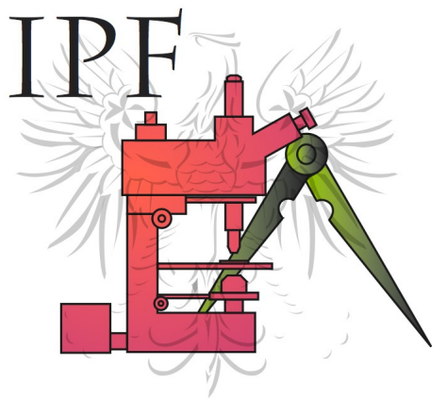
Basics of Quantitative Image Analysis

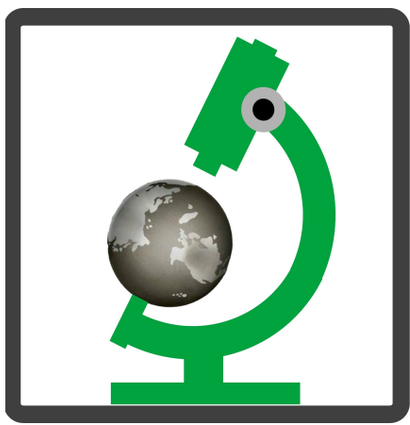
What you need to know about

Image Processing ...

... but never thought to ask

CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics





Before you start writing...

See these slides at: https://ifn.mpi-cbg.de

under: Teaching

Also available on the Fiji Wiki

- ✓ Fiji is just ImageJ – batteries included
  http://pacific.mpi-cbg.de
- ✓ Fiji tutorials
- ✓ DetectInfoLoss, ColocalisationAnalysis
  and more...
- ✓ Practicals etc. are included in online version...

Topics:

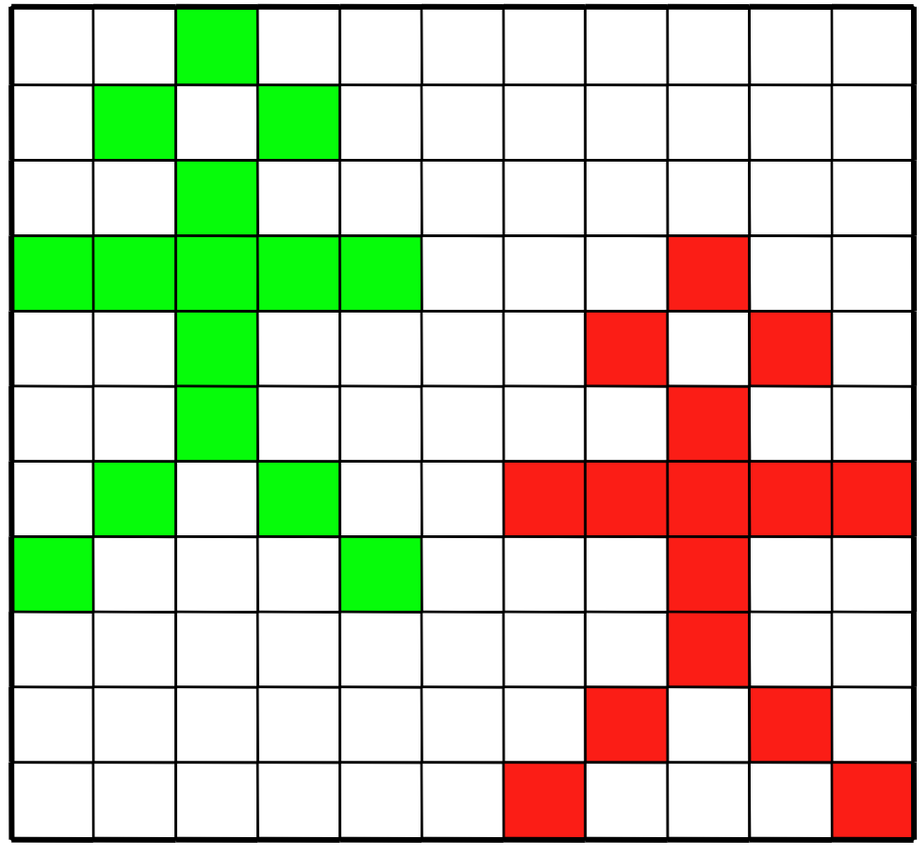
- ✓ Imaging Workflow
- ✓ Images = “Information” (Digital Images)
- ✓ What is a pixel?
- ✓ Info “about” the image = Meta Data
- ✓ Different ways to visualise / display image info

Quantitative Imaging?
…what does that mean?

Art or **Science**? **Photography** or **Spectroscopy**?

Science means to **measure** something!

- ✓Numerical Results
- ✓Statistics!
- ✓Computers become useful…



What is Image Analysis / Quantification?

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

Minimum:	50
Maximum :	255
Mean:	94.5
Std.Dev.:	93.2
Area:	10x14
Pixels:	140
Pix <255:	42

= Image Analysis/ Measurement

Object:	Stick man
Body:	1
Head:	1
Legs:	2 (1 lifted)
Arms:	2 (2 lifted)

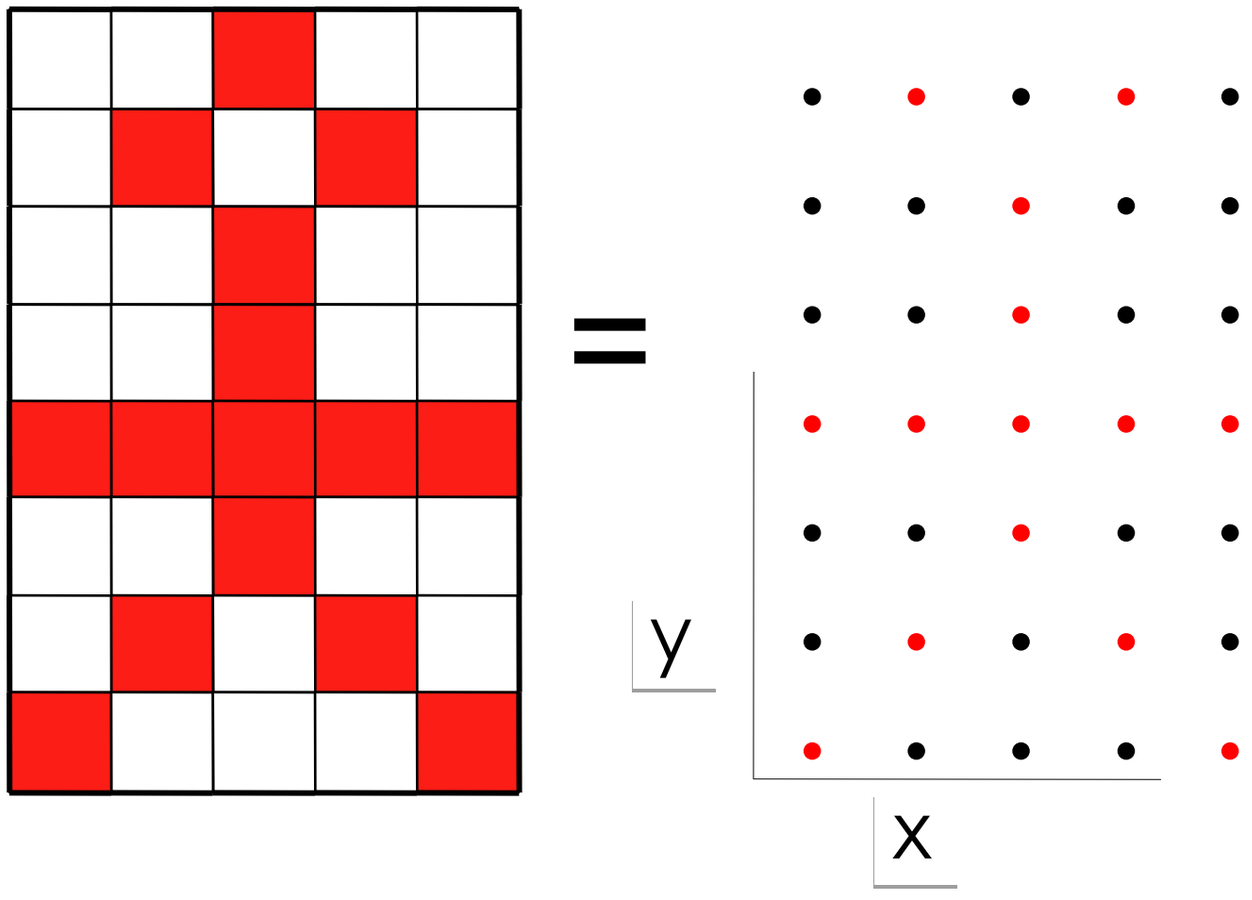
Walking left to right…

= Interpretation of Analysis Result

What is a (Digital) Image anyway..?

- ✓ it's a digital "**representation**" of reality!
- ✓ it's an **artifact** that contains less info than the object!
- ✓ it's just **numbers**! NOT analogue art!

The Image of a point is NOT a point!!!
(Point Spread Function – PSF)



A digital image of ???

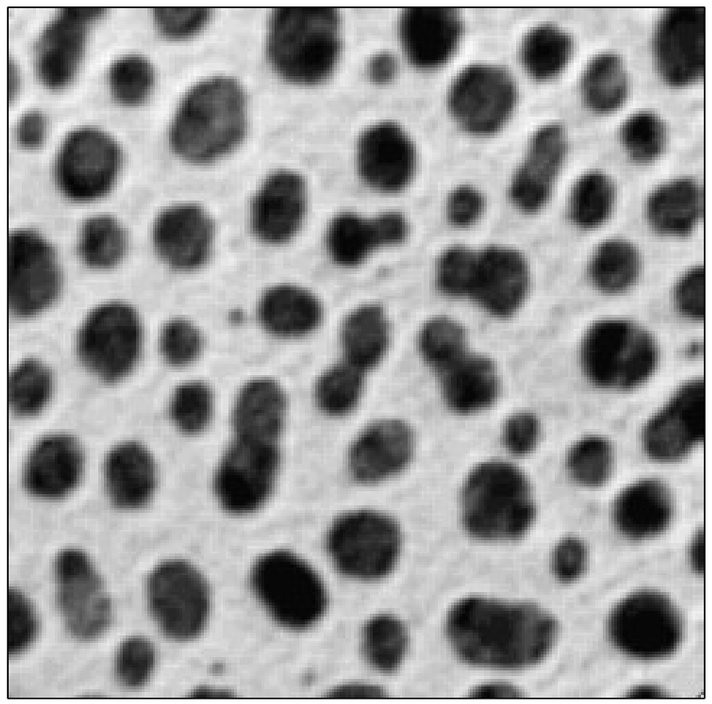
Image Analysis
(Brain or Computer)

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

Image = Information

Images contain information!!!

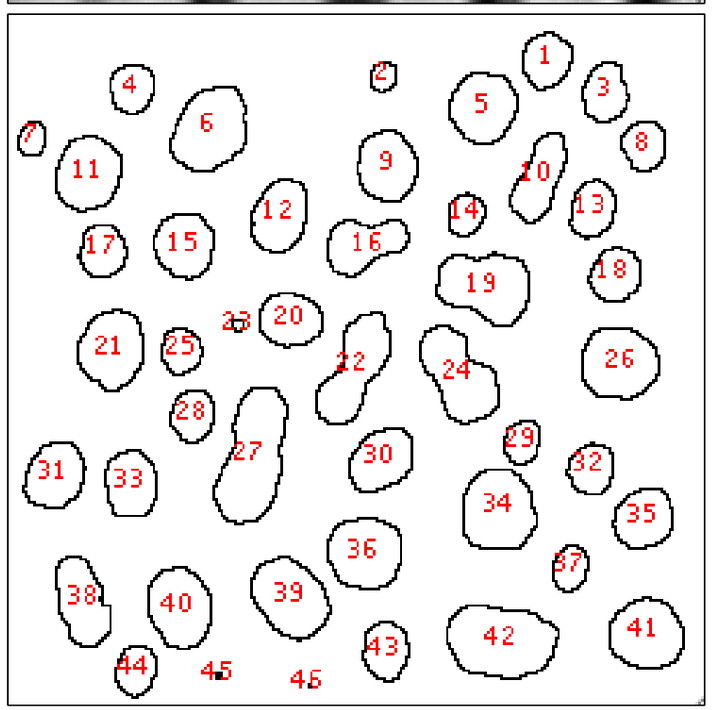
- ✓ Quantify / Measure / Analyse
- ✓Meta data (what, where, when, how)
- ✓ Noise / Background



Manipulate Image
=
Changed Info!!!

Lost Info is lost forever

	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

Image Data? What is it?

Intensity – Dye concentration??

Comparison of 2 colours / dyes / proteins??

Noisy Images?

Averaging?

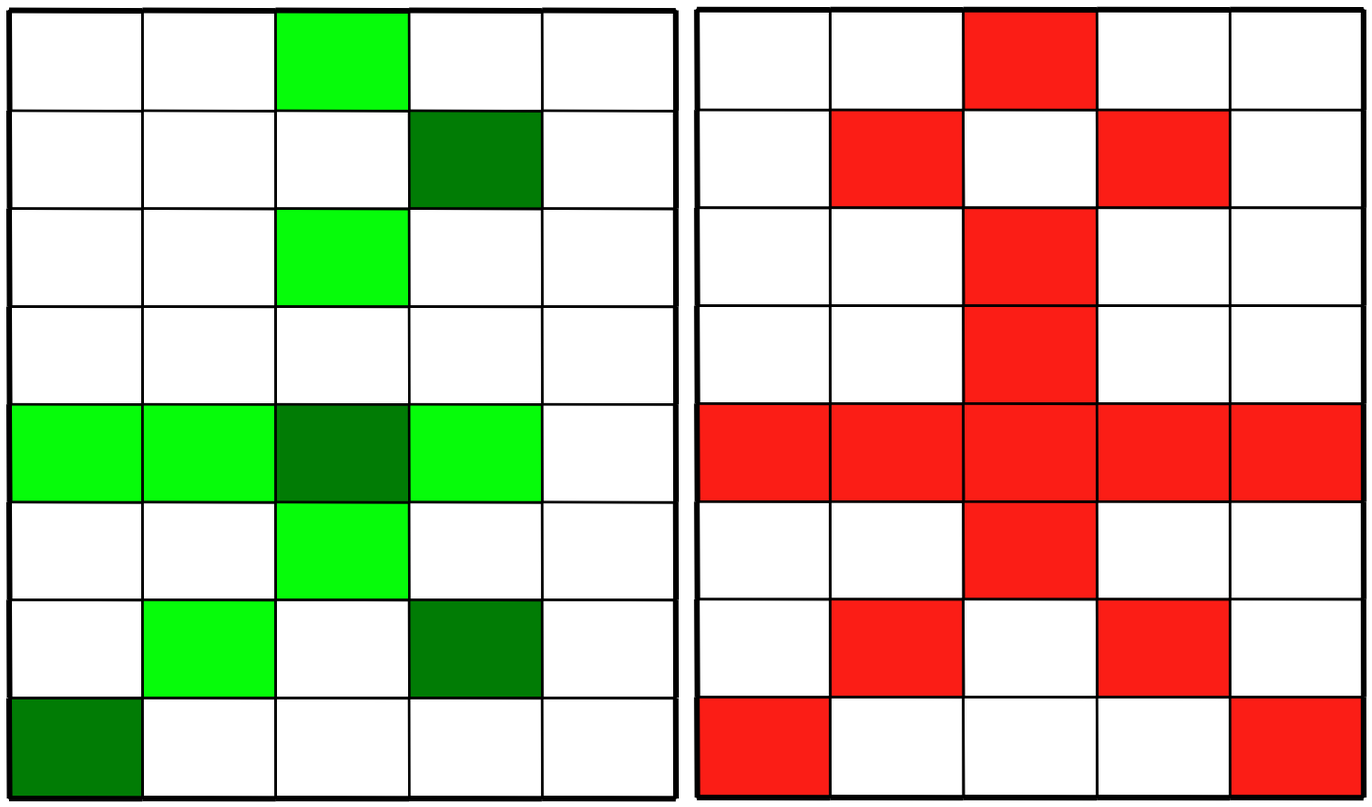
Pixel Time?

Shapes, Movement, Structure?

Internal controls!!!

A digital image with 2 channels / colours

What can you see here?



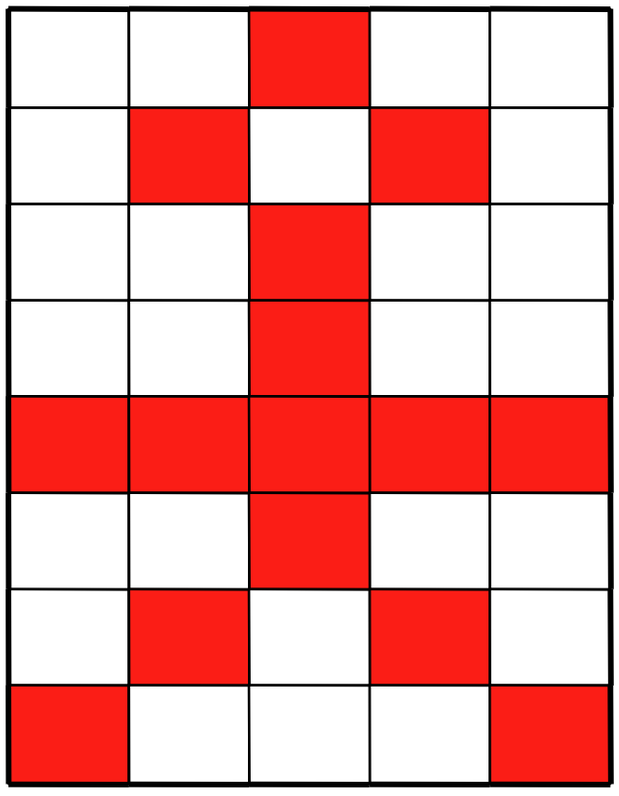
Photographer or Spectroscopist?

We can show you how to take pretty pictures (Art)

or

We can teach you how to get useful information (Science)

You choose!!



This

Is simply a way to **"Visualise"**

This

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

y

x

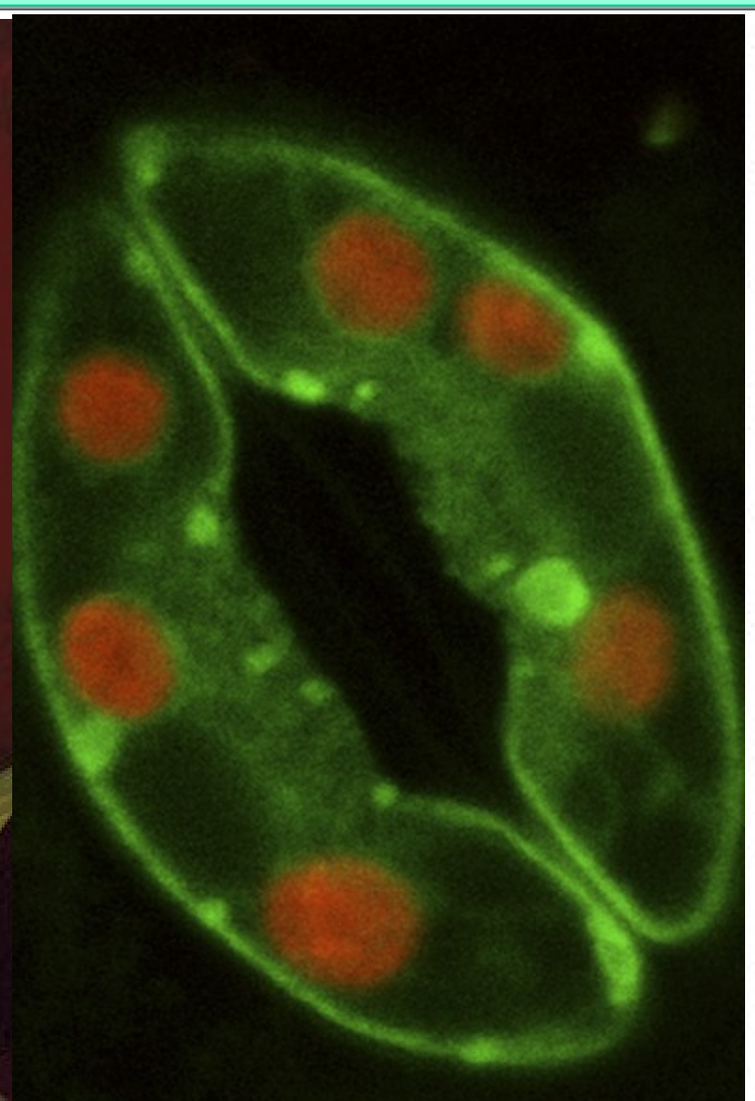
Photographer or Spectroscopist?

Science vs. Art

Objectivity vs. Subjectivity

What I "think" I see vs. What is really there

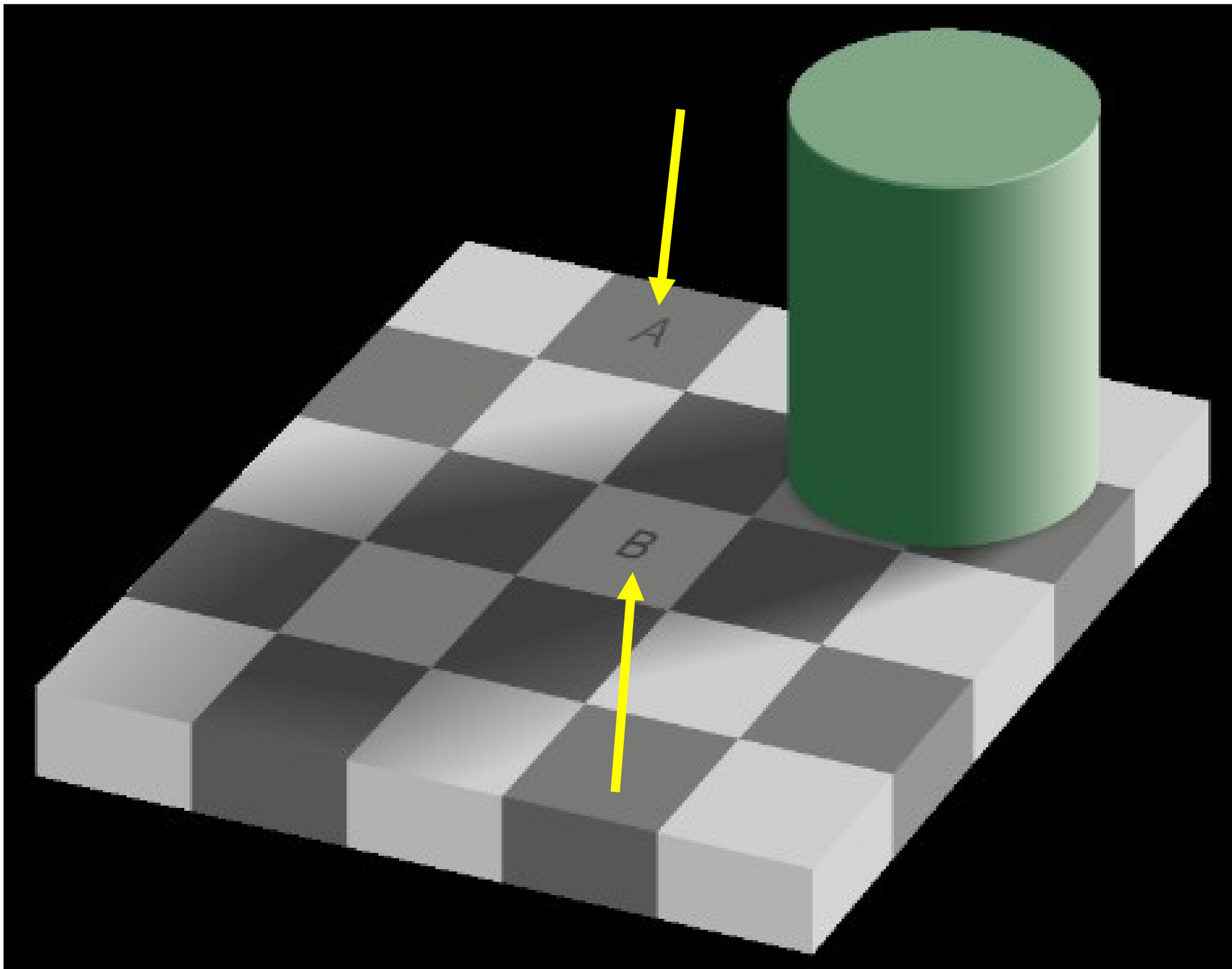
Morphology can also be quantified!



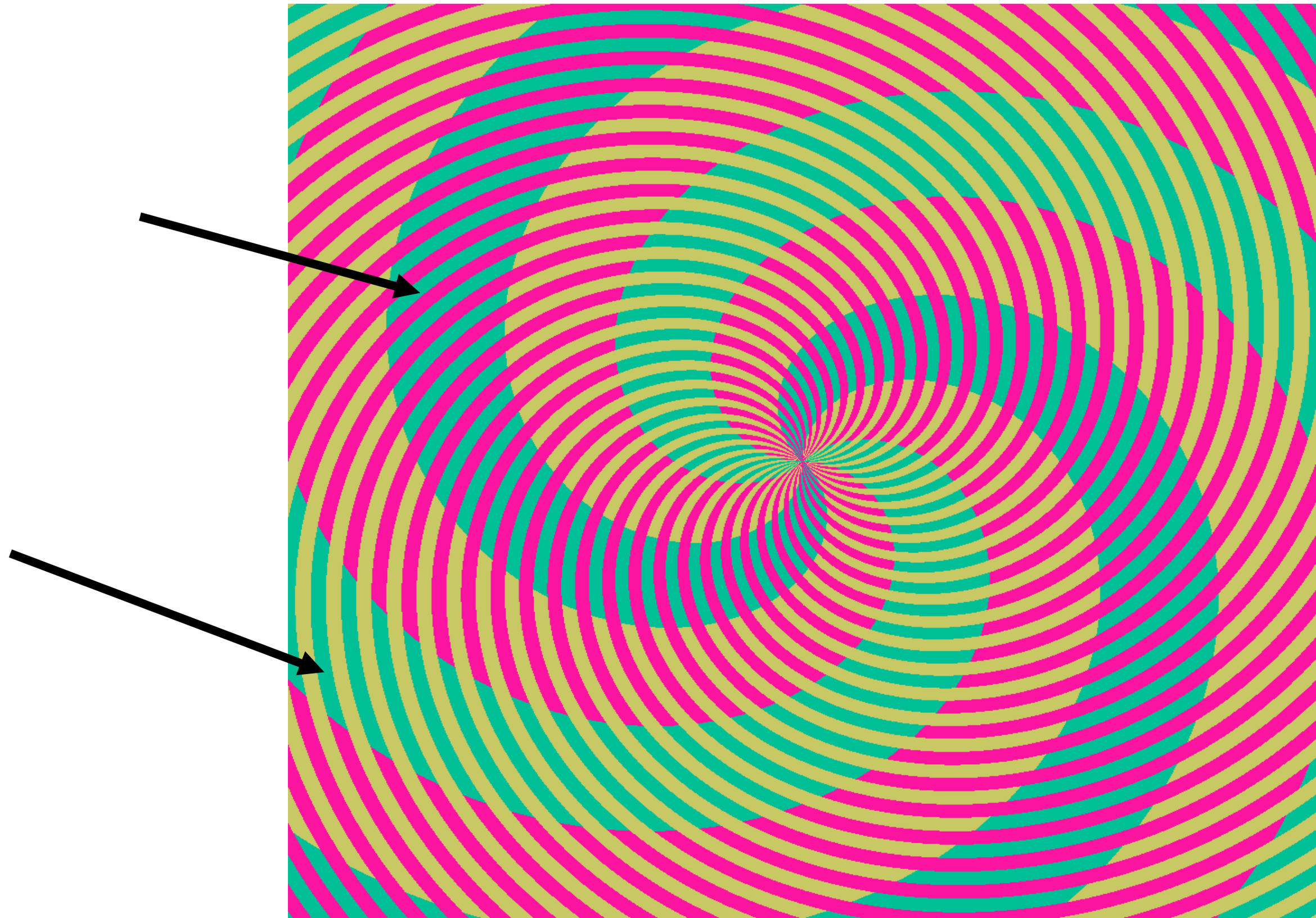
249	244	240	230	209	233	227	251	255
248	245	210	93	81	120	97	193	254
250	170	133	94	137	120	104	145	253
241	116	118	107	134	138	96	92	163
277	142	121	113	124	115	107	71	179
234	106	84	125	97	108	125	106	204
241	202	102	132	75	73	141	246	252
253	252	244	239	178	199	242	250	245
255	249	244	250	226	231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

Which is brighter A or B?

Still sure you can interpret intensities in greyscale images with your eyes?



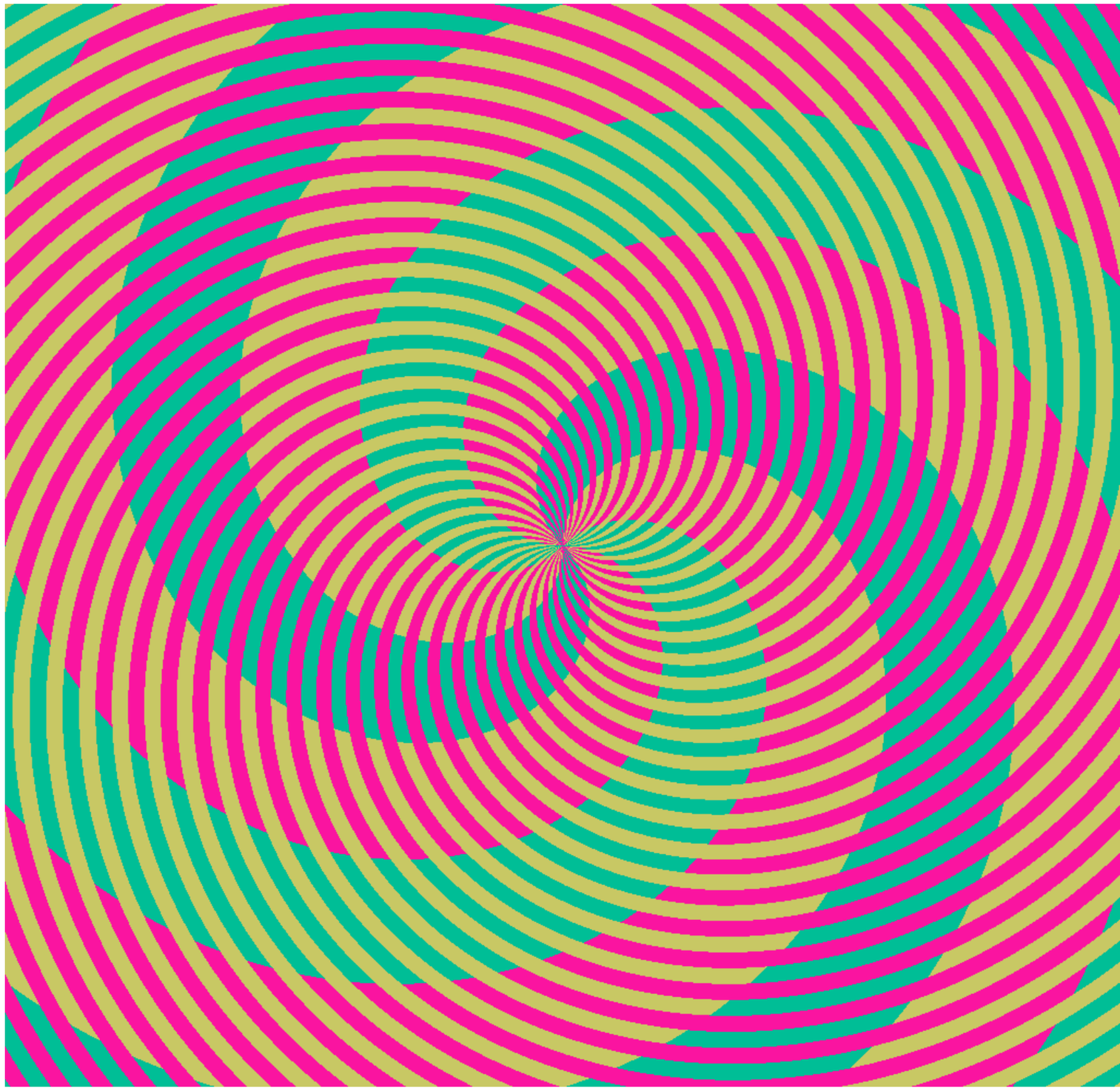
Which colours can you see???



“Colour Merge” images could ruin your life

You see spirals, of pink, orange, green and blue?

Actually,
the green and blue…
are the same color!



Moral of the story:
Don’t Trust Your Eyes!

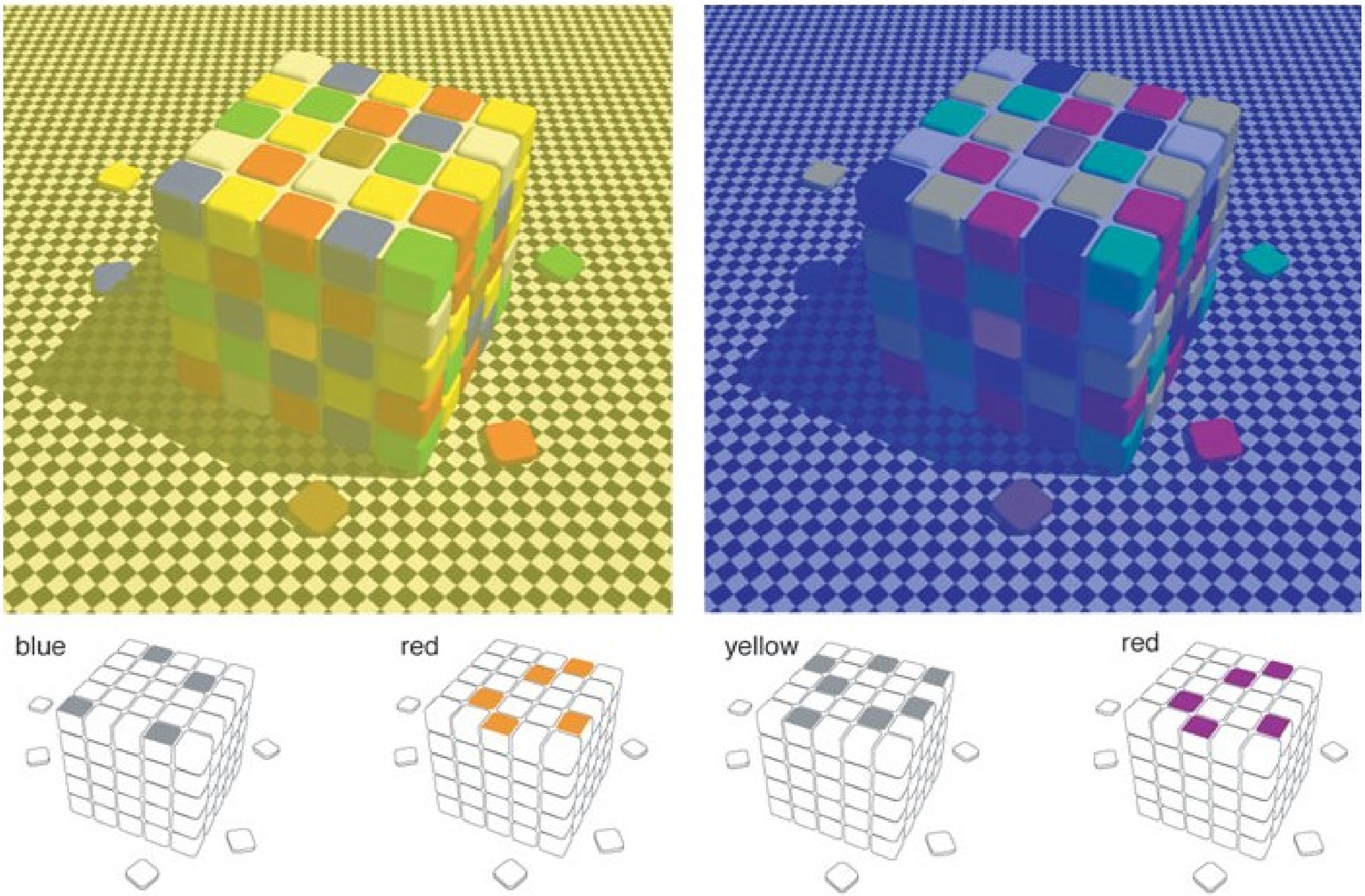


Figure 7. Color contrast and constancy arise from the same empirical generation of visual perceptions. In this computer-generated example, the authors carefully controlled the spectral information in the scene. The upper images show the cubes as if in yellowish *(top left)* or bluish *(top right)* illumination. The lower images show specific tiles of interest in the absence of these contexts. The yellow-looking tiles depicted as if under blue light and blue-looking tiles depicted as if under yellow light are actually a gray on their own—as shown in the lower boxes marked "blue" and "yellow." This is a striking example of color contrast. On the other hand, the red-looking tiles depicted as if under blue and yellow light both come from tiles that are actually purplish and orangish, respectively, as shown in the lower boxes marked "red." This demonstrates color constancy. These remarkable effects show that the same targets can be made to look like very different colors, and that different colors can be made to look the same by manipulating the context.

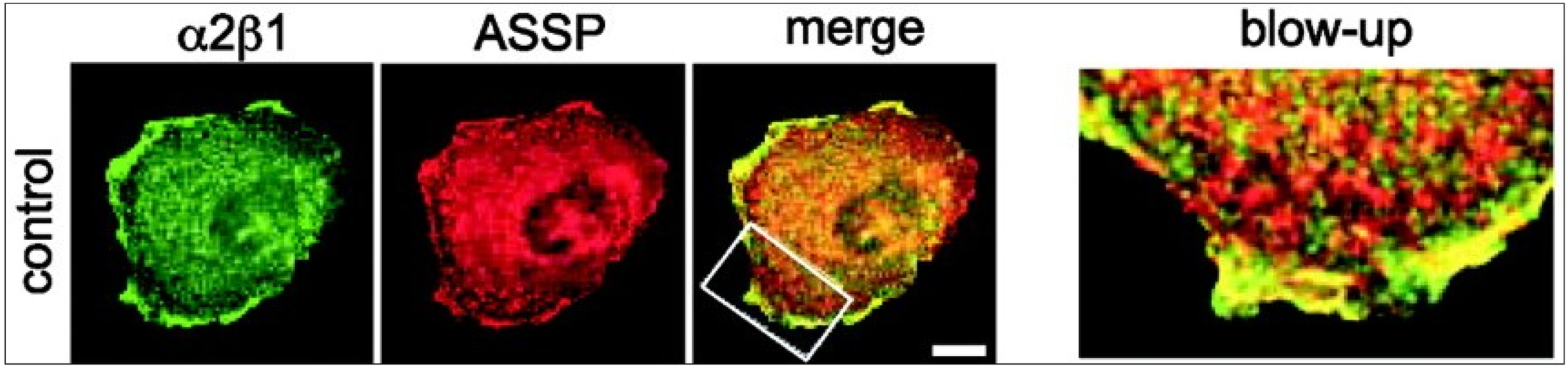
Images courtesy of Dale Purves, R. Beau Lotto and Surajit Nundy.

Colocalisation/Correlation

The past: “I see yellow - therefore there is colocalisation”

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

No colocalisation definition + No statistics = No Science



From Now On: 3D. Quantification. Correlation. Statistics.

Complementary methods: BioChemical, Optical (FRET, FLIM)

Colour Merge Images?

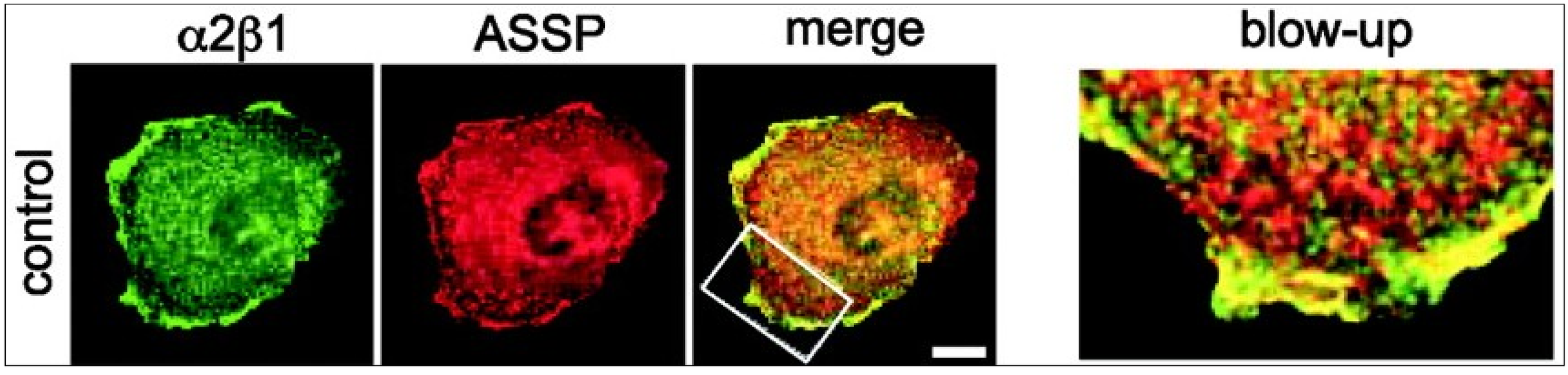
What are they good for?

Apart from looking pretty… not much.

Scientific conclusions from the image below? - No!

Colour blind people can't distinguish green and red!

So use Magenta and Green!



Publishing Images

or "how Photoshop can ruin your career"

CCD/PMT sees intensities differently than your eye/brain

LUT? Gamma correction?

Calibrate monitors

Journal Images ≠ Screen Images

Screen = RGB = Visualise

Inks = CMYK = Print

Image = data

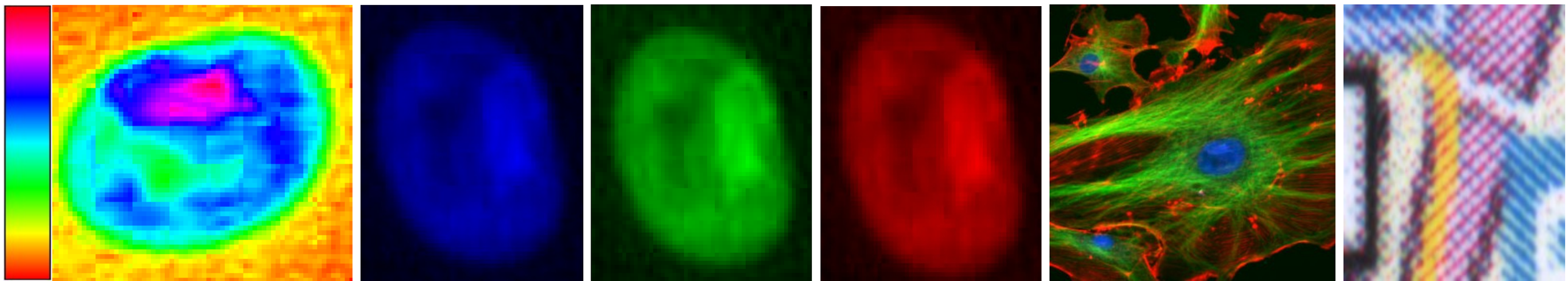
Don't corrupt information!

Compression

Lossless – Yes

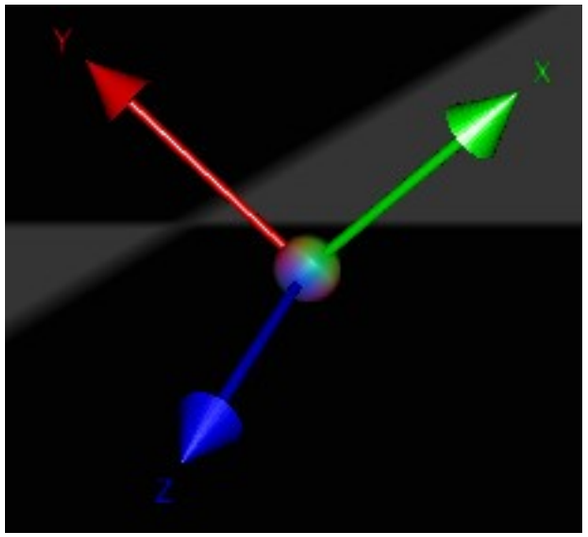
Lossy (JPEG) - NO

Always state the exact image processing done!

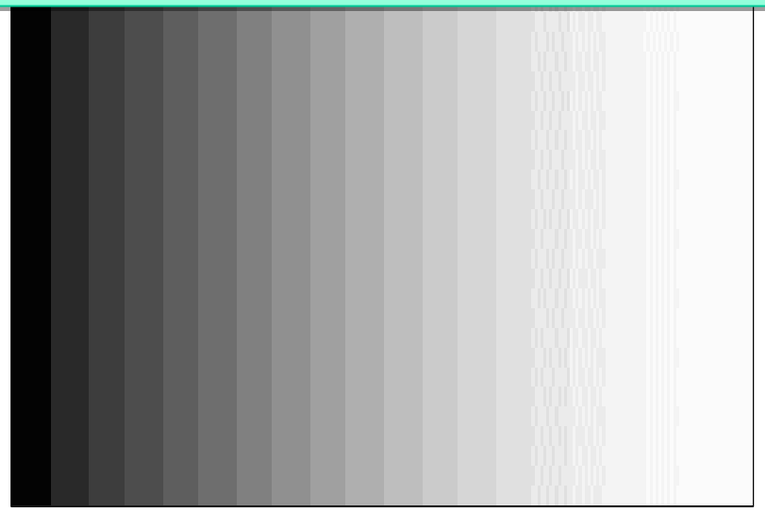


What can you digitise?

Dimensions!



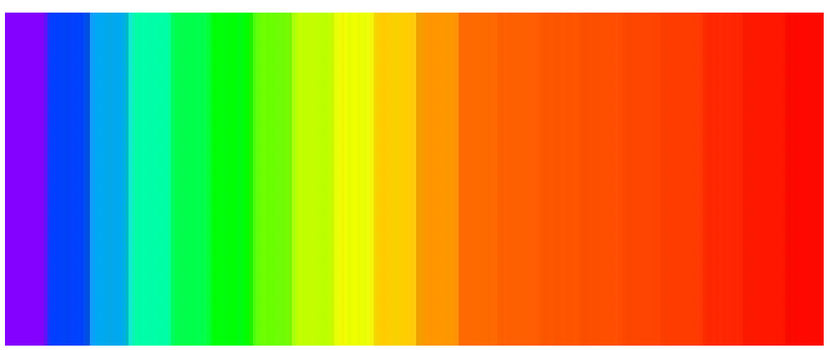
SPACE



INTENSITY

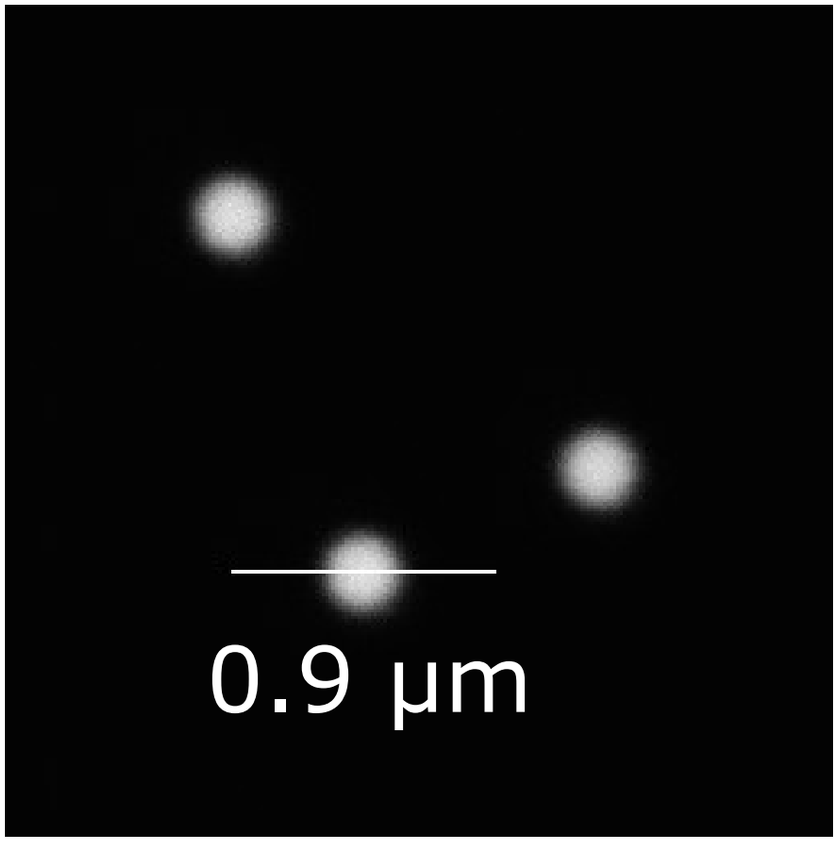
TIME

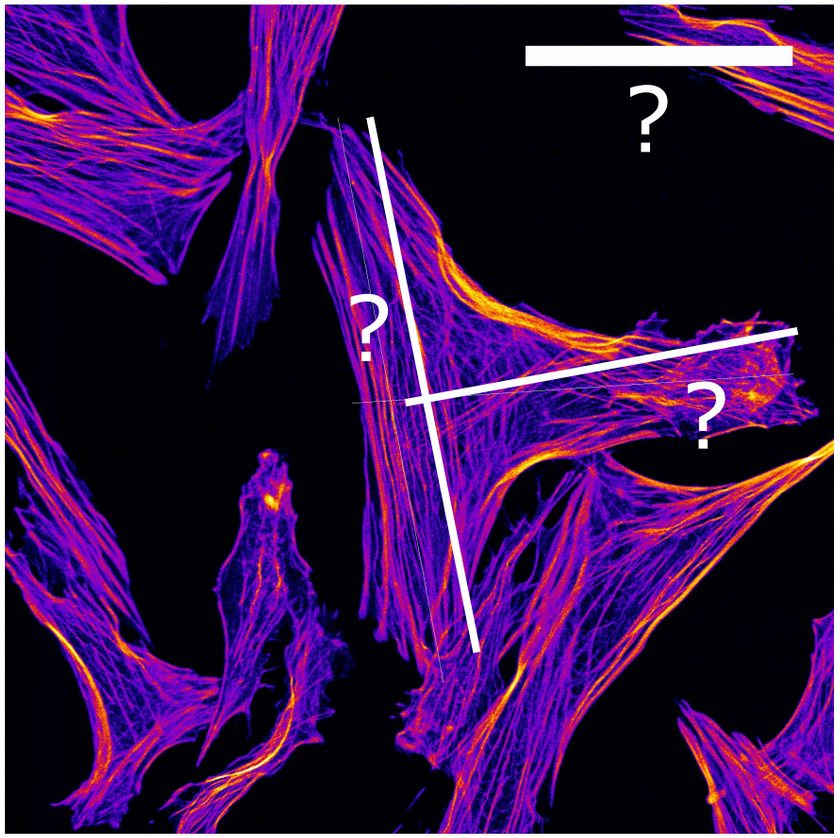
Wavelength

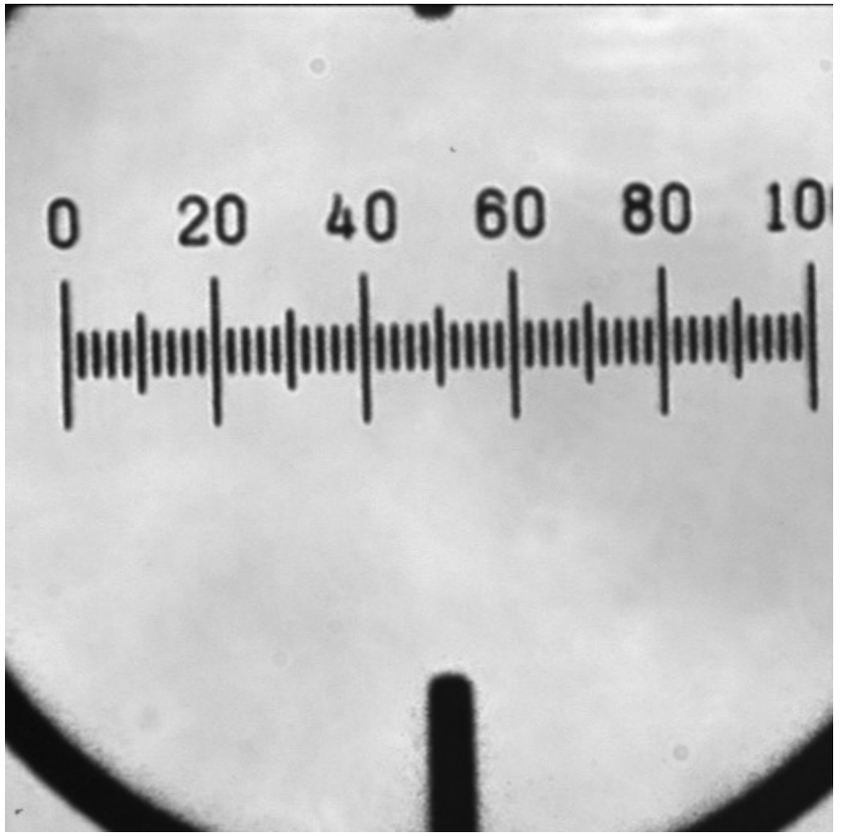


Colour

Pixel Size / Spatial Calibration





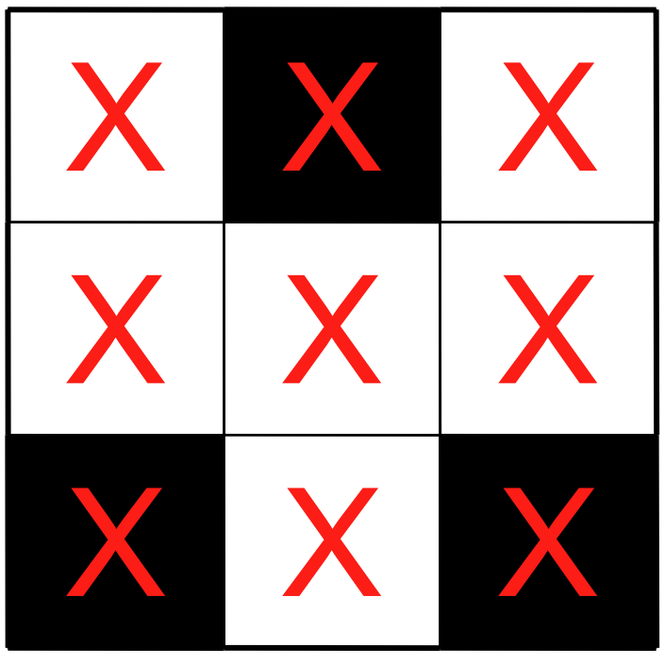


?

?

?

A pixel is NOT a little square!!!



