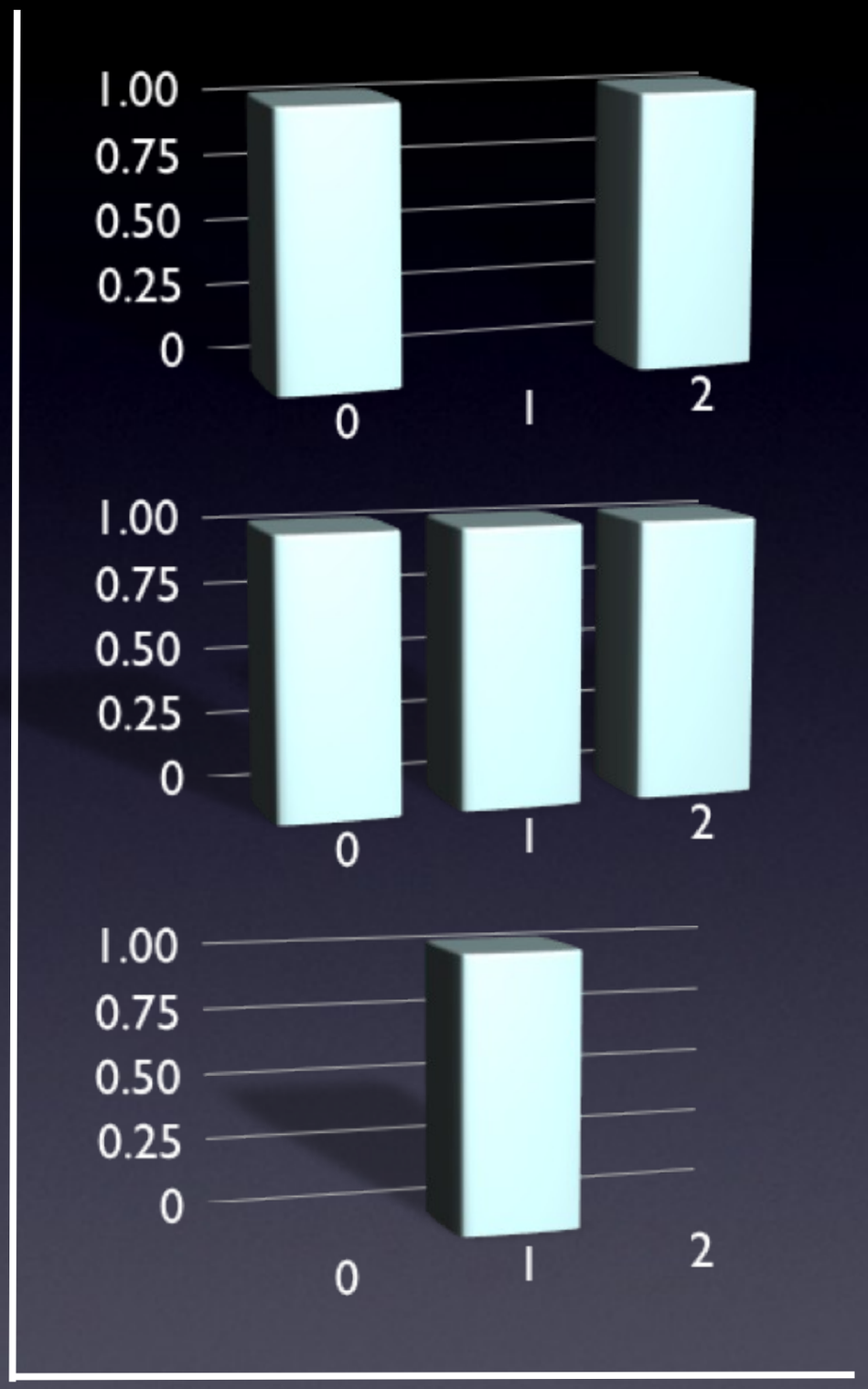
1	0	1
1	1	1
0	1	0

=

0

1

2



A pixel is a *point* sample. It exists only at a point.

Digital spatial resolution

- Projected pixel “size” at the sample/object
  - is the point sample spacing

A pixel is not a “little square”

Point sample =
Picture Element =
PixEl

Pixel Size

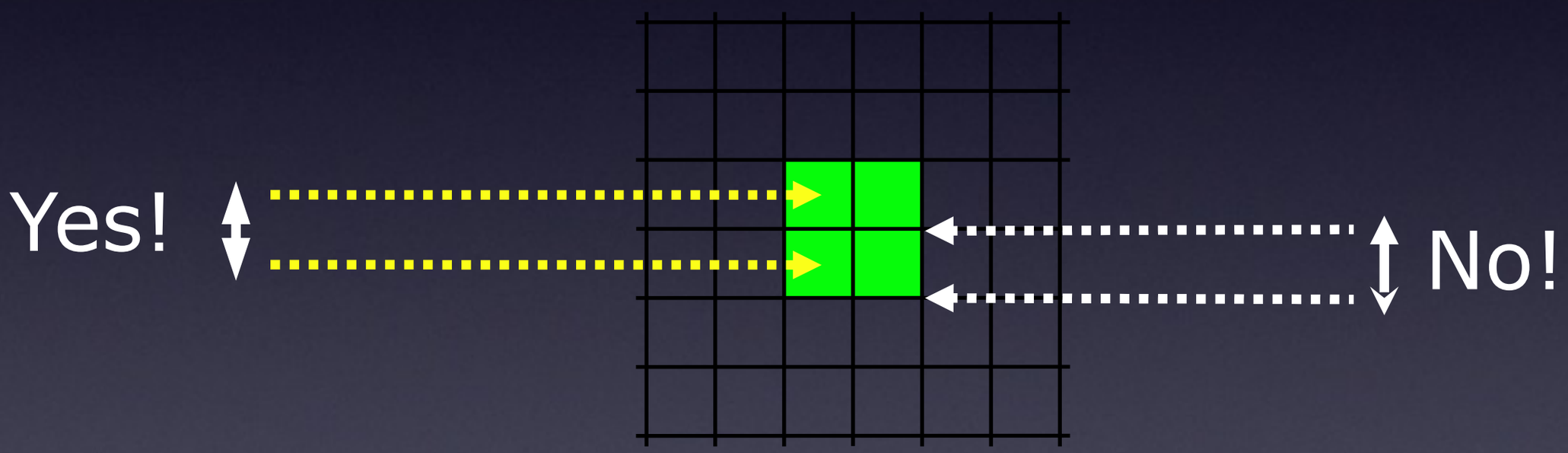
How big is a structure that is represented in my image?

=

How big is one pixel?

What is a pixel?

A pixel is NOT a little square!!!



A pixel is a sample of “intensity” from a POINT in space

“pixel size” is pixel spacing distance,

not the imaginary pixel edge length!

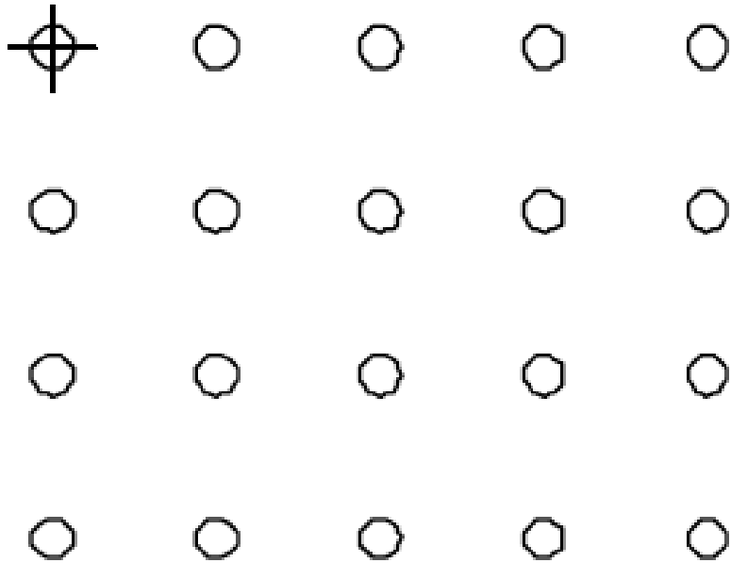
A pixel is *NOT* a little square,
A pixel is *NOT* a little square,
A pixel is *NOT* a little square!
(And a voxel is *NOT* a little cube)

ftp://ftp.alvyray.com/Acrobat/6_Pixel.pdf

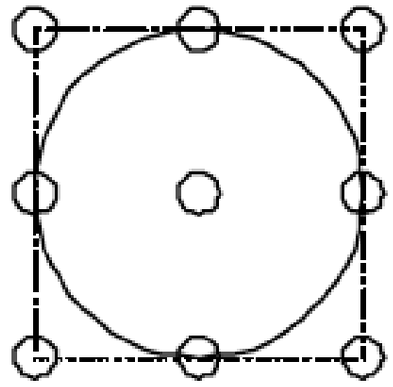
A pixel is a *point* sample. It exists only at a point.

Alvy Ray Smith
July 17, 1995

Maybe it lies on a grid pattern, but that's accidental!

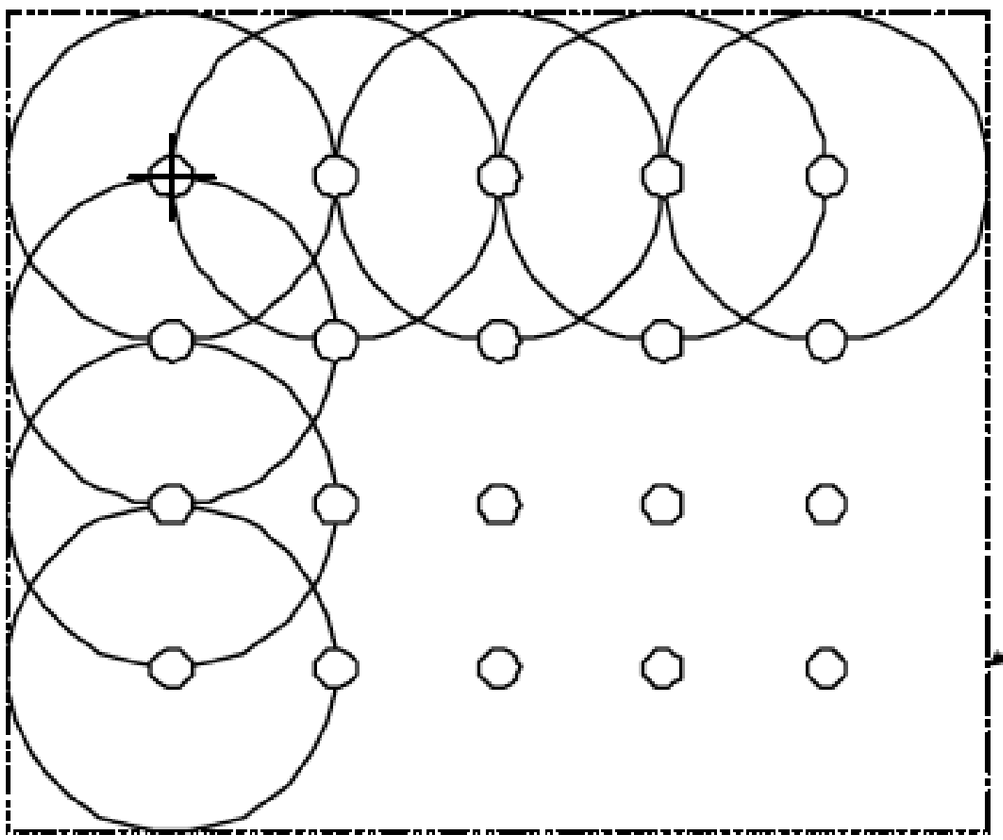


(a) A 5x4 image.



(b) The footprint of a reconstruction filter.
A truncated Gaussian, for example.

Or in our case the PSF of the microscope system!

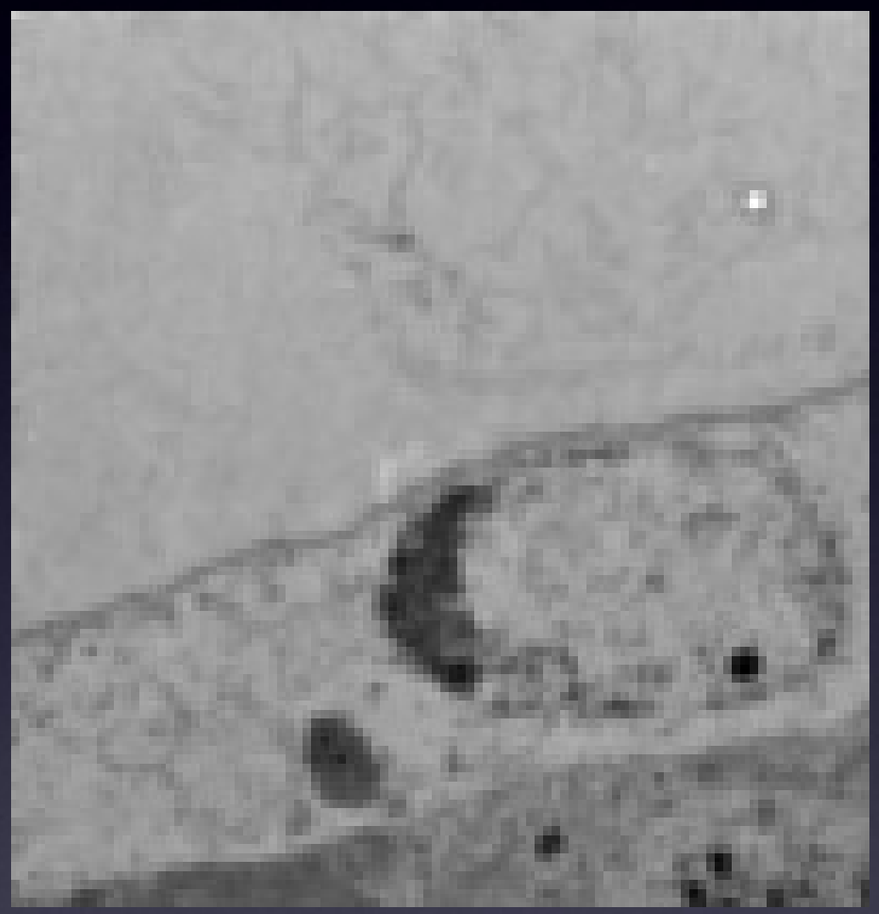


Dotted line is minimally enclosing rectangle

A pixel is not a little square...
So what! Why should I care?

Example – image shrinking
2048 x 2048 pixel electron micrograph – resized to 100 x 100

Wrong:
dumb interpolation of
square pixels (aliased)



Correct:
Gaussian smooth,
then down sample

http://pacific.mpi-cbg.de/wiki/index.php/Downsample

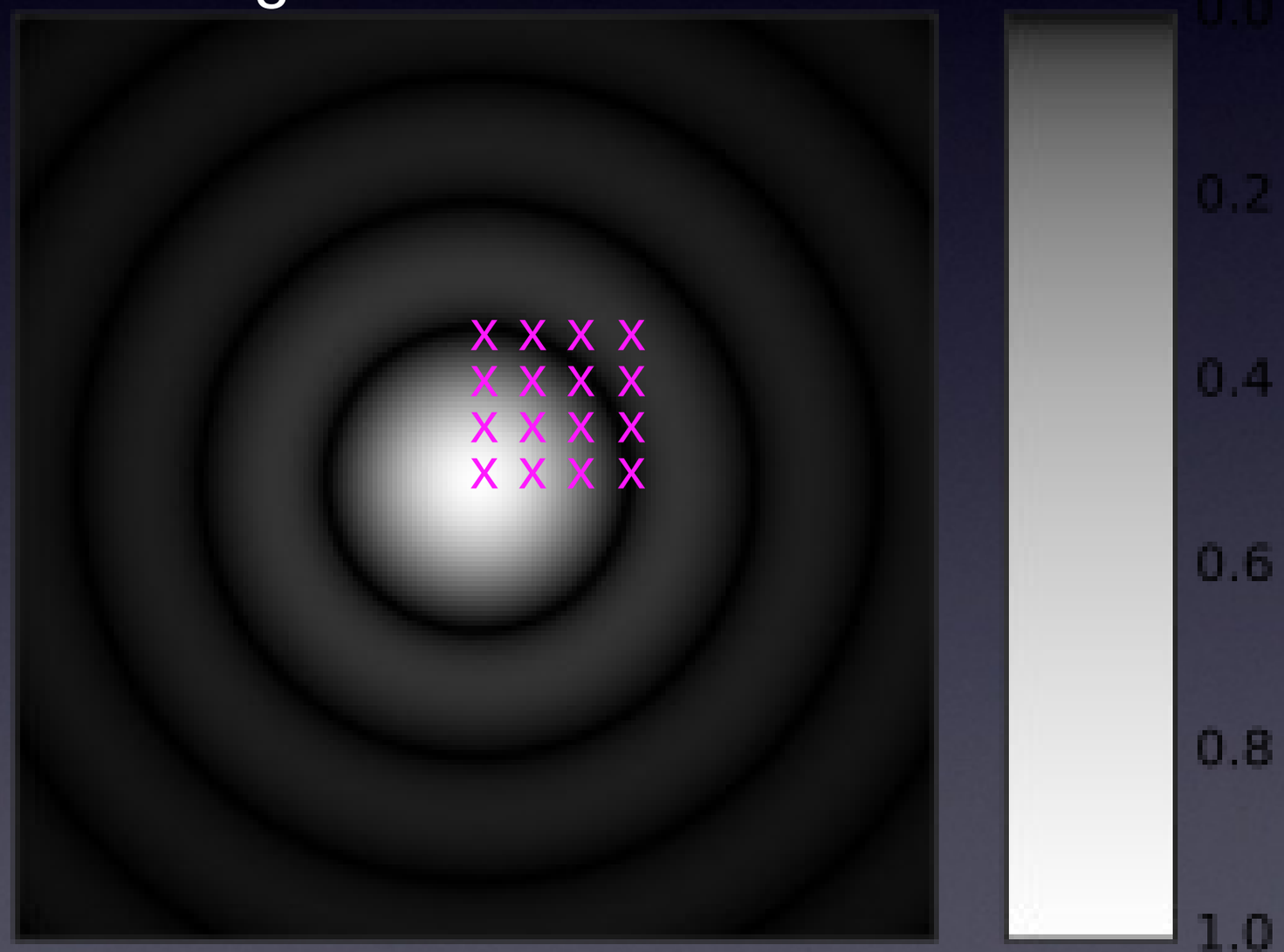
So what does a point sample from a microscope detector contain?

In the diffraction limited - high resolution case:

We sample at point X, and get an average intensity of the signal coming from all objects covered by the point spread function according to the shape of that PSF.

Because the PSF is bigger than the pixel / sample Nyquist spacing.

Image of a point light source = PSF



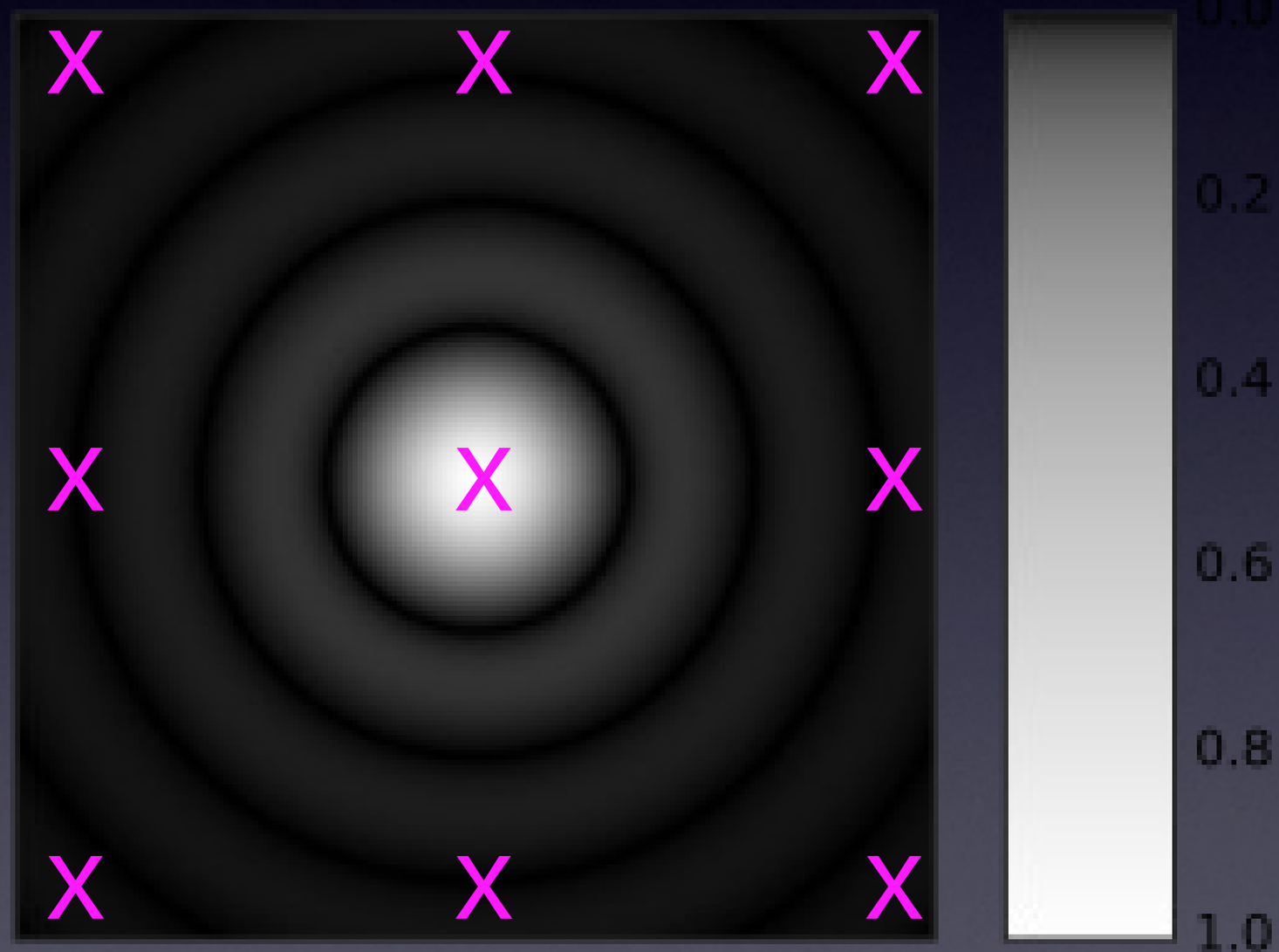
So what does a point sample from a confocal microscope detector contain?

In the low resolution - big pixels case:

We sample at a point X, but this time get intensity mainly from objects close to the sample position

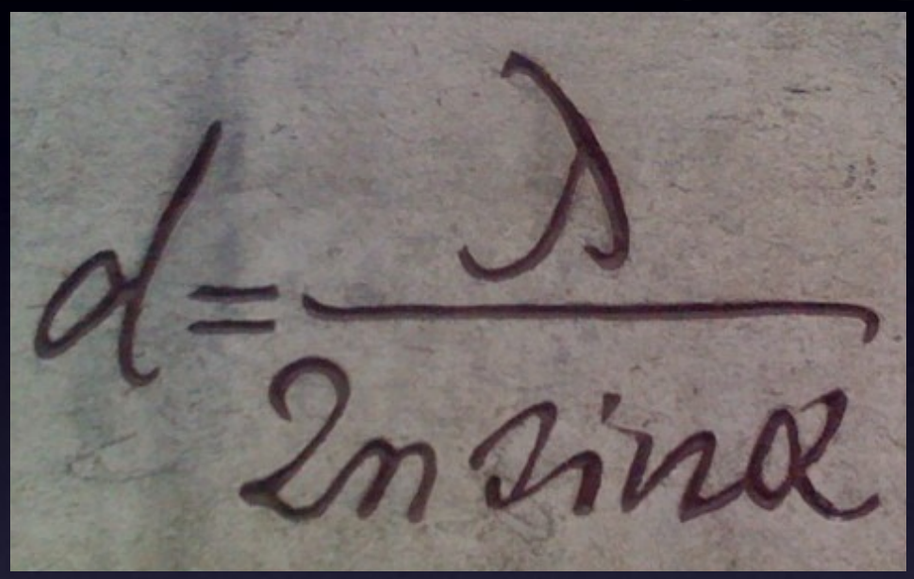
Because the PSF is much SMALLER than the pixel / sample spacing.

We miss spatial information = lower resolution

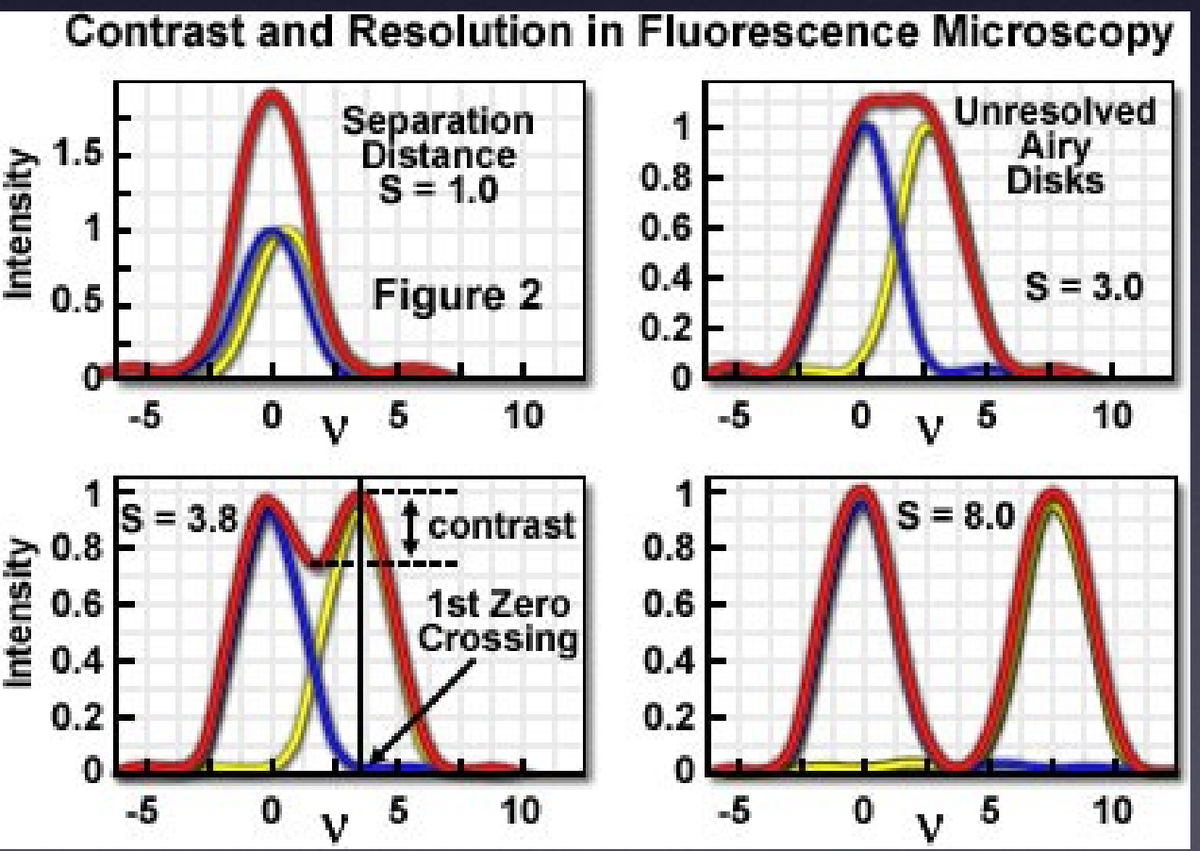


What limits light microscopy resolution?

Abbe's diffraction limit, and the Rayleigh criterion!



Optical Resolution



Airy Patterns and the Rayleigh Criterion online tutorial - Click Me!
http://www.microscopy.fsu.edu/primer/java/imageformation/rayleighdisks/index.html

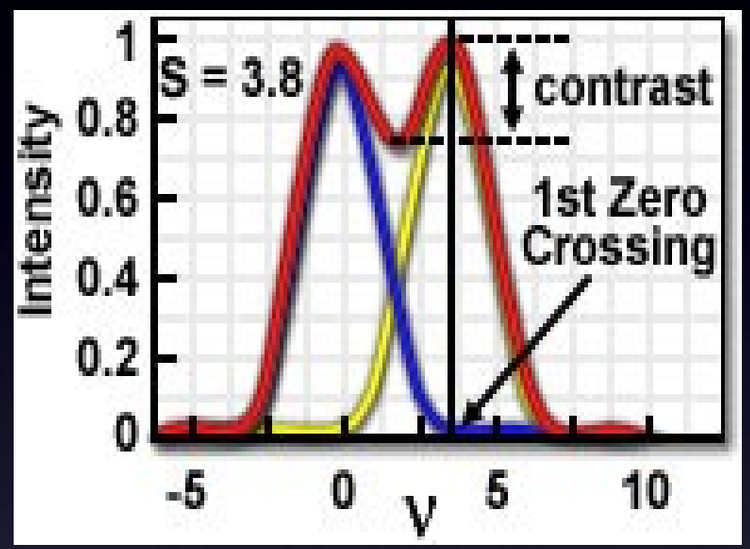
2 point light sources:

r = 0.61x wavelength / lens N.A.

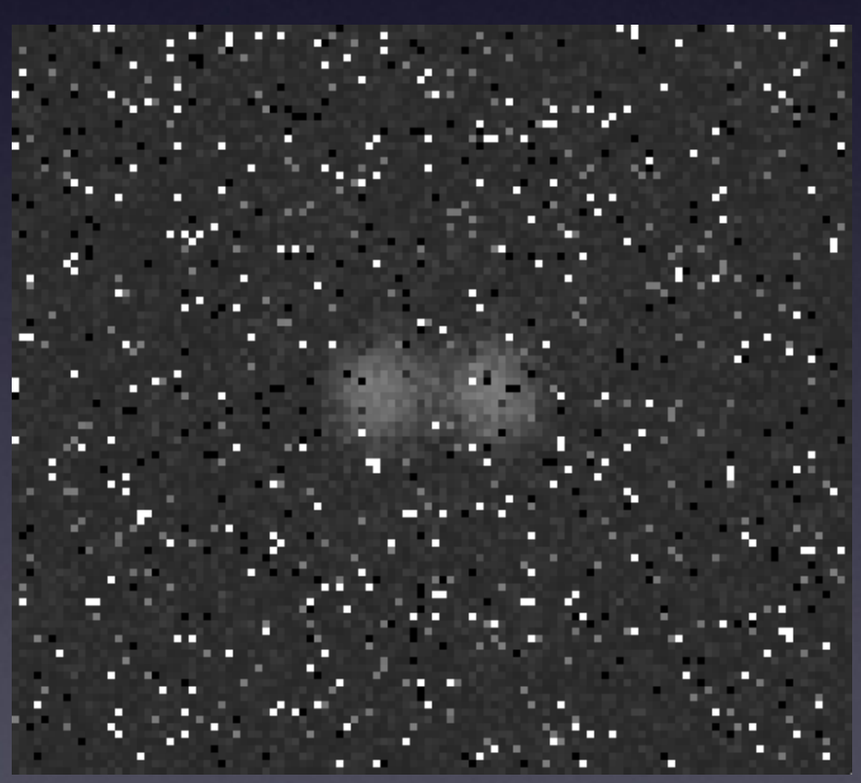
r = 0.61 x 550 nm / 1.4 ≈ 250 nm

Digital spatial resolution

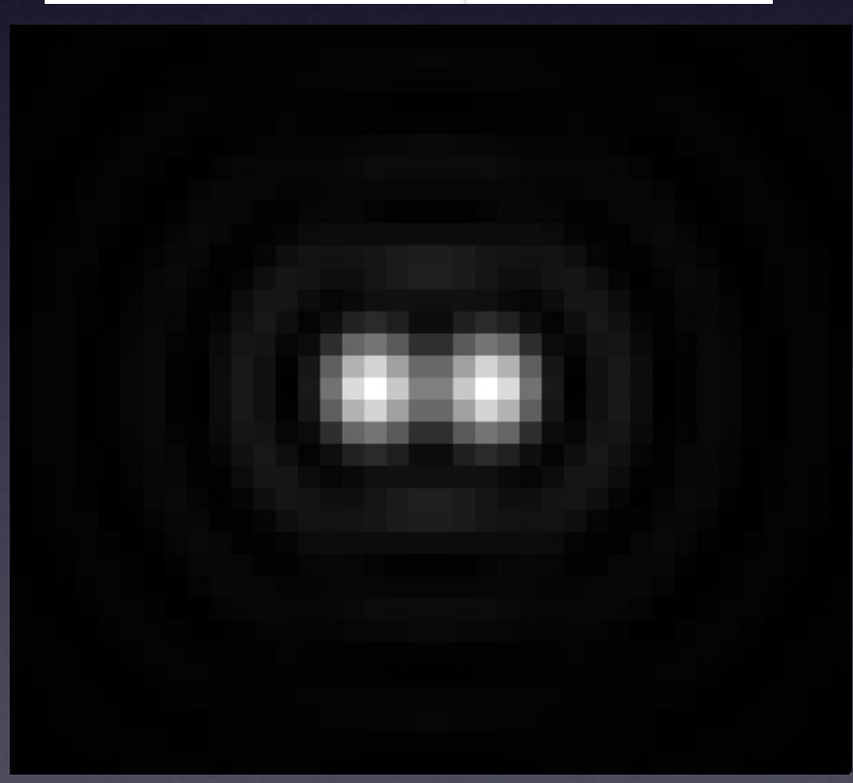
- Projected pixel “size” at the sample/object
  - The point sample spacing
  - But what “should” it be?



under sampled



over sampled

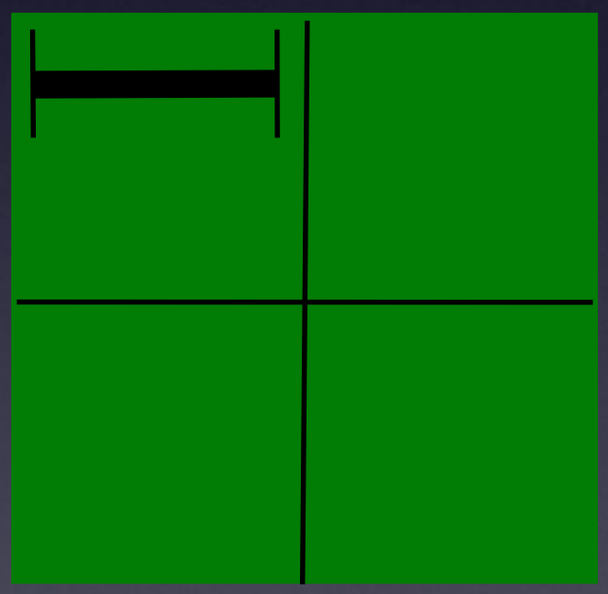


good sampling

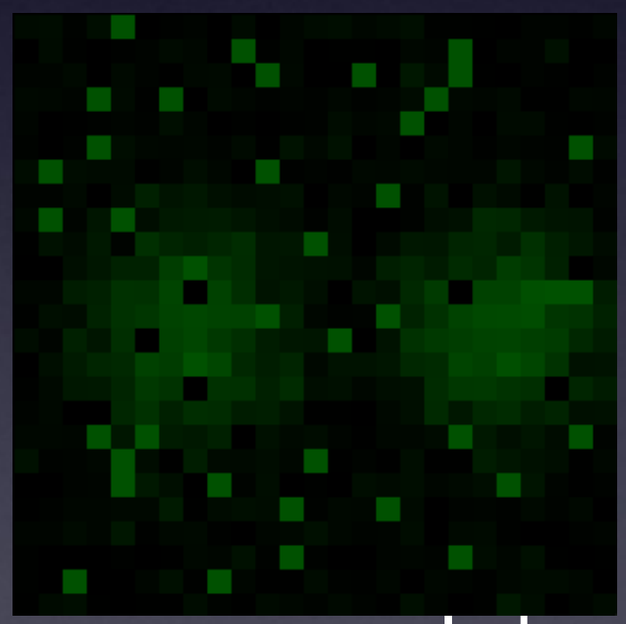
Pixel Size / Image Resolution

- "Correct" image size? (64x64, 512x512, 2048x2048)?
  - Get all information microscope can resolve, but files not too big
  - Proper spatial sampling (Nyquist-Shannon sampling theory)
  - 2.3-3 pixels over optical resolution distance. (x, y **AND** z)
  - Adjust "zoom", "binning" and image size (no of pixels).

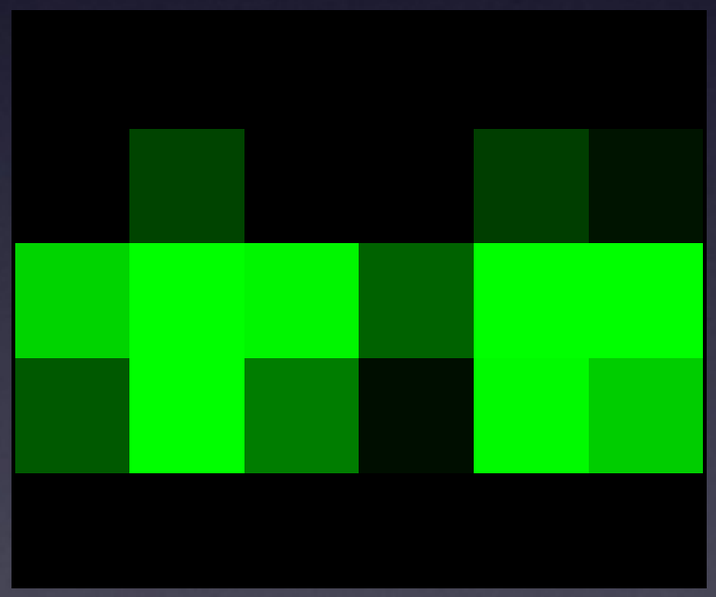
1 Airy unit



under sampled



over sampled

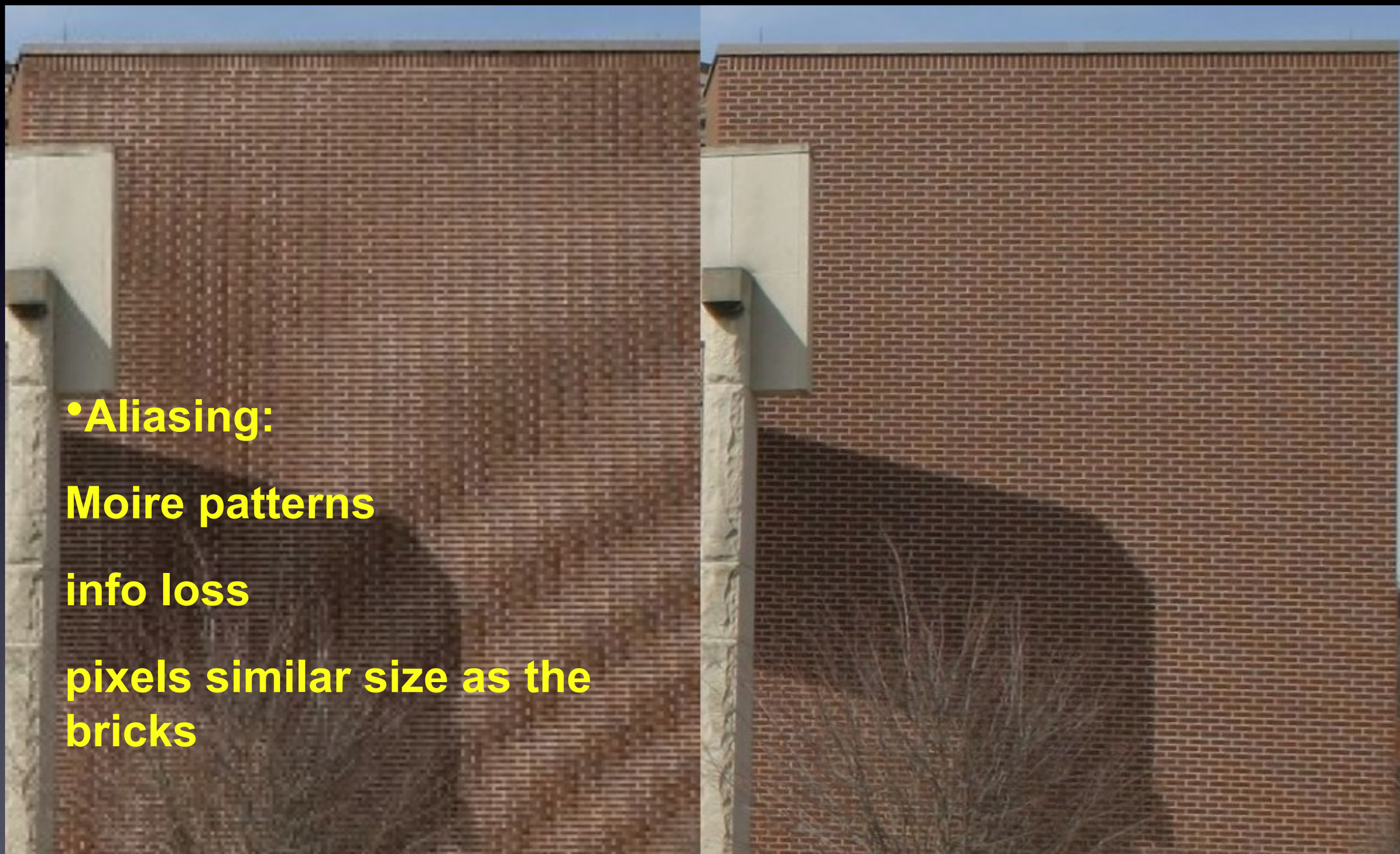


correct sampling

Harry Nyquist, 1889 - 1976

- Swedish - American
- engineer in telecommunications
- worked at Bell labs
- 138 US patents

Nyquist sampling criterion



- Aliasing:

Moire patterns

info loss

pixels similar size as the bricks

Nyquist sampling criterion

General form:

Digital sampling frequency > analogue frequency x 2

Spatial representation:

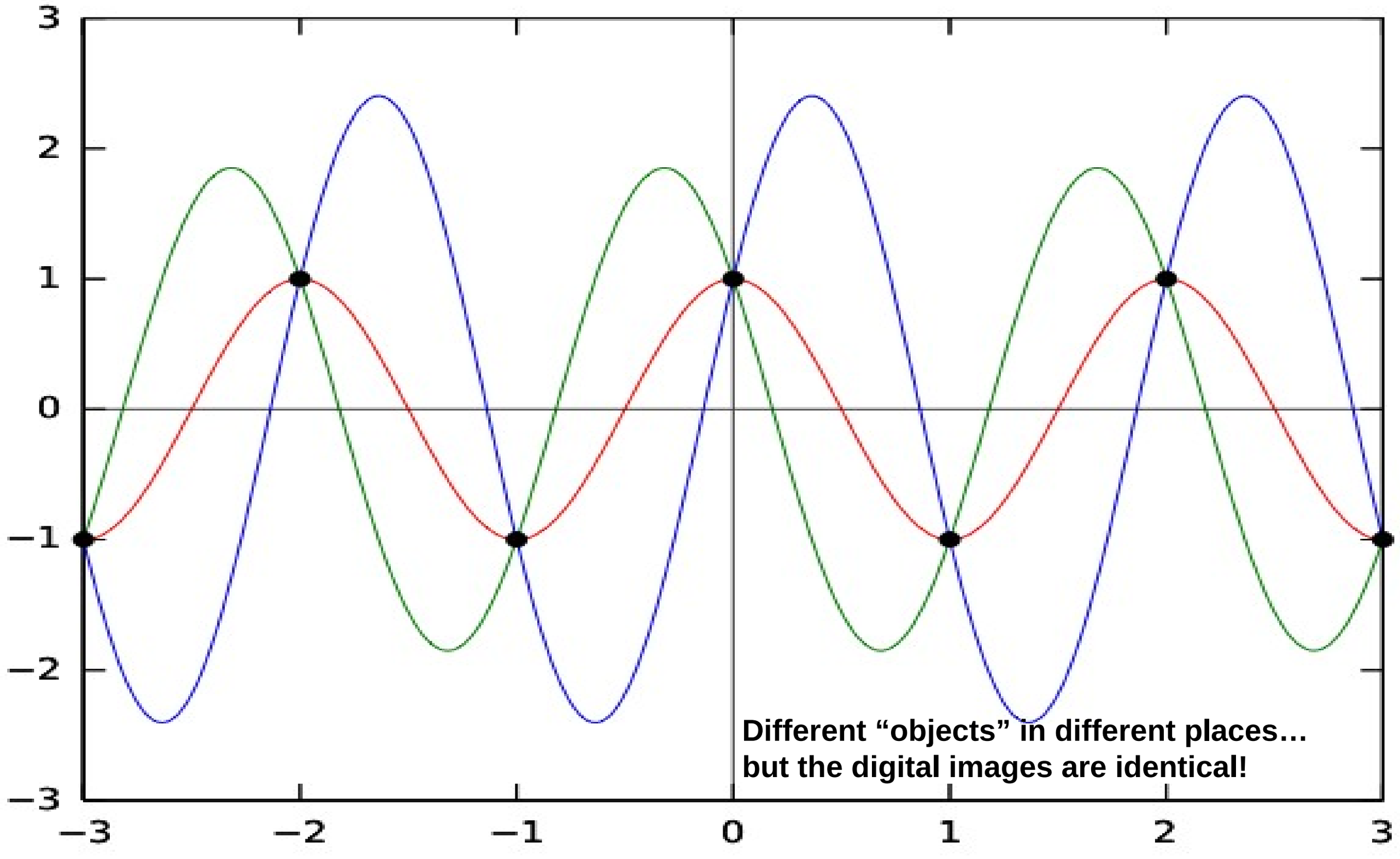
Image pixel size x 2.3 <= smallest resolvable distance

Microscopy

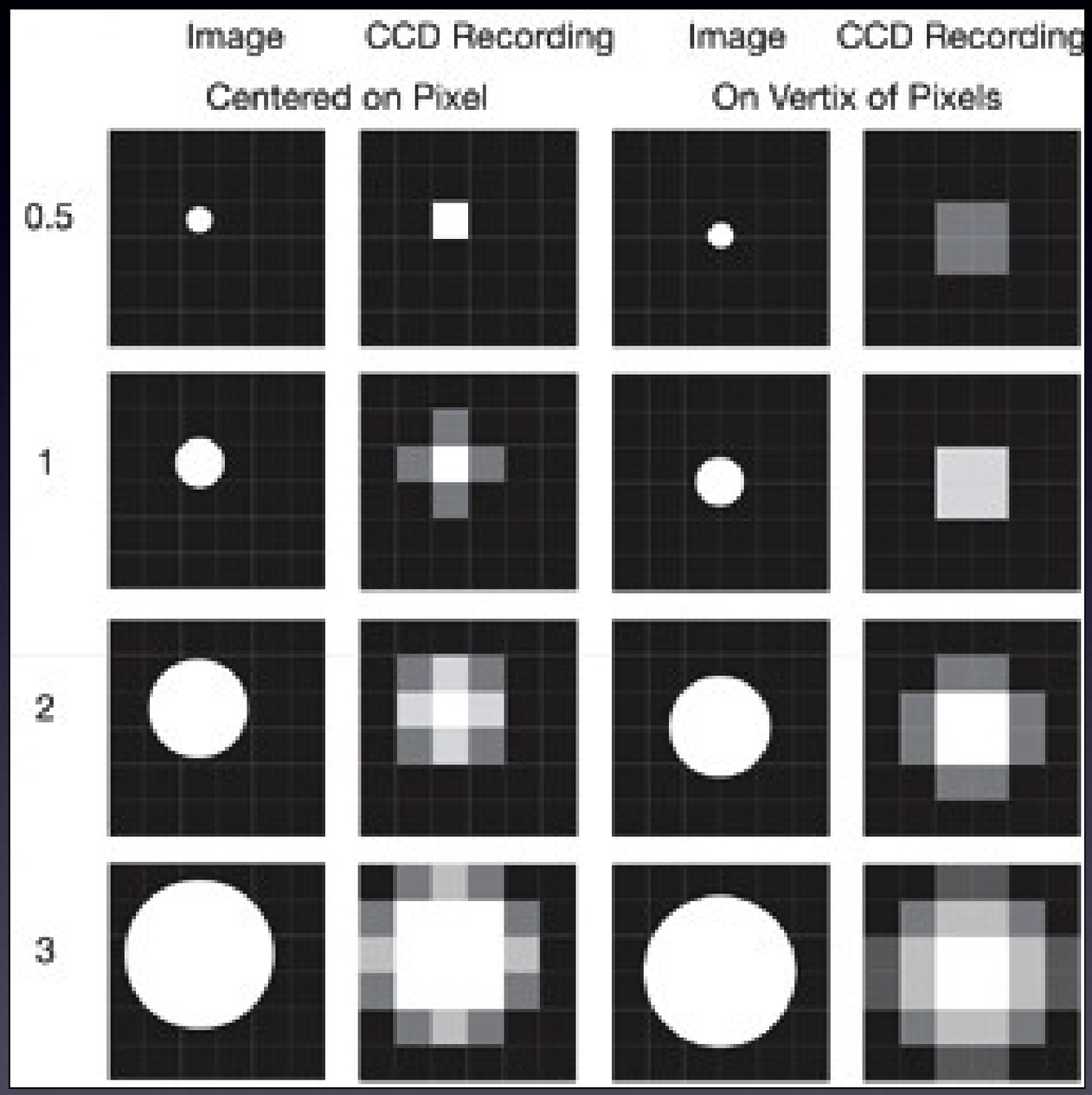
Image pixel size x 2.3 <= optical resolution (*r*)

Aliasing - Moire patterns - info loss

Nyquist sampling criterion



More aliasing problems...



