Introduction to
Digital Image Processing and Analysis

Fabrice P. Cordelières, PhD
Bordeaux Imaging Center

fabrice.cordelieres@u-bordeaux.fr
@fab_cordelieres, @BIC_Bordeaux

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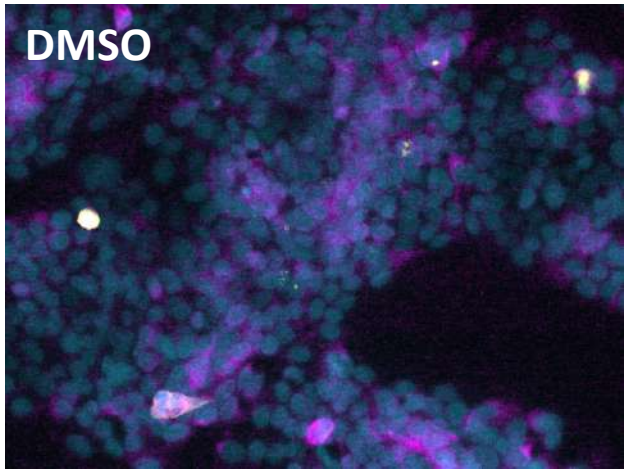
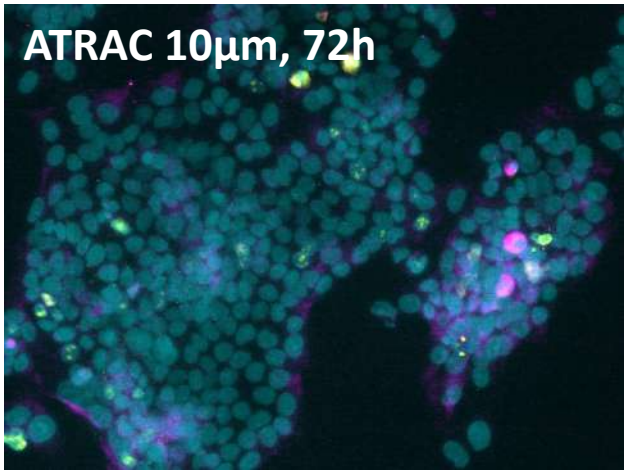
Before we start…

What are we going to do ?





Labelling, on the pictures:

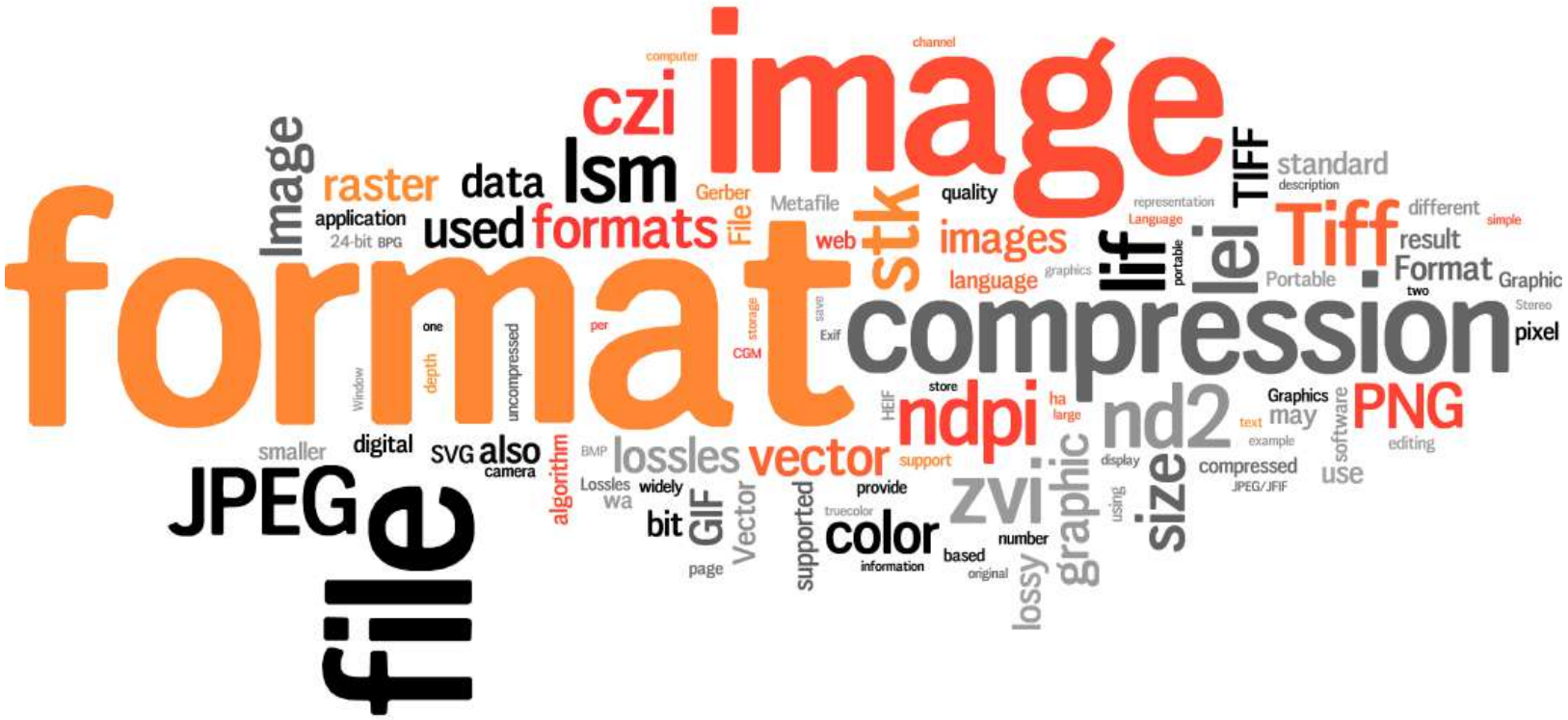
- **Cyan**: DAPI, nuclear staining
- **Magenta**: p16, inhibitor of CDK/Cyclin, preventing entry in G1/S
- **Yellow**: Ki67, expressed by cycling cells

***Our aims**: count cells and quantify p16 and Ki67 signals, cell per cell*

What will we learn during this session ?

Step 1	Prepare nuclei image for segmentation	***Play with the image's display, visualize and handle the noise***
Step 2	Differentiate signal from background	***Partition pixels into 2 classes, based on their intensities***
Step 3	Handle touching nuclei	***Use morphomathematical operations to separated objects***
Step 4	Individualise nuclei, get their outlines	***Tag positive pixels into an objects' map, extract objects' boundaries***
Step 5	Quantify p16 and Ki67 signals, cell per cell	***Use nuclei boundaries to extract the associated signal***

What is an image ?



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What is an image ?

The image IS NOT the object

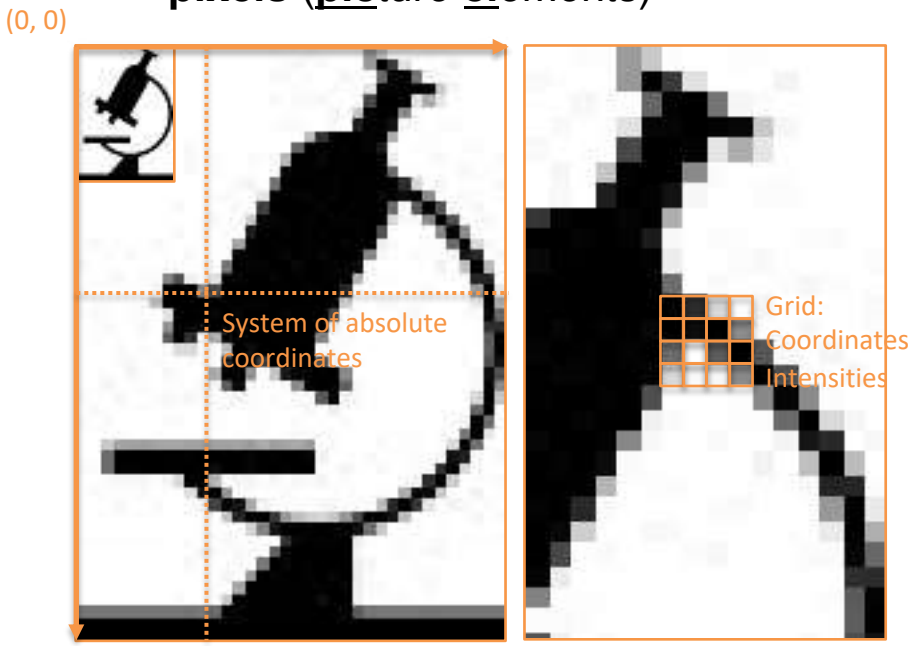
René Magritte, La trahison des images, 1928-29, huile sur toile, Los Angeles county Museum of Art, Los Angeles.

What is an image ?

The nature of data

Raster image

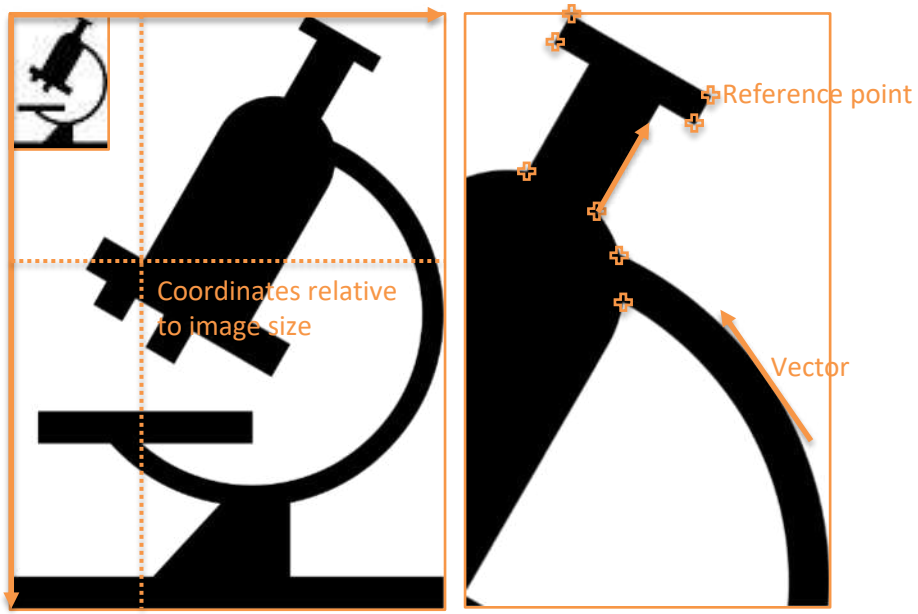
- Painted using individual elements: **pixels** (**pic**ture **el**ements)



- Printing quality **depends** on a compromise between **dimension** and **resolution**

Vector image

- Painted using **vectors** and **mathematical descriptors**



- Printing quality is **independent** of **dimension** and **resolution**

What is an image ?