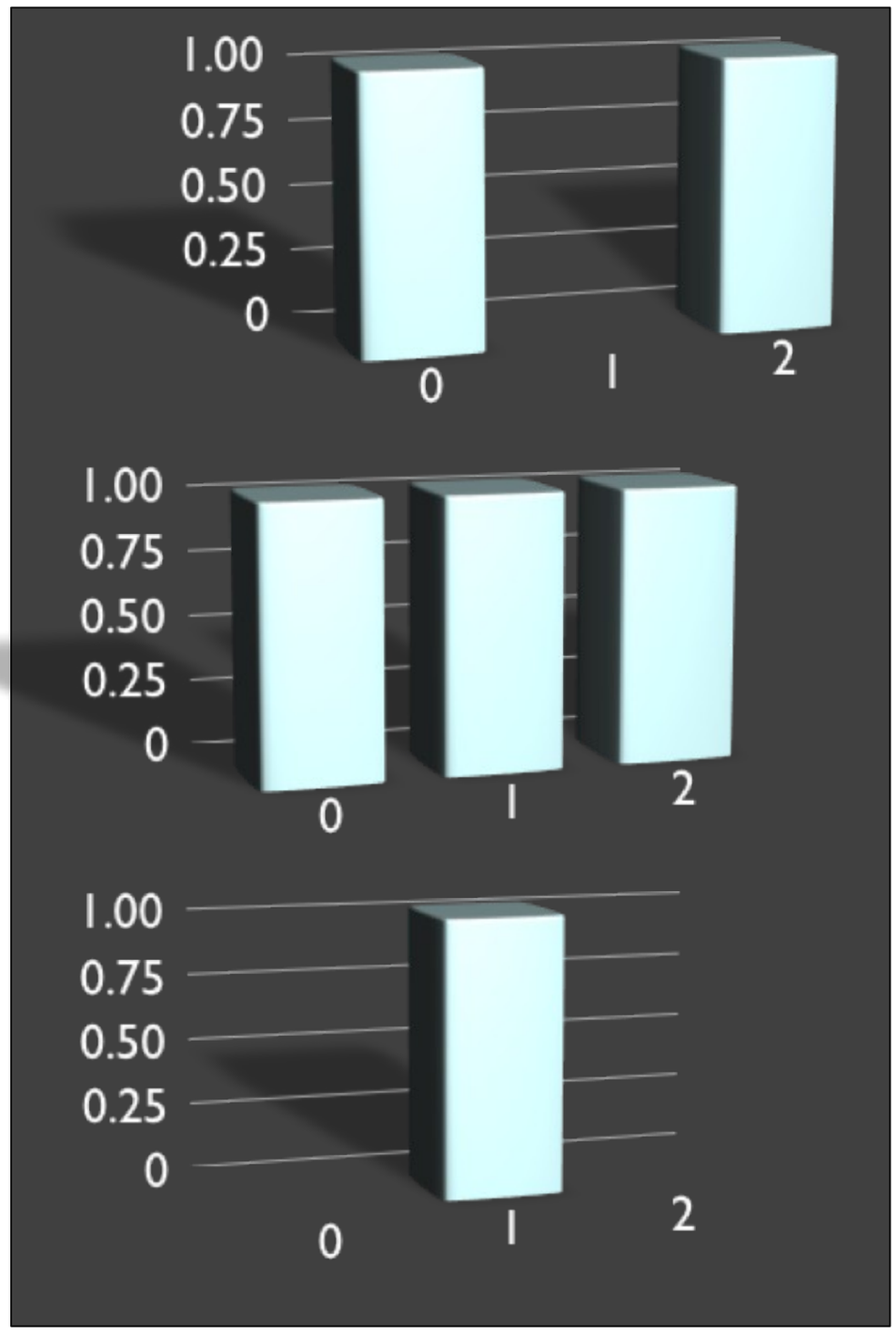
1	0	1
1	1	1
0	1	0

≡

0

1

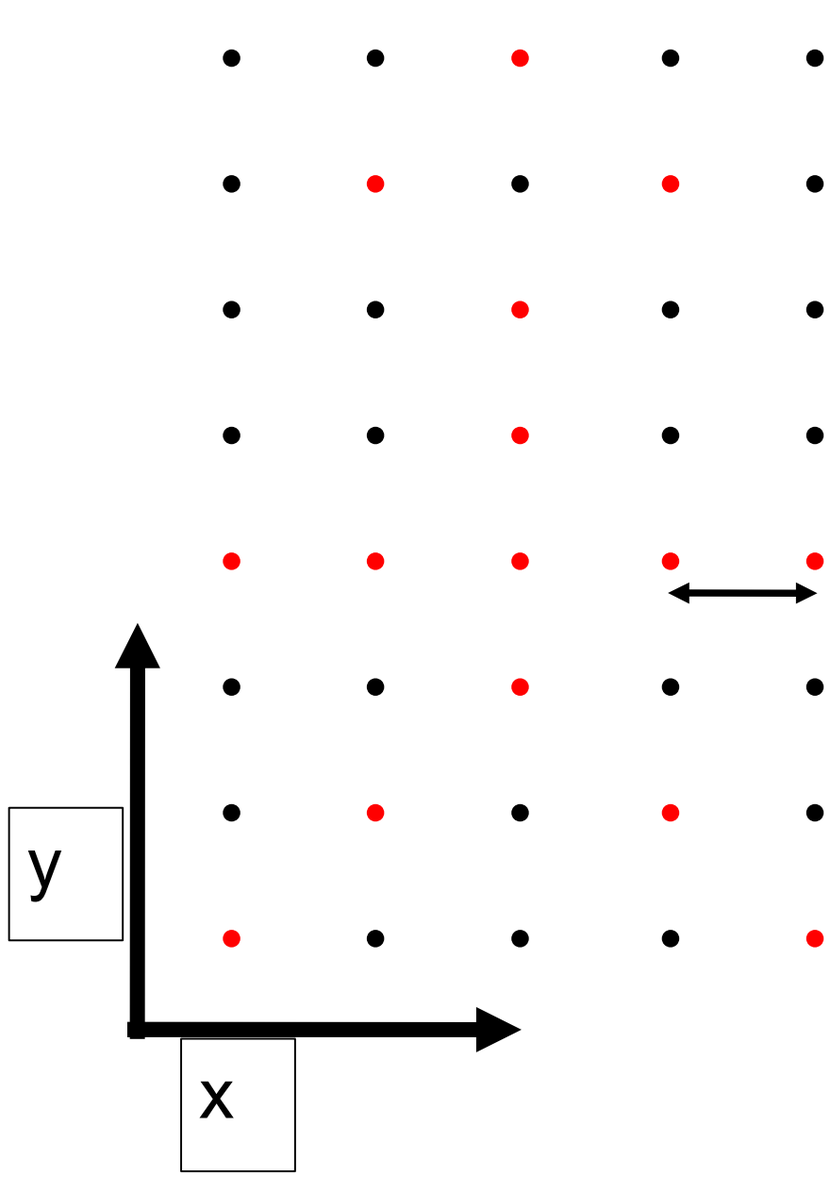
2



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

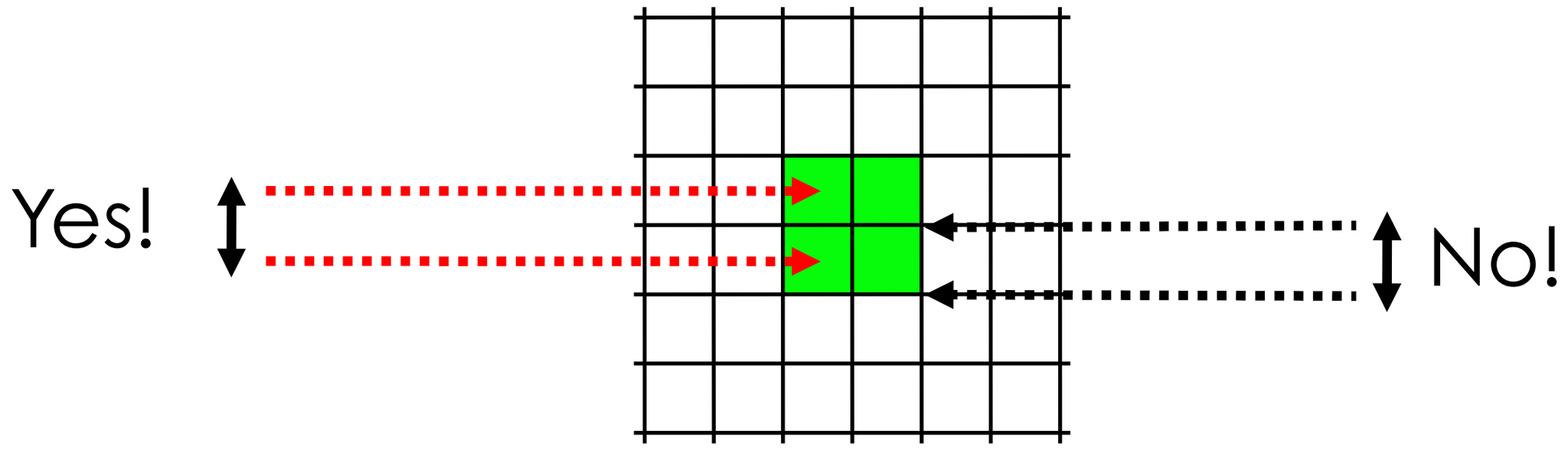
=

PixEl

Pixel Size

How big is a structure that is represented in my image?
=
How big is one pixel?

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!

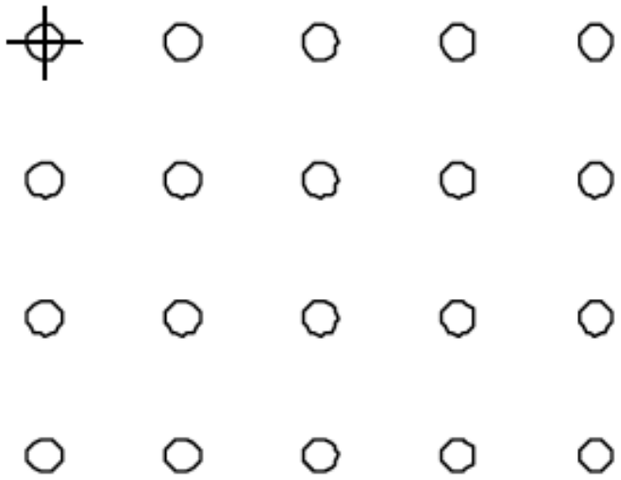
A pixel is *NOT* a little square,
A pixel is *NOT* a little square,
A pixel is *NOT* a little square!
(And a voxel is *NOT* a little cube)

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

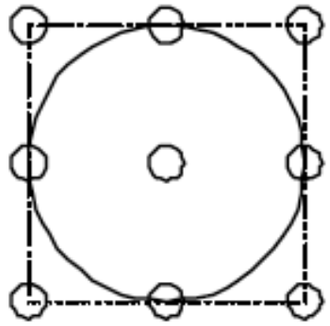
Alvy Ray Smith, July 17, 1995

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

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

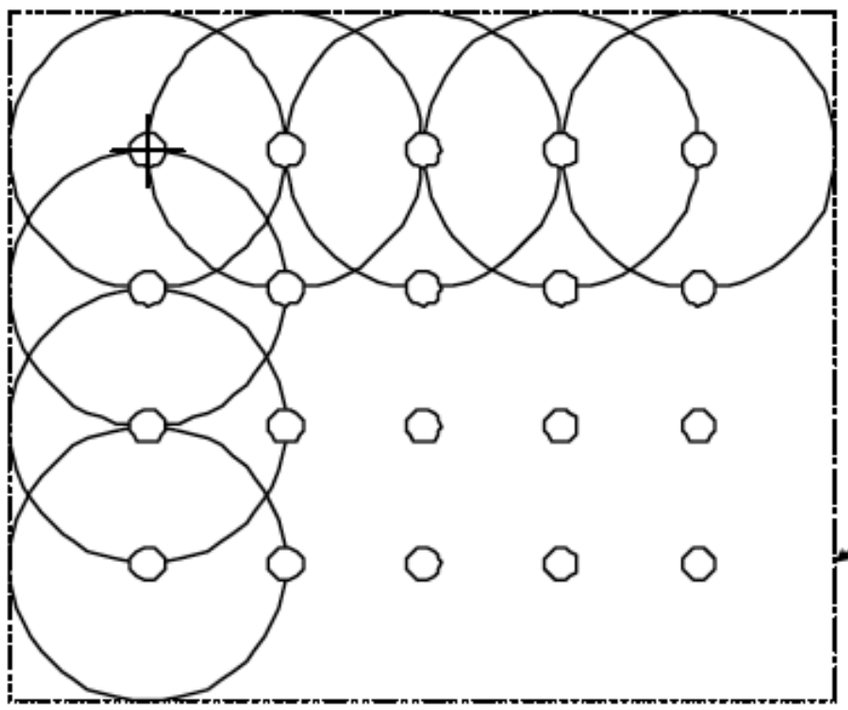


(a) A 5x4 image.



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

Or in our case the PSF (Point spread function = image of a point) of the microscope system!



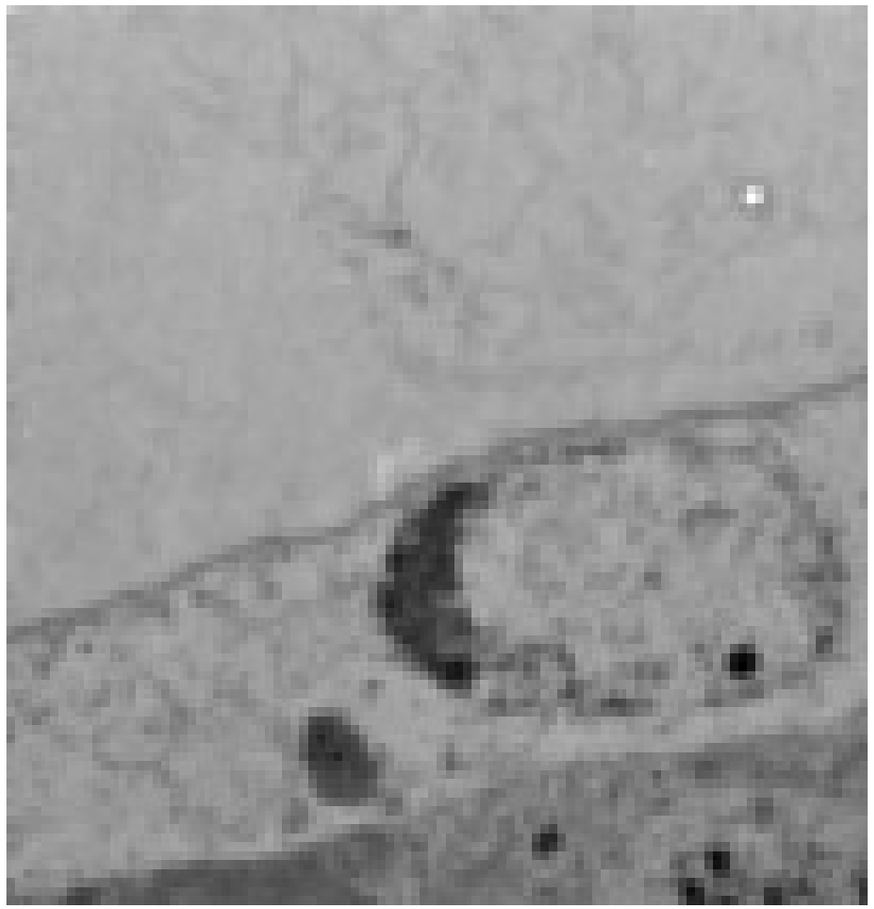
Dotted line is minimally enclosing rectangle

(c) Footprint of image under reconstruction.

A pixel is not a little square … So what?

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

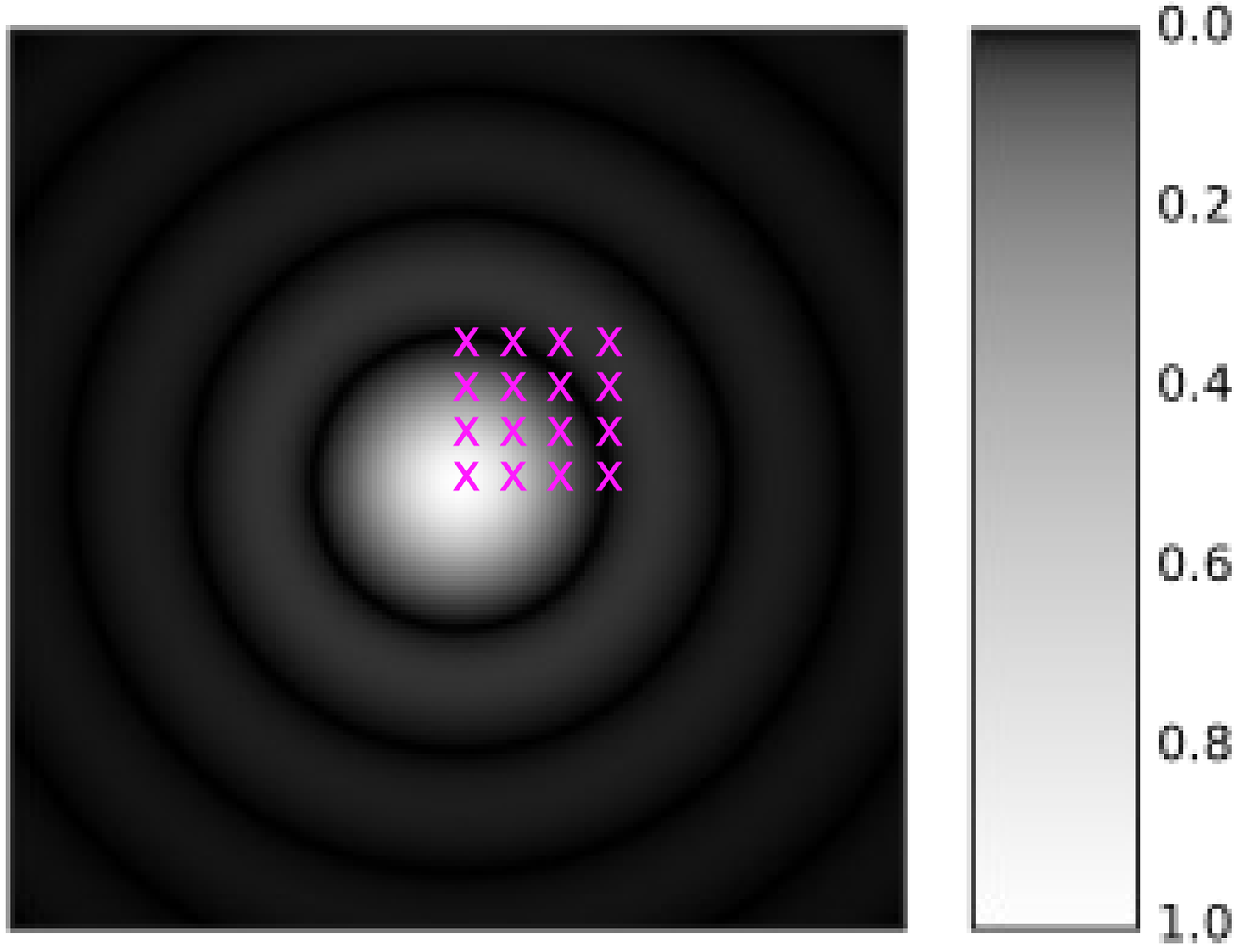
http://pacific.mpi-cbg.de/wiki/index.php/Downsample
Compare plugins-examples-downsample with Image-scale

What does a point sample from a microscope detector contain?

Image of a point light source = Point Spread Function (PSF)

In the diffraction limited, high resolution case:

The PSF is **bigger** than the pixel / sample Nyquist spacing.

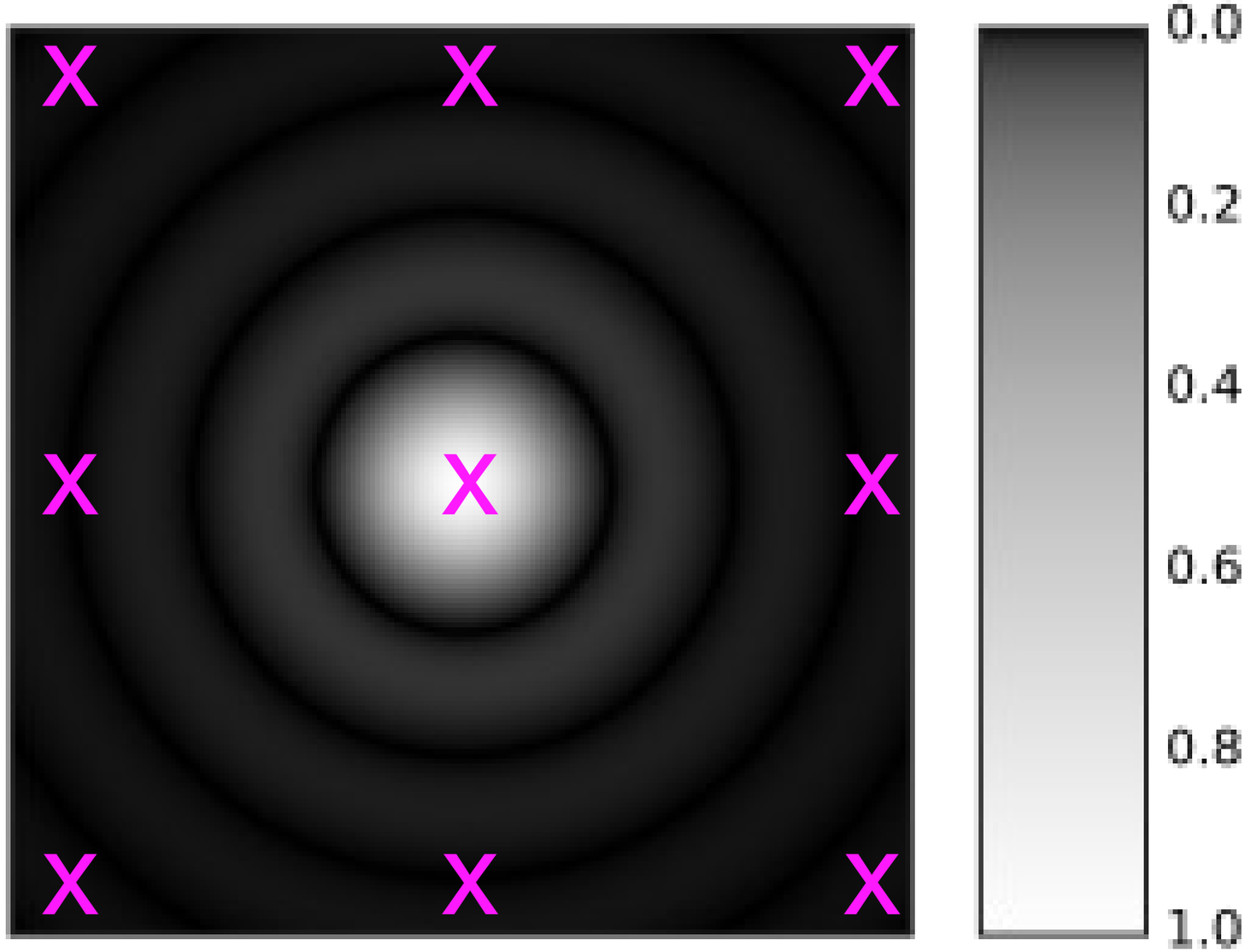


So what does a point sample from a confocal microscope detector contain?

In the low resolution, big pixel case:

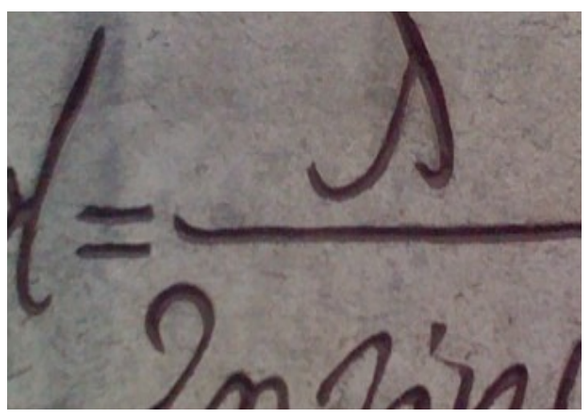
The PSF is **much smaller** than the pixel or sample Nyquist spacing.

We miss spatial information = lower resolution



Abbe's diffraction limit / Rayleigh criterion

Limit the resolution of light microscopy

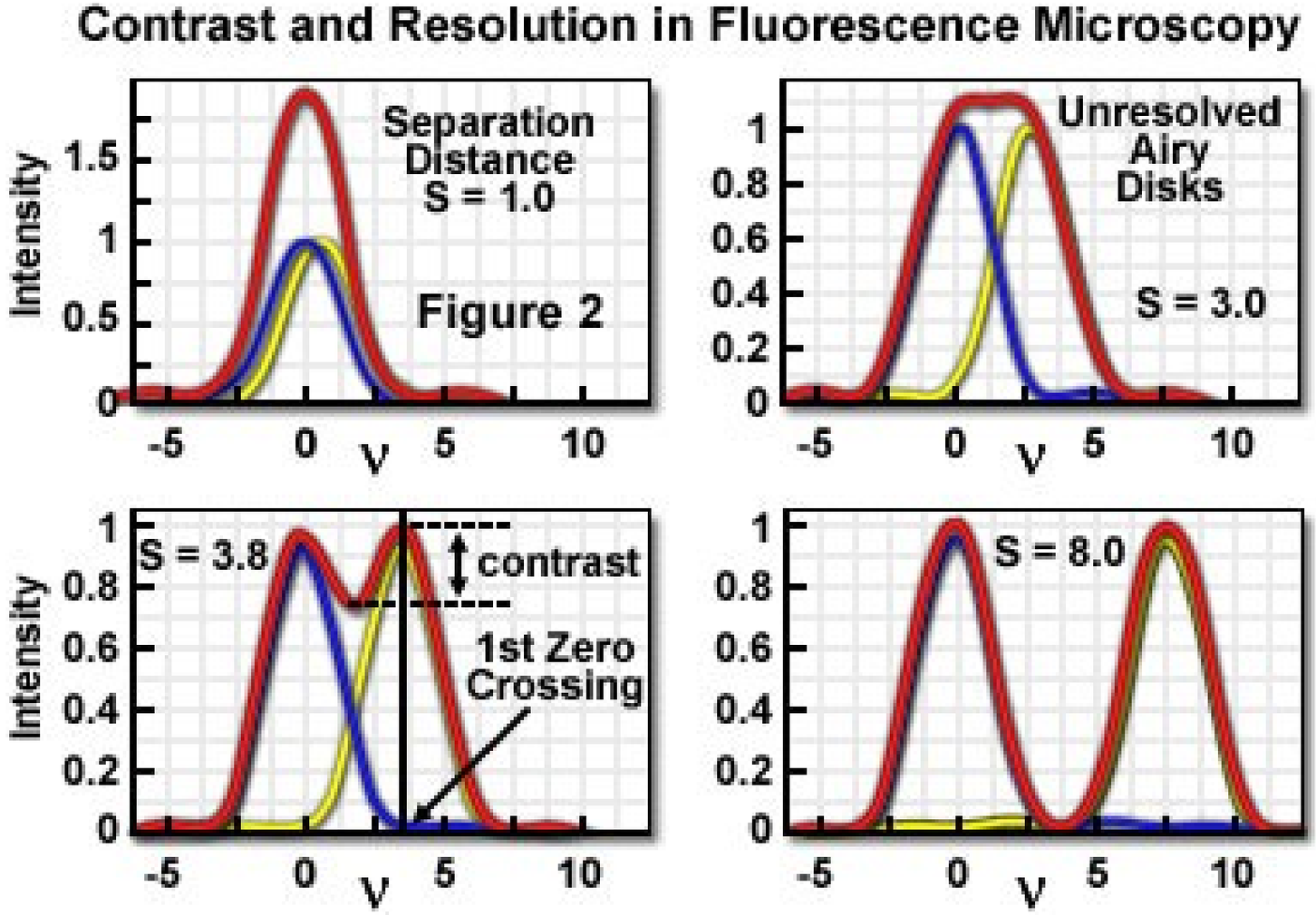


Airy Patterns and the Rayleigh Criterion online tutorial: http://www.microscopy.fsu.edu/primer/java/imageformation/rayleighdisks/index.html

2 point light sources:

$$d = \frac{0.61 \times \lambda}{\text{lens N.A.}}$$

$$d = \frac{0.61 \times 550\text{nm}}{1.4} = 240\ \text{nm}$$

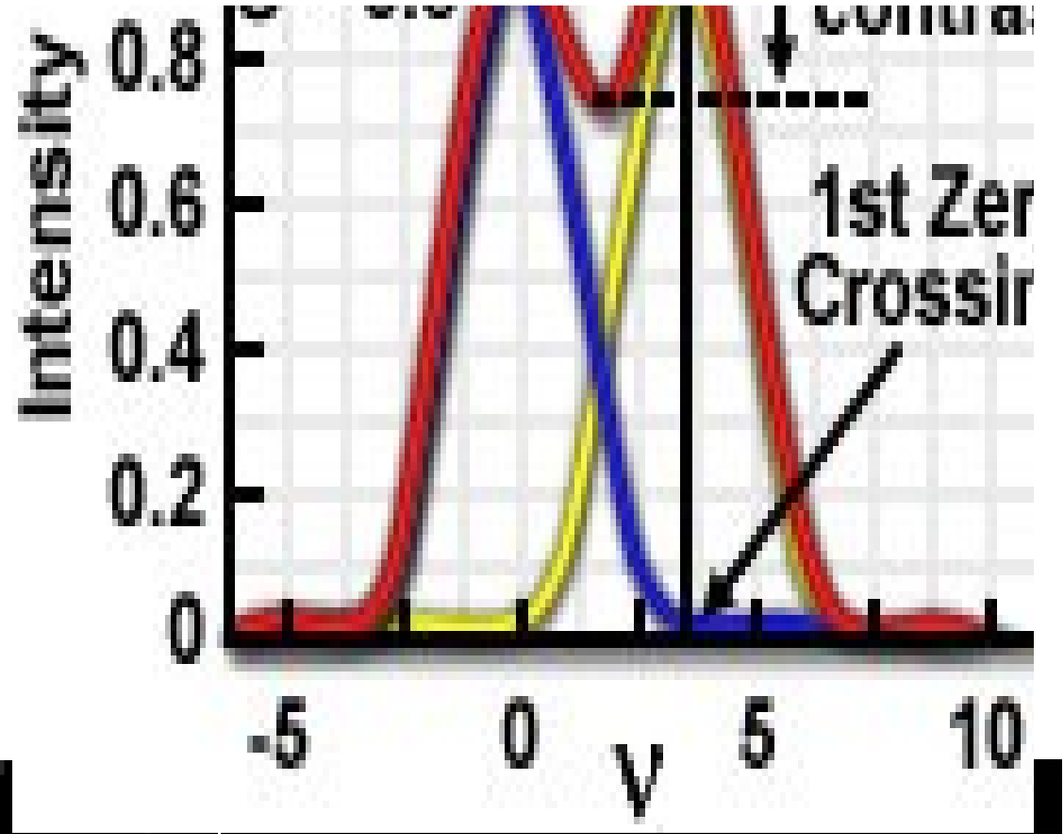


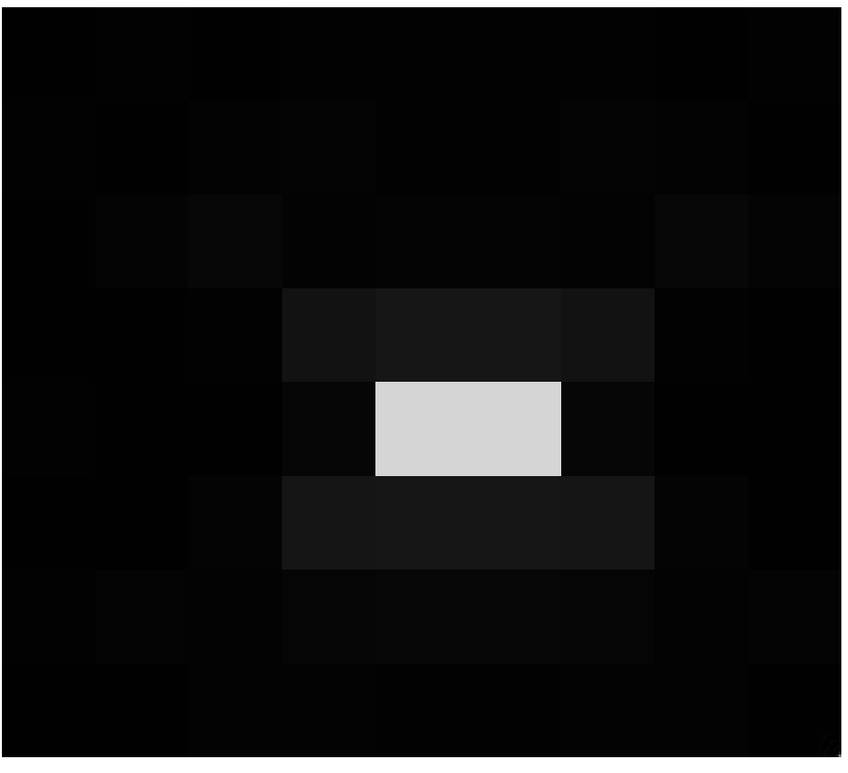
Digital spatial resolution

Projected pixel "size" at the sample/object

The point sample spacing

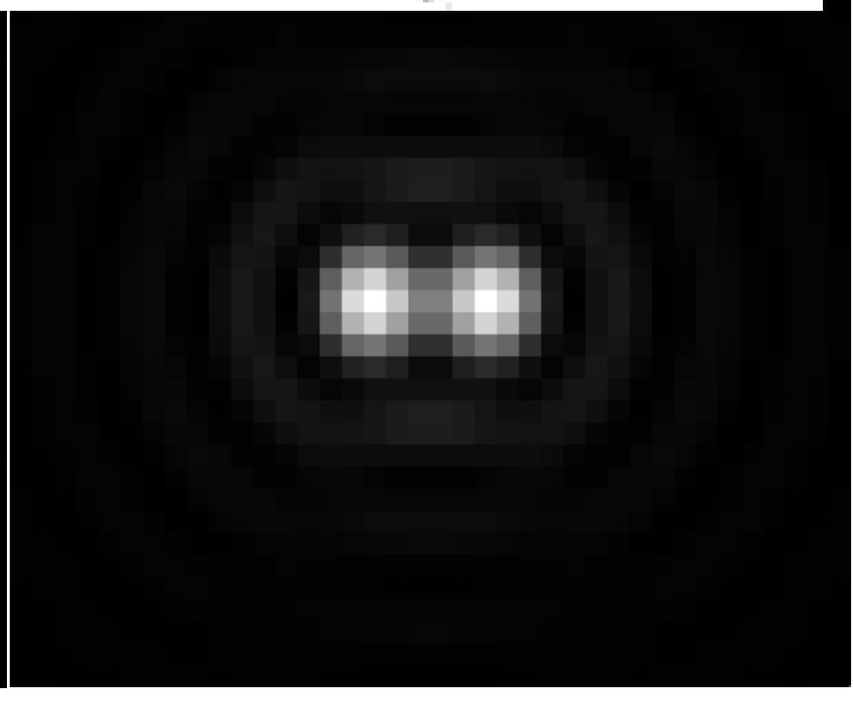
But what "should" it be?





under sampled

over sampled



good sampling

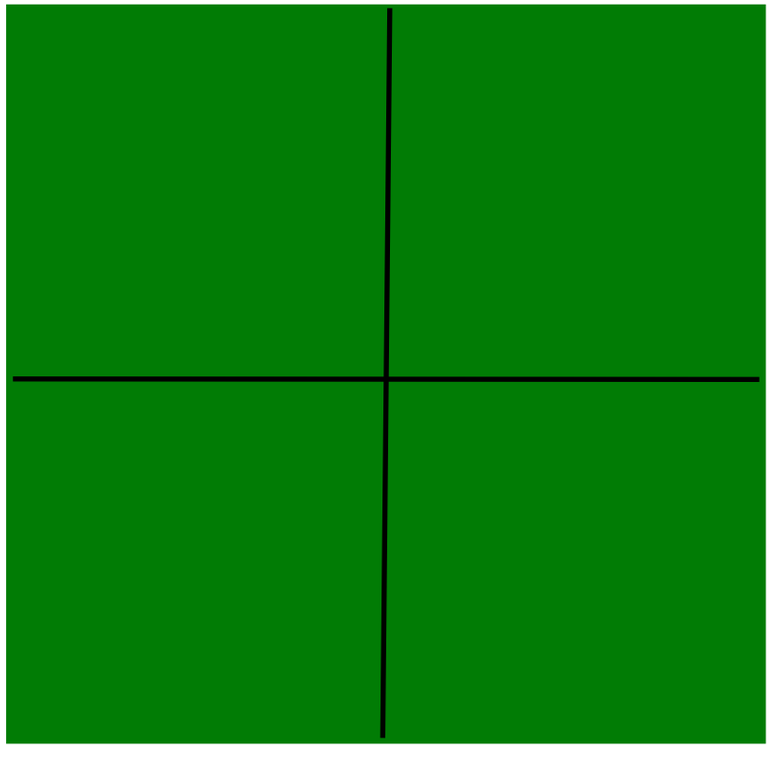
Pixel Size / Image Resolution

"Correct" image size? 64x64, 512x512, 2048x2048, …

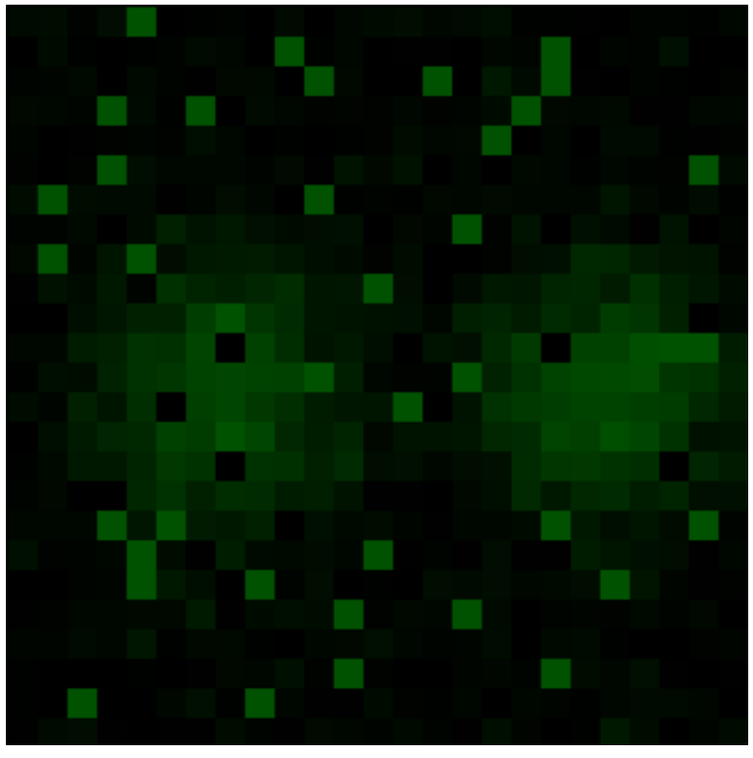
Nyquist – Shannon sampling theory: Proper spatial sampling

2.3 – 3 times smaller than optical resolution (x, y, AND z)

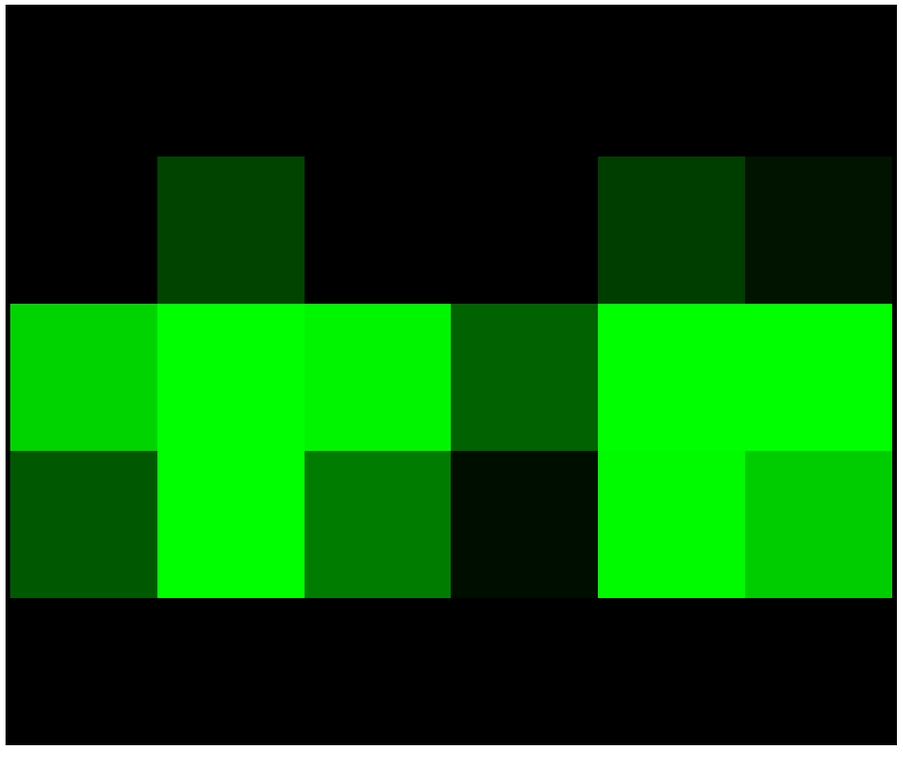
Adjust zoom, binning, and image size (no of pixels)



under sampled



over sampled



correct sampling

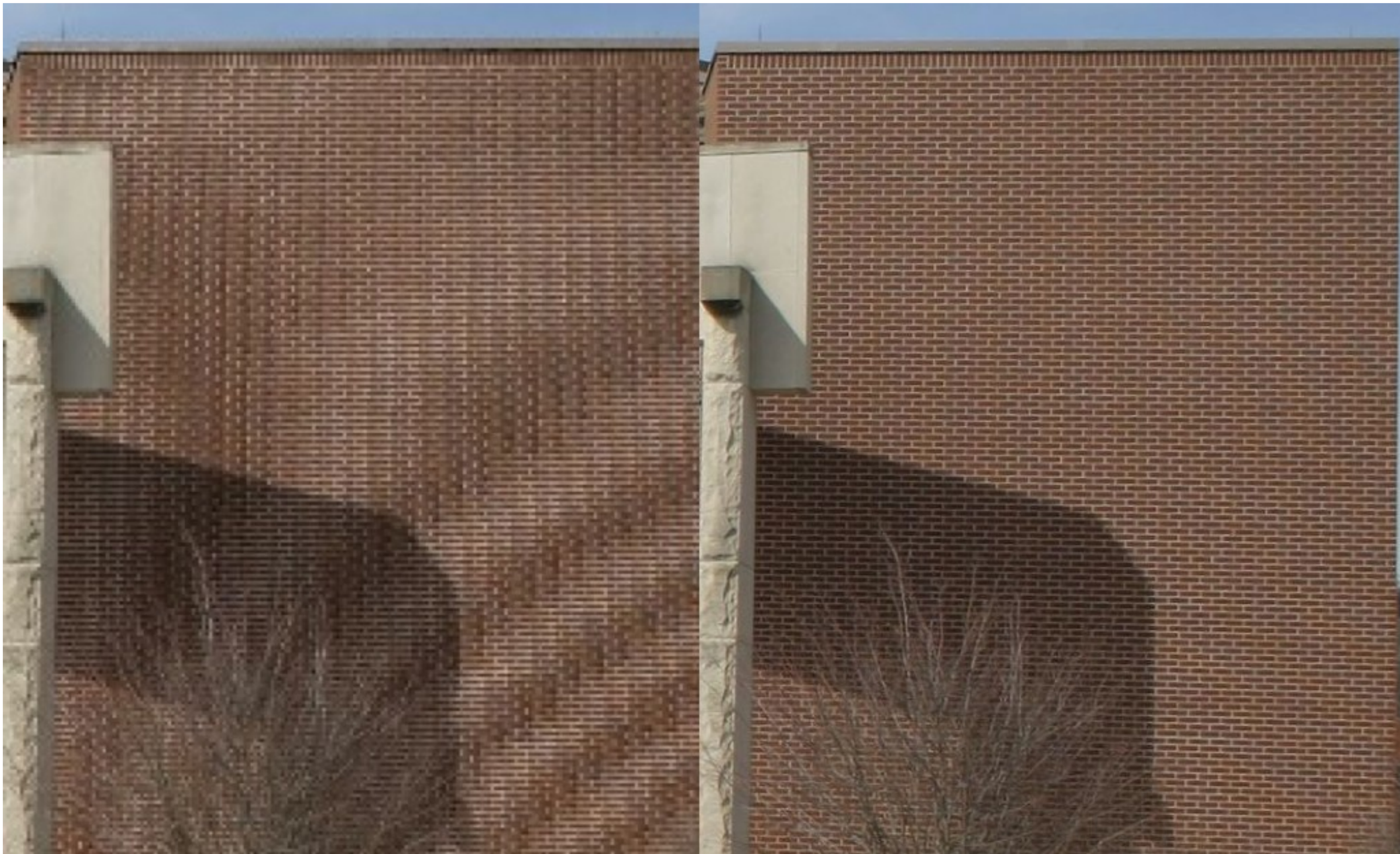
1 Airy unit

Harry Nyquist, 1889 - 1976

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

Nyquist sampling criterion

Aliasing: Moiré patterns / loss of information



Nyquist sampling criterion

Aliasing: Moiré patterns / loss of information

Nyquist sampling criterion

Aliasing: Moiré patterns / loss of information

Nyquist sampling criterion

General form

Digital sampling frequency > analogue frequency x 2

Spatial representation

Image pixel size x 2.3 = smallest resolvable distance

Microscopy

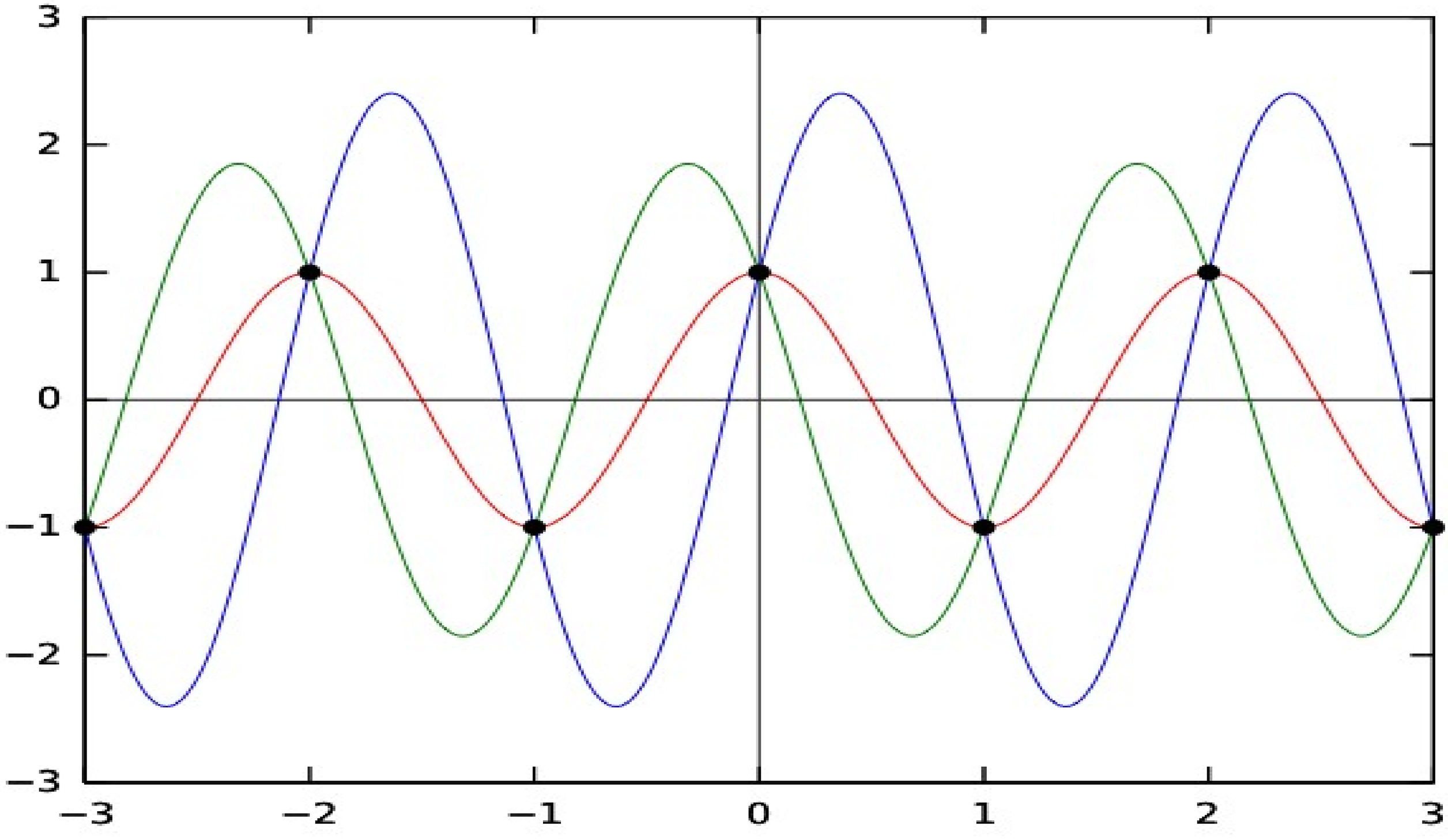
Image pixel size x 2.3 = optical resolution (d)

Aliasing

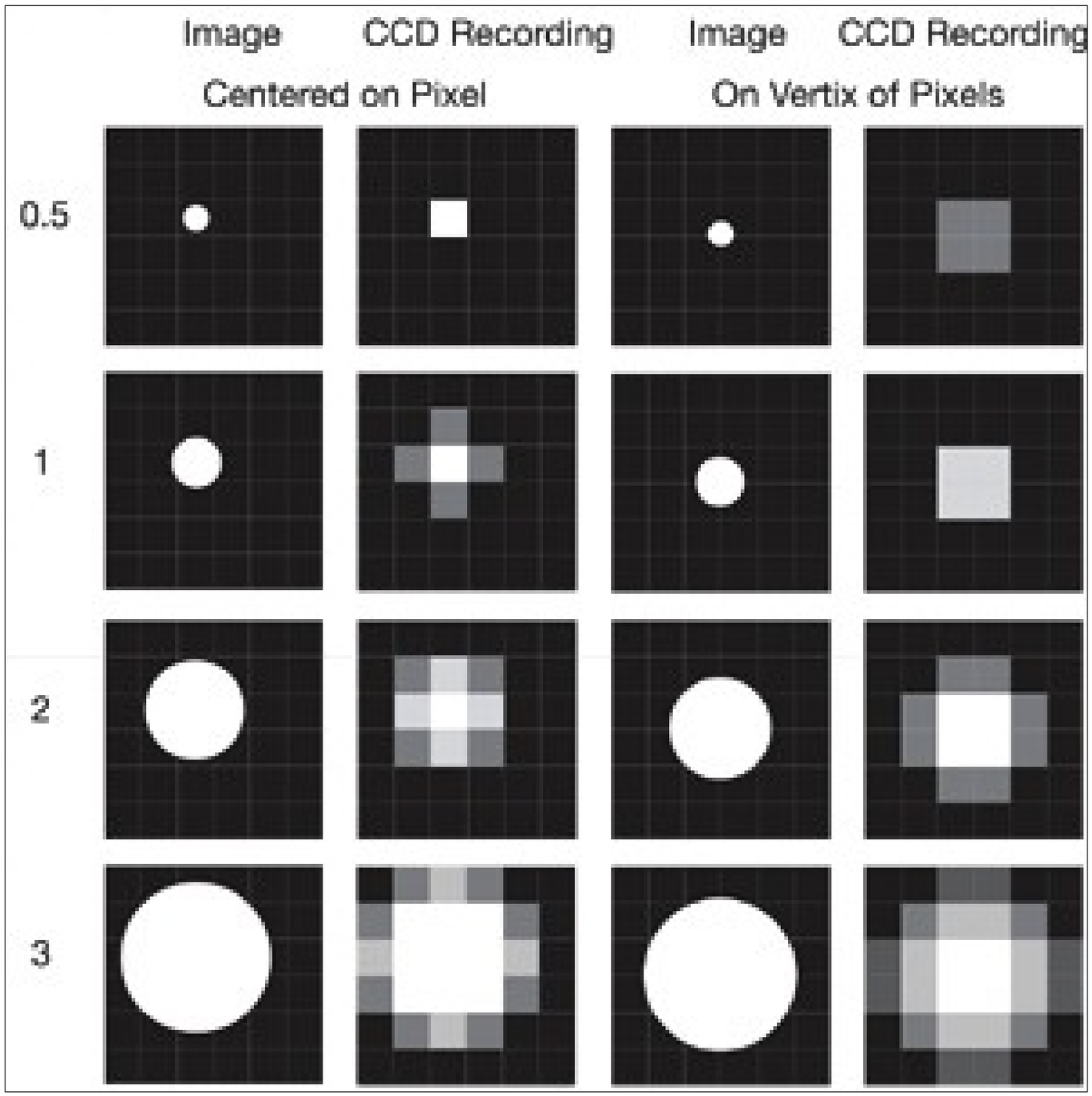
Moiré interference patterns = loss of information

Nyquist sampling criterion

Different "objects" in different places…
but the digital images are identical!



More aliasing problems...



Pixel size relative to projected image

Image of object varies, depending on where it falls on detector

-

Especially for small objects close to pixel size

Nyquist sampling criterion

Resolution - pixel size calculations:

Resolution, d = lambda / 2 NA

Required Pixel Spacing = d / 3

Nyquist sampling criterion

Optomistic pixel size calculations:

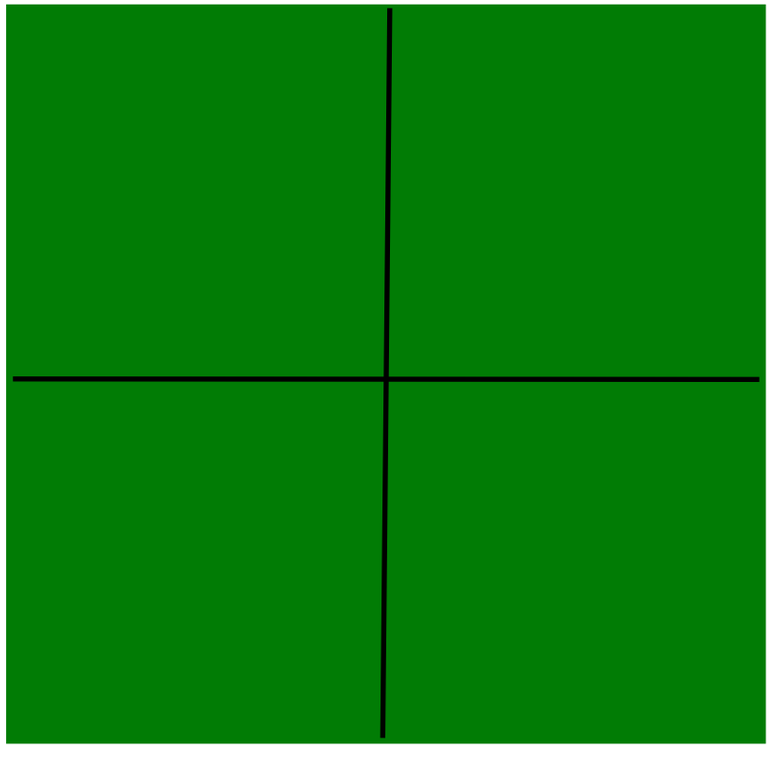
550 nm light ; d=lambda/2NA ; pix=d/3:

Objective (N.A.)	Optical Resolution limit (nm)	Projected size on CCD (um)	Required CCD pixel spacing (um)
4x (0.2)	1400	5.5	2
10x (0.4)	690	7	2
40x (0.75)	366	14.5	5
40x (1.3)	210	8.5	3
63x (1.4)	200	12.5	4
100x (1.4)	200	20	6.5

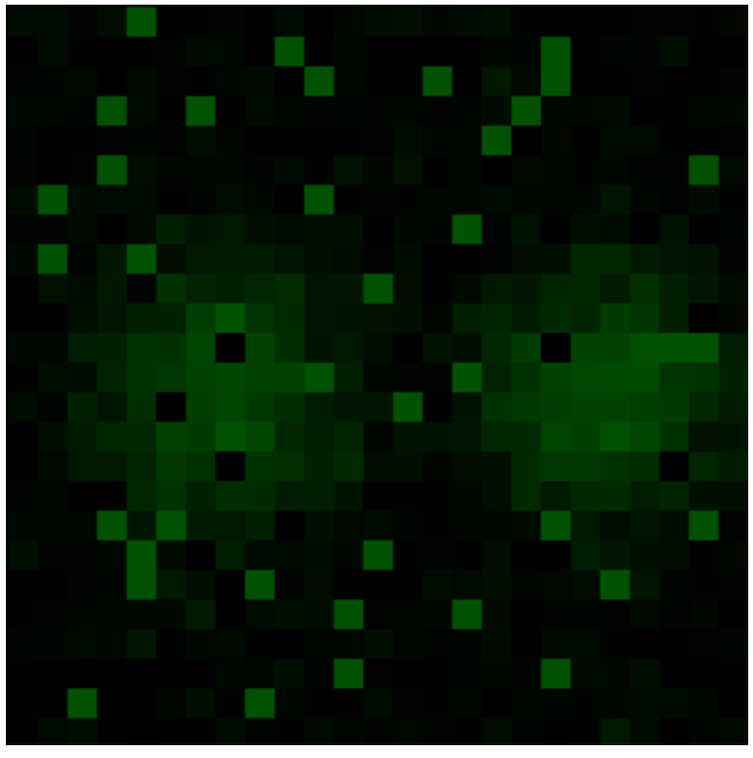
Think about your digital spatial resolution carefully!

Pixel Size / Resolution

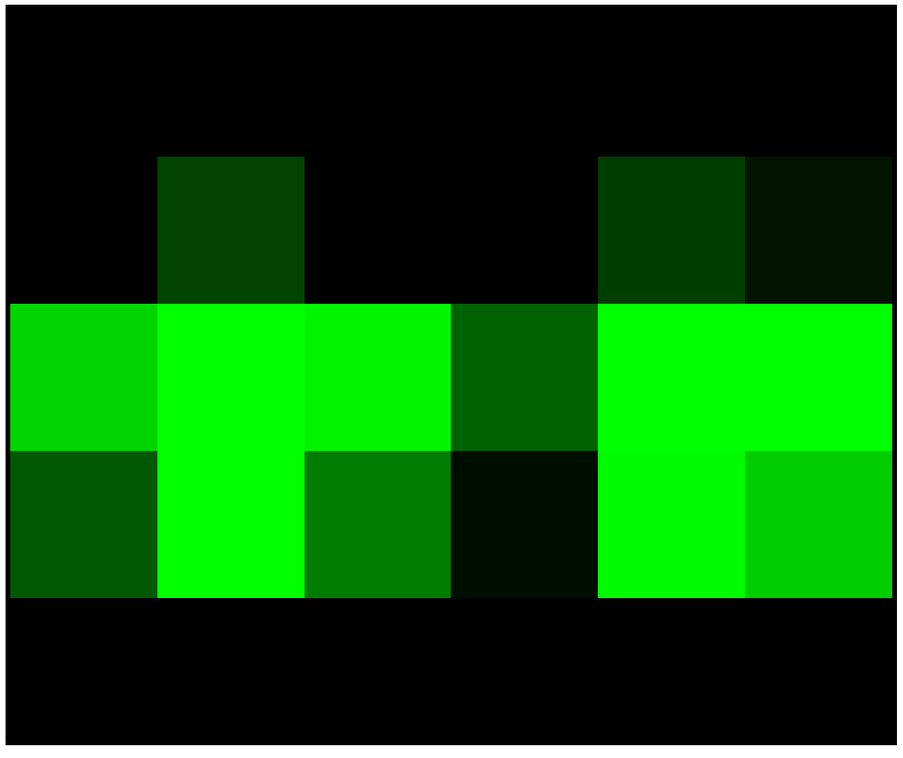
Remember !!!
Nyquist told us how to do digital sampling:
~1/3 x smallest feature.



under sampled



over sampled



correct sampling

1 Airy unit

Pixel size / Spatial Calibration

Pixel size is determined by the microscope system!