- Pixel size relative to projected image
- Image of object depends where it falls on detector
- Especially for small objects close to pixel size.

Nyquist sampling criterion

- Resolution - pixel size calculations:

Objective (N.A.)	Optical Resolution limit (um)	Projected size on CCD (um)	Required pixel size (um)
4 x (0.20)			
10 x (0.45)			
40 x (0.85)			
60 x (1.40)			
100 x (1.40)			

Nyquist sampling criterion

- Resolution - pixel size calculations:

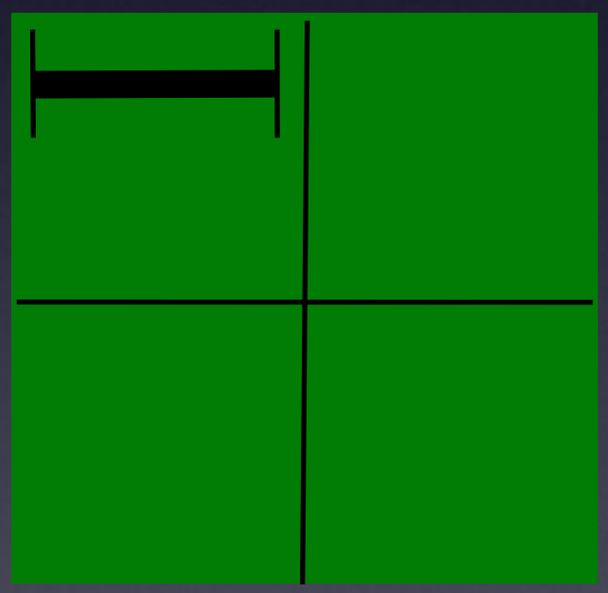
Objective (N.A.)	Optical Resolution limit (um)	Projected size on CCD (um)	Required pixel size (um)
4 x (0.20)	1.30	5.2	2.26
10 x (0.45)	0.58	5.8	2.52
40 x (0.85)	0.30	12.24	5.32
60 x (1.40)	0.19	11.14	4.85
100 x (1.40)	0.19	18.57	8.07

Think about your digital spatial resolution carefully!

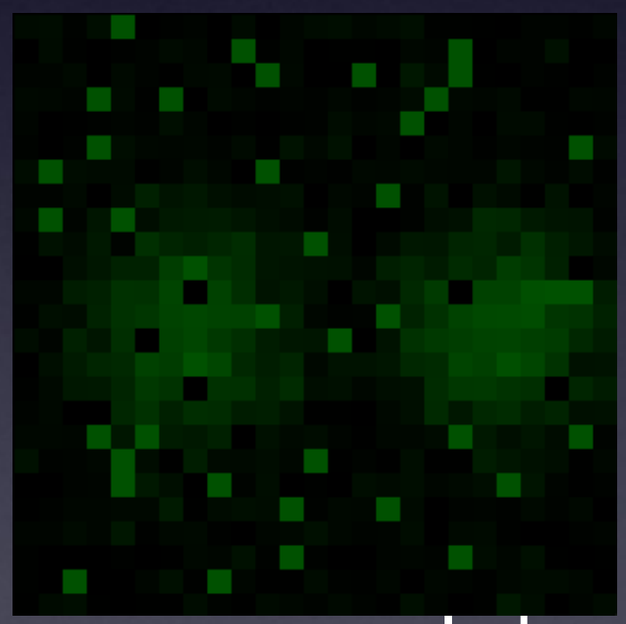
Pixel Size / Resolution

Remember !!!
Nyquist told us how to do digital sampling:
~1/3 x smallest feature.

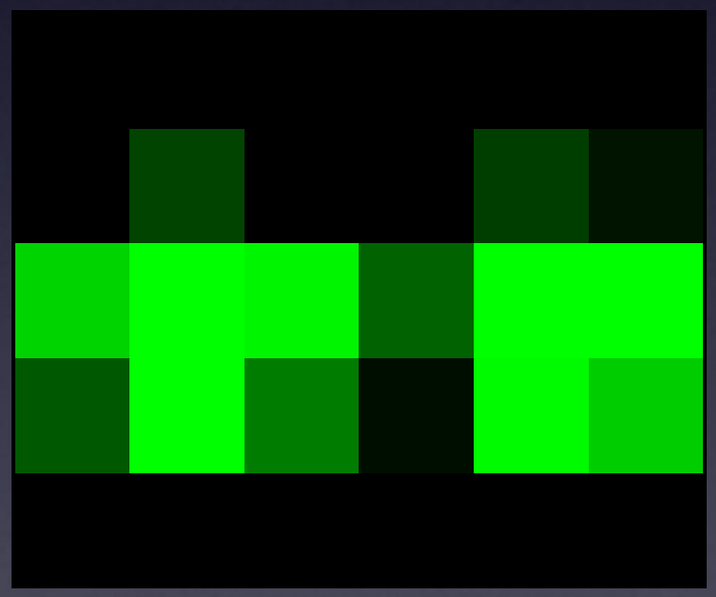
1 Airy unit



under sampled



over sampled



correct sampling

Pixel size / Spatial Calibration

- Pixel size is determined by the microscope system:
  - CCD photodiode “pixel” size / Magnif cation X
  - Point scanner settings – zoom and image size
  - Field of View Size / No. of Samples or “pixels”

- It might be changed / lost during processing

- It is stored in the “Meta Data”

- So .. a dataset for image processing =
  - Image data
  - +
  - **Meta Data!**

Practical Session 1b

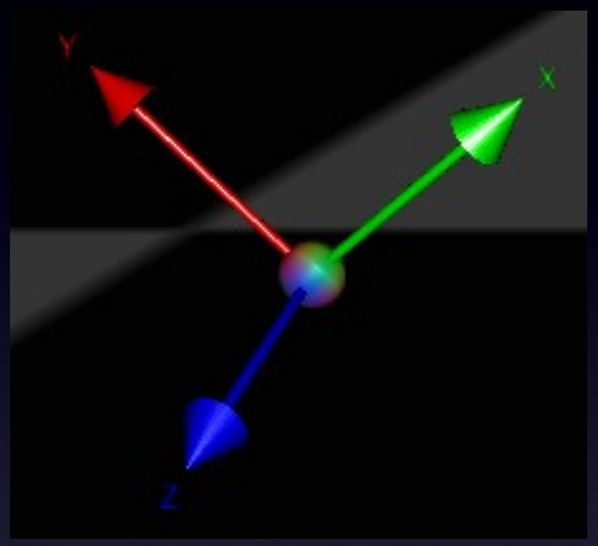
- Getting to know “Fiji” better
- (Fiji is just ImageJ)
- http://pacific.mpi-cbg.de

- Open Samples - Neuron

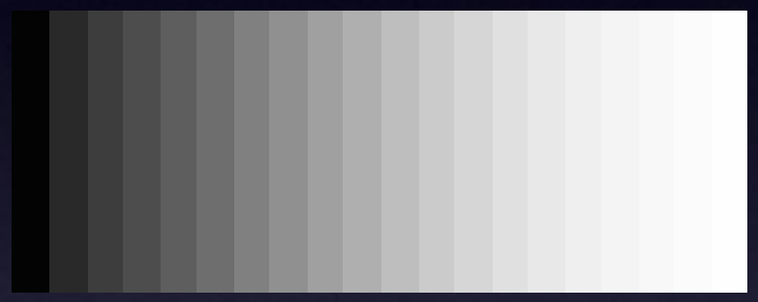
- Spatial Scaling:
- Analyze - Set Scale, Analyze-Tools-Scale Bar
- See Fiji Tutorial - SpatialCalibration (search Wiki)
- Can you measure the length and area of objects?
- Line and ROI selection - ctrl M (cmd M)
- Rectangle, Oval, Polygon, Freehand, Angle, Point, Wand.
- Analyze - Set Measurements

What can you digitise?

Dimensions!



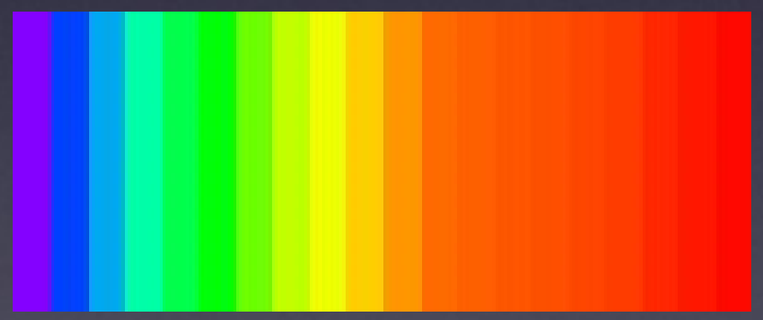
SPACE



INTENSITY

TIME

Colour Channels Wavelength



“Intensity” Digitisation

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”

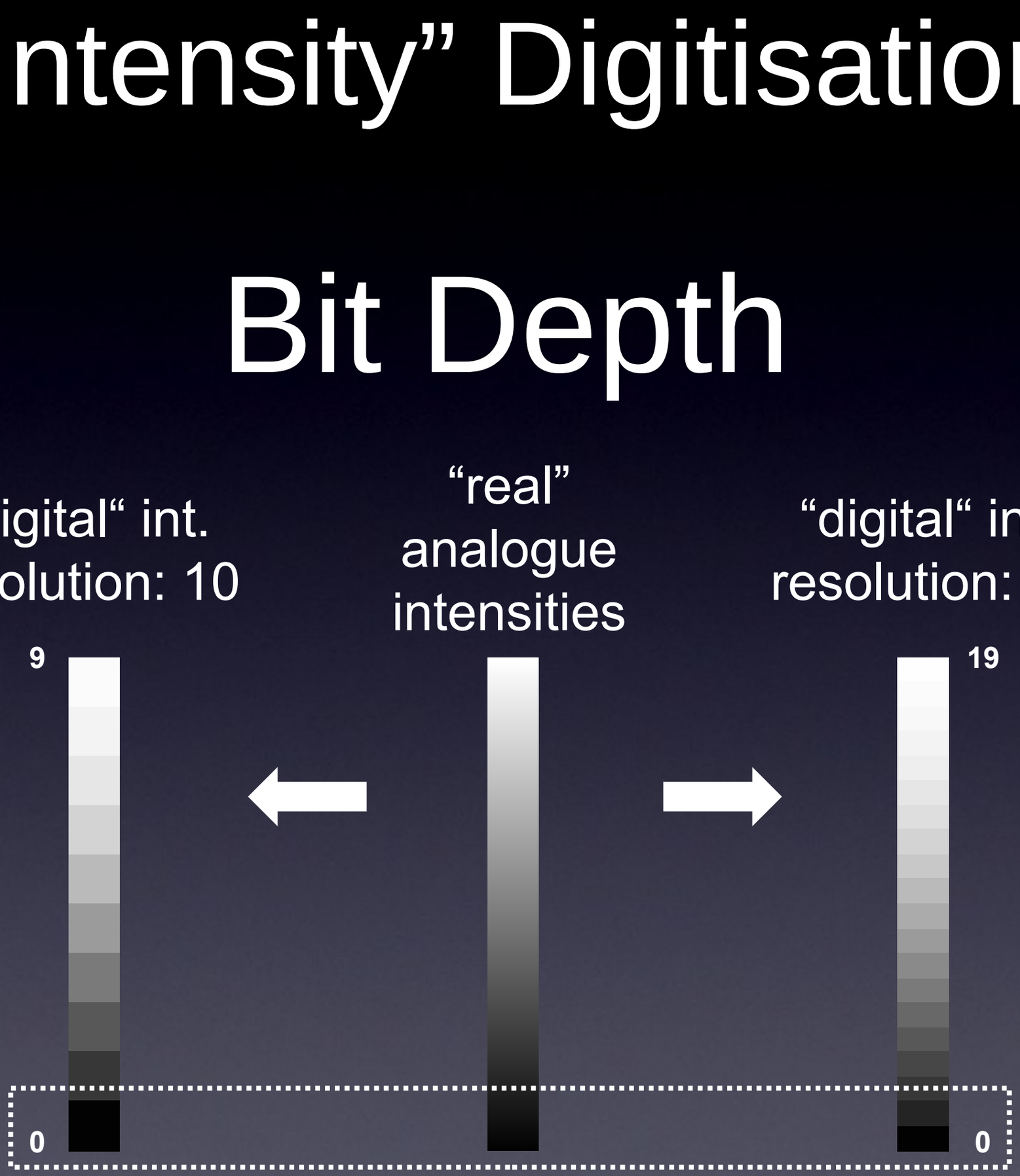
“Intensity” Digitisation

Bit Depth

“digital“ int. resolution: 10

“real” analogue intensities

“digital“ int. resolution: 20



“Intensity” Digitisation

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...

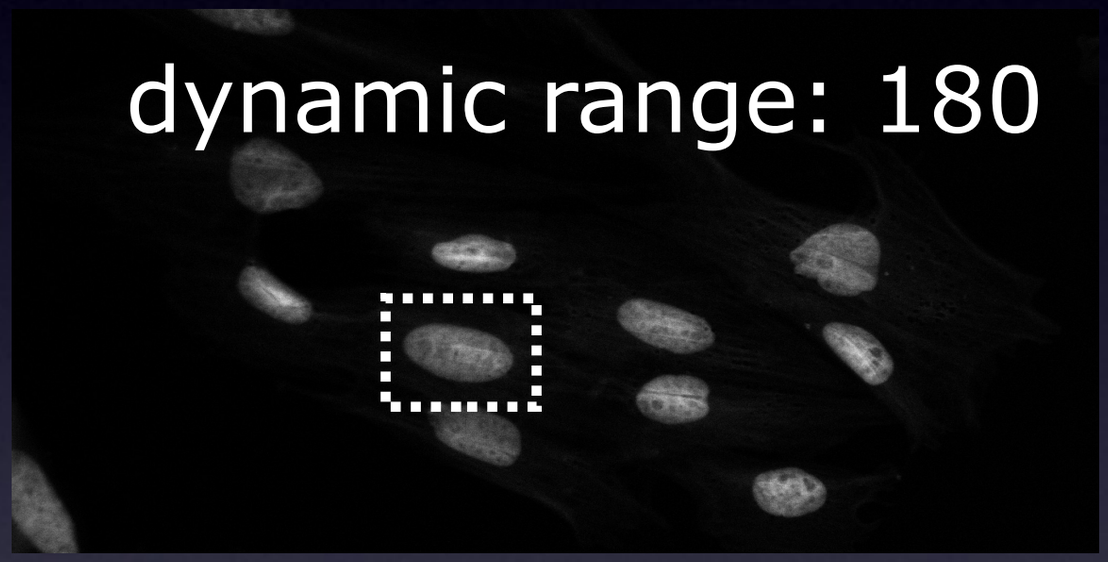
Intensity-related measurements

“Intensity” Digitisation

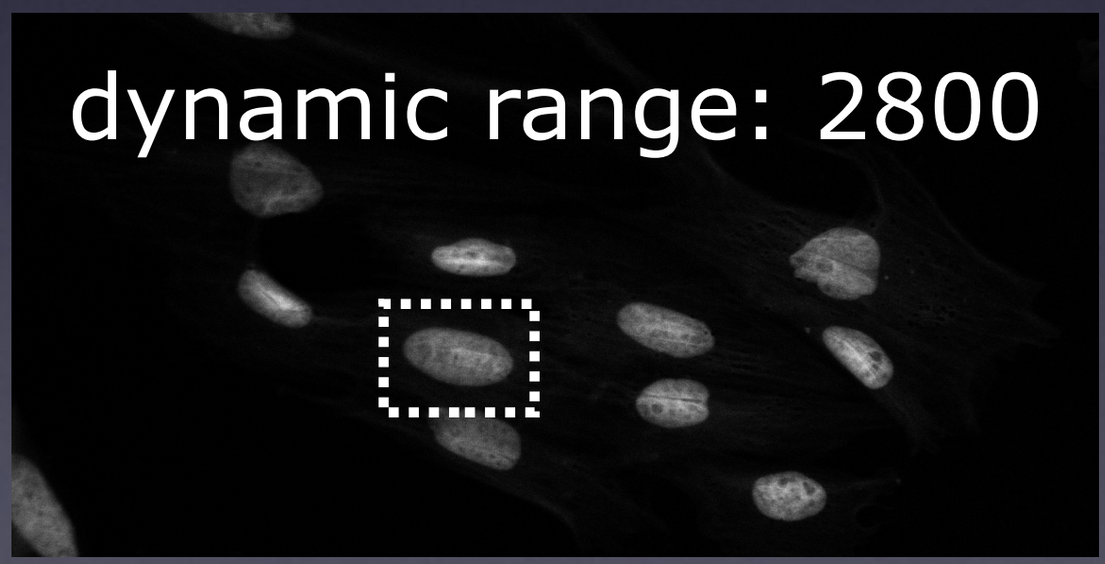
Bit Depth

for intensity-related measurements

8 bit



12 bit

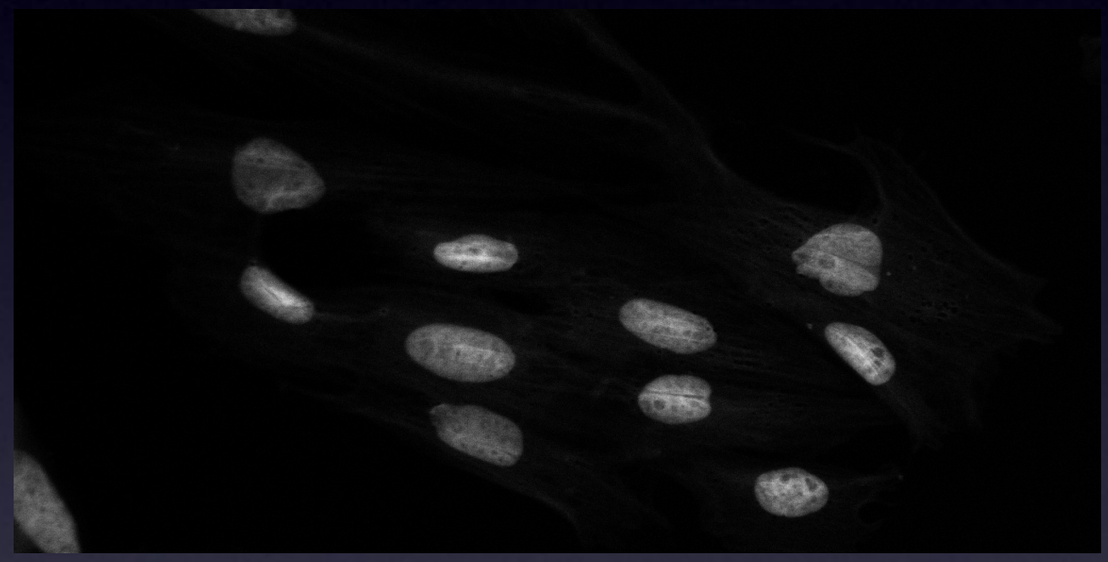


“Intensity” Digitisation

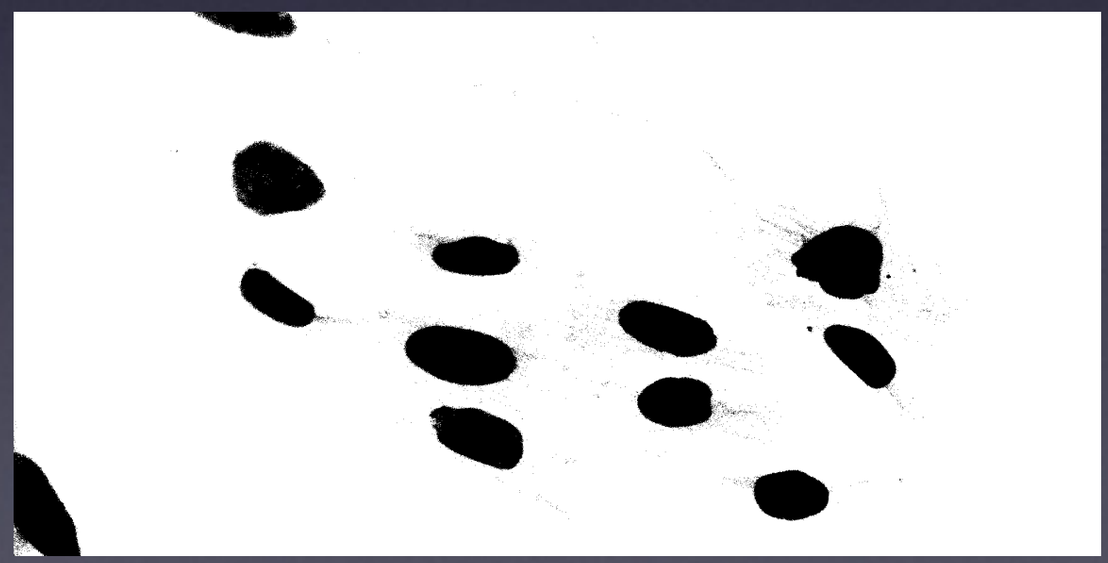
Bit Depth

for segmentation

8 bit greyscale

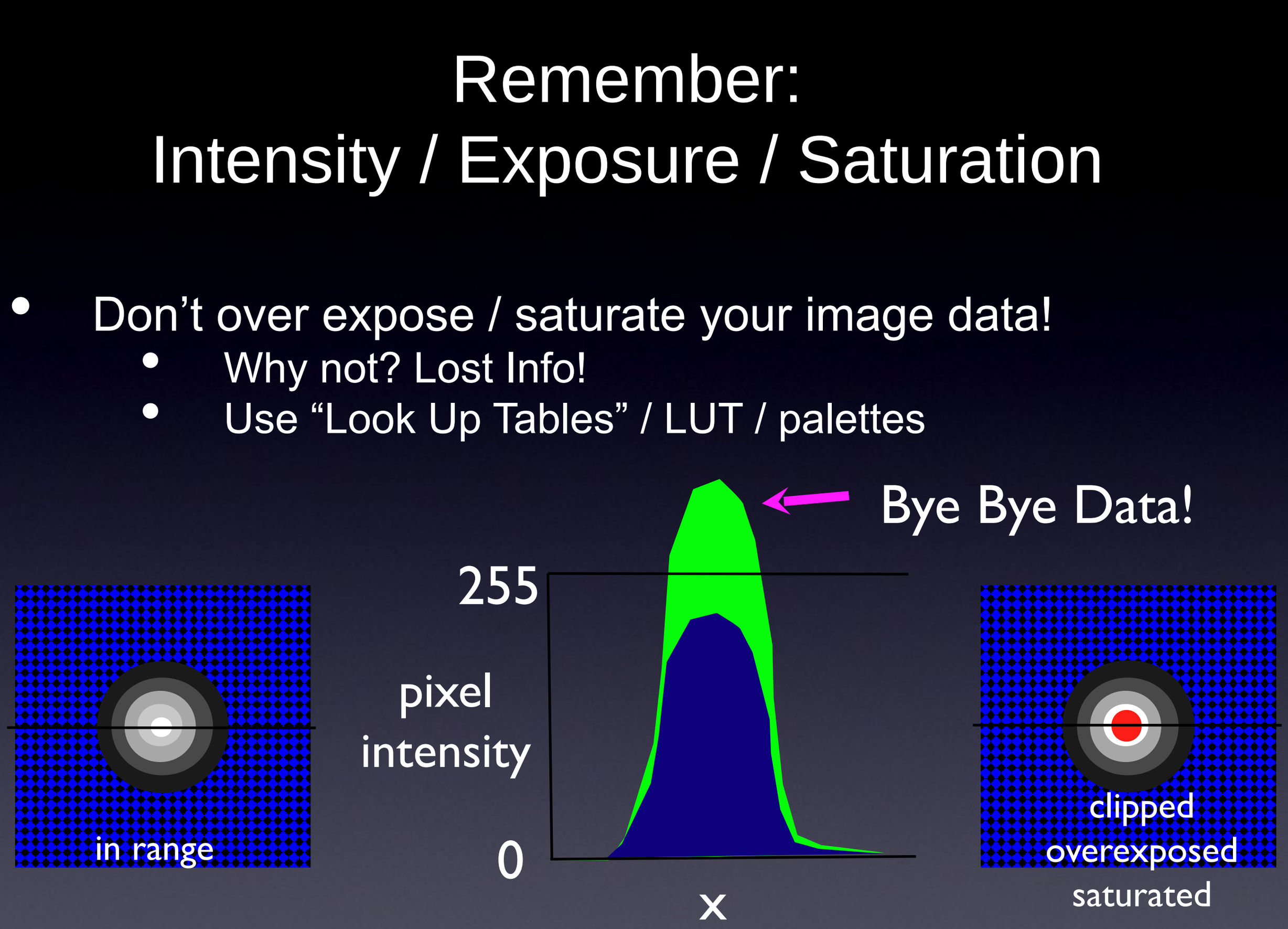


1 bit binary image



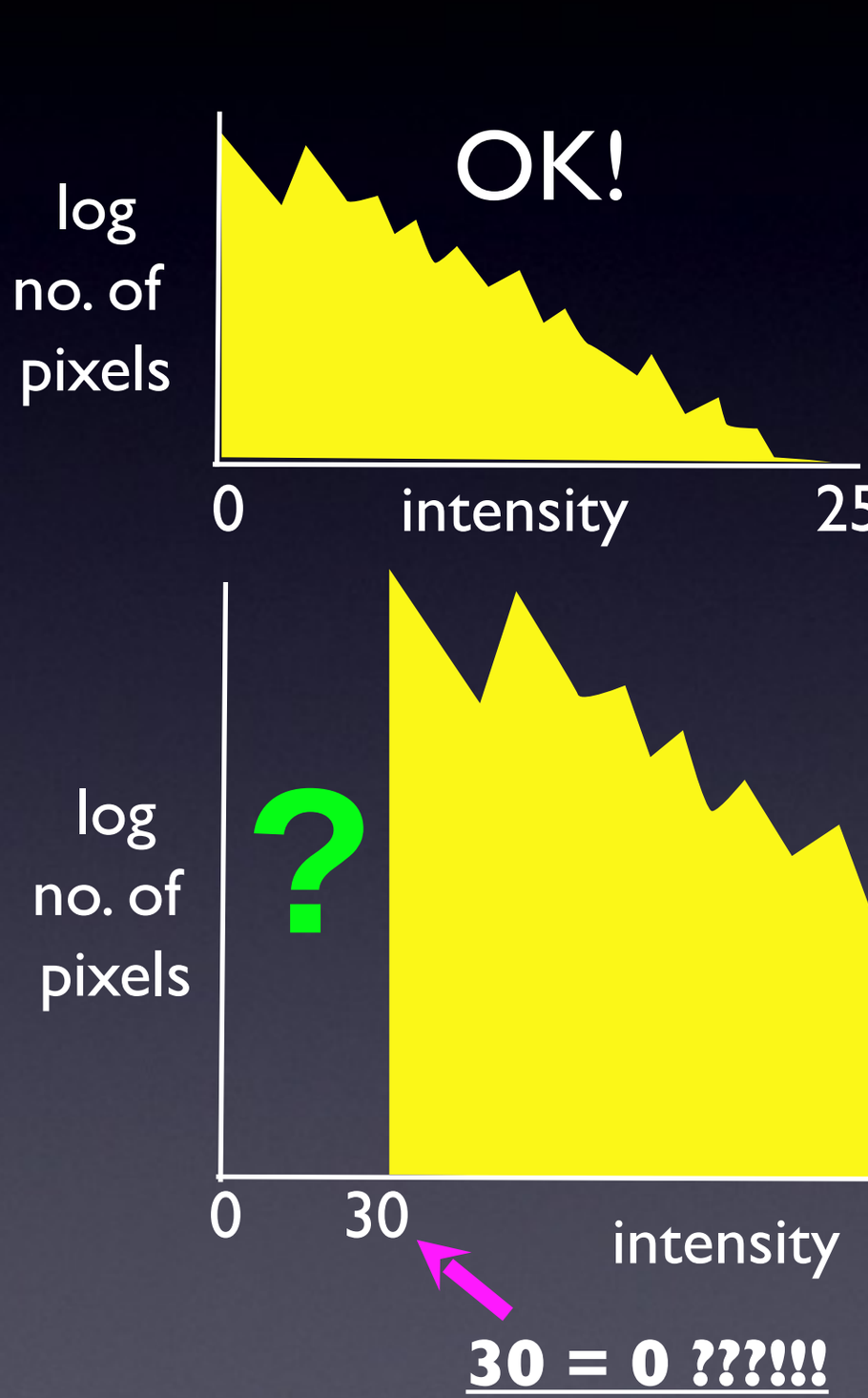
Remember:
Intensity / Exposure / Saturation

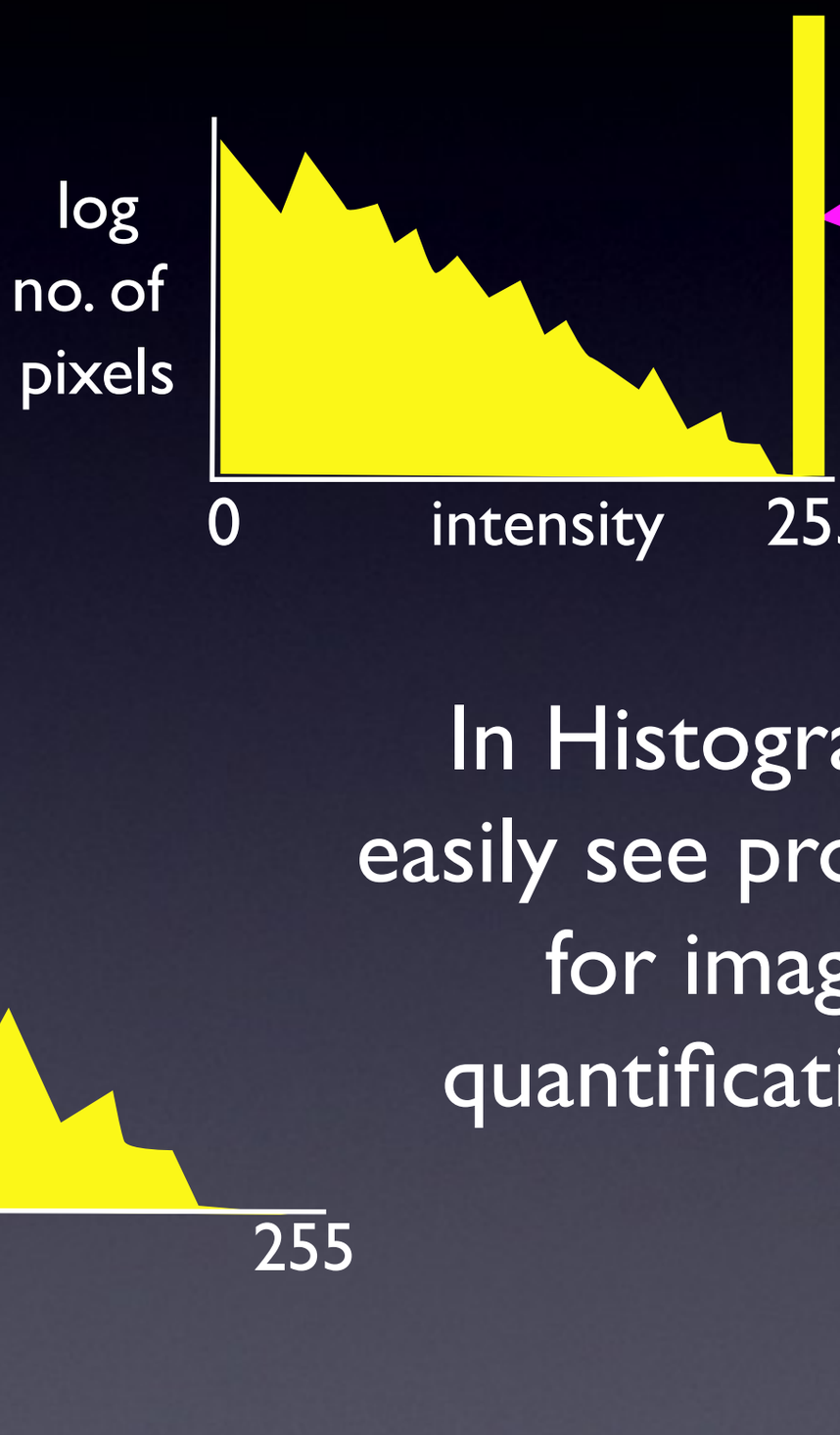
- Don’t over expose / saturate your image data!
  - Why not? Lost Info!
  - Use “Look Up Tables” / LUT / palettes



Zeiss “Range Indicator” palette

Image Intensity Histograms - Use them!

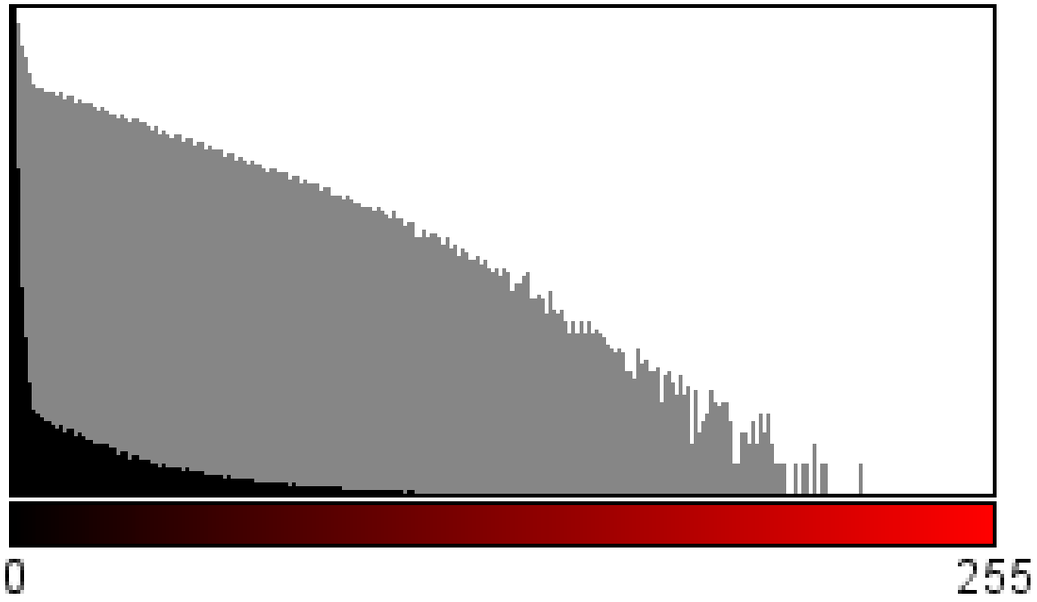




In Histograms:
easily see problems
for image
quantification!