An image is a matrix

255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	253	197	141	126	144	166	243	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	201	102	61	110	154	252	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	254	195	110	69	111	158	253	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	248	149	89	79	102	130	239	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	245	161	126	75	118	139	236	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	254	192	168	79	153	176	252	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	254	196	179	84	159	183	252	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	252	174	136	69	128	154	248	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	252	162	113	59	105	135	245	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	255	251	163	120	56	96	138	247	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	248	230	215	223	243	254	250	158	122	54	92	140	248	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	229	171	112	135	197	250	250	151	117	55	93	148	251	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	253	226	113	163	246	253	239	130	98	60	99	137	244	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	224	151	86	122	168	151	131	114	89	62	118	107	181	253	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	220	130	81	102	141	183	182	148	122	60	102	148	213	254	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	233	138	84	101	160	254	254	201	106	66	112	215	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	234	137	84	99	162	254	255	235	105	74	132	246	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	234	137	85	97	162	254	255	244	128	78	150	251	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	234	132	84	95	163	254	255	252	165	100	189	255	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	254	202	103	63	76	121	196	211	203	188	175	213	231	232	241	254	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	252	156	102	71	84	62	70	82	77	76	72	95	109	111	139	233	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	254	205	112	81	79	108	139	115	104	67	55	70	105	94	158	238	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	241	137	87	80	121	159	226	223	107	78	122	232	238	248	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	241	135	92	93	104	104	223	249	226	217	227	251	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	241	132	94	101	153	168	247	246	249	253	255	255	255	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	240	133	97	95	137	149	173	134	186	200	220	246	254	255	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	255	240	132	98	92	111	106	58		60	123	158	179	215	253	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	254	202	112	88	98	120	213	200	165	140	110	118	105	160	245	255	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	246	137	98	72	85	72	112	125	106	94	85	93	94	105	194	254	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	234	109	93	89	88	77	92	97	93	92	91	94	96	99	148	247	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	230	105	94	106	96	87	85	85	85	86	87	90	92	93	128	243	255	255	255	255	255	255	255
255	255	255	255	255	255	255	255	255	247	197	167	158	149	144	145	143	142	142	144	146	147	148	174	248	255	255	255	255	255	255	255

What is an image ?

Data handling in a computer: bits and bytes

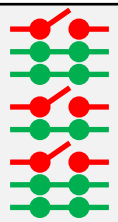
Bit
Binary digIT

0: OFF

1: ON

2 states per bit

Byte
8-bits



0 1 1 0 1 0 1 1

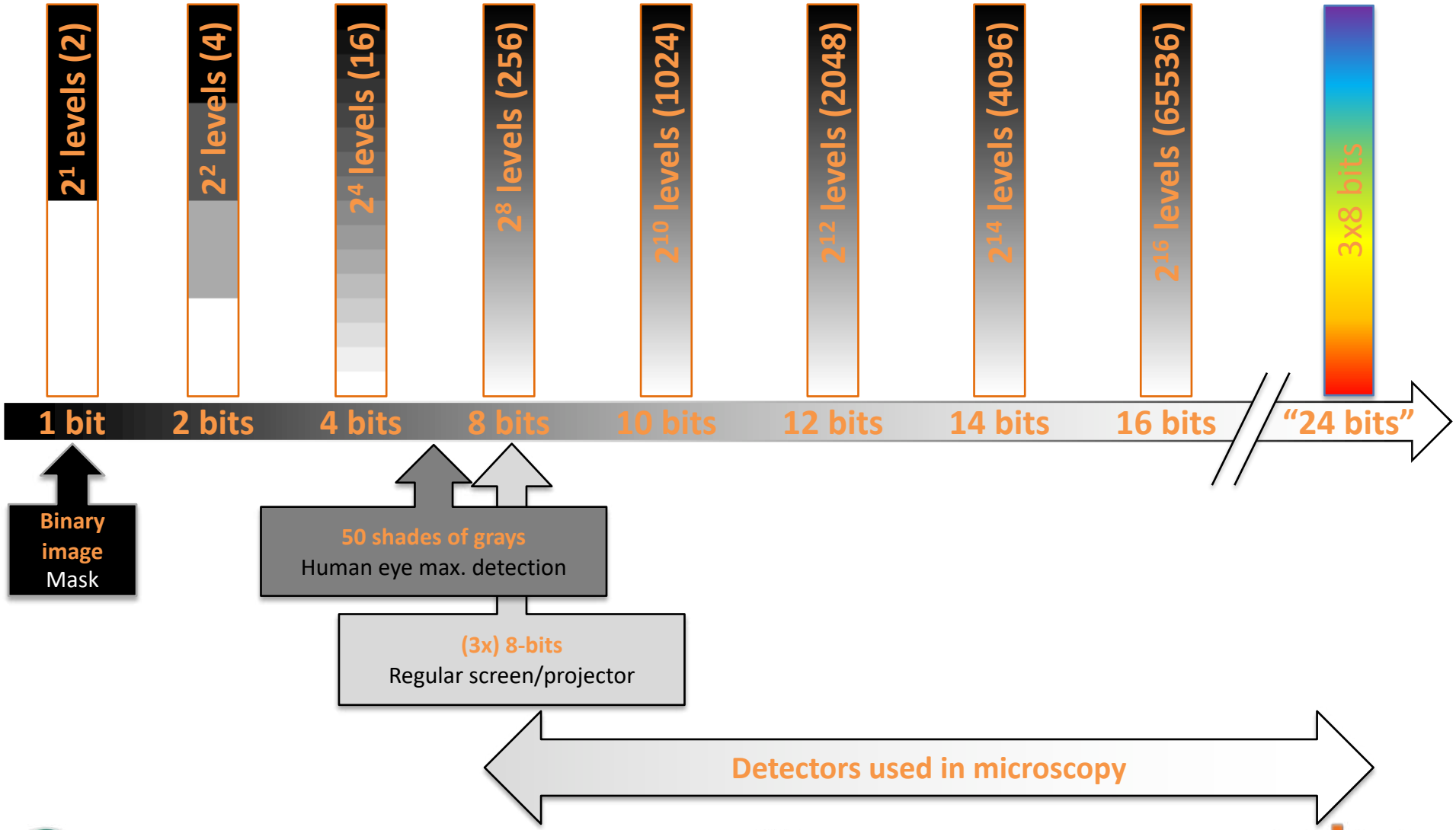
2^8 states per byte

	0	**1**	**1**	**0**	**1**	**0**	**1**	**1**	
Little endian	2^0 **1**	2^1 **2**	2^2 **4**	2^3 **8**	2^4 **16**	2^5 **32**	2^6 **64**	2^7 **128**	
	0	**2**	**4**	**0**	**16**	**0**	**64**	**128**	**214**
Big endian	2^7 **128**	2^6 **64**	2^5 **32**	2^4 **16**	2^3 **8**	2^2 **4**	2^1 **2**	2^0 **1**	
	0	**64**	**32**	**0**	**8**	**0**	**2**	**1**	**107**

"There are only 10 types of people in the world: those who understand binary, and those who don't."

What is an image ?

Dual sampling: intensity and bit depth



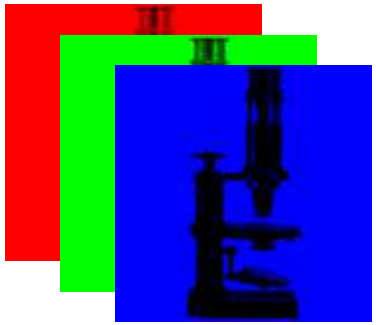
What is an image ?

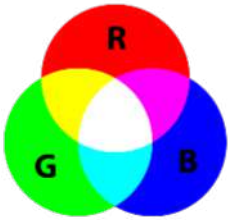
Color versus colorised image

Color image

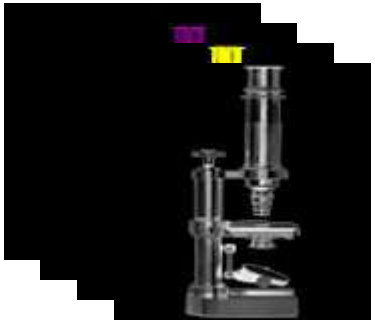
- A reference is selected
- One channel is generated per reference's component
- Each color is expressed as a weighted sum of each component

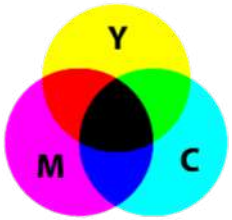
Additive mixing





Subtractive mixing



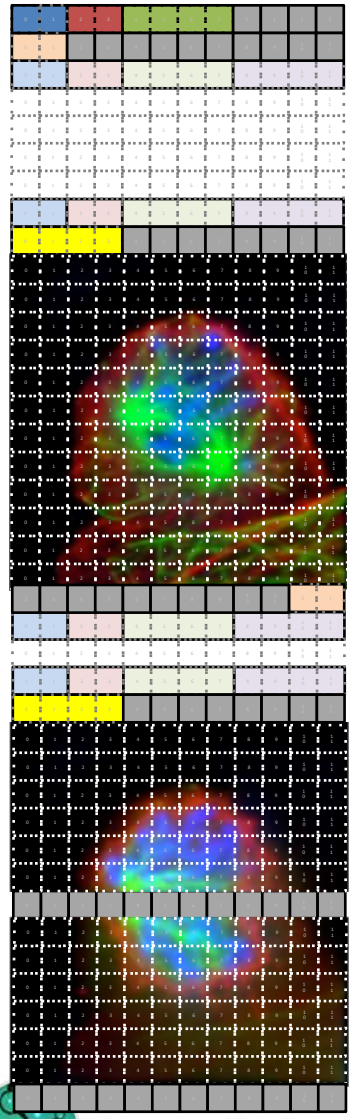


Indexed colors

- Build a dictionnary: 1 color=1 reference
- Replace each pixel's color by its reference

What is an image ?