- ✓ CCD photodiode “pixel” size - Magnification 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 1a

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

File - Open Samples - Embryos or Bridge

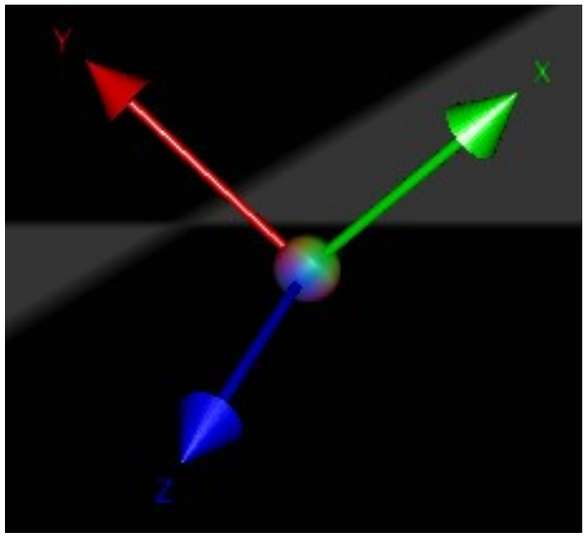
Spatial Scaling:

Can you measure the length and area of objects?
→ See Fiji Tutorial - SpatialCalibration (search Wiki)

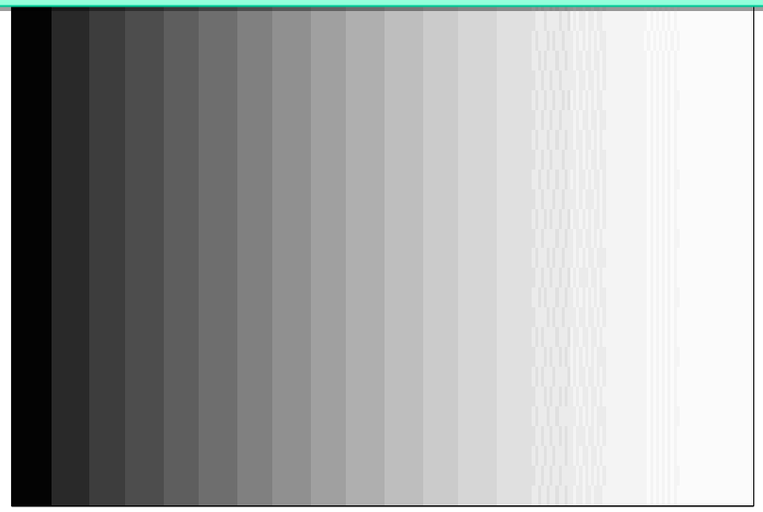
- ✓ ***Analyze - Set Scale, Analyze-Tools-Scale Bar***
- ✓ ***Line and ROI selection - ctrl M (cmd M)***
- ✓ ***Rectangle, Oval, Polygon, Freehand, Angle, Point, Wand.***
- ✓ ***Analyze - Set Measurements (Results – Edit - summarize)***

What can you digitise?

Dimensions!



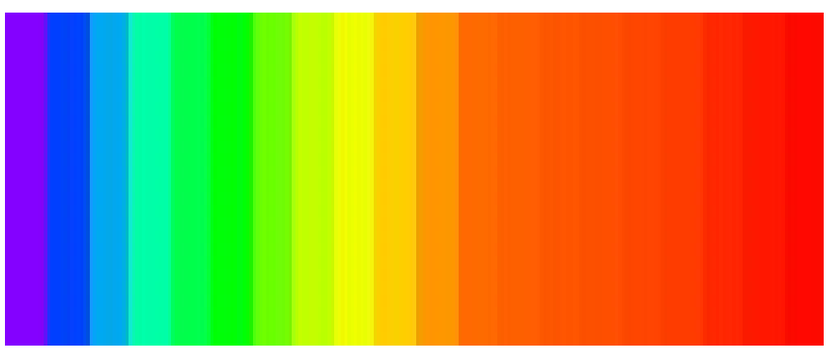
SPACE



INTENSITY

TIME

Wavelength



Colour

“Intensity” Digitisation

Remember: Bit Depth

Measured intensity by detector

digitization

Corresponding level in image

“Bucket” holds 0-9 electrons

5 electrons counted

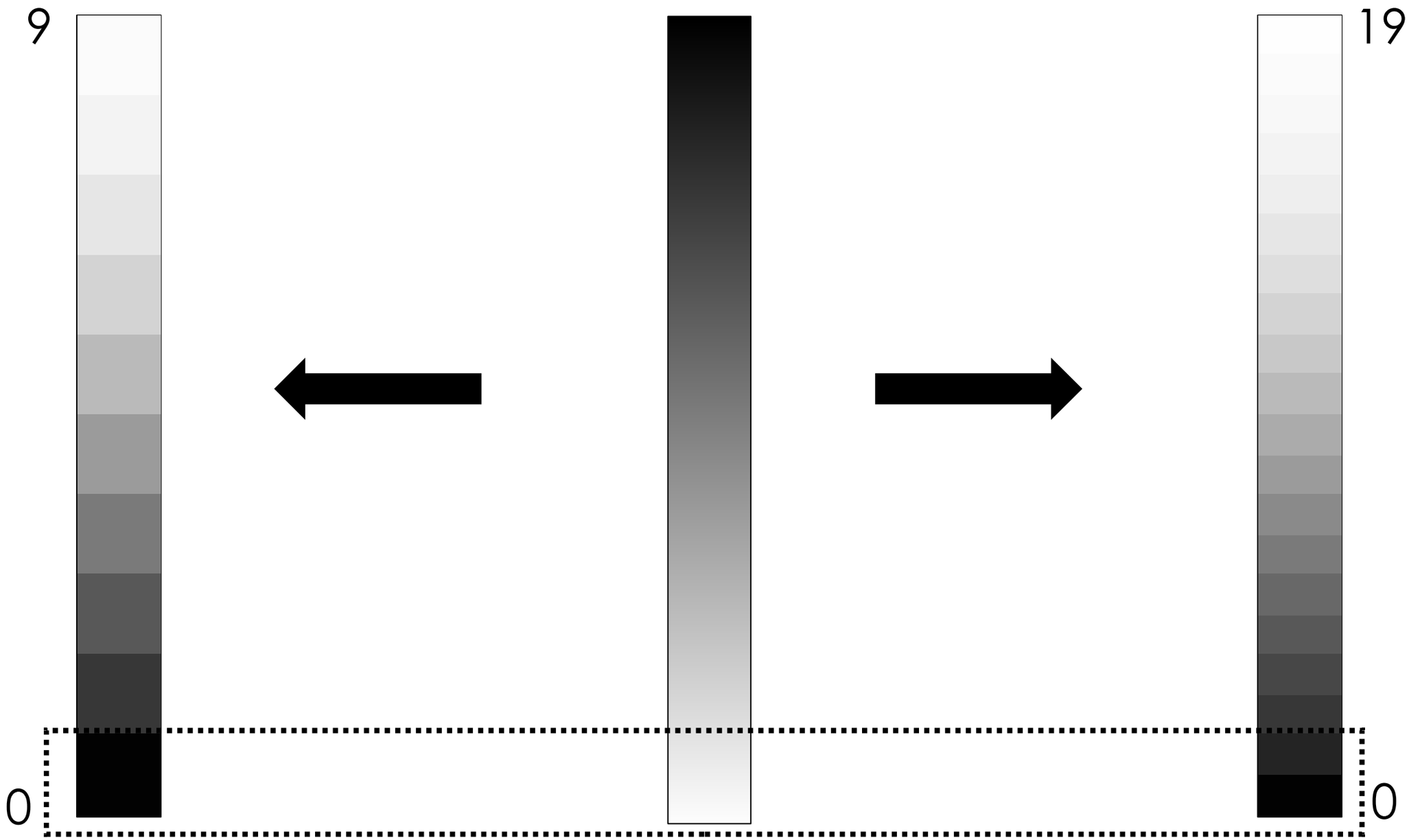
Bit depth: 10 (0 to 9)levels

Level 5 selected for RAW data “image”

“Intensity” Digitisation

Bit Depth

“digital“ intensity resolution: 10	“real” analogue intensities	“digital“ intensity 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

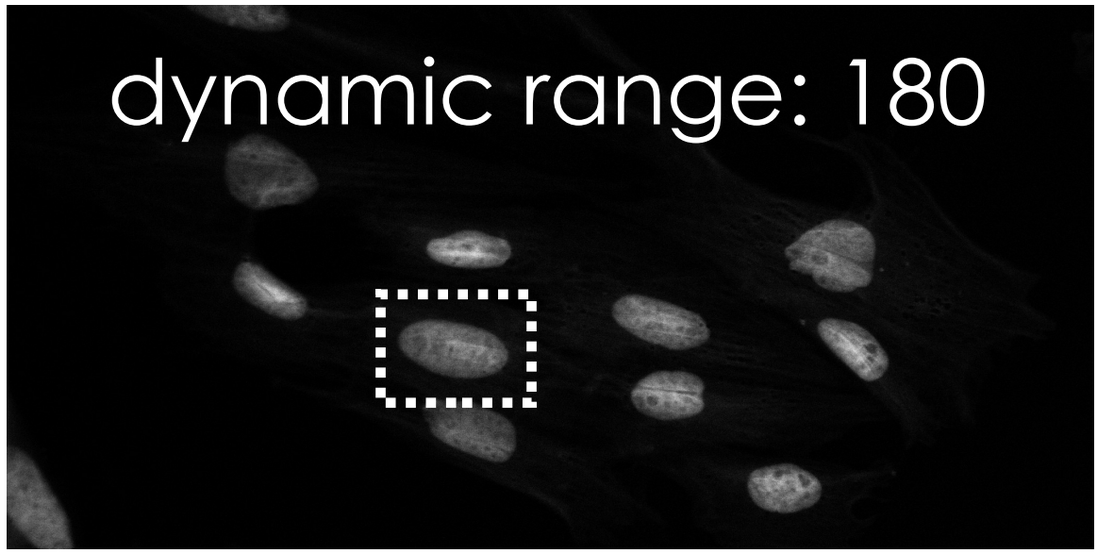
~ limit of human eye, displays...

Intensity-related measurements

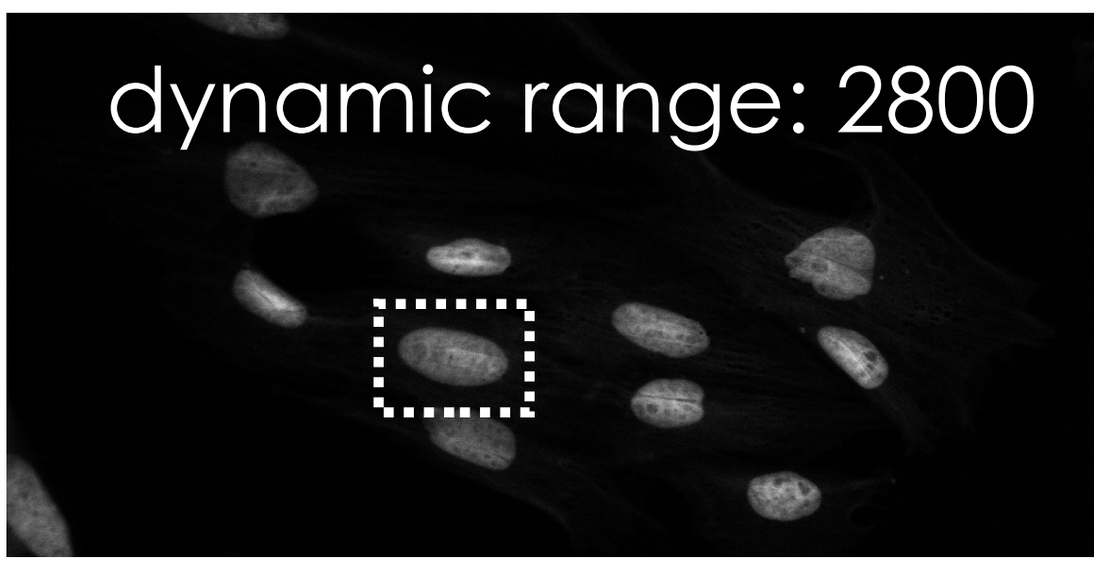
“Intensity” Digitisation

Bit Depth
for intensity-related measurements

8 bit



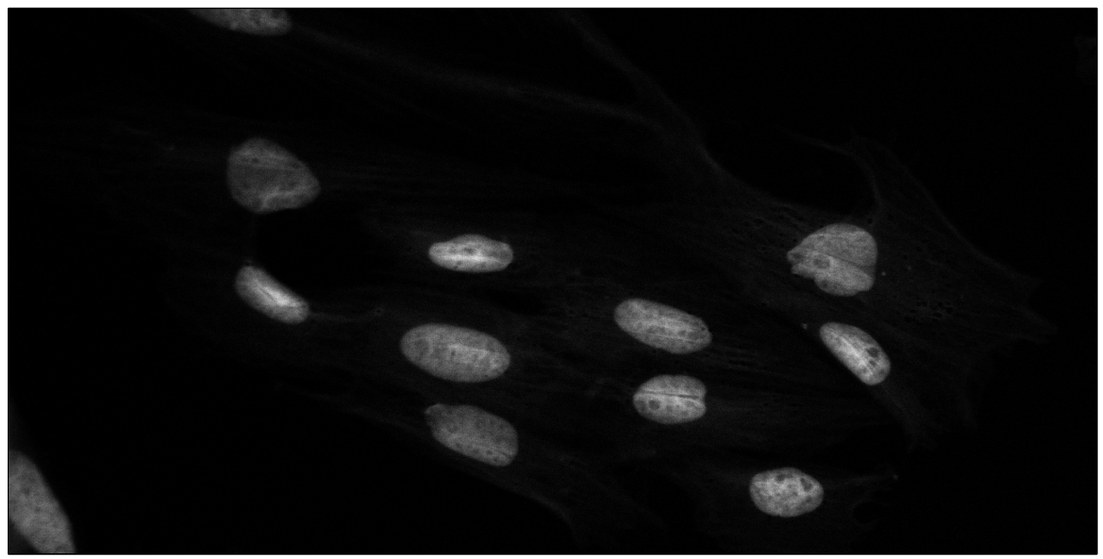
12 bit



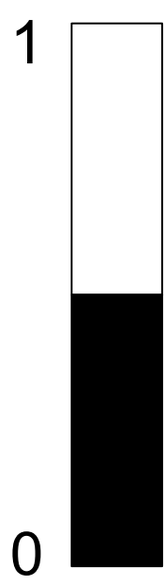
“Intensity” Digitisation

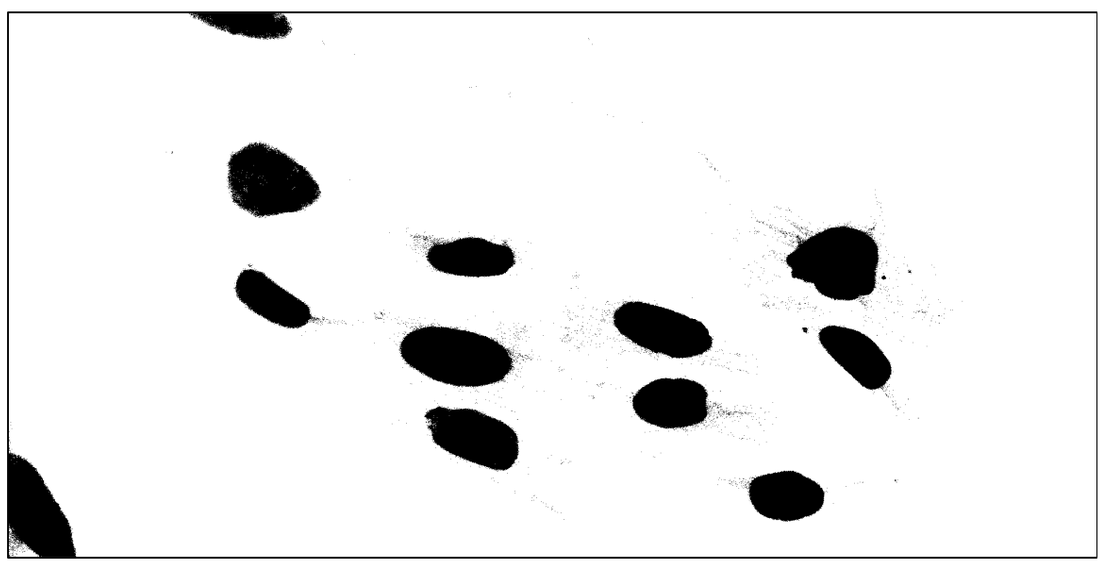
Bit Depth
for segmentation

8 bit
greyscale



1 bit
Binary image



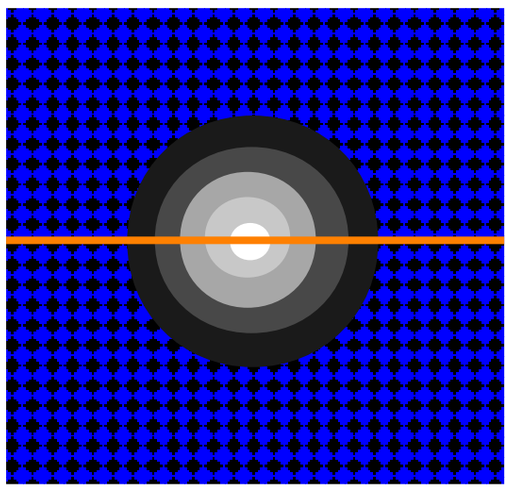


Remember:
Intensity / Exposure / Saturation

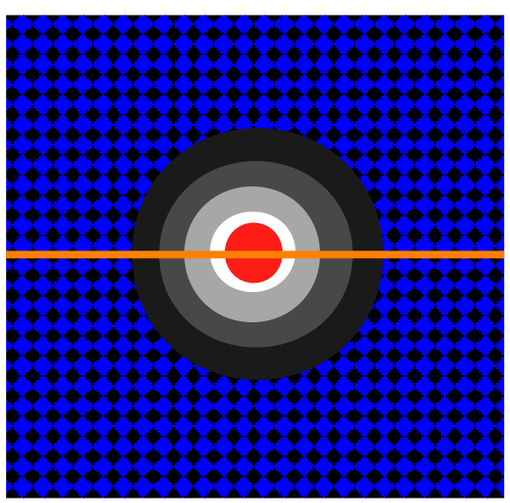
Do NOT over expose / saturate your image!!!

Why not? → Lost Information!

Use “Look Up Tables (LUT) / palettes to check the saturation



in range



clipped
overexposed
saturated

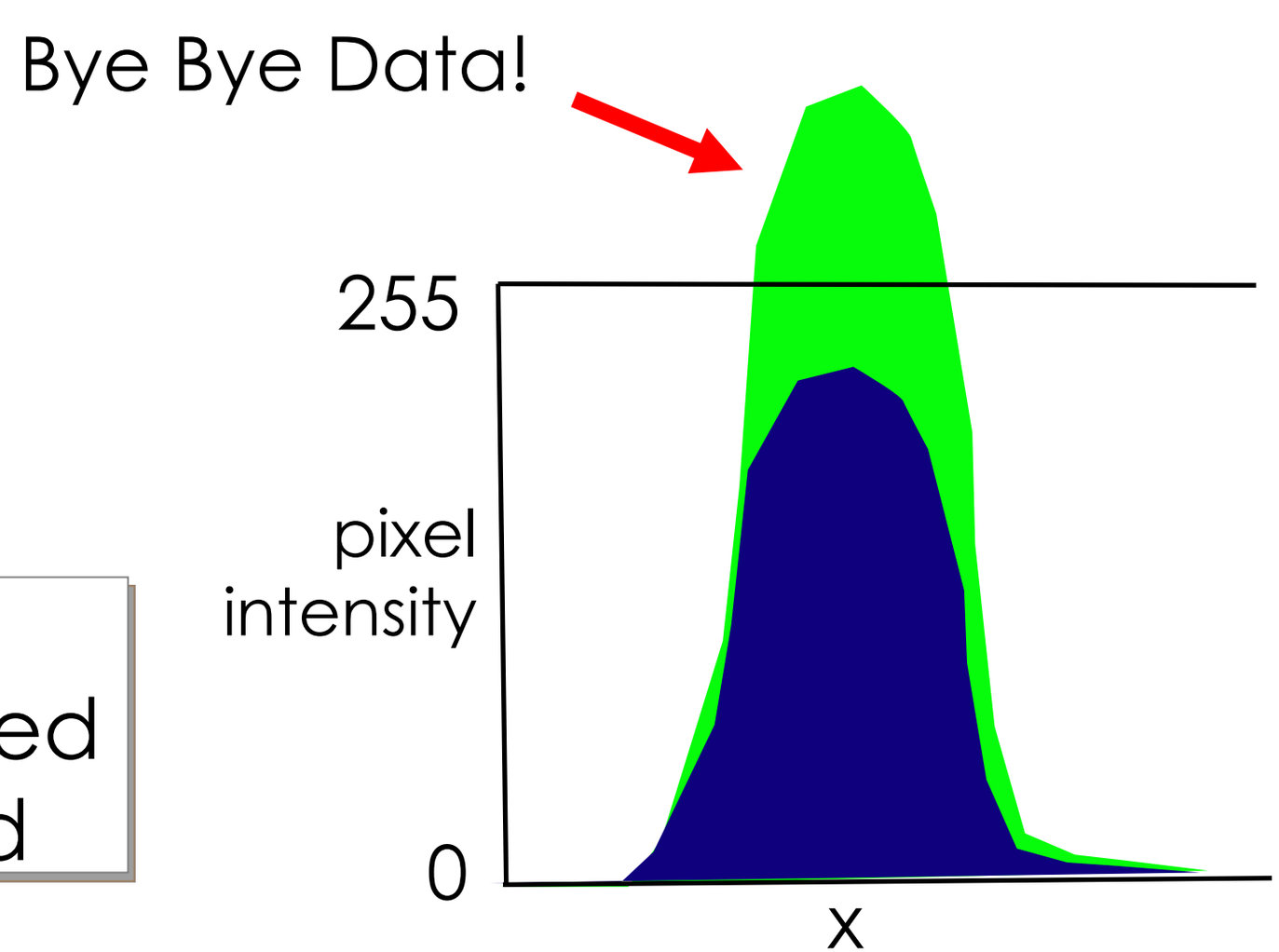
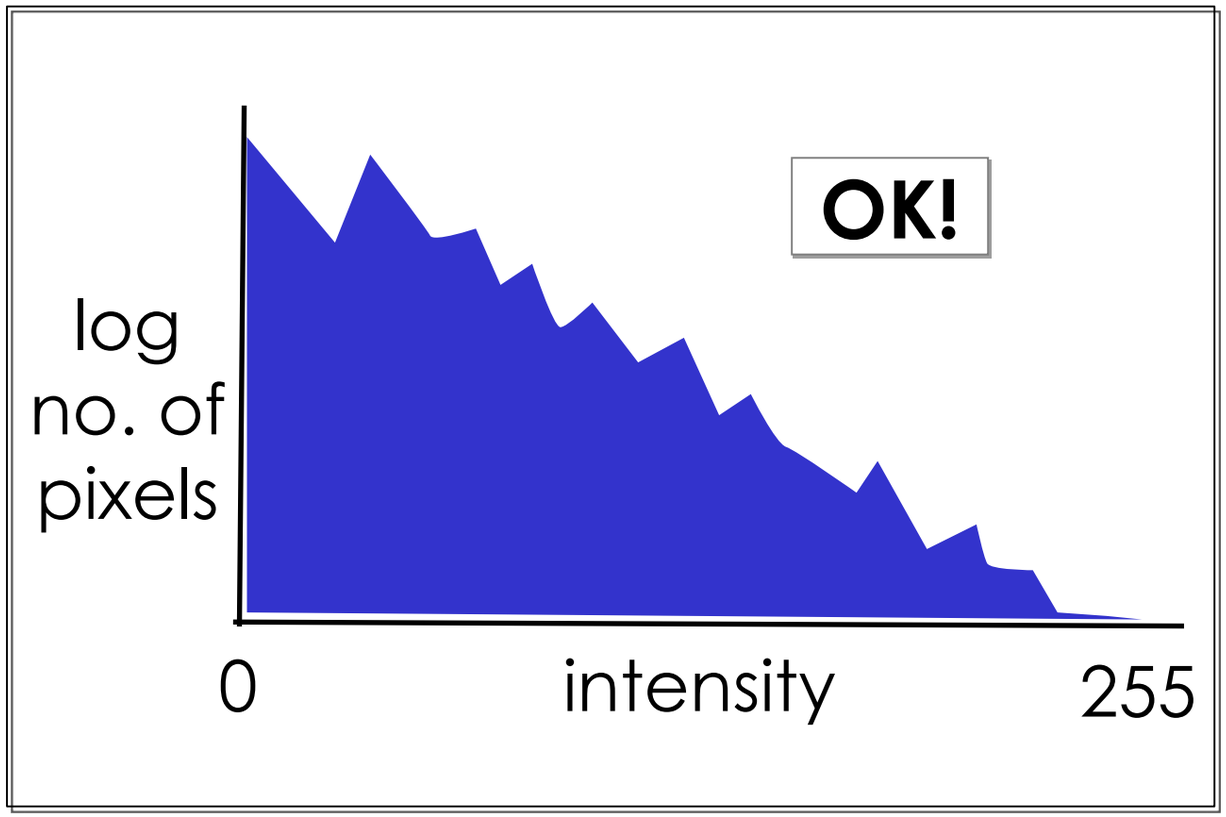
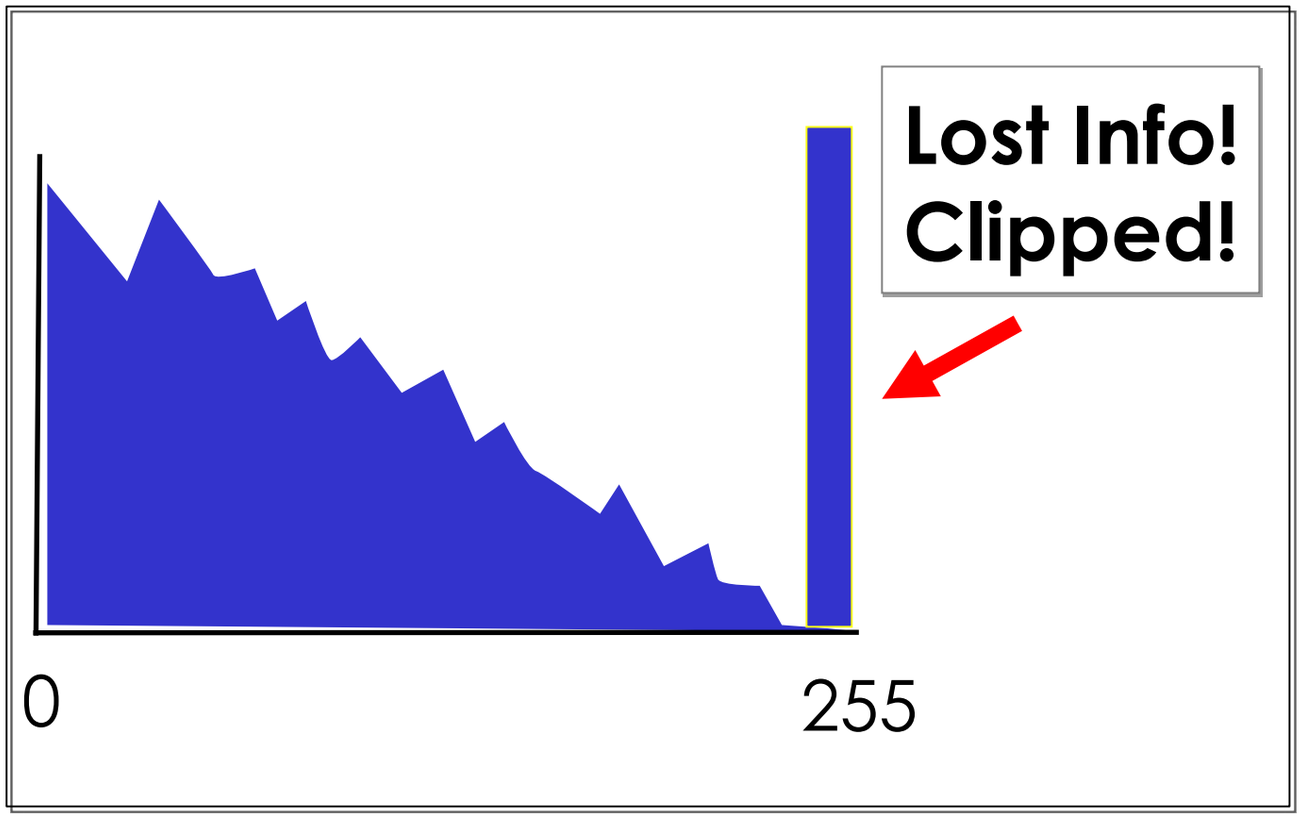
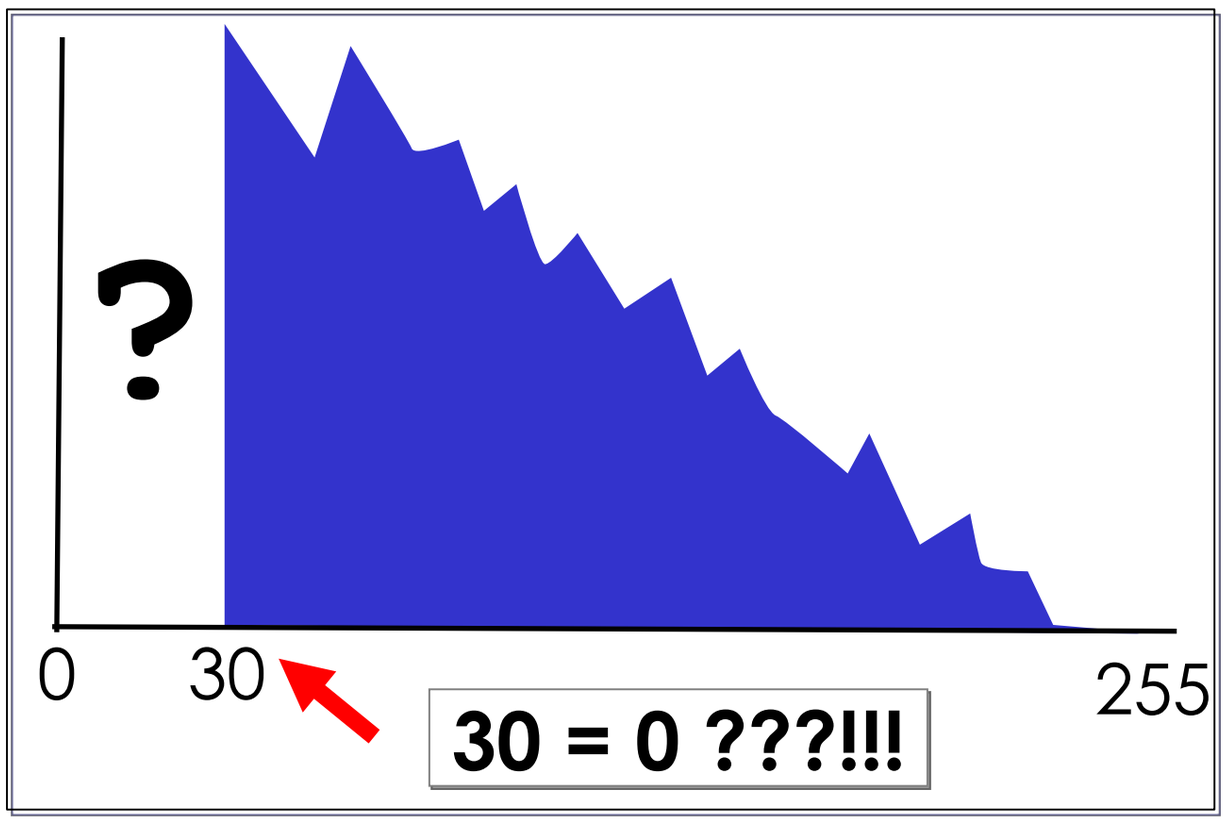


Image Intensity Histograms - Use them!



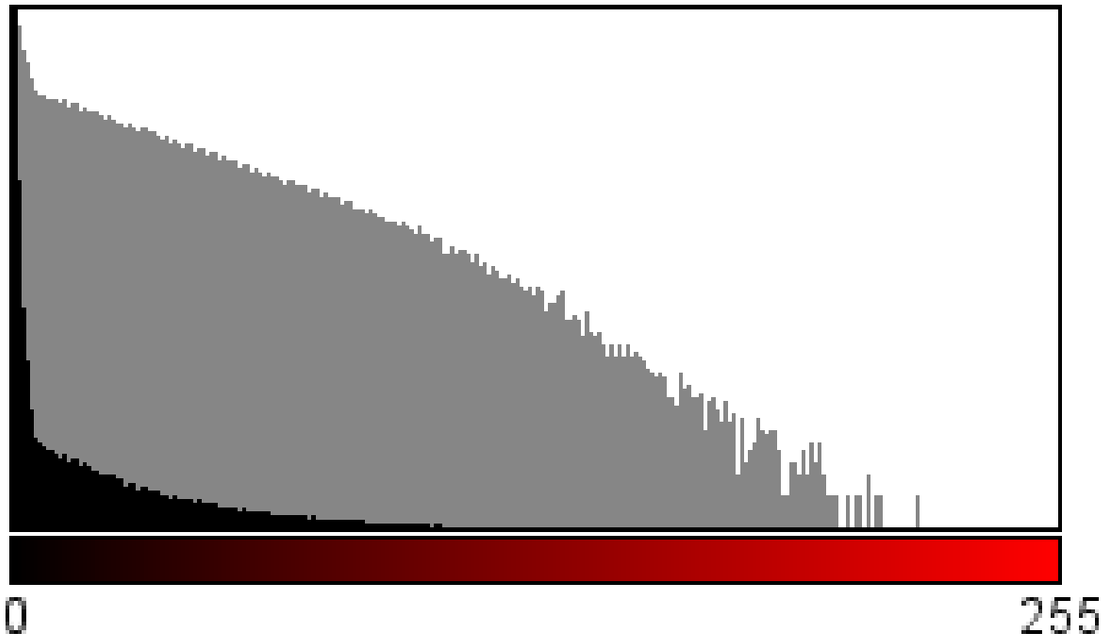




In Histograms:
easily see problems
for image
quantification!

Intensity Histogram

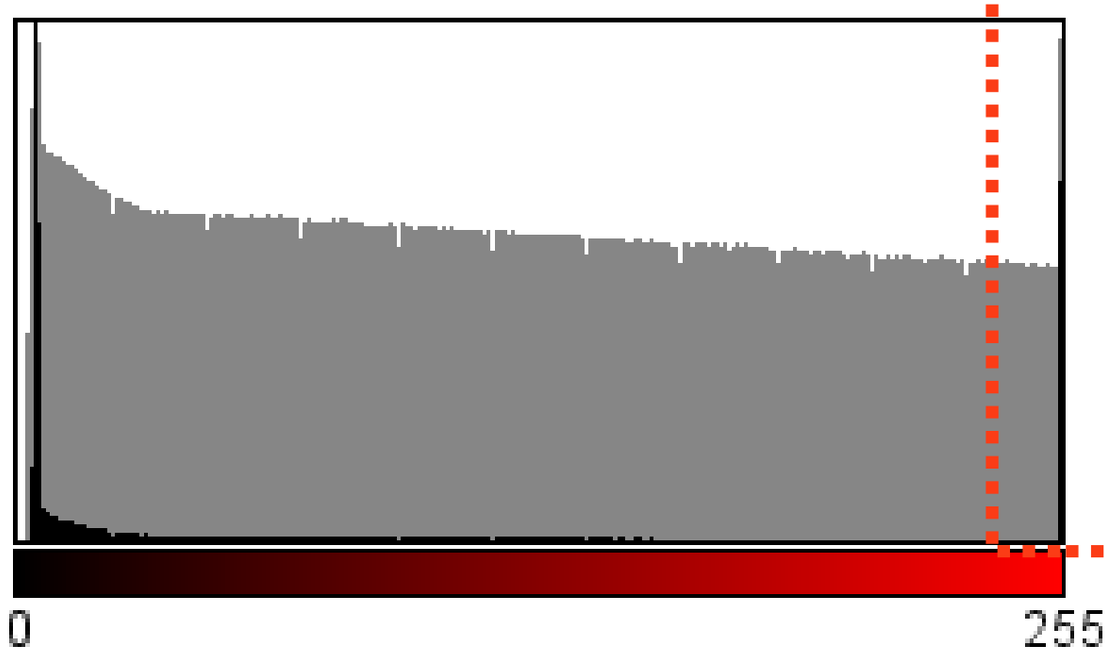
Fluorescence Microscopy



Count: 524288
Mean: 18.561
StdDev: 26.465

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

OK



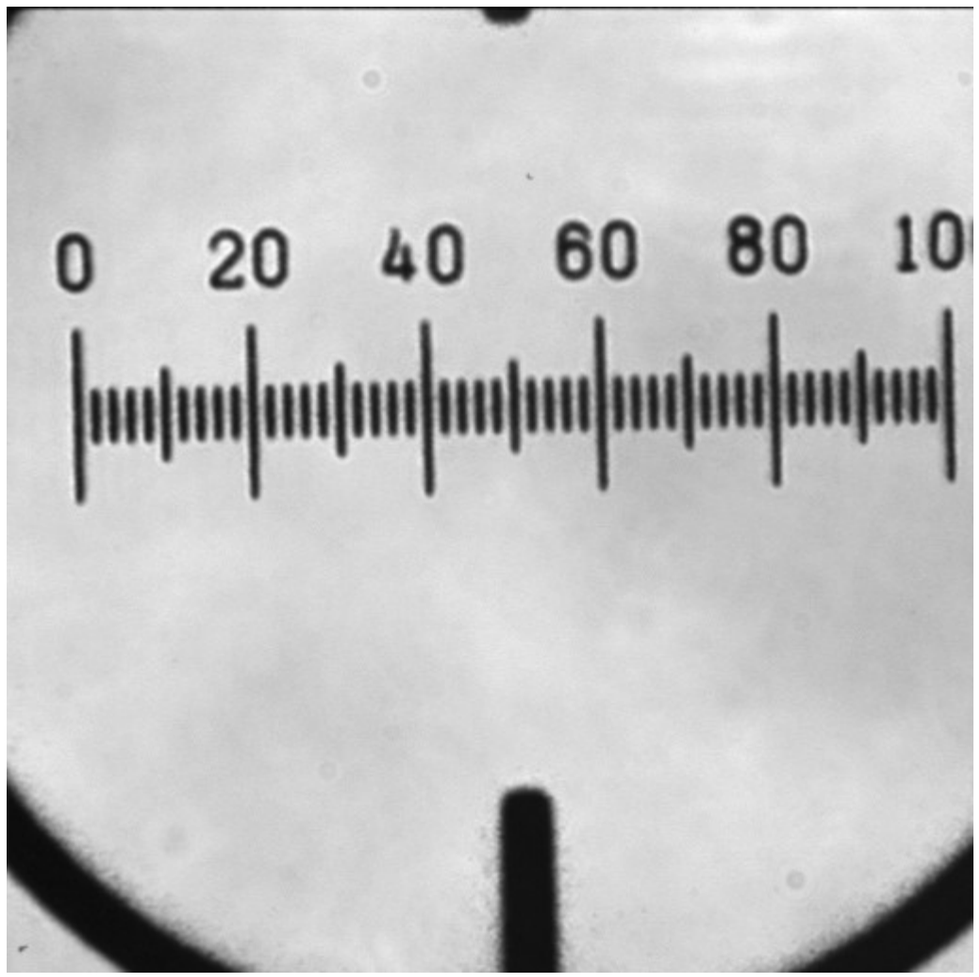
Count: 524288
Mean: 82.504
StdDev: 93.452

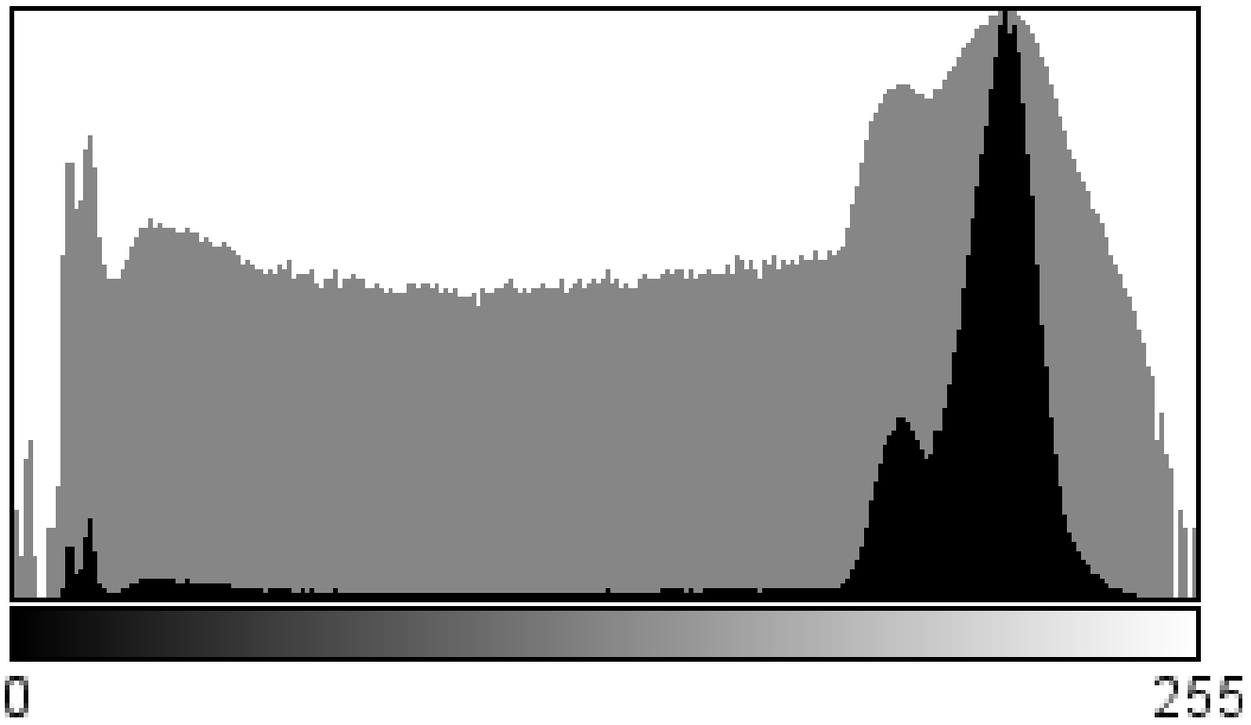
Min: 2
Max: 255
Mode: 4 (101652)

not OK - why?

Intensity Histogram

Brightfield Microscopy

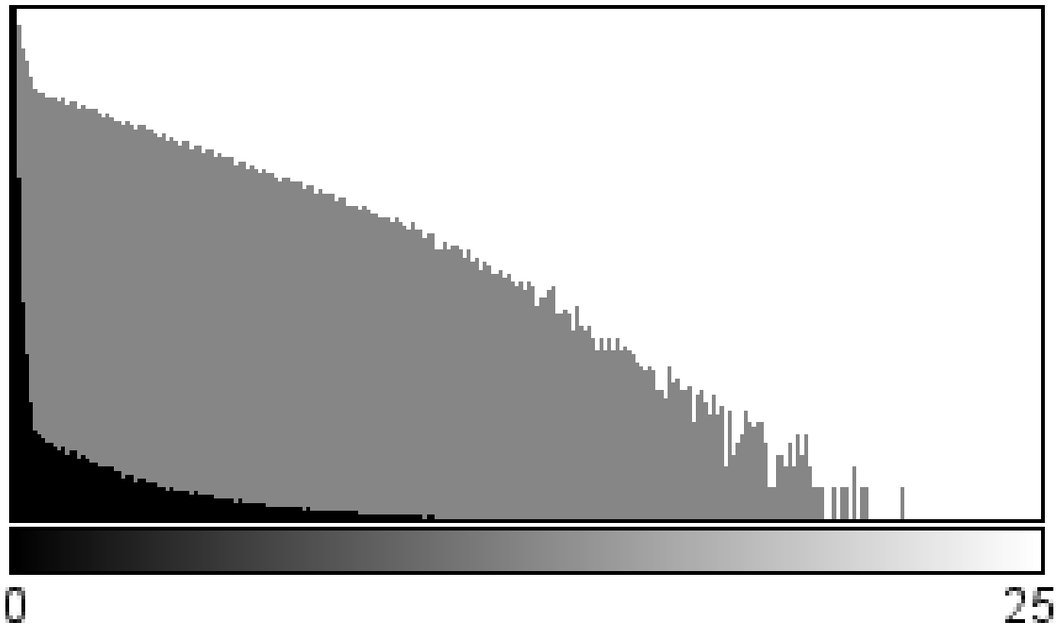




Count: 262144
Mean: 191.793
StdDev: 50.337

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

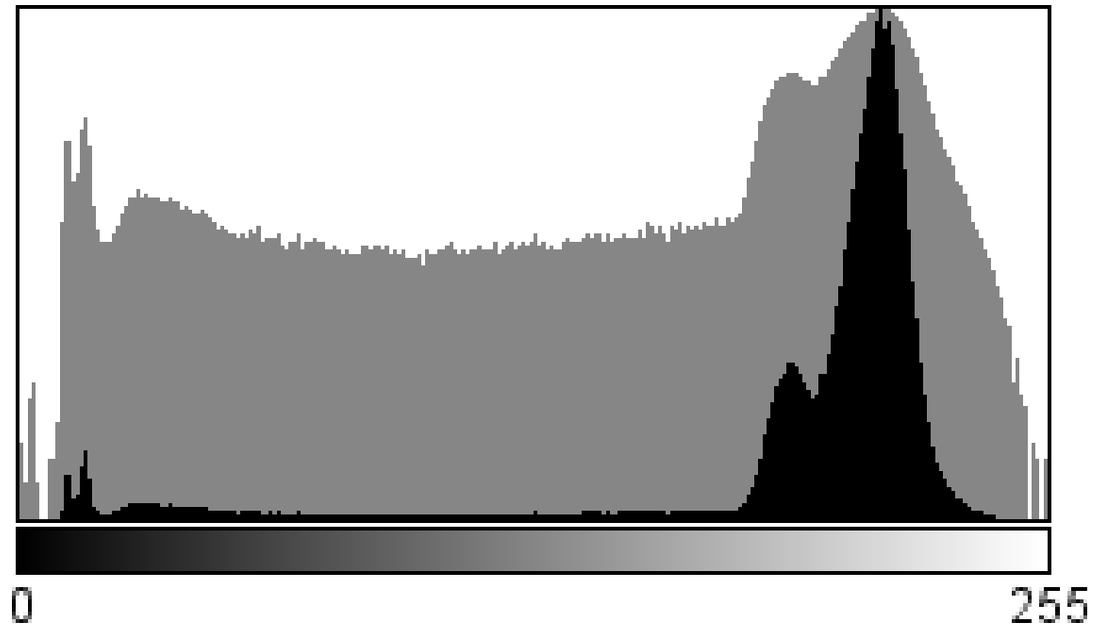
Intensity Histogram



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 1b

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

File - Open Samples - Neuron

Intensity clipping/ saturation and offset:

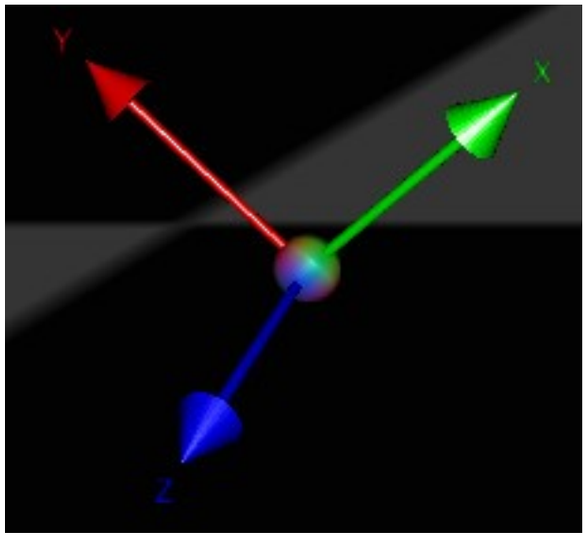
✓ **Bit Depth** – *change from 16 to 8. What happens to the numbers?*

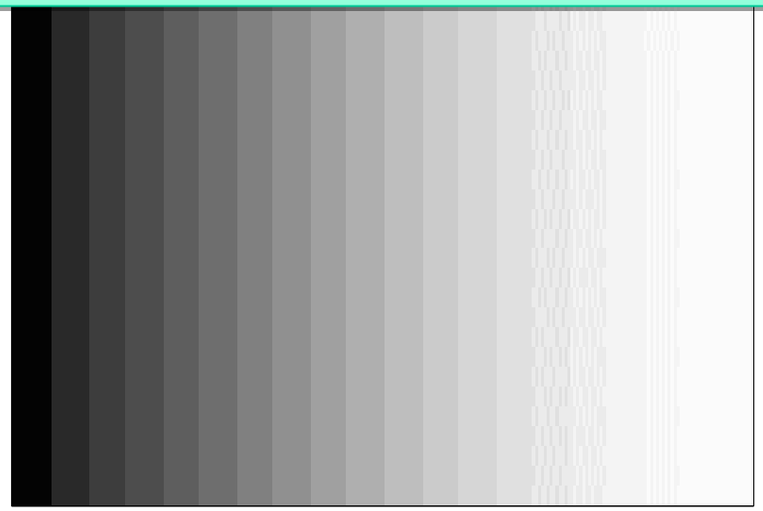
✓ **Brightness/Contrast**: *Image-Adjust-Brightness/Contrast. Realize: you can loose data using “Apply”!*

✓ **Intensity Histograms**: *log scale for fluorescence*

What can you digitise?

Dimensions!



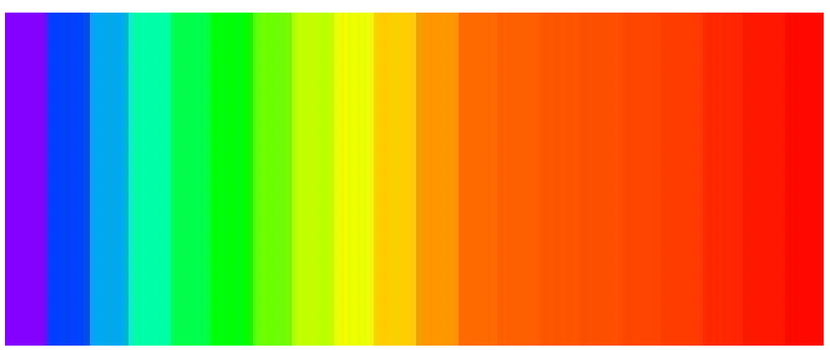


SPACE

INTENSITY

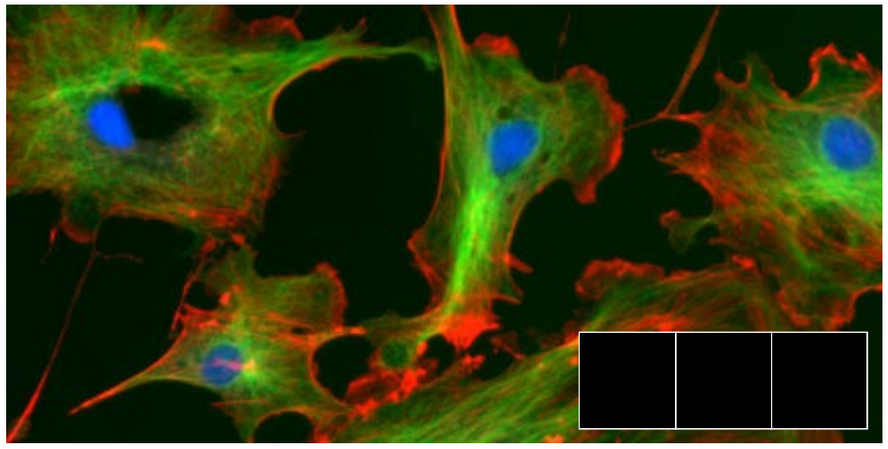
TIME

Wavelength

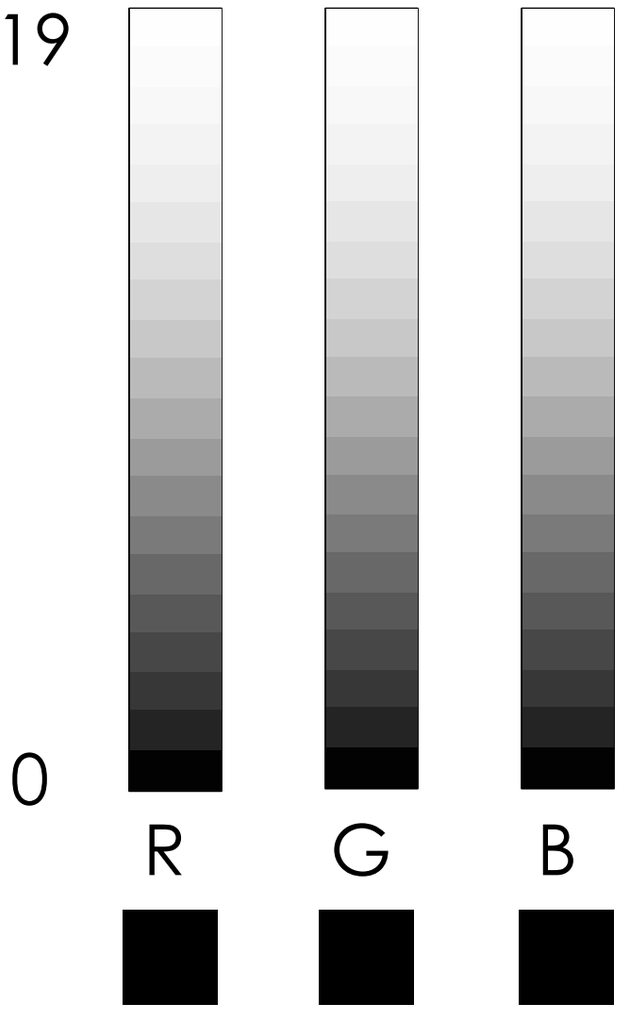


Colour

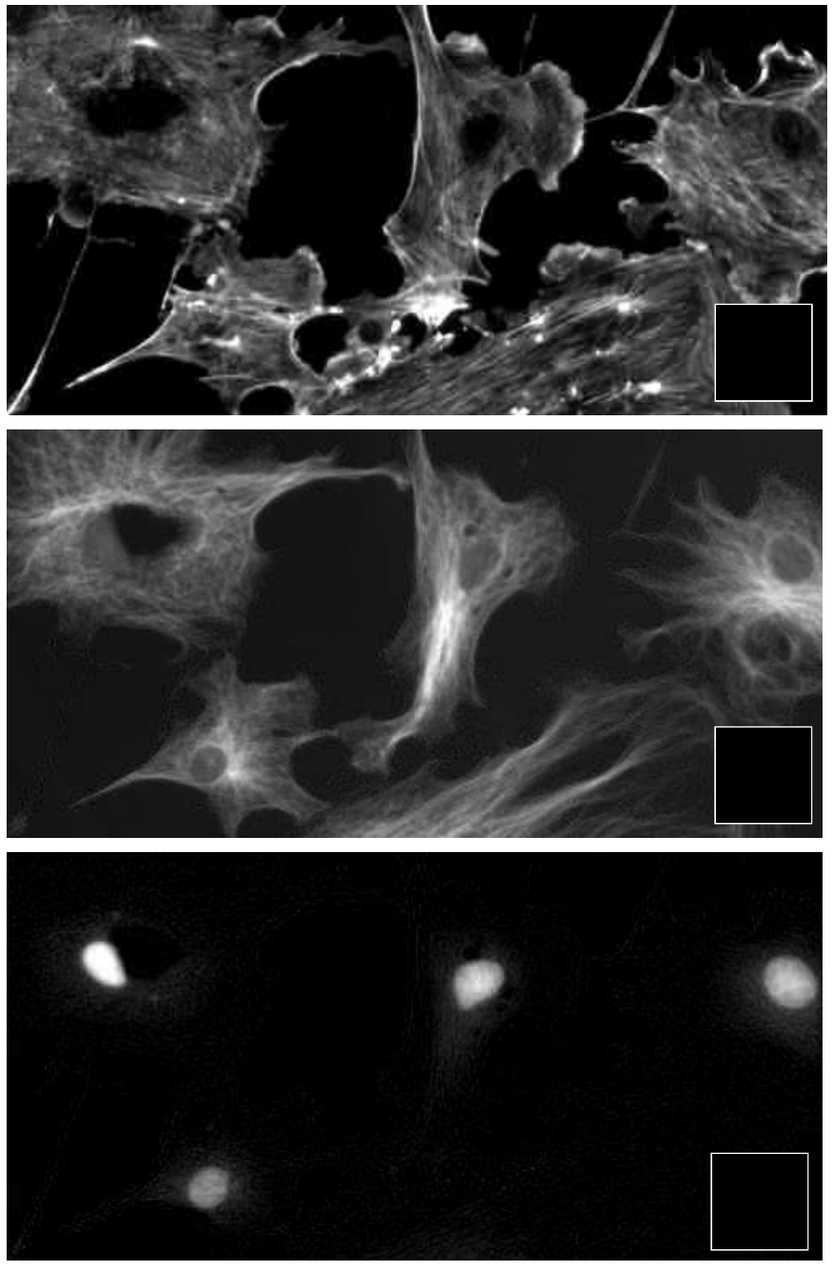
RGB Color Space



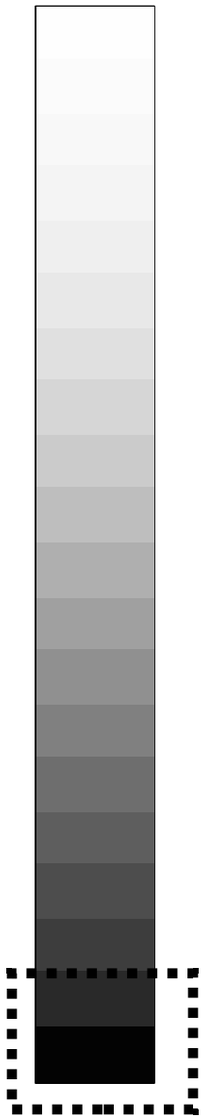
Why RGB? ... because we have red, green and blue sensitive photo receptors in our eyes!



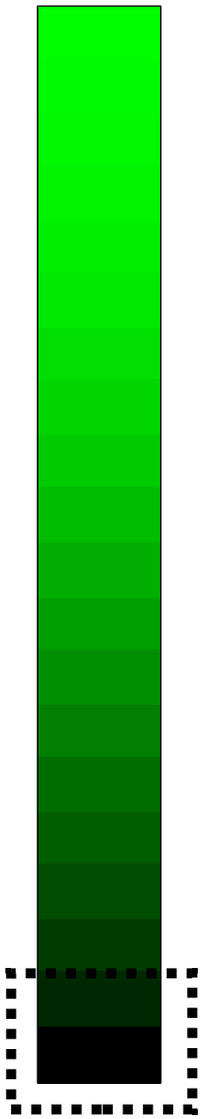
Each "colour" is really just single greyscale numbers!