Intensity Histogram

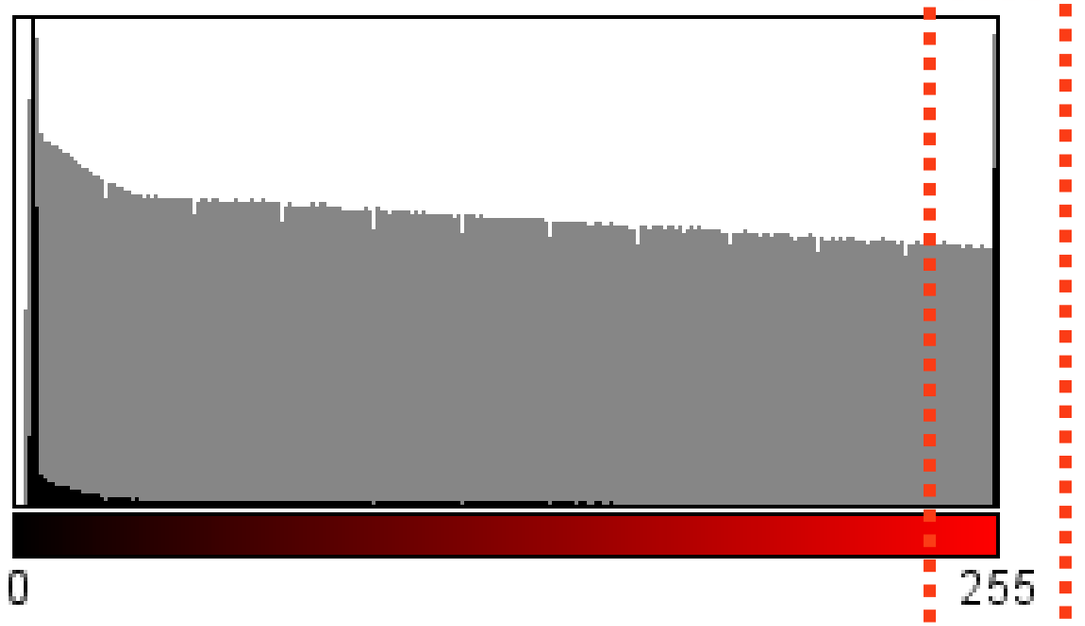
fluorescence microscopy



Count: 524288
Mean: 18.561
StdDev: 26.465

Min: 0
Max: 235
Mode: 0 (174427)

OK



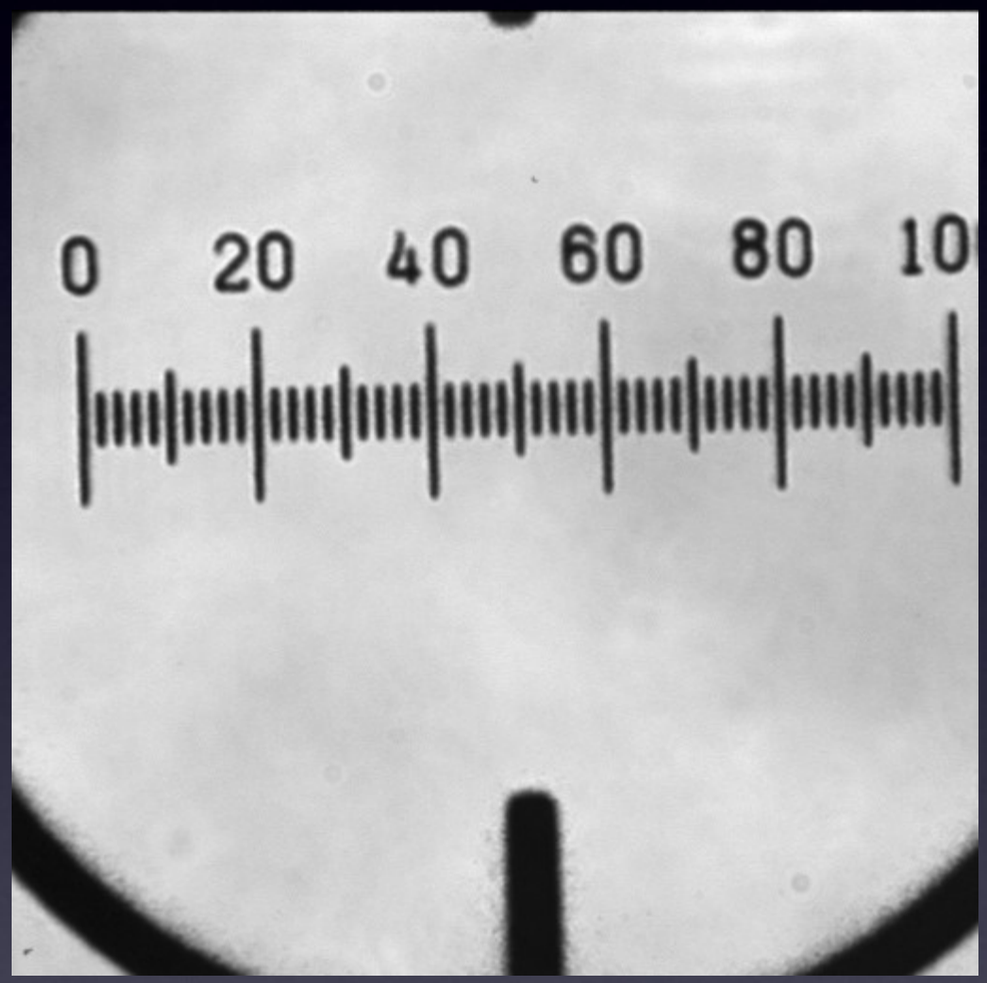
Count: 524288
Mean: 82.504
StdDev: 93.452

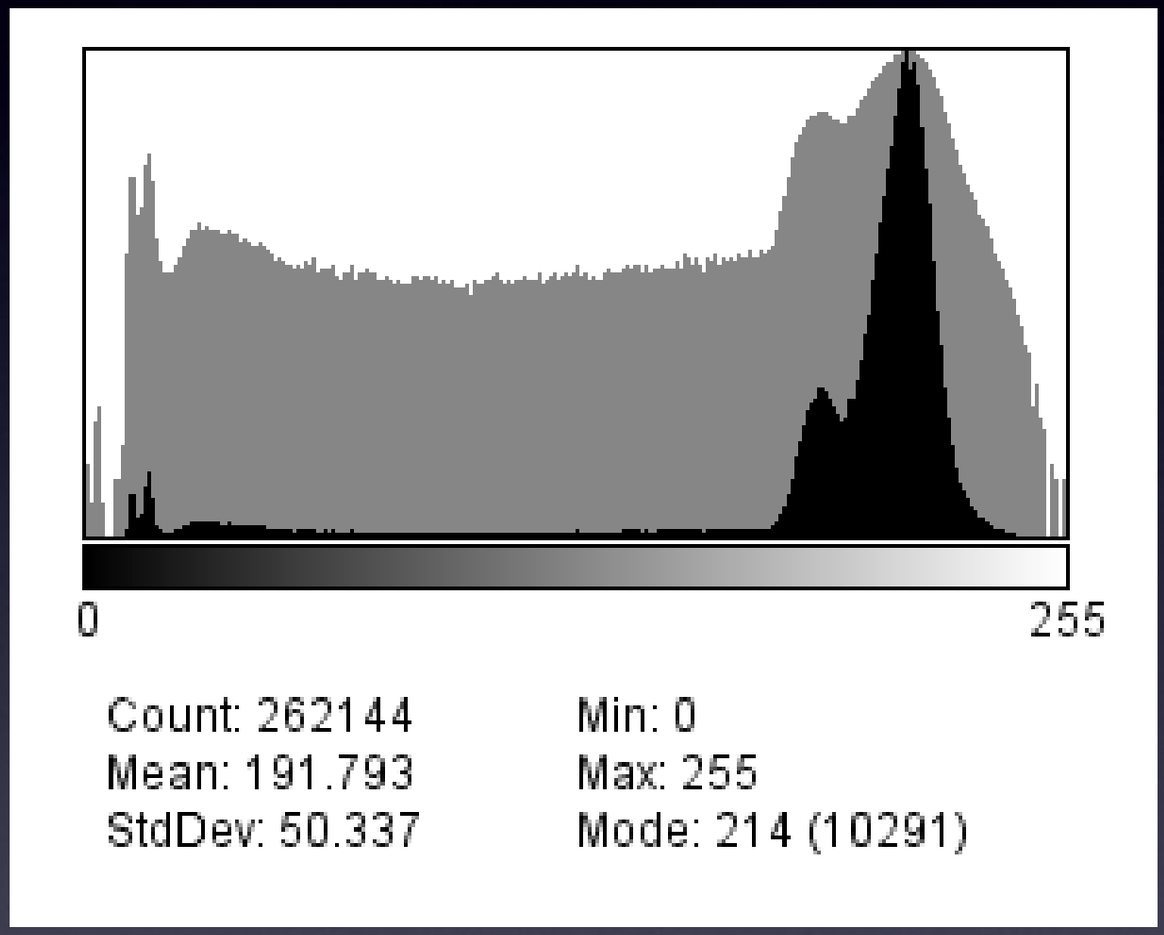
Min: 2
Max: 255
Mode: 4 (101652)

not OK - why?

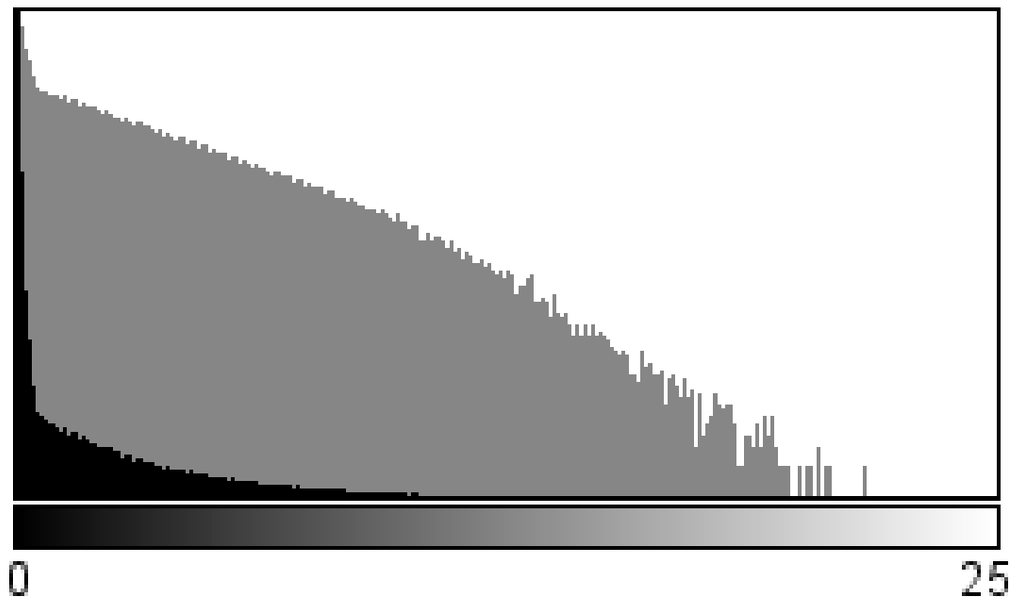
Intensity Histogram

brightfield microscopy





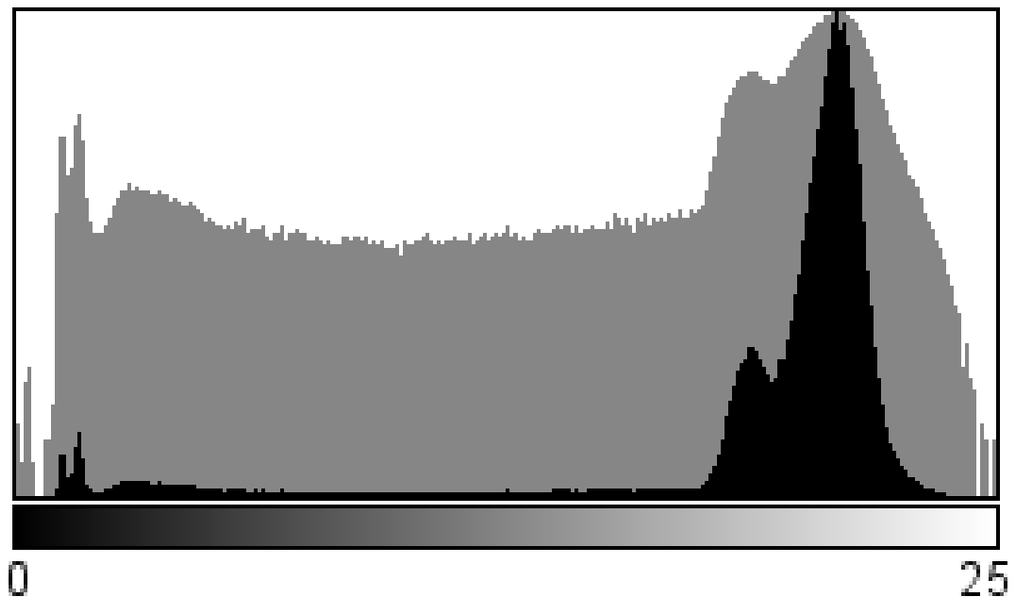
Intensity Histogram



Count: 524288
Mean: 18.561
StdDev: 26.465

Min: 0
Max: 235
Mode: 0 (174427)

fluorescence



Count: 262144
Mean: 191.793
StdDev: 50.337

Min: 0
Max: 255
Mode: 214 (10291)

brightfield

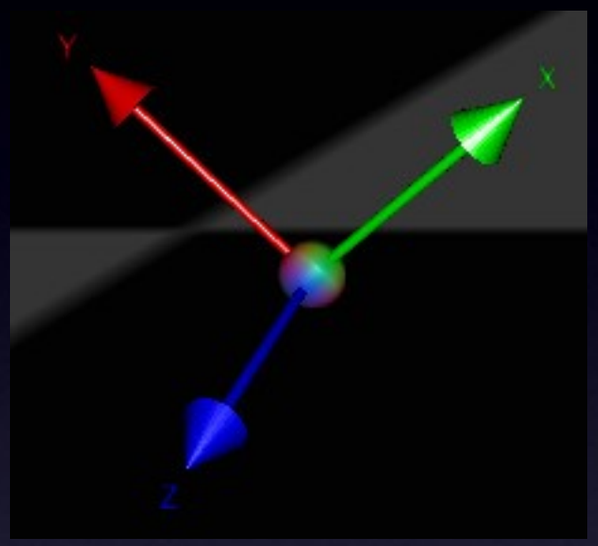
Practical Session 1c

- Getting to know “Fiji” better
- (Fiji is just ImageJ)
- http://pacific.mpi-cbg.de

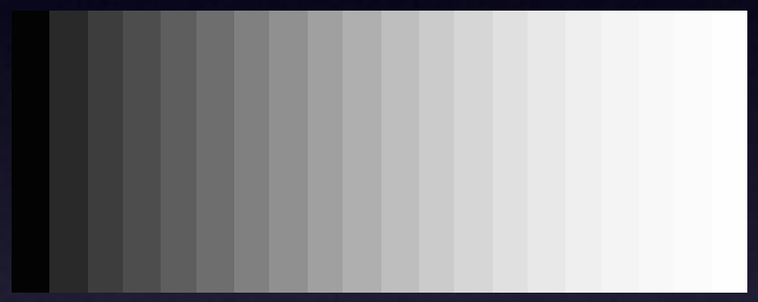
- Bit Depth - change from 16 to 8?
- Neuron - What happens to the numbers?
- Brightness / Contrast
- Image - Adjust - Brightness/Contrast
- LOOK! You Can Lose Data!
- Intensity Histograms
- *log* scale for fluorescence
- Look for Intensity clipping / saturation and offsets.

What can you digitise?

Dimensions!



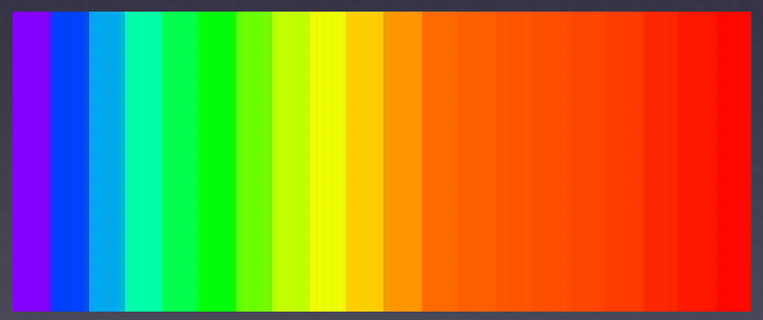
SPACE



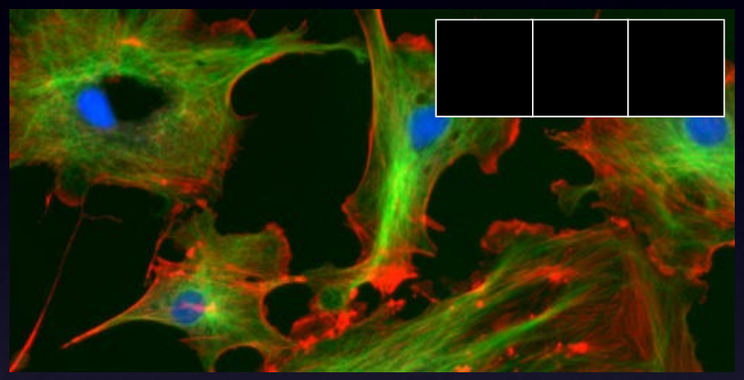
INTENSITY

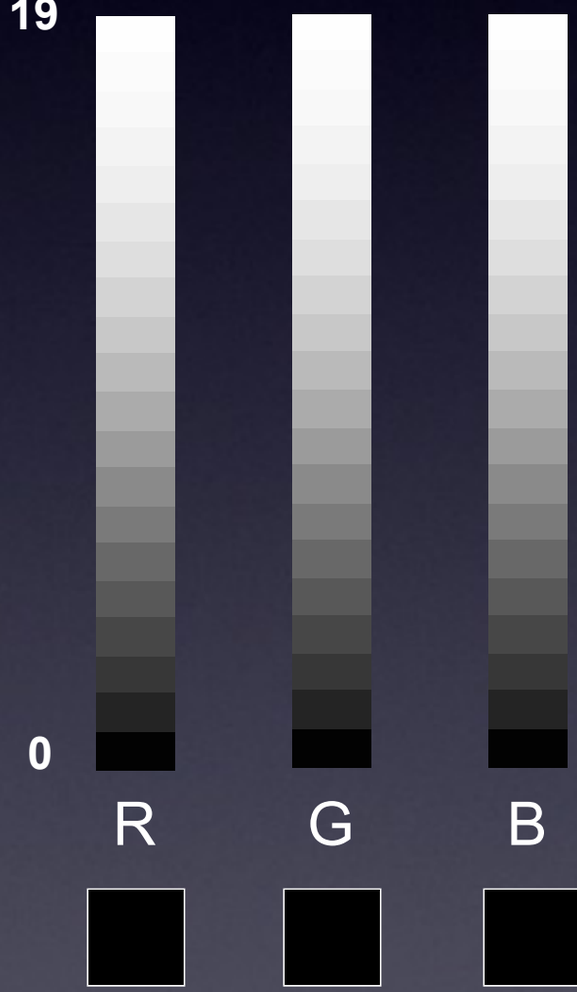
TIME

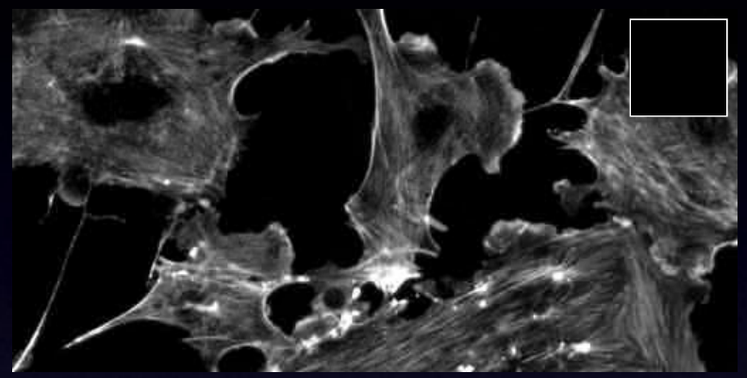
Colour Channels Wavelength

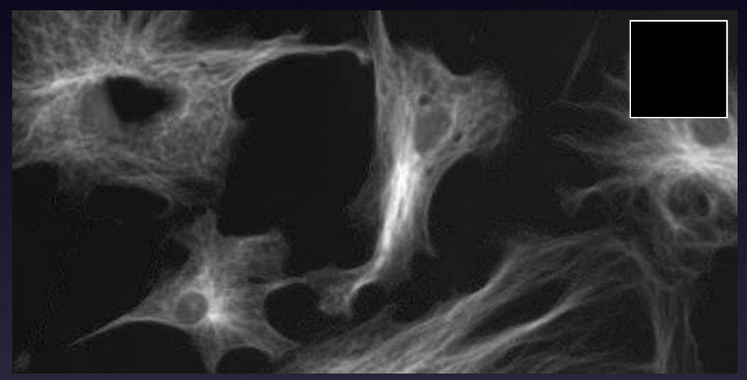


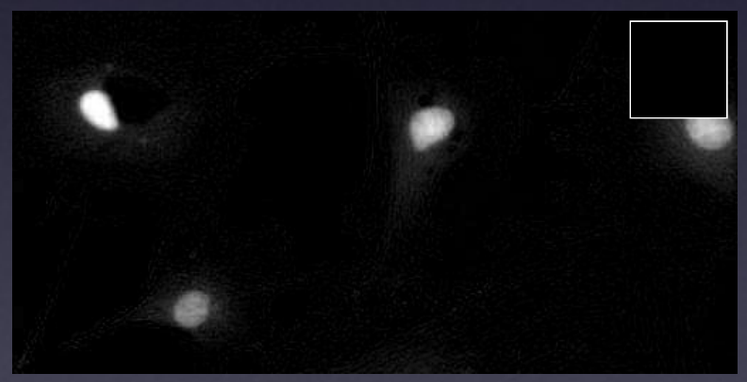
RGB Color Space







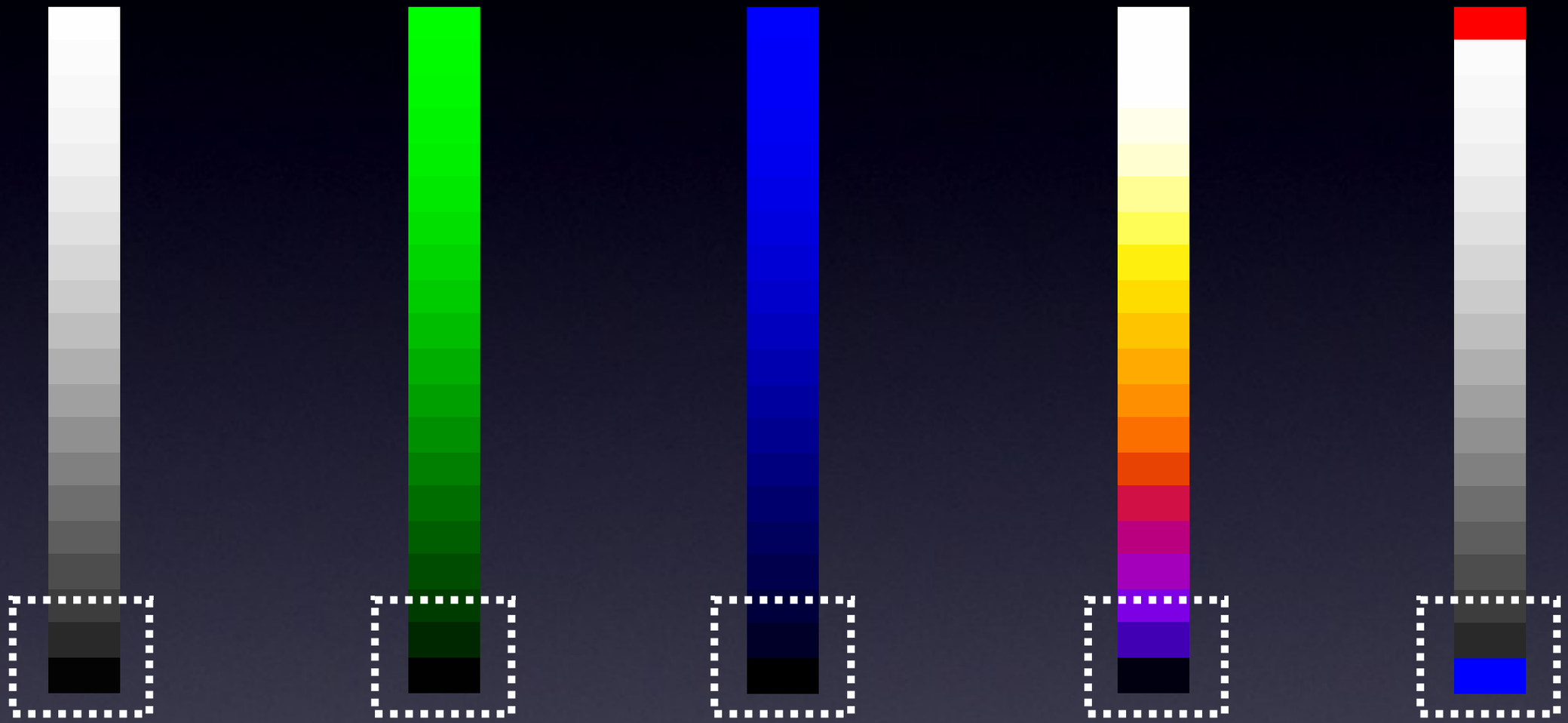




Why RGB?
... because we have red, green and blue sensitive photo receptors in our eyes!

Each “colour” is really just single greyscale numbers!

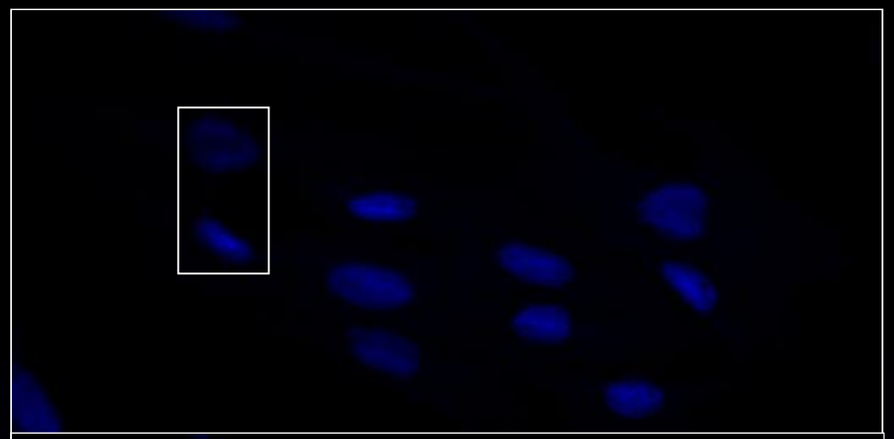
Lookup Tables / Palettes



“grey” “green” “blue” “fire” “HiLo”

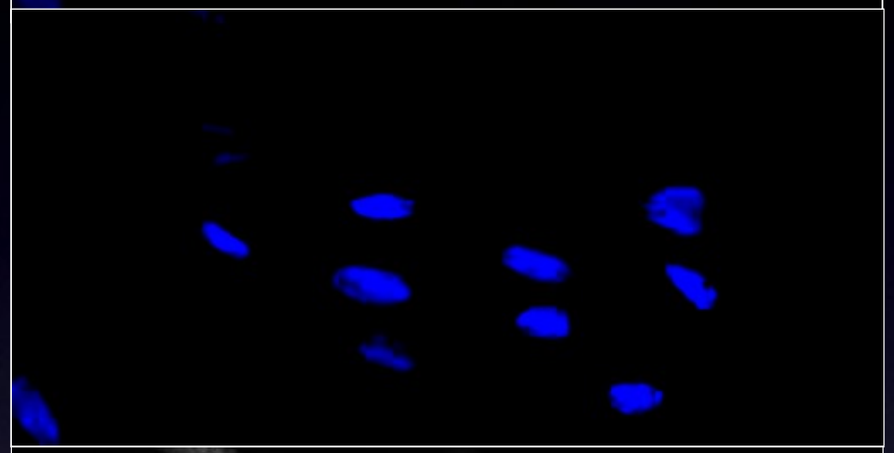
Each “colour” is really just single greyscale numbers!

So we can represent that information however we like!



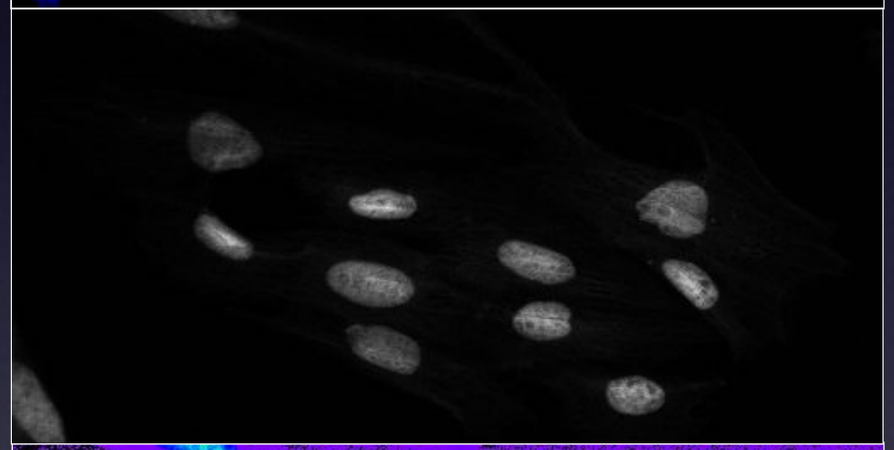
"original"

linear blue



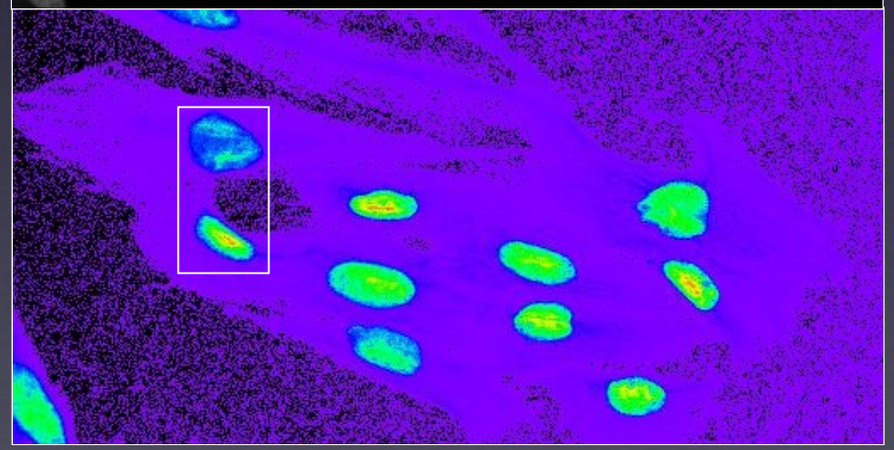
brightness + contrast

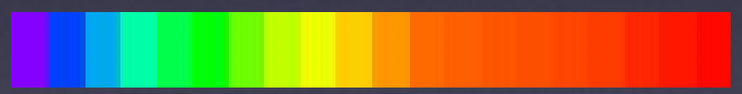
data changed/lost!



grayscale

linear

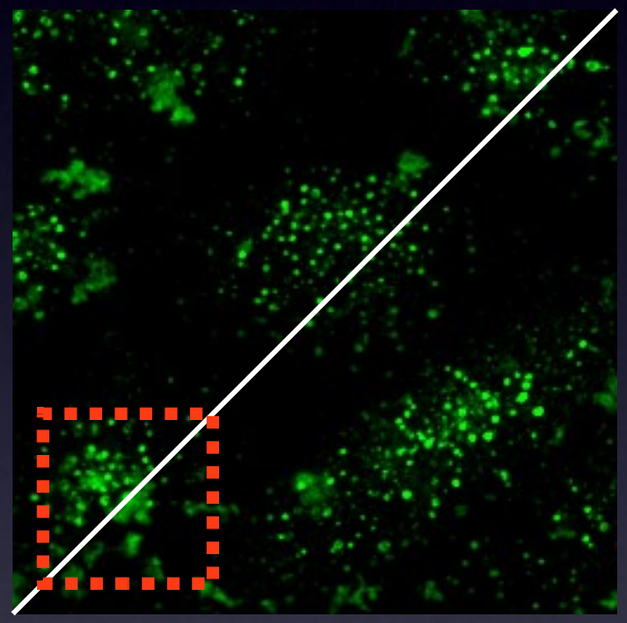


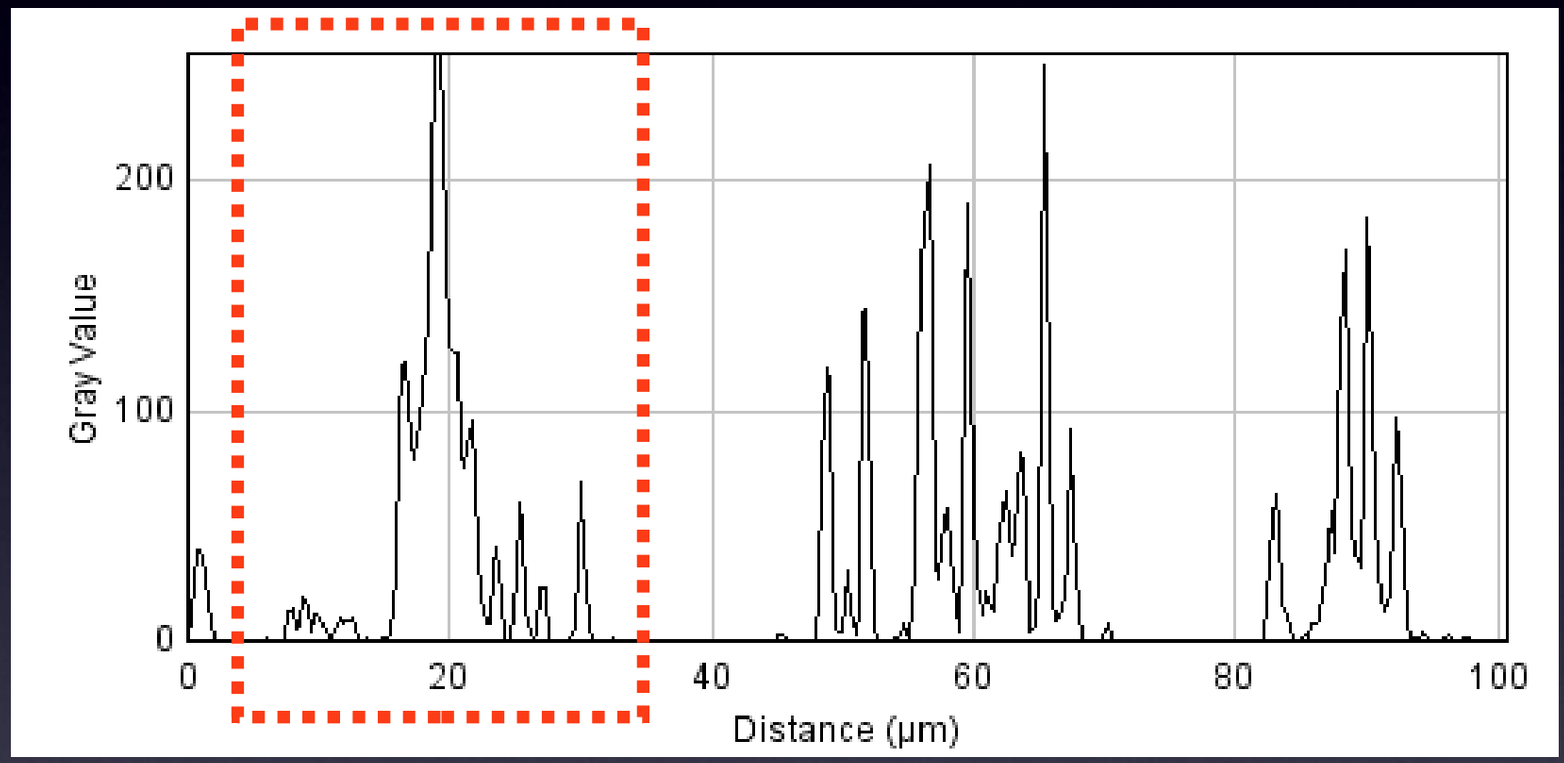


rainbow lookup table

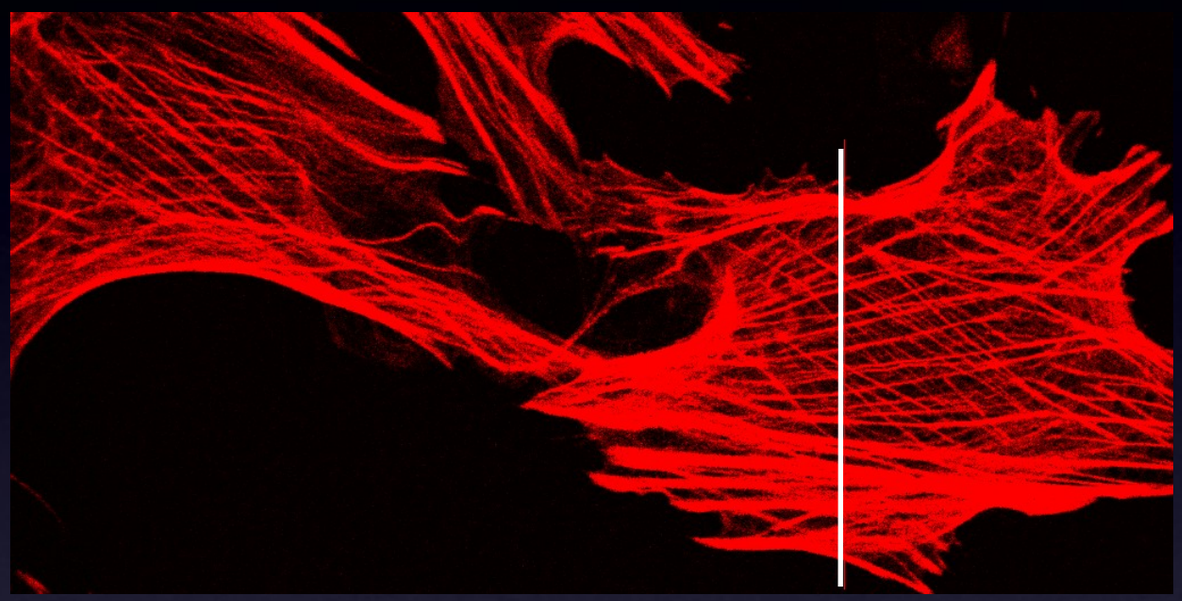
better see and also compare different intensity levels