Storing an image: the container



File format

- TIFF (Tagged Image File Format)
- ~~JPEG (Joint Photographic Experts Group)~~
- JFIF (JPEG File Interchange Format)
- PNG
- CZI, LIF, ND2, NDPI, OIB, ZVI…

Metadata

ImageWidth	ImageDescription
ImageLength	Make
BitsPerSample	Model
Compression	XResolution
PhotometricInterpretation	YResolution
FillOrder	…
DocumentName	

Image

Compression

***CODEC** (**CO**mpression/**DEC**ompression)*

- RLE (Run Length Encoding, PackBits)
- LZW (Lempel-Ziv-Welch)
- JPEG (Joint Photographic Experts Group)
- Modified Huffman compression (CCITT Group 3 1D)

What is an image ?

Storing an image: saving space, compression strategies

Run-length encoding

Sentence:

AAABBBBBBAAACCCCCAAAAAB

- For each value, count the number of occurrence

Compressed sentence:

3A6B3A5C5A1B

Dictionary-based compression

Sentence:

ABCDDEFABCEFEABC

- Identify individual words

  ABCDDEFABCEFEABC

- Build a dictionary:

  1=ABC; 2=DD; 3=EF; 4=E

- Re-write the sentence using the dictionary:

Compressed sentence:

1231341

Used in LZW/ZIP compressions

Only** non destructive compressions **should be used for image processing and analysis

JPEG is a destructive compression: to be ONLY used for mail or presentation purposes