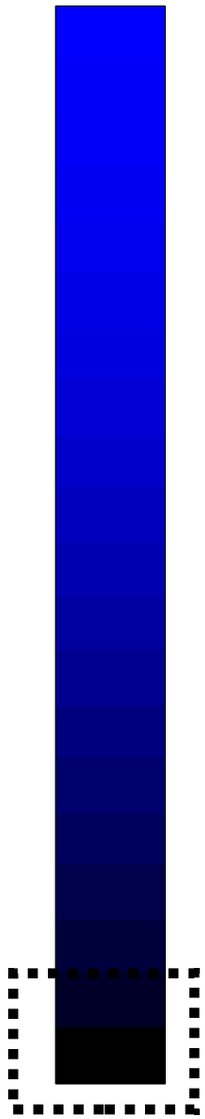
Lookup Tables / Palettes



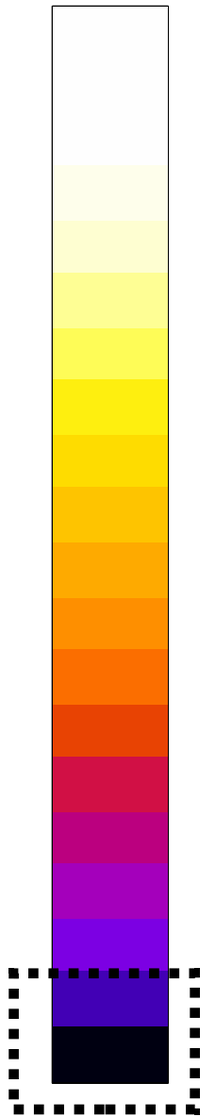
“grey”



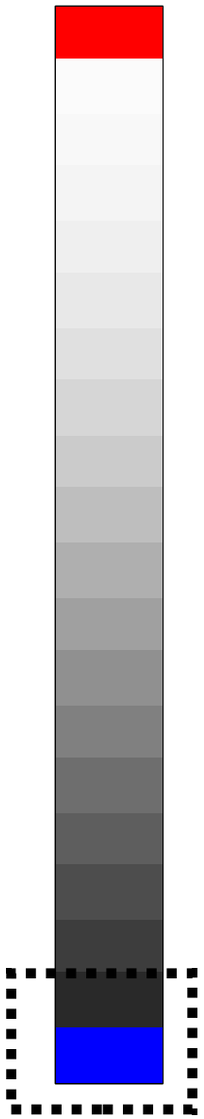
“green”



“blue”



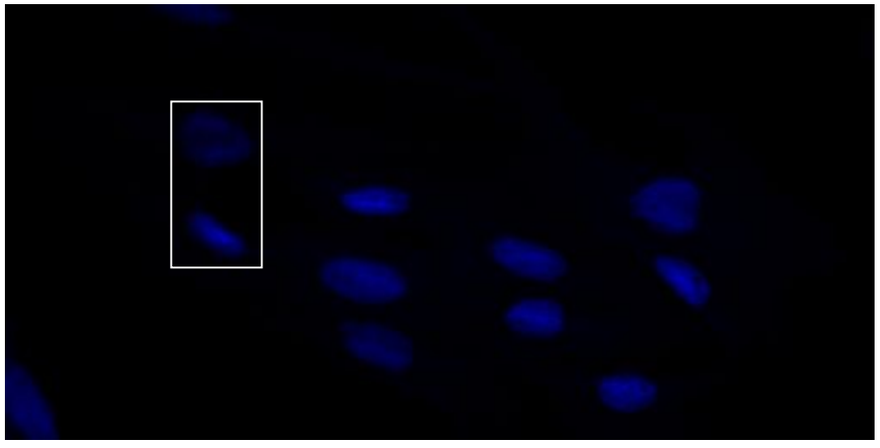
“fire”



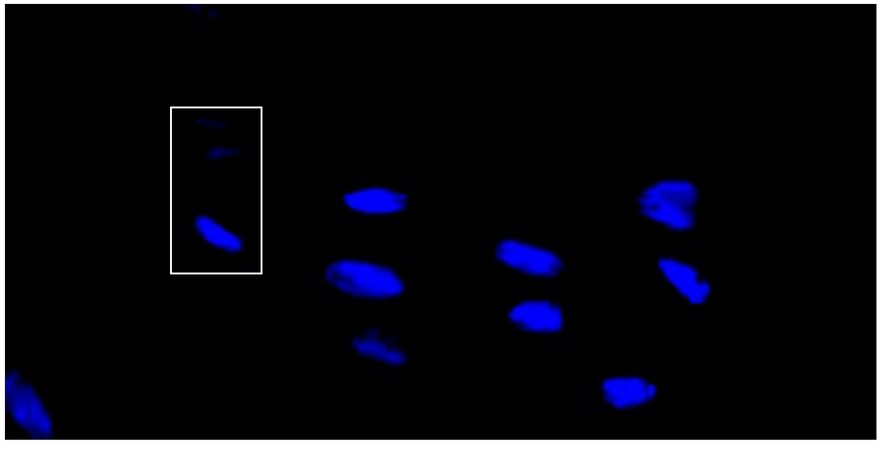
“HiLo”

Each “colour” is really just single greyscale numbers!

So we can represent that information however we like!

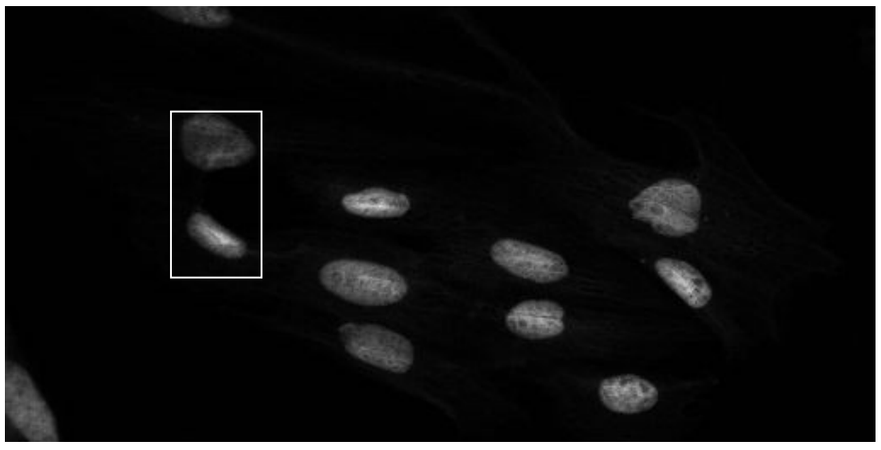


“original” - linear blue

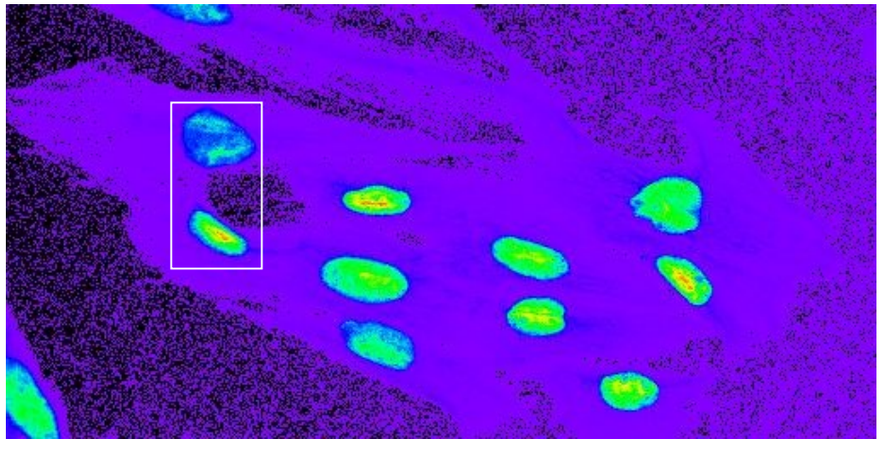


altered brightness/contrast

data changed/lost!



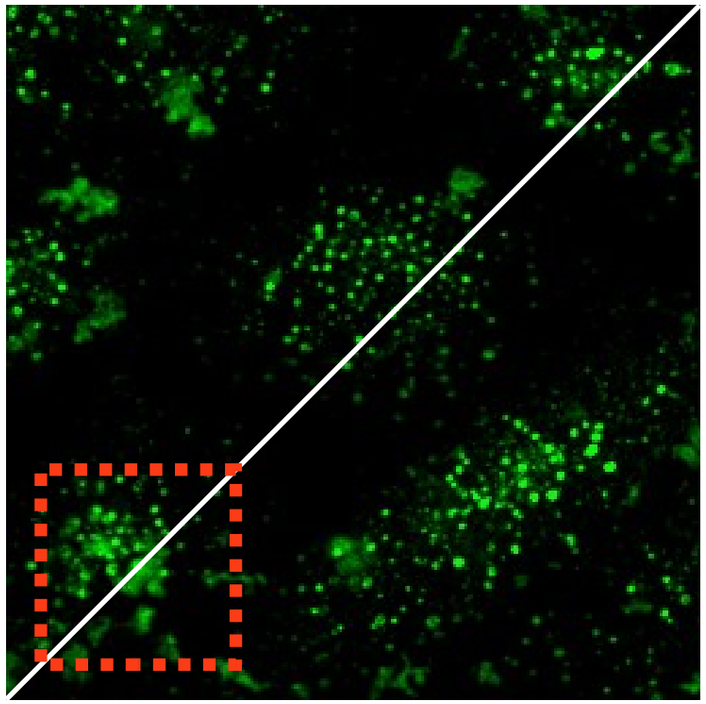
Grayscale - linear

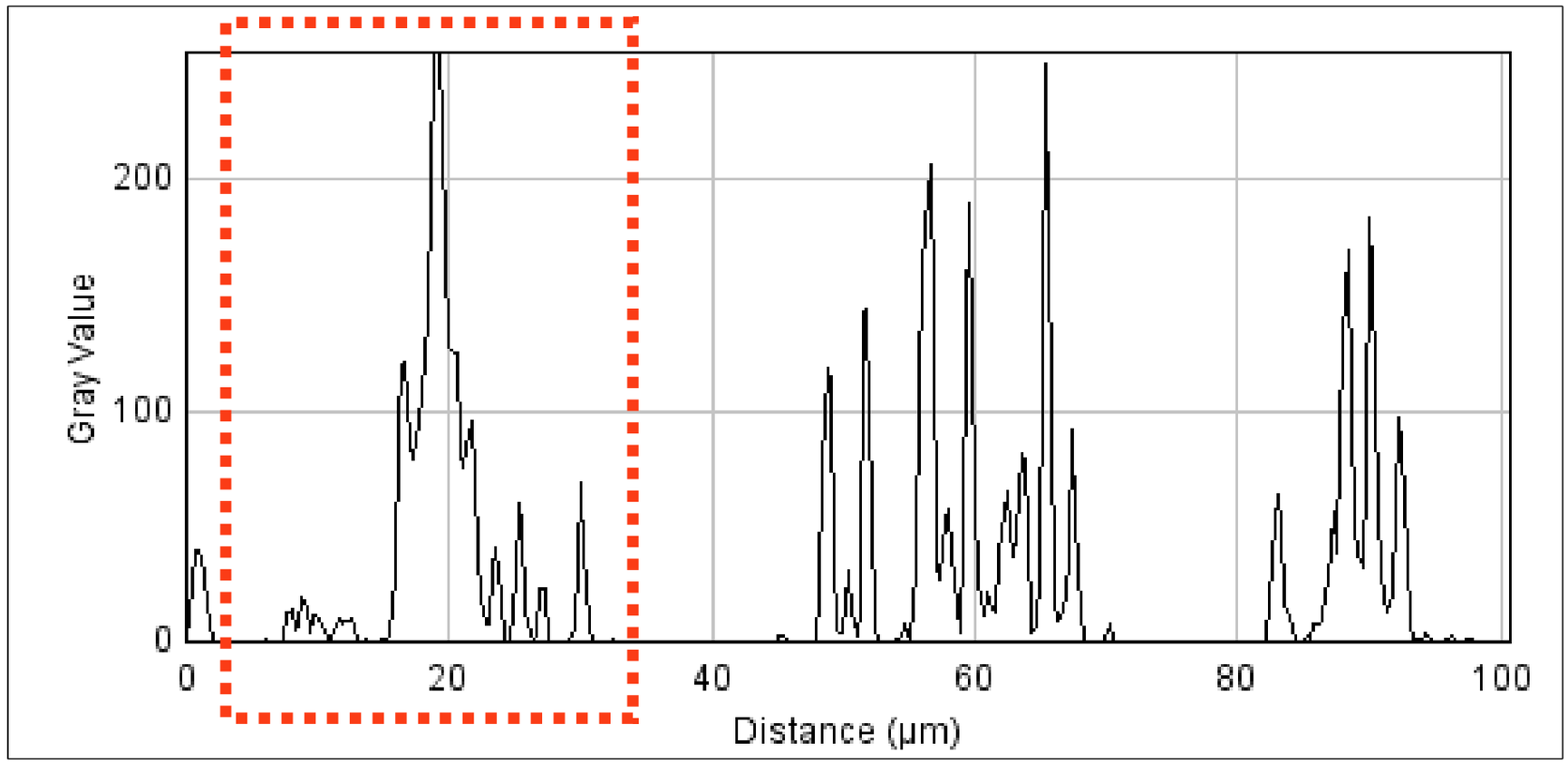


Rainbow lookup table

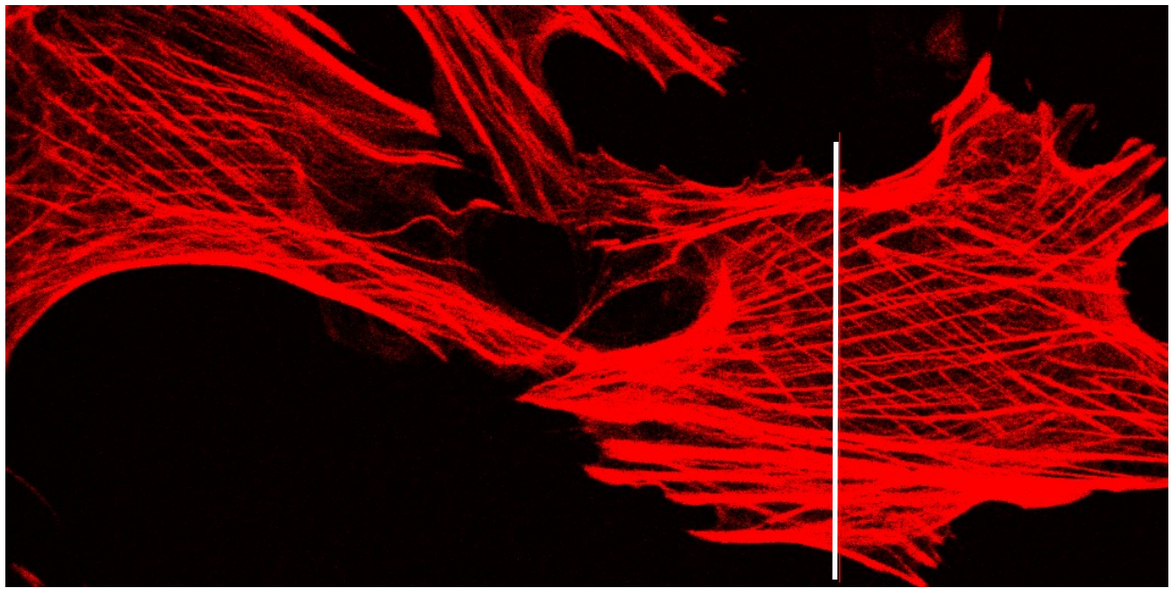
better see and also compare different intensity levels

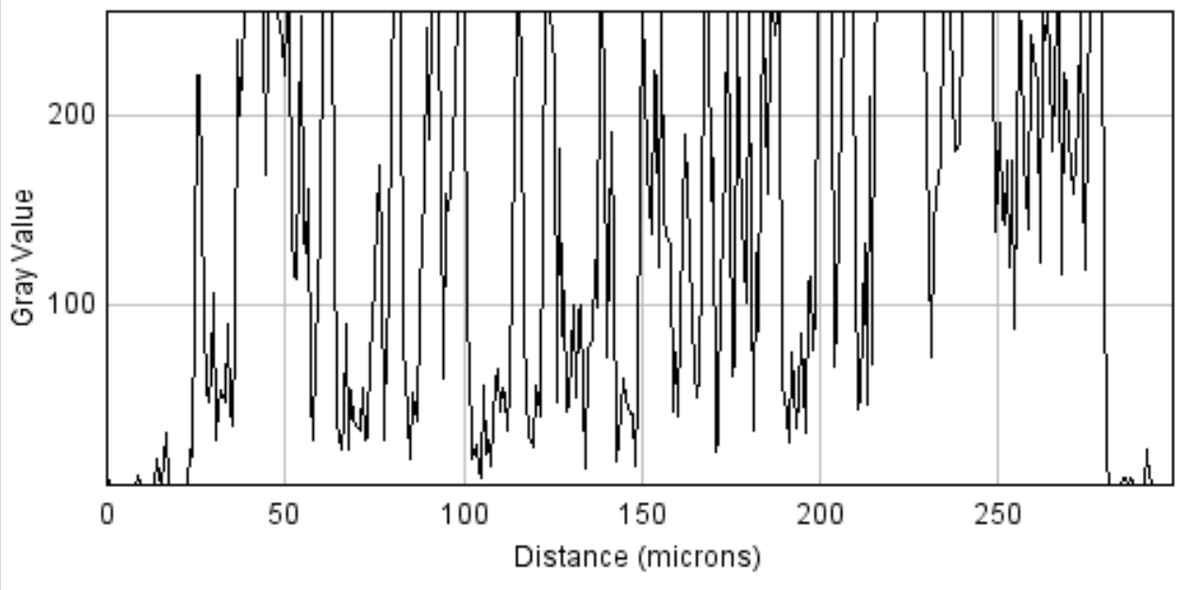
Line Profile

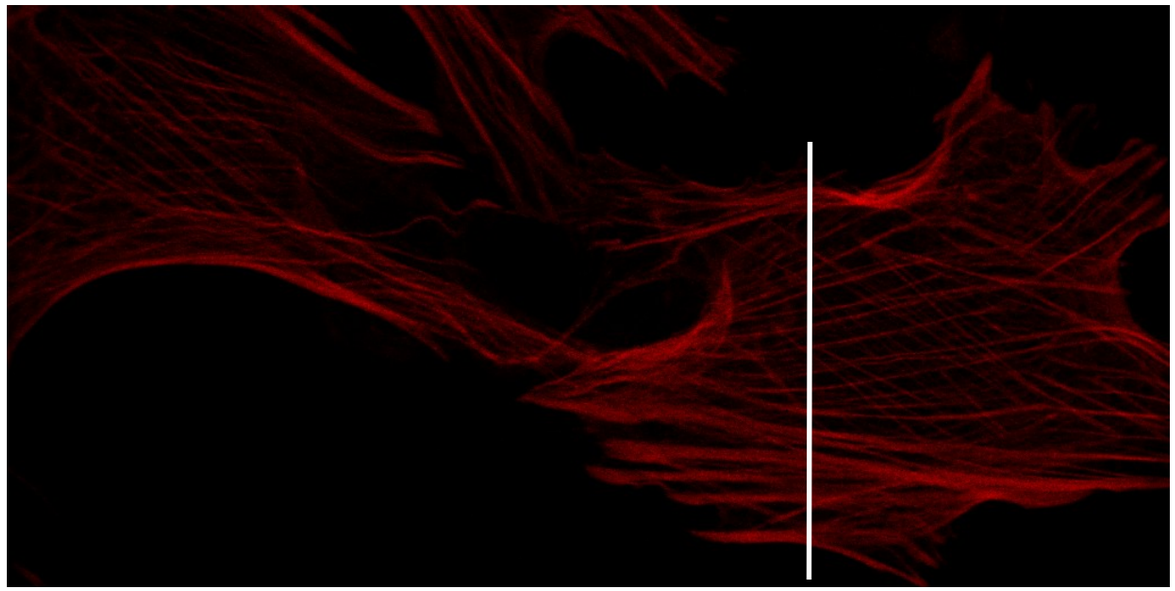


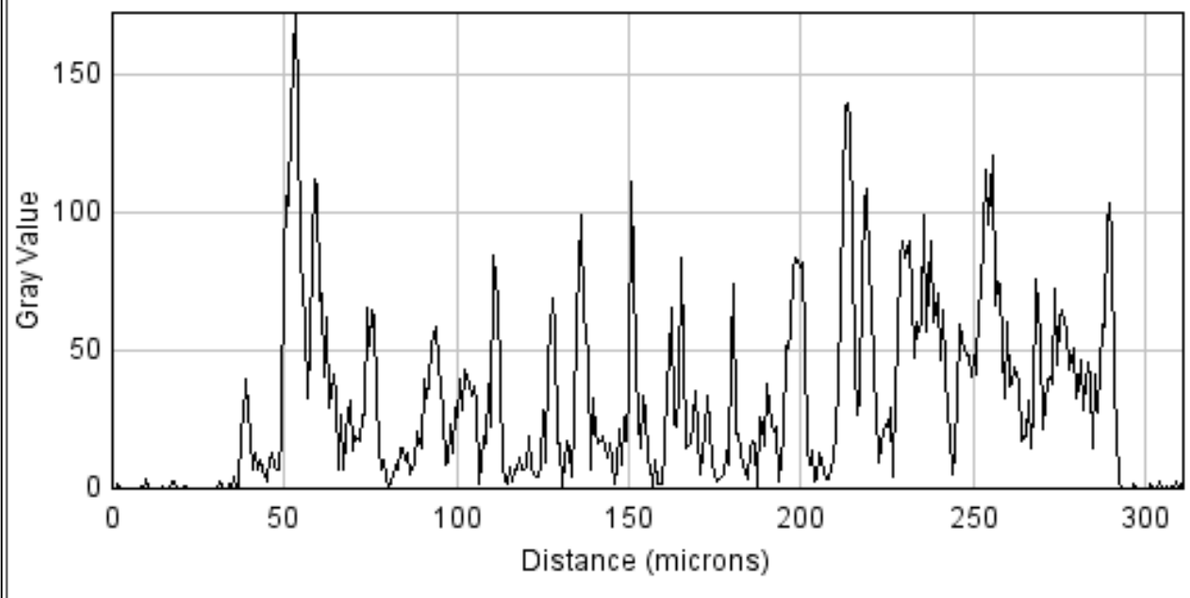


Line Profile



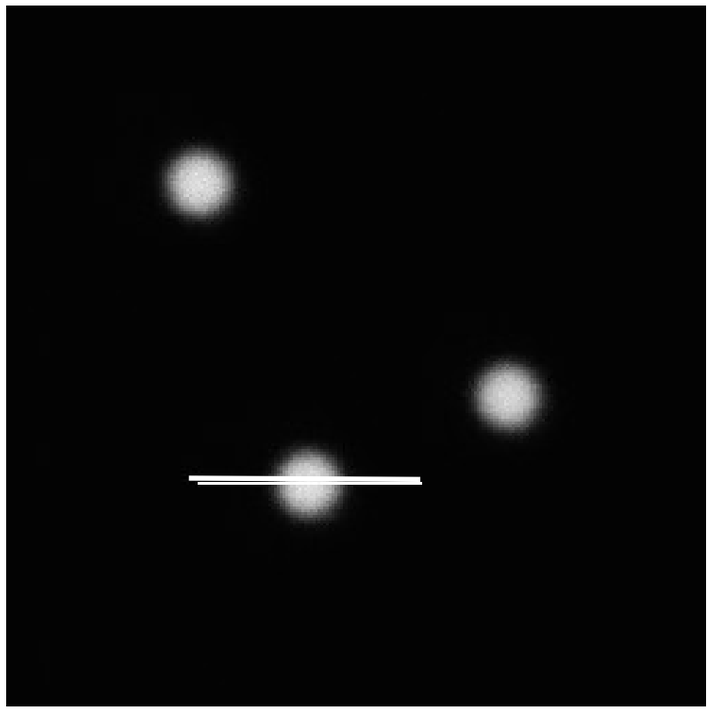


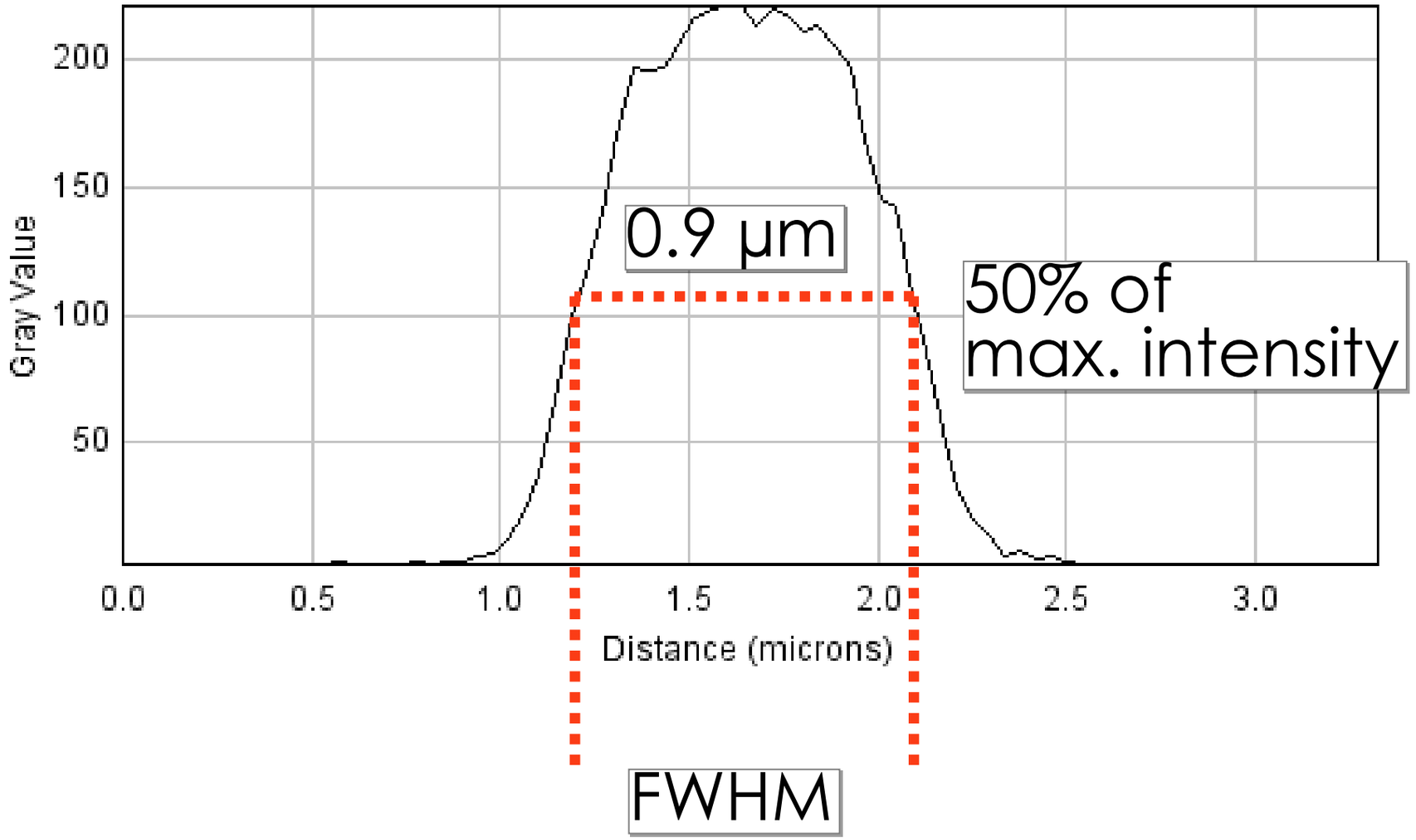




Line Profile

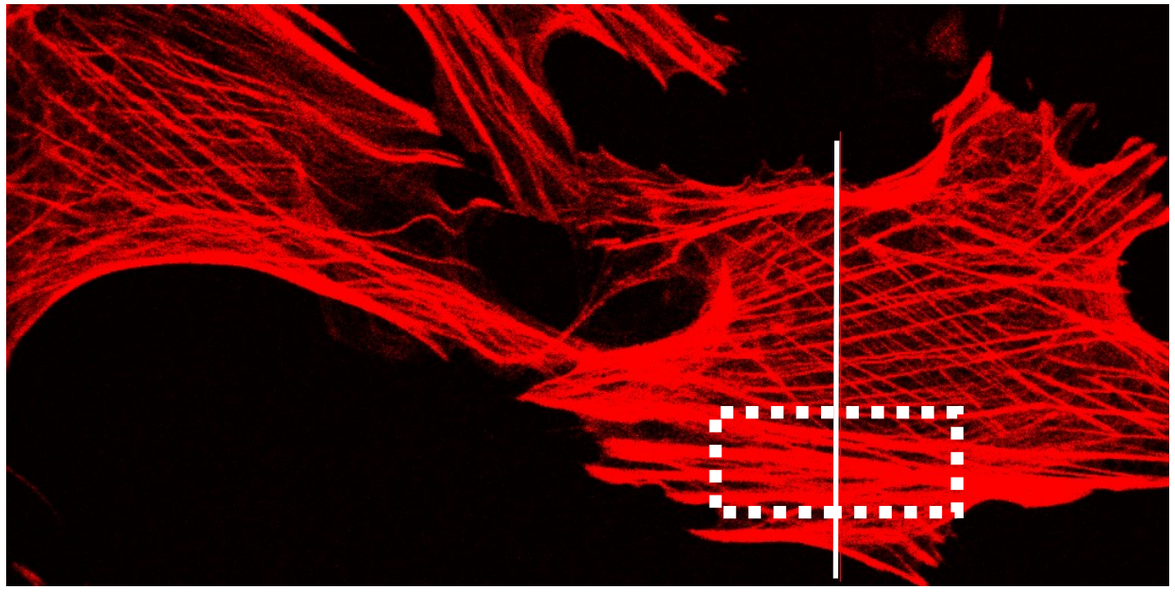
for measurements

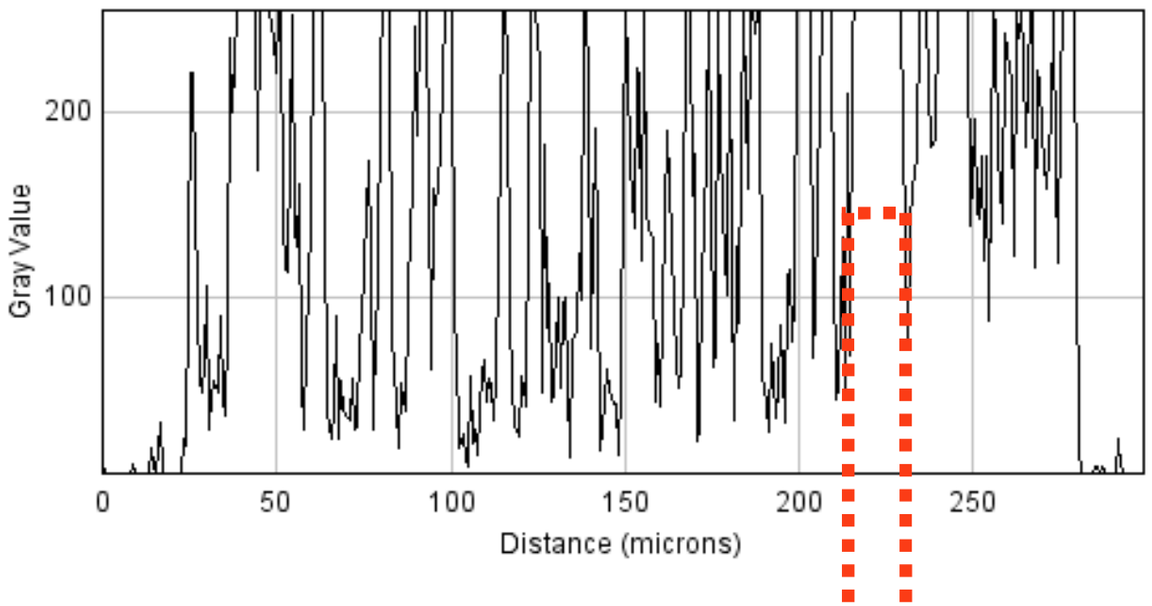




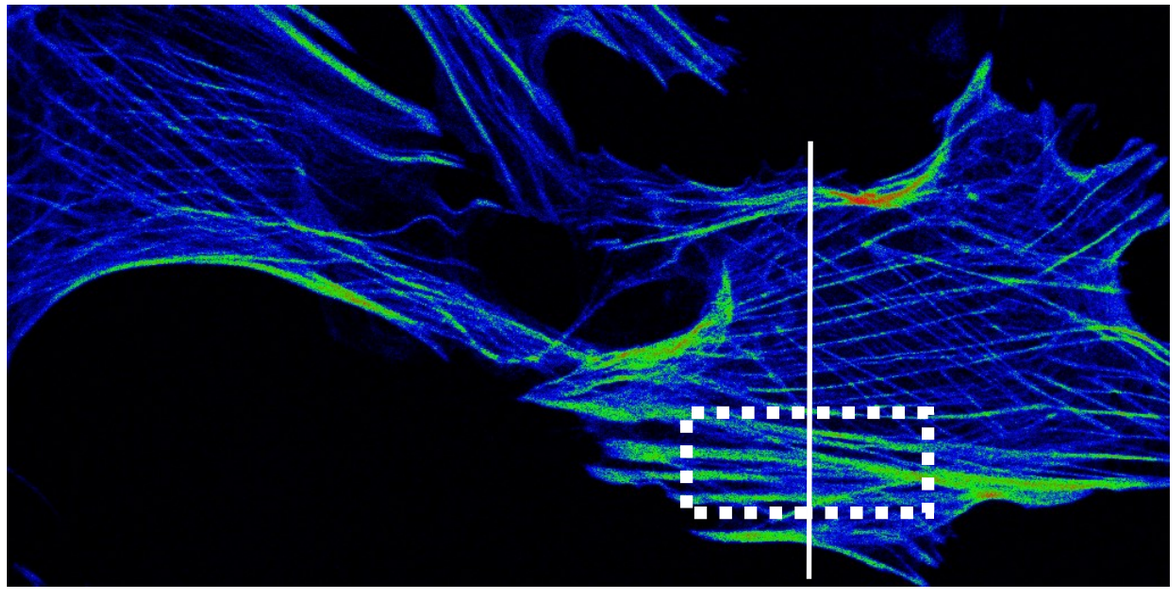
FWHM= “Full Width at Half Maximum”

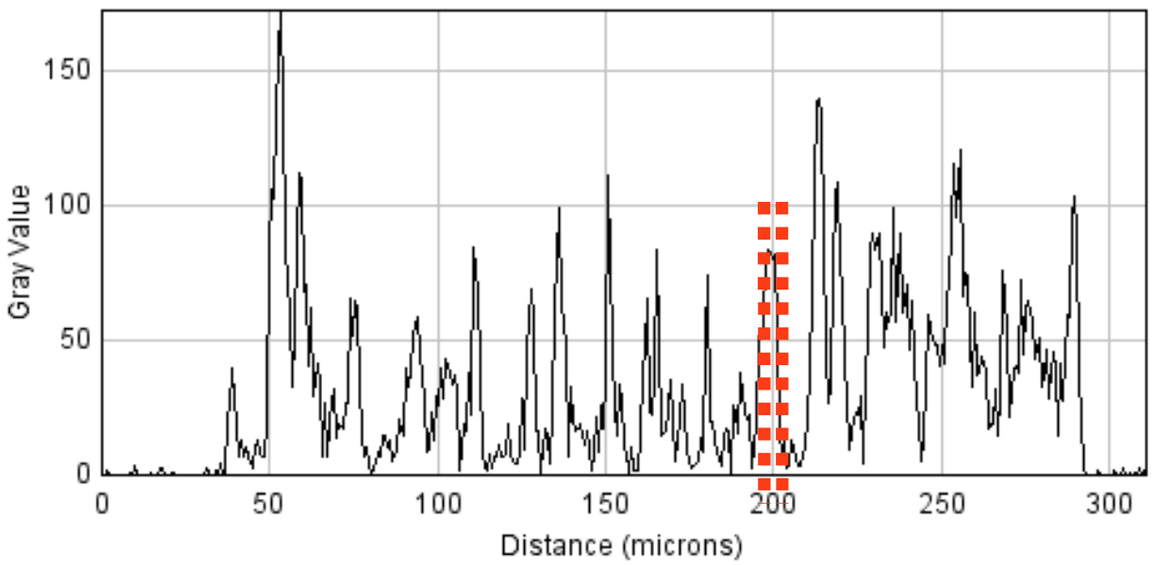
Line Profile





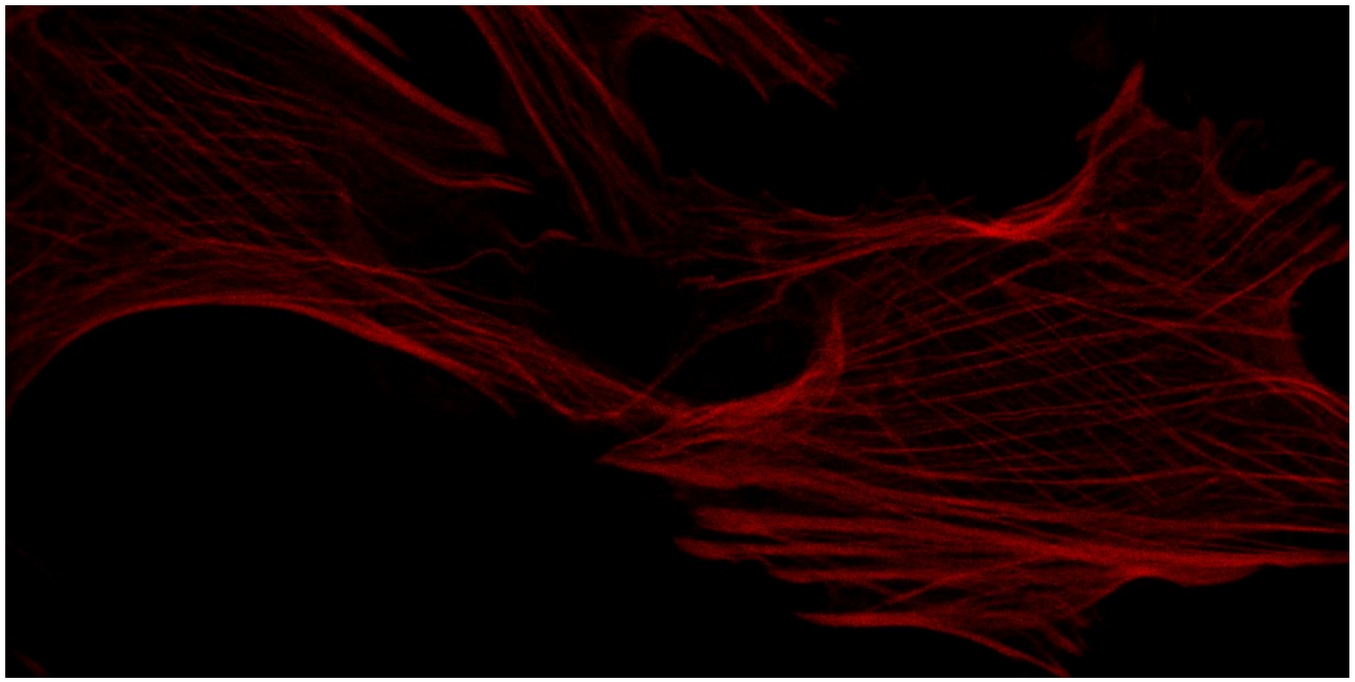
correct ?

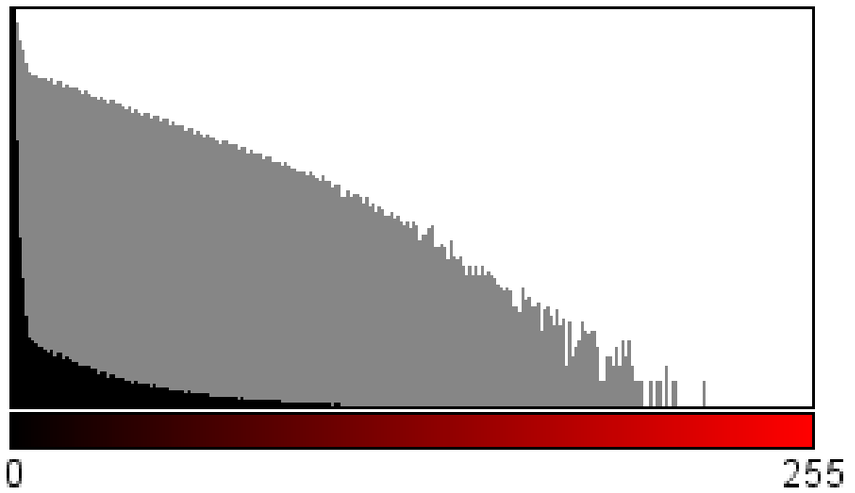




correct !

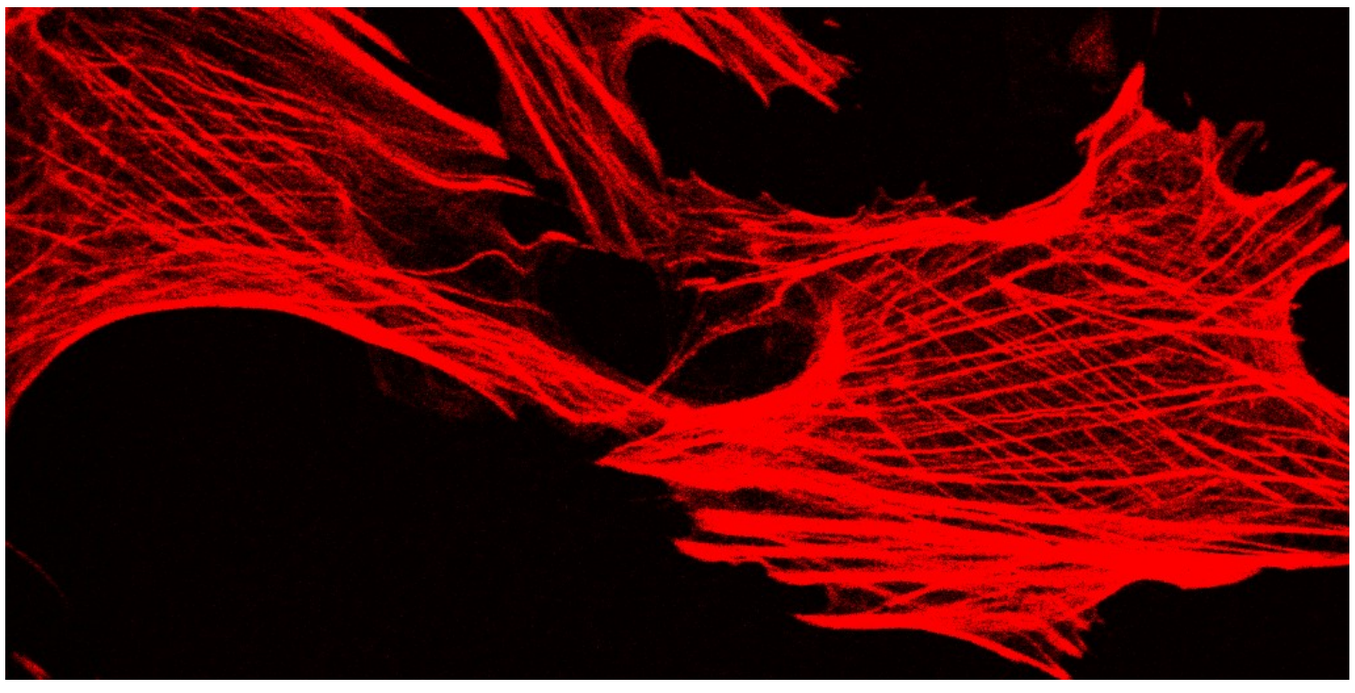
Intensity Histogram

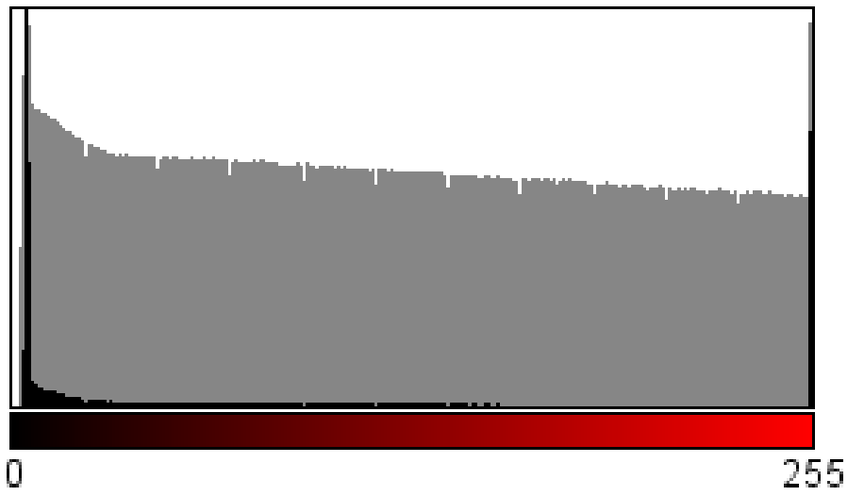




Count: 524288
Mean: 18.561
StdDev: 26.465

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

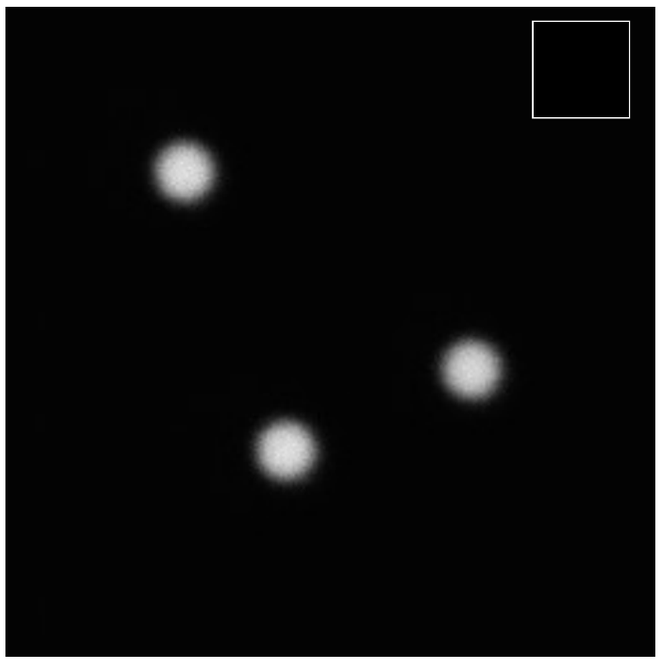


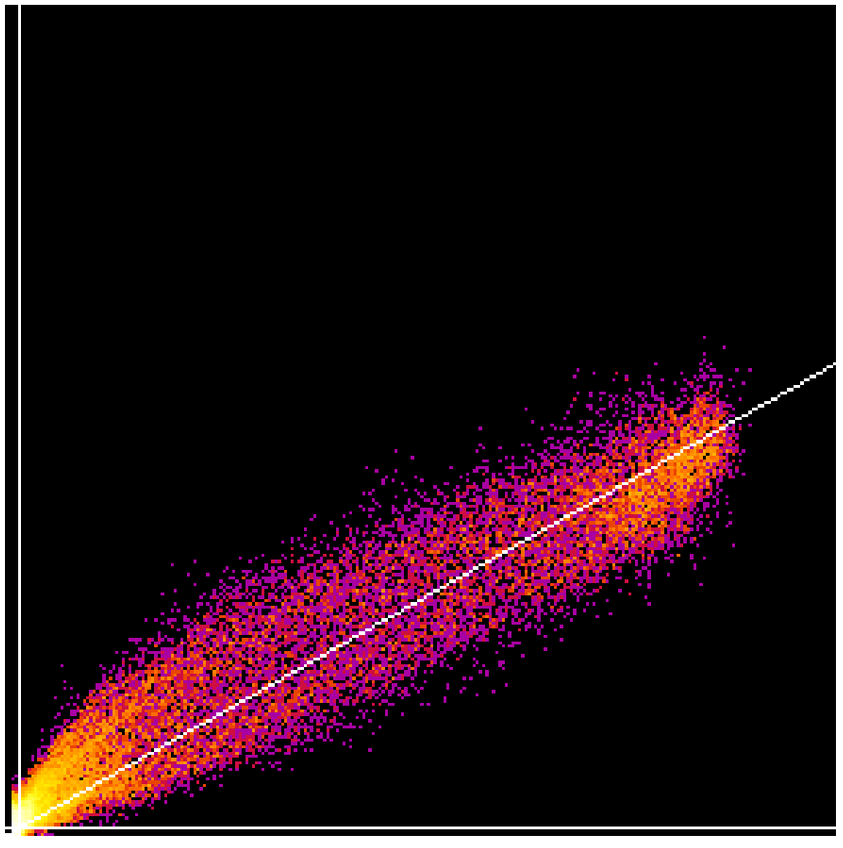


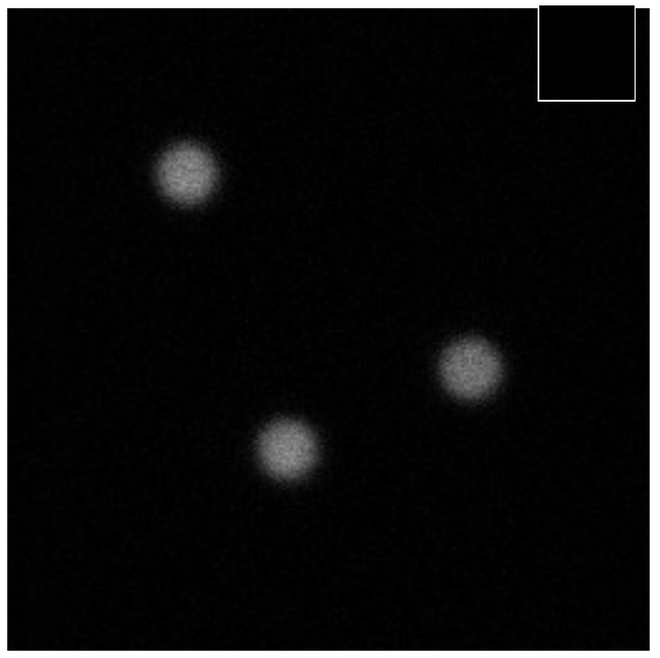
Count: 524288
Mean: 82.504
StdDev: 93.452

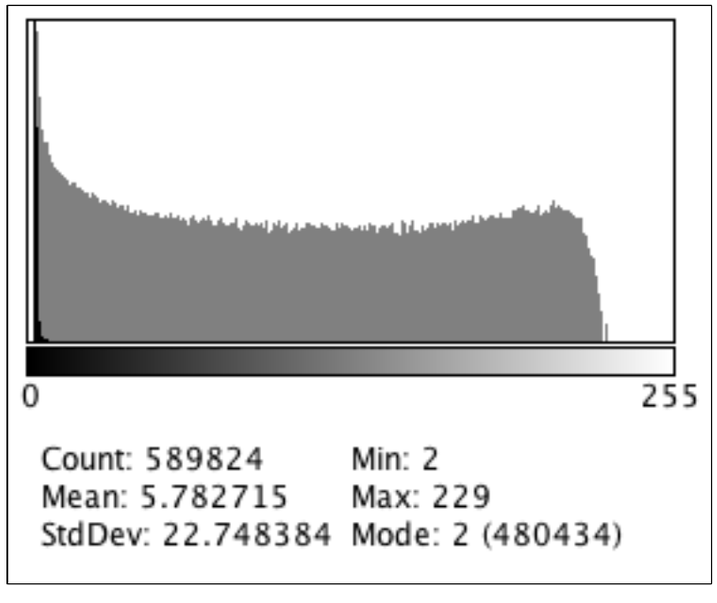
Min: 2
Max: 255
Mode: 4 (101652)

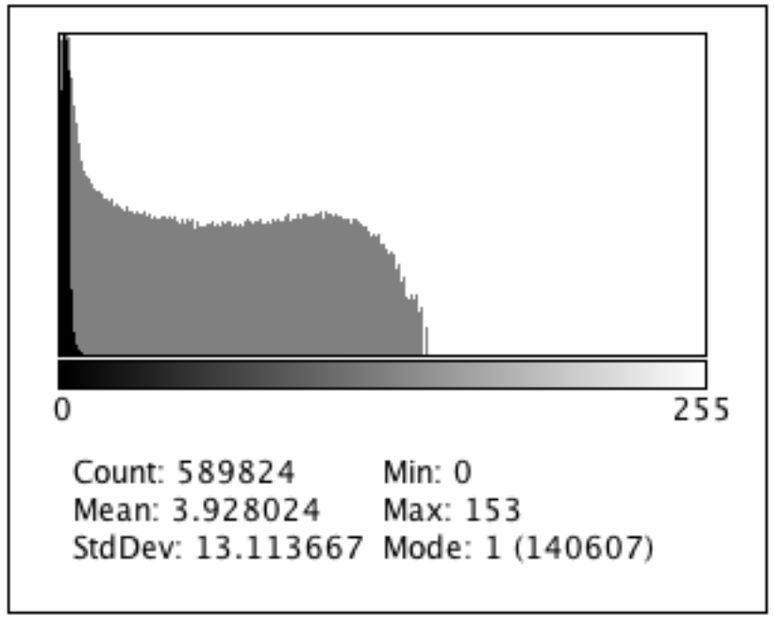
2D Histogram
=
Scatterplot or cytofluorogram



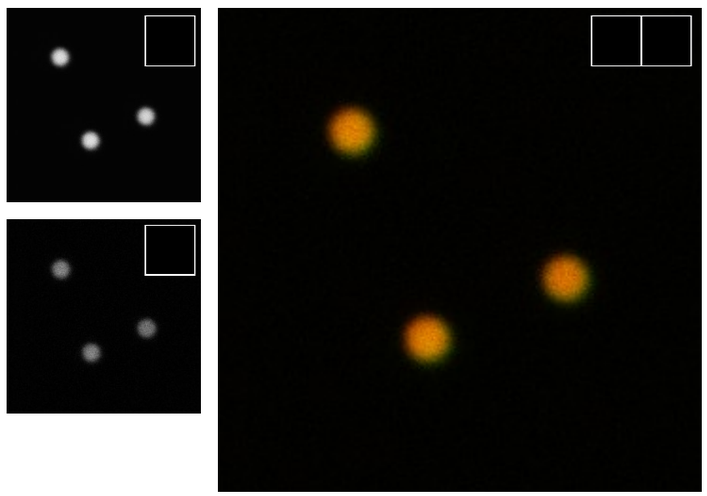




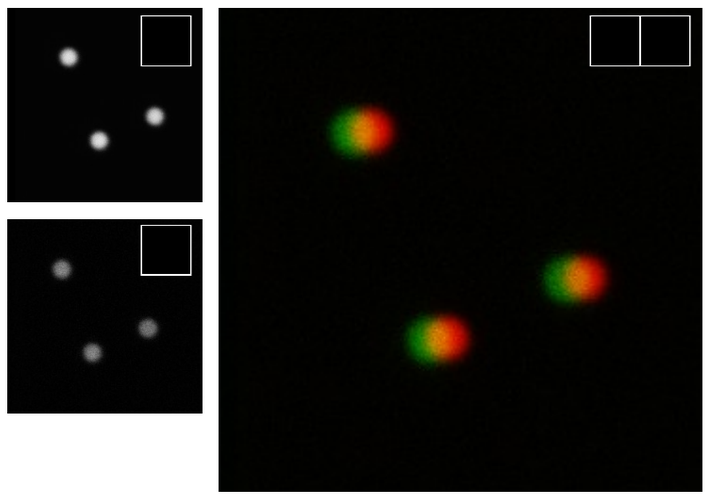




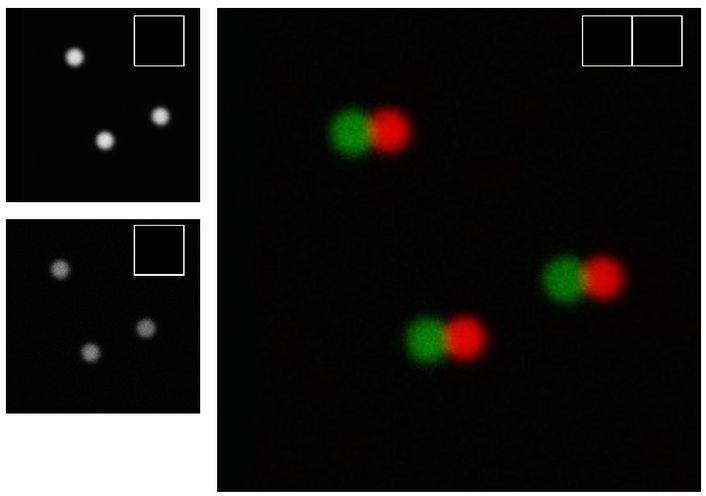
Scatterplot / 2D Histogram



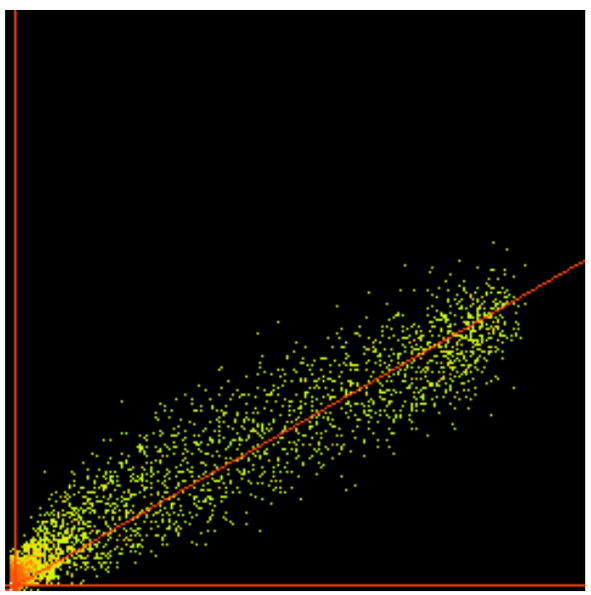
original R+G

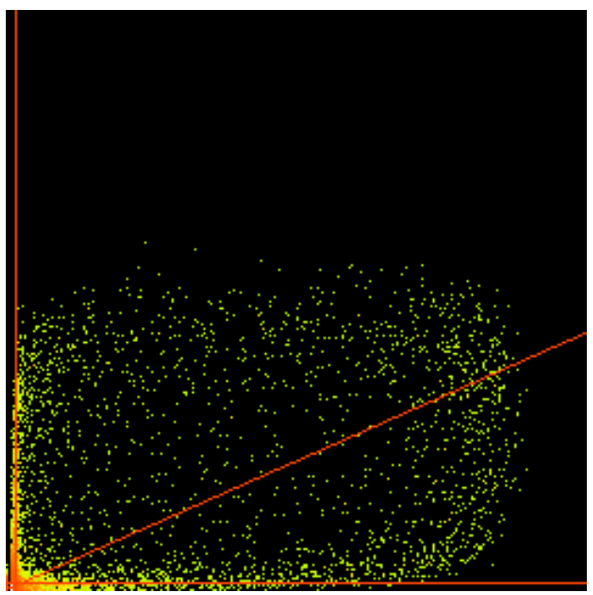


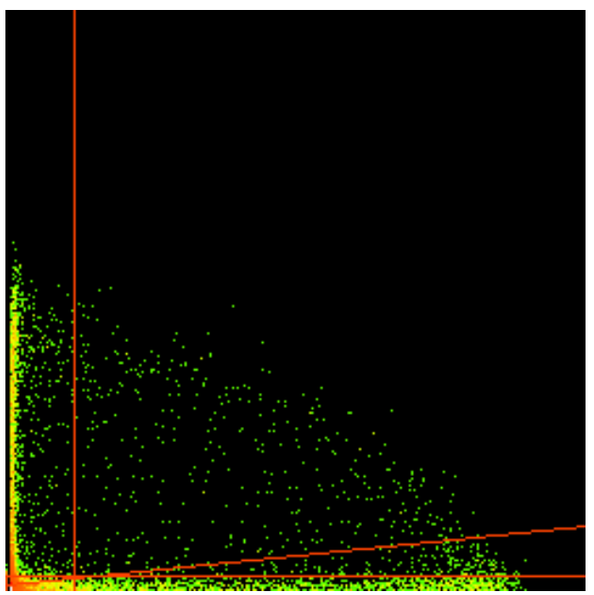
R shifted +10 pix



R shifted +20 pix



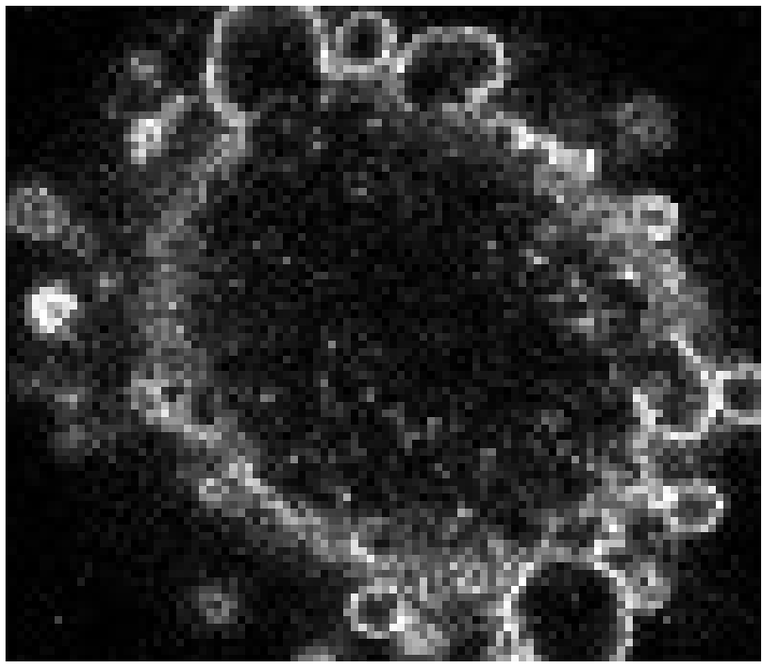


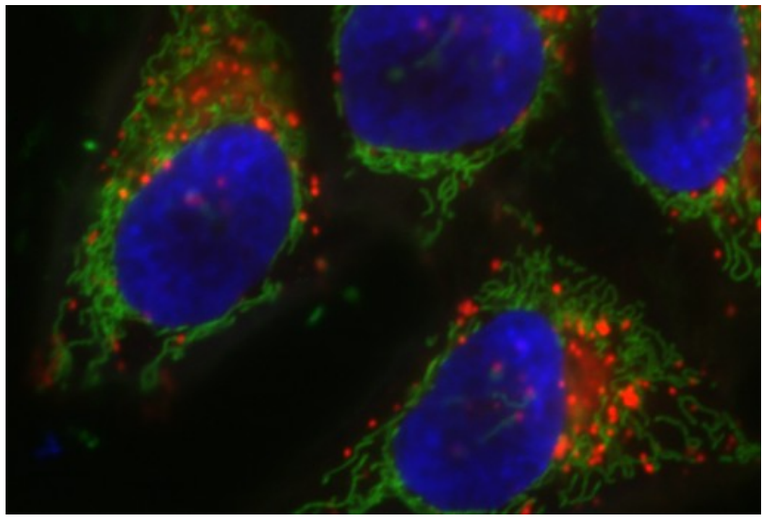


Find a way to visualise what you actually want to see:
Here, we don't care WHERE the beads are;
We care if they are in the same place or not!