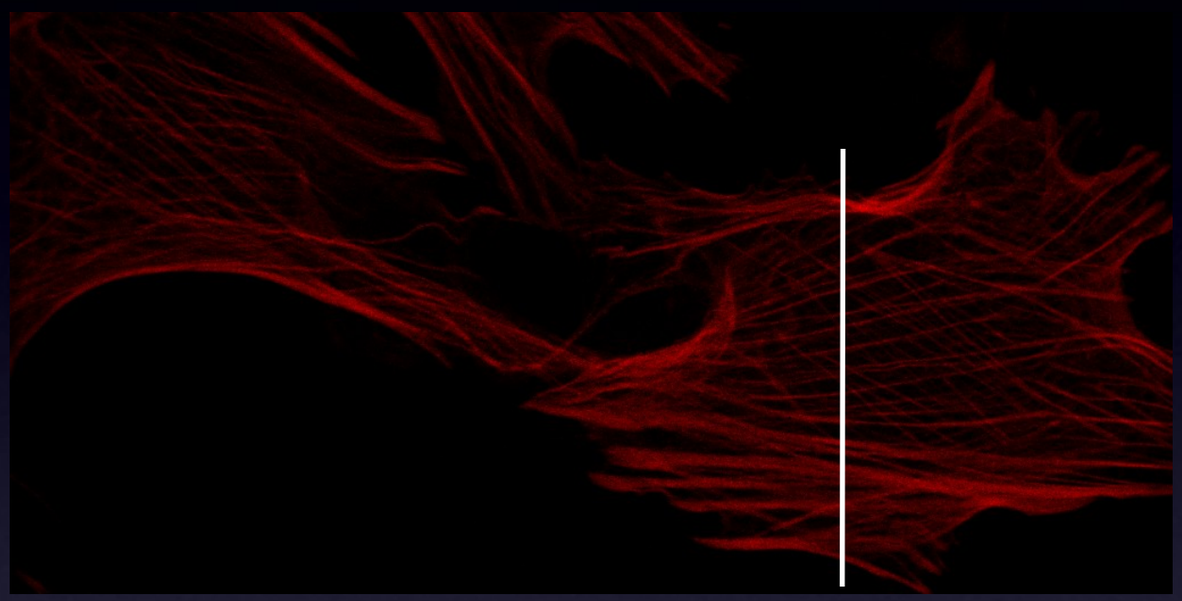
Line Profile

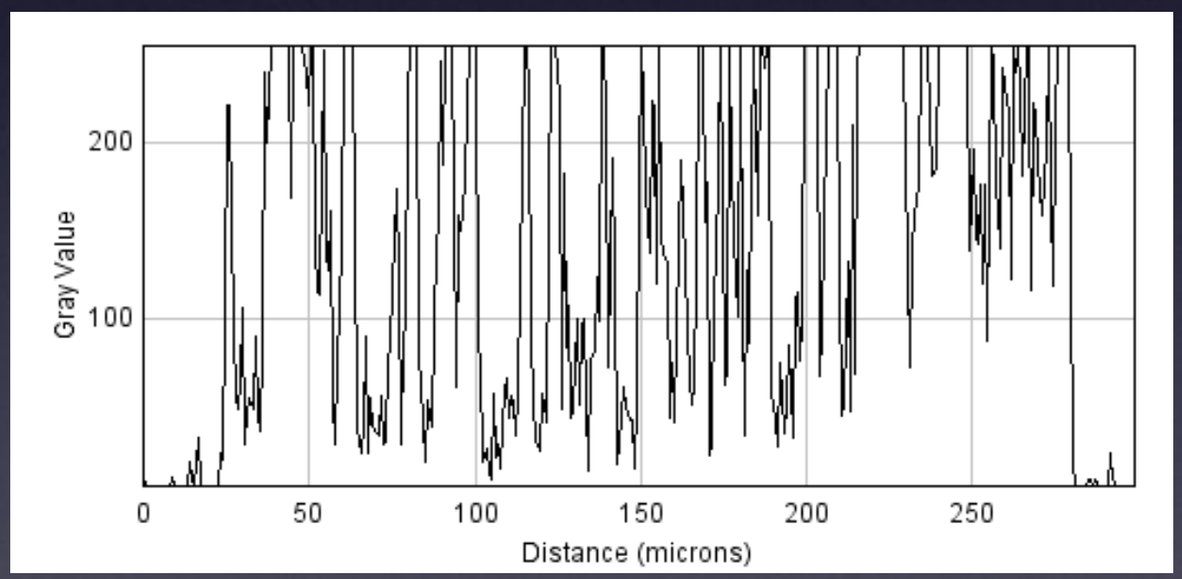


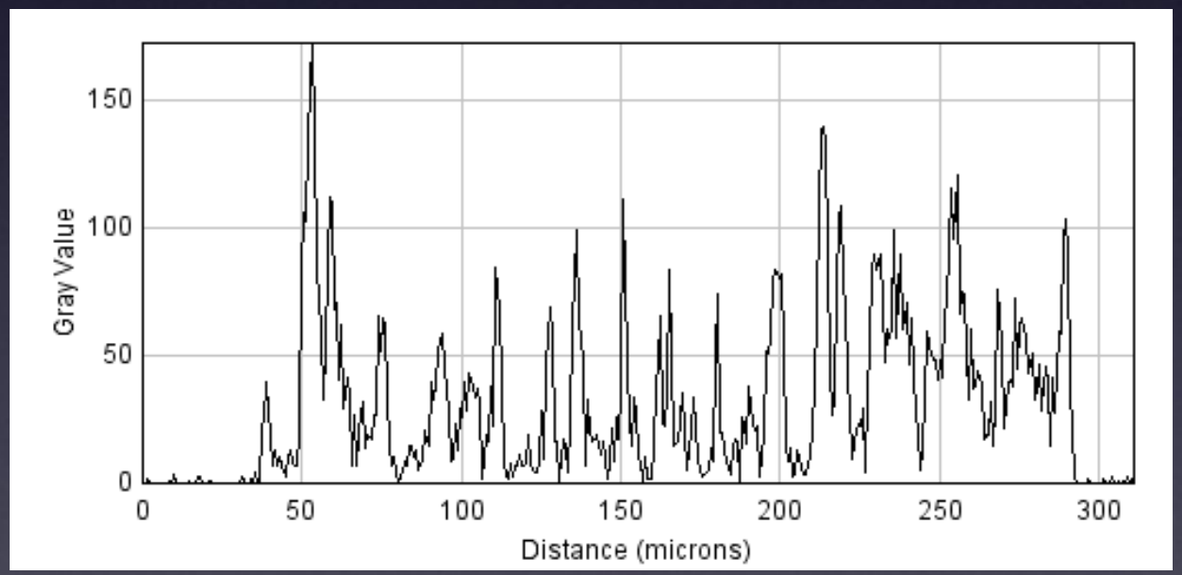


Line Profile



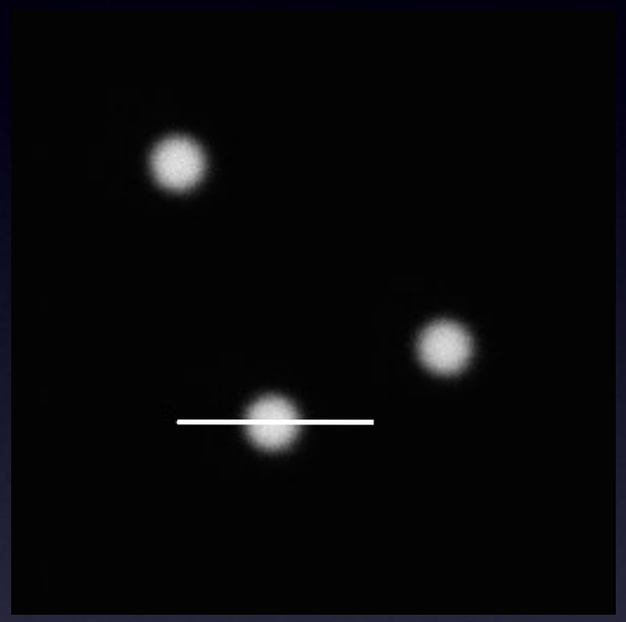


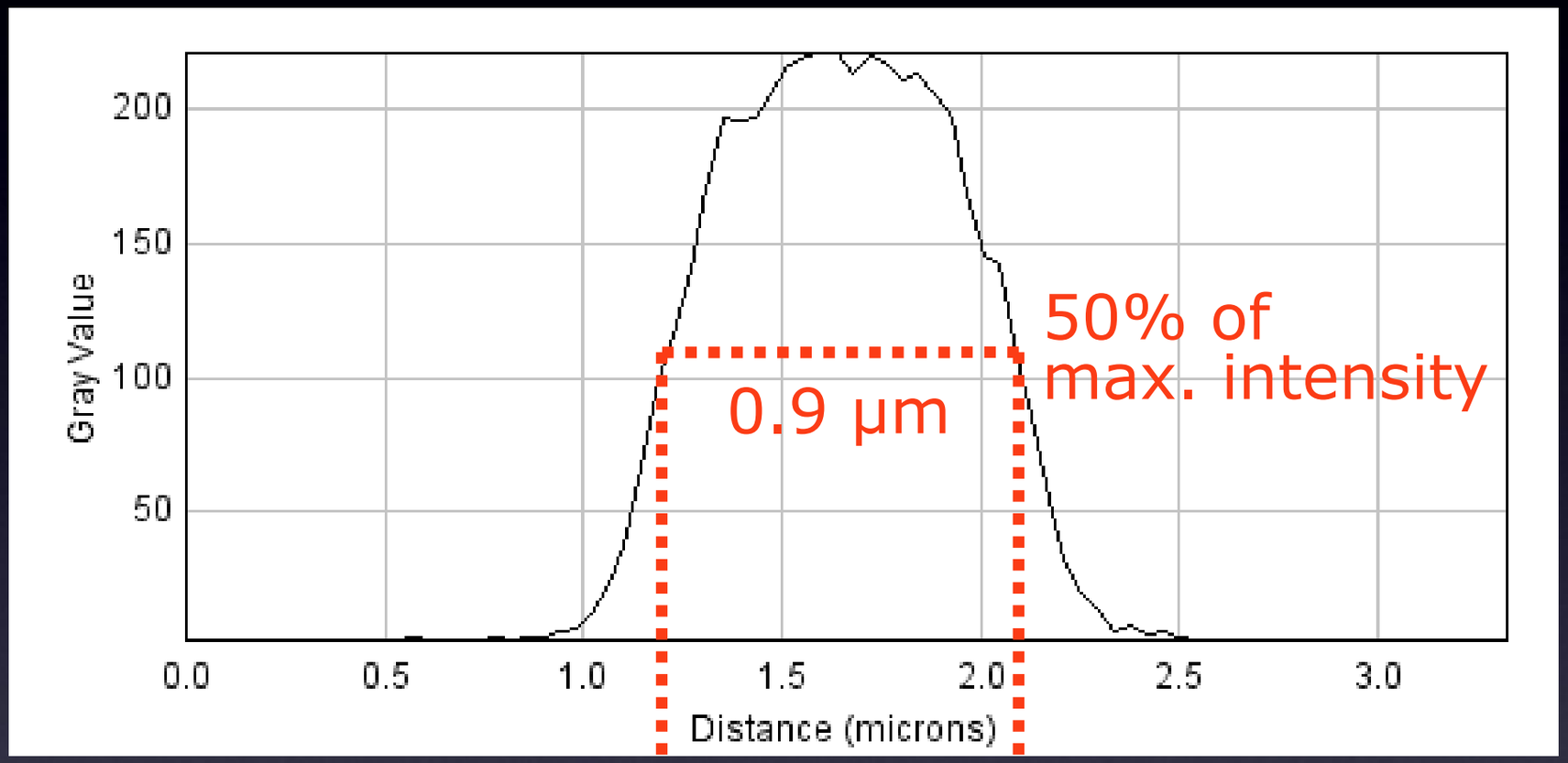




Line Profile

for measurements

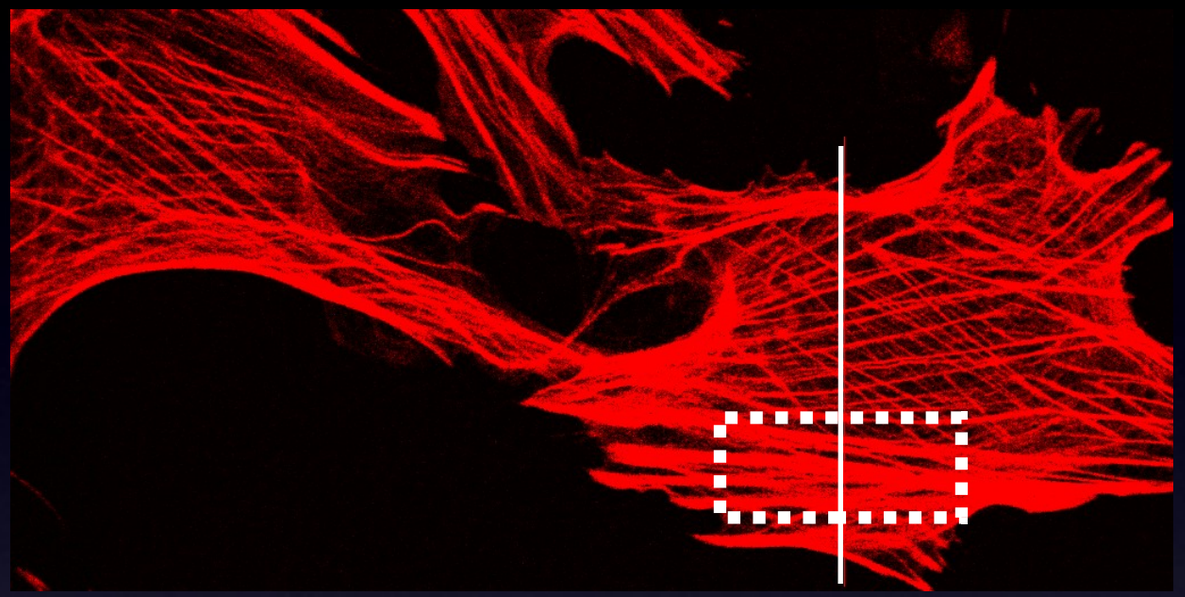


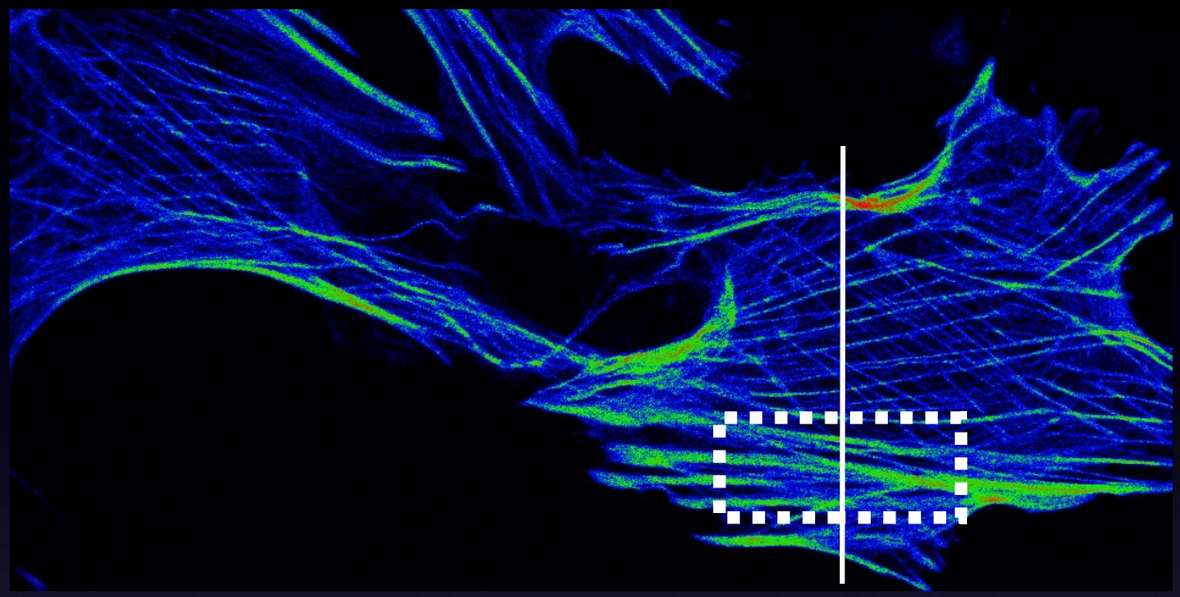


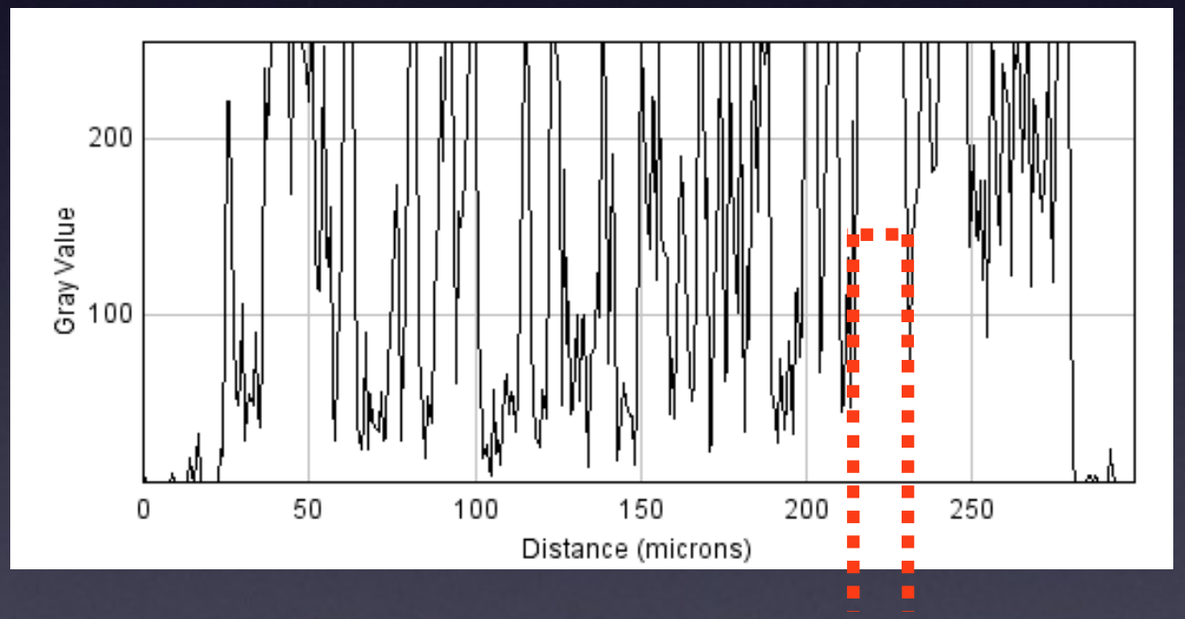
FWHM

= “Full Width at Half Maximum”

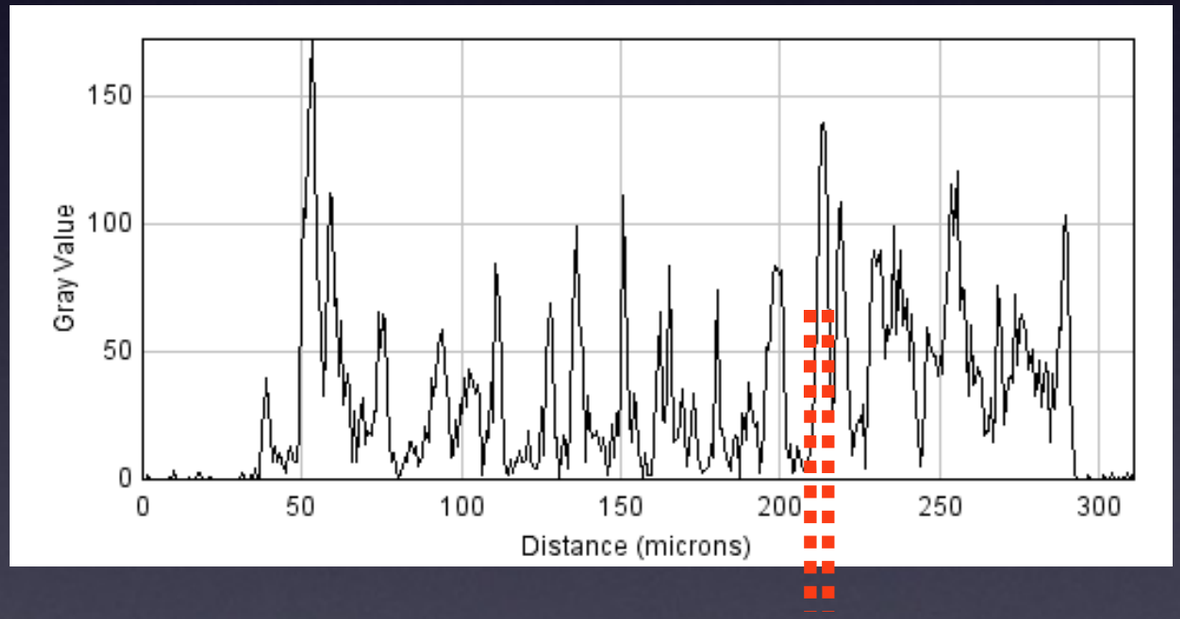
Line Profile





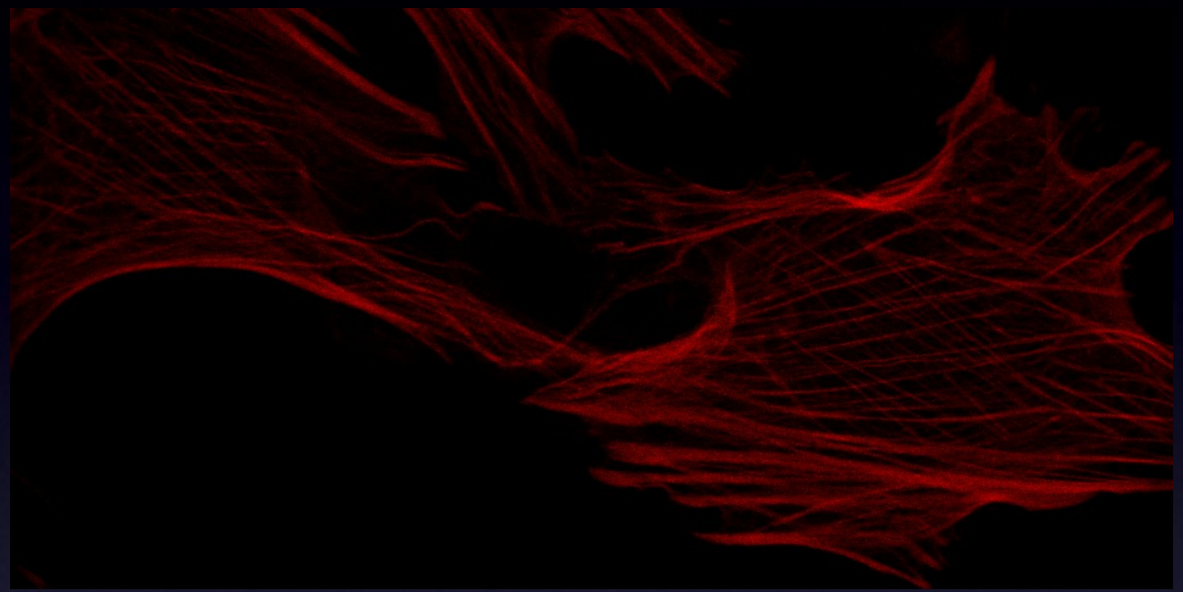


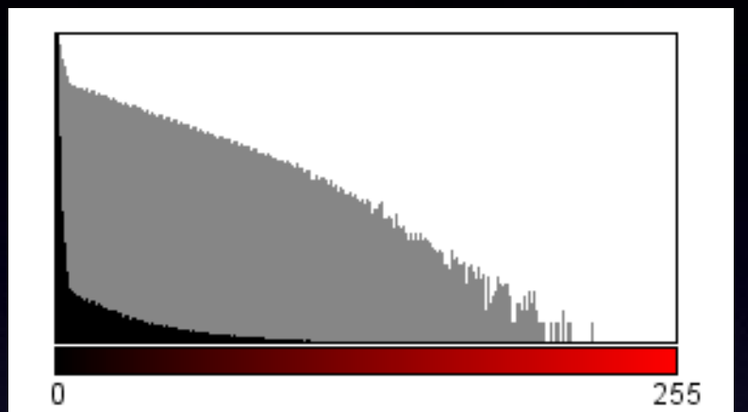
correct ?

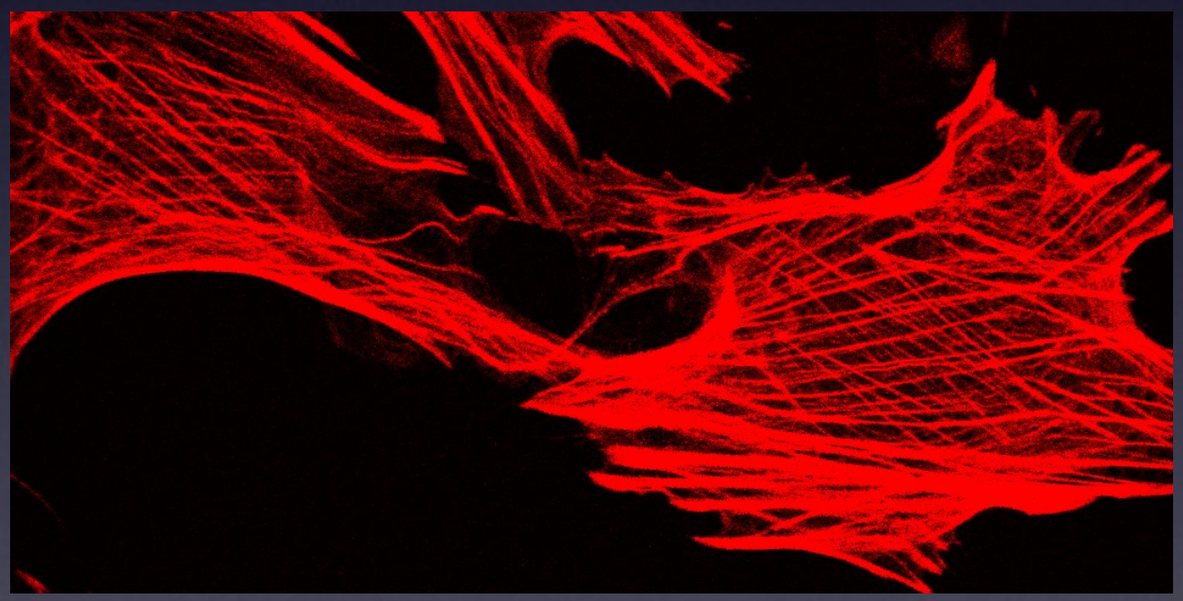


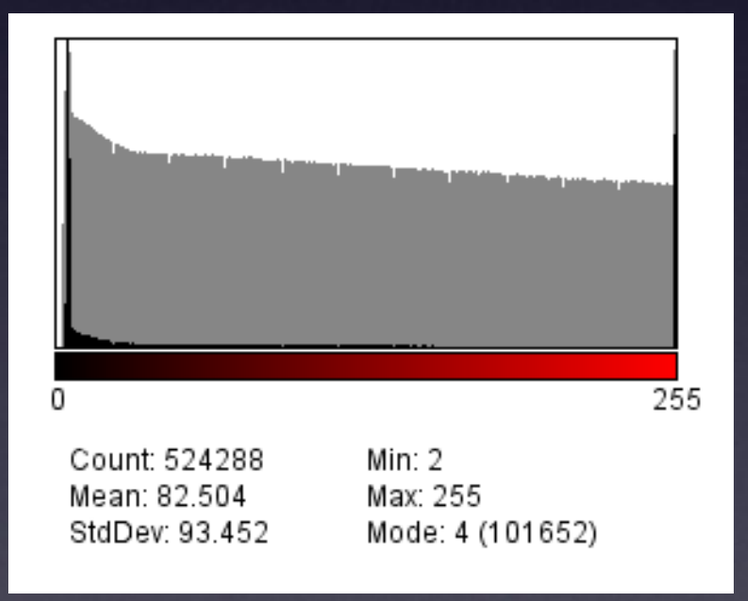
correct !

Intensity Histogram

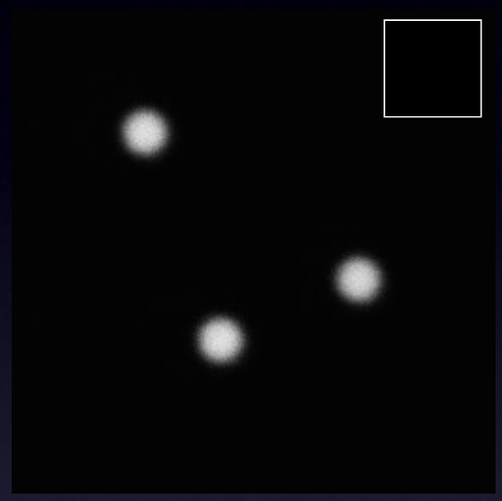


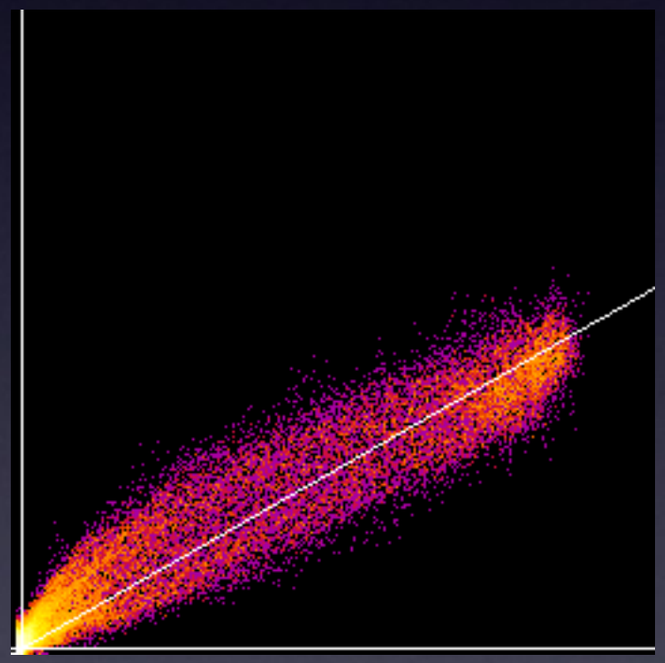


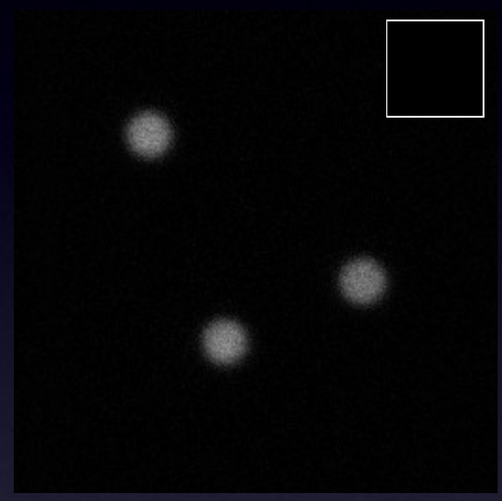


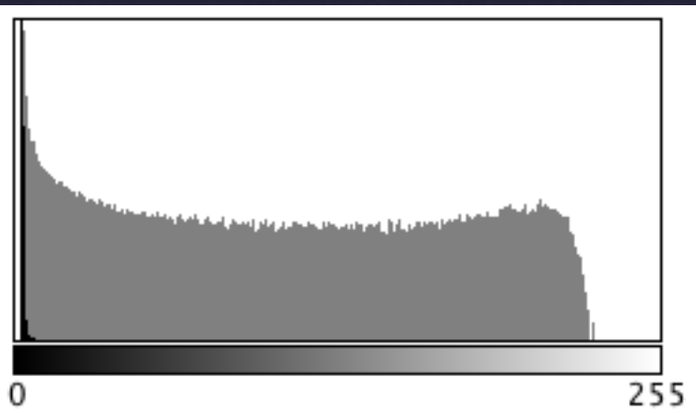


2 Histograms
=
Scatterplot
or
2D Histogram

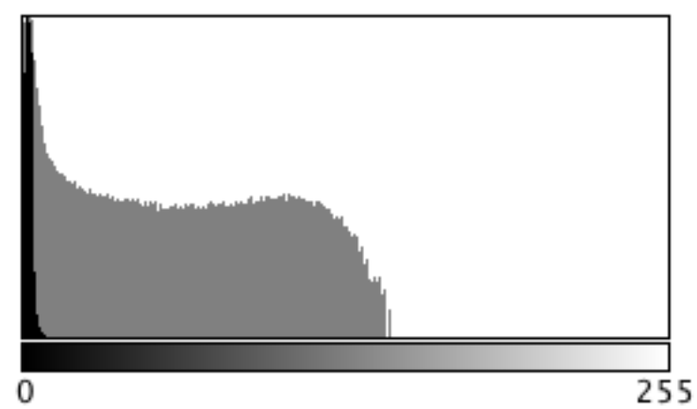






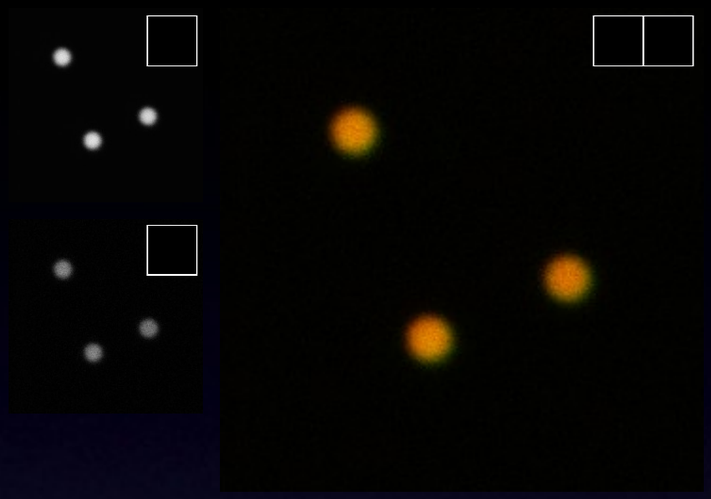


Count: 589824 Min: 2
Mean: 5.782715 Max: 229
StdDev: 22.748384 Mode: 2 (480434)

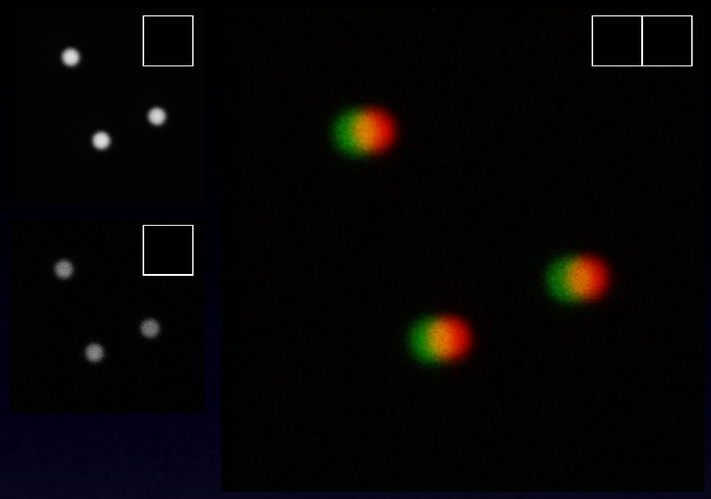


Count: 589824 Min: 0
Mean: 3.928024 Max: 153
StdDev: 13.113667 Mode: 1 (140607)

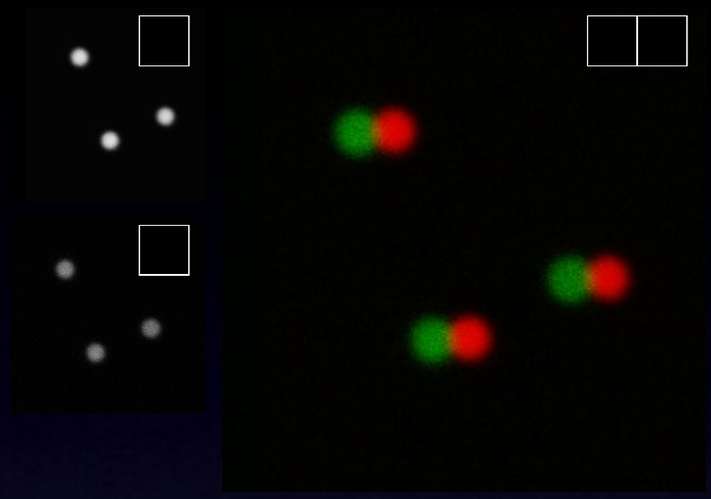
Scatterplot / 2D Histogram



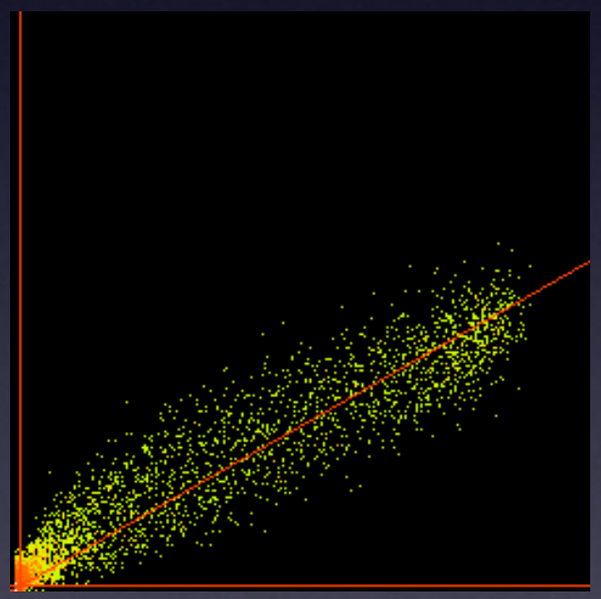
original R+G

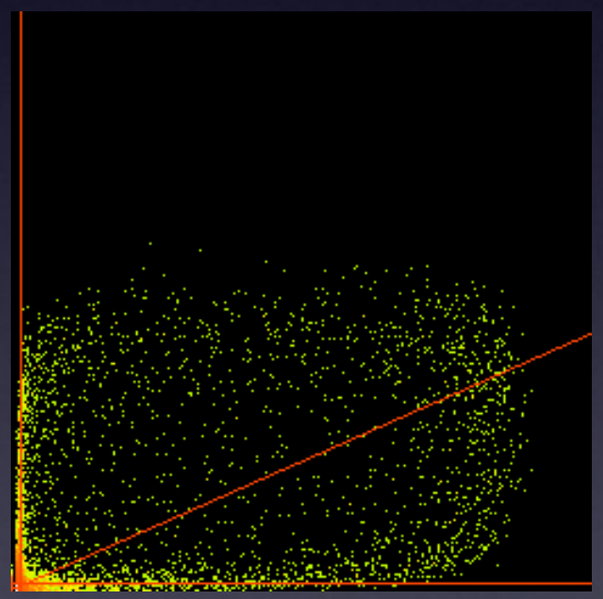


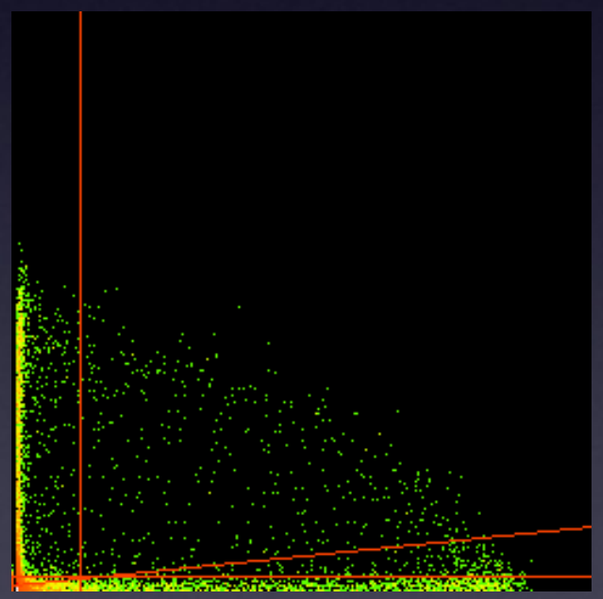
R shifted +10 pix



R shifted +20 pix



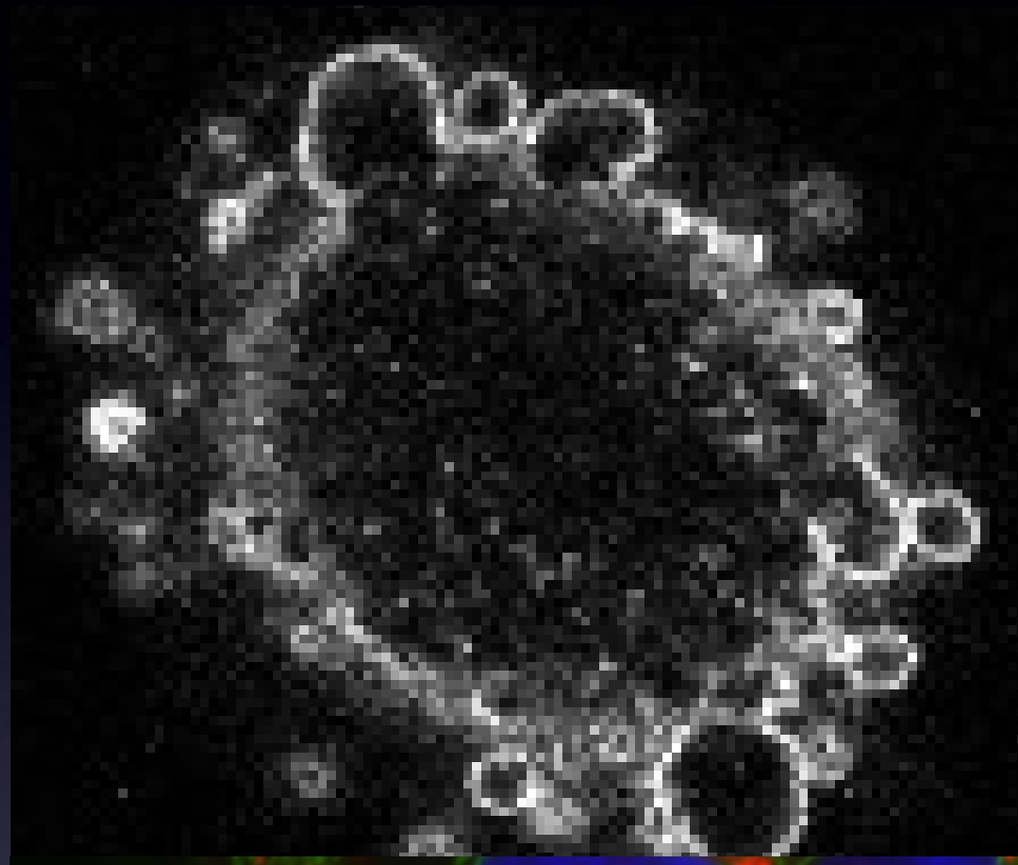


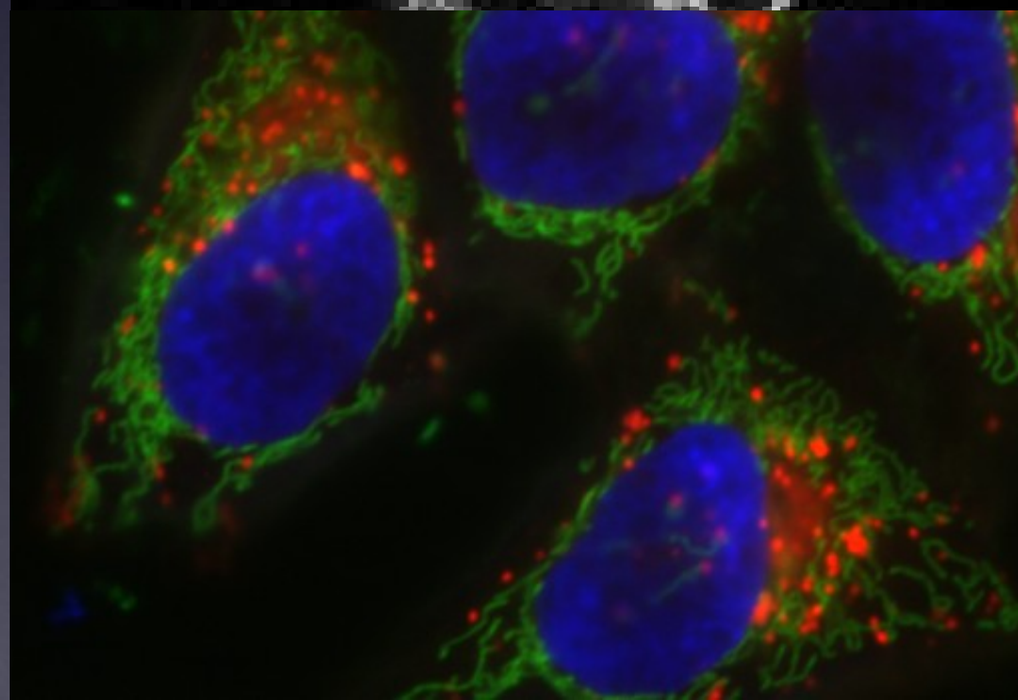


Find a way to visualise what you actually want to see:
In this case we don’t care where the beads are;
We care if they are in the same place or not!

Imaging Experiment Planning:

- What **BIOLOGY** an I trying to **measure**?
  - What is the hypothesis under test?
- Do I need 3D, 4D, xD information
  - Resolution? Sampling: Space, Time, Intensity
- Choose appropriate microscope
  - Don't use Confocal LSM just because its the newest or most expensive or because that what others in your lab use
- Optimise microscope system
  - get best data from your sample
- Do the right controls
- Measure Something
  - Statistics to test hypothesis
  - How many data points/images/cells?





Imaging Experiment Work Flow

EXPERIMENT HYPOTHESIS
- What is my experimental hypothesis?
- How can I test my hypothesis?

WHAT INFO/DATA DO I NEED
- Dimensions
- Resolution
- Precision

CONTROLS DESIGN
- What controls do I need?

PLAN INFO ANALYSIS
- How can I get that info?
- How will the statistical tests work?

EQUIPMENT CHOICE + SETUP
- What type of equipment/imaging System is needed
- System learning
- Process optimization (Imaging equipment + sample prep)

DATA ACQUISITION

IMAGE PROCESSING
- Noise removal
- Deconvolution
- etc.

MEASUREMENTS
- Intensities / objects

STATISTICAL ANALYSIS
- Hypothesis is true or false?

Practical Session 1d

- RGB Color Space
- Colour Channels: Image - Colour - Channels Tool, Split channels etc.
- Lookup Tables / Palettes: Image - Lookup tables or LUT tool icon
- Line Profile: Analyze - Plot Profile
- Histogram: 1) Analyze - Histogram. 2) Plugins-Analyze-2D Histogram)
- Intensity Scale: Analyze - Tools - Calibration Bar

File - Open Samples - Neuron

What you need to know about Image Analysis...but never thought to ask continued....

Session 2

Filtering images in the spatial, frequency and time domains

Segmentation - finding and measuring objects in images

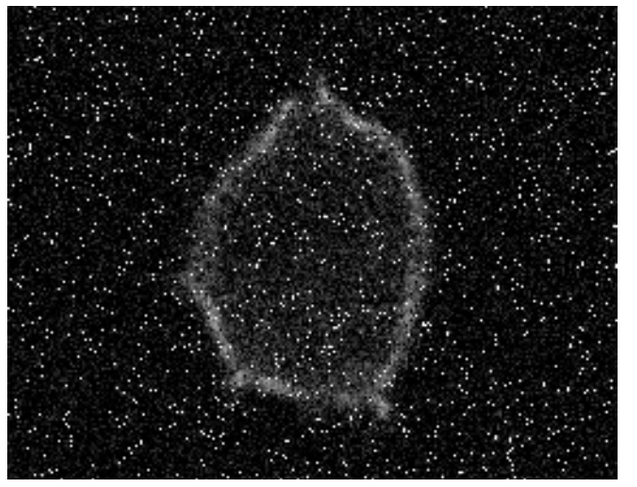
Session 3

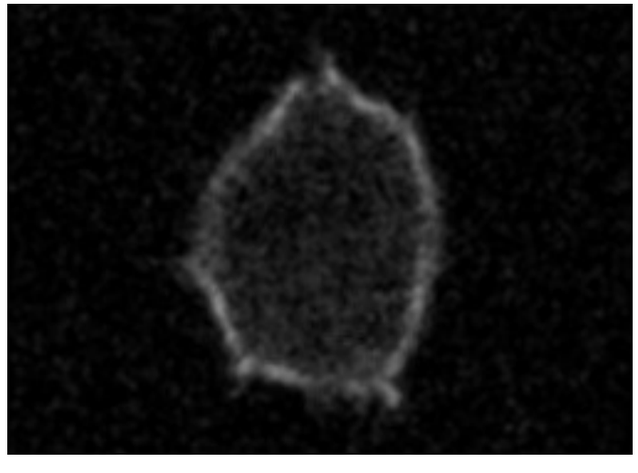
Fiji tutorials

DetectInfoLoss, ColocalisationAnalysis and more...

Whatever you find interesting...