Tutorial 1

From individual images to an overlay

- Transtype each RGB image into an 8-bits image

  Image>Type>8-bits

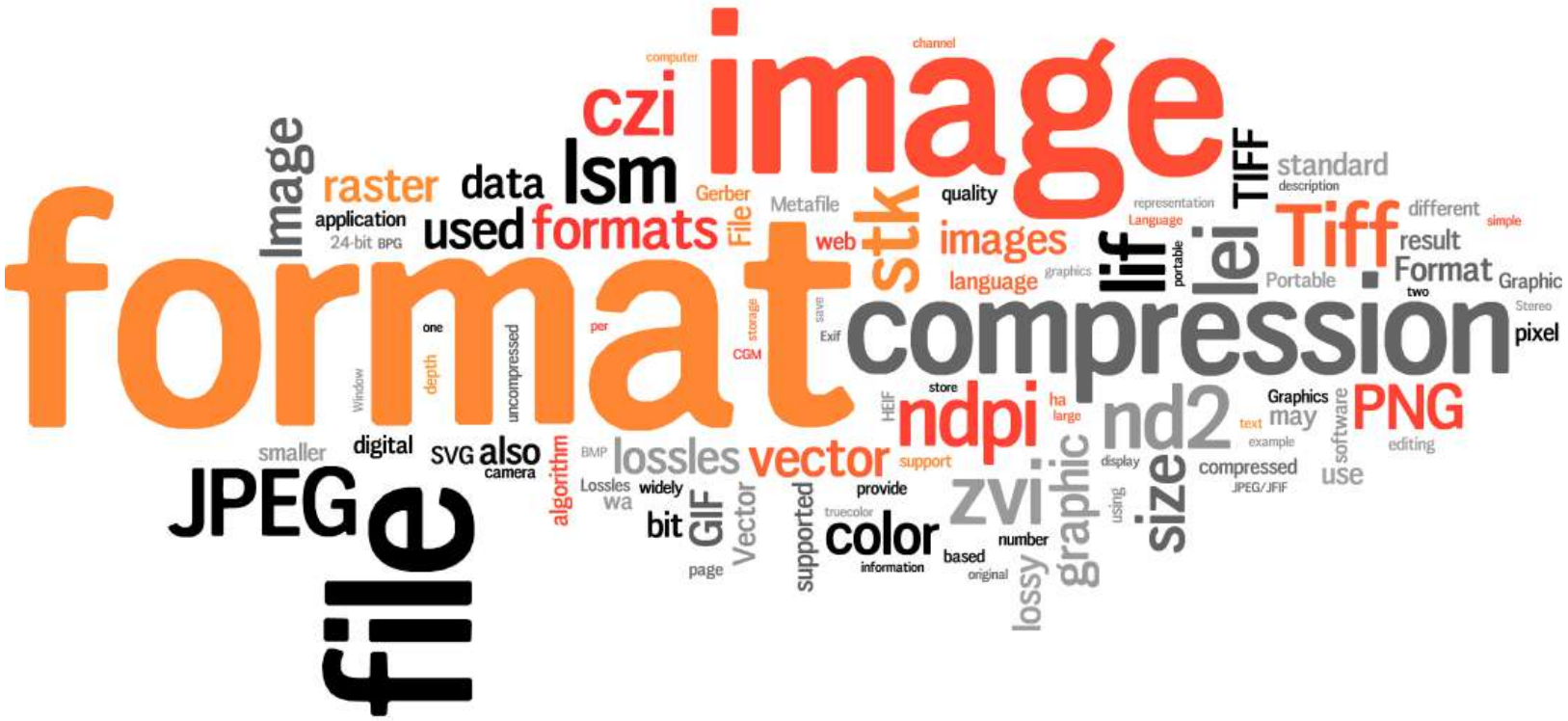
- Overlay images

  Image>Color>Merge channels

- Interact with the display

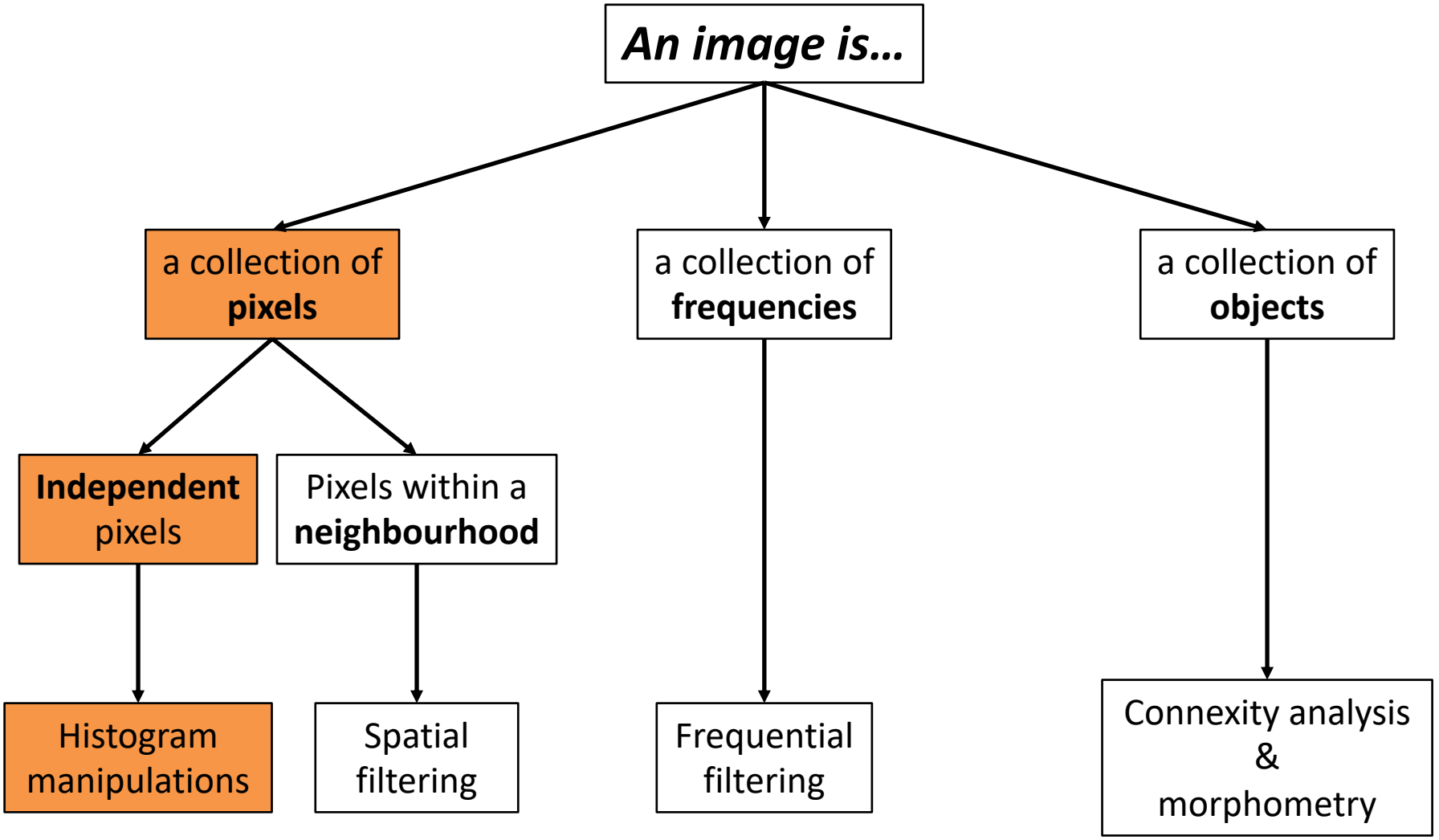
  Image>Color>Channels tool

Image Processing



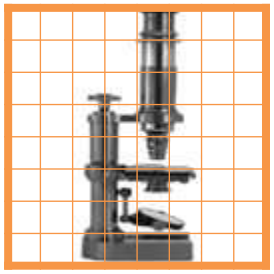
Several way to consider a single image

And associated processing techniques

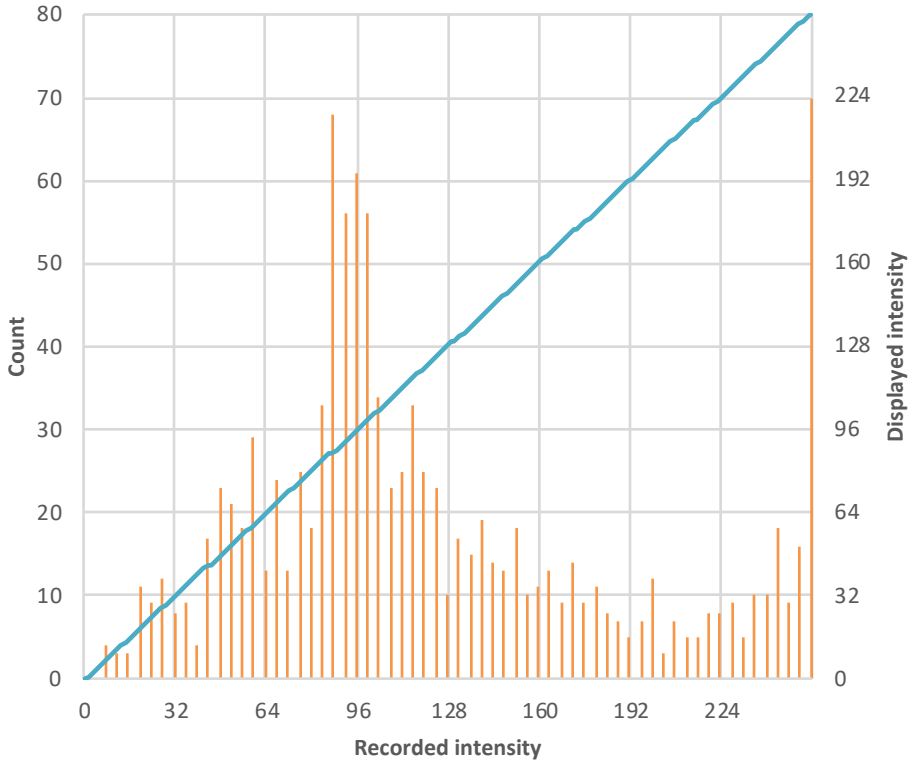


The image is a collection of intensities

Working with the image's histogram



1. Group pixels per increasing intensity
2. Count pixels per group
3. Plot count as a function of intensity



*This is a **REALLY BAD** histogram ! But a good support to illustrate histogram modifications…*

Tutorial 2

Explore data display

- Have a look at the intensities' distribution

  Analyze>Histogram

- Place a rectangular ROI and activate live mode

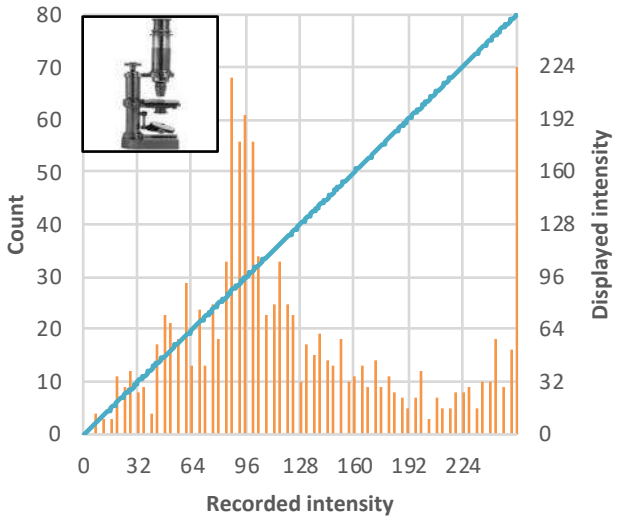
  Histogram window>Click « live »

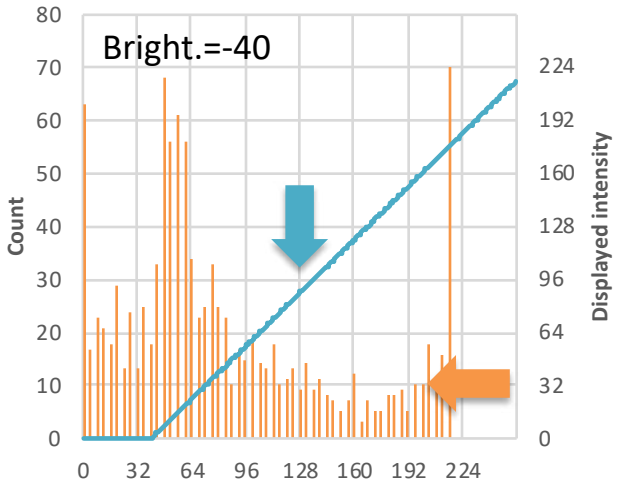
- Modify the image's display

  Image>Adjust>Brightness and contrast

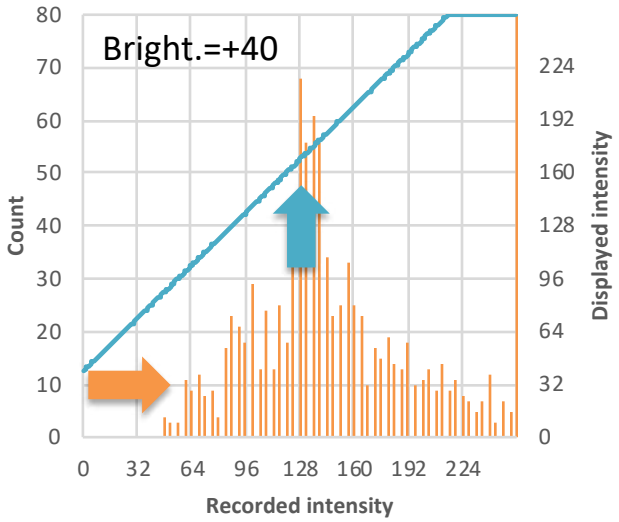
The image is a collection of intensities

Linear histogram modification: brightness





Brightness:
The same value is added to all intensities

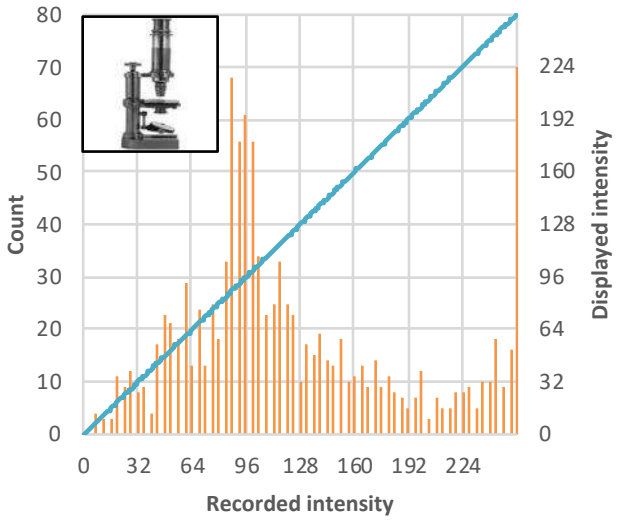


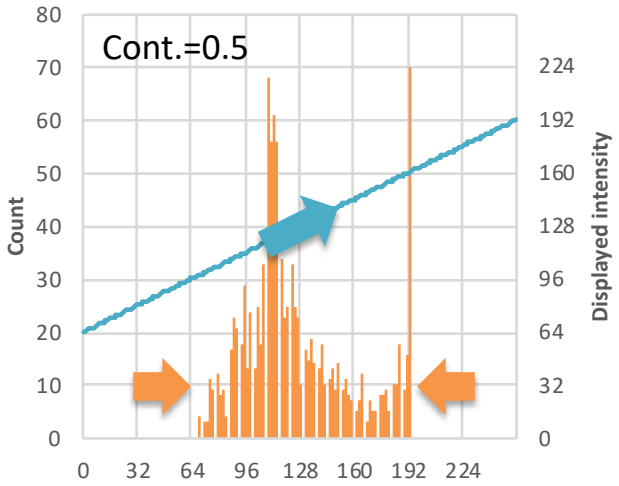
Thresholding:
Negative values are shifted to zero

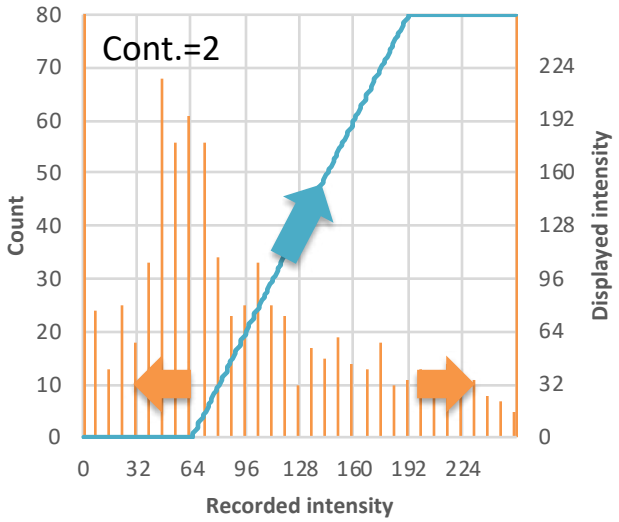
Saturation:
Values over the maximum of the range are clipped to the maximum of the range

The image is a collection of intensities

Linear histogram modification: contrast







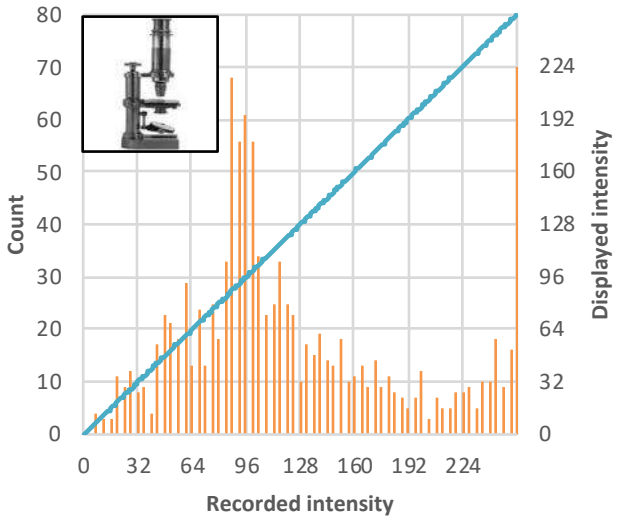
Contrast:
Response line: the slope is changed
The mid-range value remains constant