Imaging Experiment Planning:

- ✓ What <u>BIOLOGY</u> am I trying to <u>measure</u>?
  - *Hypothesis?!!?*
- ✓ Do I need 3D, 4D, xD information
  - *Resolution?*
  - *Sampling: Space, Time, Intensity*
- ✓ Choose appropriate microscope
  - Don't always use Confocal LSM
- ✓ 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 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 1c

Getting to know "Fiji" better – Fiji is just ImageJ
http://pacific.mpi-cbg.de

File - Open Samples - Neuron

RGB colour space:

✓ **Colour channels** – ***Image-Colour-Channels Tool, Split channels etc.***

✓ **LookUp Tables/Paletts**: ***Image - Lookup tables, or LUT toolbar icon***

✓ **Line Profile**: ***Analyze – Plot Profile***

✓ **Histogramm**: **Analyze-Histogram or Plugins-Analyze-2D Histogram**

✓**Intensity Scale**: ***Analyze – Tools - Calibration bar***

Basics of Quantitative Image Analysis

What you need to know about Image Processing… but never thought to ask

… *continued*

Session 2
- ✓ Filtering Images in the spatial, frequency and time domain
- ✓ Segmentation – finding and measuring objects in images

Session 3
- ✓ Detect Info Loss, Colocalization Analysis and more
- ✓ Whatever you find interesting

CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics

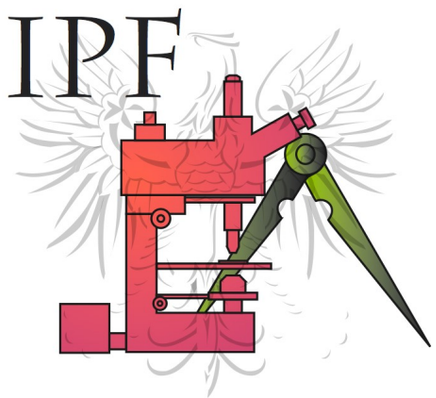
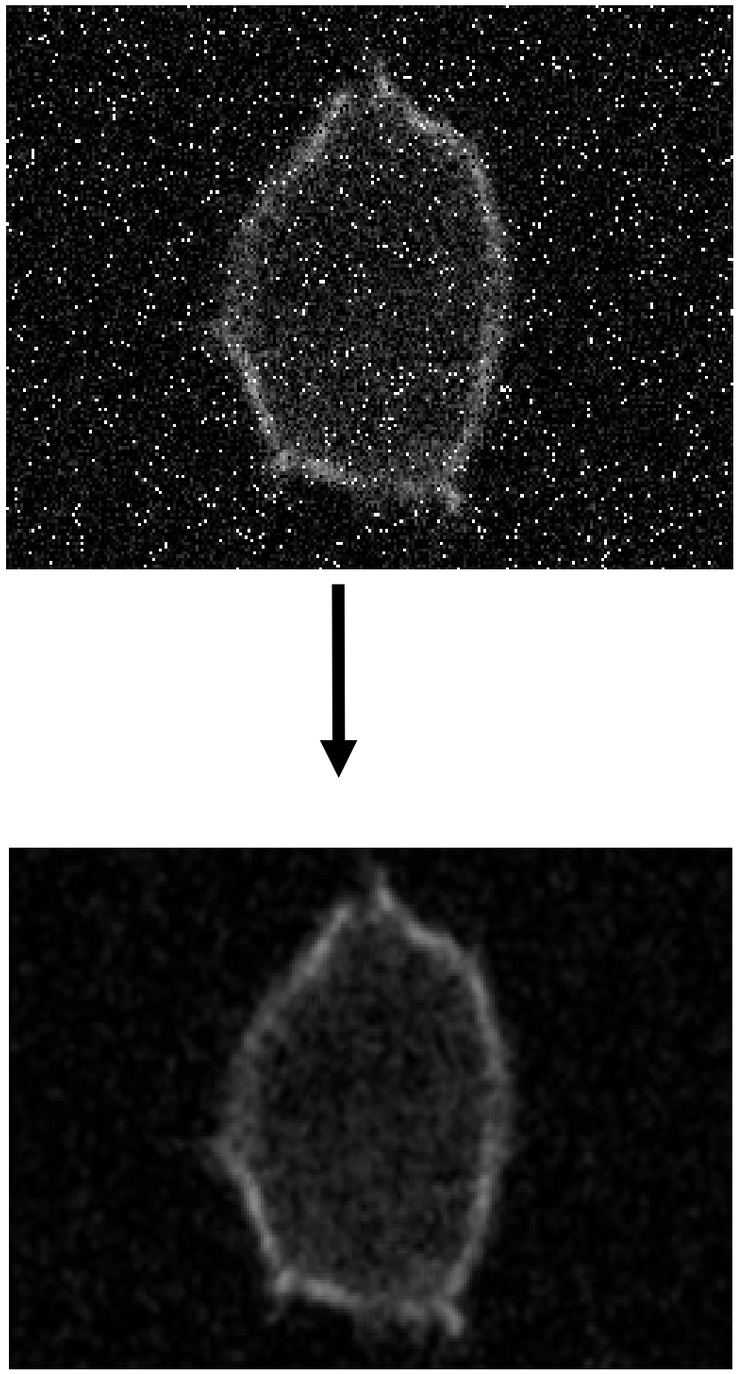


Image processing in the spatial domain

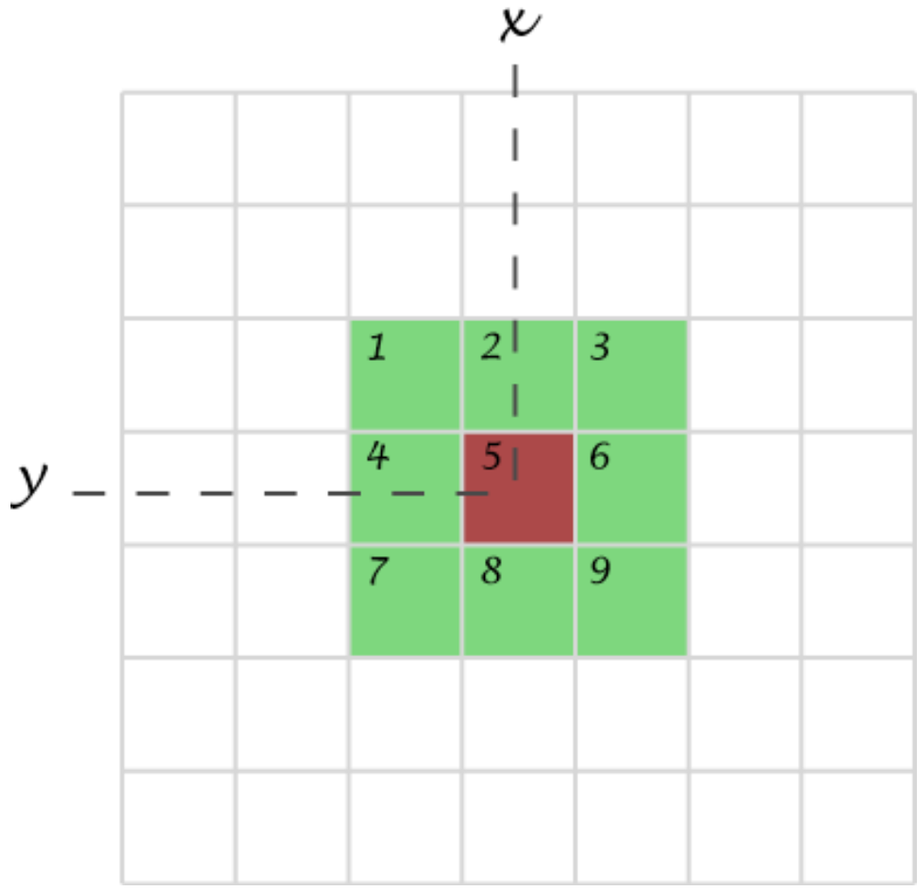


A) Introduction
   - Neighbourhood
   - Operation on neighbourhood

B) Spatial filters
   - Mean and Median filter
   - Edge detection

A. Introduction

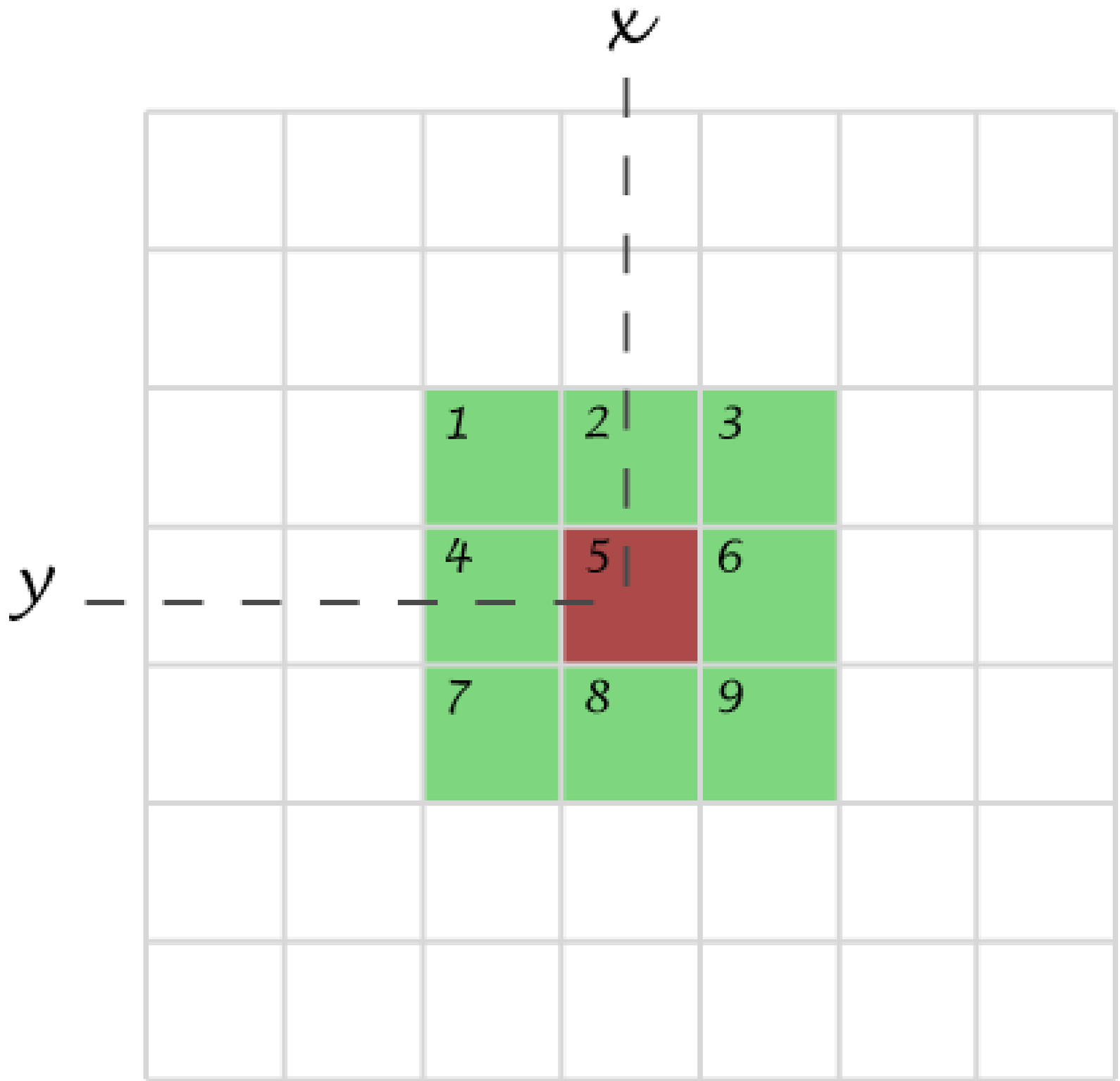
"Transformation or set of transformations where a new image is obtained by neighbourhood operations."



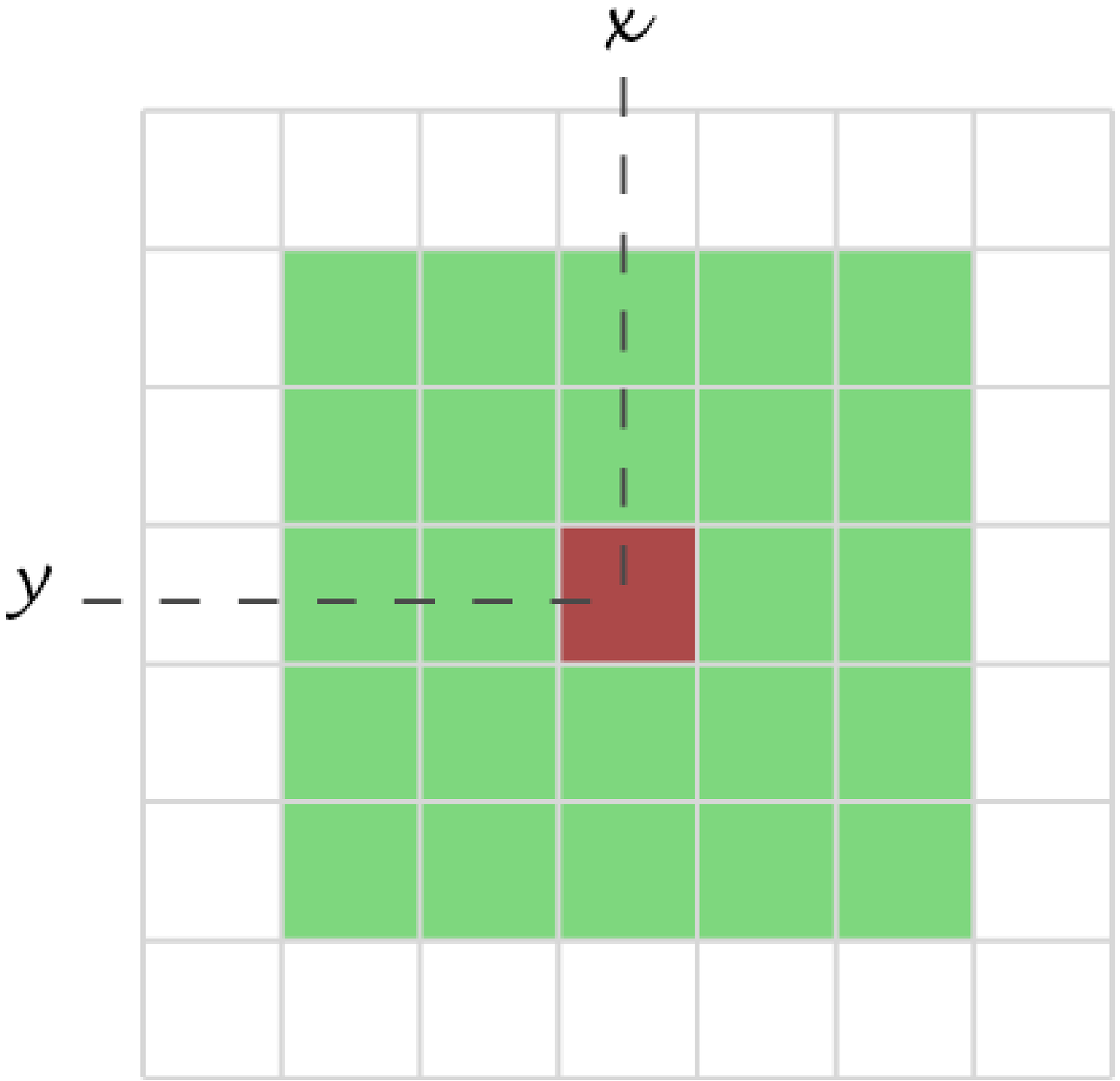
The Intensity of a pixel in the new image depends on the intensity values of "neighbour pixels"

Neighbourhood (or kernel): pixels that matter

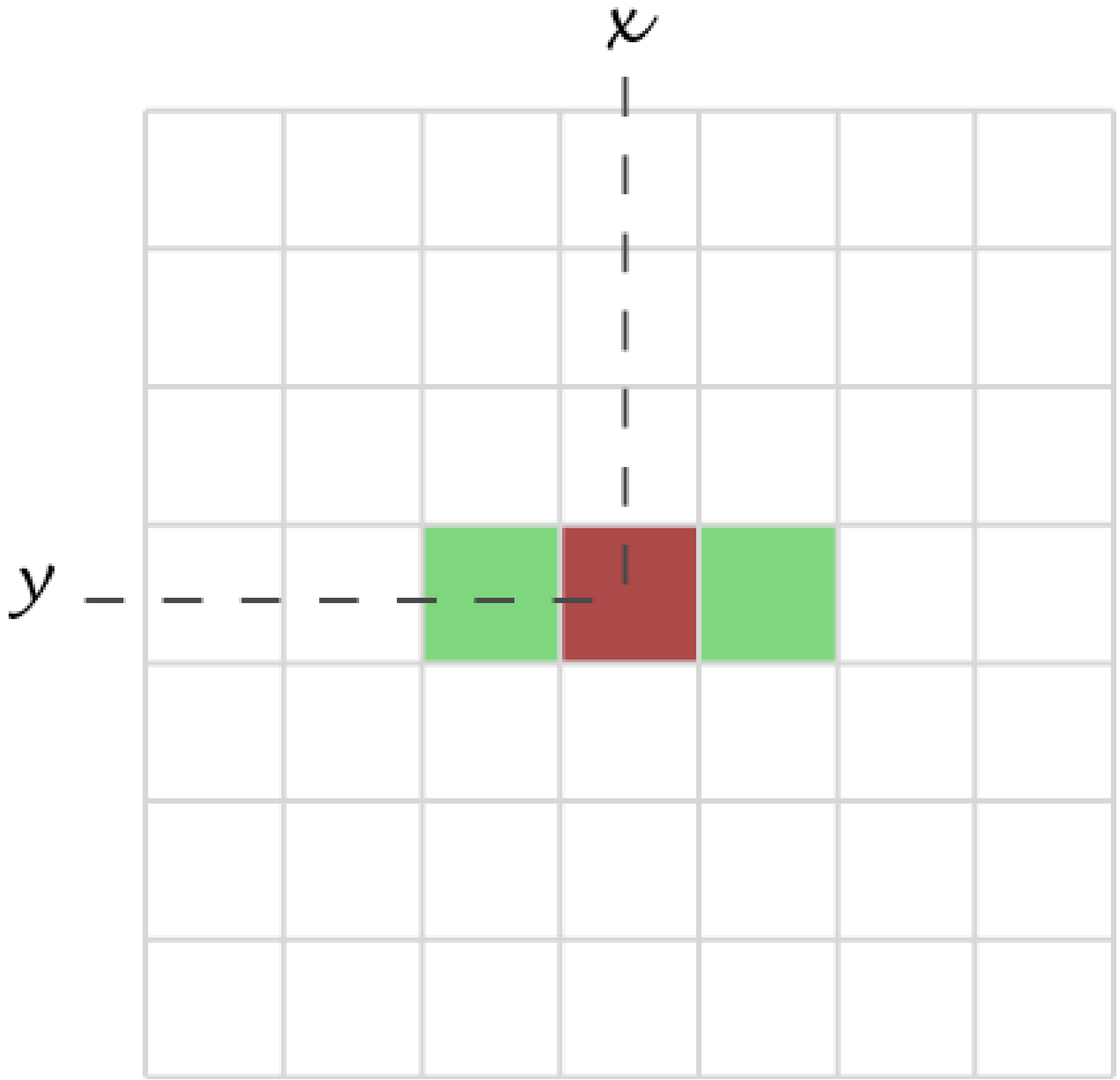
3 x 3



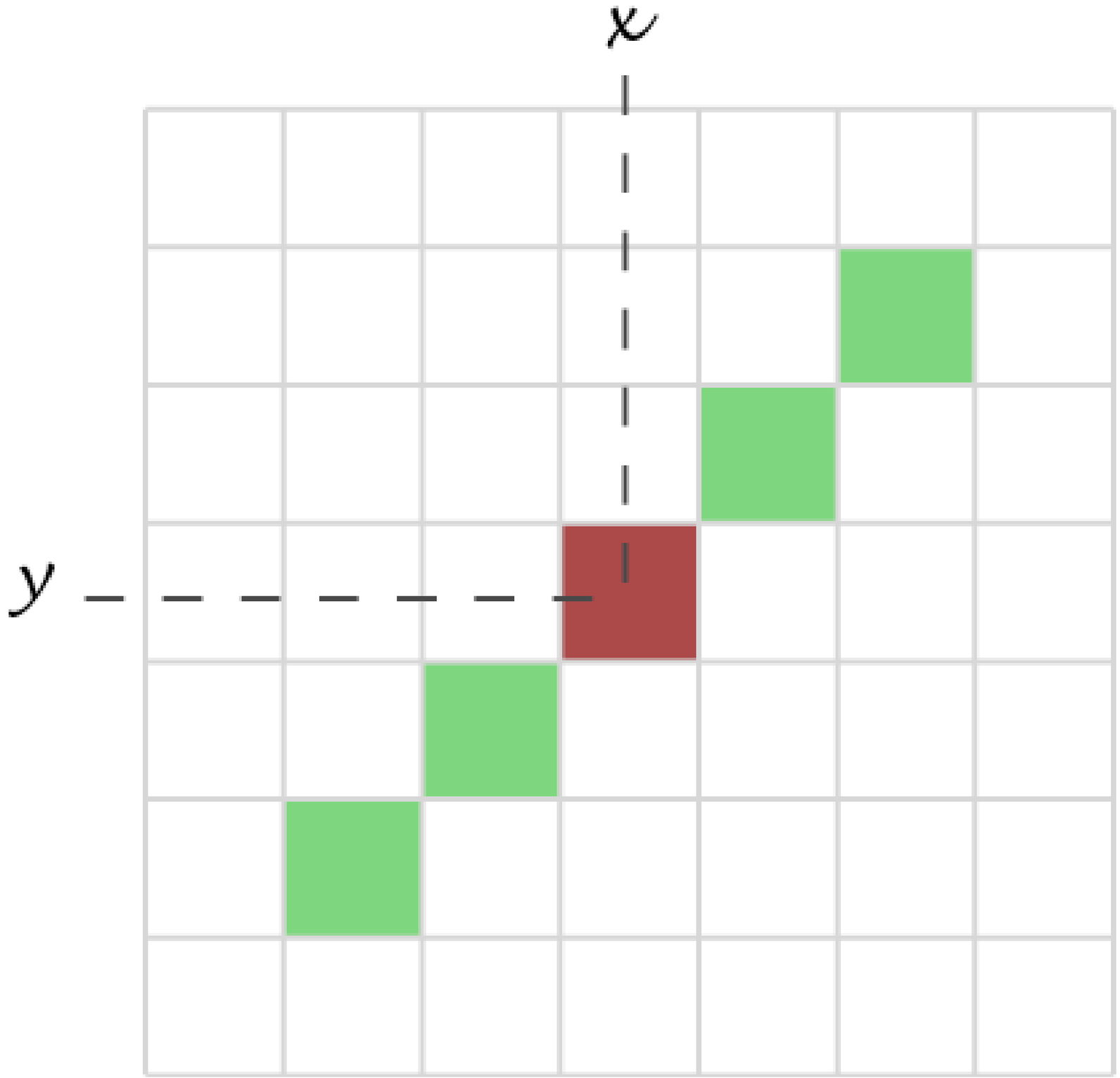
5 x 5



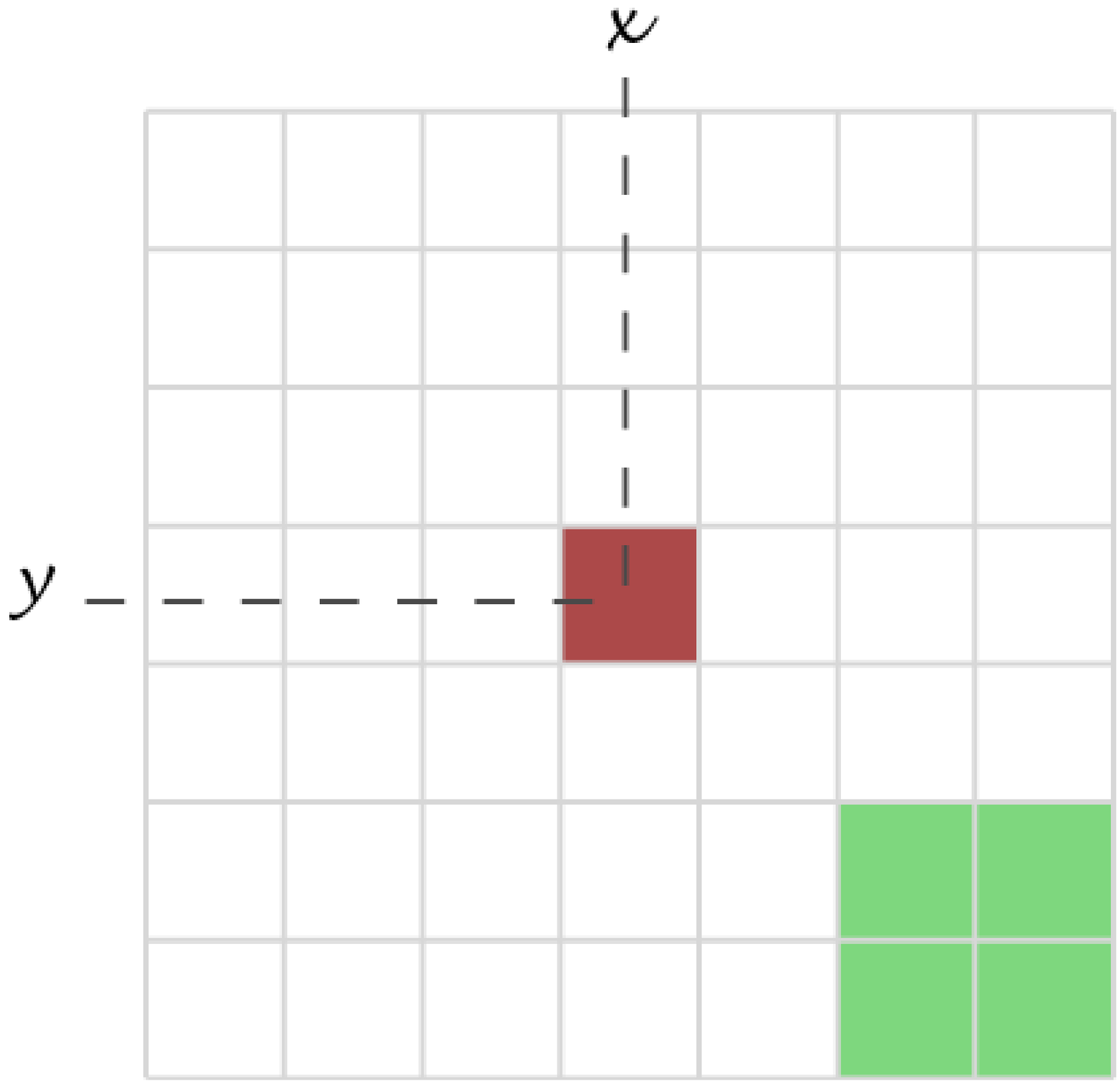
1 x 3



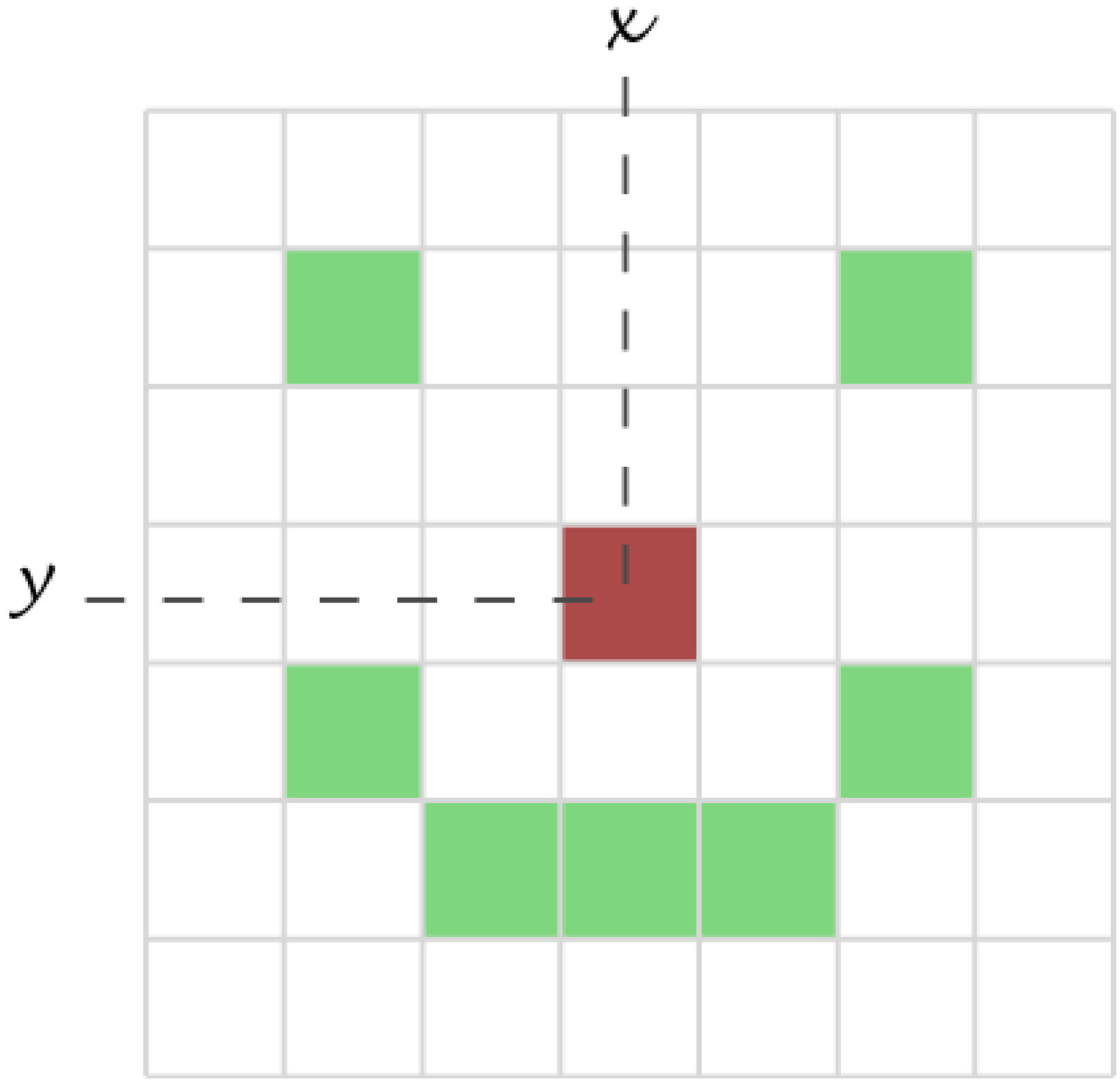
1 x 5



2 x 2 ; shift

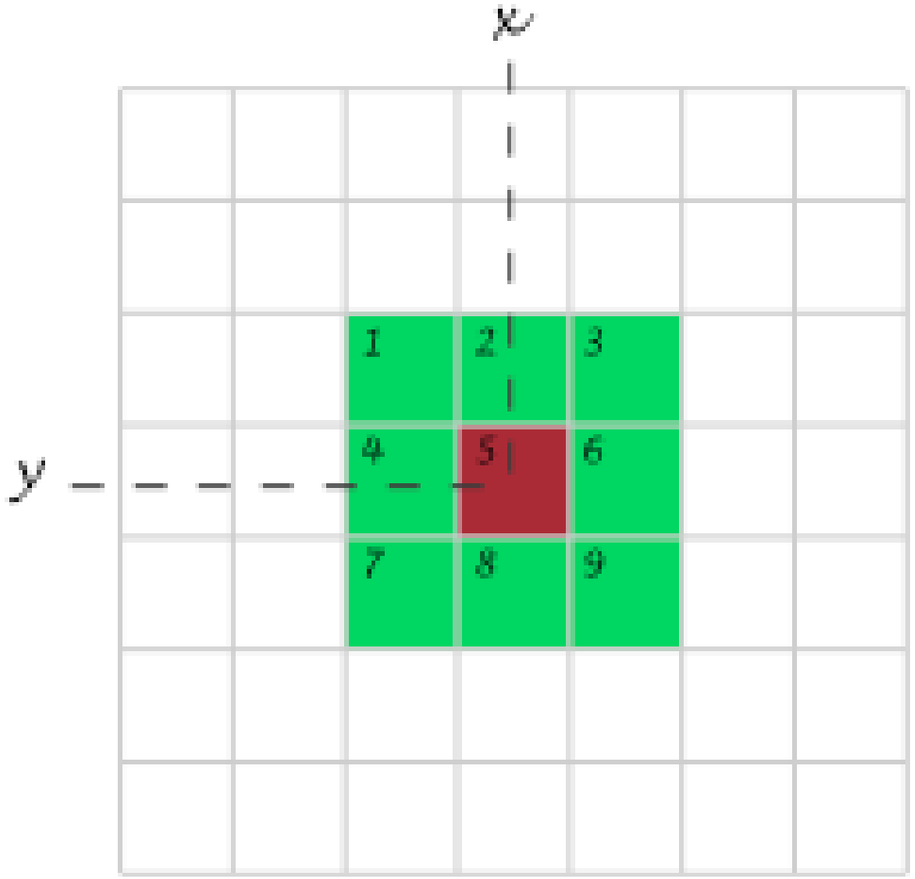


Misc.



B: Filtering - the mean filter

Simplest filter: The value of a pixel is replaced by the intensity mean of the neighbourhood pixels.

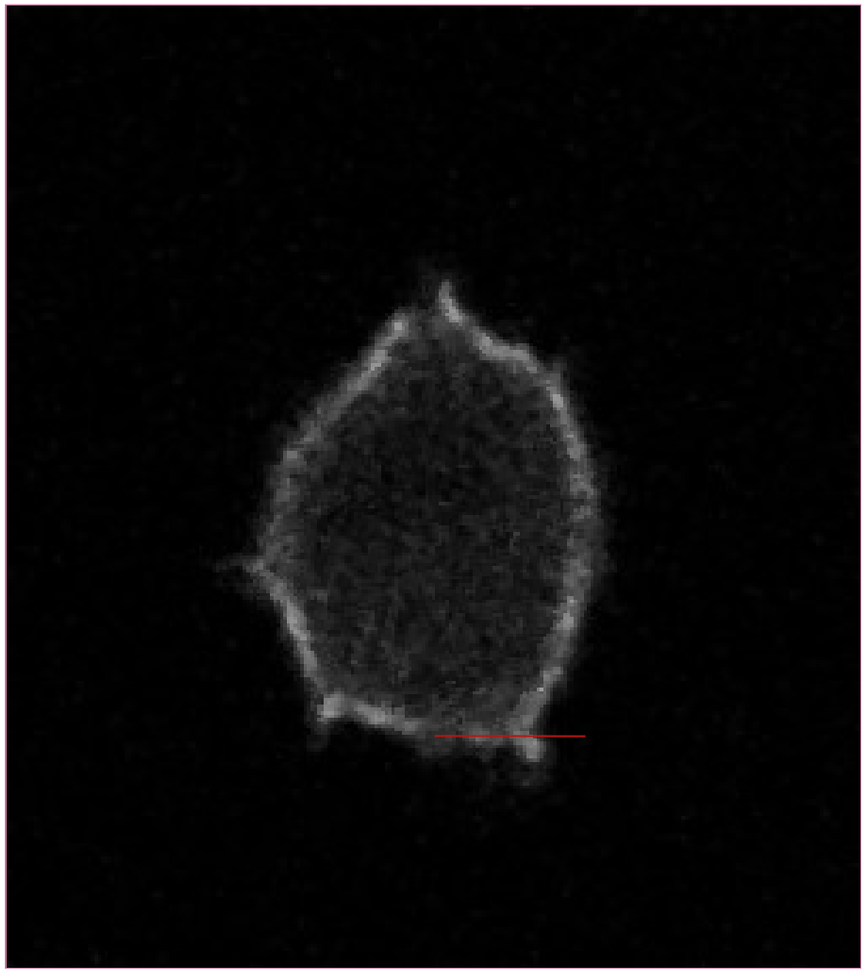


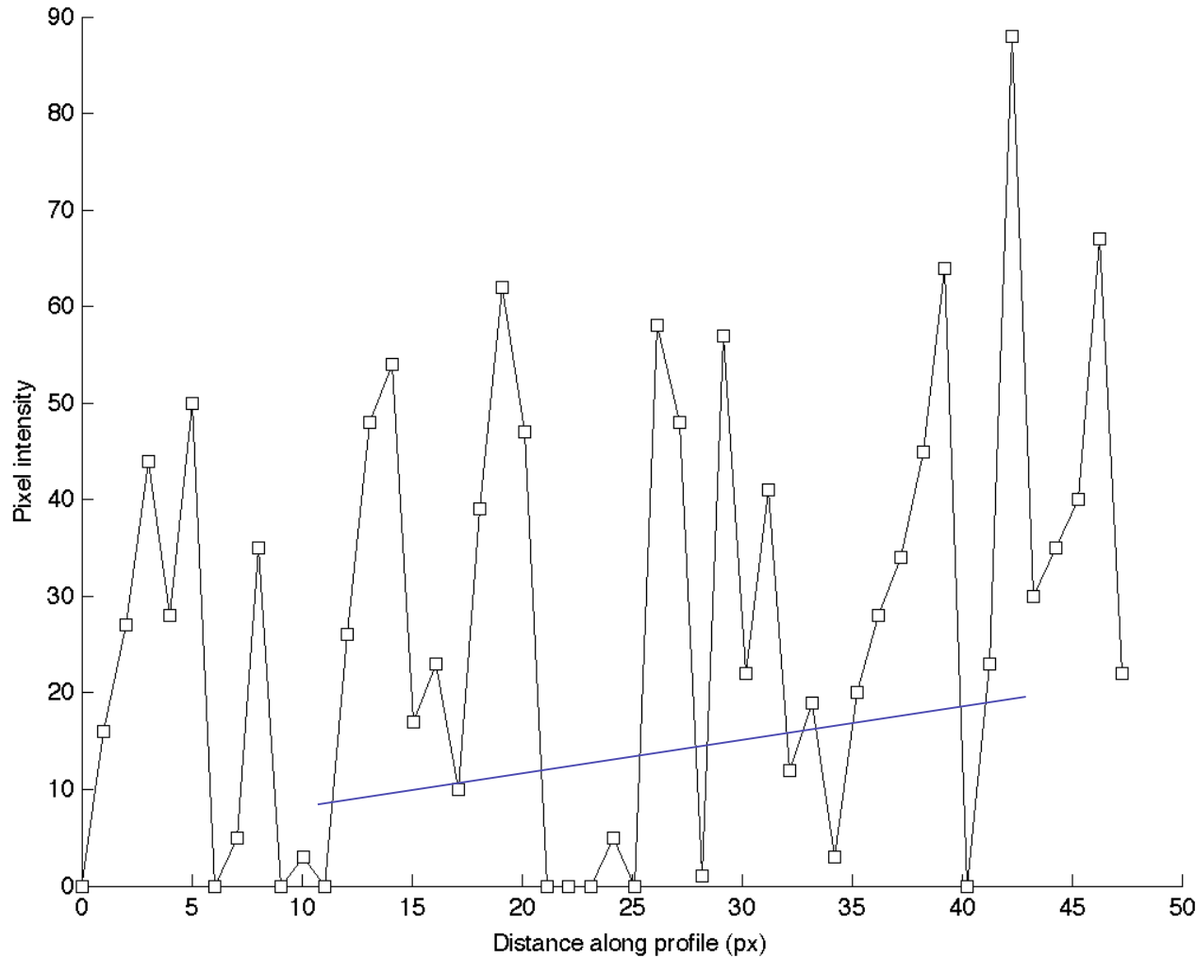
3x3 example:

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

The mean filter

Noise removal - typically Gaussian or Poisson noise.





Appears for weak labeling, short exposure time, confocal = few photons detected

The mean filter

The mean filter is a linear filter!

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

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

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

The mean filter

Main property: low-pass filter (smooths small objects)
- kernel size influence
- number of successive applications

+ simplest filter – fast

+ it's a linear filter

+ averages noise, does not eliminate it

+ works against Gaussian and Poisson noise

- blurs images - small details are lost (low pass filter)

- smoothes edges dramatically

- fails for salt & pepper noise

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.

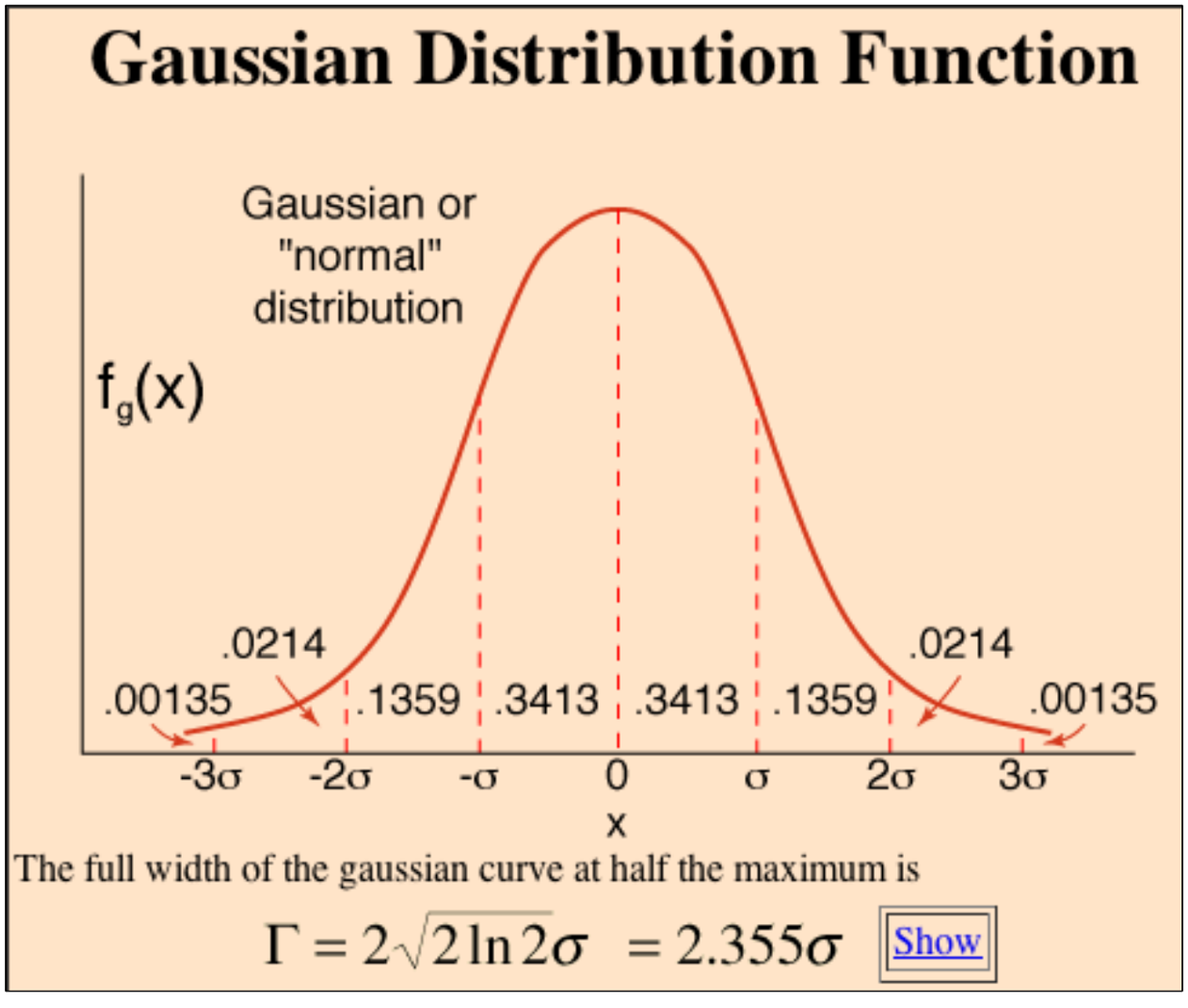
✓ Mathematical framework underlying it: Convolution.

✓ We can also reverse the process : Deconvolution

The Gaussian filter

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
- ✓ varying degree of blur.



The median filter

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

Take neighbourhood (e.g. 3x3)

Sort it

Take median

5	112	86
235	88	211
137	233	108

5
86
88
108
112
137
211
233
235

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

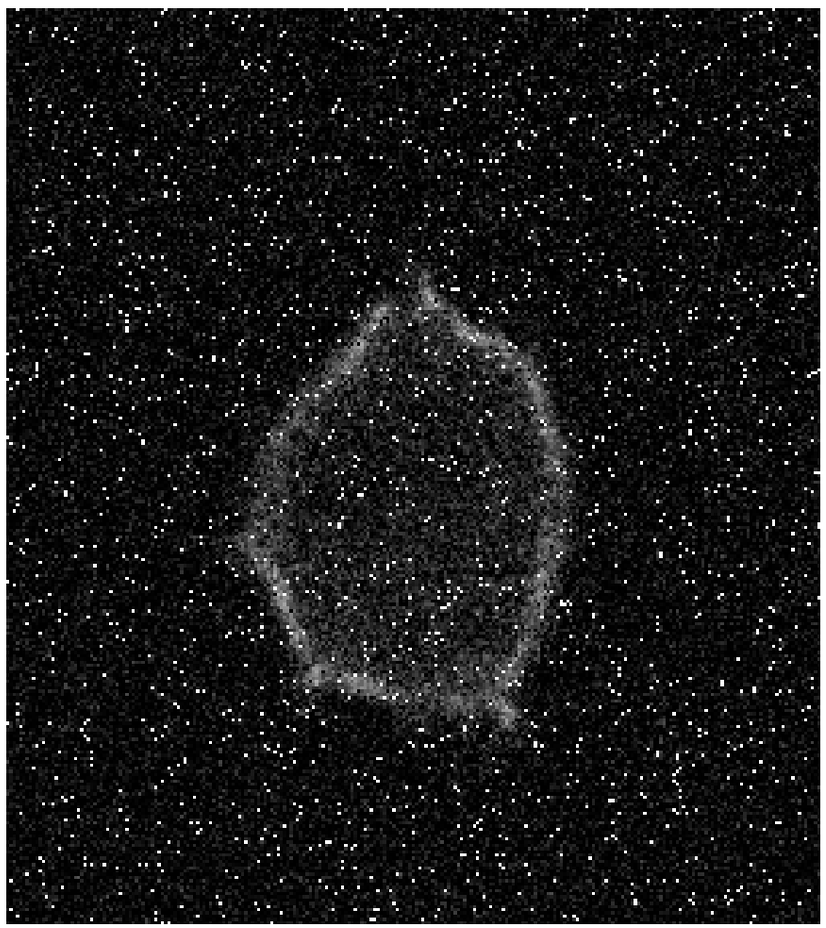
The outlier value has been completely removed from the dataset

The median filter - what is it good for?

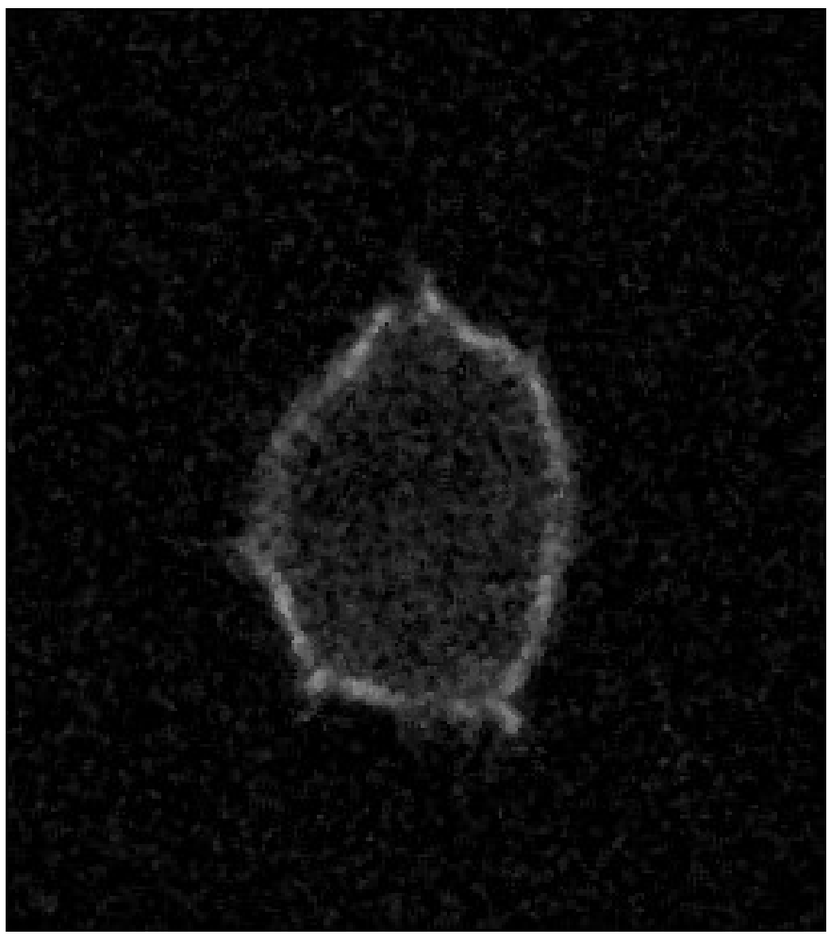
"Salt & pepper" noise * removal

* Typically appears for very weak labeling, high detector gain etc.

Original:



Median filtered:



The median filter

+ Typically good for “Salt & pepper” noise removal

+ Eliminates noise

+ Edge-preserving

-Slower than mean (not such a problem anymore... computers are fast)

- NOT linear

Practical Session 2a

Simple Image Filtering

(1) File - Open Samples – bat cochlea volume

(2) File – Import – URL…

http://pacific.mpi-cbg.de/samples/colocsample1bRGB_BG.tif

(1) Convolve a simple binary image

✓ Process – Filters – Convolve (play with different kernels)

✓ *Process – Filters – Gaussian Blur (change sigma, in px)*

(2) Noisy sample image

✓ Mean and Median Filter (change pixel number, kernel size)

✓ Gaussian Blur … and Gaussian Blur again… and…

Morphological Filters

Binary Images
(plus variants for grayscale images)

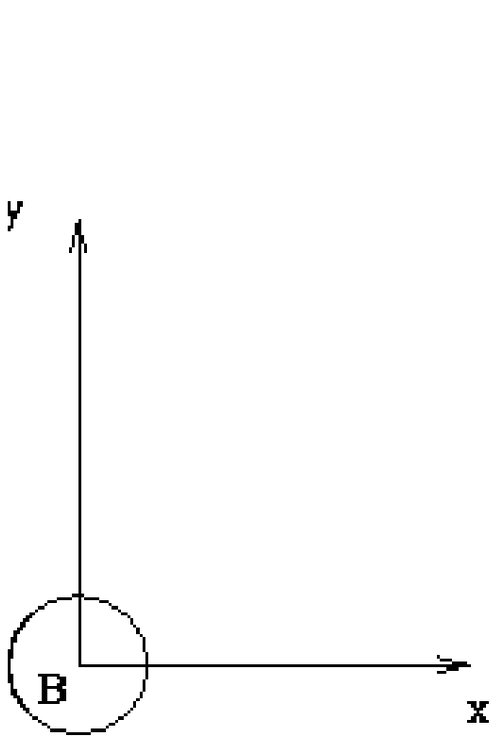
Erode
Dilate
Open
Close

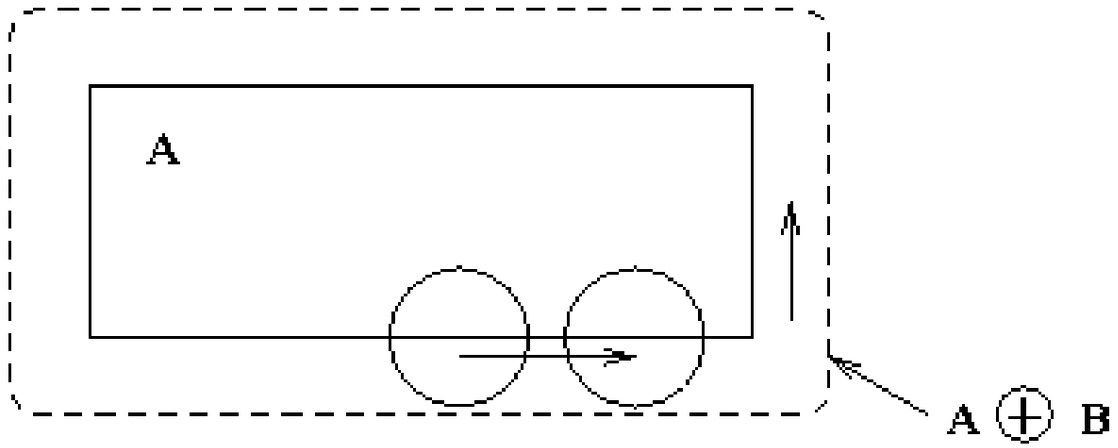
QuickTime™ and a
decompressor
are needed to see this picture.

... done using spatial filters - kernels

Morphological Filters

Erode: Removes pixels from the edges of objects.