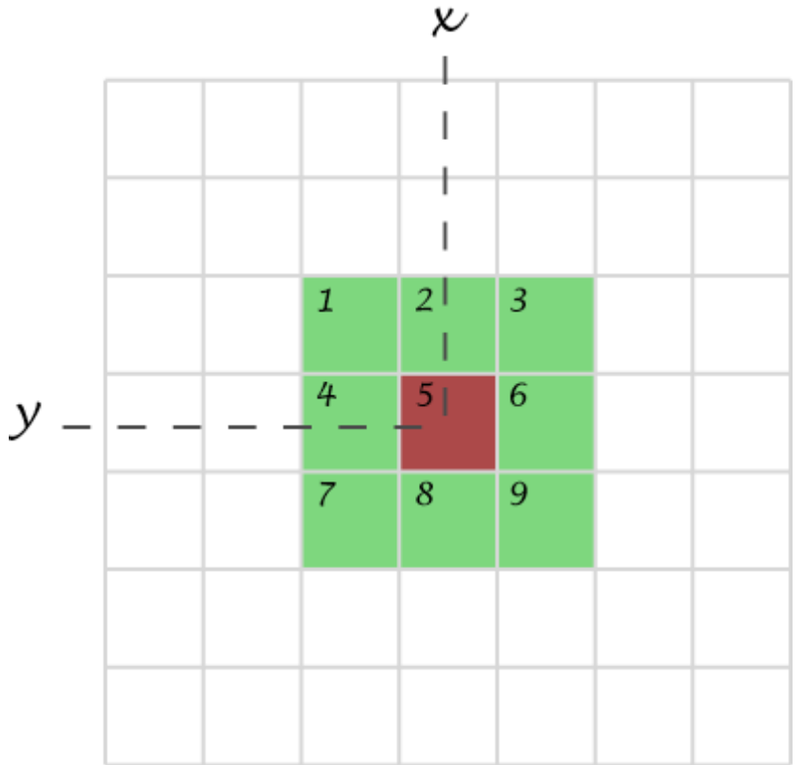
Image processing in the spatial domain





- A. Introduction
  - Neighbourhood
  - Operation on neighbours
- B. Spatial filters
  - Mean filter
  - Median filter
  - Edge detection

A. Introduction



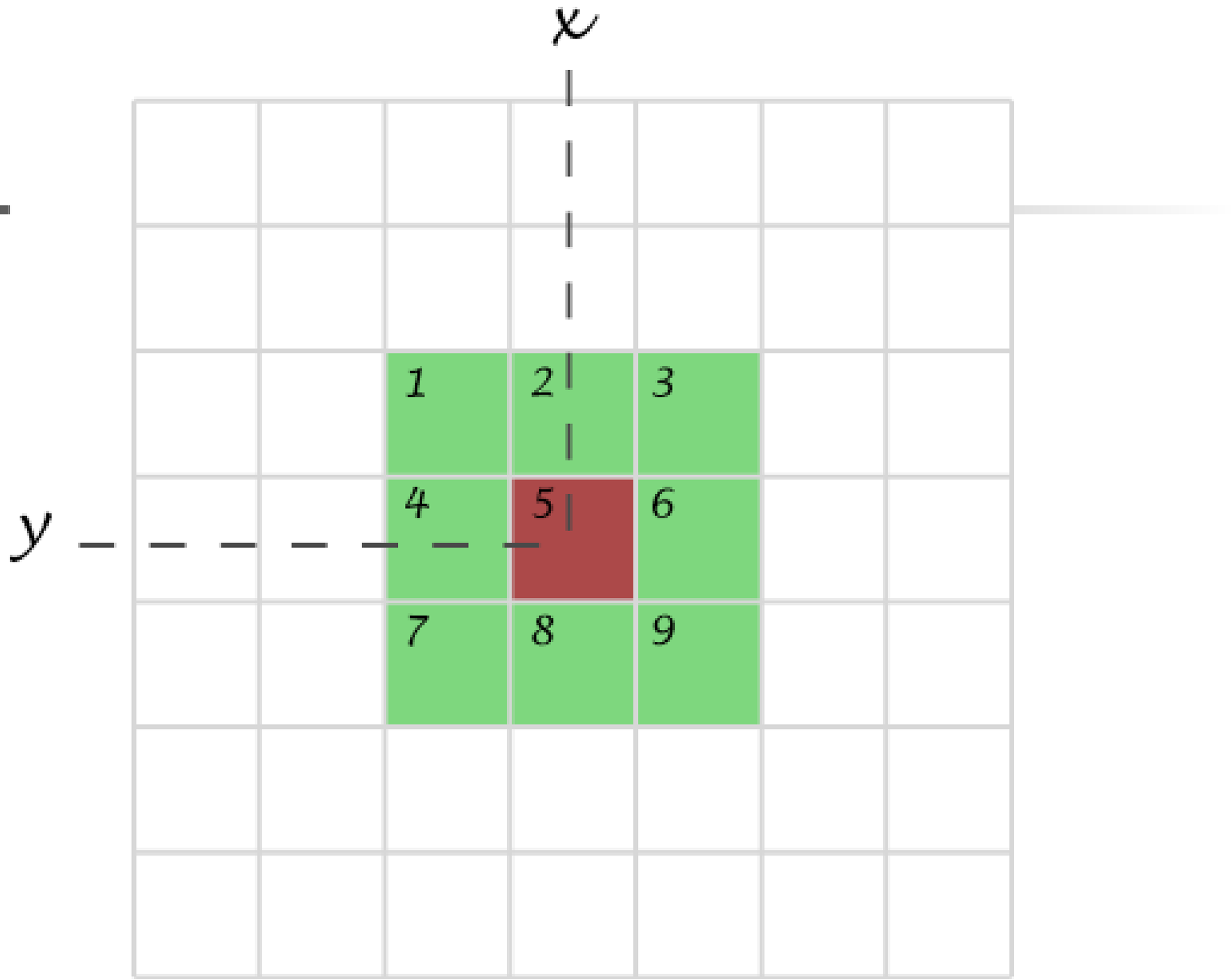
Neighbourhood (or kernel): pixels that matter

- <u>Definition</u>

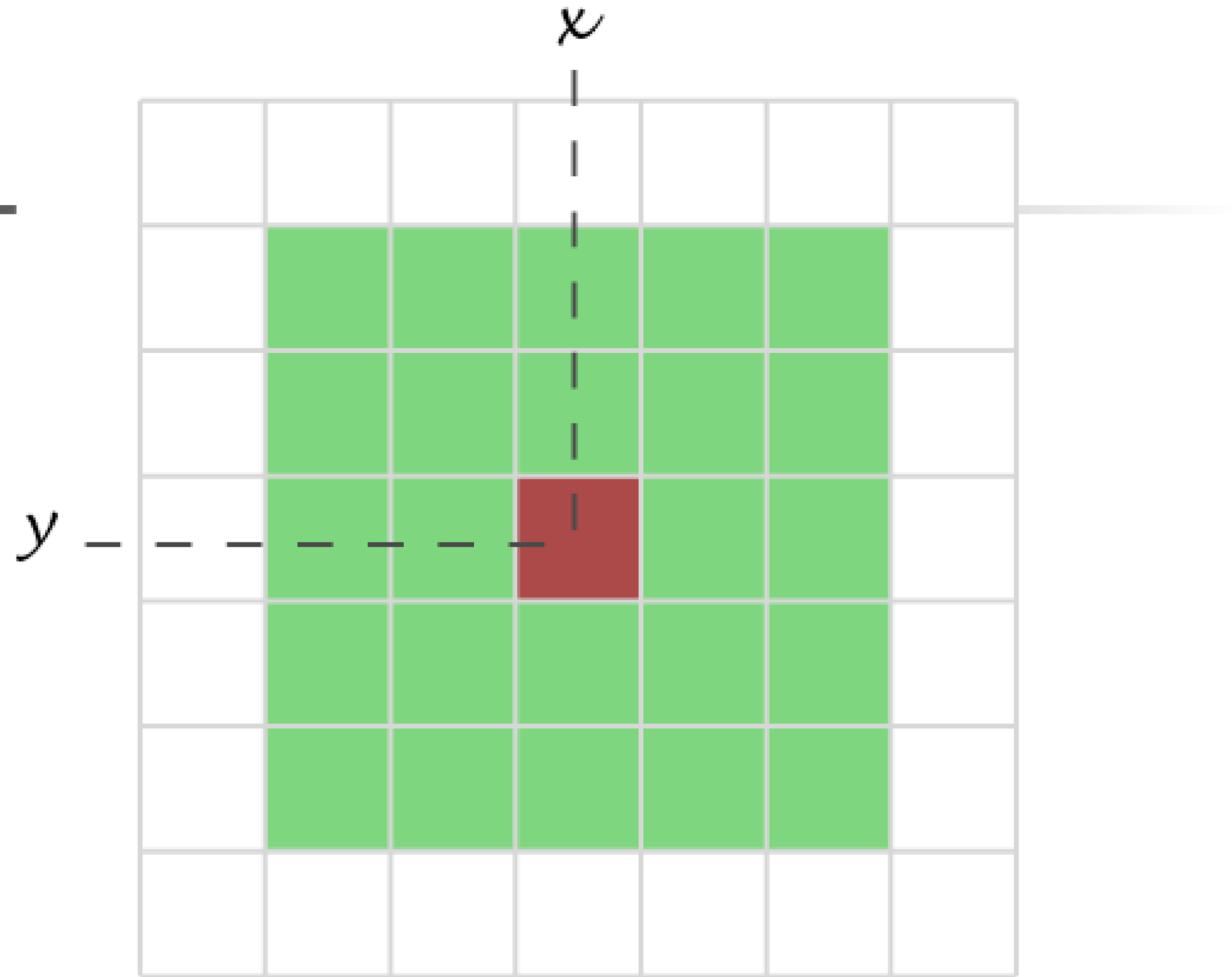
“ Transformation or set of transformations where a new image is obtained by *neighbourhood operations*.”

→ The intensity of a pixel in the new image depends on the intensity values of “neighbour pixels”.

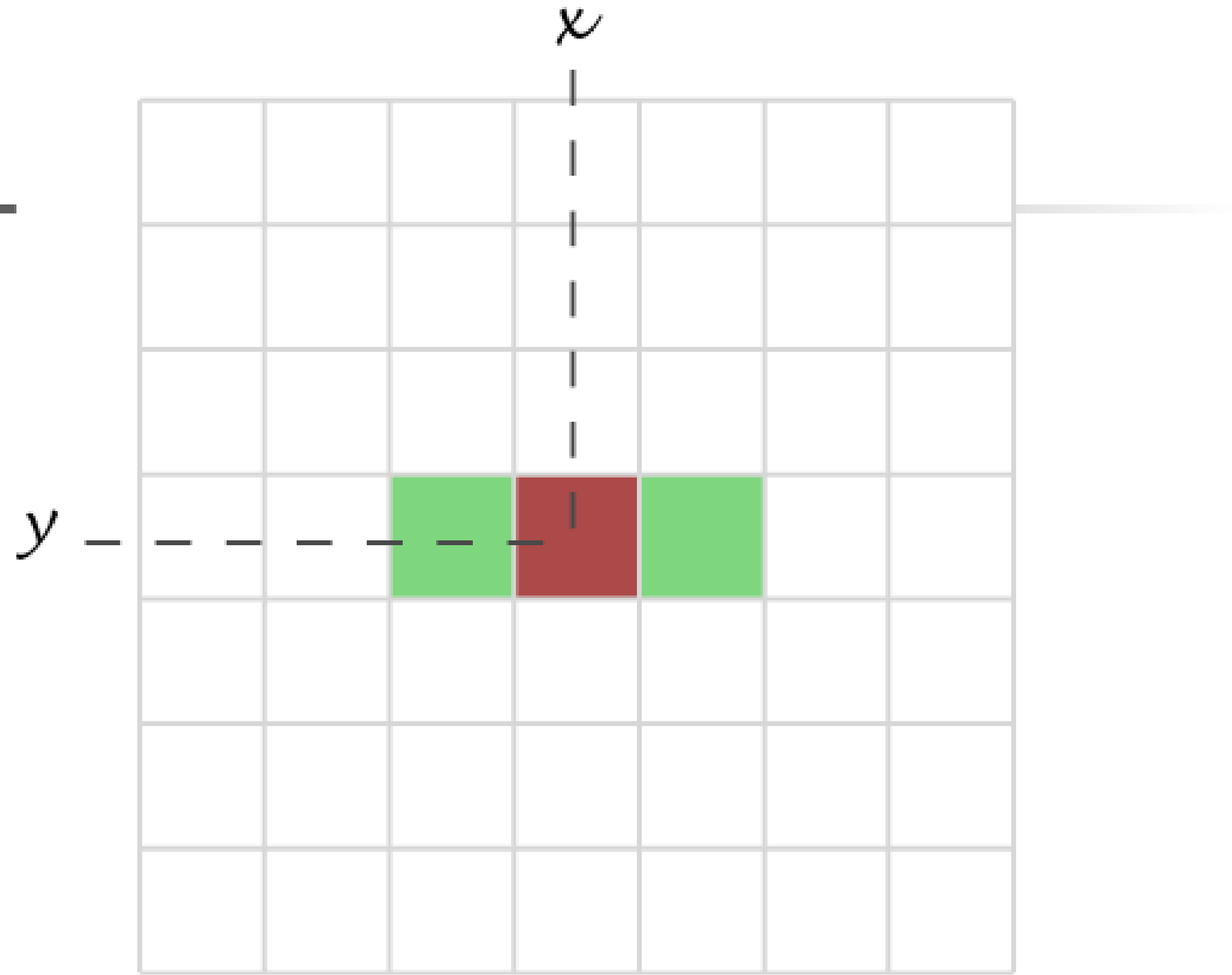
3x3



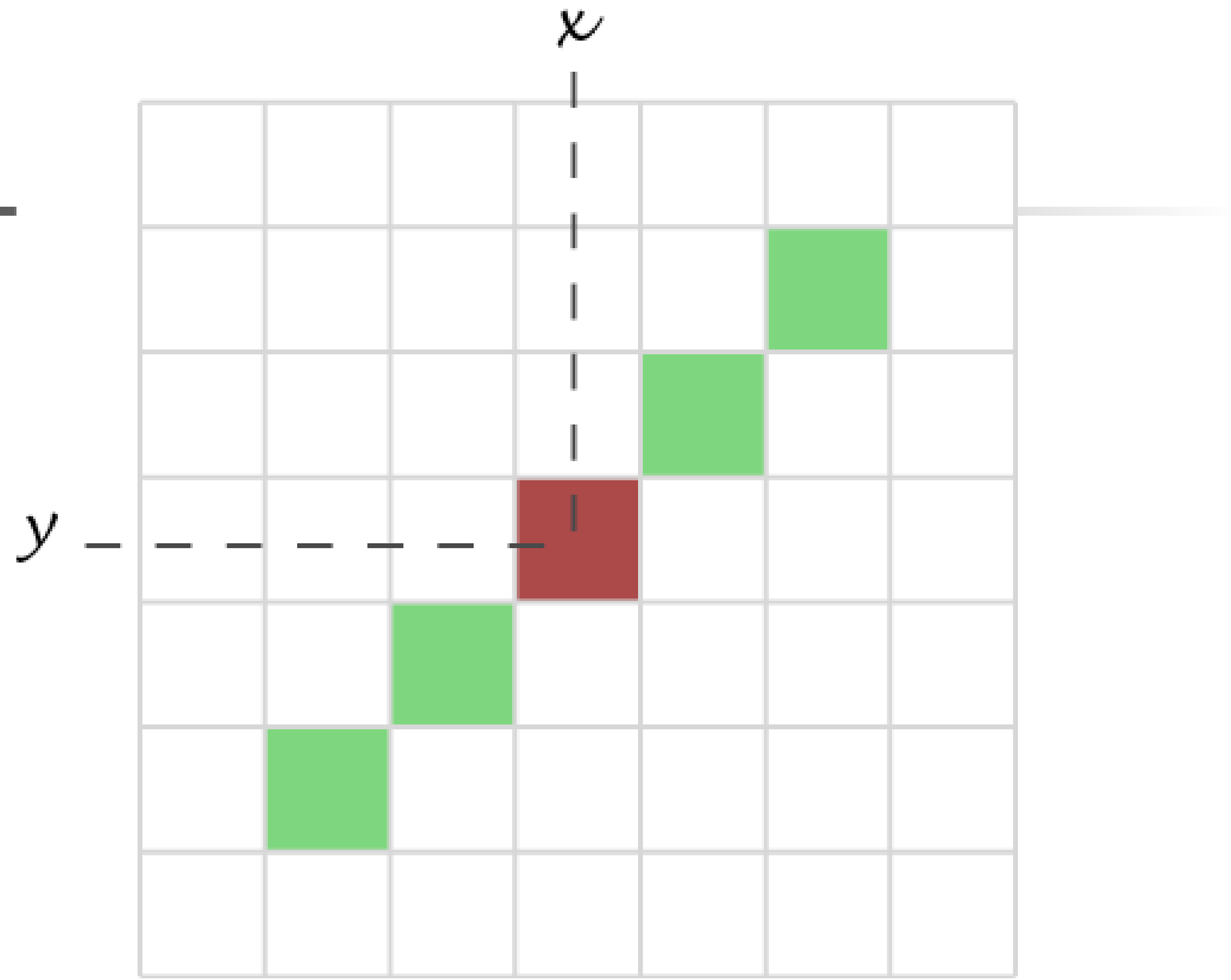
5x5



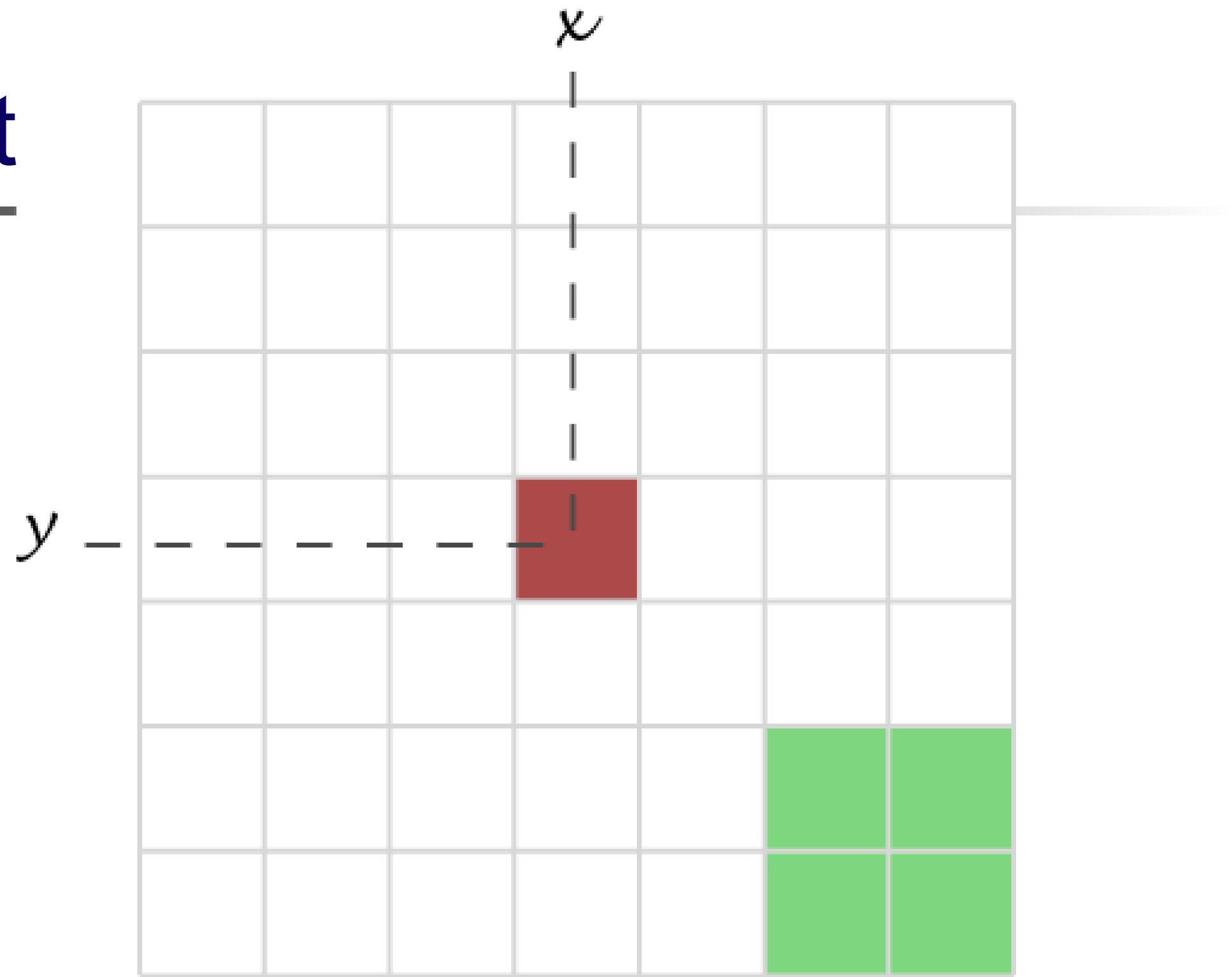
1x3



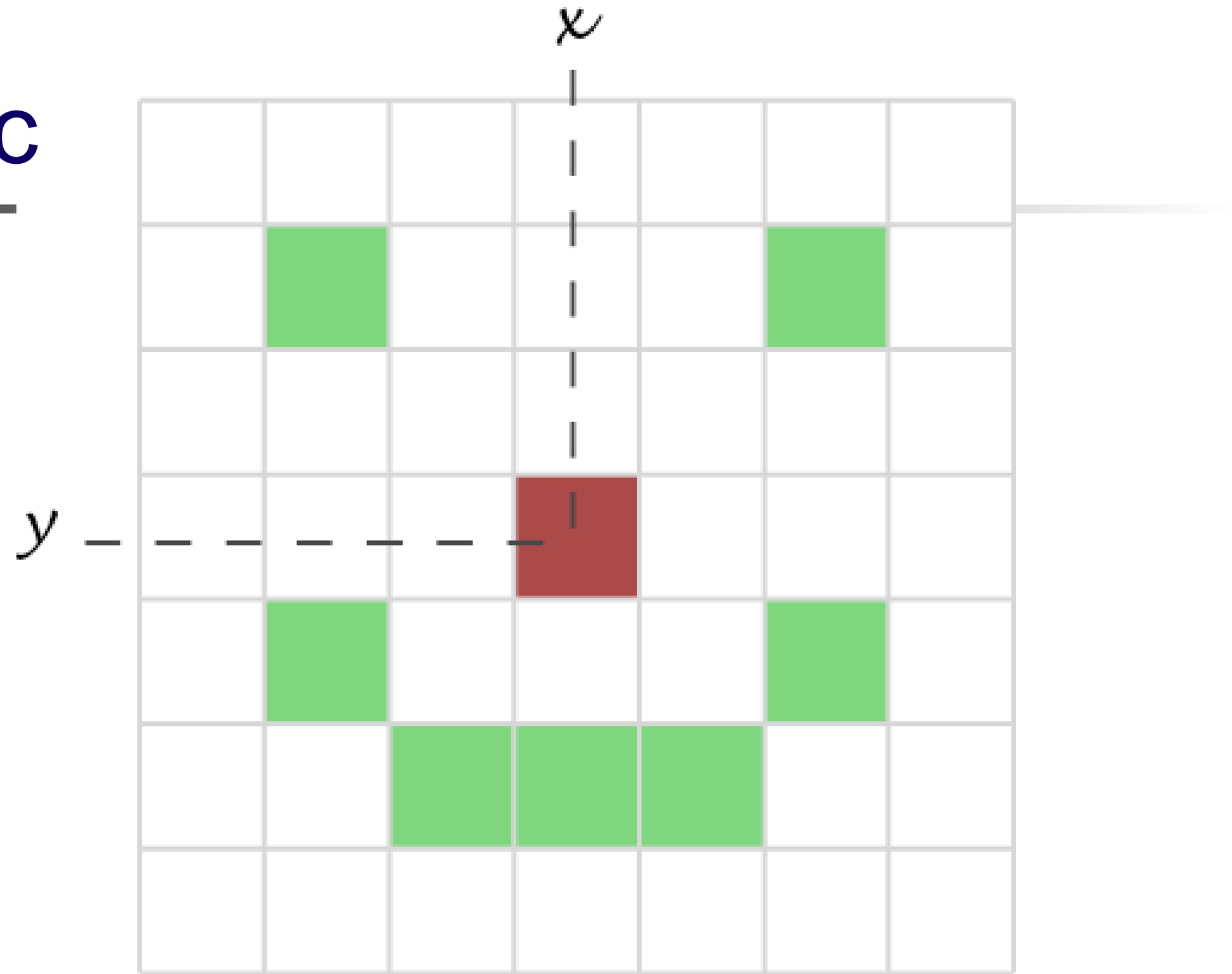
1x5



2x2 shift



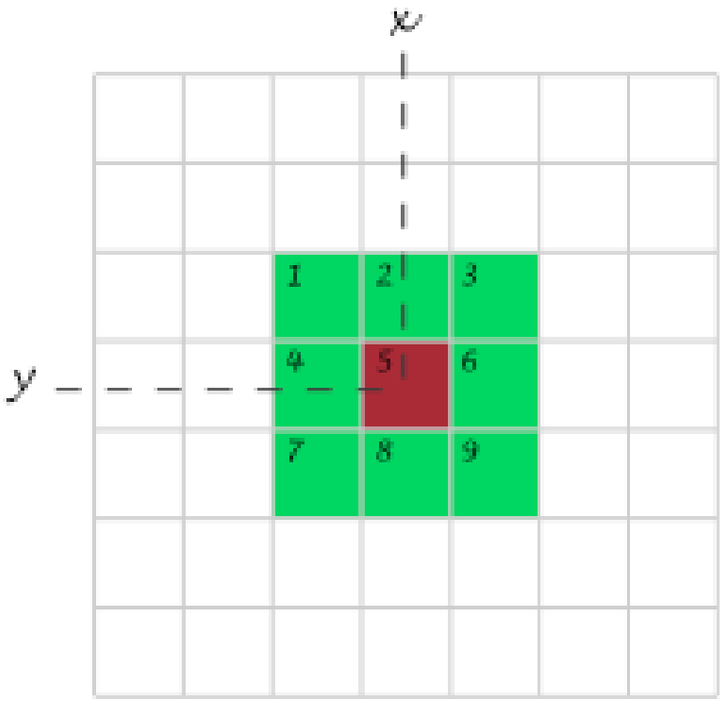
Misc



B: Filtering
The mean filter
The mean filter

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

$$\alpha_i^* = \frac{1}{N_{\mathrm{W}\,j\;\mathrm{W}}} \sum a_j$$



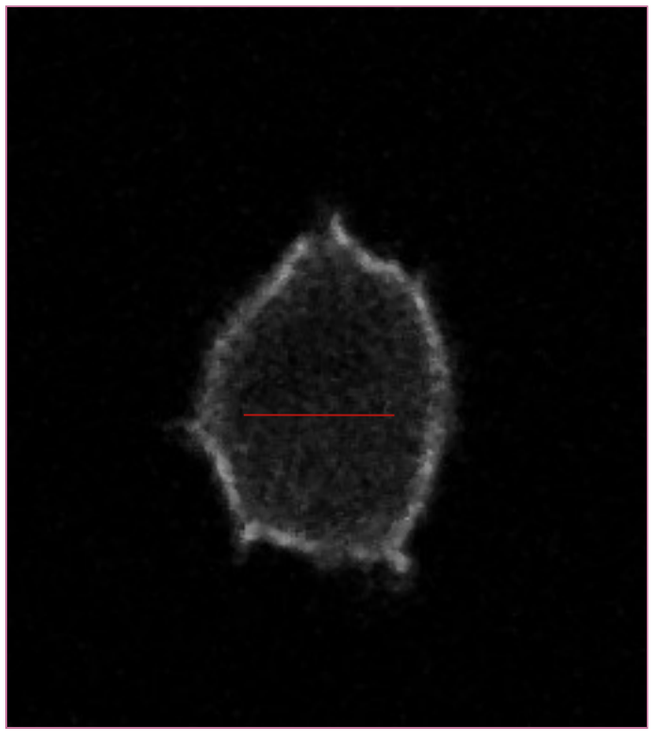
3x3 example:

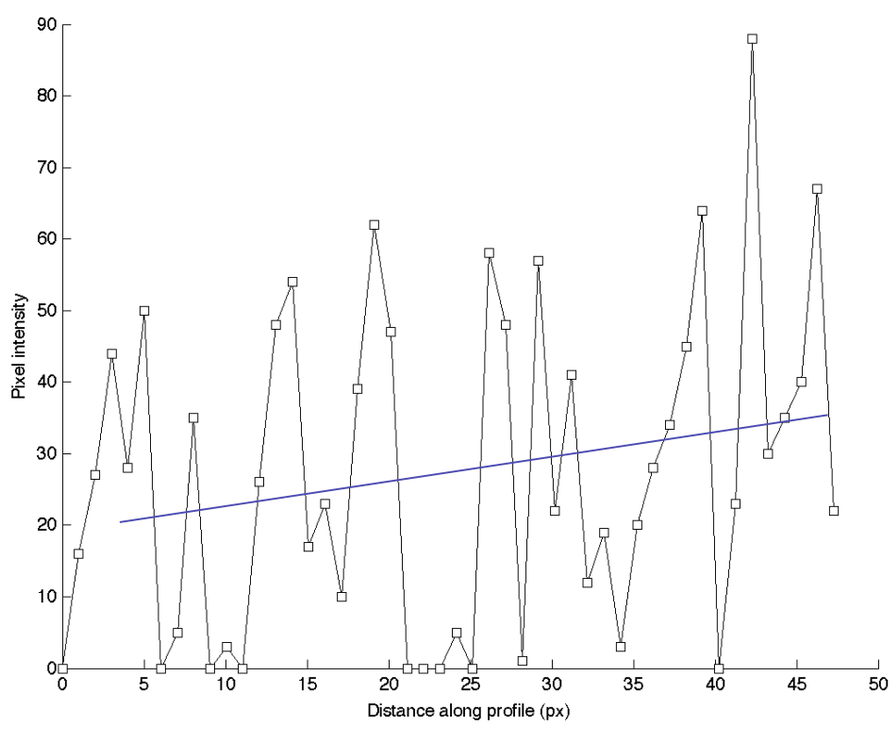
$$\alpha_i^* = \frac{1}{9}(a_1 + a_2 + a_3 + a_4 + a_5 + a_6 + a_7 + a_8 + a_9)$$

The mean filter

what is it good for?

Noise removal - typically Gaussian / Poisson noise.





(typ. Appears for weak labeling, short exposure time, confocal = 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)

$\alpha_{1,1}$	$\alpha_{1,2}$	$\alpha_{1,3}$
$\alpha_{2,1}$	$\alpha_{2,2}$	$\alpha_{2,3}$
$\alpha_{3,1}$	$\alpha_{3,2}$	$\alpha_{3,3}$

➔ 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
- works 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.

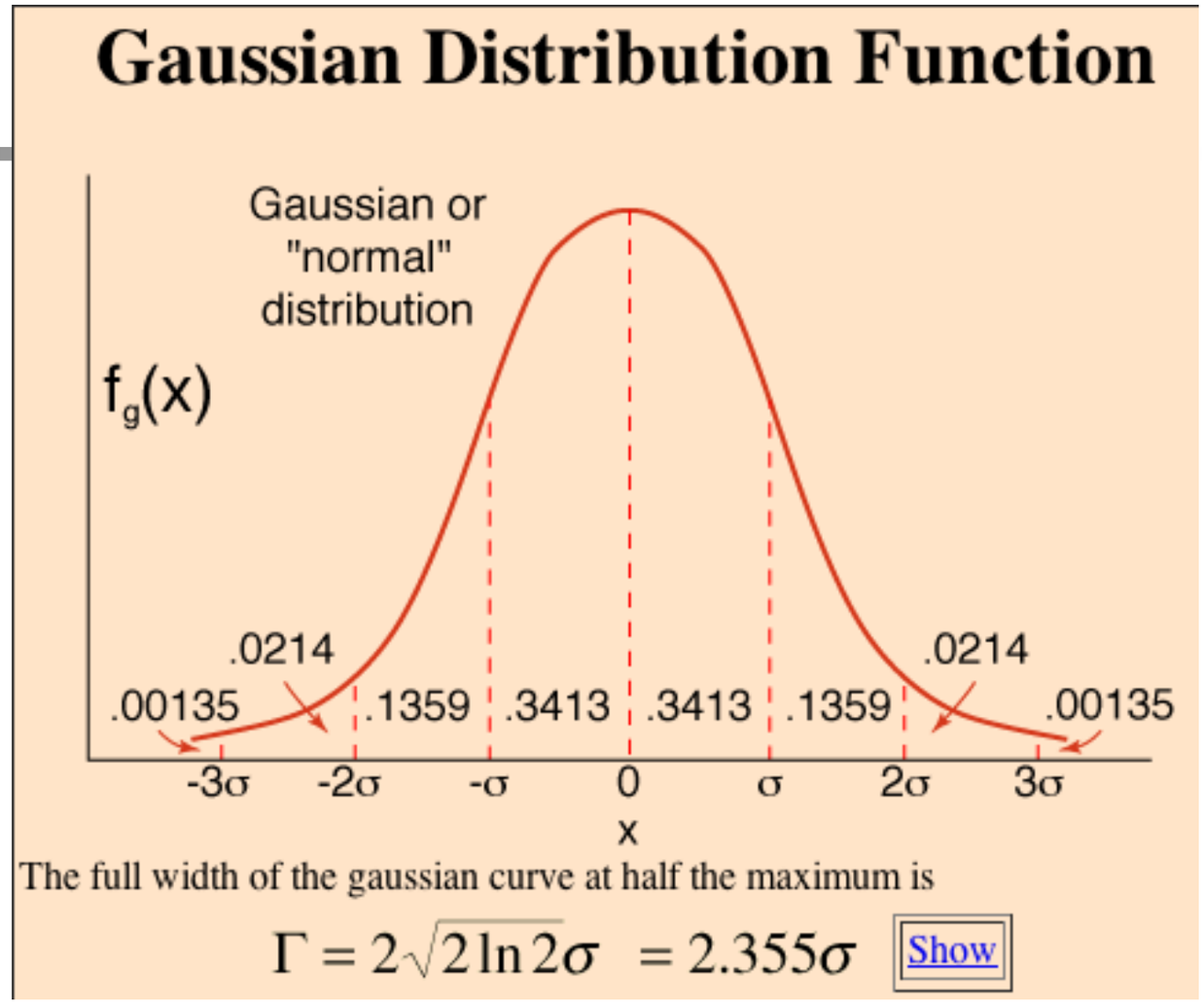
- When applying a succession of linear filters:
the order filters are applied in does not matter.

- Mathematical framework underlying it:
Convolution.

We can also reverse the process : Deconvolution

The Gaussian filter

properties



- Gaussian Curve
- Bell Shaped function

- Smooths poisson noise

- Linear Filter

- Makes more mathematical sense than mean filter?
- ...properly spatially sampled image, looks like PSF

- Can vary the sigma value: number of pixels
- vary degree of blur.

Filtering: The median filter

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

Take neighbourhood (*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

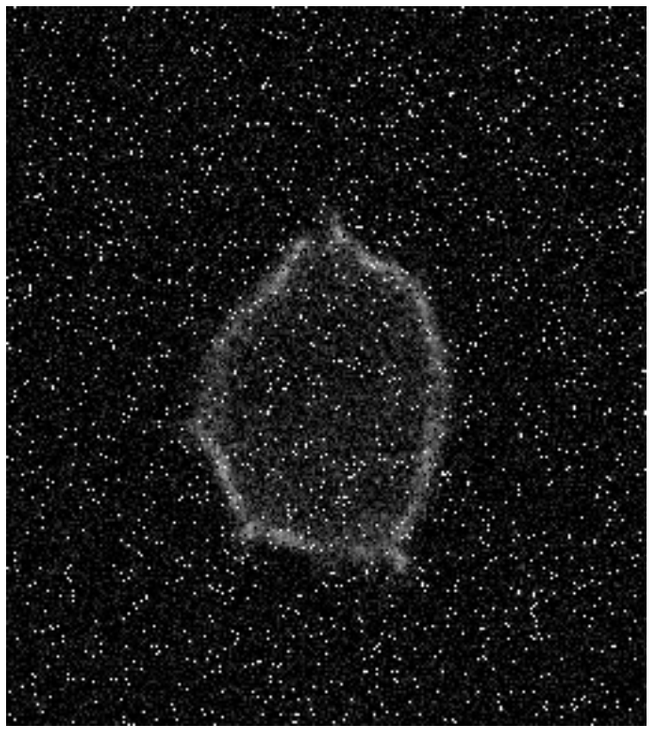
The outlier value has completely been removed from the dataset

The median filter

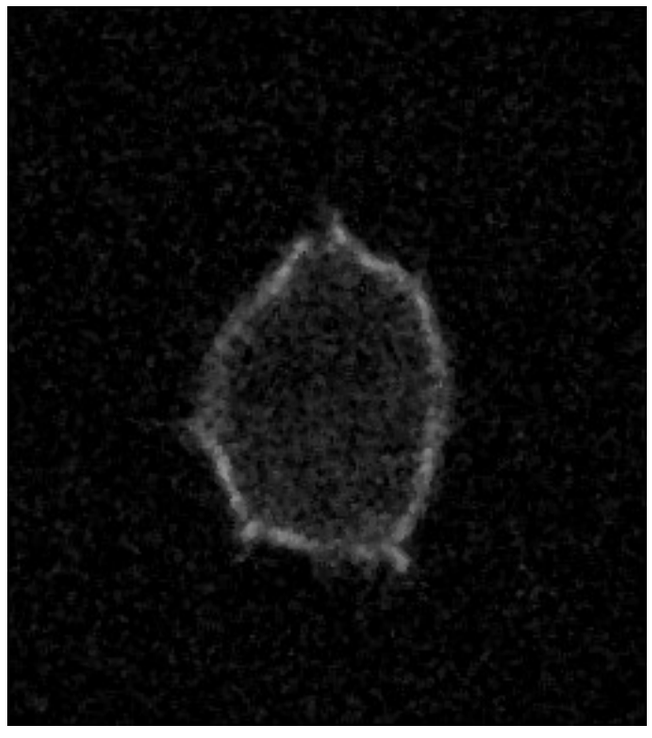
what is it good for?

“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
(not such a problem anymore... computers are fast)

- NOT linear

- Edge-preserving

Practical Session 2a

- Simple Image Filtering

- Convolve a simple binary image - bat cochleaProc
Convolve (play with different kernels)Process - Filters - Gaussian Blur (change value of sigma, in px)

- Open a noisy sample image:
- File - Import - URL...
- http://pacific.mpi-cbg.de/samples/colocsample1bRGB_BG.tif
- Mean Filter (change no of pixels - kernel size)
- Median Filter (change no of pixels - kernel size)
- Gaussian Blur - again
- Gaussian Blur - again

The Fourier transform

- The Fourier transform is a way to obtain a new *representation* of the data.
- A bit like the 2D histogram from yesterday.

- It is best suited for data with *repetitive patterns*
- Highlights these patterns.

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

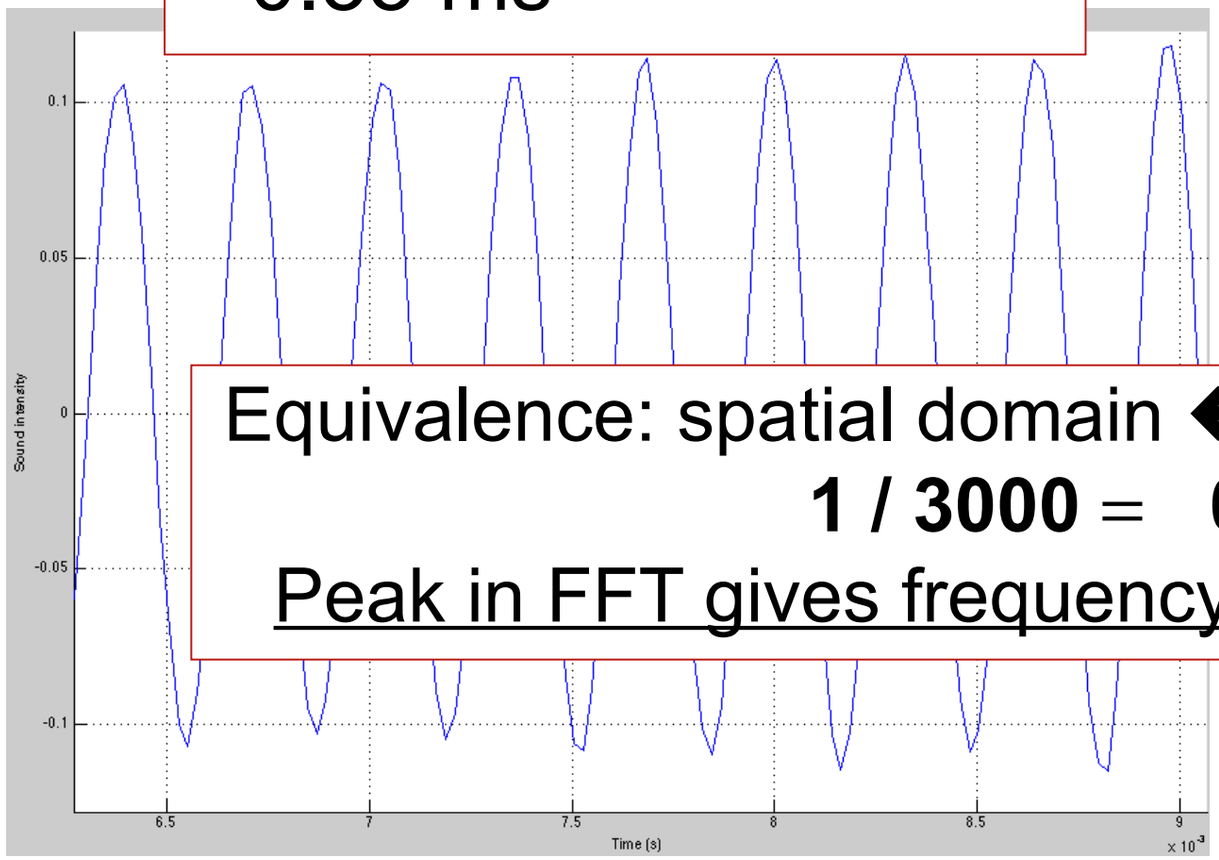
The Fourier transform

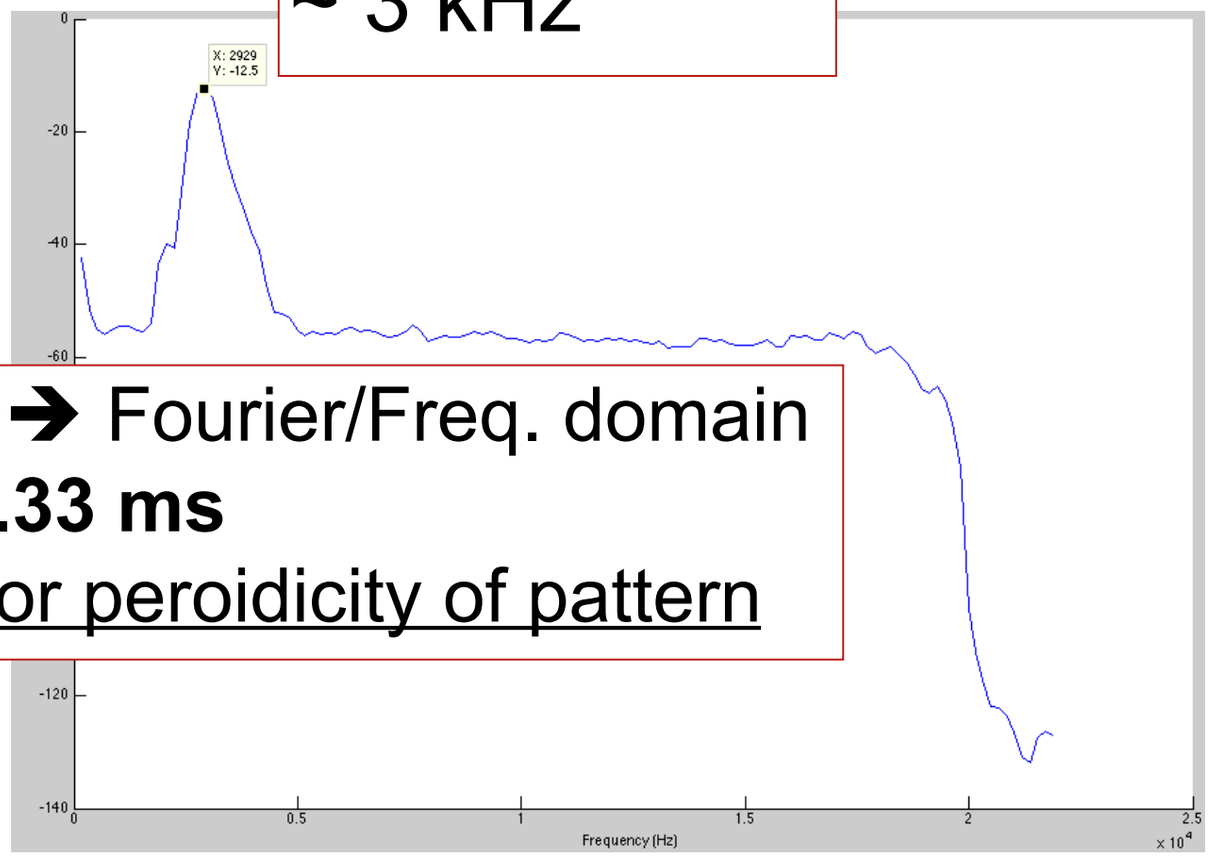
Bird song.
Detail of the signal:

Delay between peaks:
~ 0.35 ms

FFT of this looks like:

Peak in FFT:
~ 3 kHz





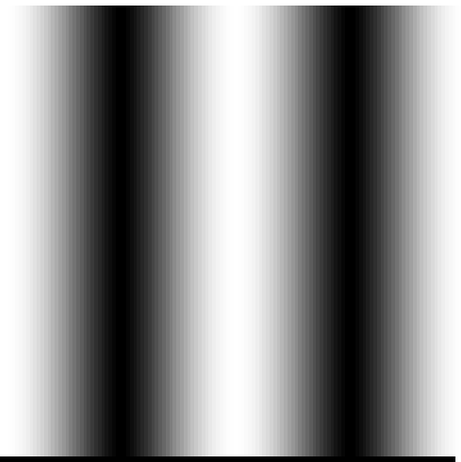
Equivalence: spatial domain ⟷ Fourier/Freq. domain

1 / 3000 = 0.33 ms

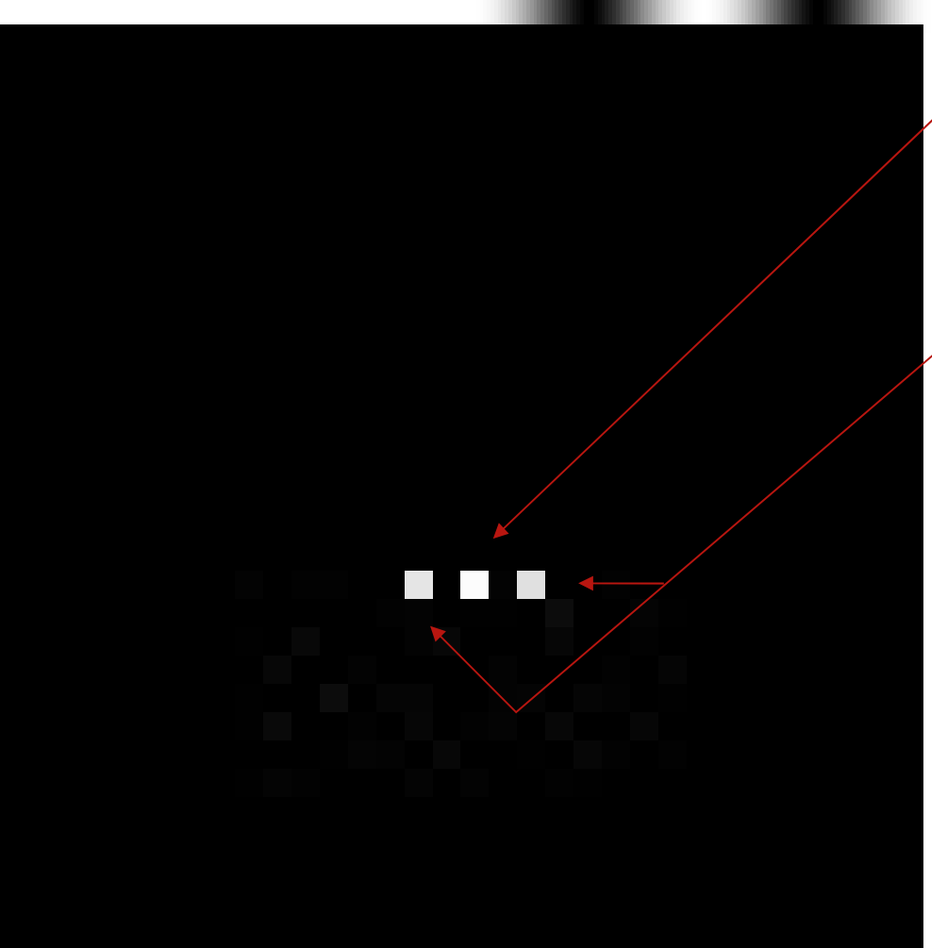
Peak in FFT gives frequency or peroidicity of pattern

The Fourier transform
in 2D (images)

orig



FFT (zoomed)



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

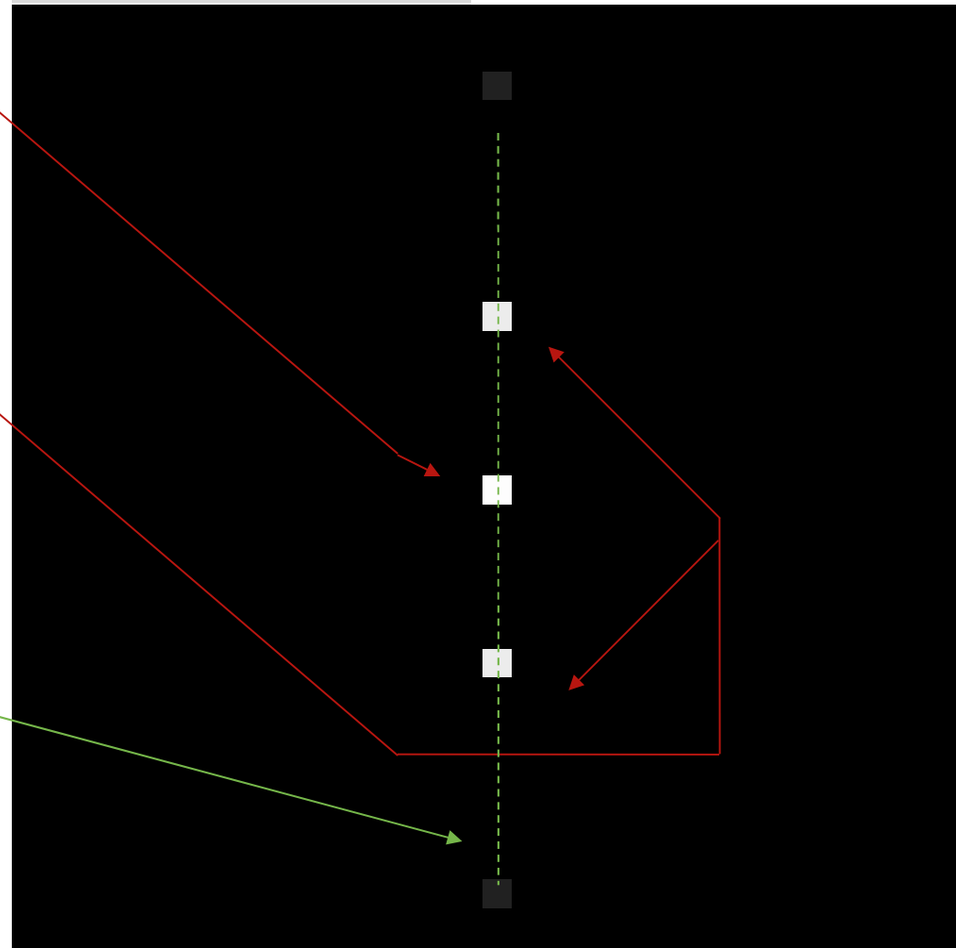
Pattern of points:
always symmetrical,
the further = the smaller
higher freq. = smaller object

Angle of pattern point gives pattern orientation

Diffraction pattern?

orig

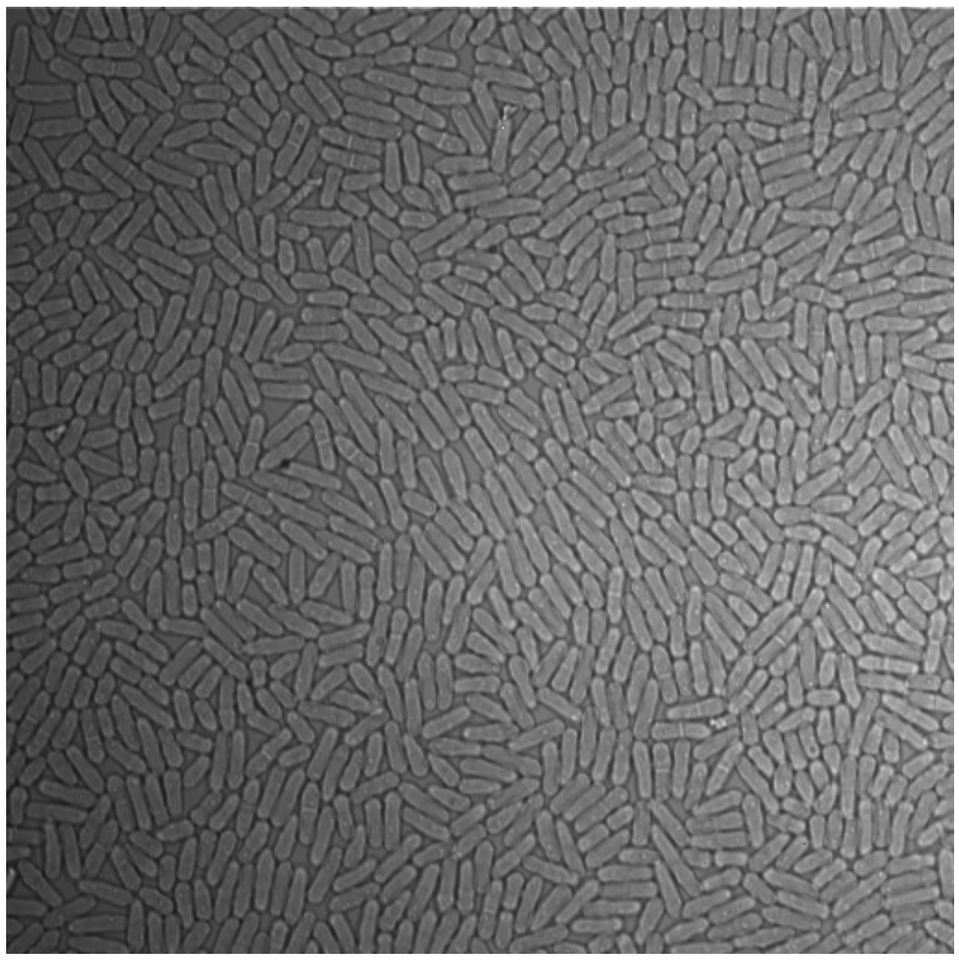
FFT (zoomed)



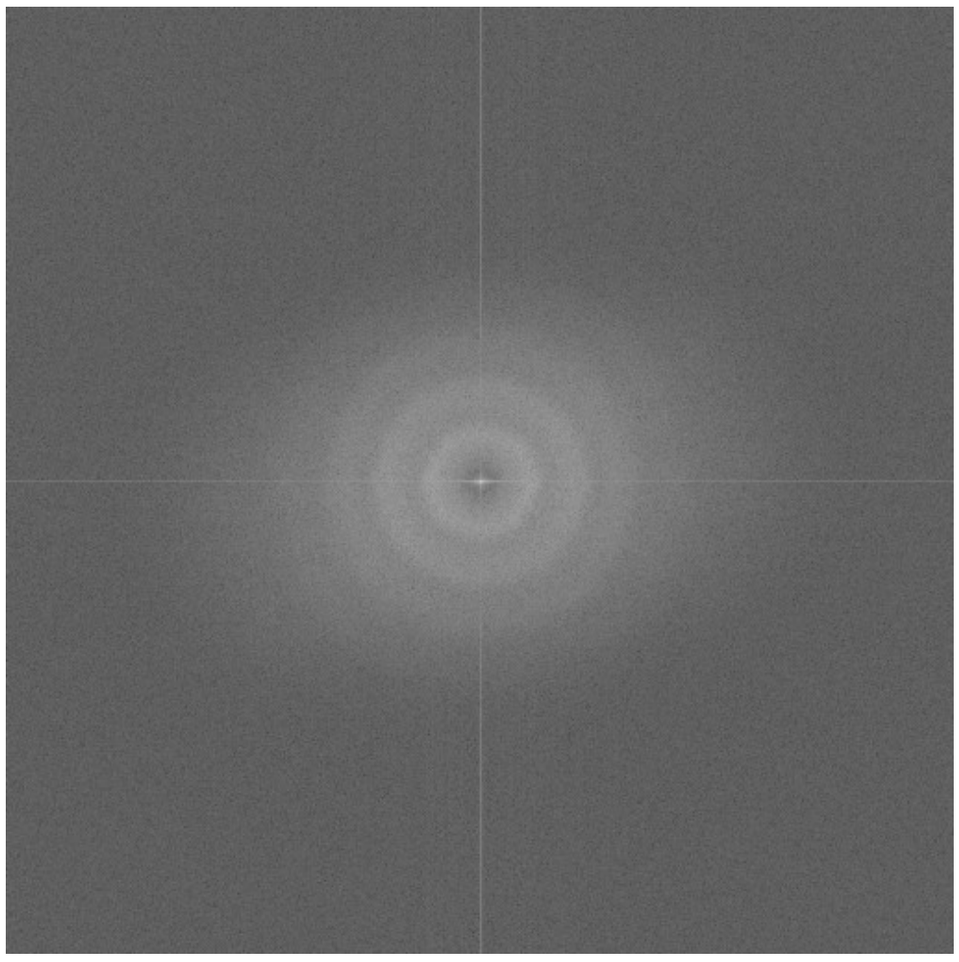
The Fourier transform

real images

… are rarely that clear



S. pombe cells (*Tolic lab*)



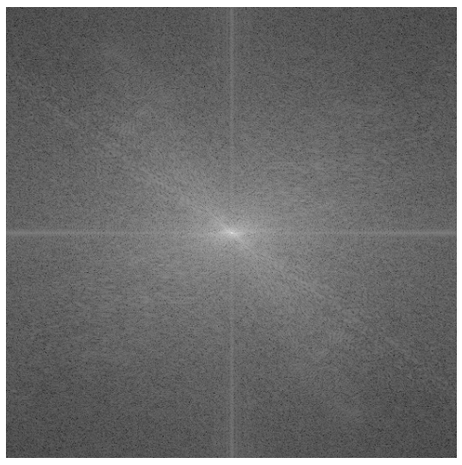
FFT

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:

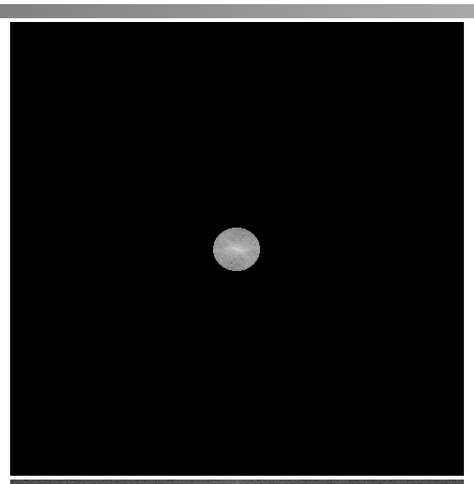


Changed her mind:

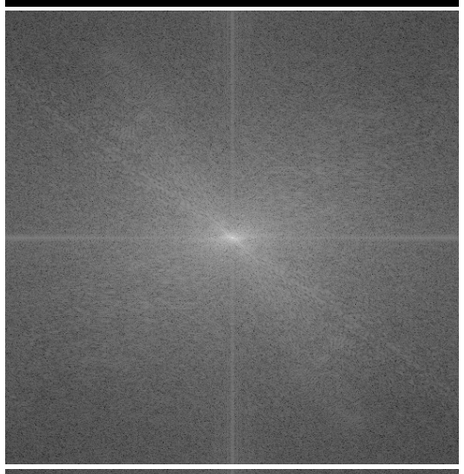
Basically, the same thing happens physically in a microscope. FT image is in the Back Focal Plane of Obj.!

Can use as a filter for detail:

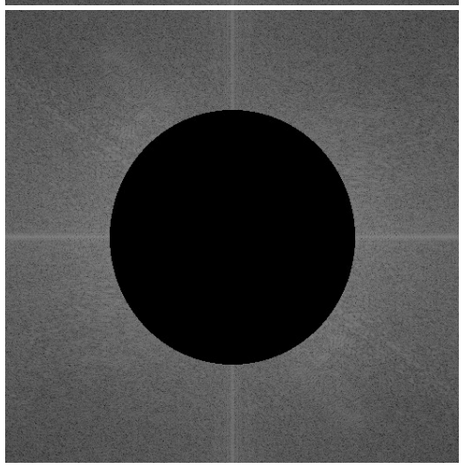
Low freq pass



FT



High freq pass



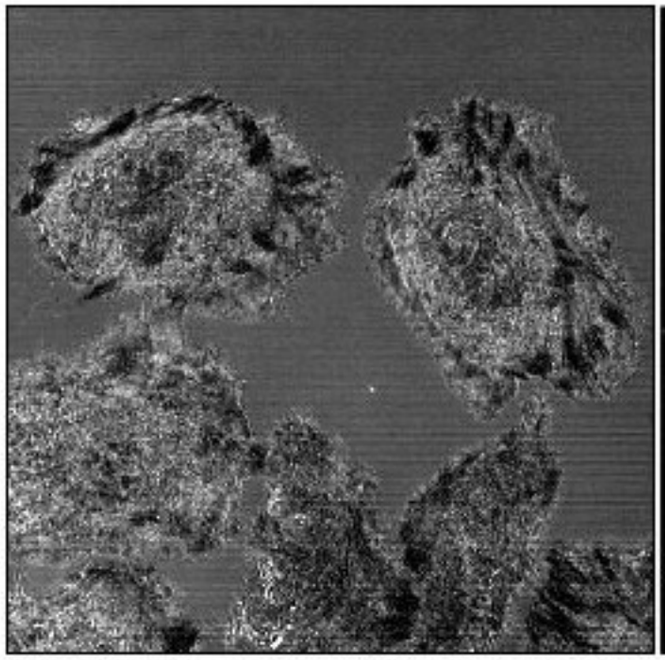
IFT

IFT

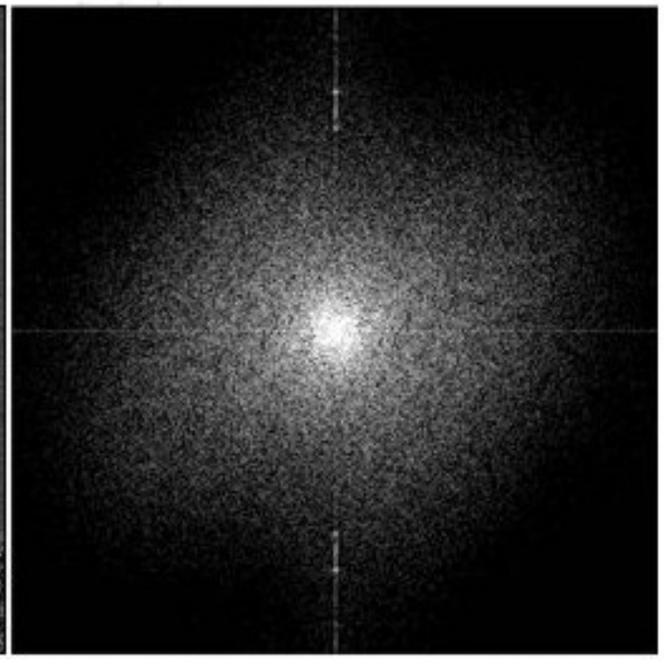
… a filter for periodic noise:

Laser intensity noise from a bad AOTF...

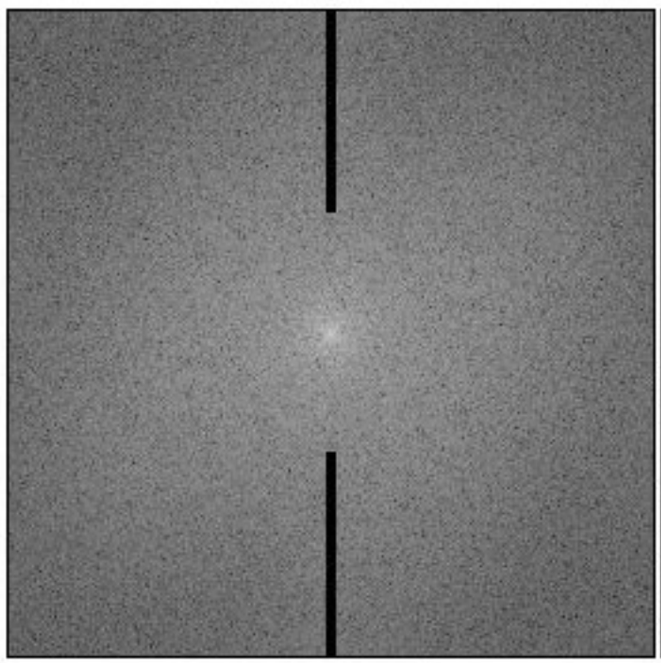
can be removed by frequency filtering in the correct spatial direction.



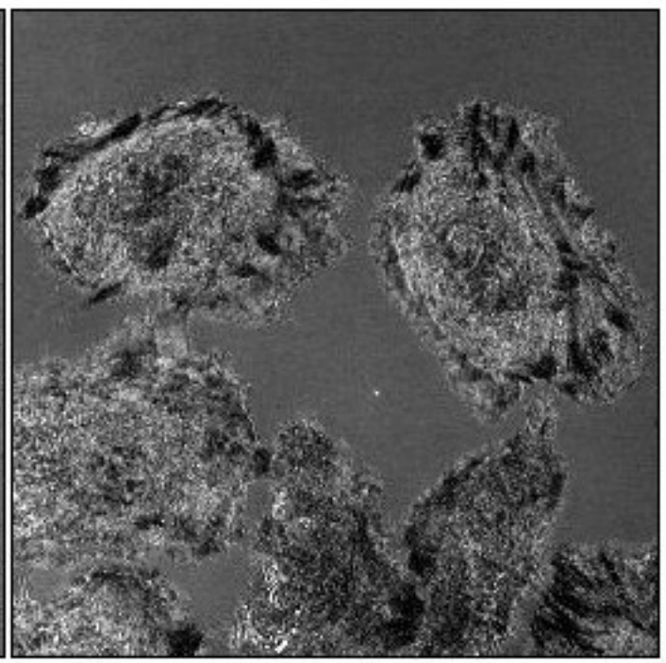
The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.



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.

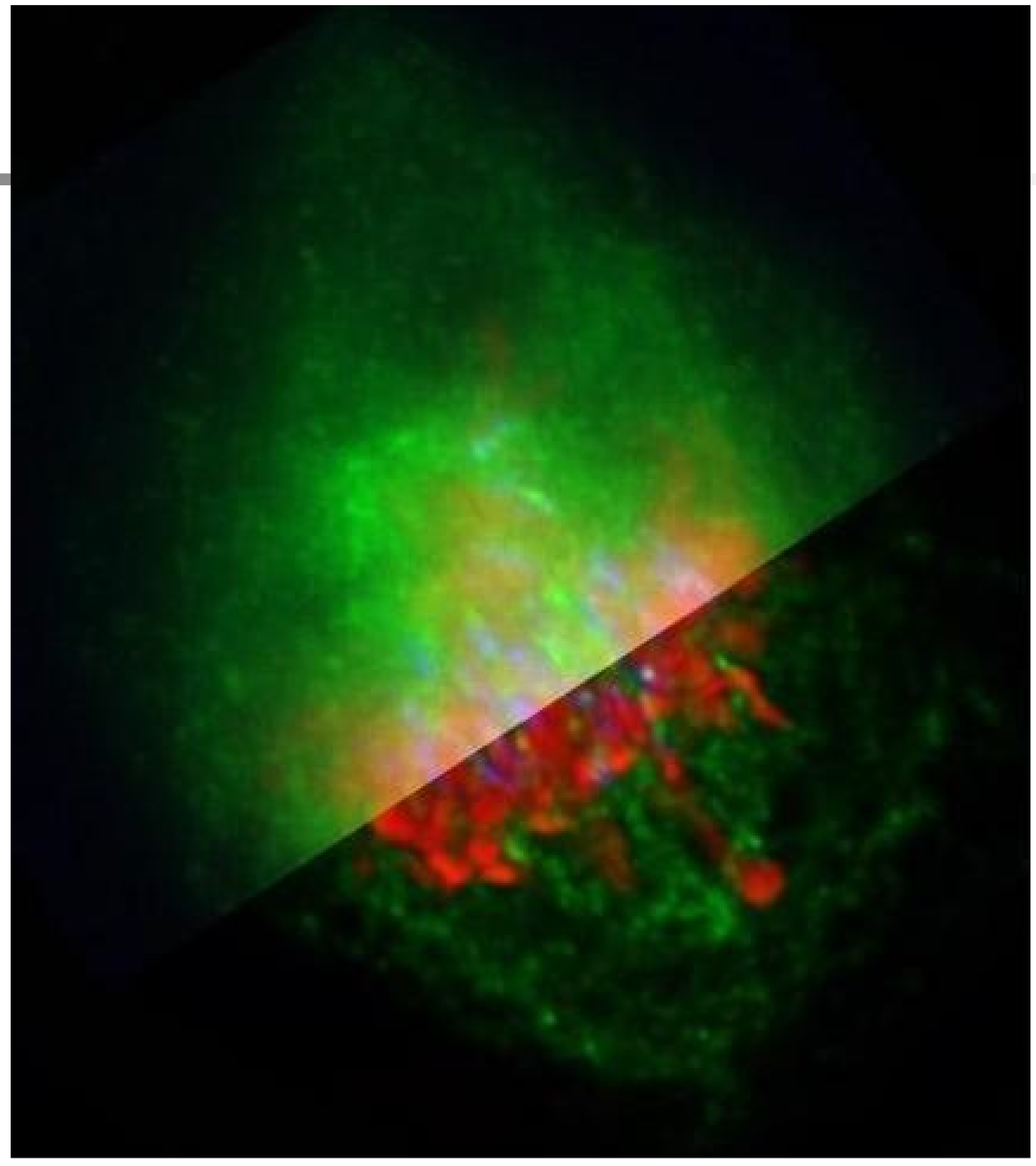
… during “Deconvolution”:

Take Image and PSF image

Do Fourier transforms

Image FT / PSF FT

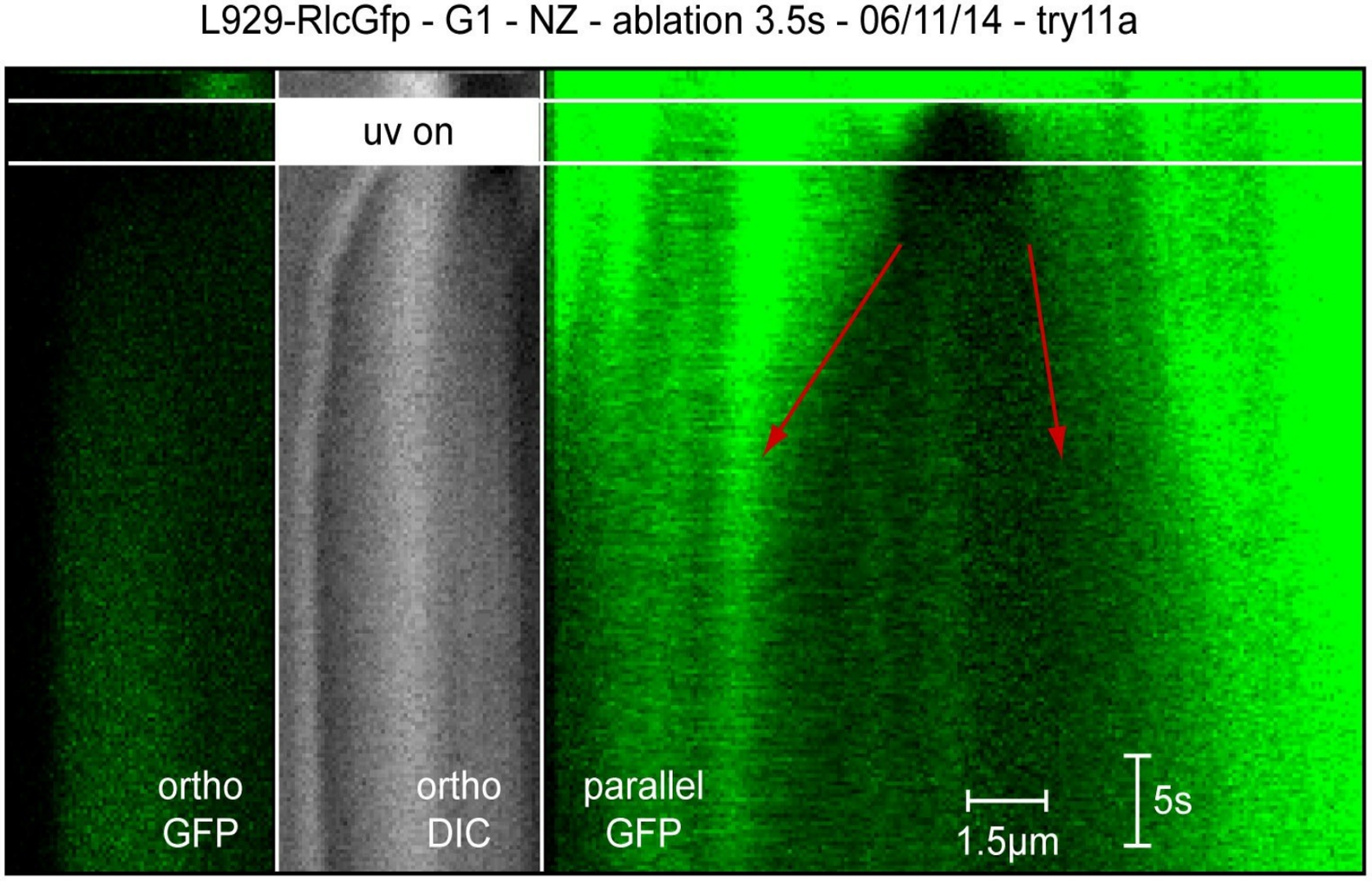
Reverse FT of result
=
Deconvolved image with much improved contrast and less out of focus signal.



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, …



total speed of
cortex movement:
17.0 µm/mn

- Intensity over time
- Kymographs

Motion blur

Motion blur = average over time

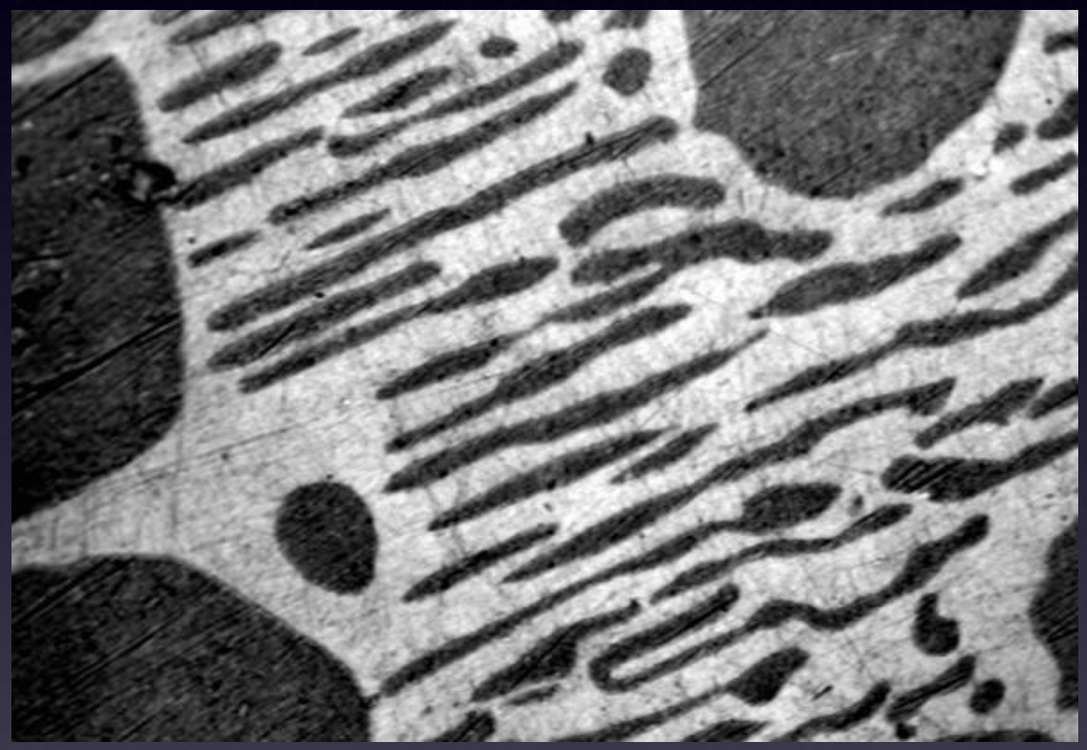
Does this happen in your sample? Frame Rate?

Practical Session 2b

- Less Simple Image Filtering

- FFT, filter out parts, Inverse FFT
- mess up the Bridge sample image
- can you extract high and low frequency information in the image?
- Use circle selection and Edit - Fill
- Set the foreground colour to black.

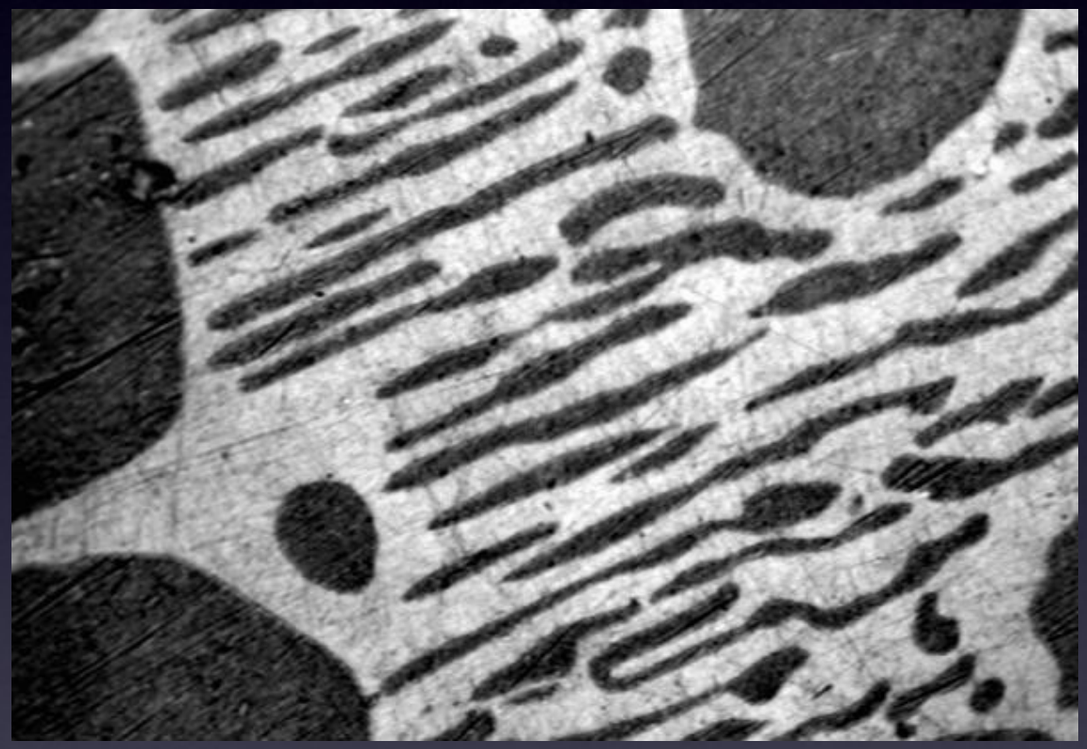
What is “Image Segmentation”?



“Greyscale” image

foreground background

What is “Image Segmentation”?

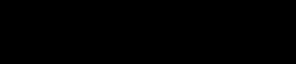


“Scalar Intensity” image

“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

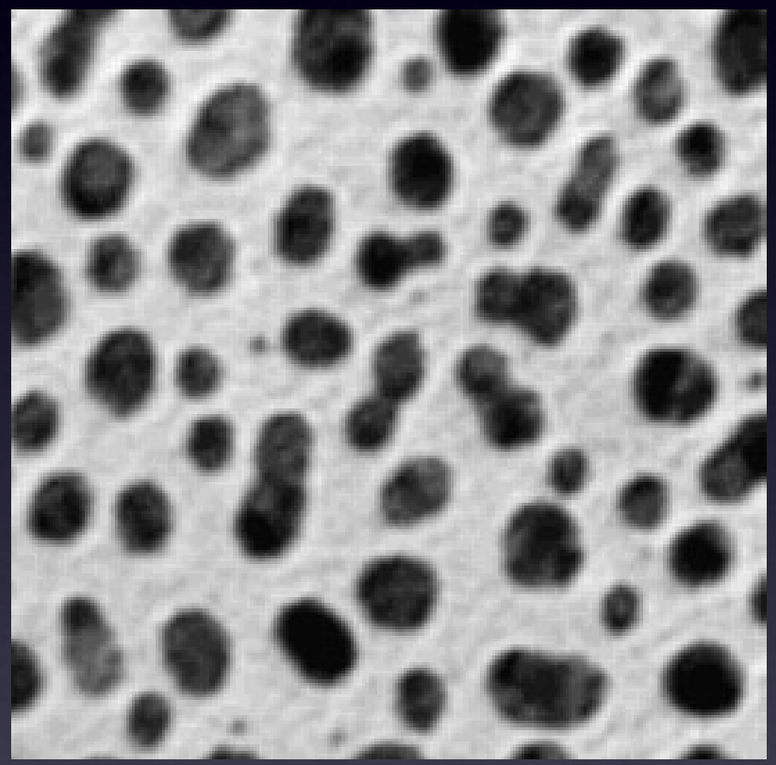


0	1	1	1	0
0	0	1	0	0
0	0	1	0	0
0	1	0	1	0
1	0	0	0	1

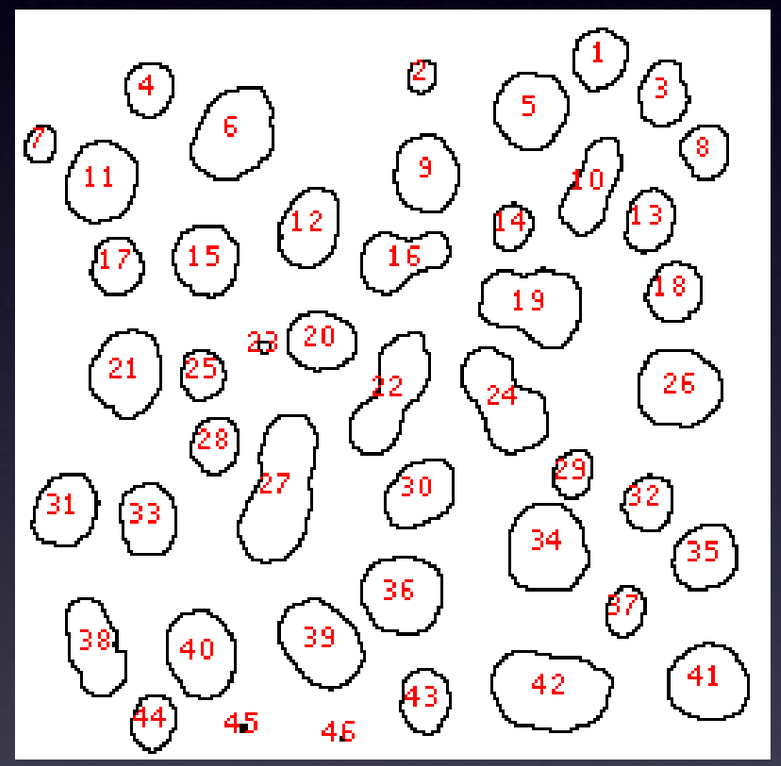
“Scalar Intensity” image

“Binary” image

What is “Image Segmentation”?

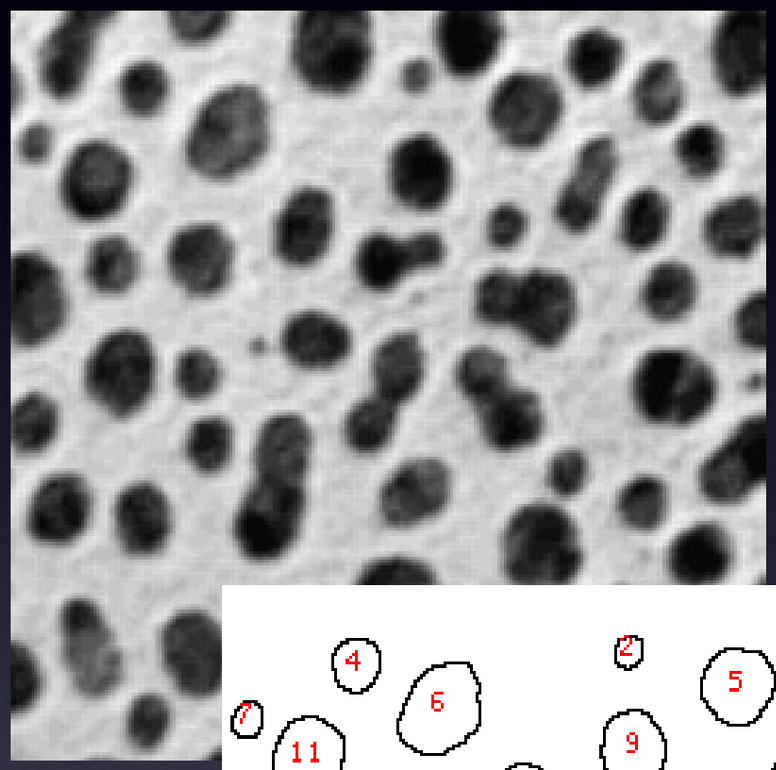


“Scalar Intensity” image

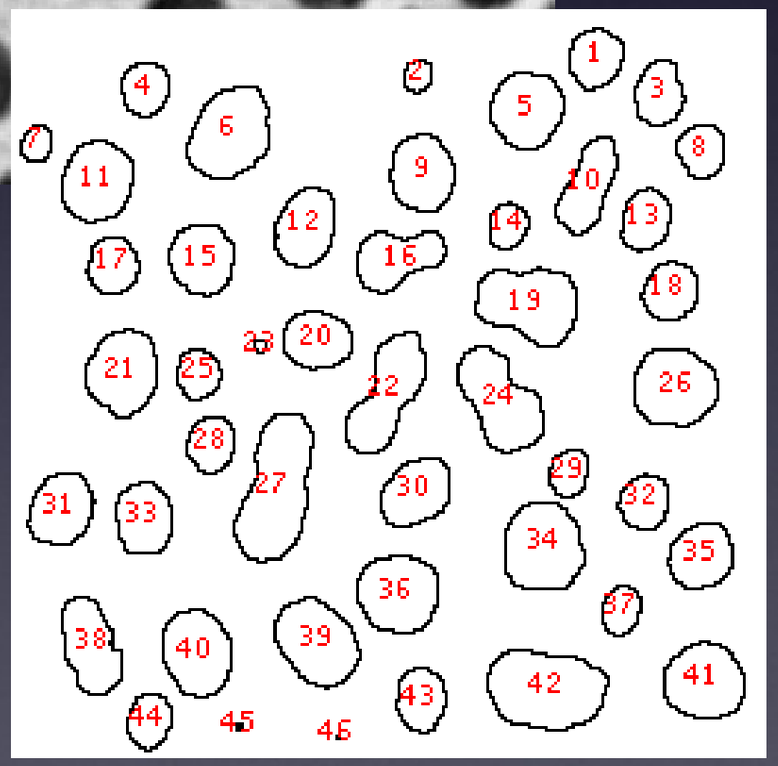


“Labelled Objects”

What is “Image Segmentation”?