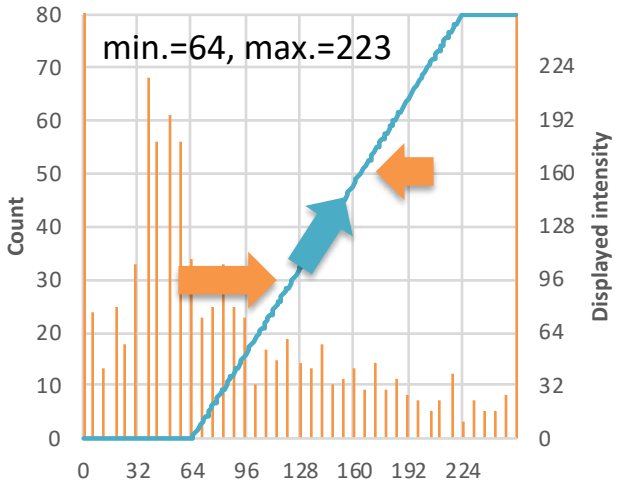
Thresholding:
Negative values are shifted to zero

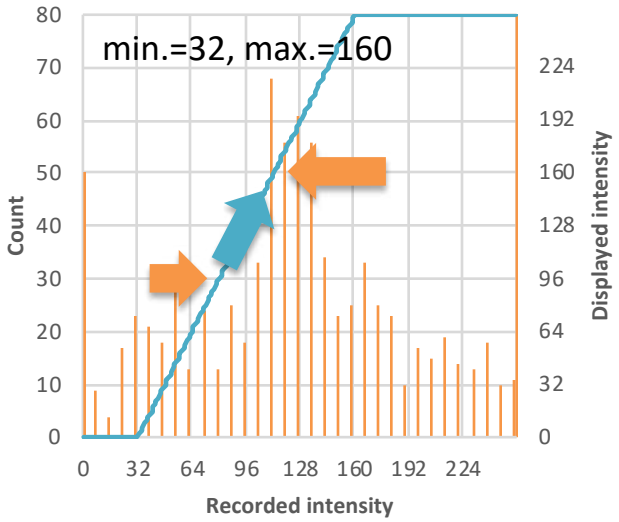
Saturation:
Values over the maximum of the range are clipped to the maximum of the range

The image is a collection of intensities

Linear histogram modification: min-max







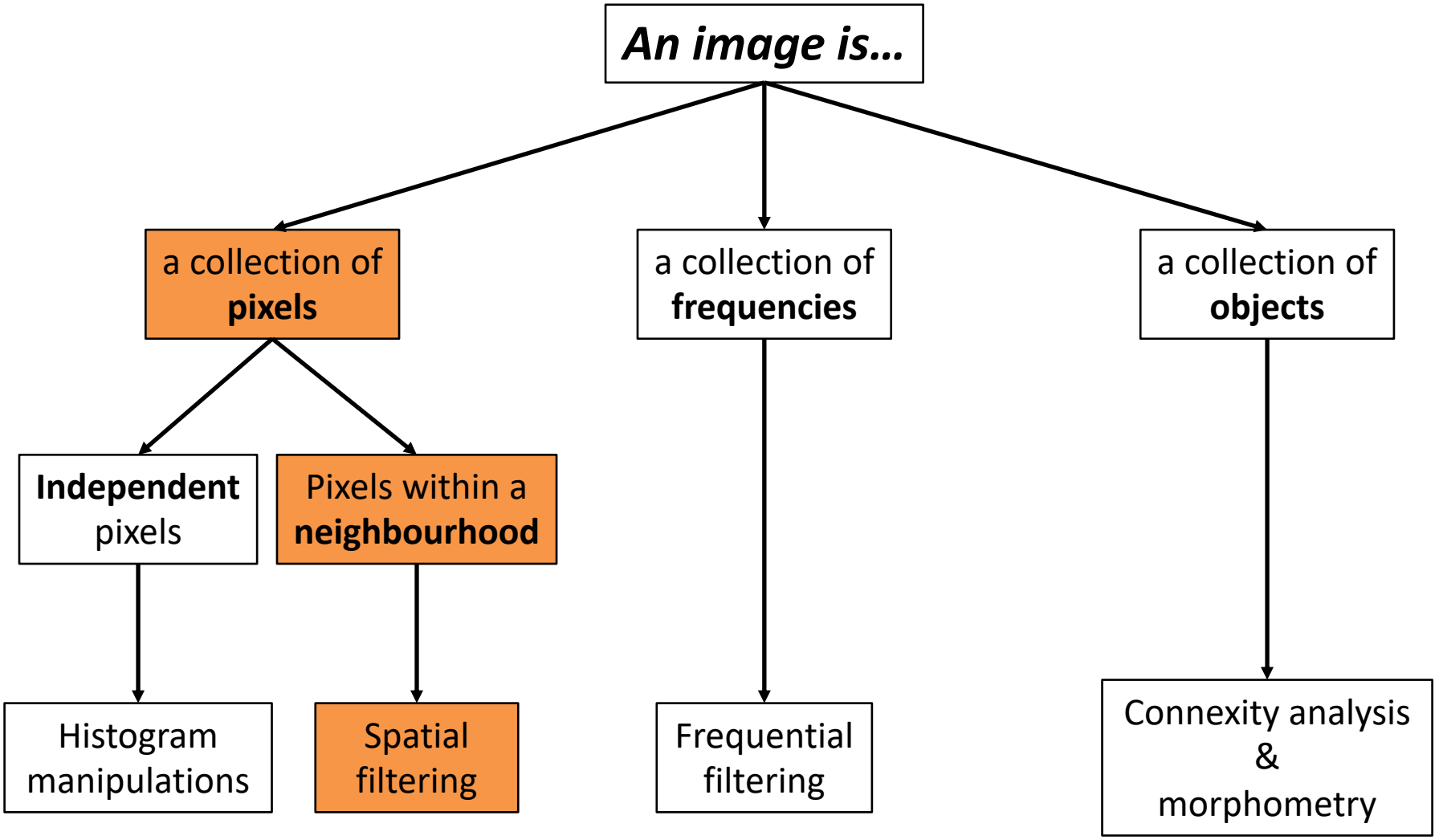
Min-Max:
Intensities are linearly distributed between the two newly set limits

Thresholding:
Negative values are shifted to zero

Saturation:
Values over the maximum of the range are clipped to the maximum of the range

Several way to consider a single image

And associated processing techniques



Tutorial 3

Explore local intensities

- Place a linear ROI over the image
- Modify the line's width

  Double click on the line tool
- Plot the intensity profile

  Analyze>Plot profile
- Activate the live mode

  Profile window>Click « live »

Spatial filtering

Linear filtering : taking care of the neighbours, based on values

Original image

168	151	131	114	89	62	118
141	183	182	148	122	60	102
160	254	254	201	106	66	112
162	254	255	235	105	74	132
162	254	255	244	128	78	150
163	254	255	252	165	100	189
121	196	211	203	188	175	213

Filter

1	1	1
1	1	1
1	1	1

168x1+151x1+131x1+
141x1+183x1+182x1+
160x1+254x1+254x1
=1624

1624/9=180.44444
➔**180**

168	151	131	114	89	62	118
141	**180**	182	148	122	60	102
160	254	254	201	106	66	112
162	254	255	235	105	74	132
162	254	255	244	128	78	150
163	254	255	252	165	100	189
121	196	211	203	188	175	213

Destination image

168	151	131	114	89	62	118
141	183	182	148	122	60	102
160	254	254	201	106	66	112
162	254	255	235	105	74	132
162	254	255	244	128	78	150
163	254	255	252	165	100	189
121	196	211	203	188	175	213

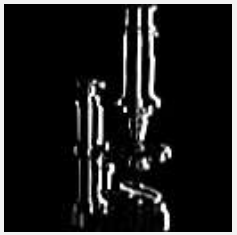
Spatial filtering

Most currently used filters

Original

Sobel Horizontal

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



Laplacian 4-connected

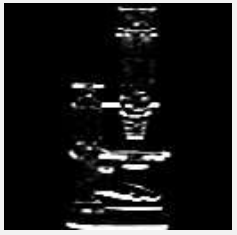
0	-1	0
-1	4	-1
0	-1	0

Gaussian (σ=1)

1	4	7	4	1
4	16	26	16	4
7	26	41	26	7
4	16	26	16	4
1	4	7	4	1

Sobel Vertical

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



Laplacian 8-connected

-1	-1	-1
-1	8	-1
-1	-1	-1

Sharpen

-1	-1	-1
-1	12	-1
-1	-1	-1

Sobel

$$\sqrt{Horizontal^2 + Vertical^2}$$

Your turn: be creative !

?	?	?
?	?	?
?	?	?