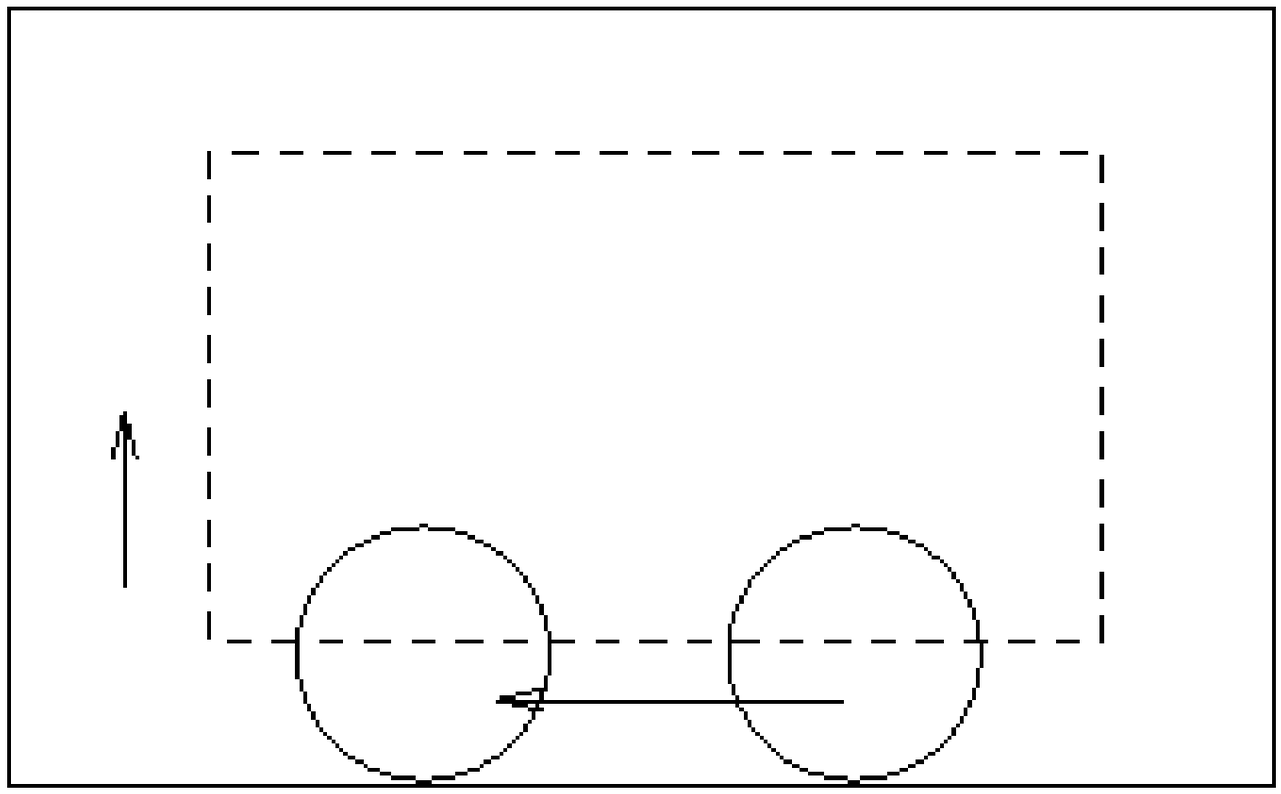


QuickTime™ and a
decompressor
are needed to see this picture.

The size and shape of the kernel matters!

Morphological Filters

Dilate: Adds pixels to the edges of objects.



QuickTime™ and a
decompressor
are needed to see this picture.

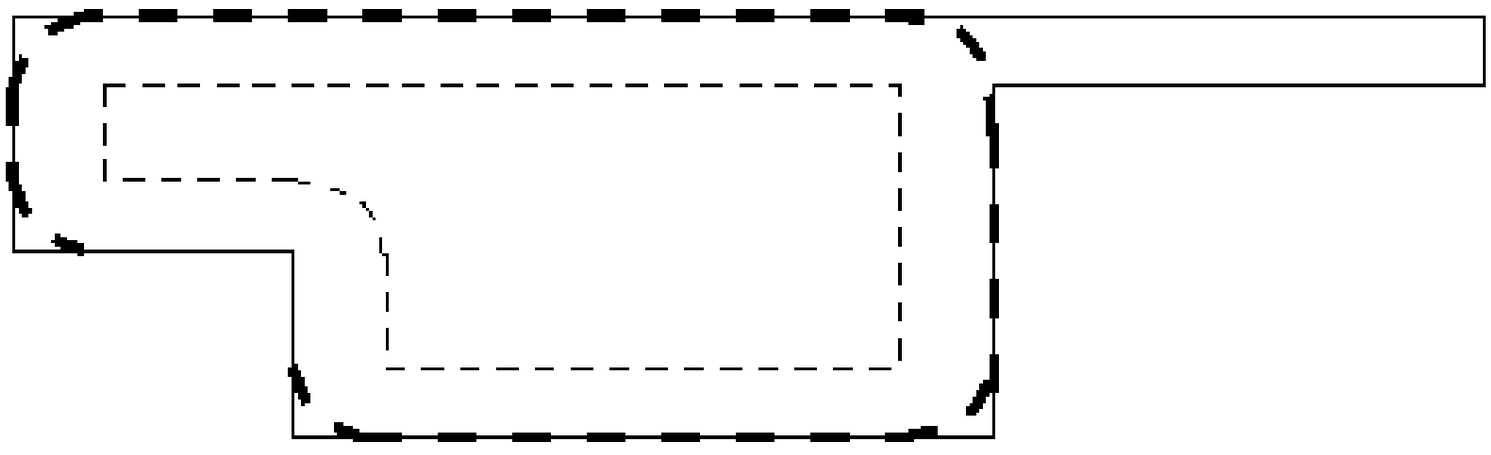
Again, the size and shape of the kernel matters!

Morphological Filters

Open:

Performs an erosion operation,
followed by dilation.
This smoothes objects
and removes isolated pixels.

$$A \circ B = (A \ominus B) \oplus B.$$

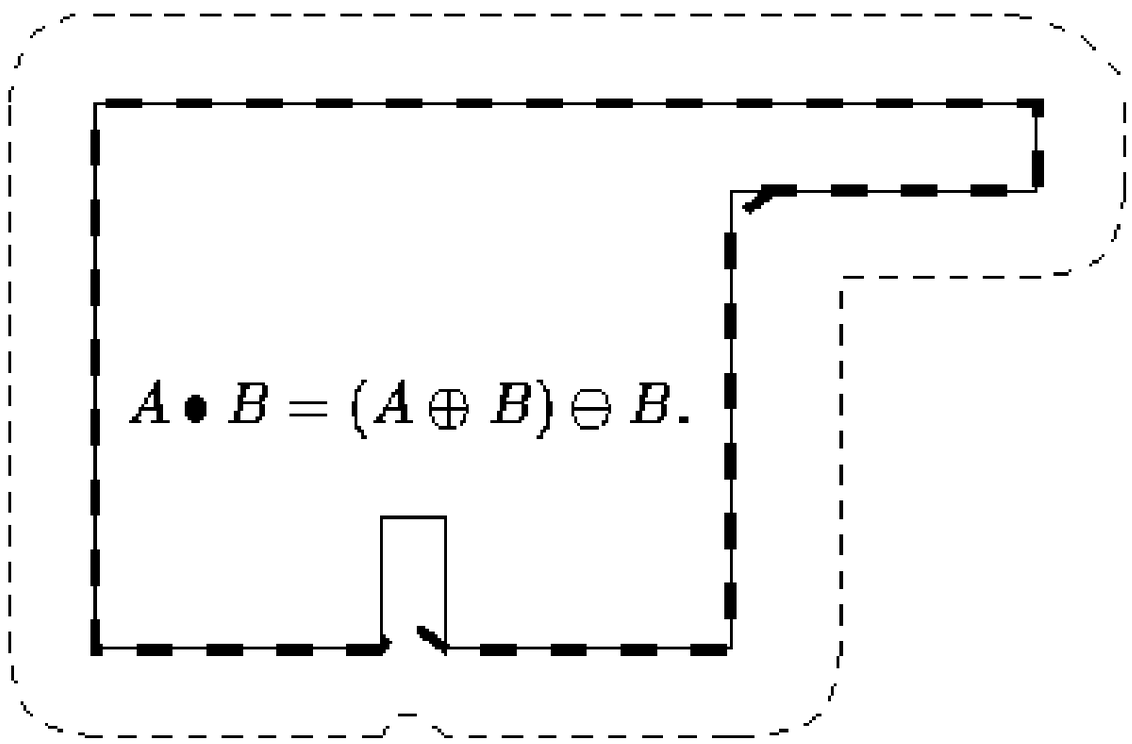


Again, the size and shape of the kernel matters!

Morphological Filters

Close:

Performs a dilation operation,
followed by erosion.
Again, this smoothes objects
and fills in small holes, but differently.



QuickTime™ and a
decompressor
are needed to see this picture.

Again, the size and shape of the kernel matters!

Morphological Filters

In Fiji/ImageJ - Greyscale images:

Use Maximum and Minimum filters for Dilate and Erode respectively.

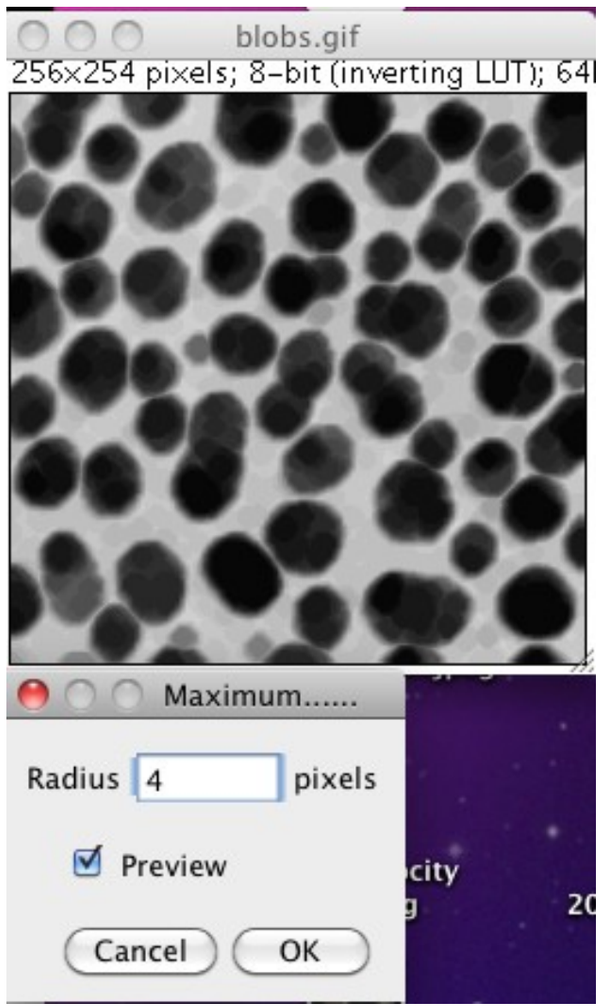
QuickTime™ and a decompressor are needed to see this picture.

Minimum...

grayscale erosion: replace each pixel with the min pixel value of pixel's neighborhood.

Maximum...

grayscale dilation: max pixel value of pixel's neighborhood.



Morphological Filters

Options...

Settings for Binary submenu commands

Iterations: the number of times performed.

Count: the number of adjacent "other" pixels necessary before a pixel is + or - from object edge

Check **Black background** if the image has white objects on a black background.

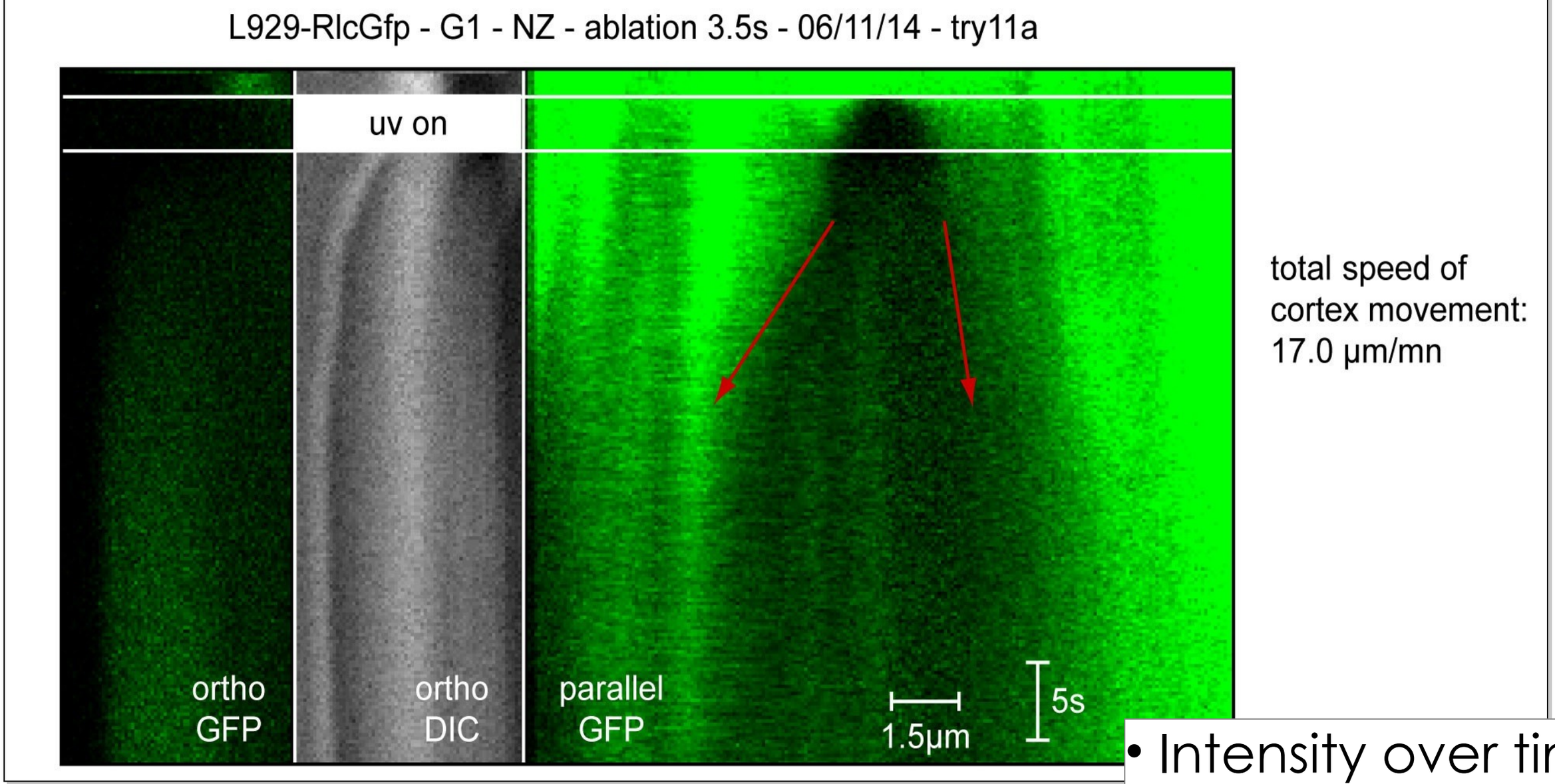
If **Pad edges** when eroding is checked, Process>Binary>Erode does not erode from the edges of the image.

Also affects Process>Binary>Close: erodes from the edges unless this checkbox is selected.

QuickTime™ and a
decompressor
are needed to see this picture.

Time? Just another dimension

Dealing with multiple images files:
time stacks, timelapse movies, 3D stacks, …



- Intensity over time
- Kymographs

Motion blur

Motion blur = average over time

Does this happen in your sample? Frame Rate?

Practical Session 2b

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

File - Open Samples - Bridge

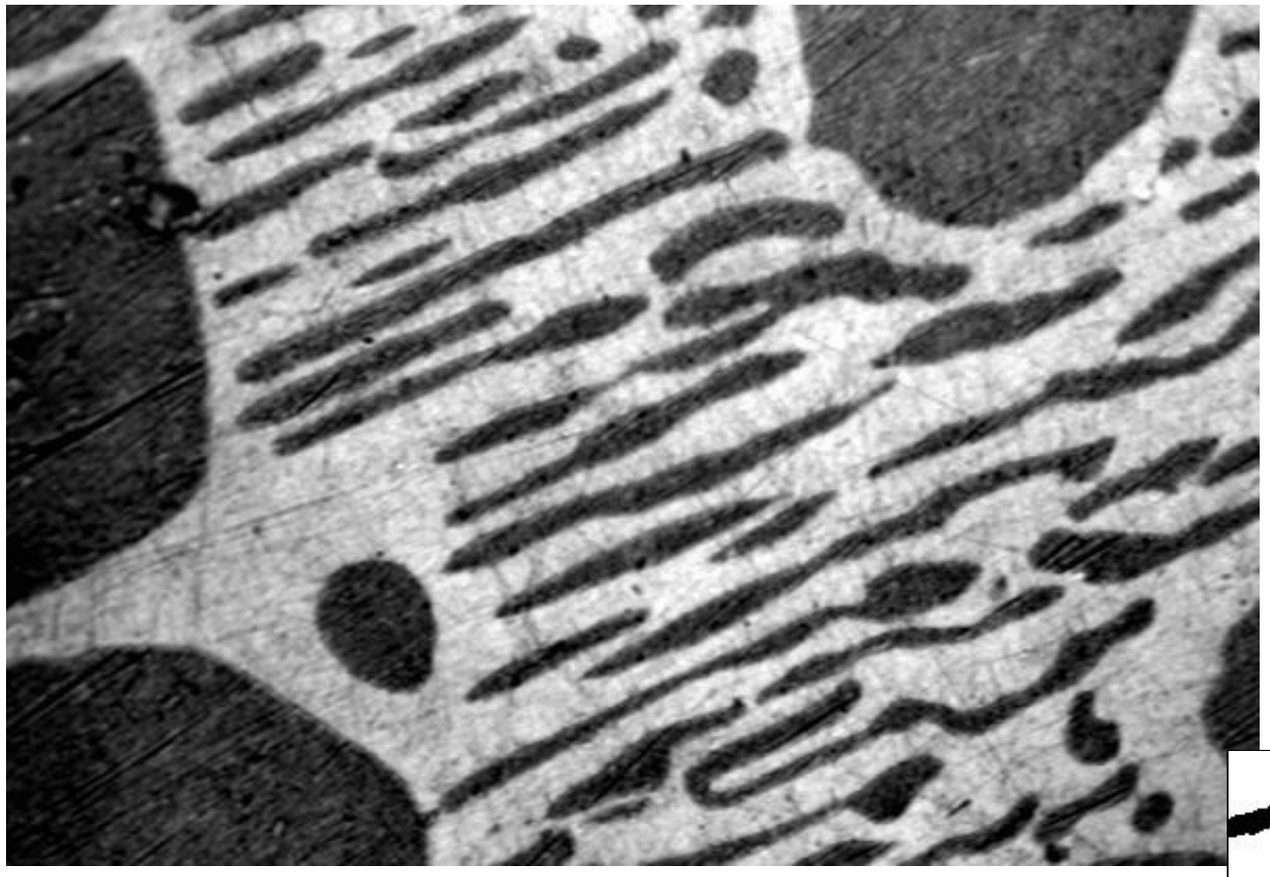
Fourier Image Filtering

✓ FFT, filter out parts, Inverse FFT: *Mess up the image. Can you extract high and low frequency information?*

✓ Use circle selection and Edit - Fill: *Set foreground colour to black.*

✓ FFT bandpass filter

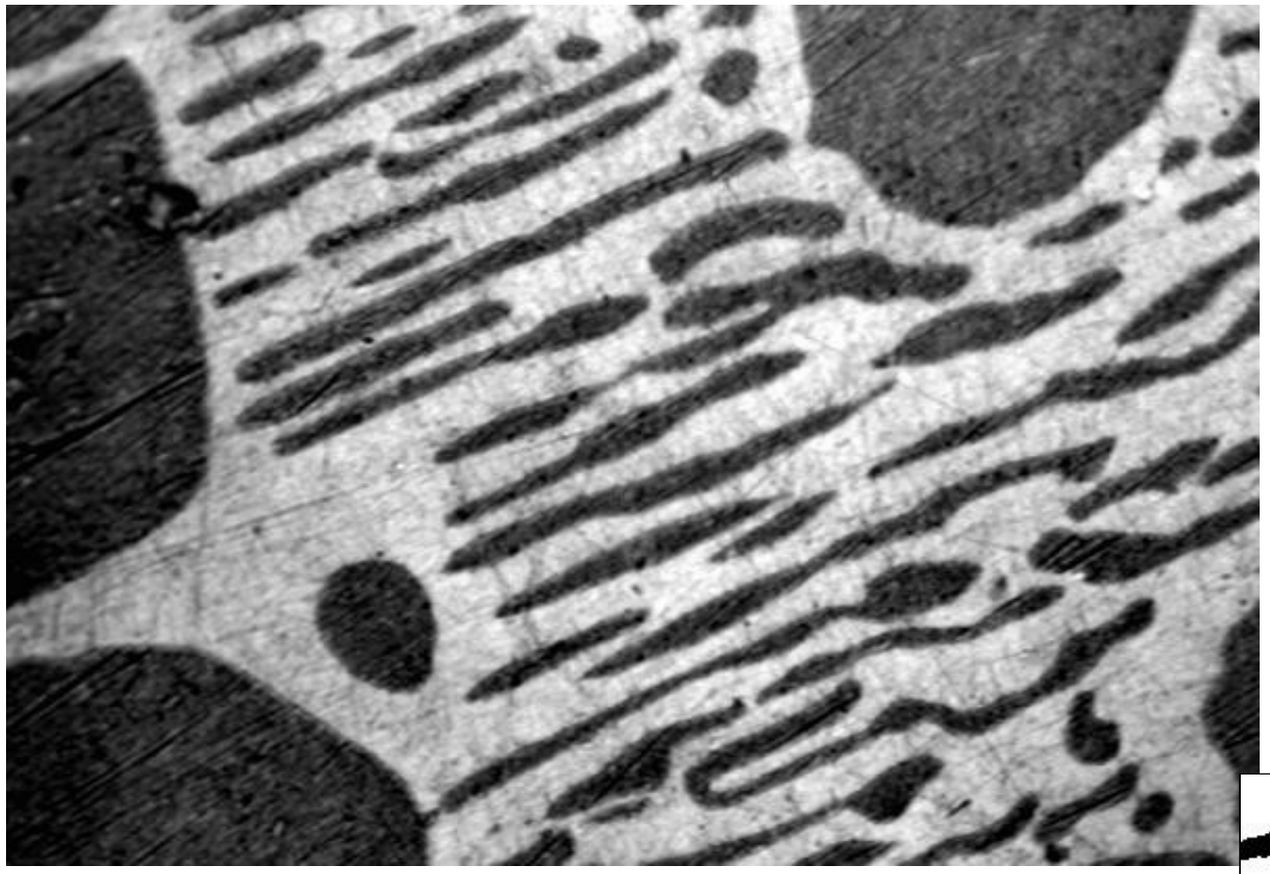
What is “Image Segmentation”?



“Greyscale” image

Foreground background

What is “Image Segmentation”?



“Scalar Intensity” image

“Binary” image

What is “Image Segmentation”?

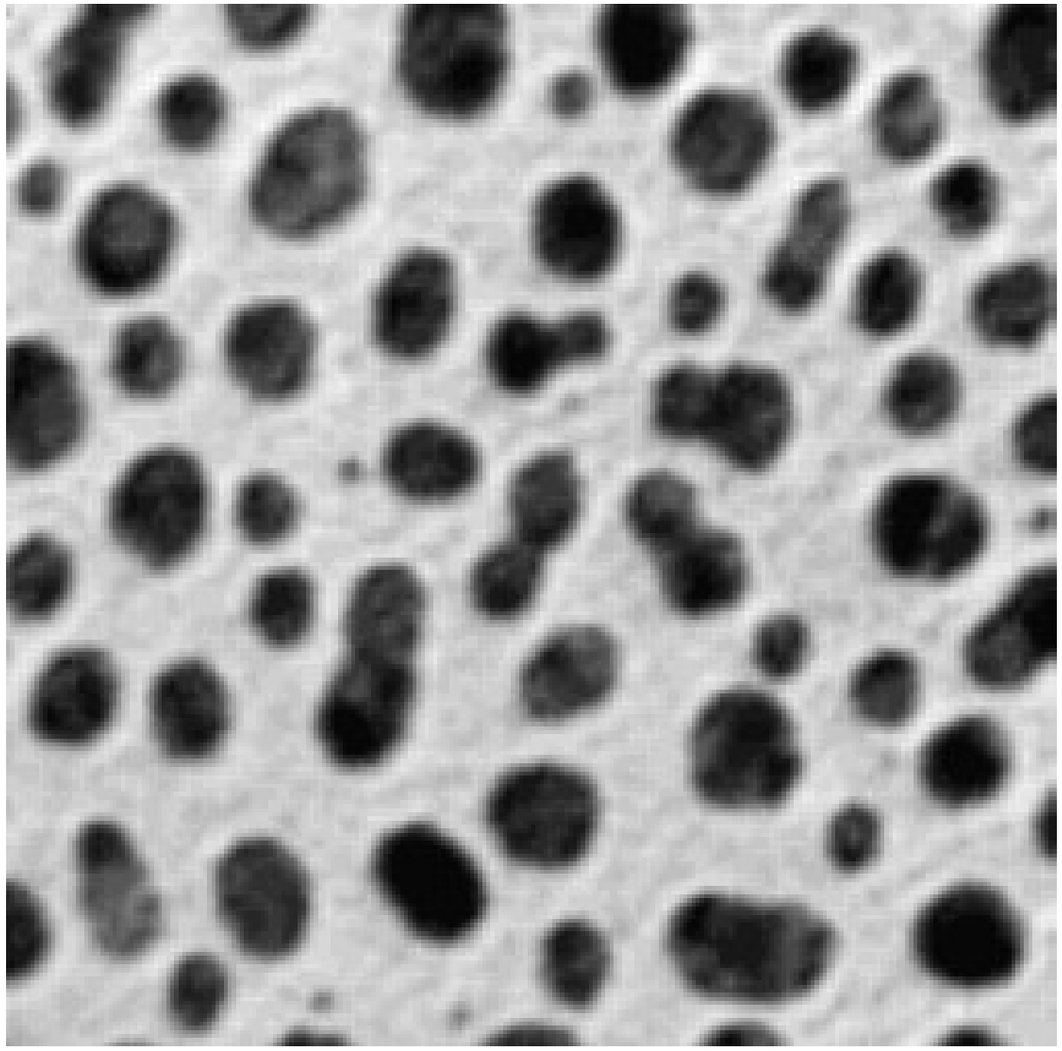
1	65	13	55	2
2	3	34	2	1
4	0	31	1	2
1	33	3	54	3
56	3	2	1	34

“Scalar Intensity” image

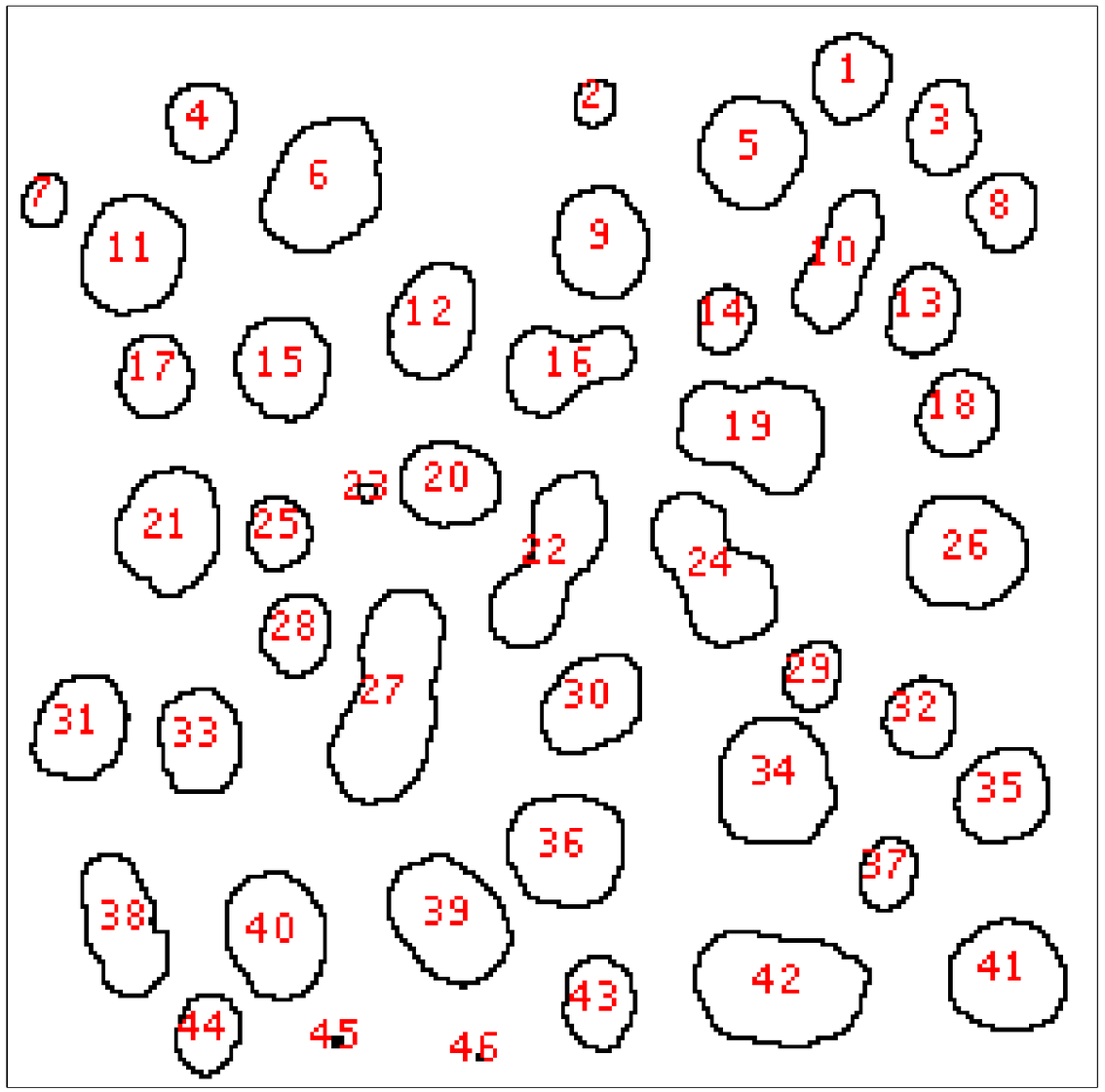
0	1	1	1	0
0	0	1	0	0
0	0	1	0	0
0	1	0	1	0
1	0	0	0	1

“Binary” image

What is “Image Segmentation”?

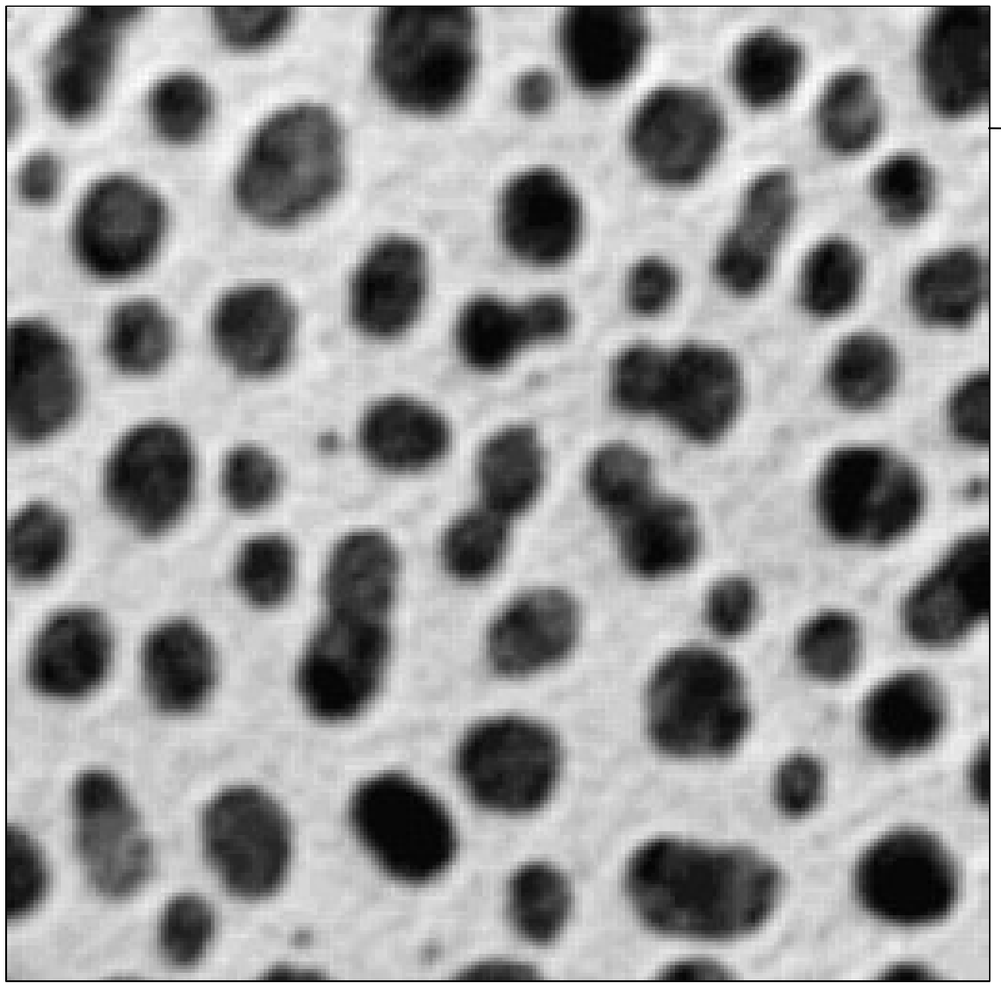


“Scalar Intensity” image

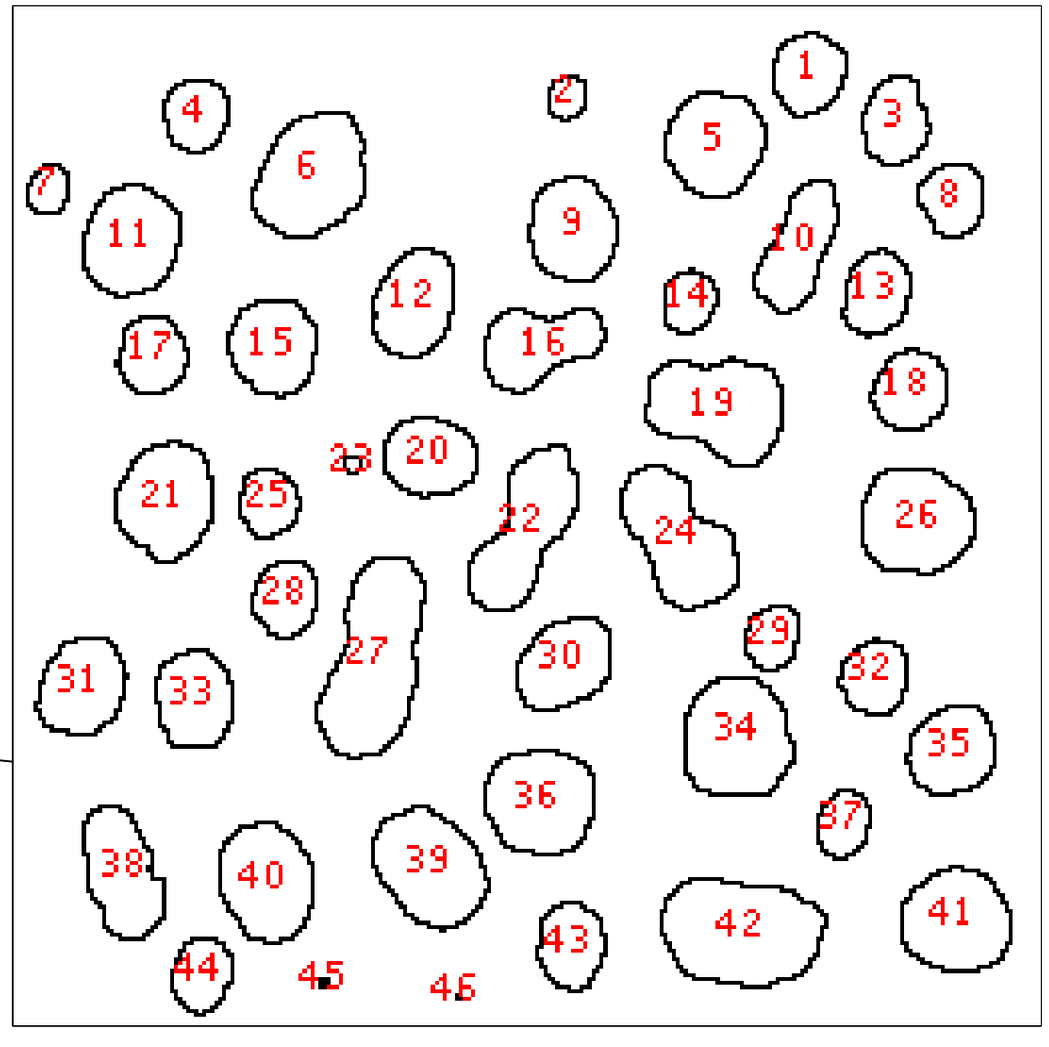


Labeled objects

What is “Image Segmentation”?

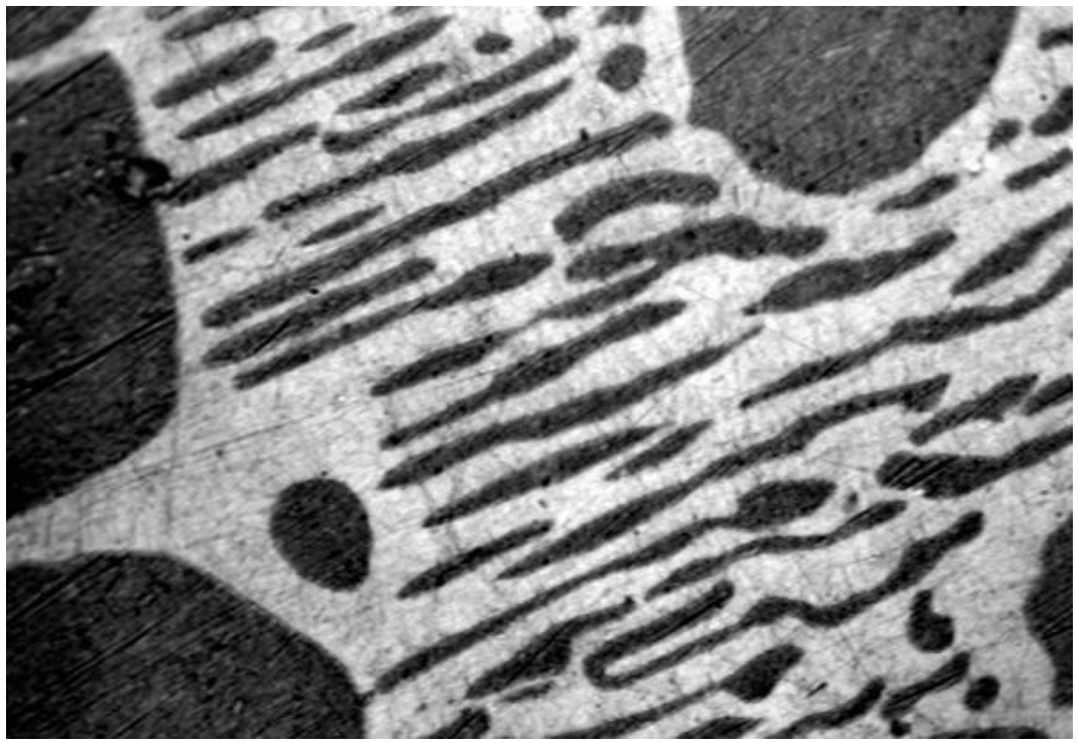


High Information Content
65536 pixels, 0-255 value



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

“Thresholding” (Intensity Histogram Split)



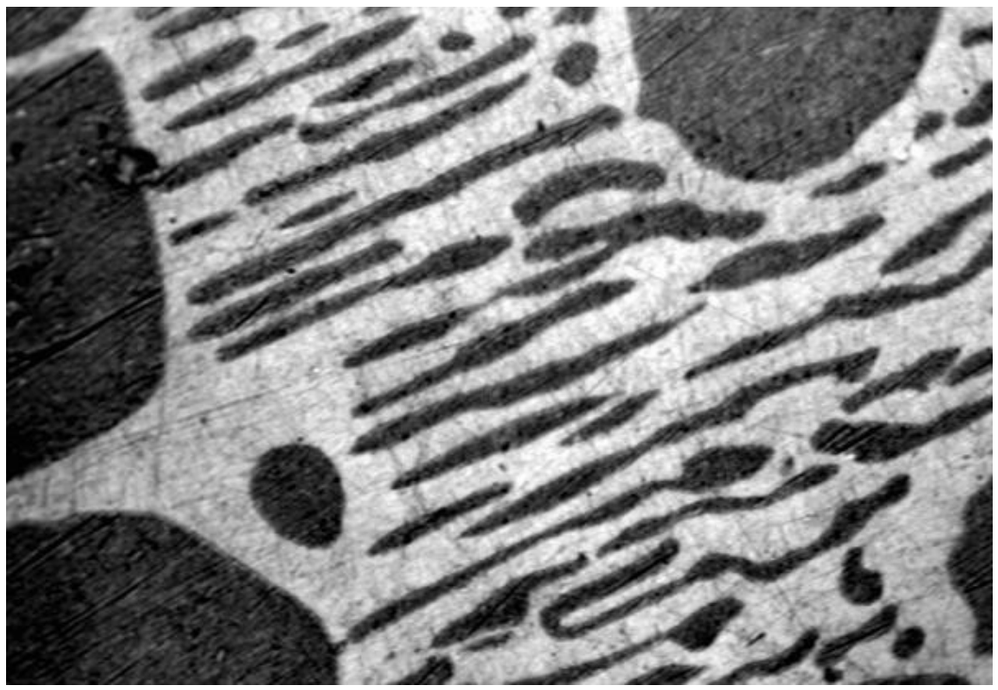
Clear difference between foreground and background?

Image not very noisy?

Choose an intermediate grey value = “threshold”

Determines foreground and background.

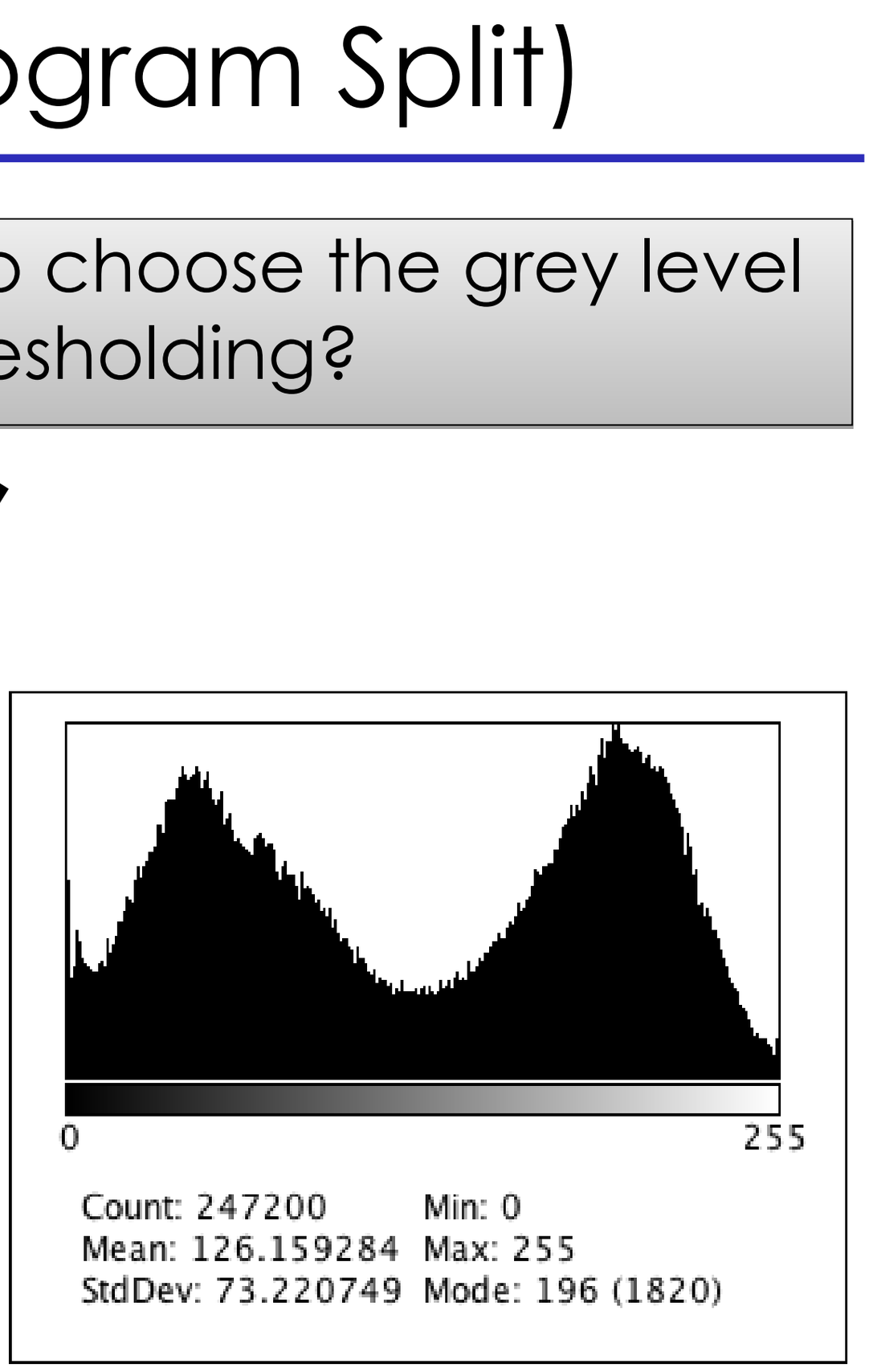
"Thresholding" (Intensity Histogram Split)



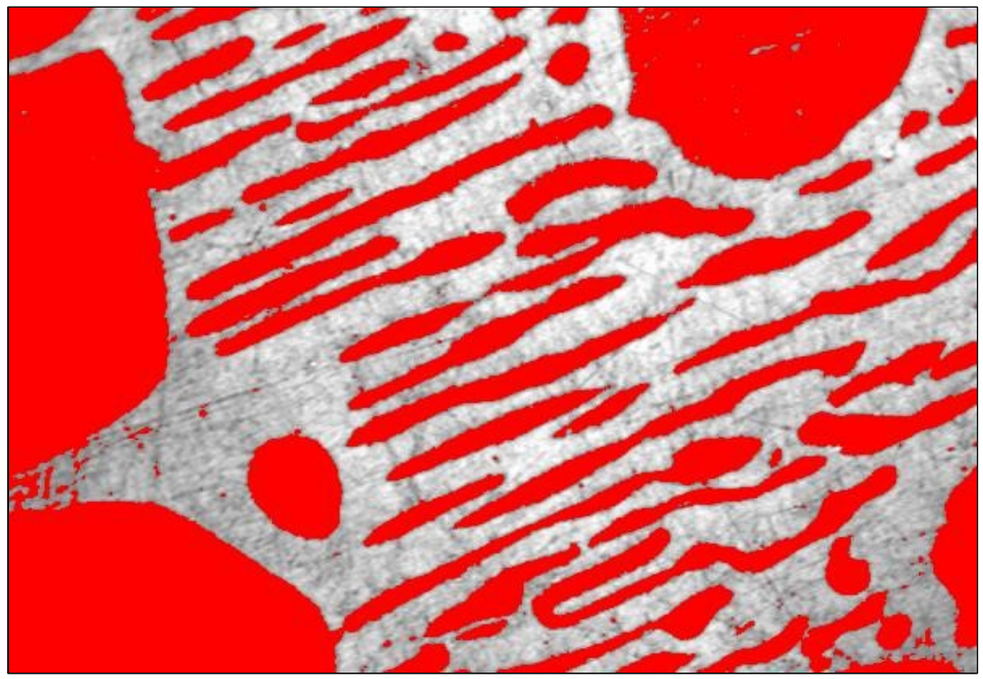
How to choose the grey level for thresholding?

Look at pixel intensity histogram of whole image…

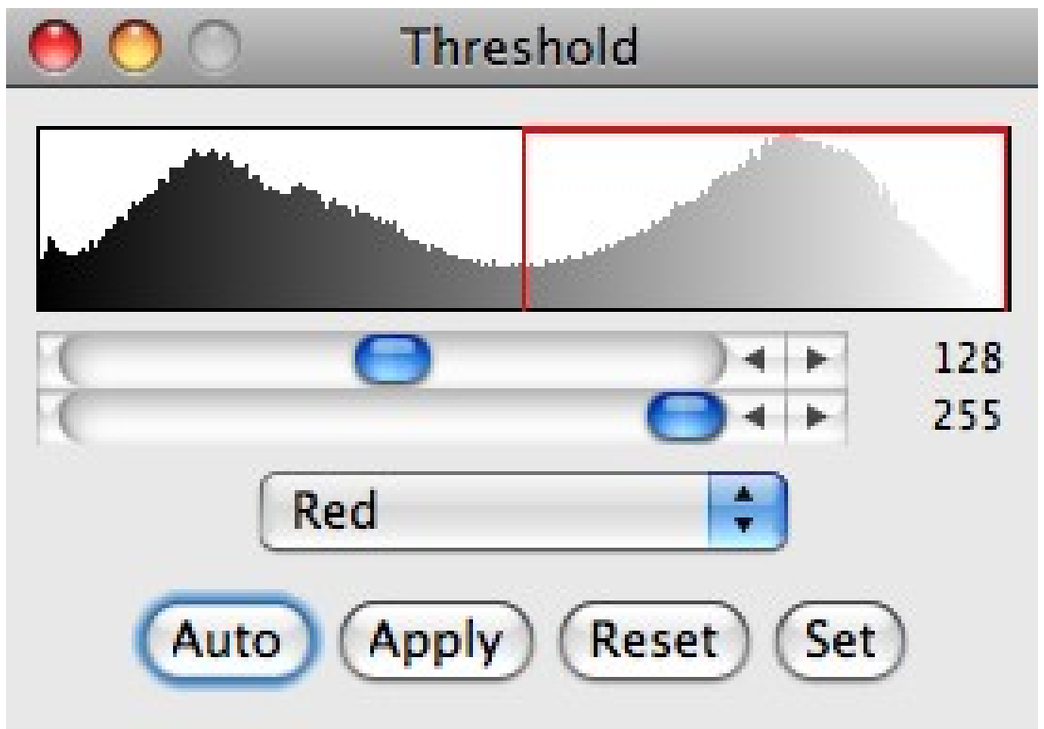
Is there an obvious place?



"Thresholding" (Intensity Histogram Split)

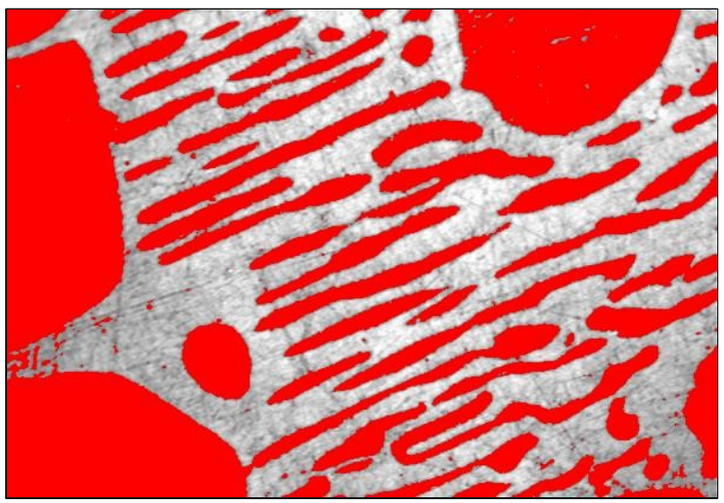


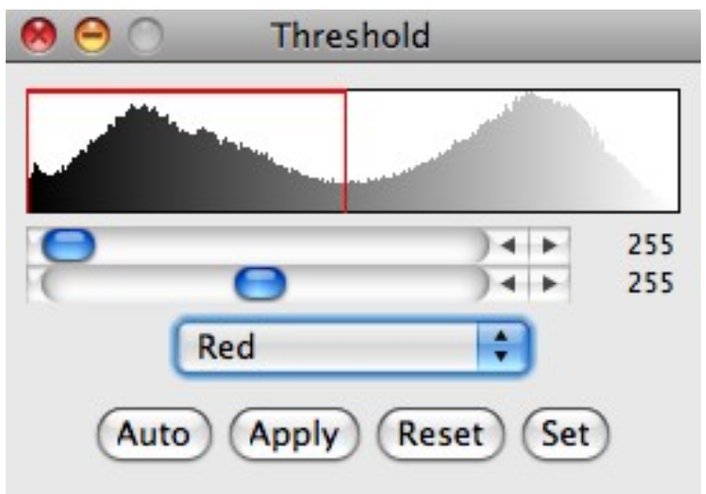
Histogram is bimodal, so put threshold in the trough between the peaks!

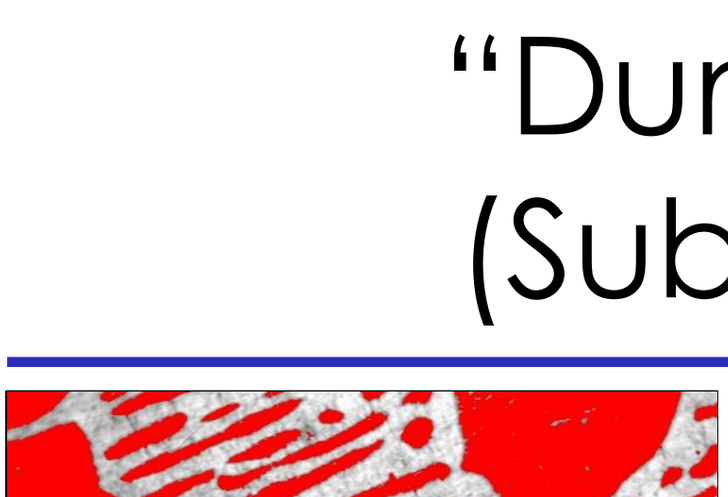


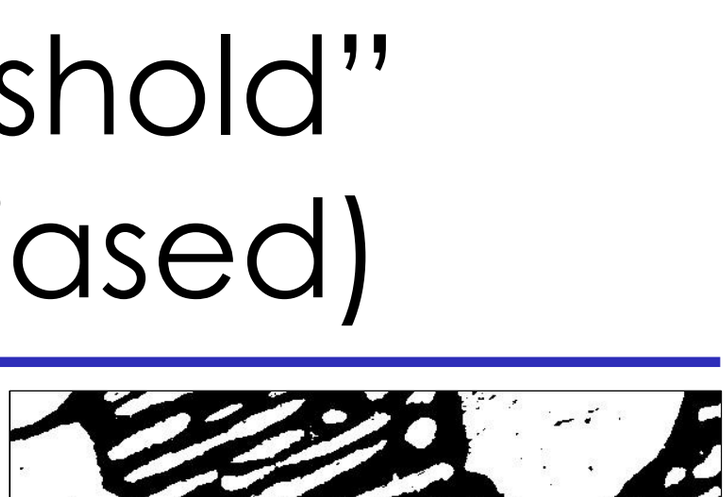
Note, in this case:
Foreground = "dim" objects
Background = "bright" objects

“Dumb Global Threshold” (Subjective - User Biased)



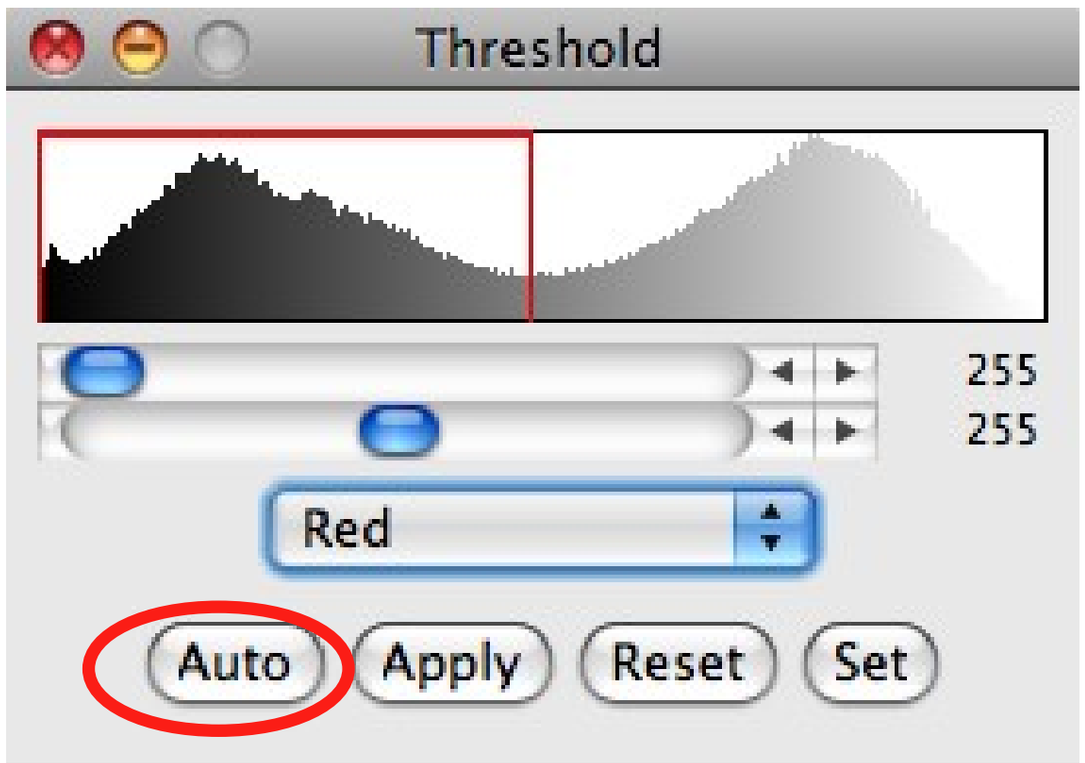






Computed Global Threshold Objective - Reproducible

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

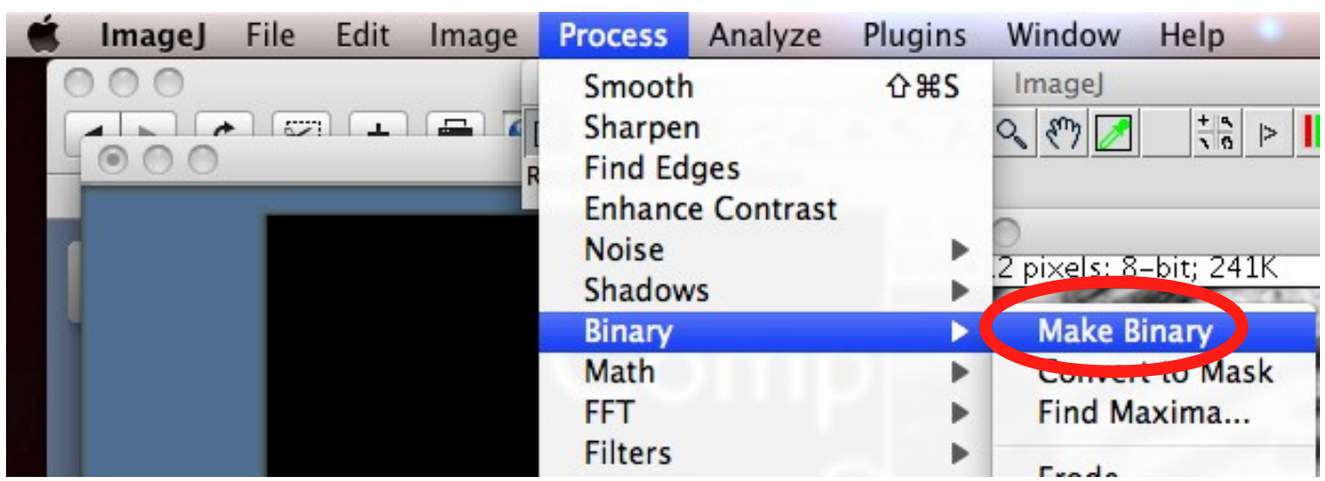


Initial guess of Threshold, T

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!