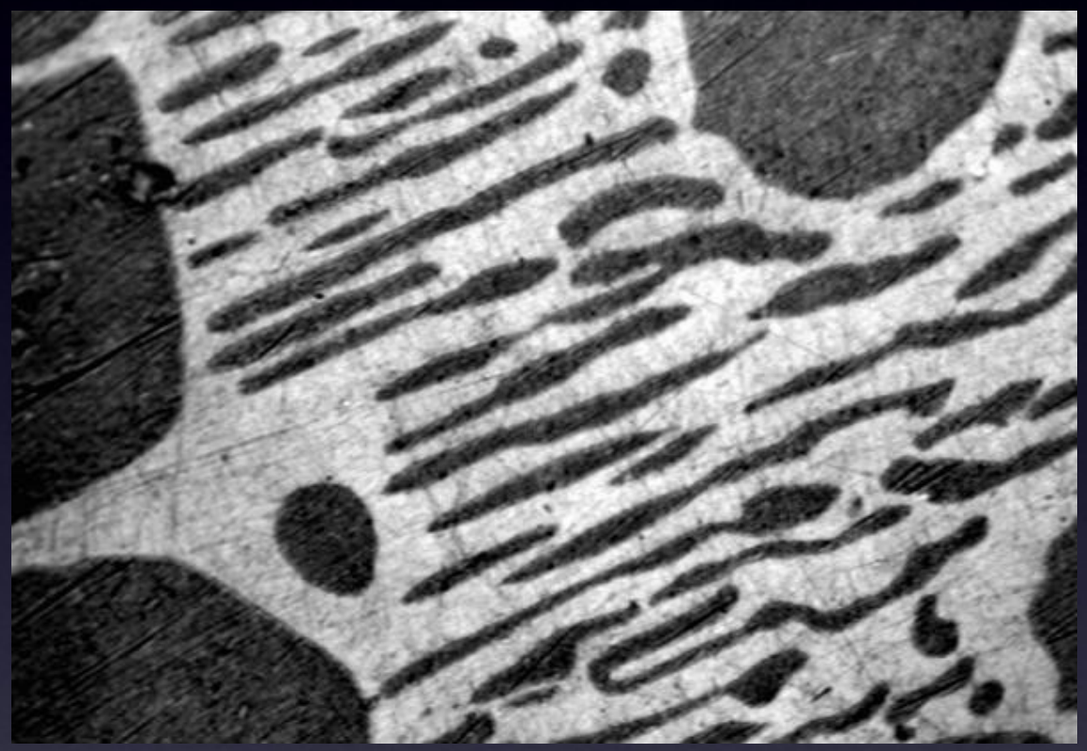


High Information Content
65536 pixels, 0-255 value



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

“Thresholding” (Intensity Histogram Split)

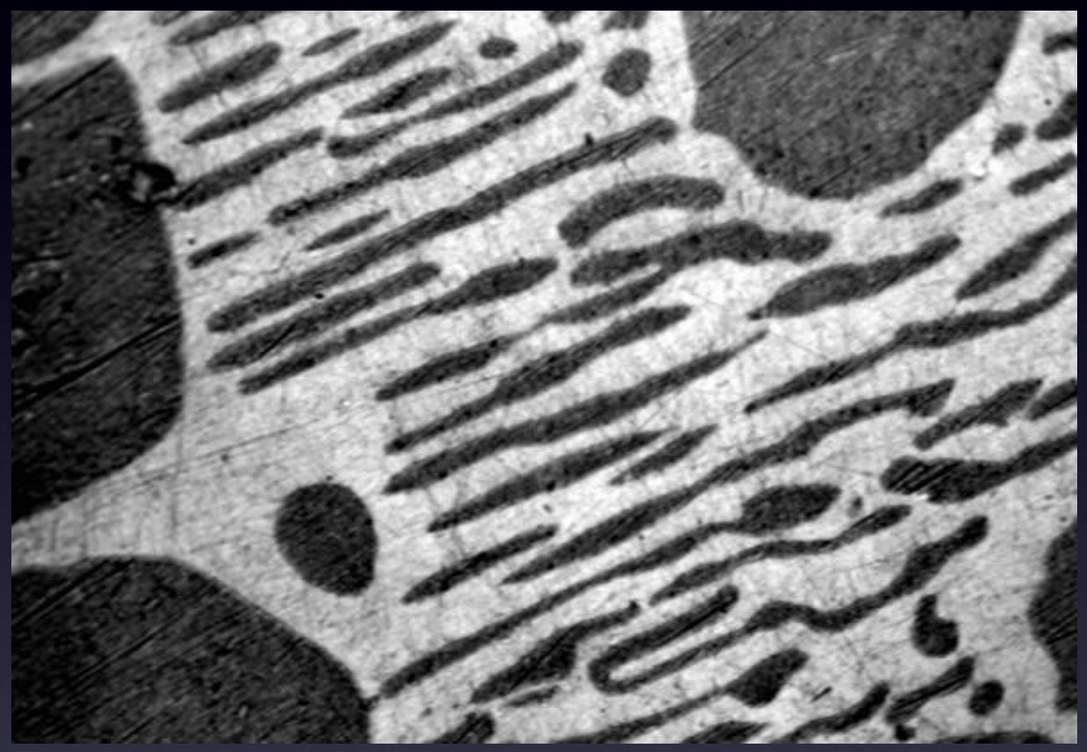


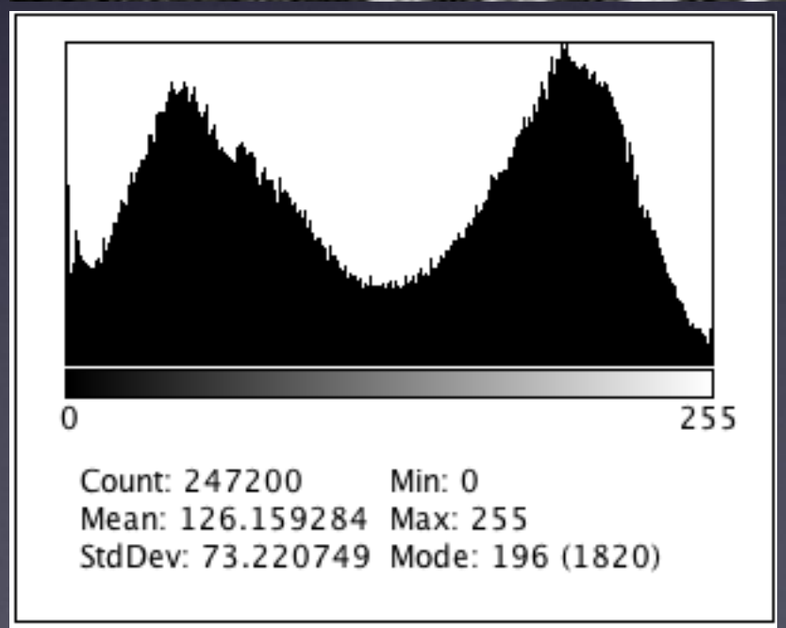
Clear difference between foreground and background?
Image not very noisy?

Choose an intermediate grey value = “threshold”

Determines foreground and background.

“Thresholding” (Intensity Histogram Split)



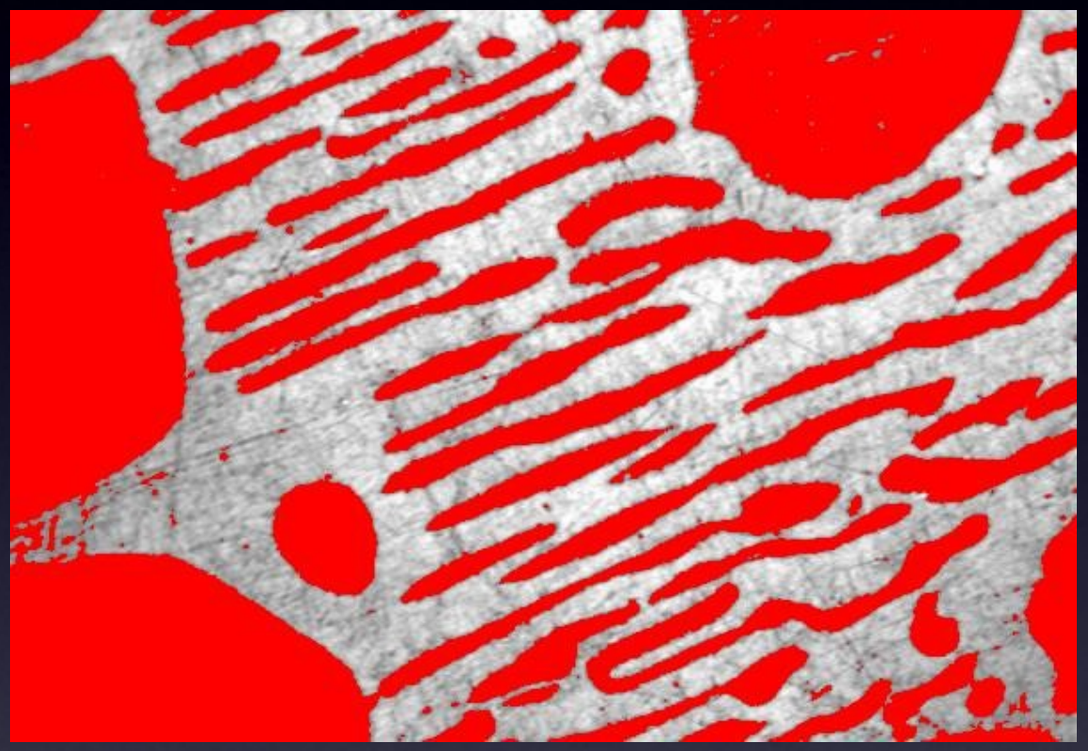


How to choose the grey level for thresholding?

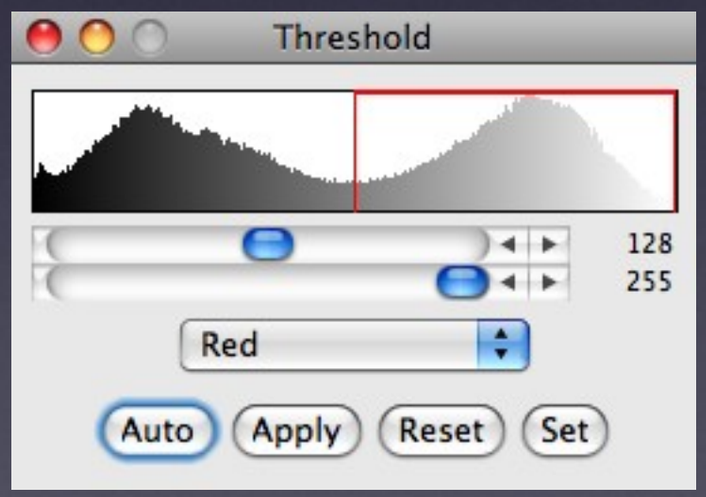
Look at pixel intensity histogram of whole image…

Is there an obvious place?

“Thresholding” (Intensity Histogram Split)

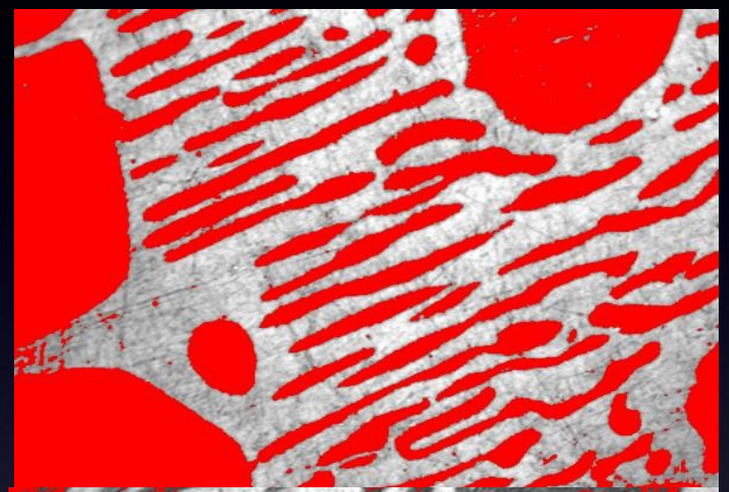


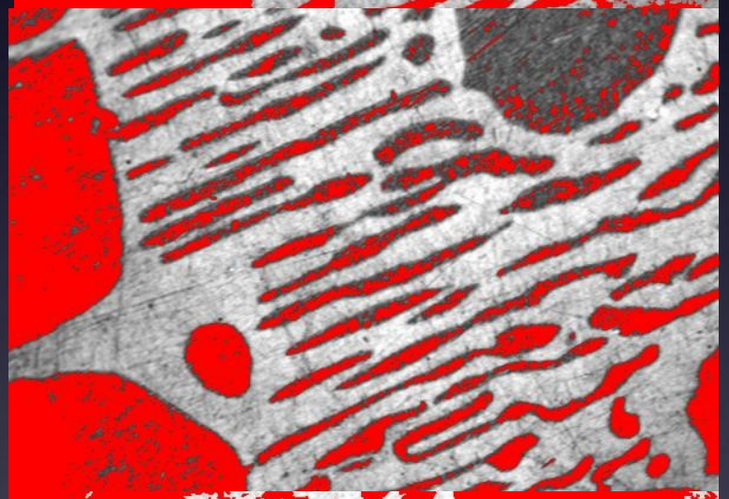
Histogram is bimodal, so put threshold in the trough between the peaks!

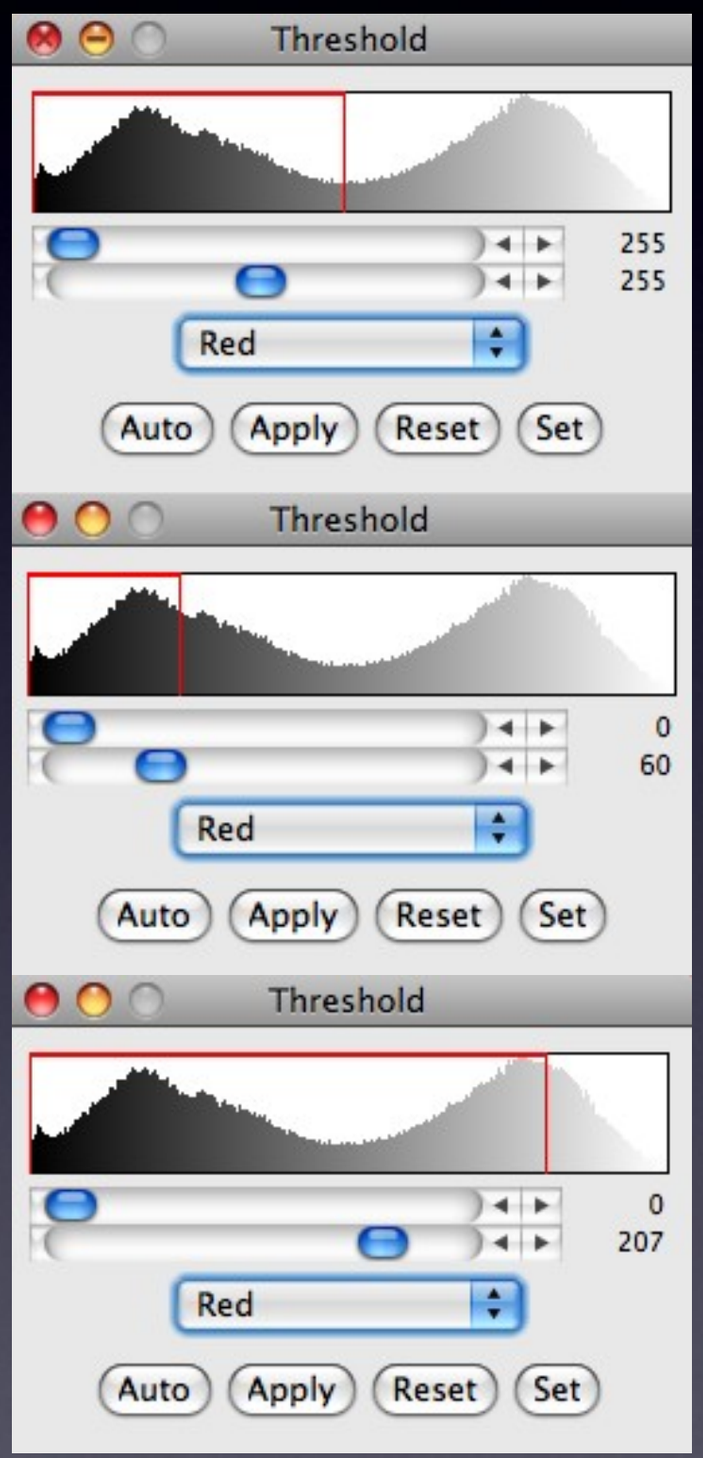


Note, in this case:
Foreground = “dim” objects
Background = “bright” objects

“Dumb Global Threshold”
(Subjective - User Biased)

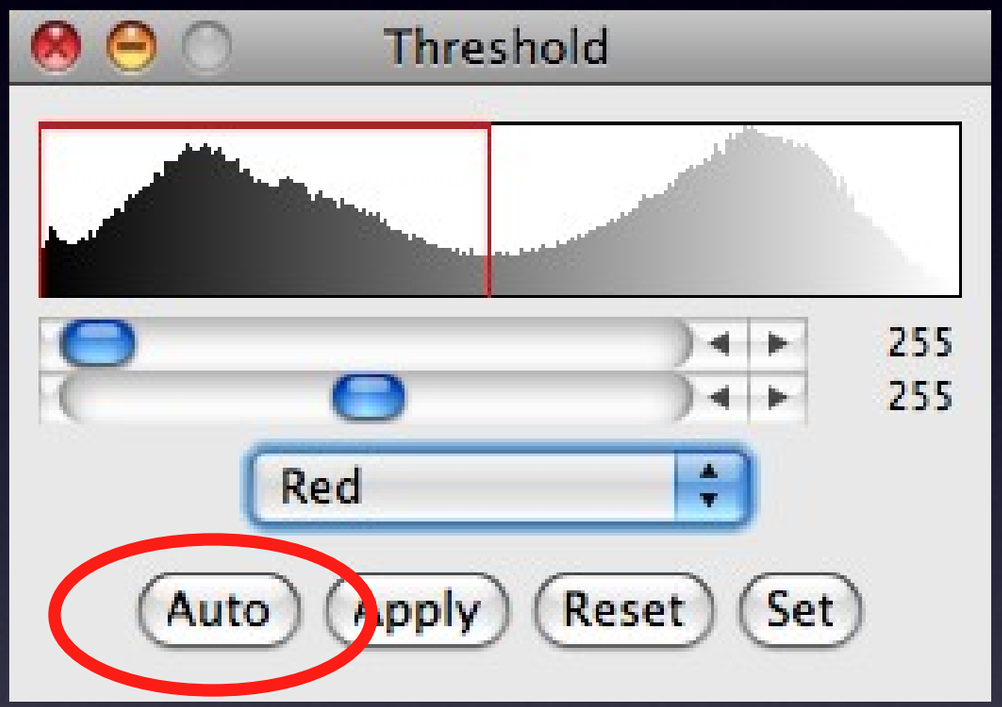






Computed Global Threshold

Objective - Reproducible



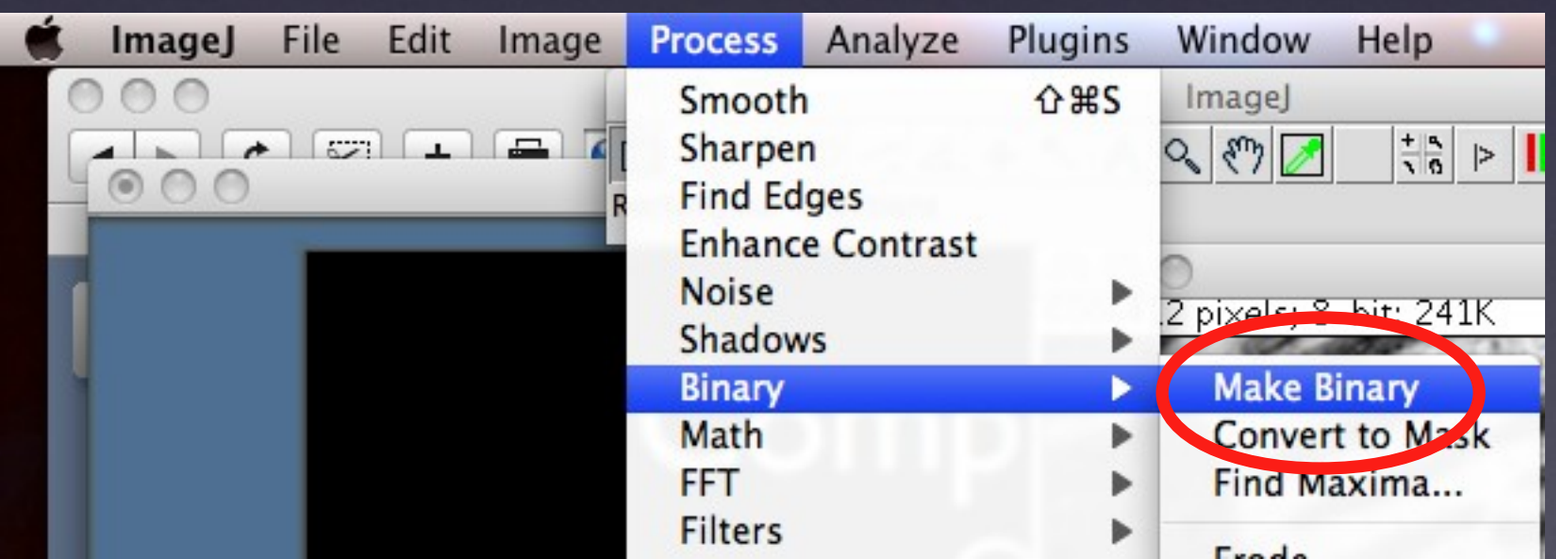
ImageJ - Image - Adjust - Threshold - Auto (=Make Binary):

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 Fiji - Image - Adjust - Auto Threshold for more methods.

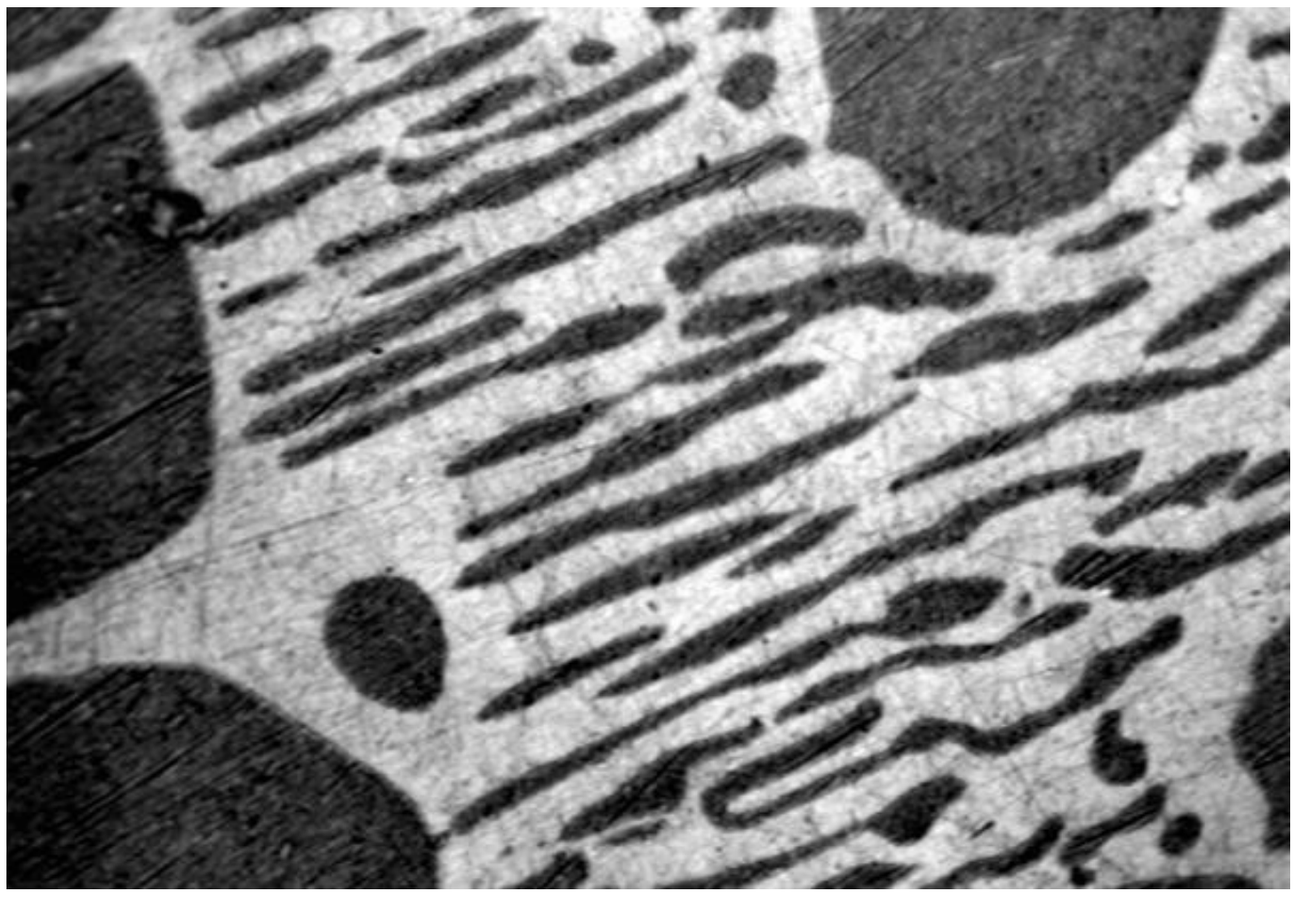
Practical Session 2c

- Simple Image segmentation - Blobs (inverse LUT)
- Process - Binary - Make Binary (default method)
- Image - Adjust - threshold
- Adjust the thresholds, then set them to make binary.

- Image - Adjust - Auto Threshold and Auto Local Threshold
- Many more methods, and “local” method

- Statistical Region Merging - Clown.

Edge Detection:
The Sobel filter
The Sobel filter



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

Edge Detection

What is an “edge” ?

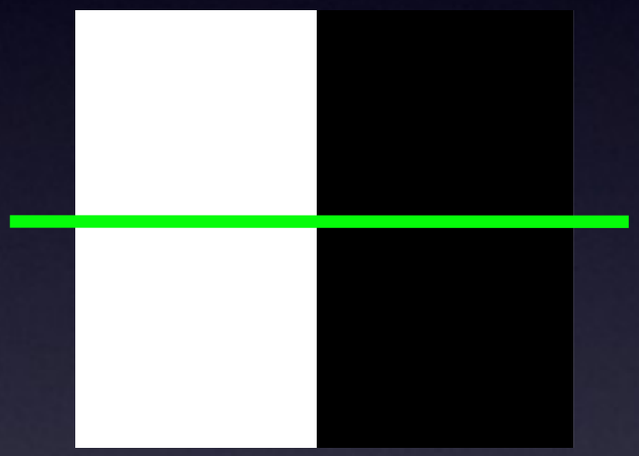
- “Hard Edge” - Adjacent black - white pixels
- “Soft / Fuzzy Edge” - common in images
- Especially for small diffraction limited objects (vesicles / membranes)
- Noise makes edges look softer

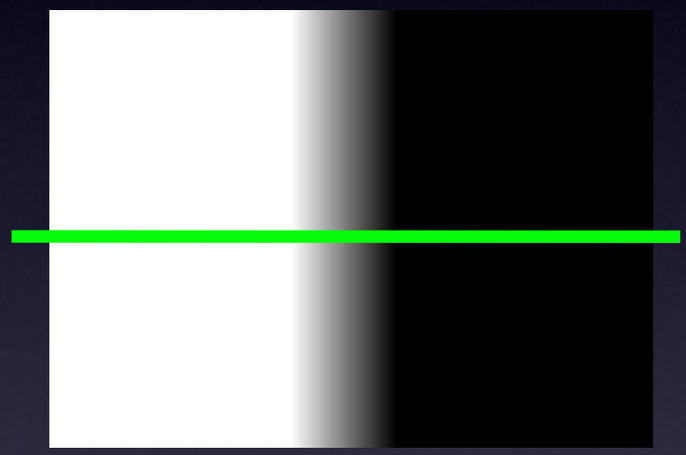
Edge Detection

”Image Gradient”

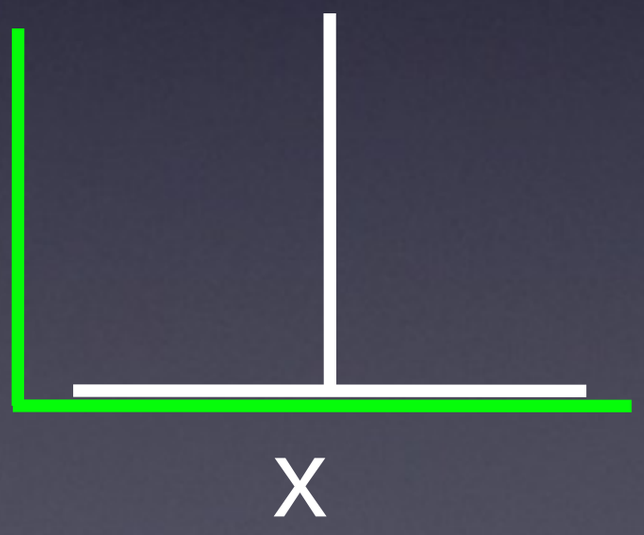
What is a “Gradient Image” ?

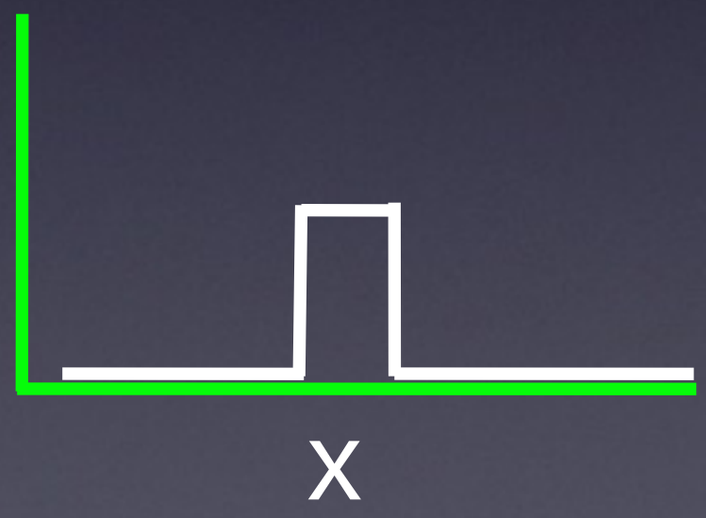
Rate of change of pixel intensity (1st derivative)





pixel intensity gradient



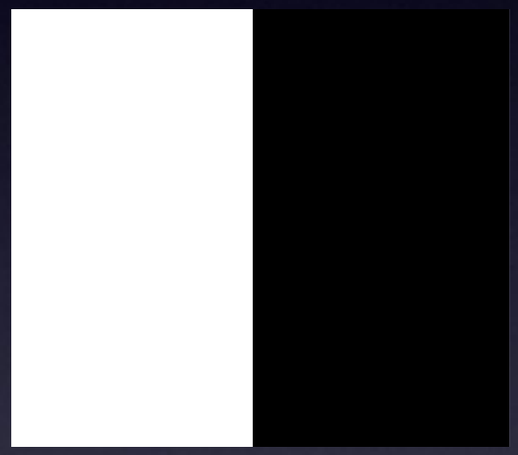


Edge Detection

”Image Gradient”

What is a “Gradient Image” ?

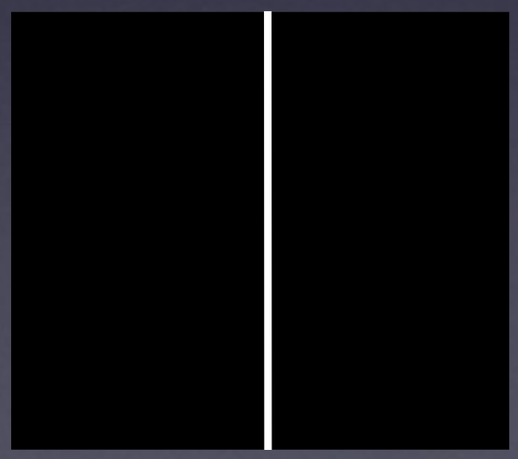
Rate of change of pixel intensity (1st derivative)



Image

hard edge

soft edge



Gradient Image

”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

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

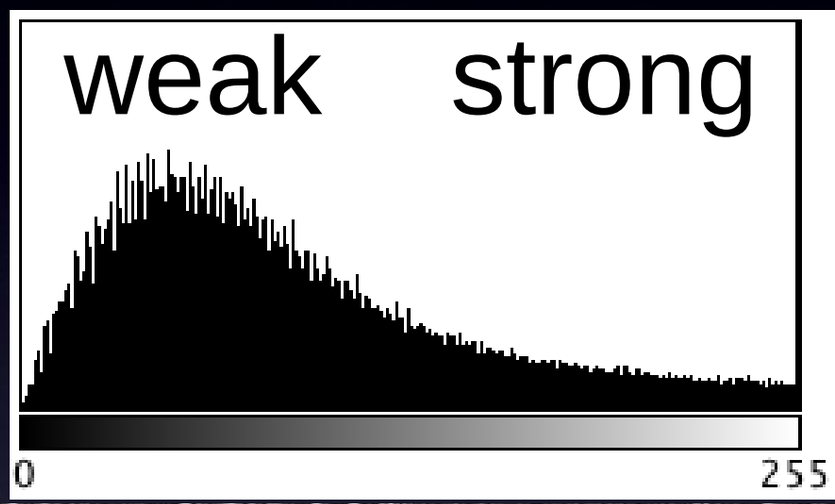
$$|g_x| + |g_y| = |g|$$

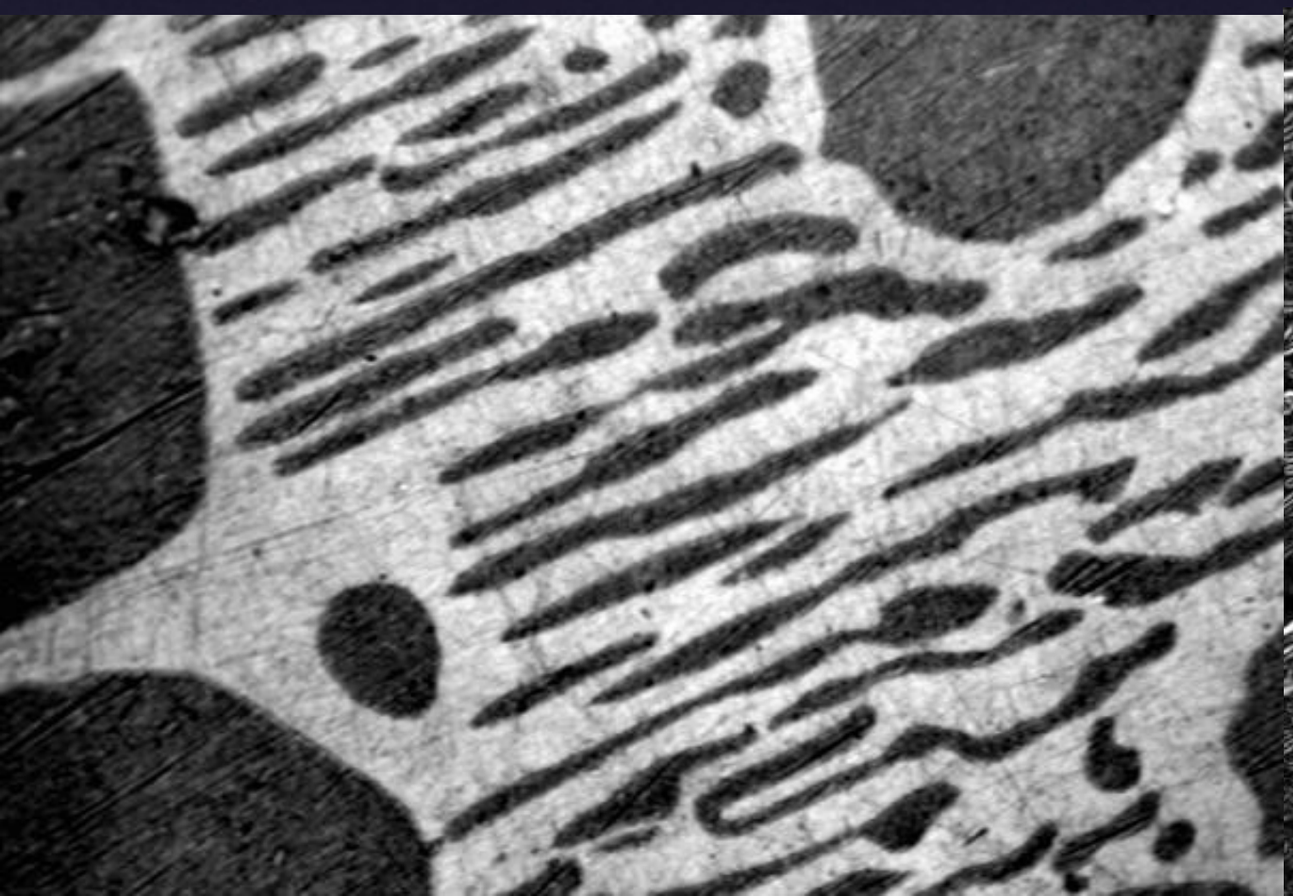
Gradient Image - Real Sample:

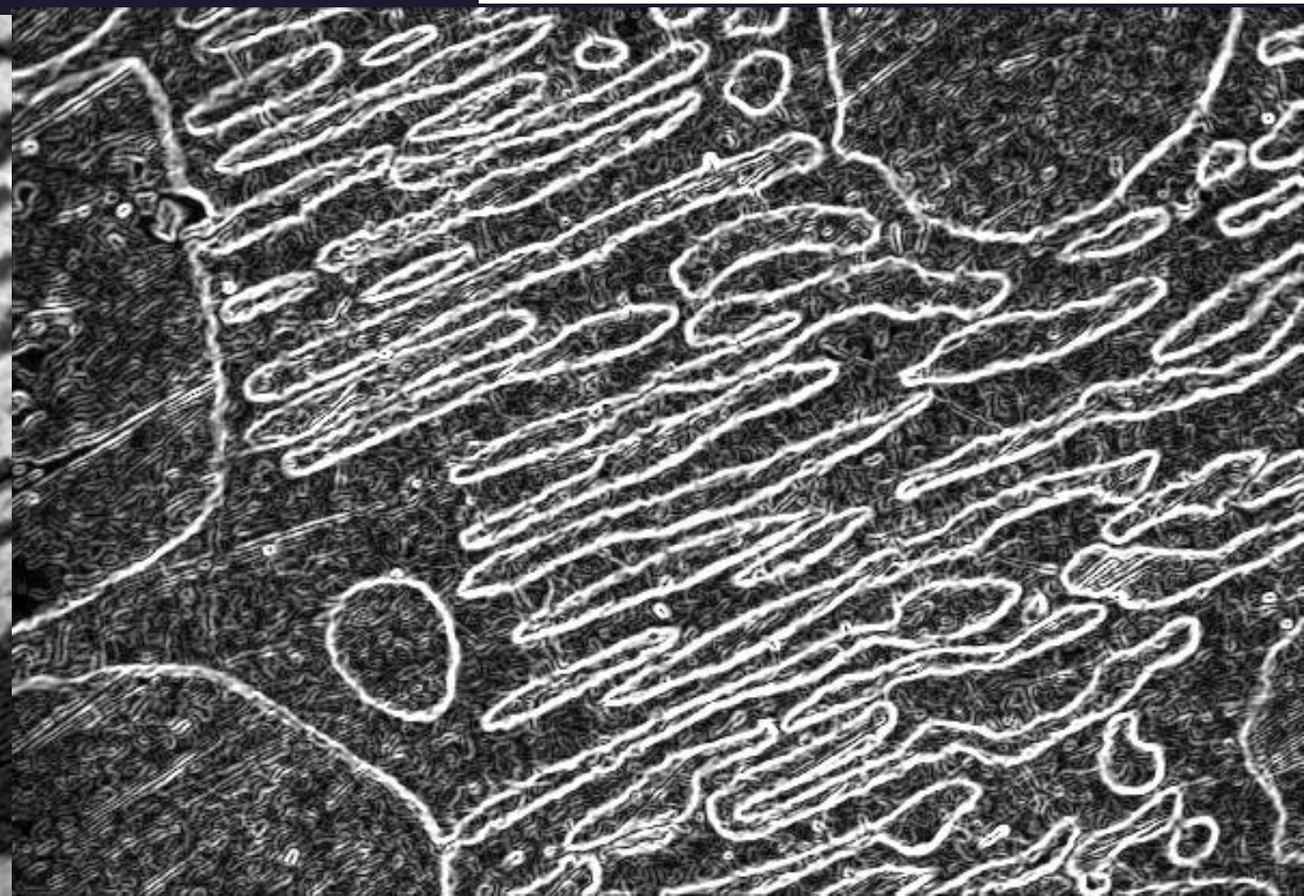
Real / Biological images:

- Sobel f lter
- many edges
- many weak edges from noise

gradient image histogram



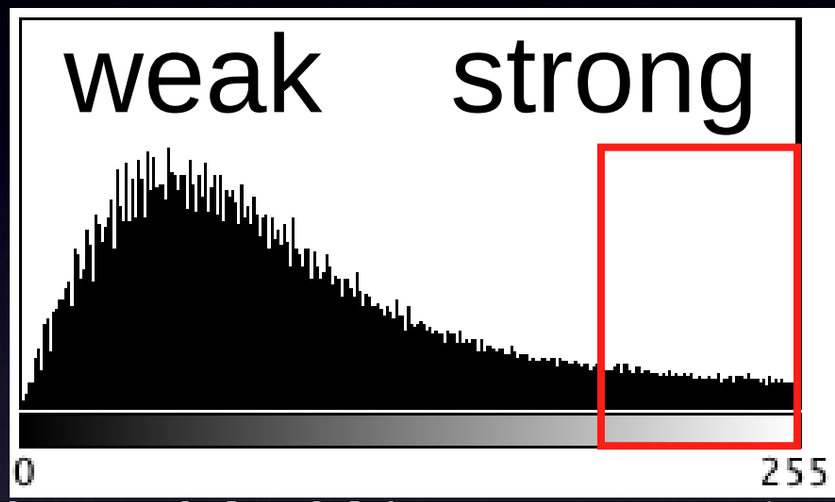


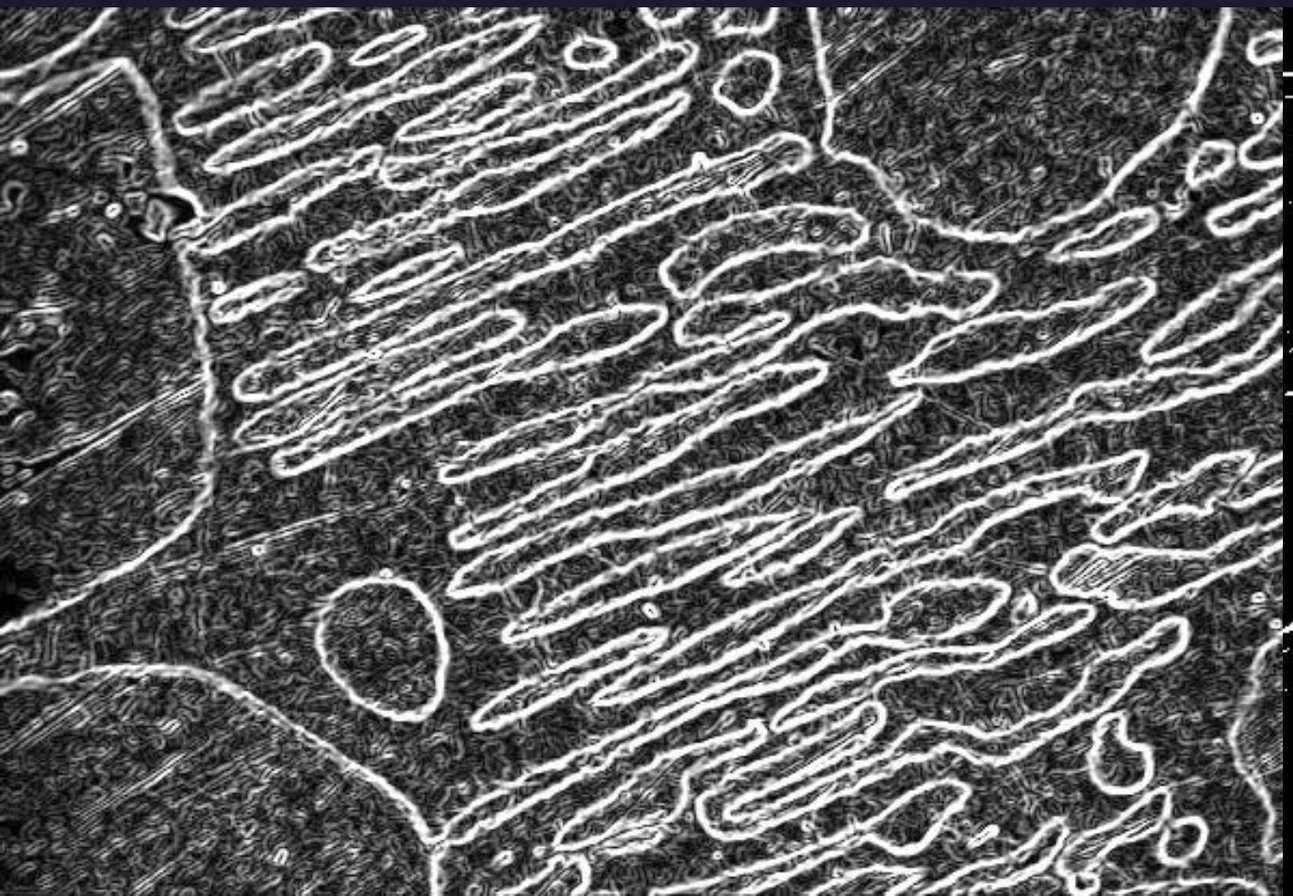


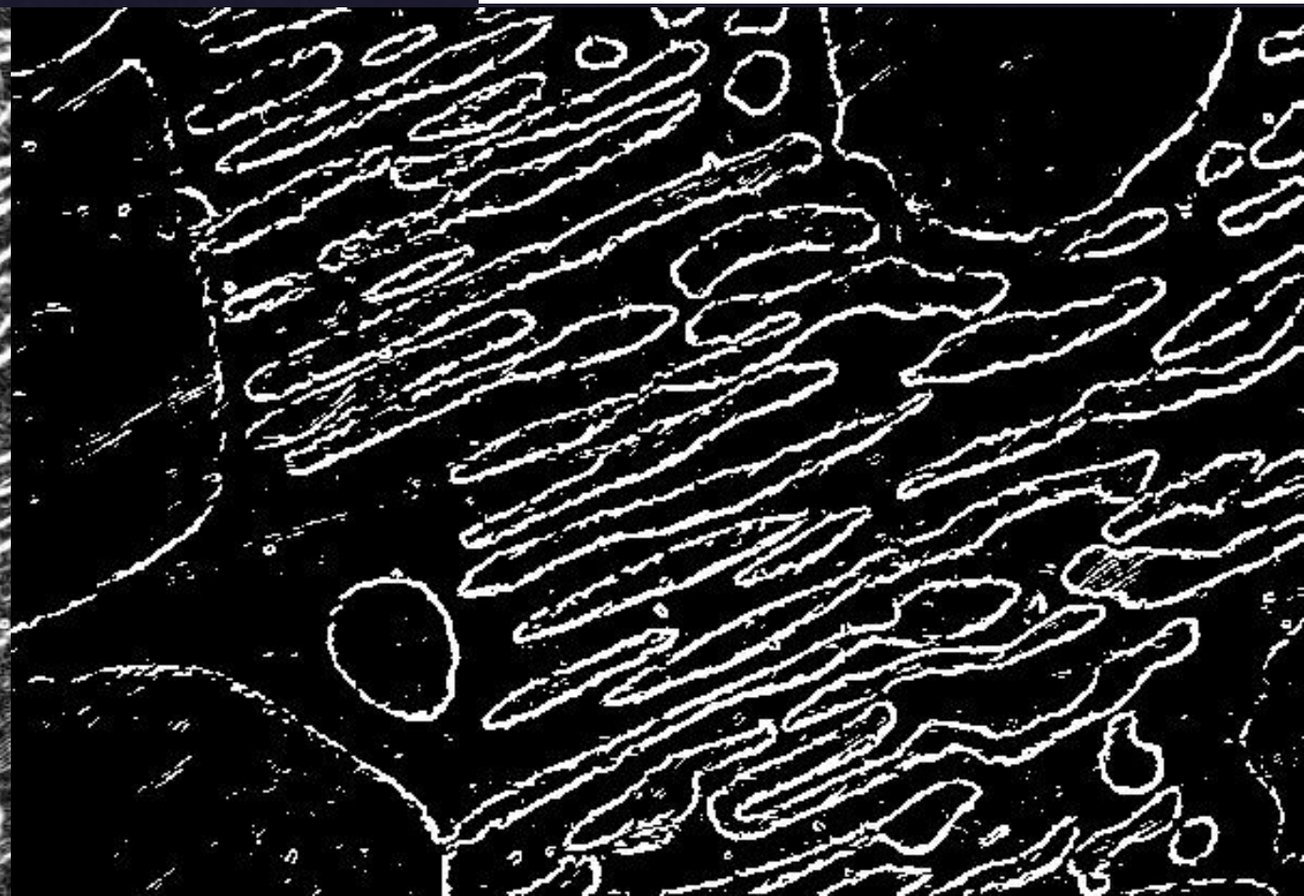
Gradient Image - Strong Edges?