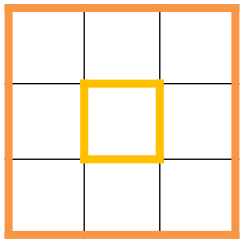
Spatial filtering

Rank filtering: taking care of the neighbours, based on order

Original image

168	151	131	114	89	62	118
141	183	182	148	122	60	102
160	254	254	201	106	66	112
162	254	255	235	105	74	132
162	254	255	244	128	78	150
163	254	255	252	165	100	189
121	196	211	203	188	175	213

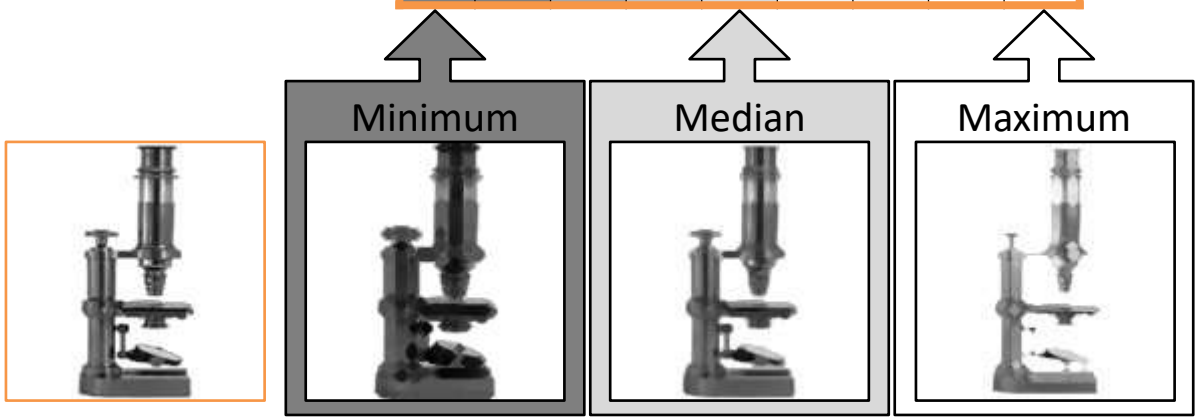
Filter



Destination image

168	151	131	114	89	62	118
141	183	182	148	122	60	102
160	254	254	201	106	66	112
162	254	255	235	105	74	132
162	254	255	244	128	78	150
163	254	255	252	165	100	189
121	196	211	203	188	175	213

131	141	151	160	168	182	183	254	254



Tutorial 4

Trying to smooth intensities, locally

- Always duplicate the image BEFORE trying anything

  Image>Duplicate

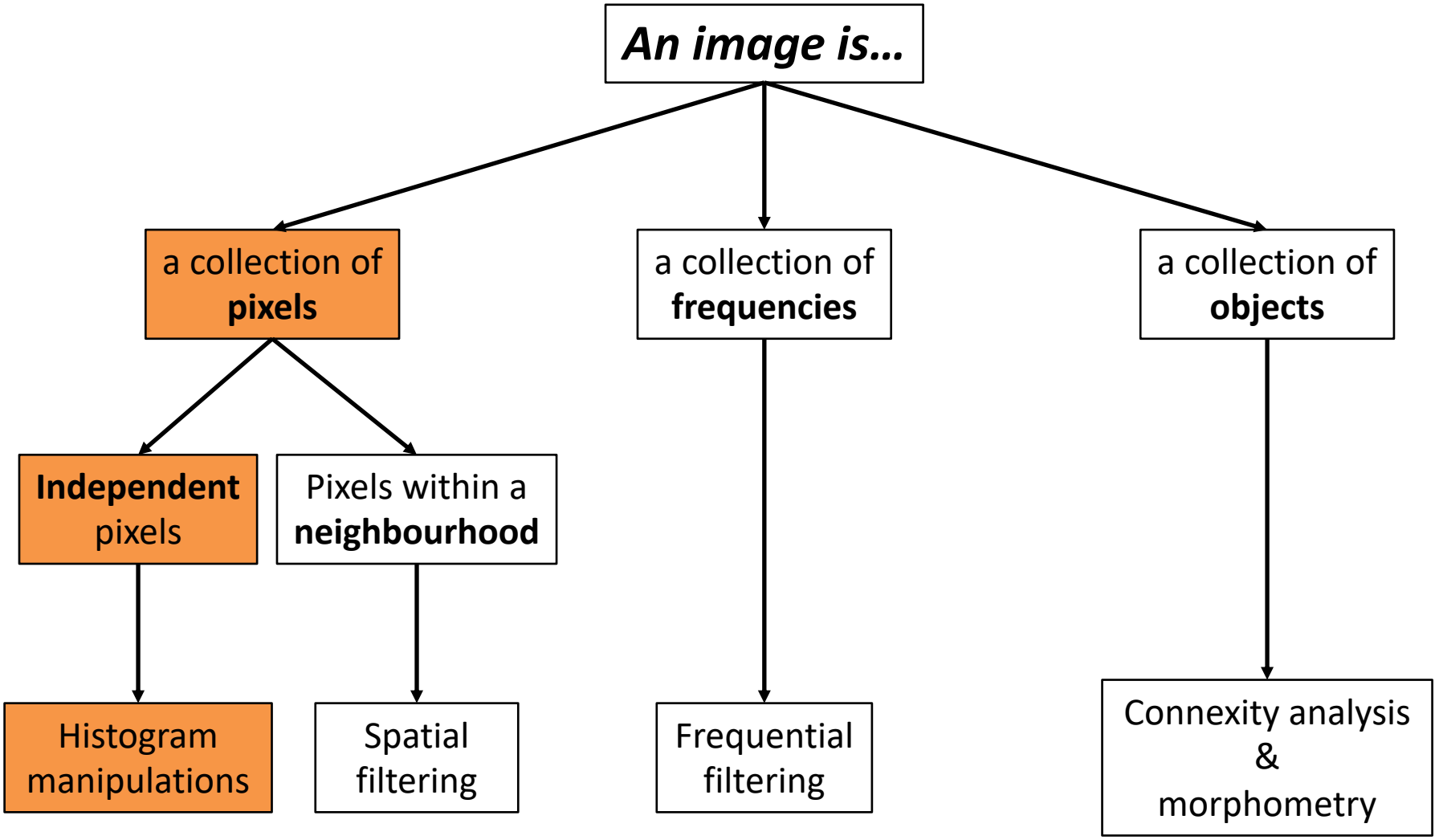
- Try the linear/rank filters on the image

  Process>Filters

- Control the effects using the profile plot in live mode

Several way to consider a single image

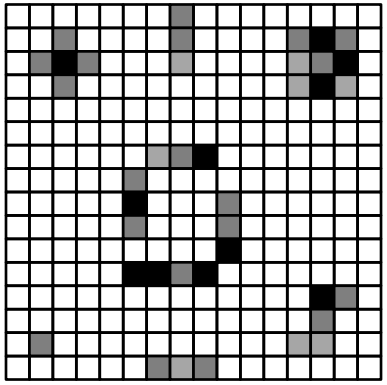
And associated processing techniques



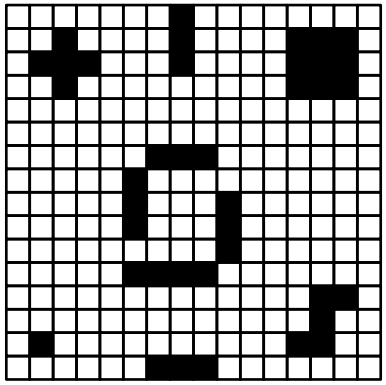
Morphomathematics

From intensity image to binary image (mask)

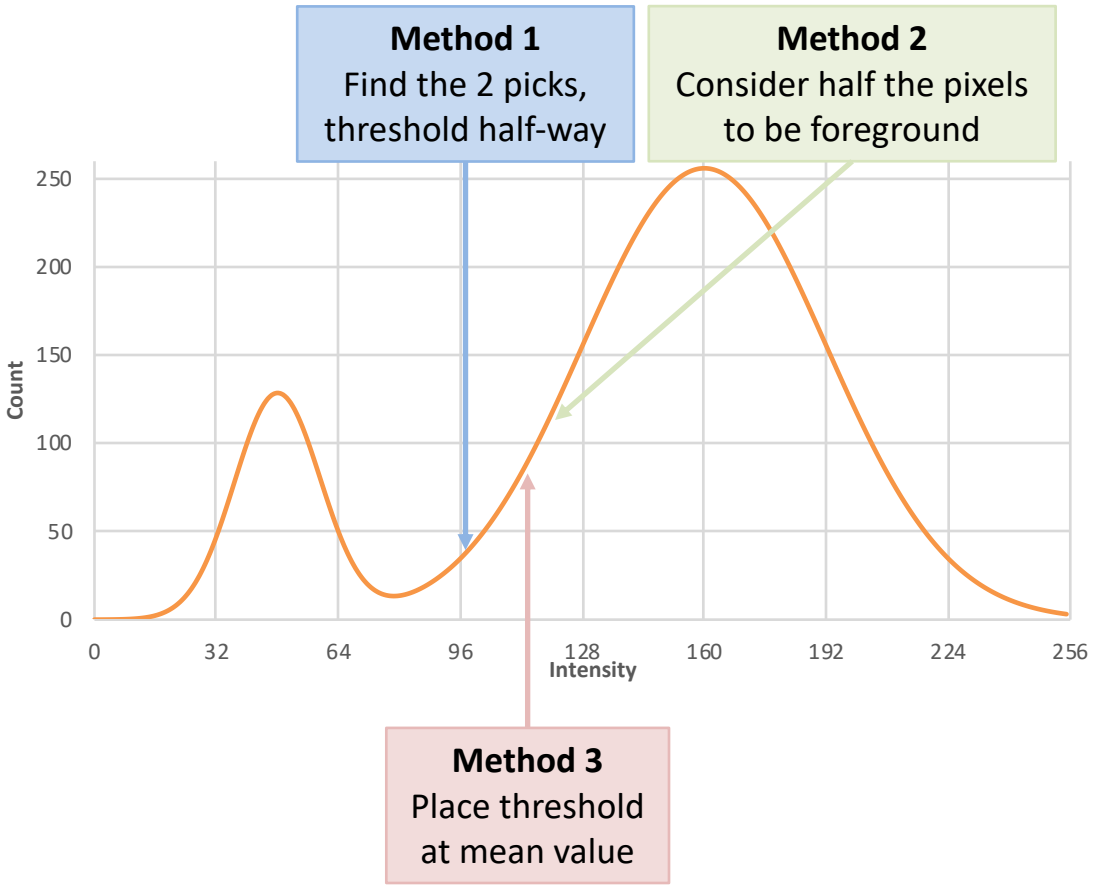
Intensity image



Binary image



How to partition image in object vs background pixels ?