Practical Session 2c

Simple Image Segmentation

(1) File - Open Samples – Blobs (inverse)

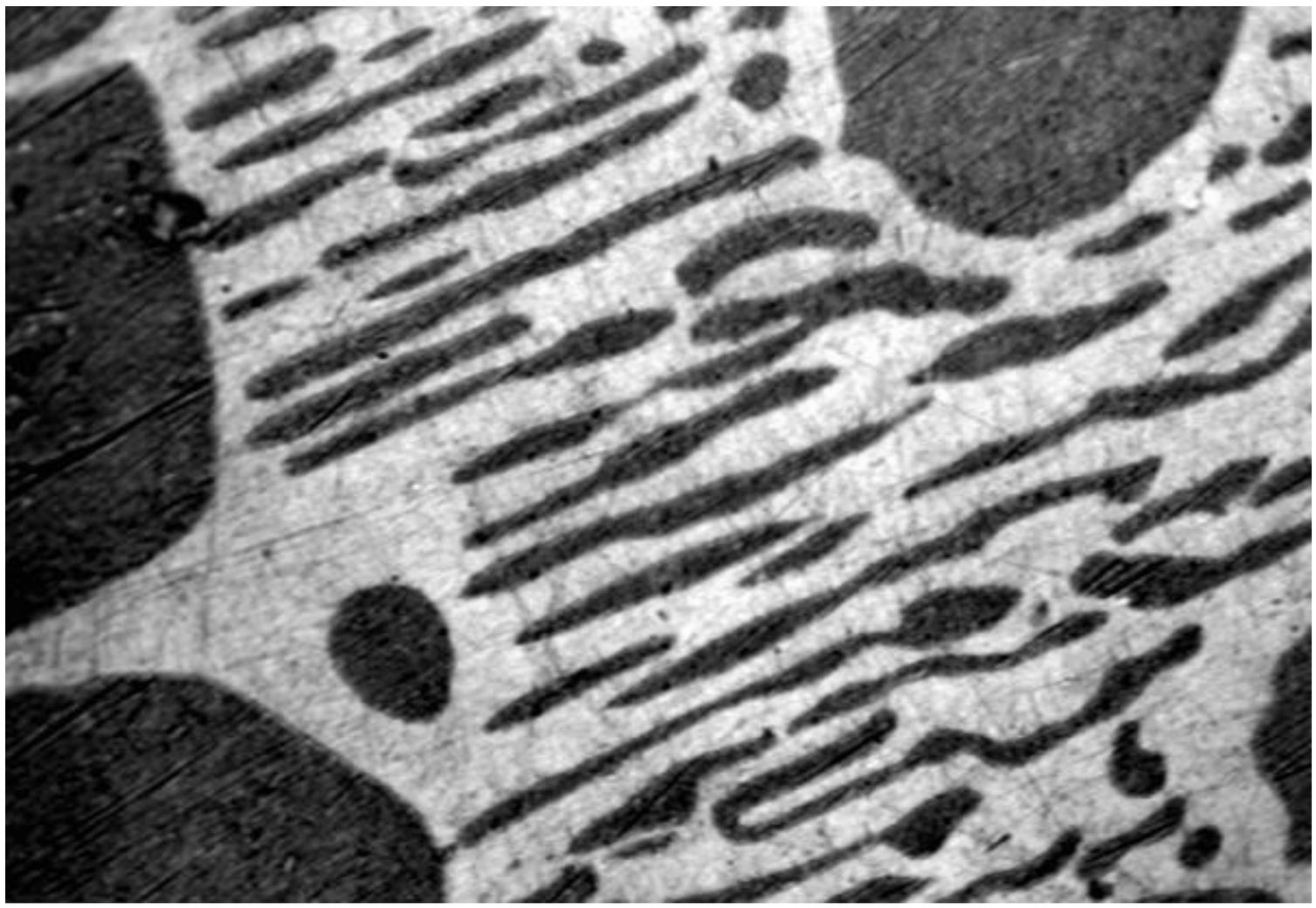
(2) File – Open Samples – Clown

(1) Thesholds

- ✓ Image – Lookup Tables – Invert 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

(2) Statistical Region Merging

Edge Detection: The Sobel filter



Images may contain objects

These objects have edges

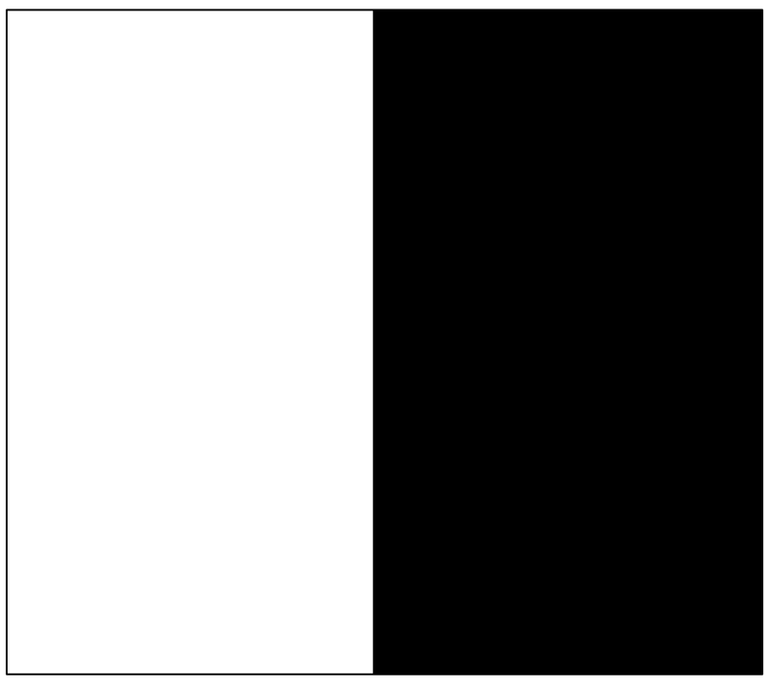
How can we find the edges?

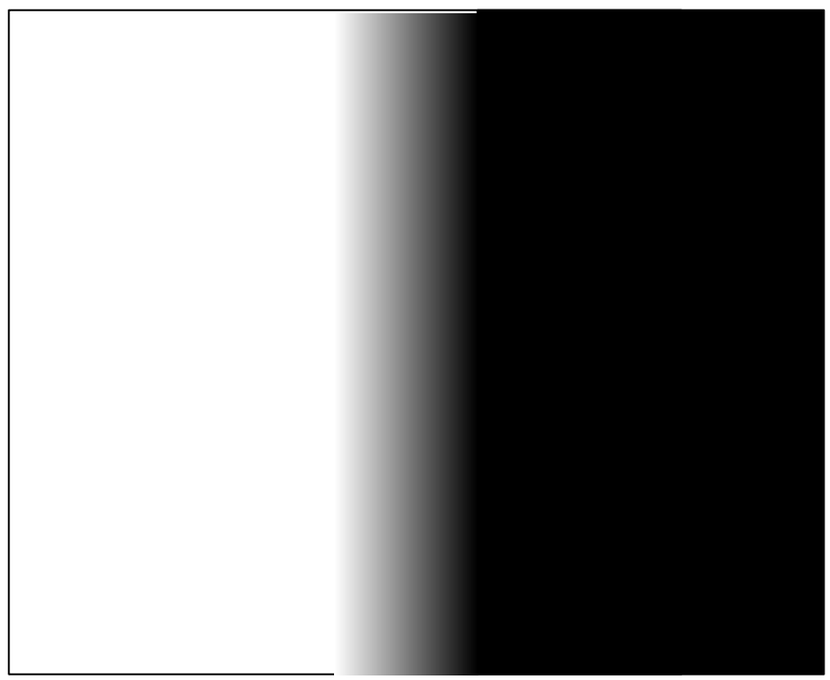
Edge Detection

What is an “edge” ?

“Hard Edge” - Adjacent black / white pixels

“Soft / Fuzzy Edge” - common in images. Especially for small diffraction limited objects like vesicles/membranes. Noise makes edges look softer

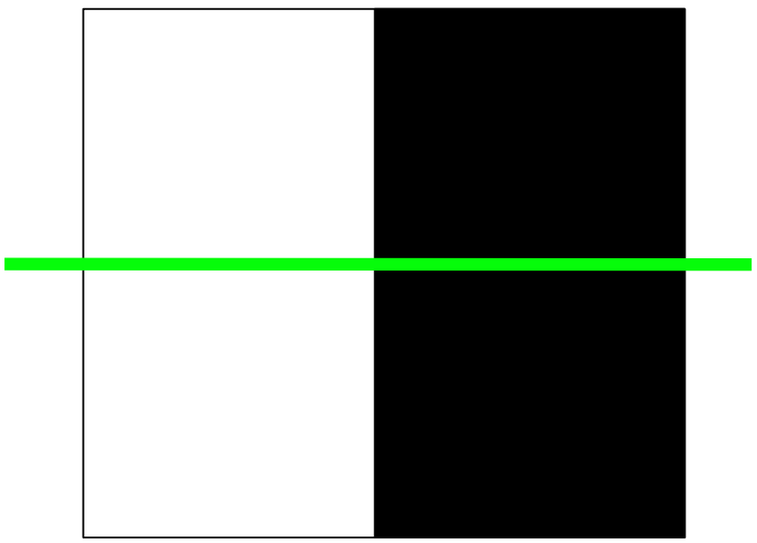


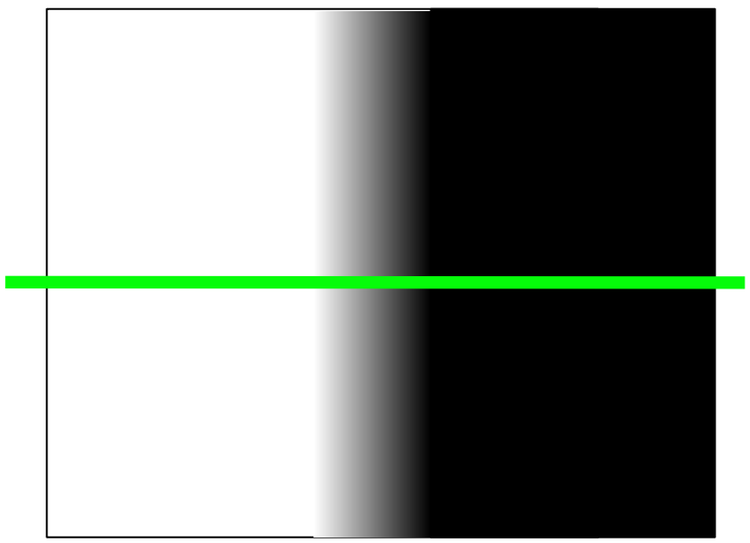


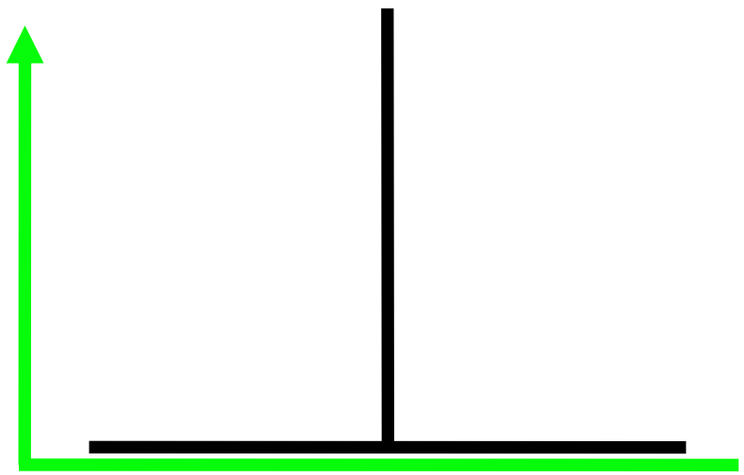
Edge Detection "Image Gradient"

What is a "Gradient Image" ?

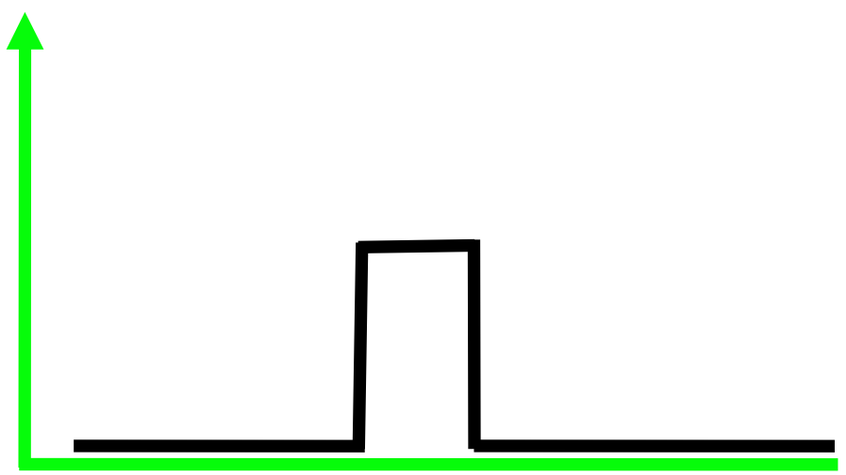
Rate of change of pixel intensity (1st derivative)







Y=pixel intensity gradient



Edge Detection "Image Gradient"

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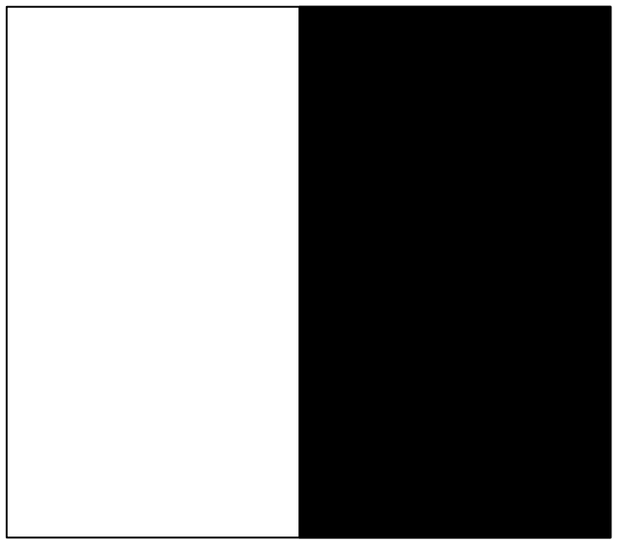
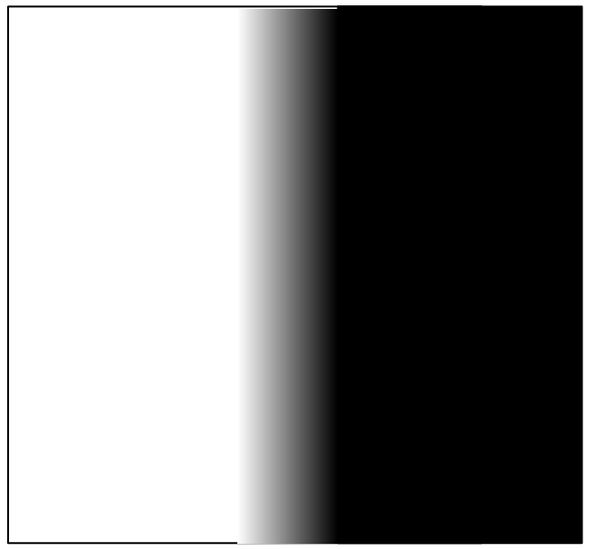
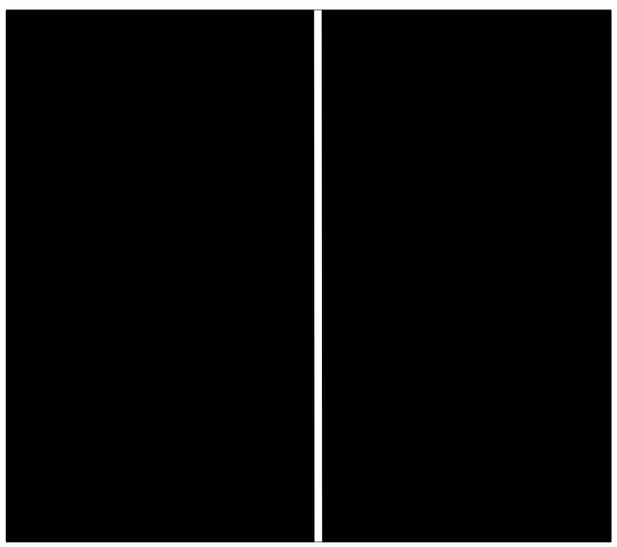


Image



Hard edge

Soft edge



Gradient Image

"Image Gradient" - How?

Sobel filter - 3x3 convolution filter pair 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

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

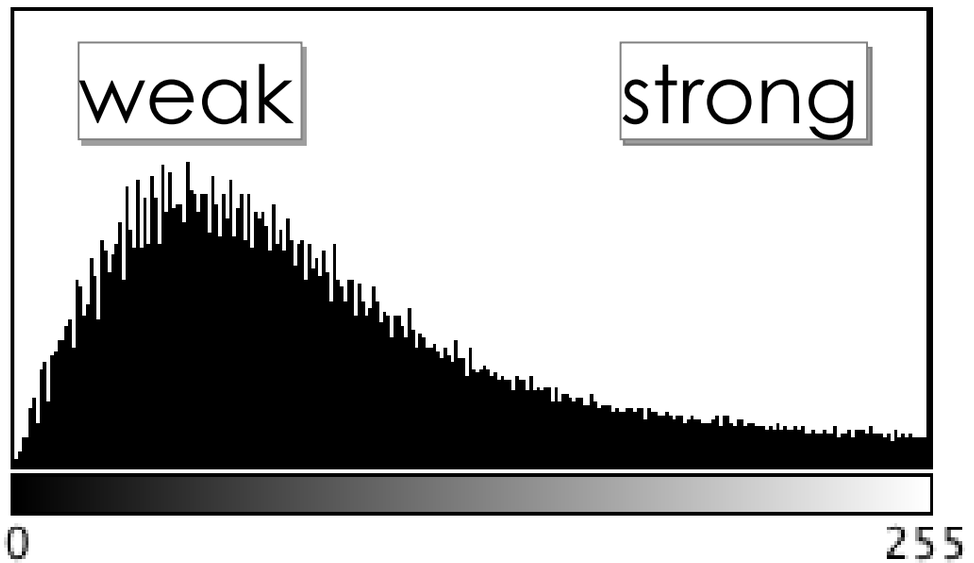
$|\ g_x\ |$ + $|\ g_y\ |$ = $|\ g\ |$

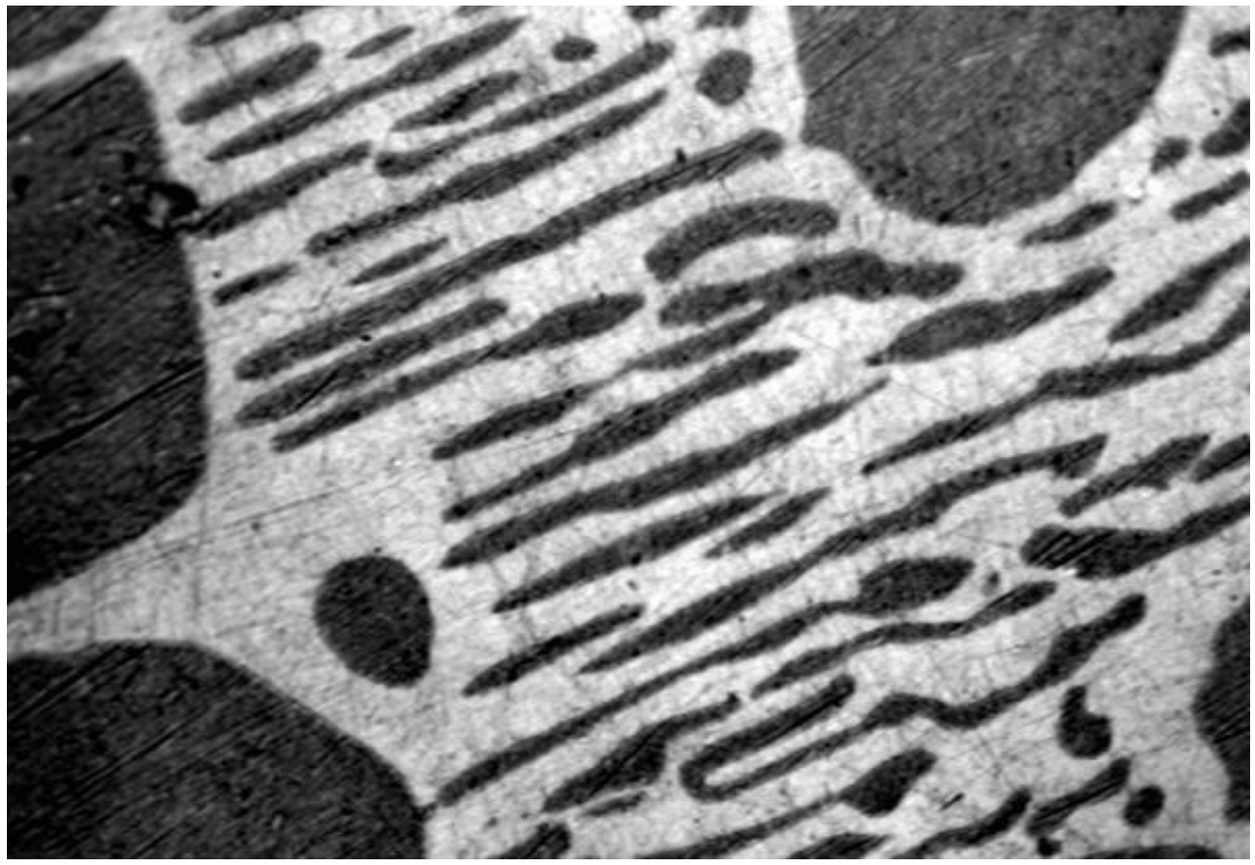
Gradient Image - Real Sample:

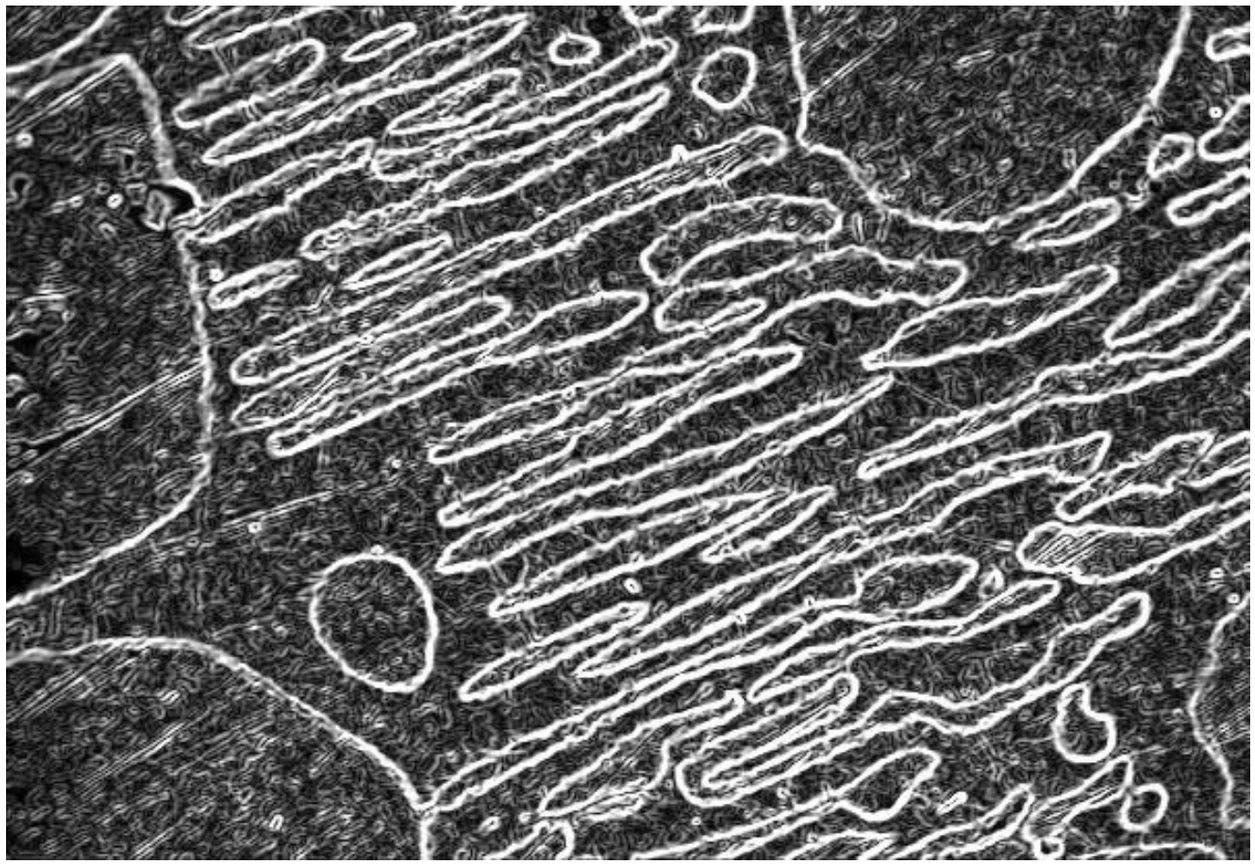
Real / Biological images:

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

gradient image histogram





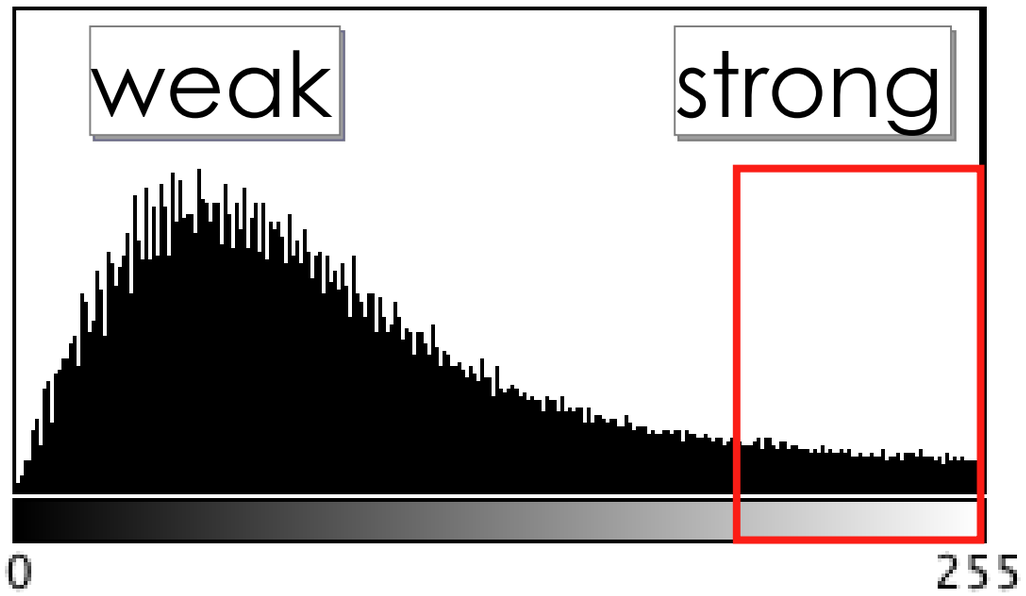


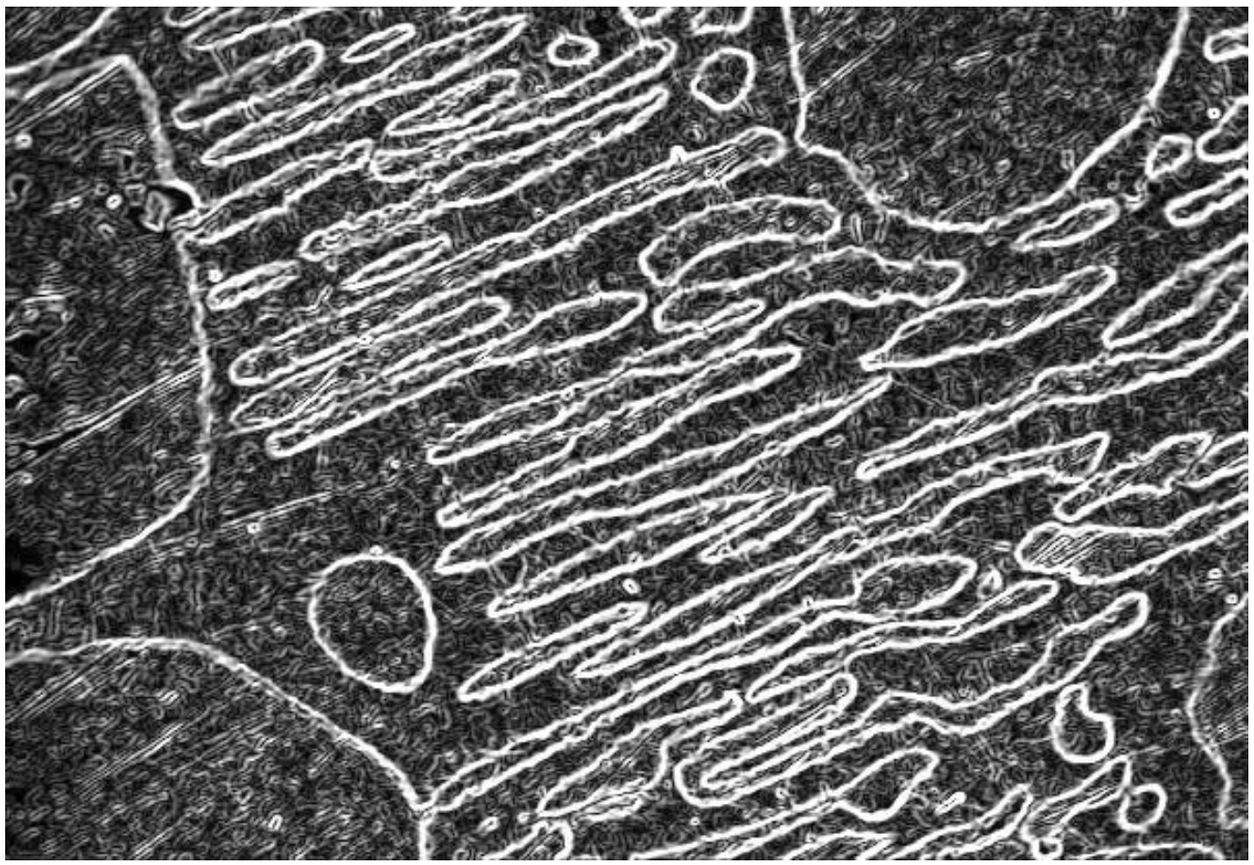
Gradient Image - Strong Edges?

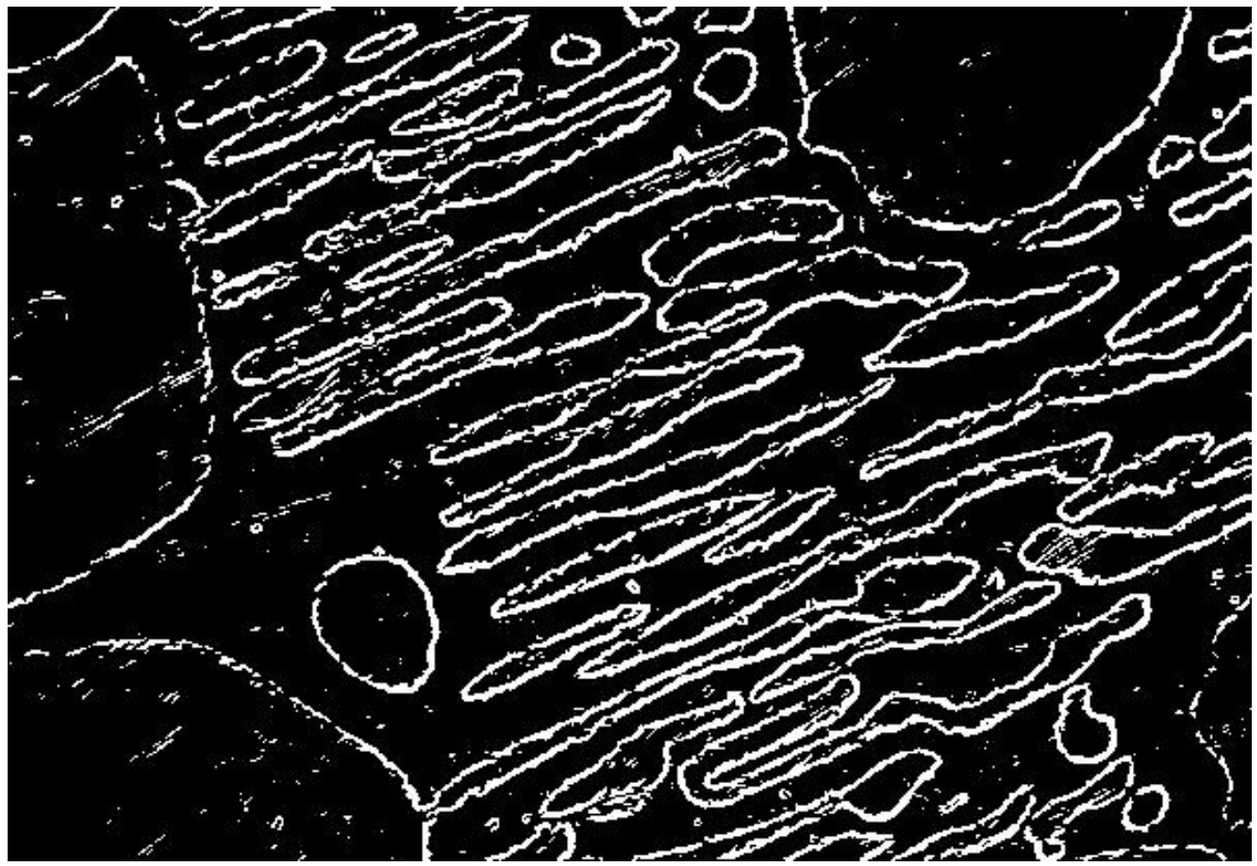
Remove weak edges?

✓ Threshold the gradient image

✓ Smoothing filter beforehand







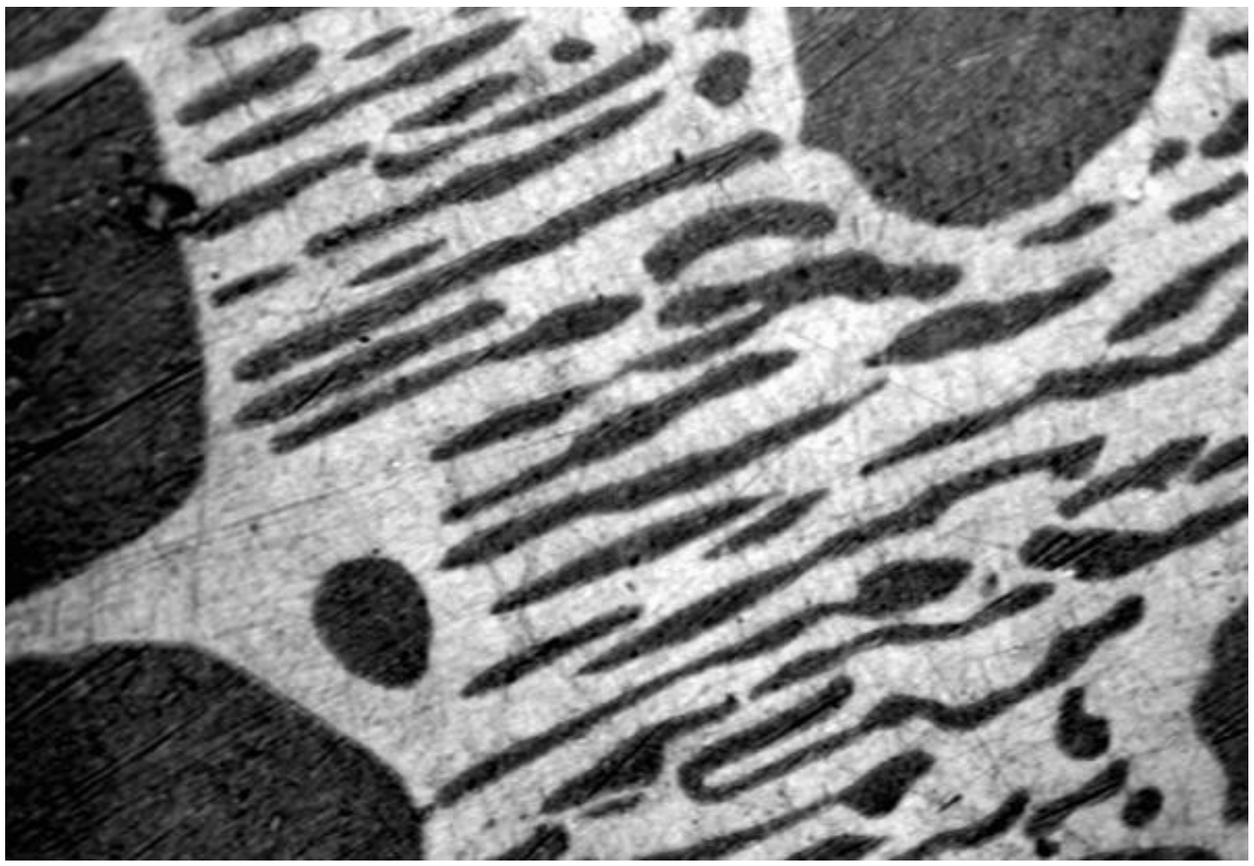
“Canny” Edge Detection

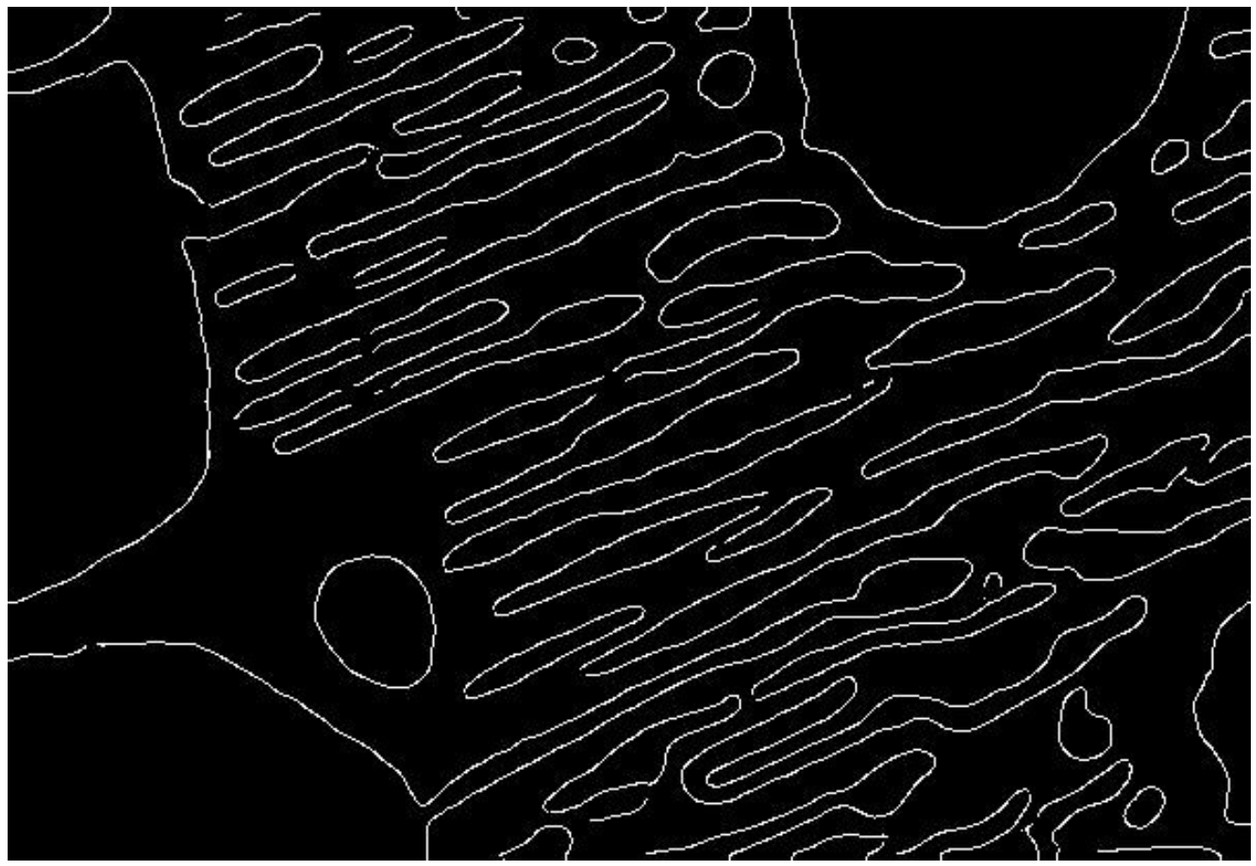
Remove weak/noisy edges - keep strong

Gaussian smooth image + hysteresis threshold gradient image

Make edges sharp - 1 pixel wide

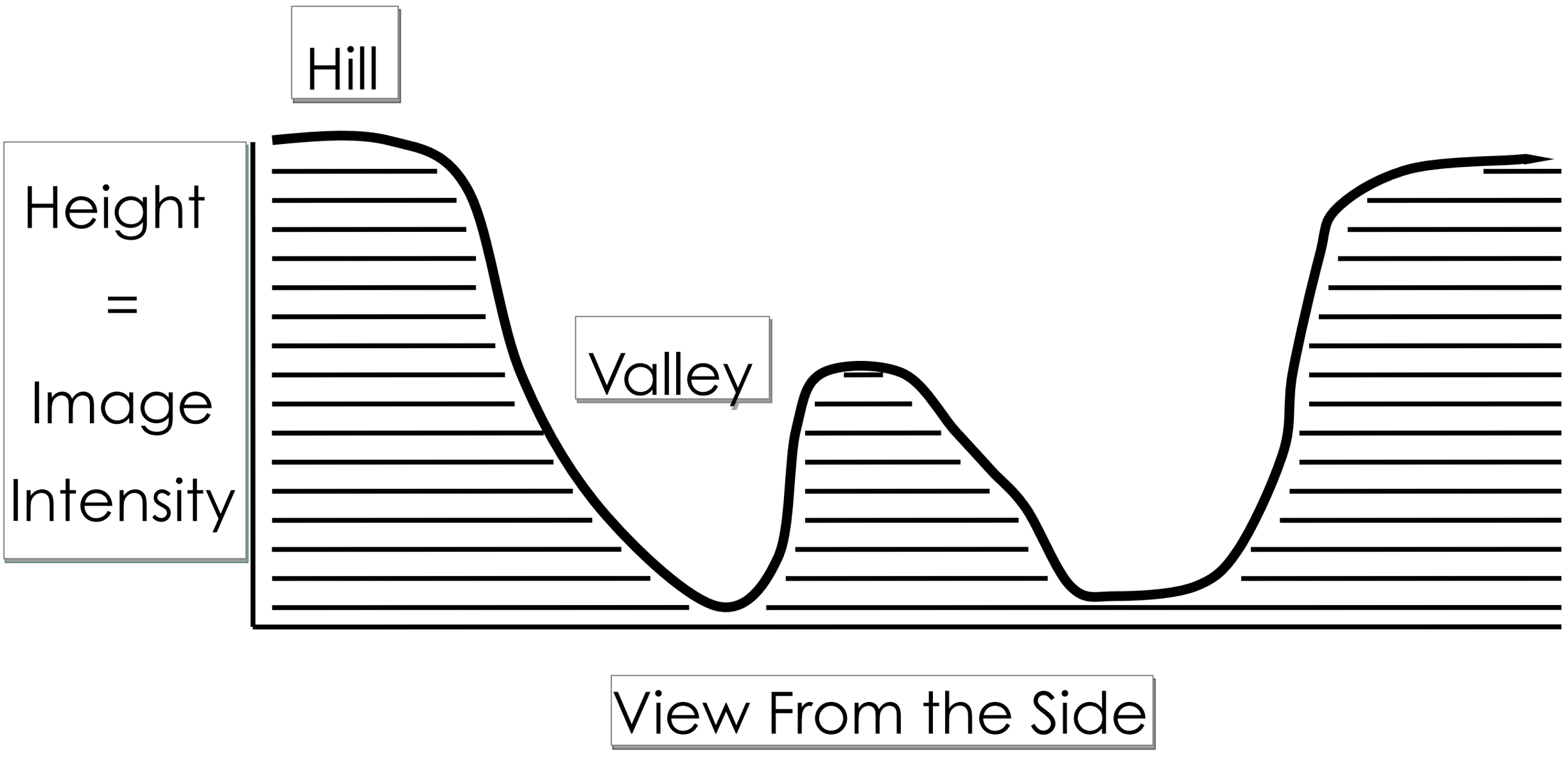
Non maximal suppression of gradient image





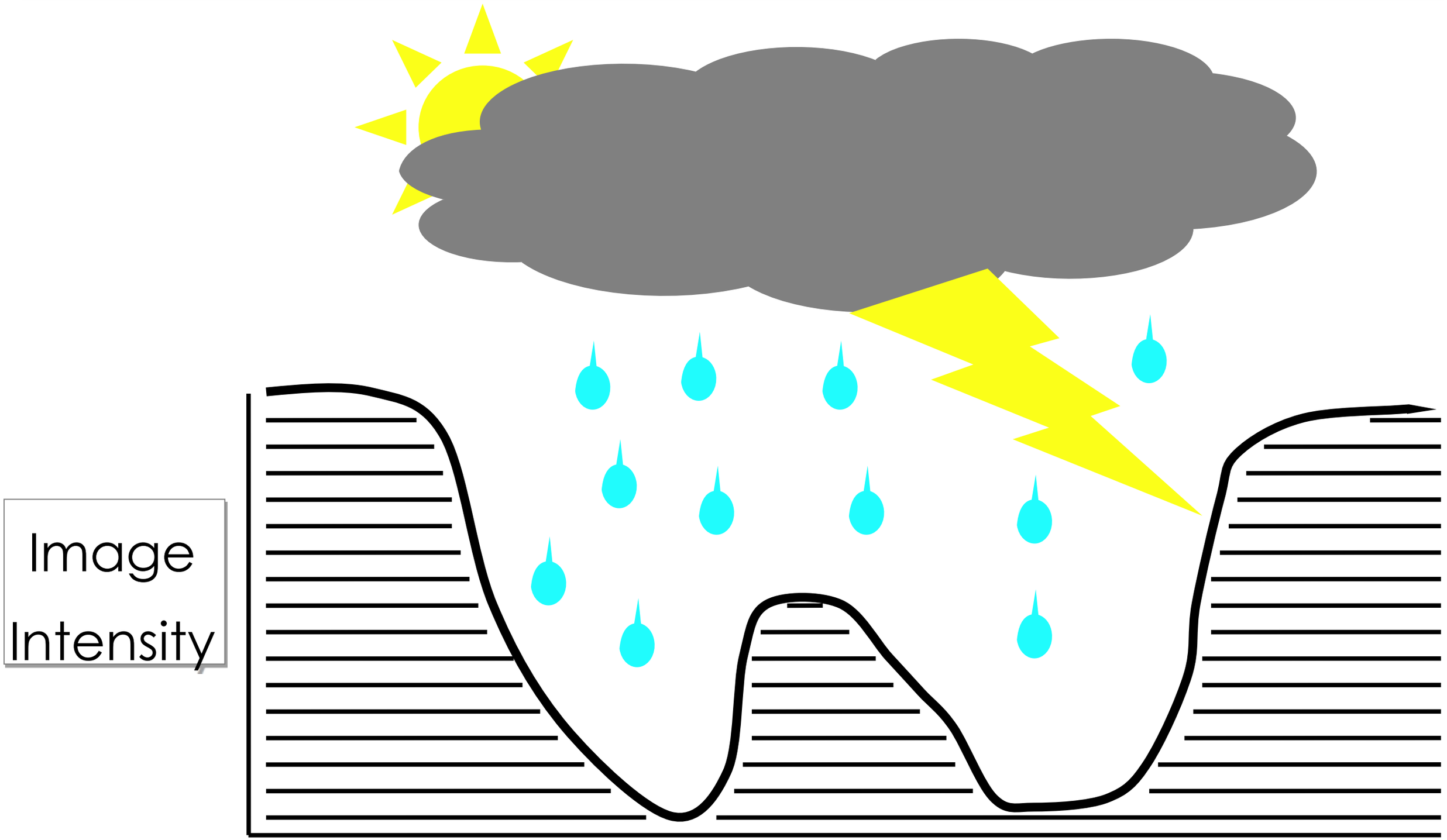
Watershed Algorithm:

… mountains, lakes and oceans



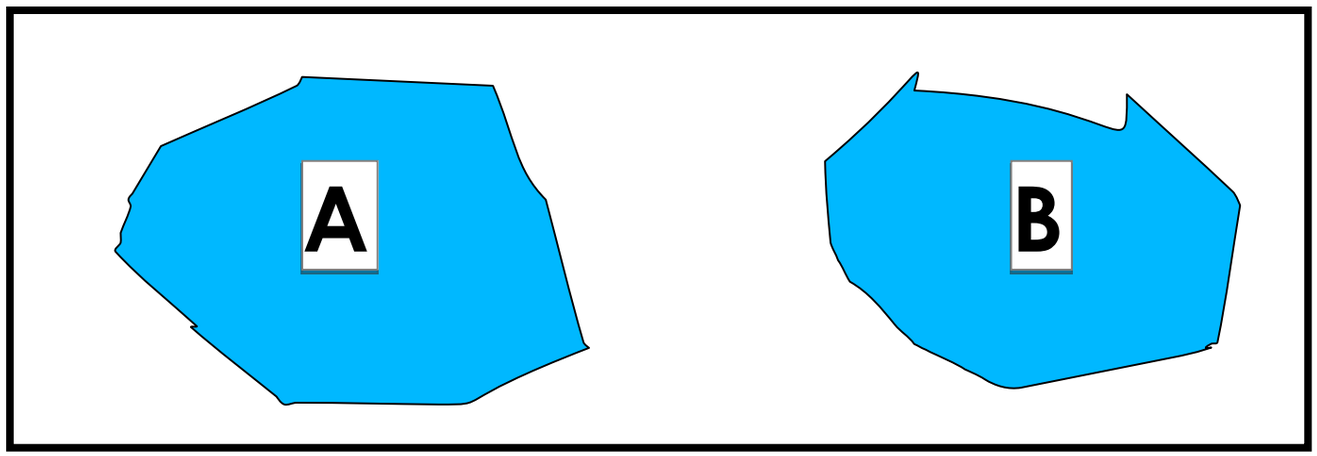
Watershed Algorithm:

… mountains, lakes and oceans

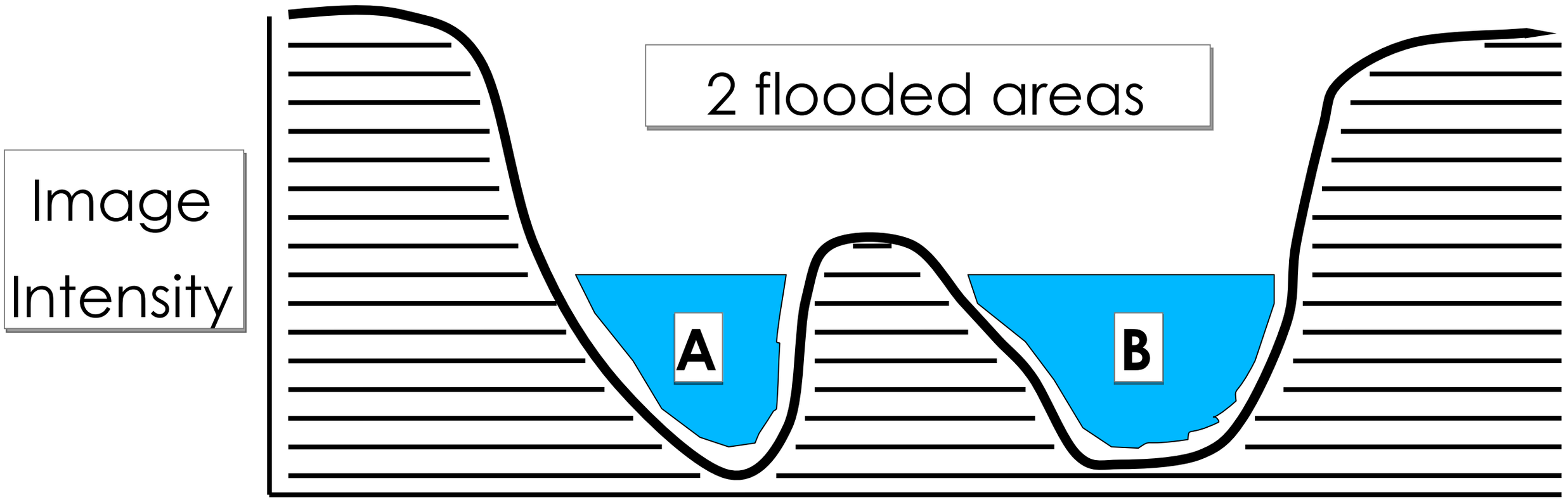


Watershed Algorithm:

… mountains, lakes and oceans



View from above



Watershed Algorithm:

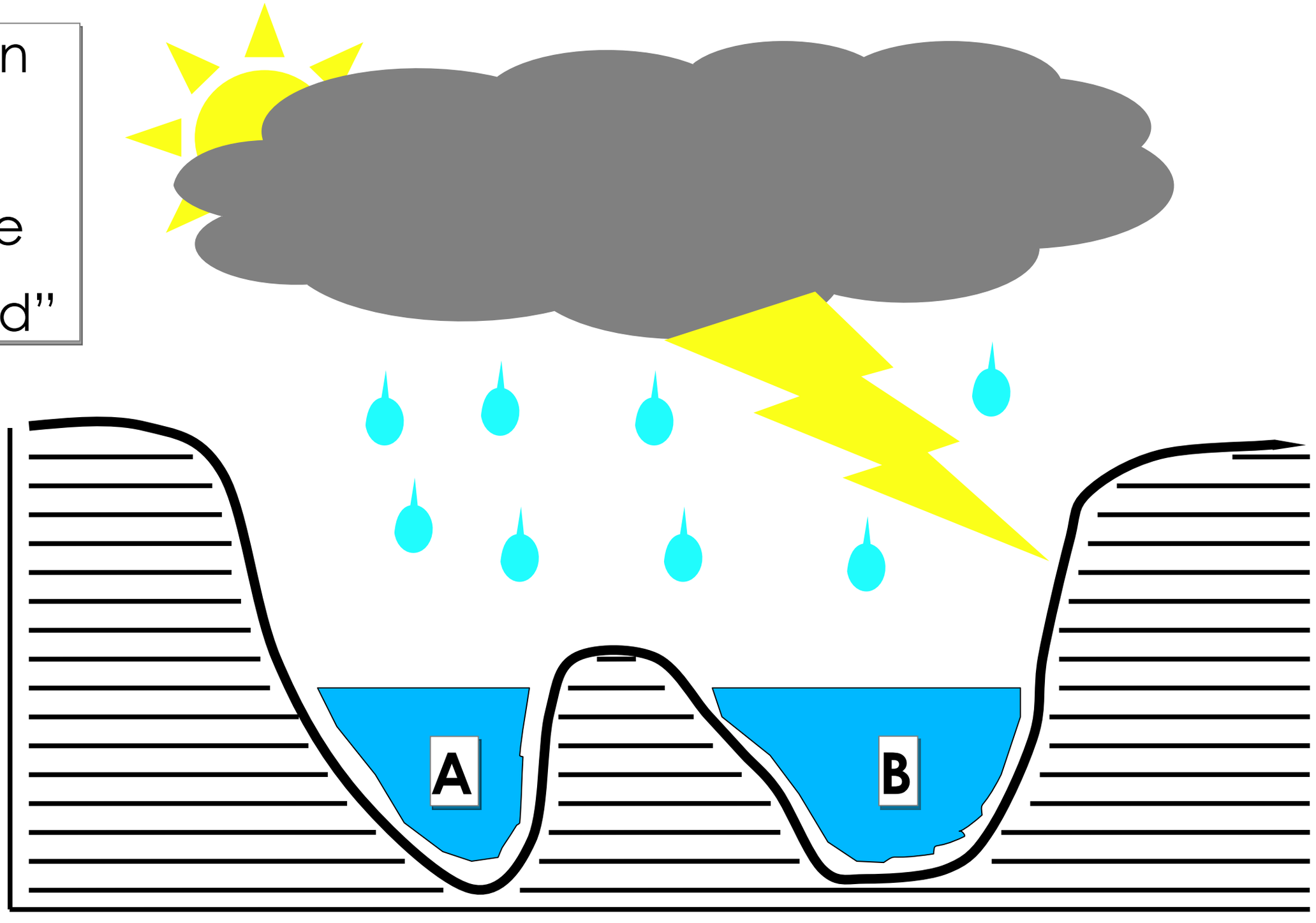
… mountains, lakes and oceans

More rain

=

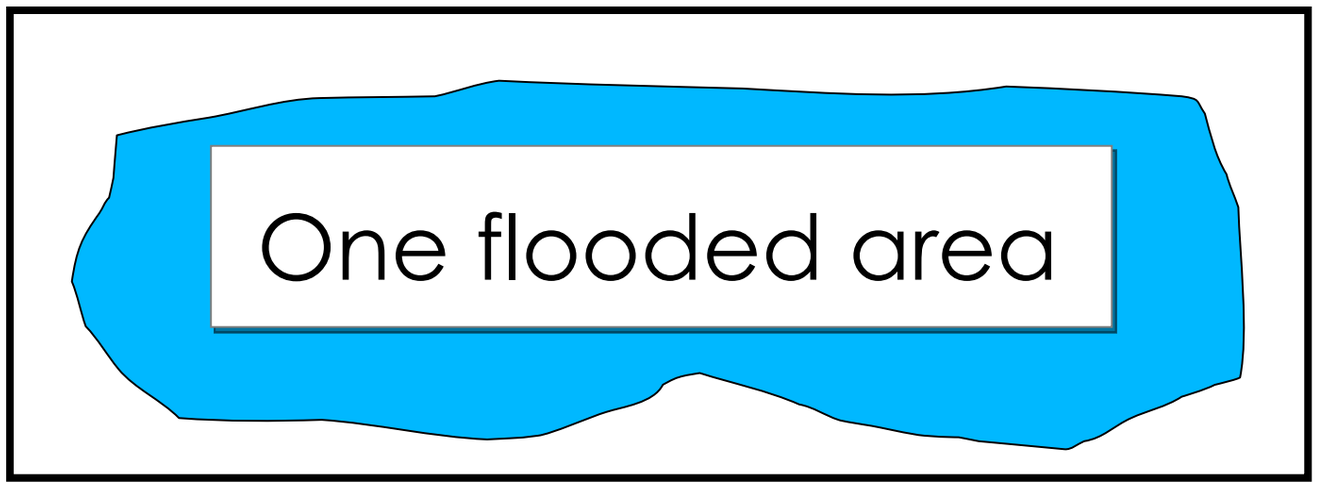
Increase

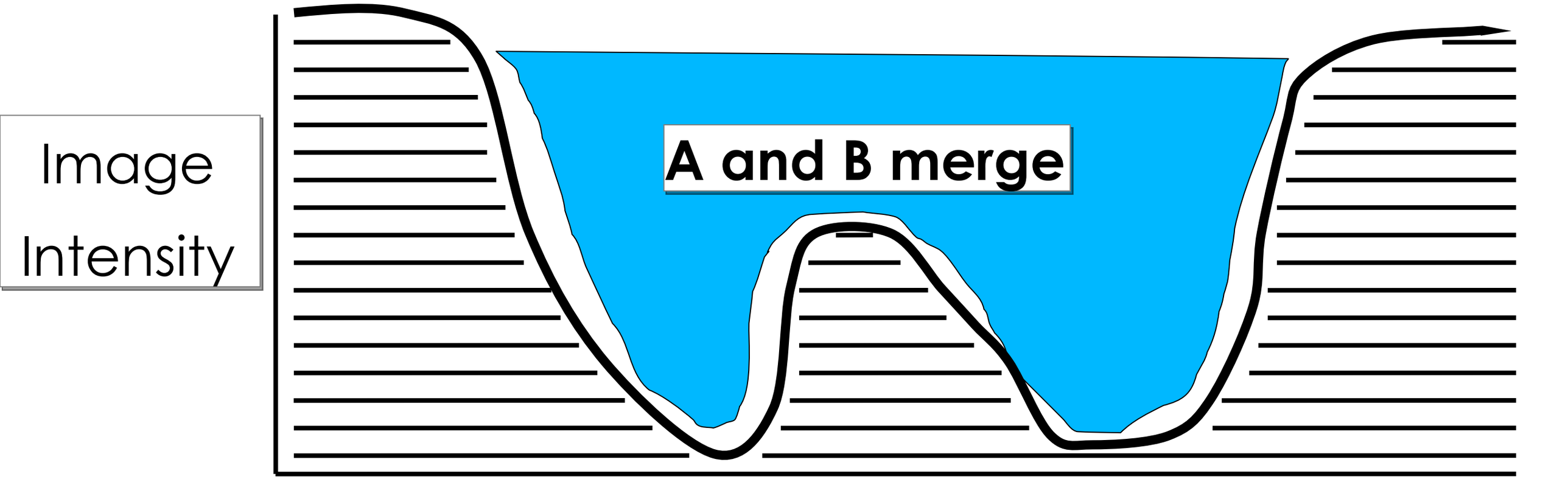
“threshold”



Watershed Algorithm:

… mountains, lakes and oceans

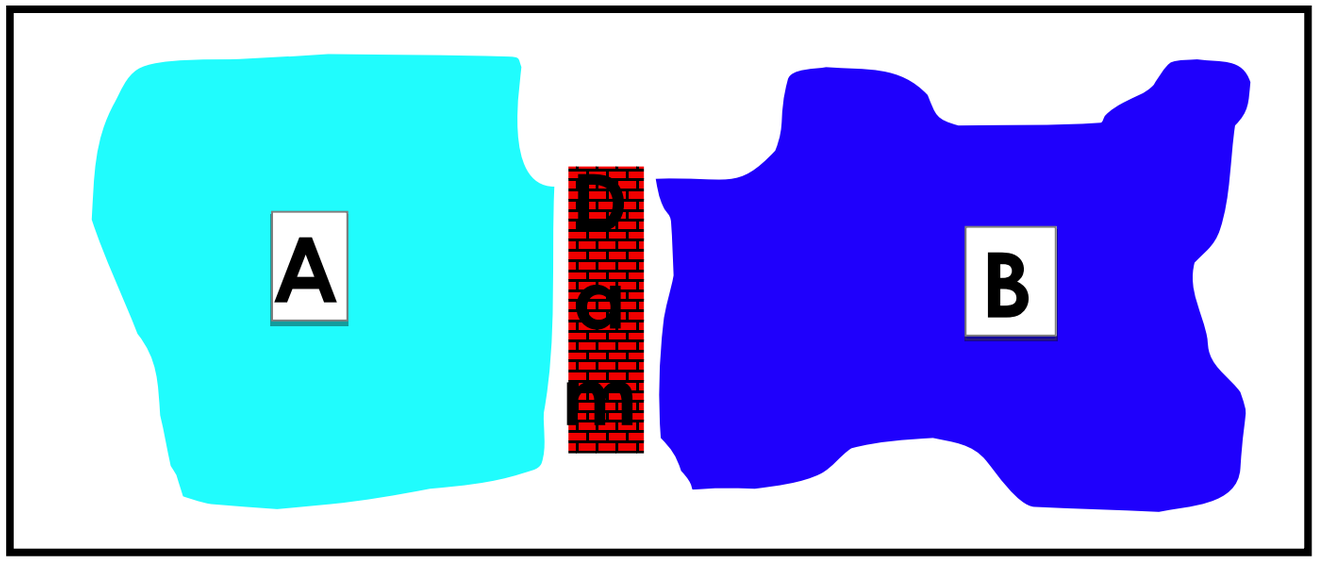


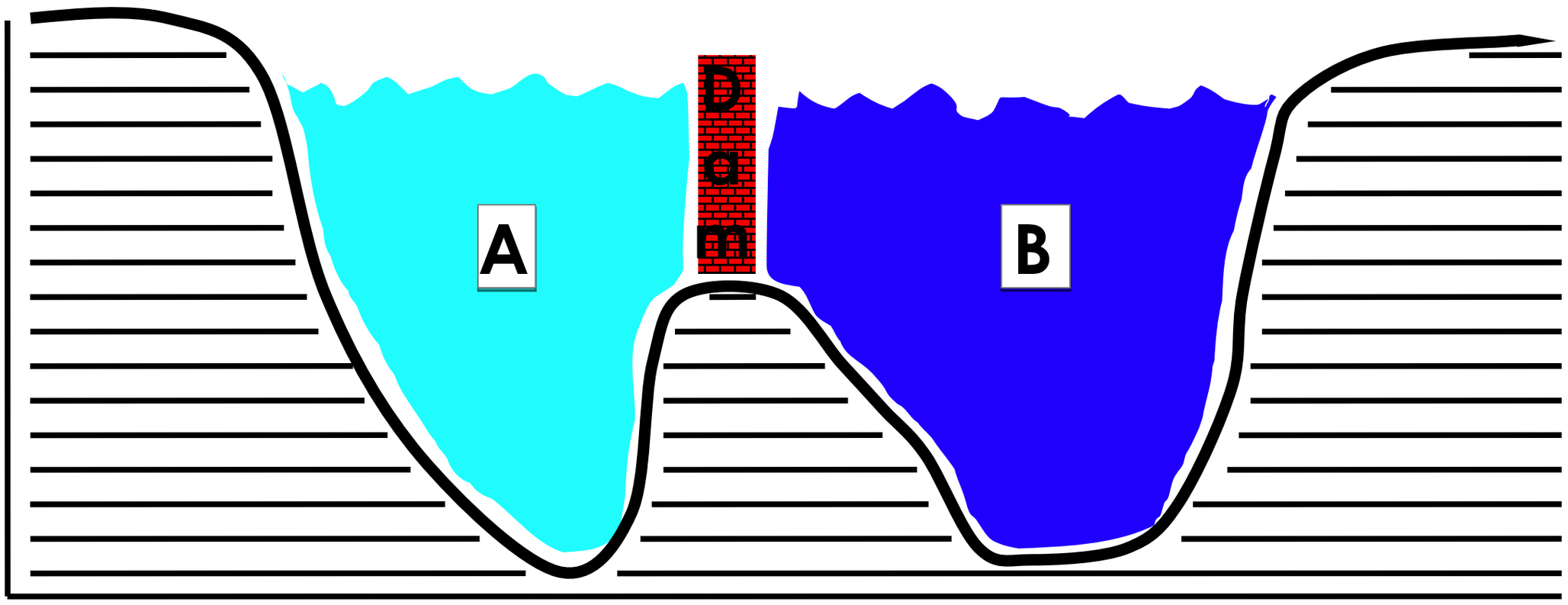


Watershed Algorithm:

… mountains, lakes and oceans

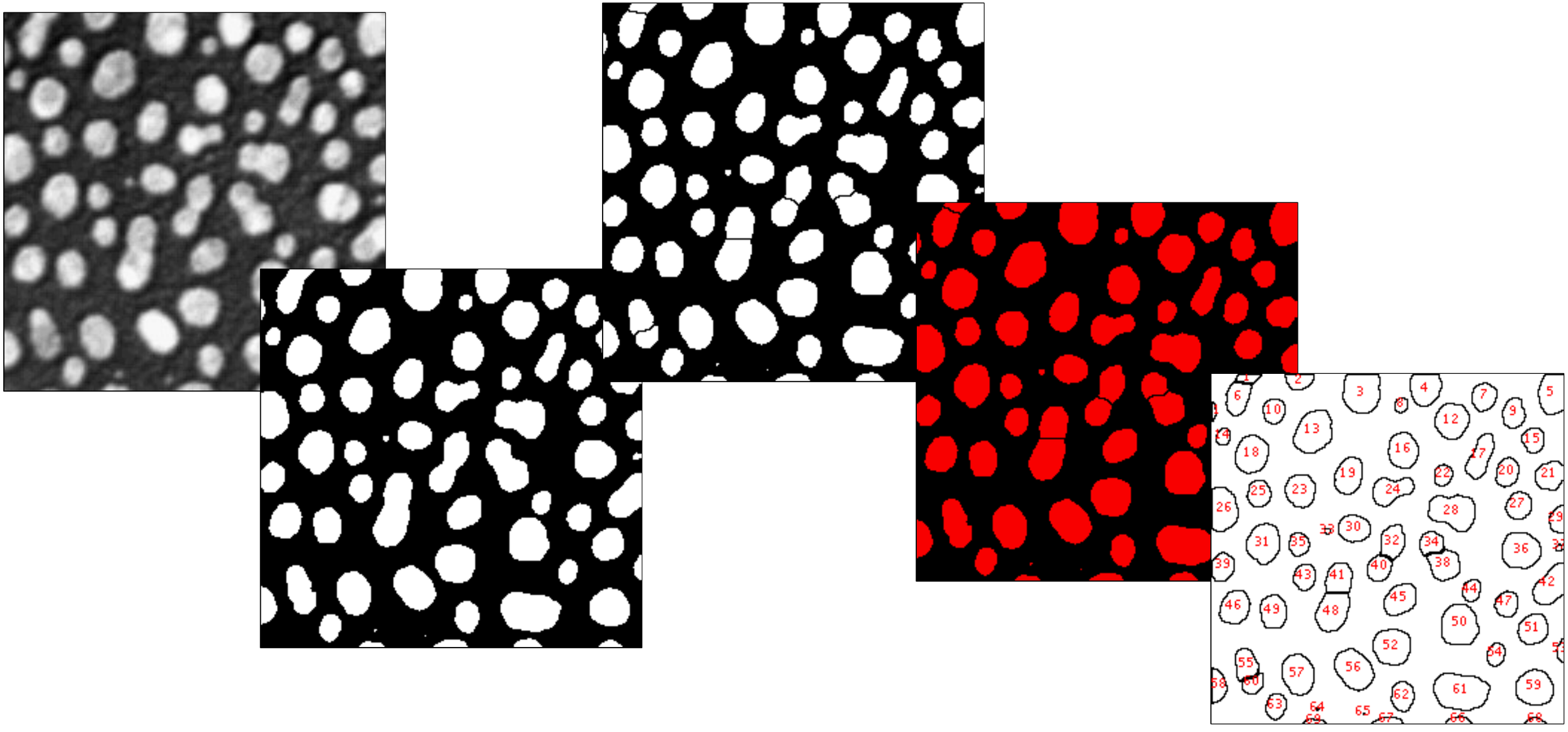
Make a “Dam” at the “Watershed line”





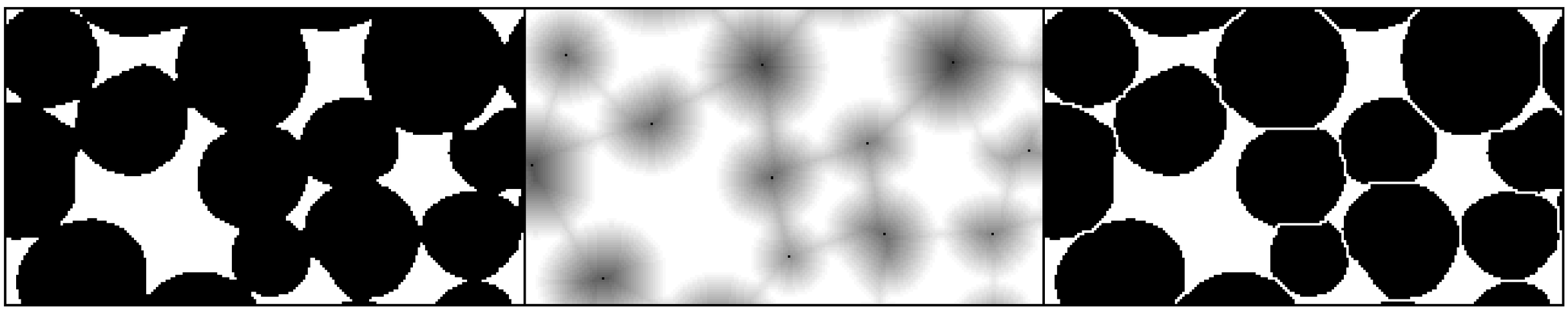
Watershed - to find object number

Sometimes there are just too many to count by hand …



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



Thresholded Cells | EDM and UEPs | After Watershed Segmentation

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

Practical Session 2d

Getting to know “Fiji” better –

Fiji is just ImageJ (Batteries included)

http://pacific.mpi-cbg.de

File - Open Samples - Blobs

Watershed Segmentation and Analysis

✓ Invert, Make Binary, Watershed, Threshold, Analyze Particles:
Separate and measure touching objects

✓ Search the Wiki for NucleiWatershedSegmentation tutorials

Links and Further Reading

Standard Text Book:

Digital Image Processing 2nd Ed., Gonzalez and Woods, Prentice Hall

Fiji and ImageJ:

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

Image Processing Facility

- ✓ **Intranet** - Services and Facilities - Image Processing Facility
- ✓ **Wiki - info for beginners - tips - software documentation:**
  https://wiki.mpi-cbg.de/wiki/imagepro/index.php/Main_Page

Imaging Facility Network (IFN): https://ifn.mpi-cbg.de

Email: ipf(at)mpi-cbg.de

The Fourier transform

The Fourier transform is a way to obtain a new representation of the data (a bit like the 2D histogram from earlier)

It is best suited for data with repetitive patterns, as it highlights those

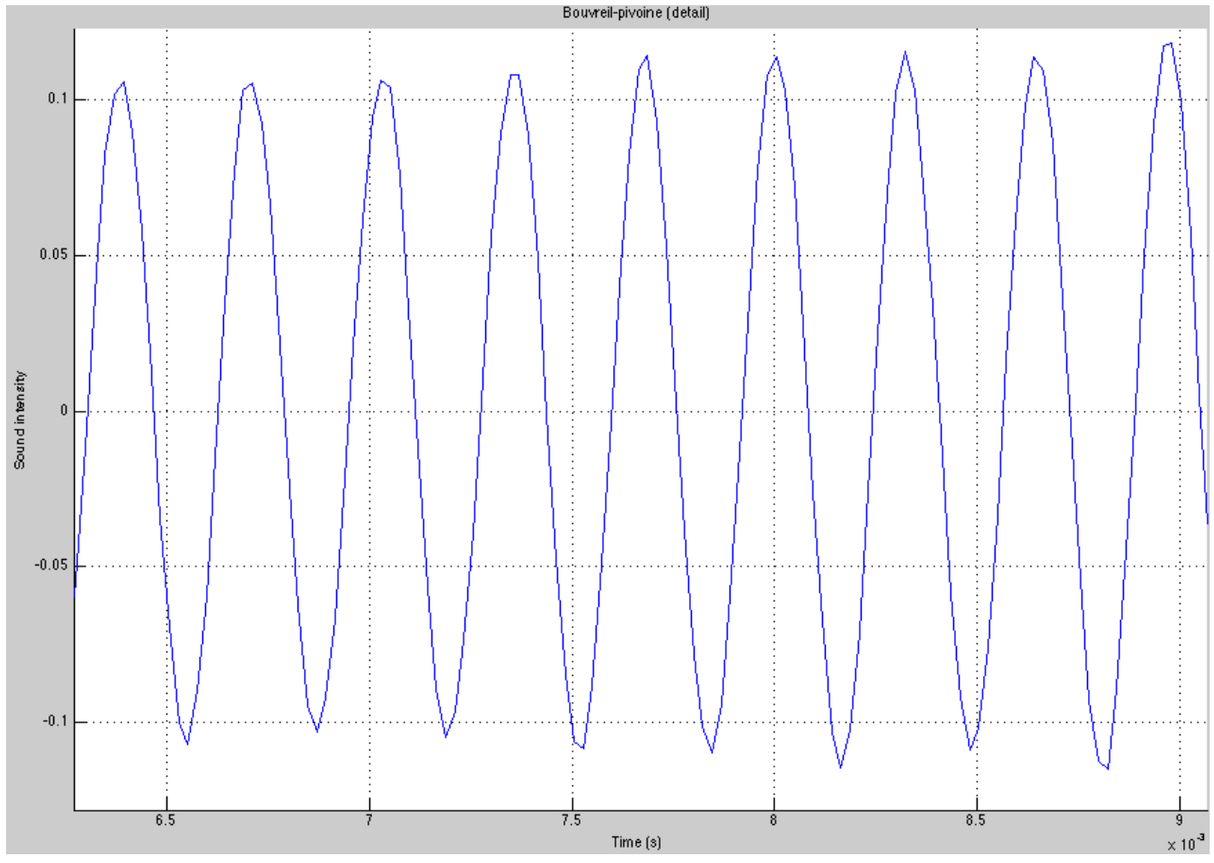
And … don't worry about the maths for now...

The Fourier transform

Bird song

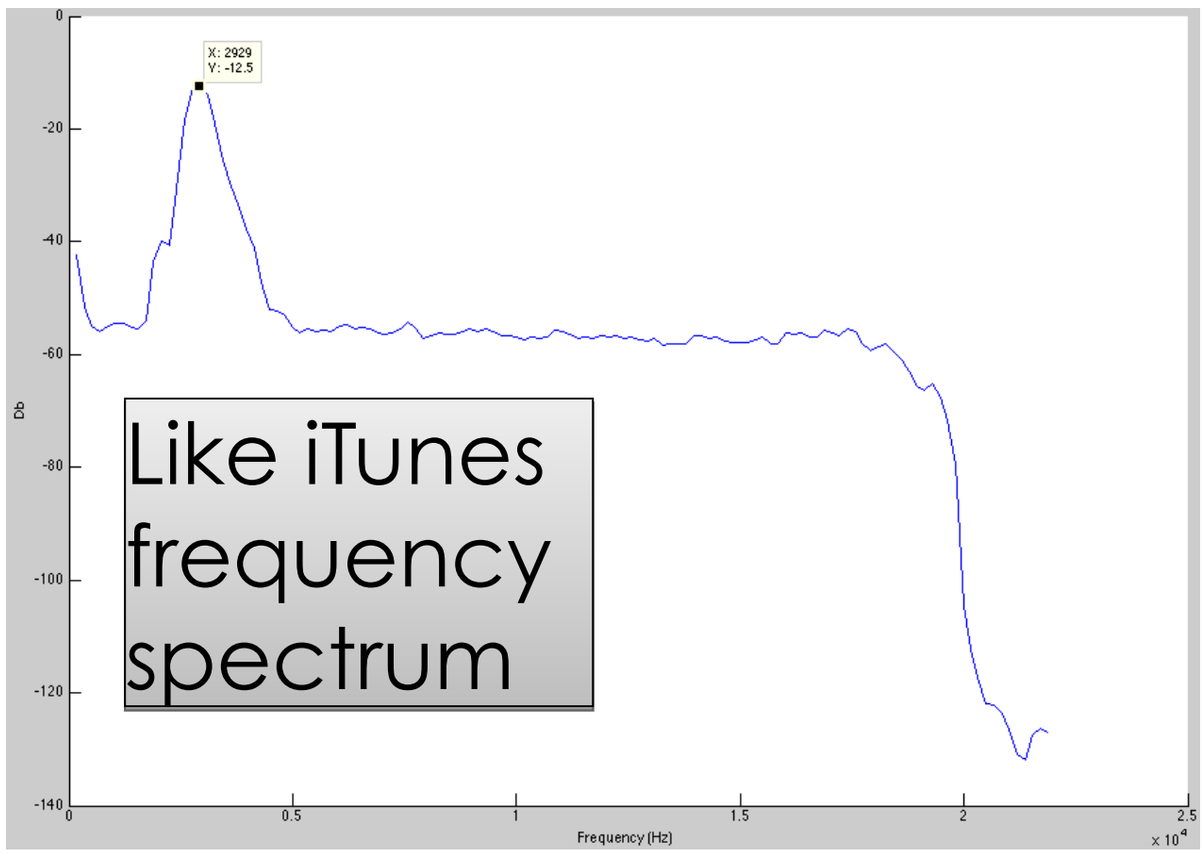
Detail of the signal:

Delay between peaks:~ 0.35 ms



FFT of this looks like:

Peak in FFT: ~ 3 kHz



Like iTunes frequency spectrum

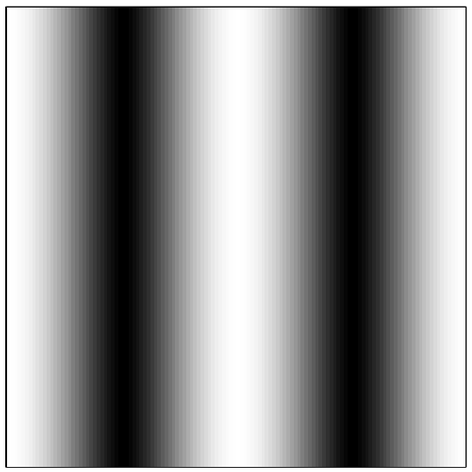
Equivalence: spatial domain vs. Fourier or Freq. domain

1 / 3000 ü 0.33 ms

Peak in FFT gives frequency or peroidicity of pattern

The Fourier transform – in 2D images

Real image



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

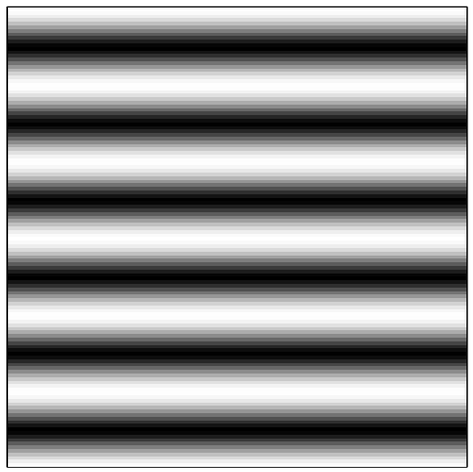
Pattern of points:

always symmetrical!

further = smaller

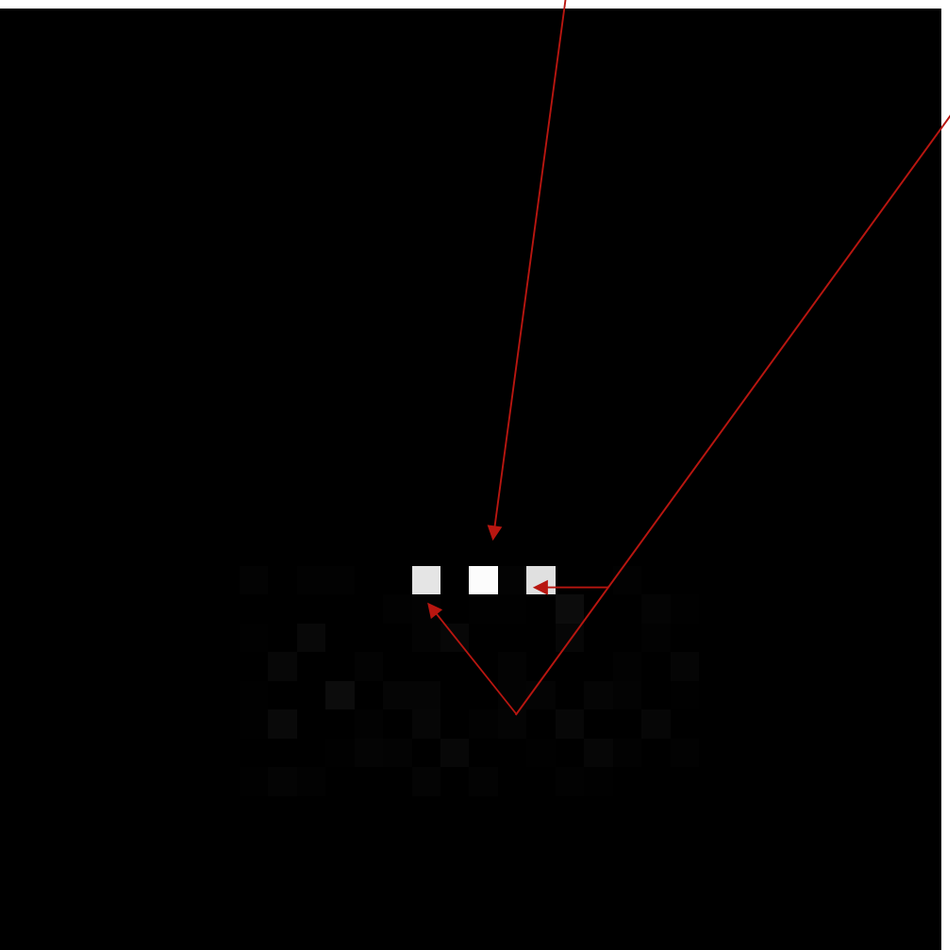
higher freq = smaller object

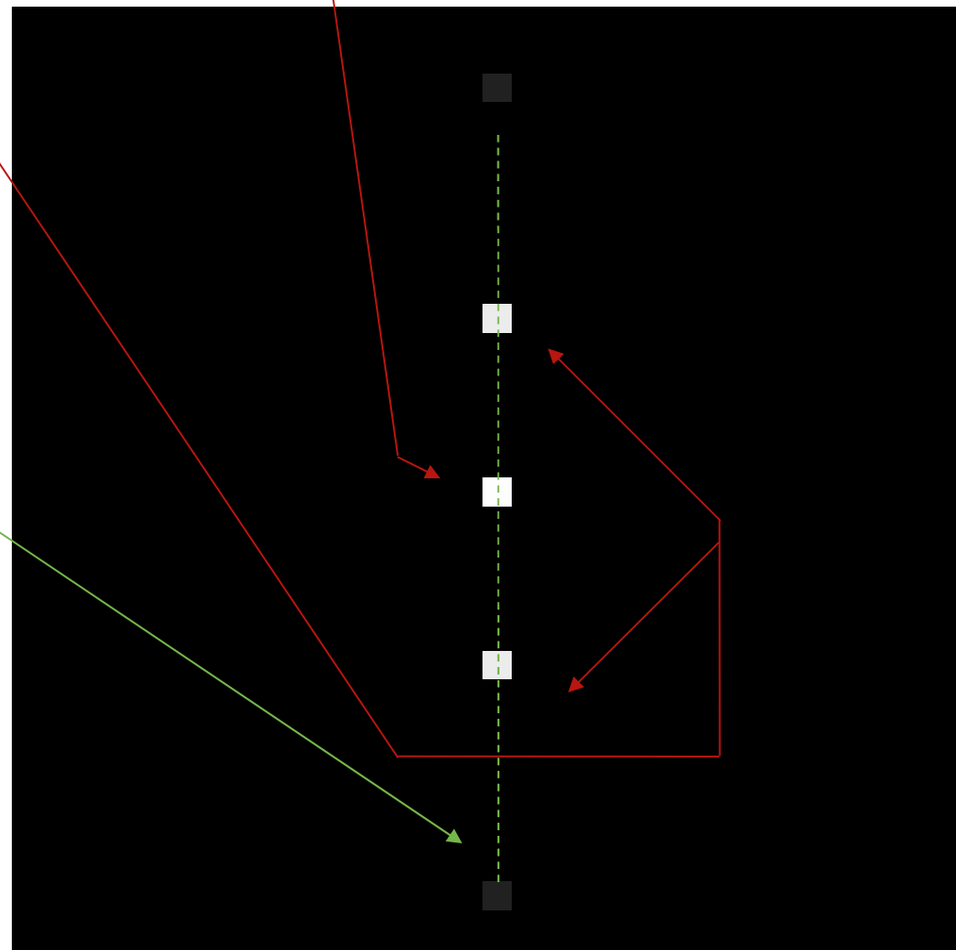
Real image



Angle of pattern gives pattern orientation

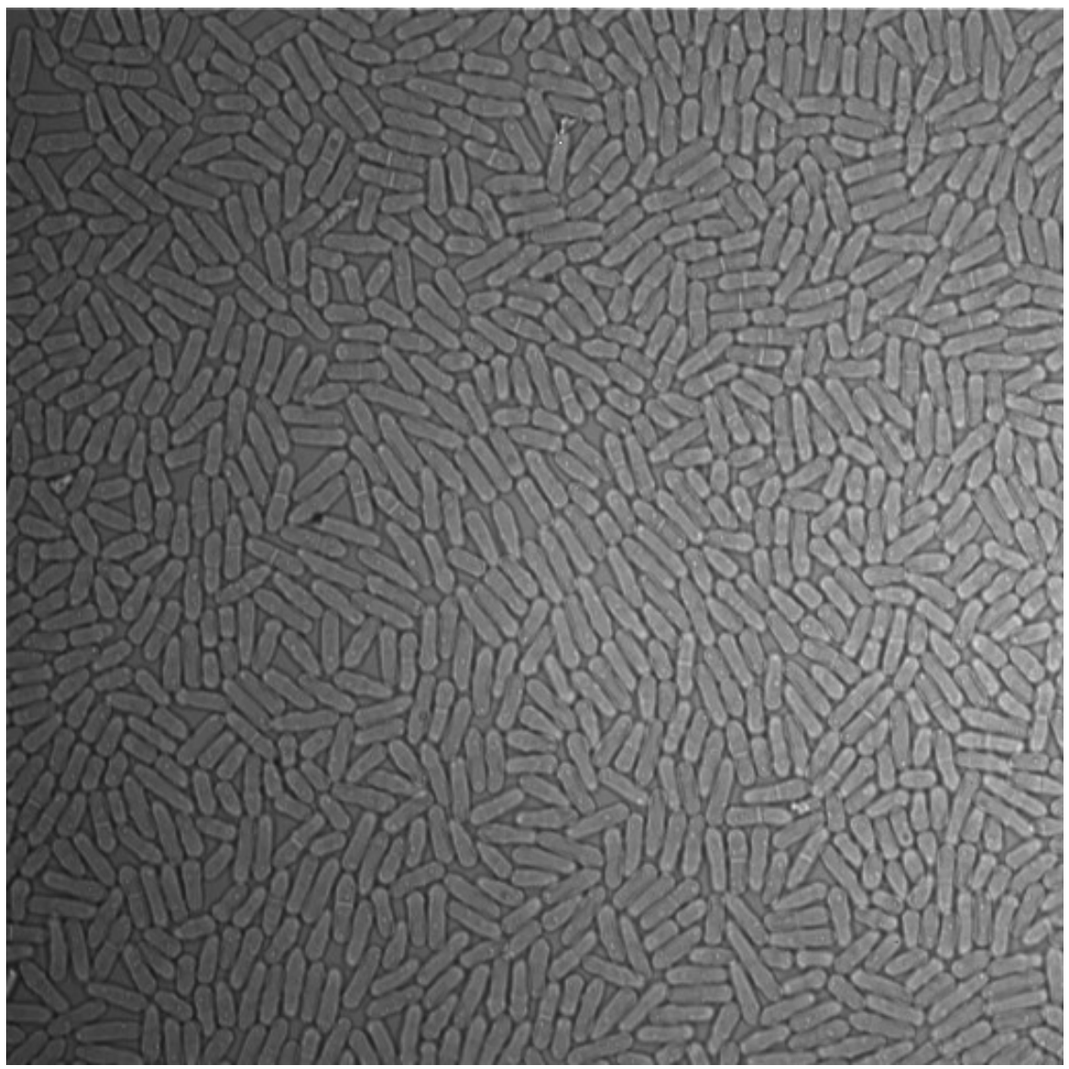
Diffraction pattern?



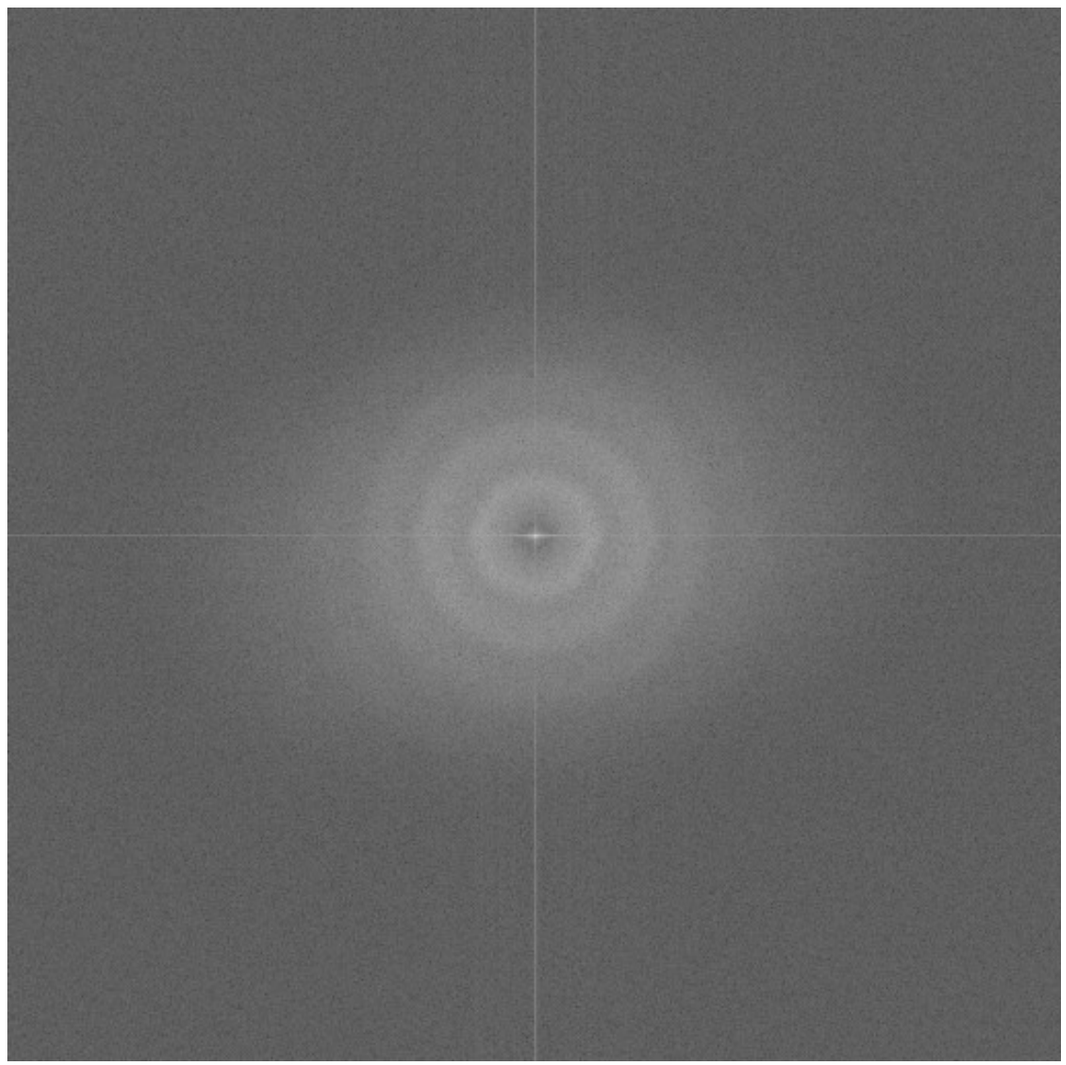


The Fourier transform – in 2D images

Real images… are rarely that clear



S. pombe cells (Tolic lab)



FFT

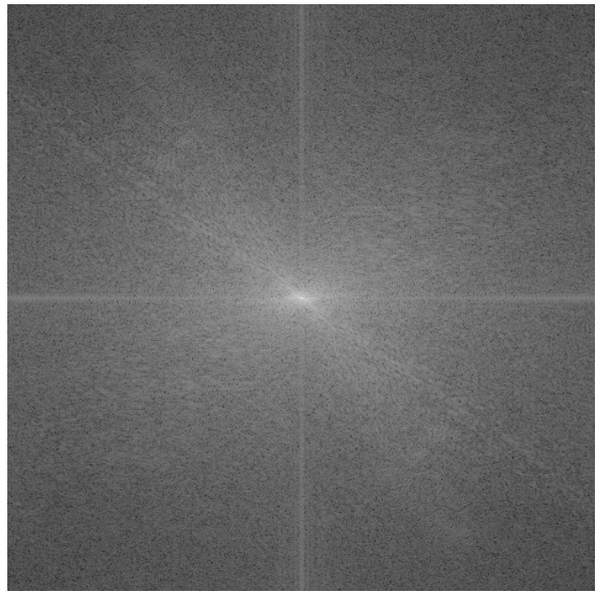
The inverse Fourier transform

Fourier image and real image contain same information → so it's possible to reverse the process:

Before:

FFT

After:



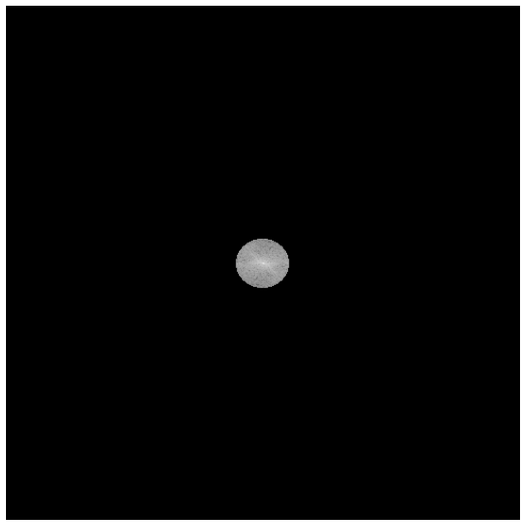
Reverse FFT

Changed her mind:

Same thing happens physically in a microscope.
FT image is in the Back Focal Plane of Objective!

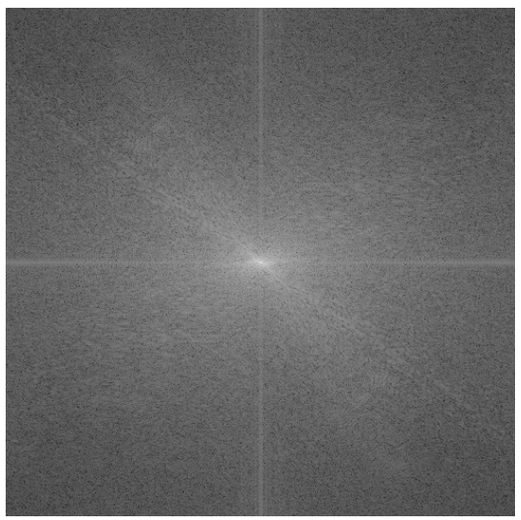
Can use as a filter for detail:

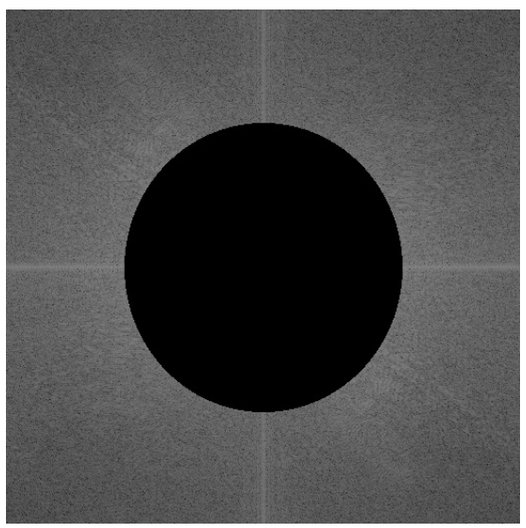
Low frequence pass



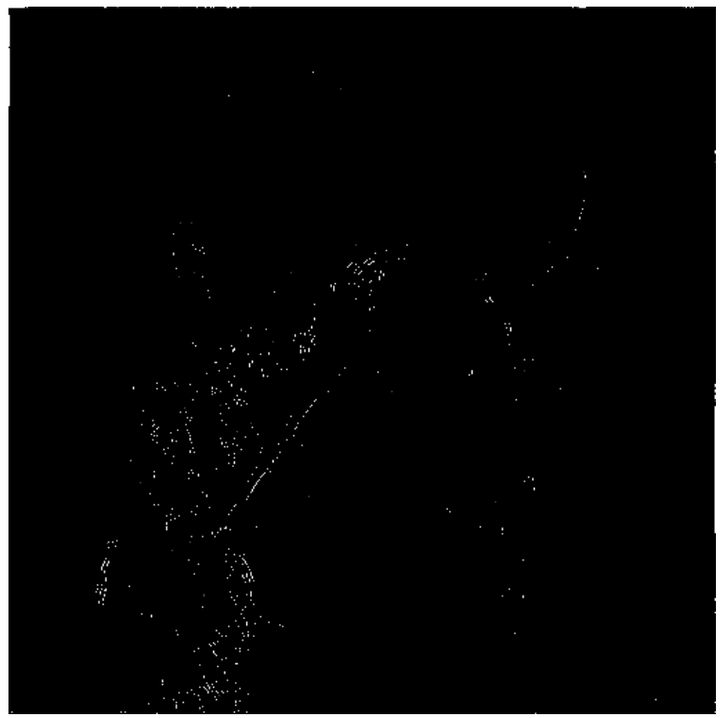
IFT

FT



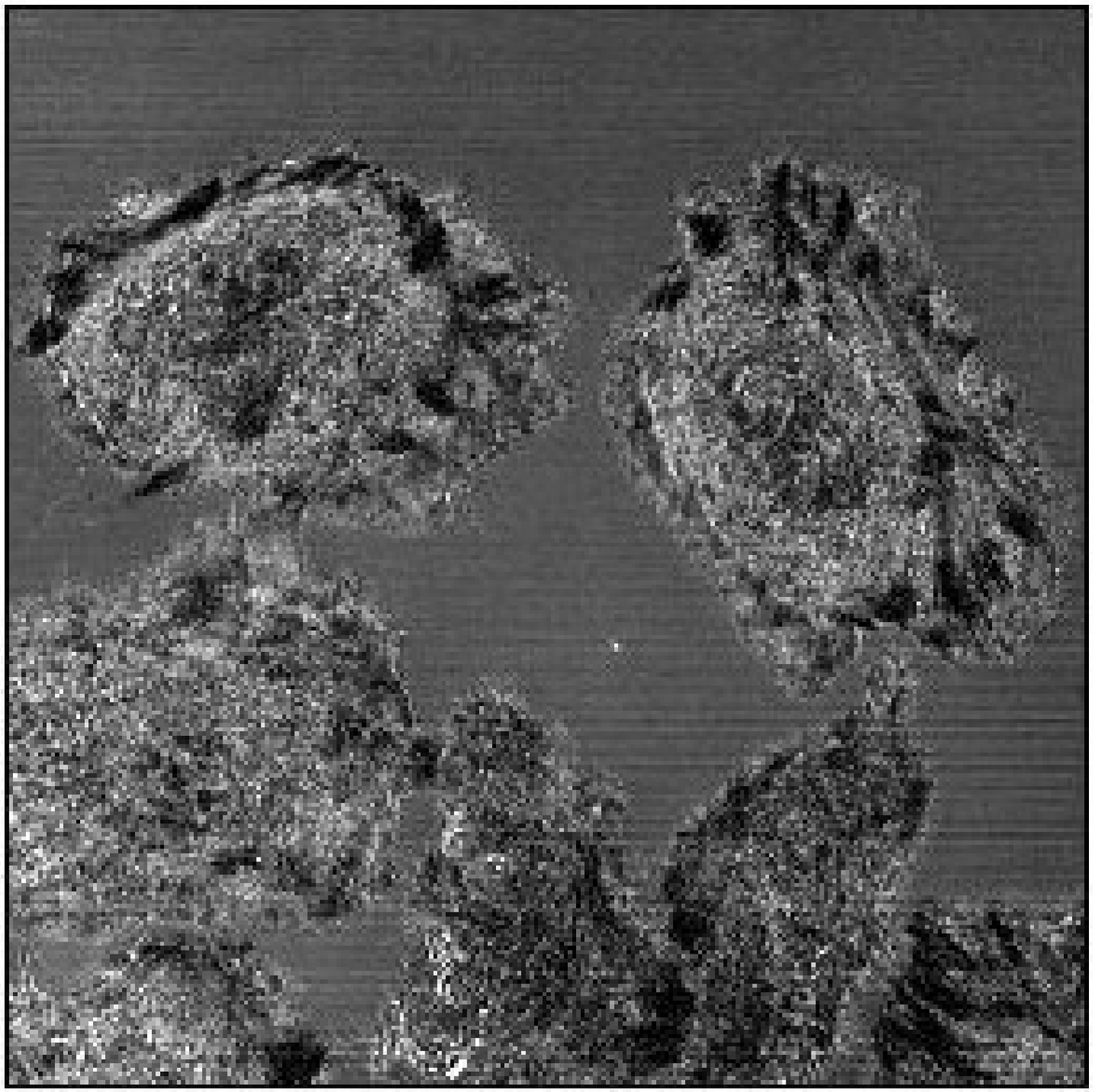


IFT



High frequence pass

… a filter for periodic noise:

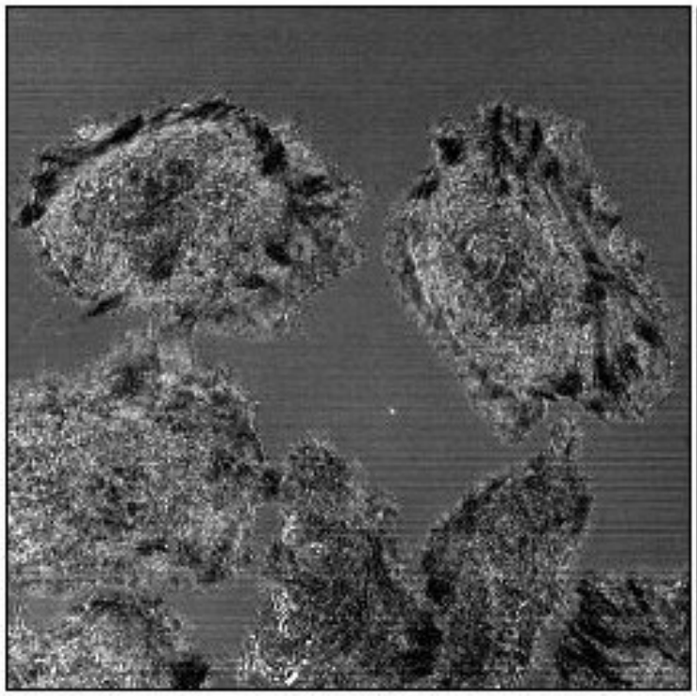


The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.

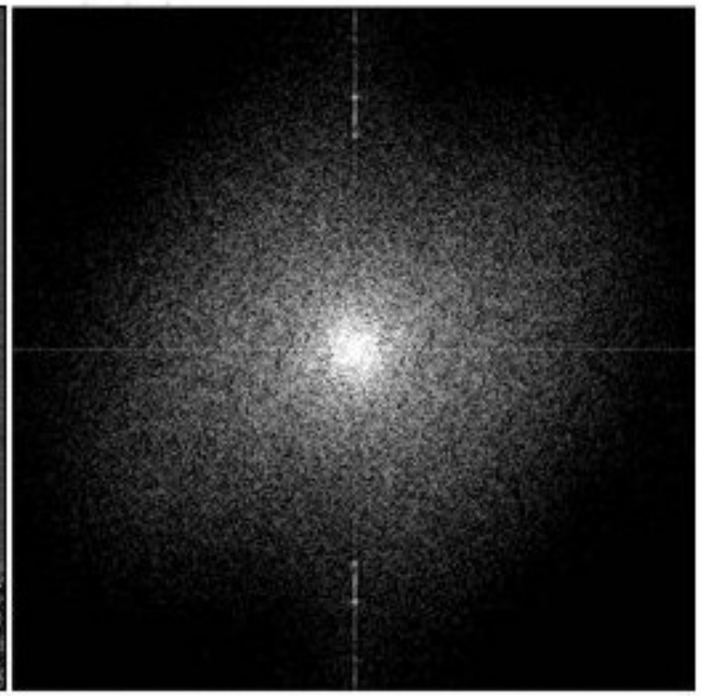
… a filter for periodic noise:

Laser intensity noise from a bad AOTF…

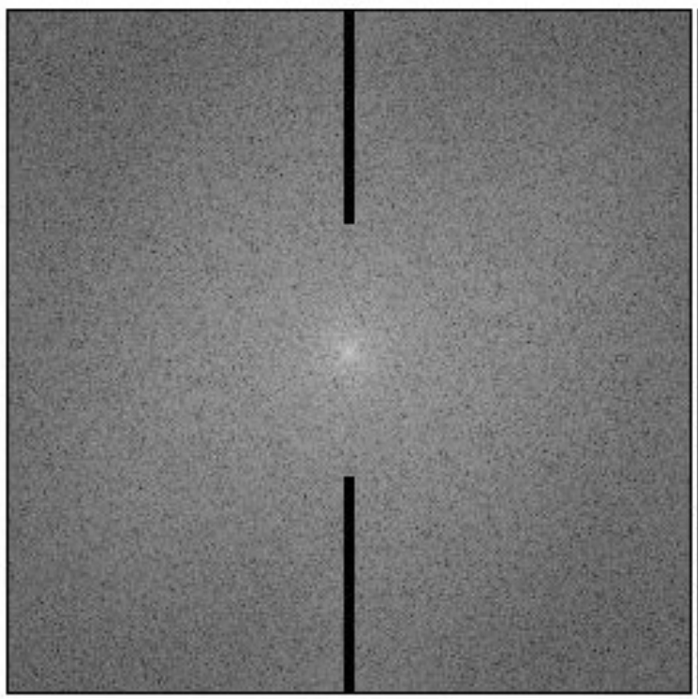
can be removed by frequency filtering in the correct spatial direction.



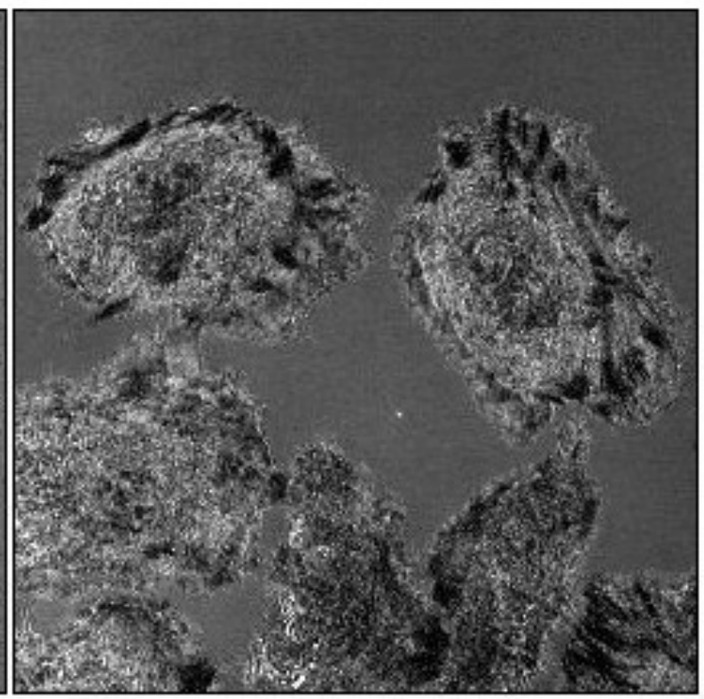
The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.



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

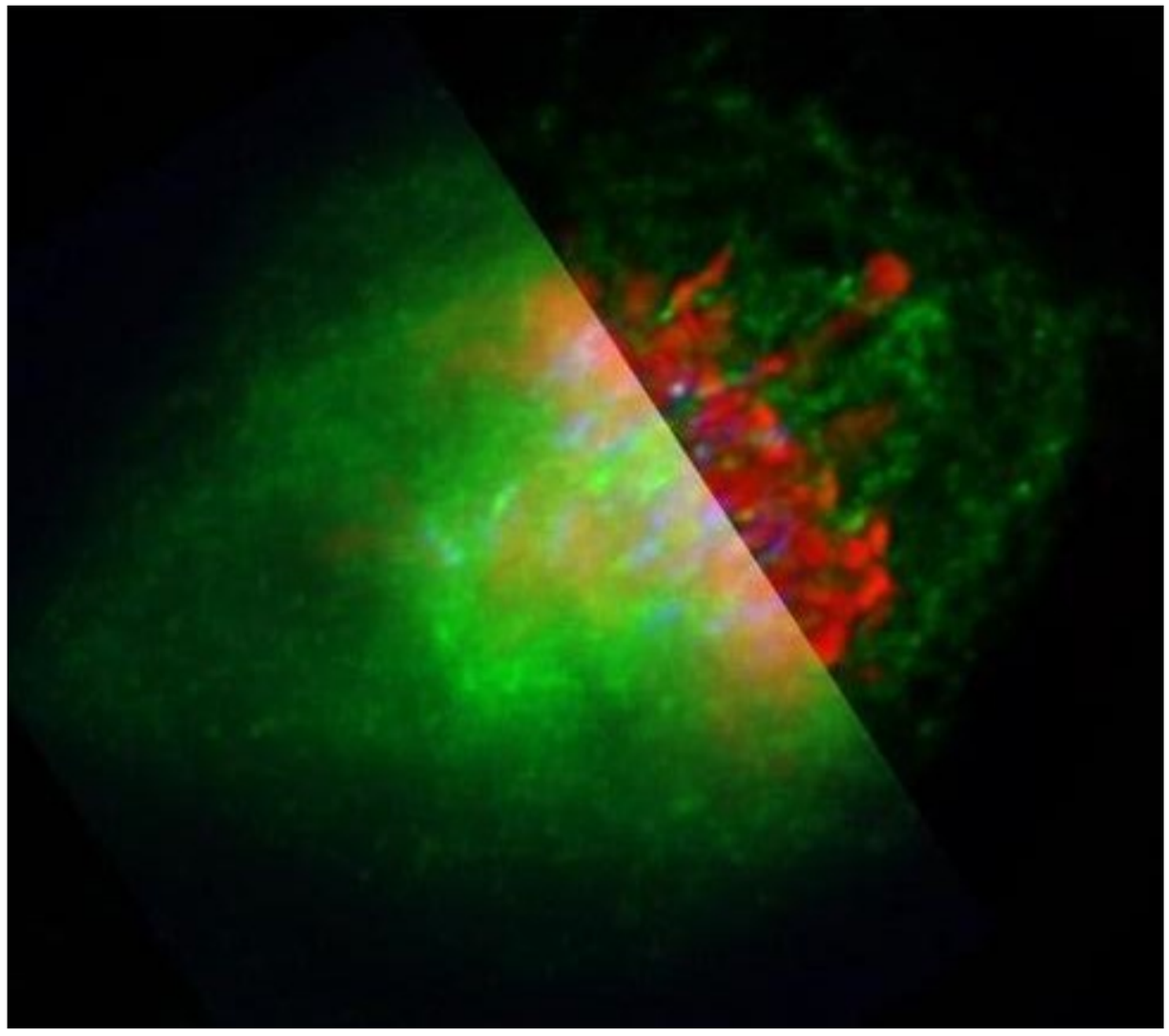
+ Do Fourier transforms

+ Image FT / PSF FT (kinda…)

+ 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). Recorded by Claire Acquaviva, Pines lab

Left part: original data

Right part: deconvolved with Huygens Professional.