Remove weak edges?

- Threshold the gradient image
- Smoothing filter beforehand

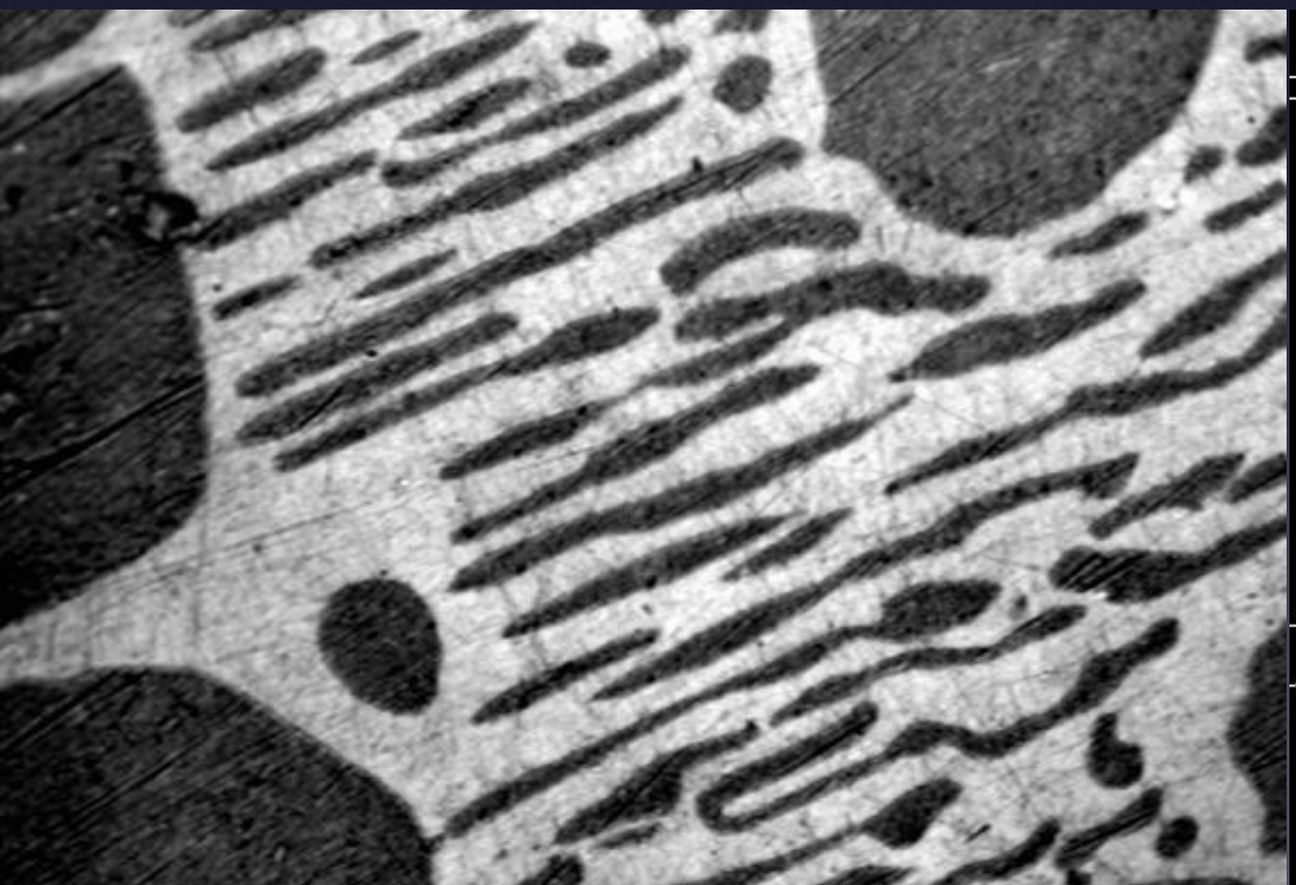




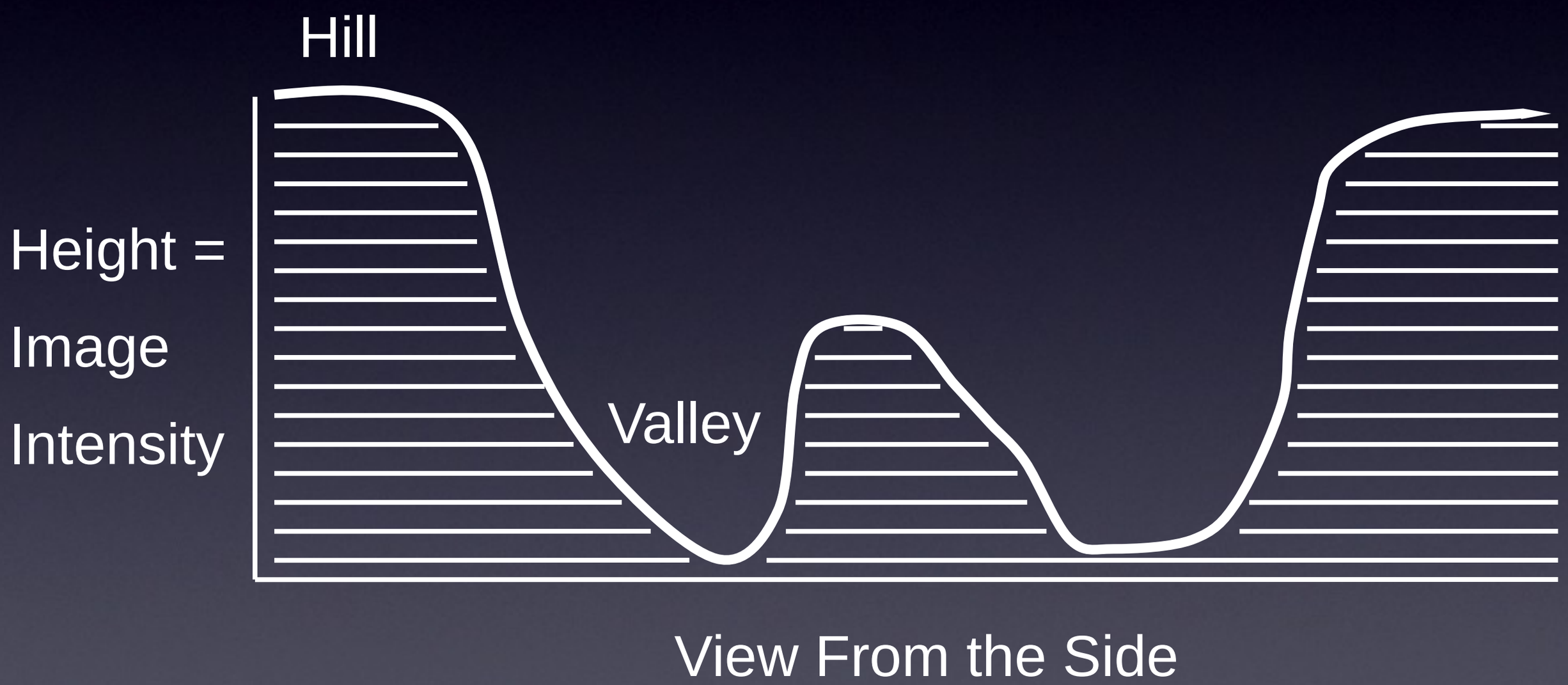


“Canny” Edge Detection

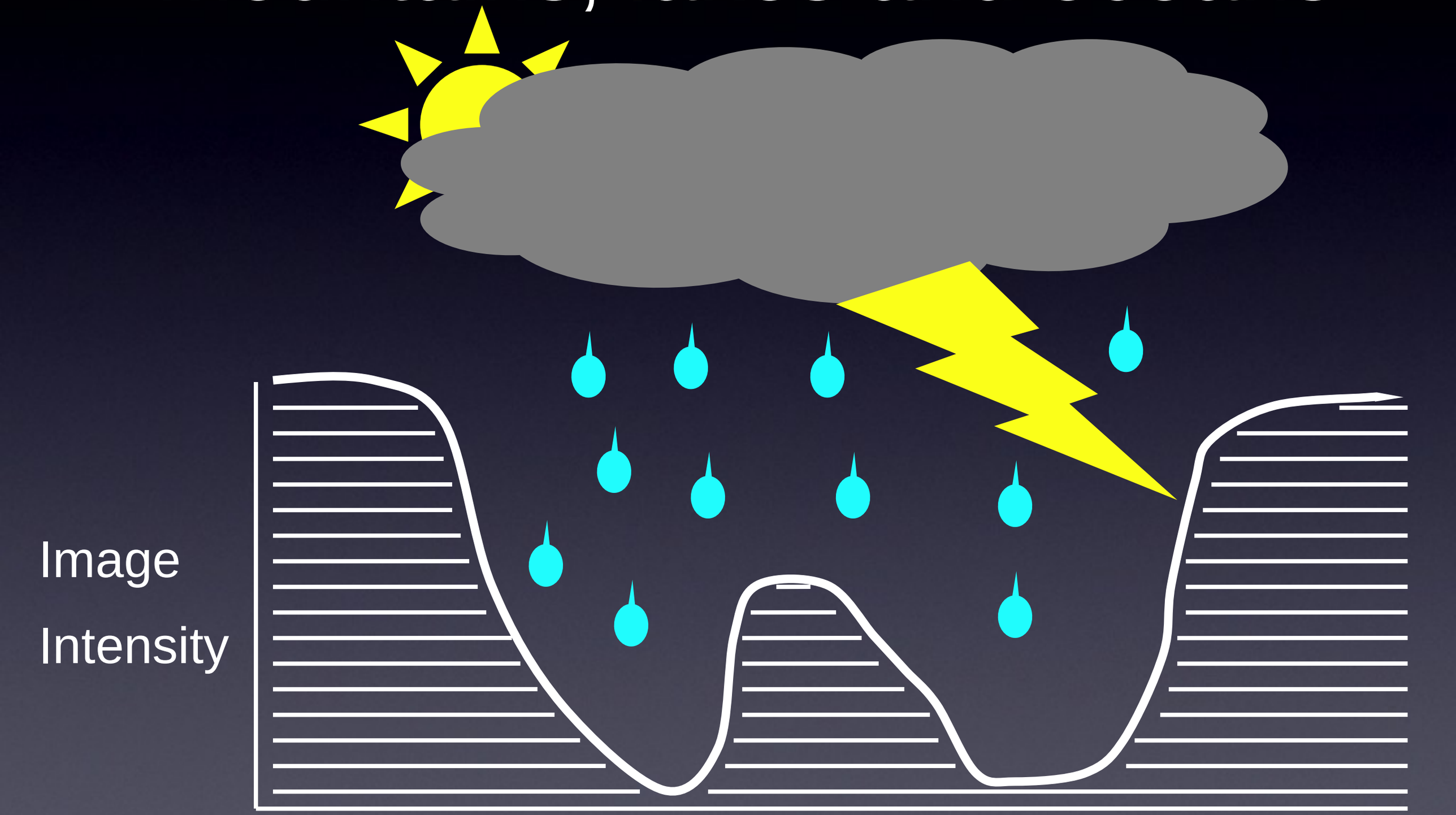
- Remove weak/noisy edges - keep strong
  - Gaussian smooth image + hysteresis threshold gradient image
- Make edges sharp - 1 pixel wide
  - Non maximal suppression of gradient image



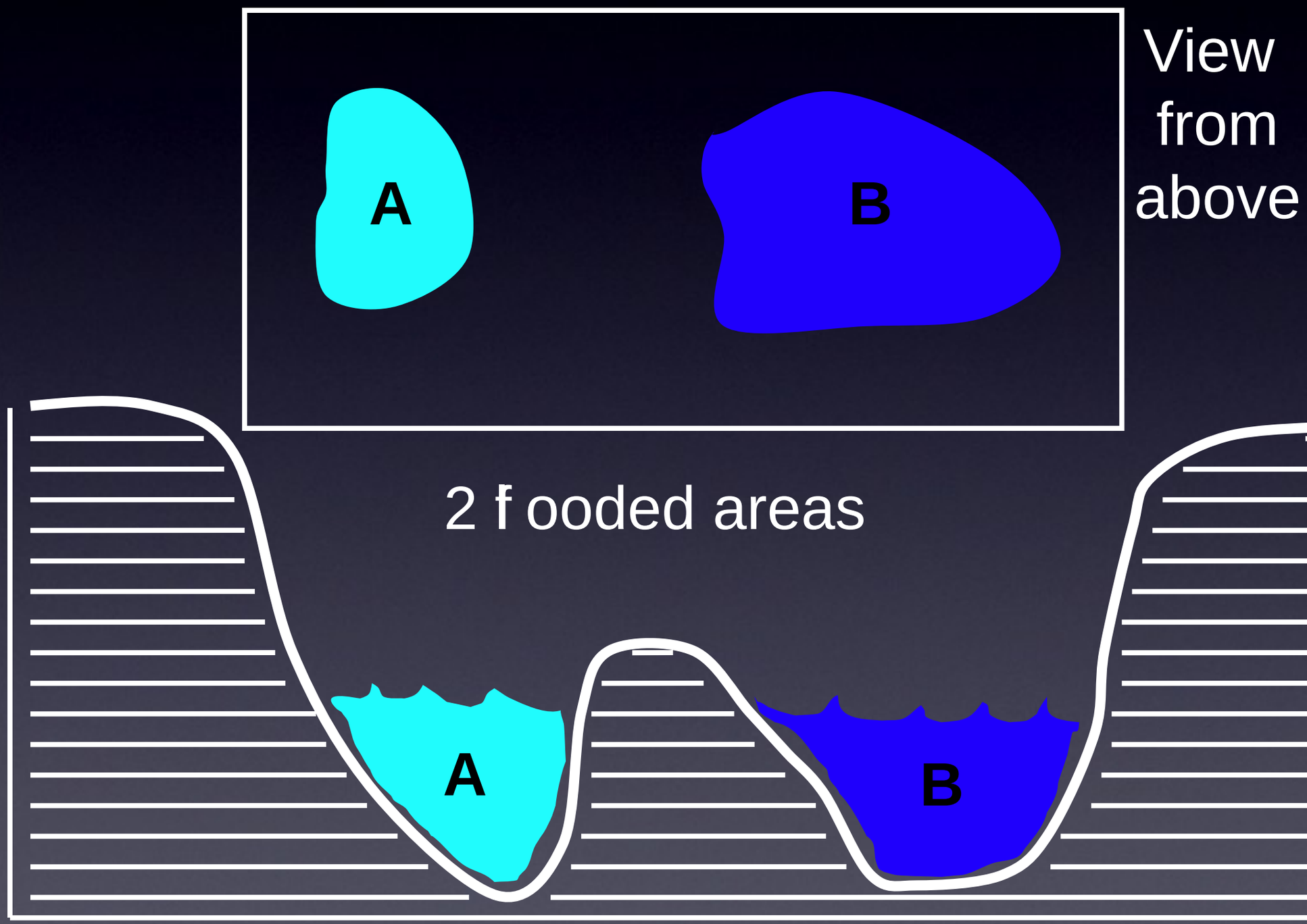
Watershed Algorithm: mountains, lakes and oceans



Watershed Algorithm: mountains, lakes and oceans

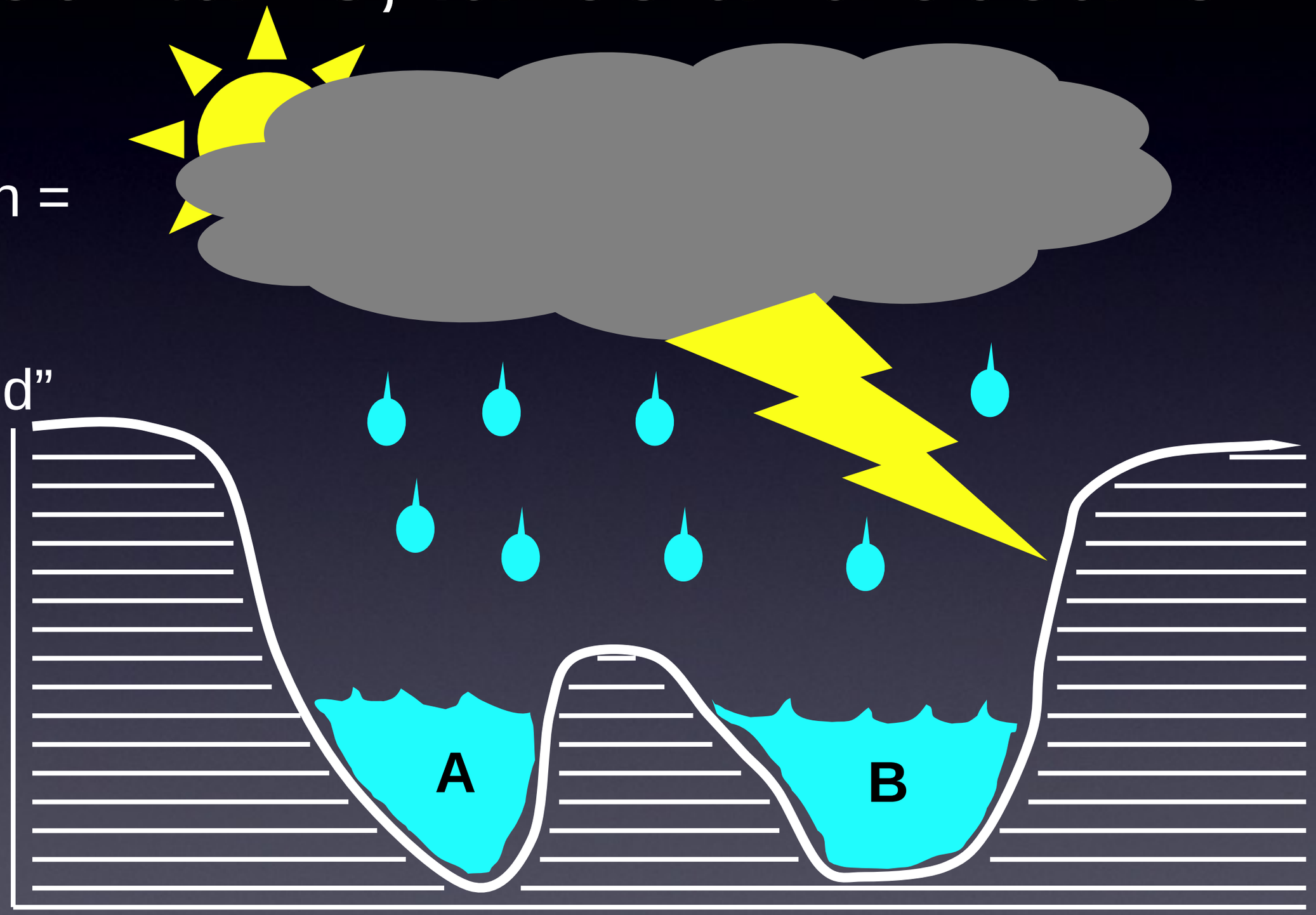


Watershed Algorithm: mountains, lakes and oceans

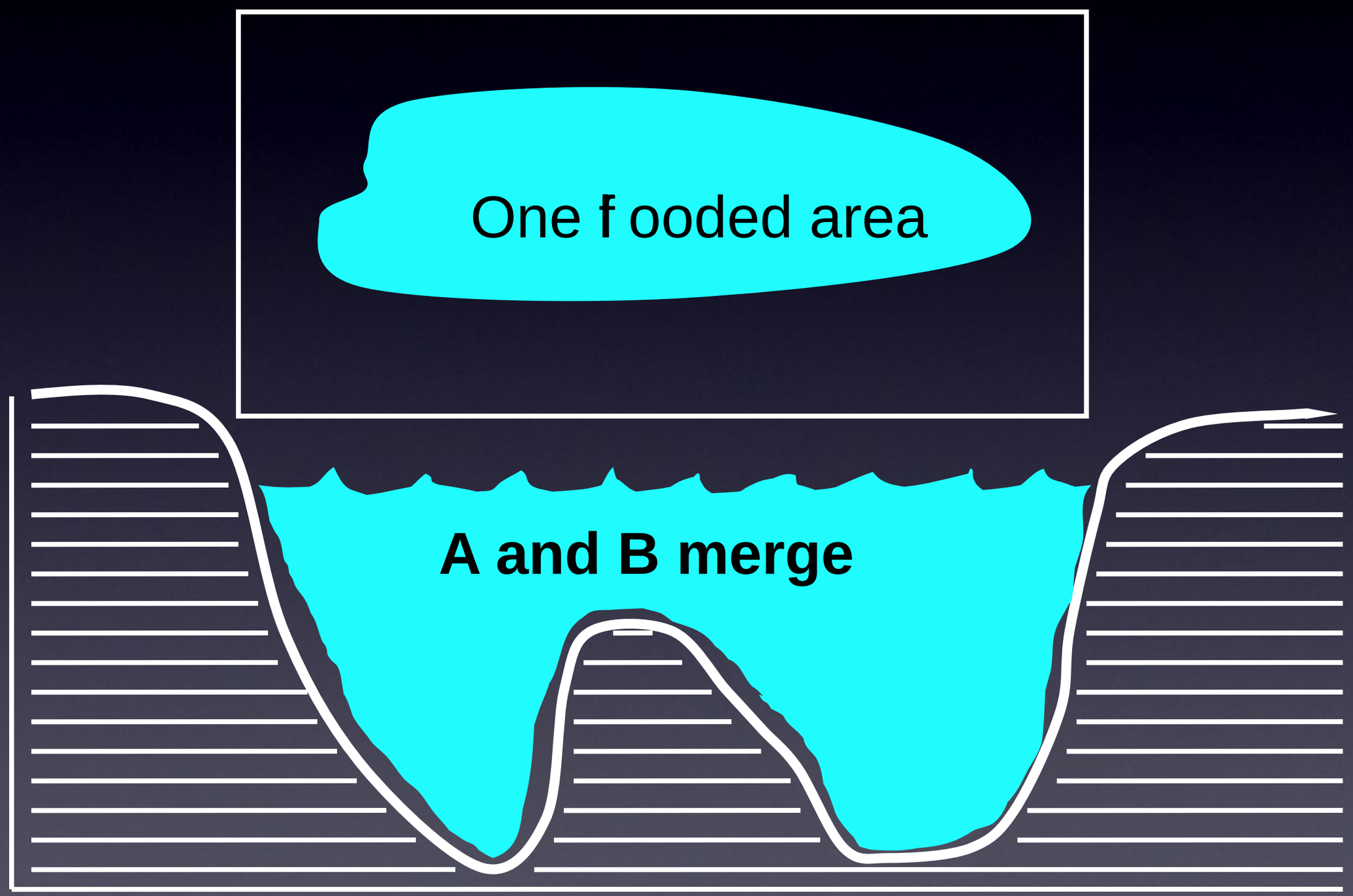


Watershed Algorithm: mountains, lakes and oceans

More rain = increase “threshold”

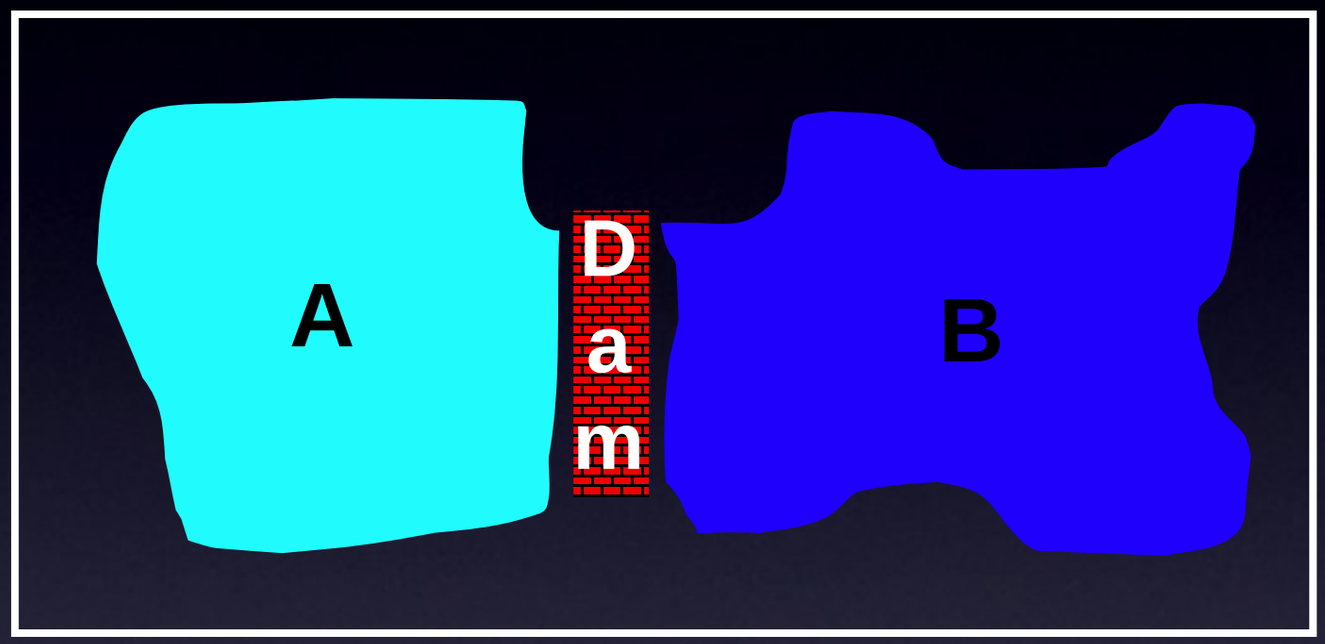


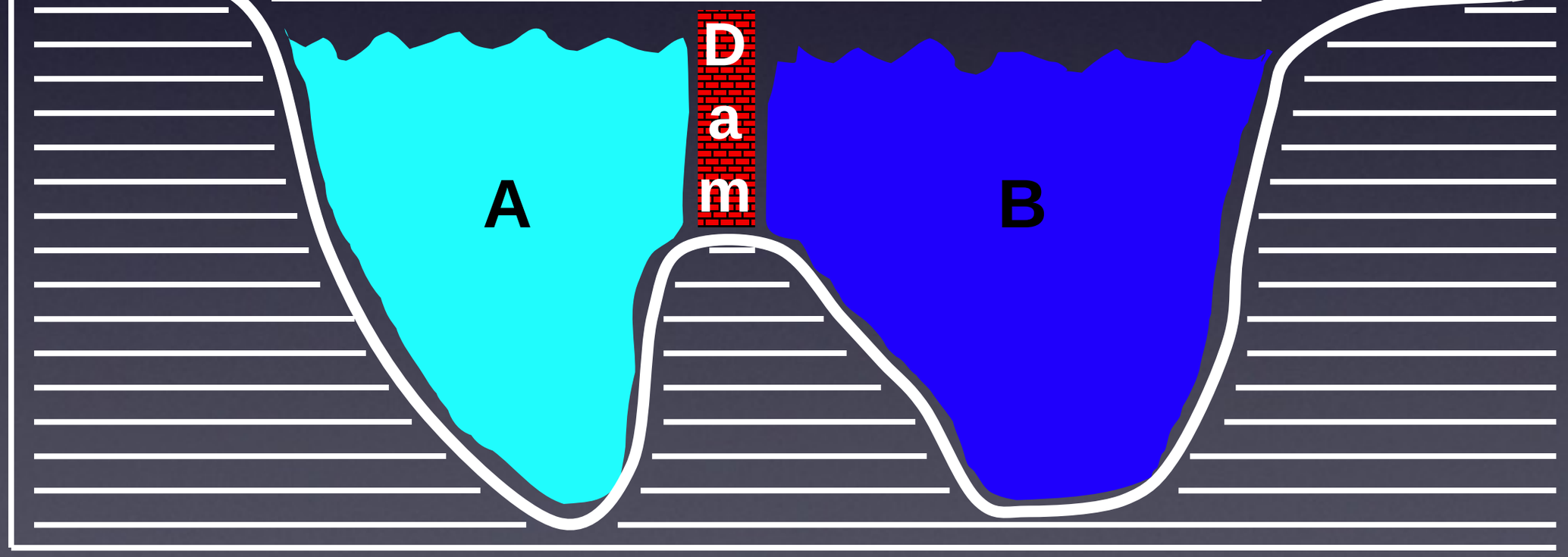
Watershed Algorithm: mountains, lakes and oceans



Watershed Algorithm: mountains, lakes and oceans

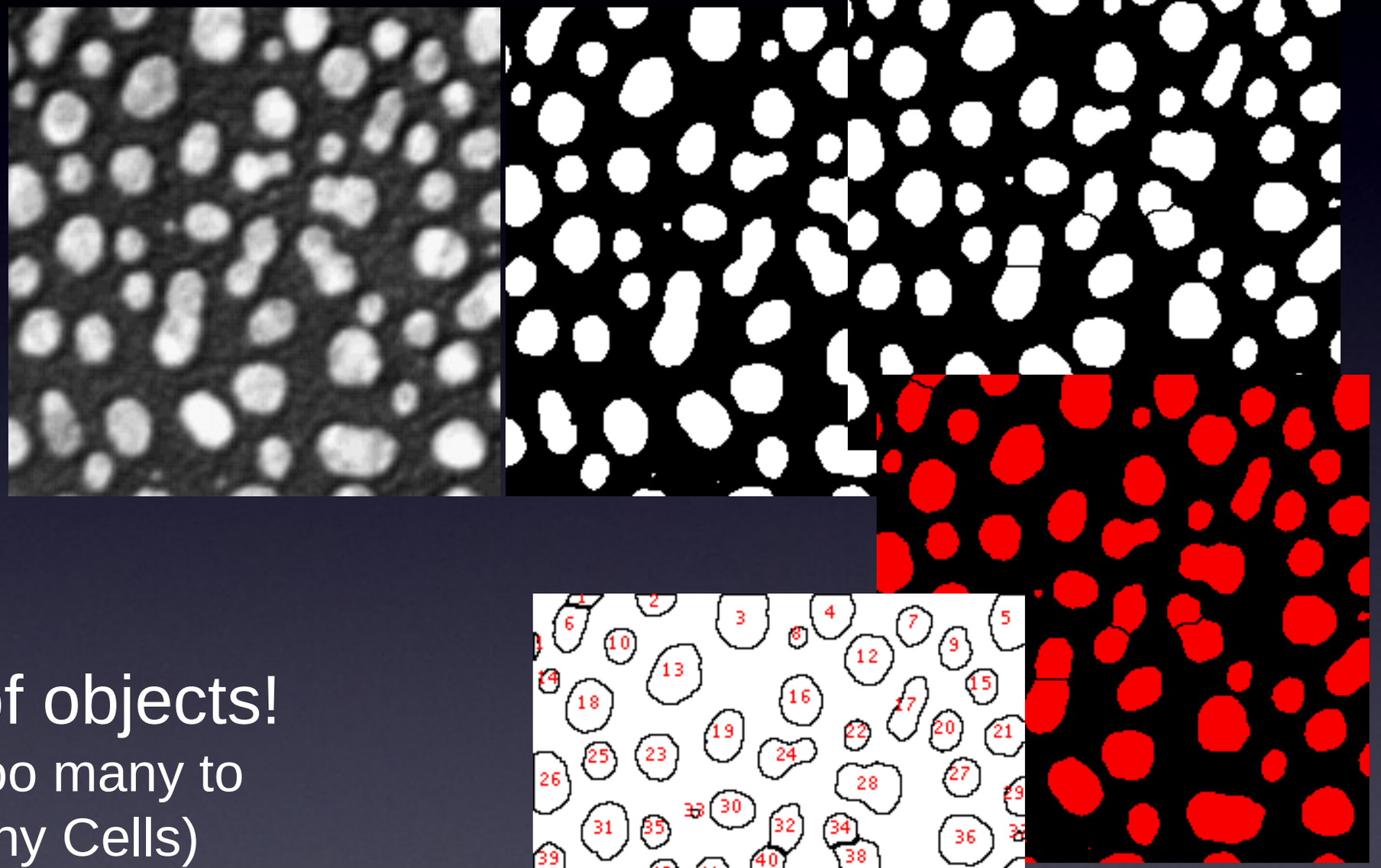
Make a “Dam” at the “Watershed line”





Watershed
to f nd object number

- Blobs.gif
- Invert
- Make Binary
- Watershed
- Threshold
- Analyze Particles

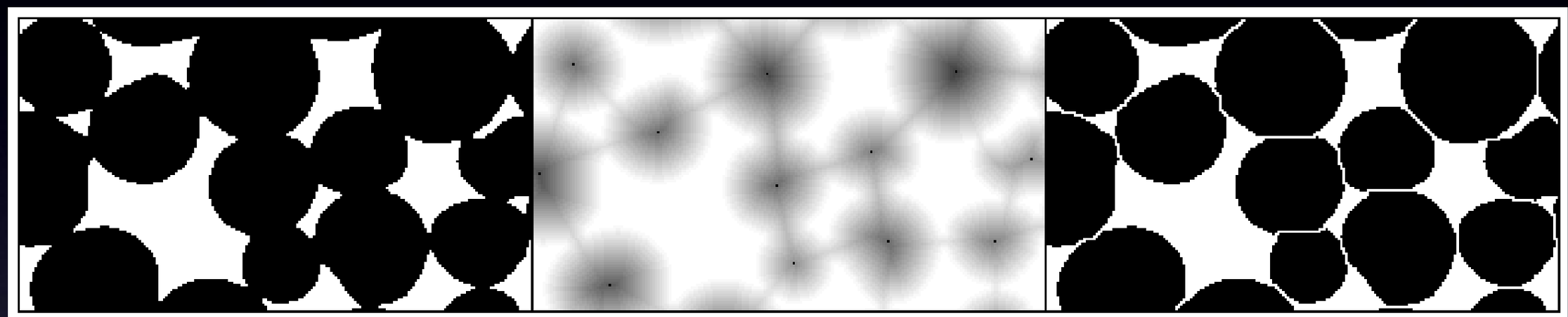


- Gives number of objects!

(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

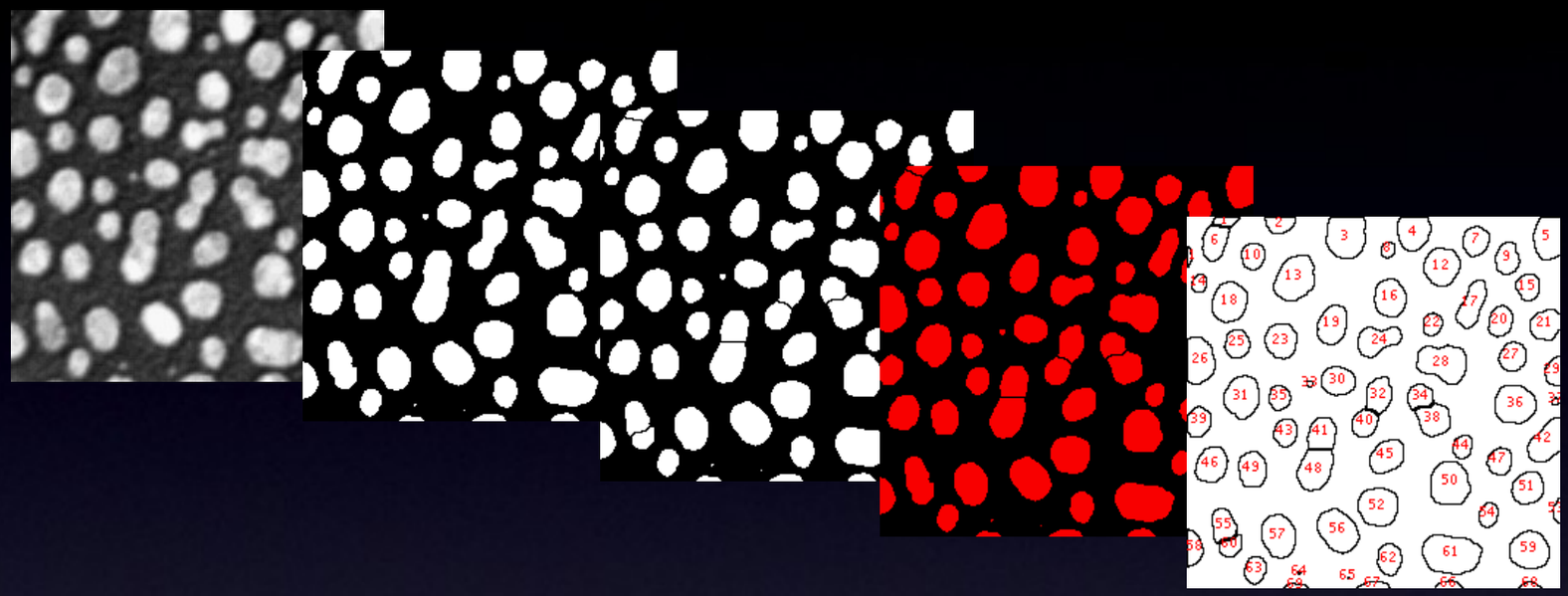
Watershed to separate touching objects



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

QUICKTIME 2? AND A DECOMPRESSOR ARE NEEDED TO SEE THIS PICTURE.

Practical Session 2d



- Watershed Segmentation and Analysis
- Separate and measure touching objects - DAPI stained nuclei
- Tutorial is on the Fiji WIKI
- Look in Tutorials for NucleiWatershedSegmentation
- or Just search the Wiki for NucleiWatershedSegmentation

Links and Further Reading

- Standard Text Book

Digital Image Processing 2nd Ed.

Gonzalez and Woods, Prentice Hall

- Fiji and ImageJ
  Fiji - http://pacif c.mpi-cbg.de Fiji Wiki and docs.
  Fiji Installation: http://pacif c.mpi-cbg.de/Installation
  http://rsb.info.nih.gov/ij/ ImageJ home
  http://imagejdocu.tudor.lu/doku.php ImageJ Doc. Wiki
  MacBioPhotonics plugins collection for microscopy
  http://www.macbiophotonics.ca/downloads.htm

- Image Processing Facility

  Intranet - Services and Facilities - Image Processing Facility
  https://zope.mpi-cbg.de/intranet/services/image-processing-facility
  Wiki - info for beginners - tips - software documentation
  https://wiki.mpi-cbg.de/wiki/imagepro/index.php/Main_Page
- Email: ipf(at)mpi-cbg.de