Tutorial 5

Partitionning the intensities into 2 sets: signal and background

- Always duplicate the image BEFORE trying anything

  Image>Duplicate

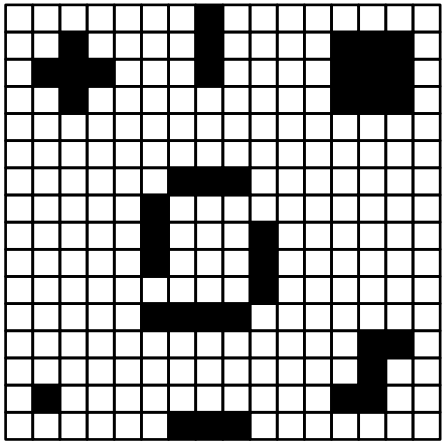
- Try several algorithm to highlight the nuclei in red

  Image>Adjust>Threshold

Mathematical morphology

Morphological operators: 4-connected case

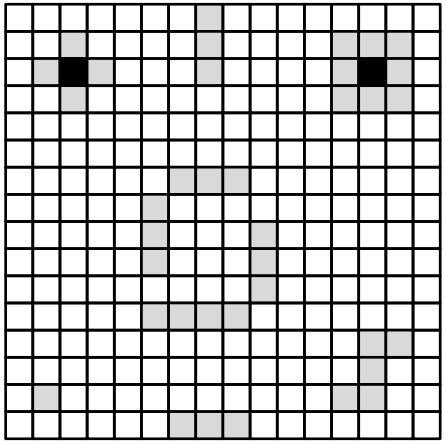
Structuring element



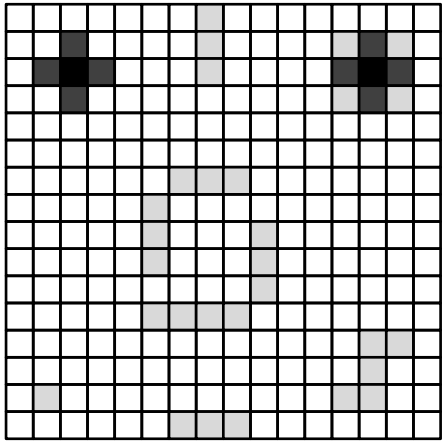
Binary image

Erode: propagate background

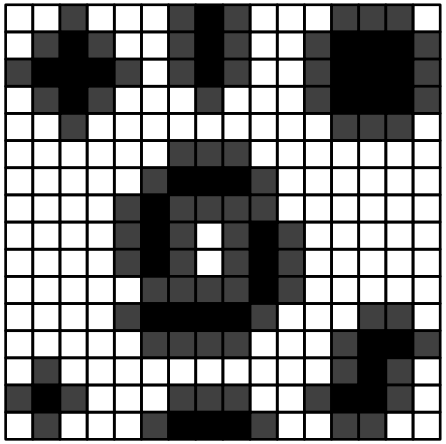
Dilate: propagate objects



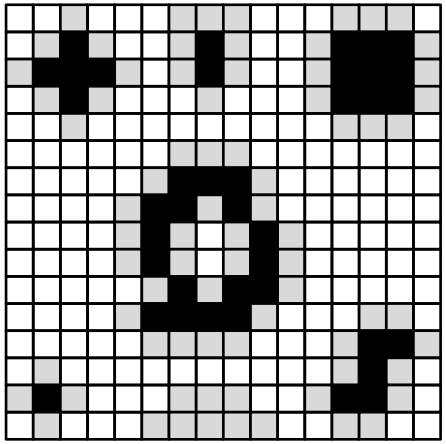
Erosion



Erode → Dilate = Open



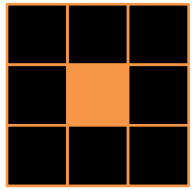
Dilation



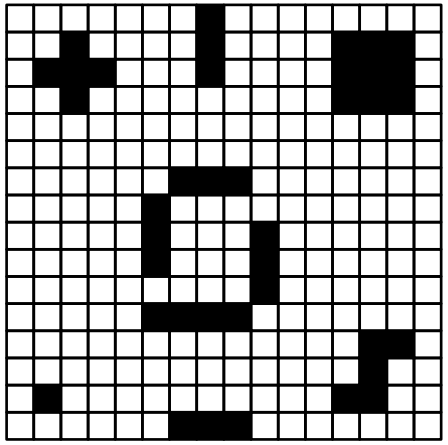
Dilate → Erode = Close

Mathematical morphology

Morphological operators: 8-connected case



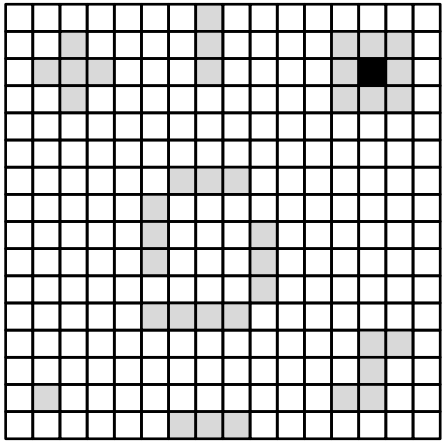
Structuring element



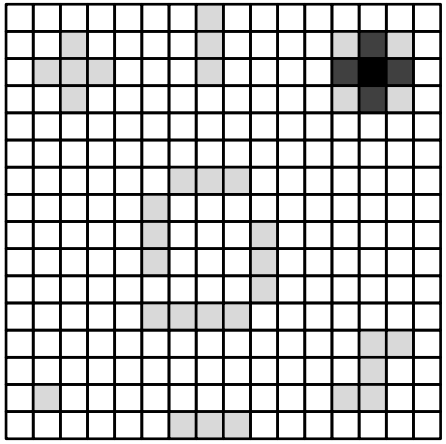
Binary image

Erode: propagate background

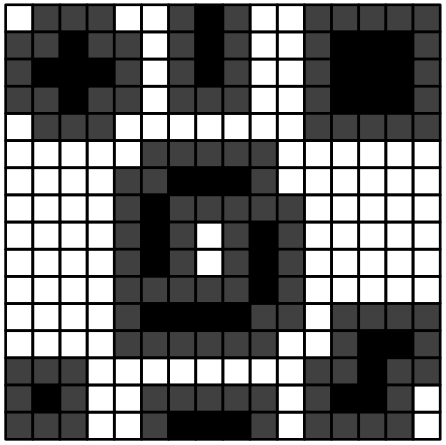
Dilate: propagate objects



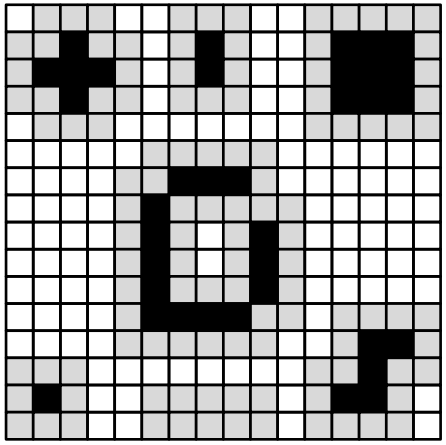
Erosion



Erode → Dilate = Open



Dilation

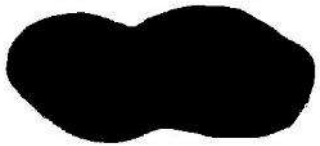


Dilate → Erode = Close

Mathematical morphology

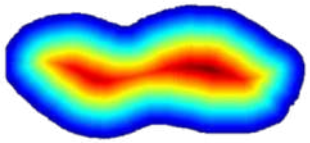
How to separate peanuts? Watershed transform

Original image



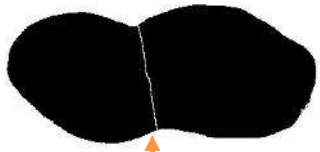
Mask

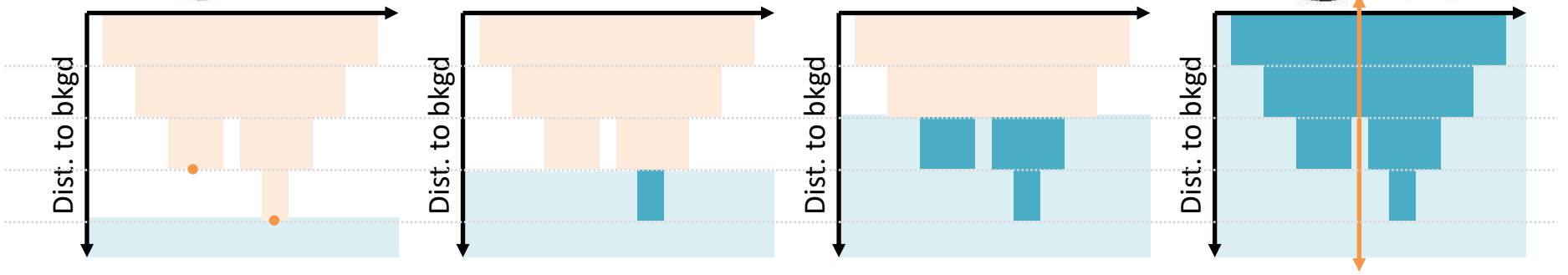
Euclidian Distance Map
Ultimate Erode Points



Flooding

Watershed





Combined
Watershed AND original image

Tutorial 6

Separating touching pixels' blocks

- Always duplicate the image BEFORE trying anything

  Image>Duplicate

- Try several algorithm to highlight the nuclei in red

  Image>Adjust>Threshold

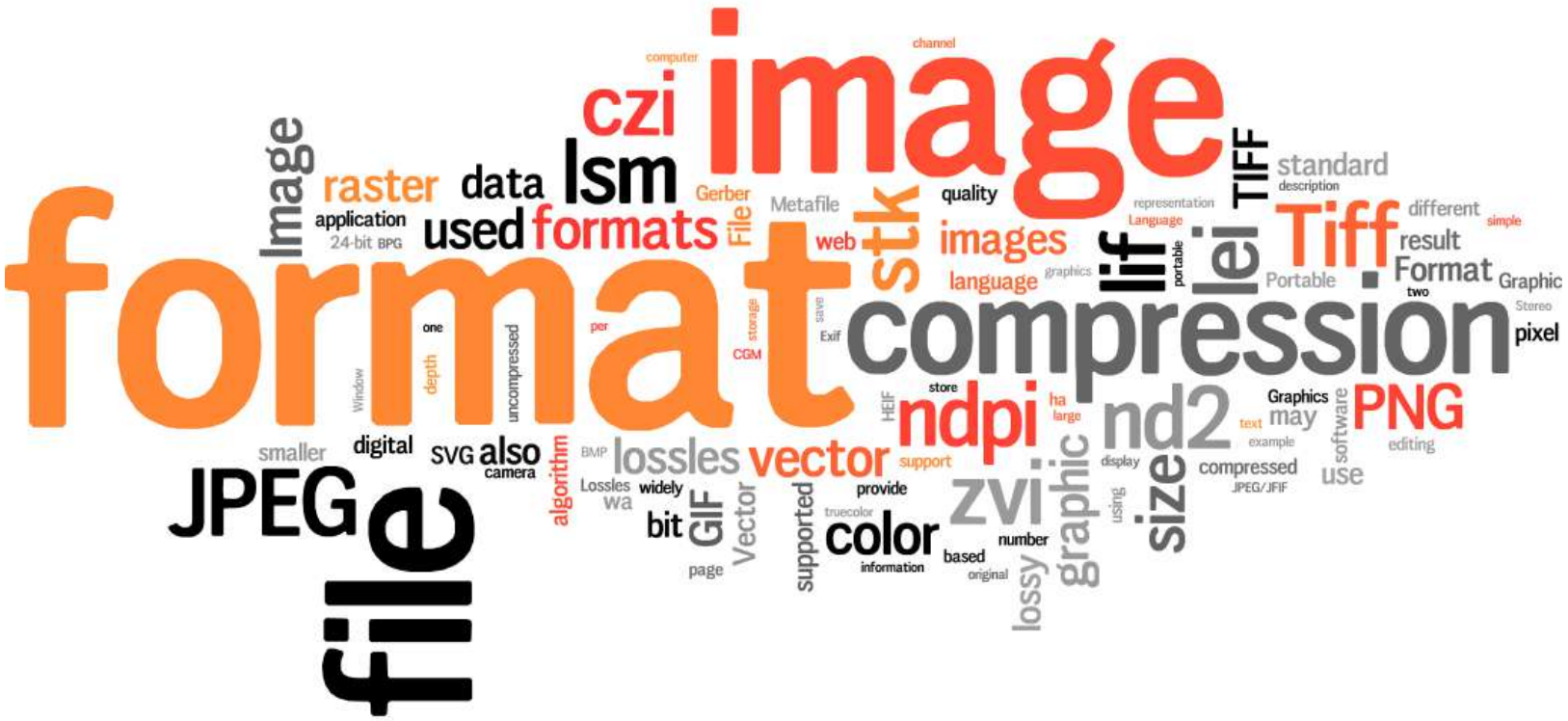
- Apply the threshold

  Threshold window>Apply

- Use the watershed processing

  Process>Binary>Watershed

2D morphometry



Several way to consider a single image

And associated processing techniques

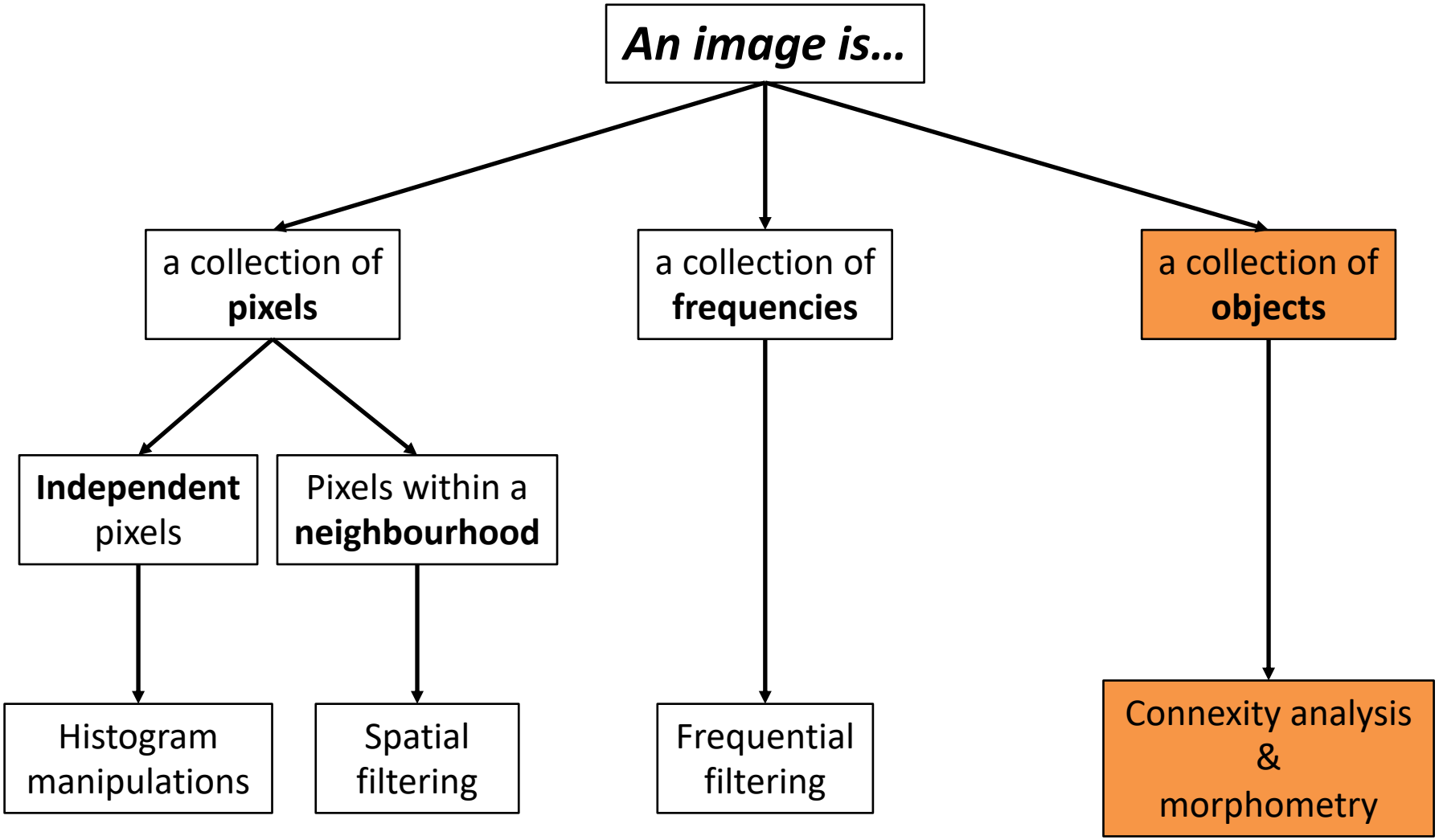
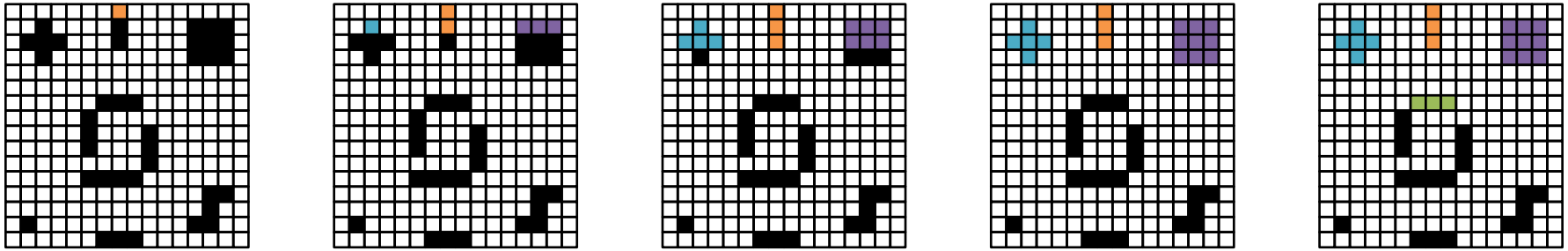


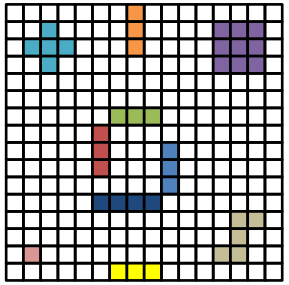
Image as a collection of objects

Connexity analysis

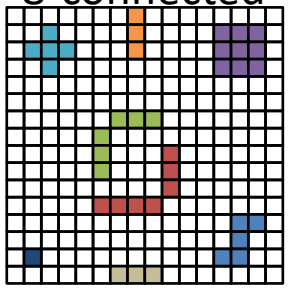
Pixel tagging, line per line ➔ Objects' map



4-connected



8-connected



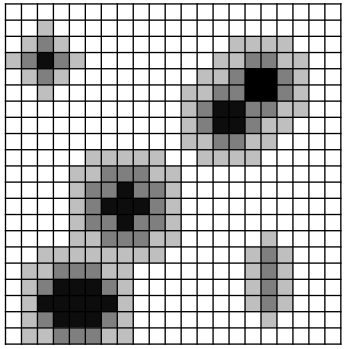
Features extraction:

-*Count objects*: maximum of all tags
-*Total intensity*: sum intensities for each tag
-*Area*: number of pixels per tag x pixel surface
-*Perimeter*: number of pixels lacking at least 1 neighbour x pixel size

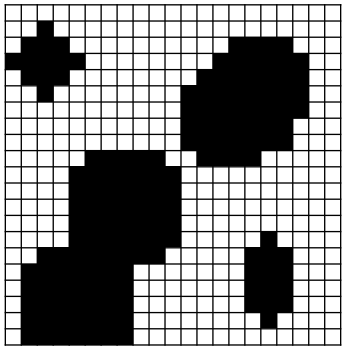
Extension to the 3D case:
6 or 26-connected

Image as a collection of objects

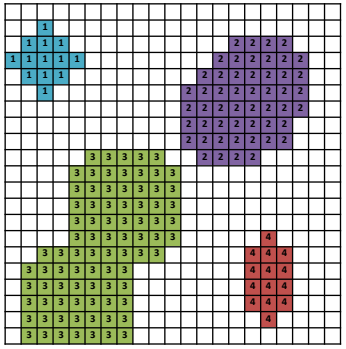
Influence of the threshold



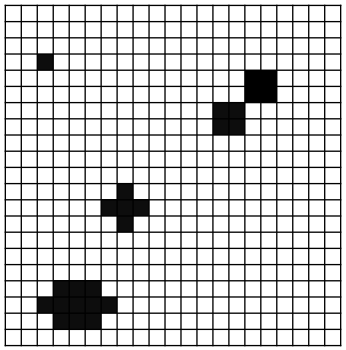
Original



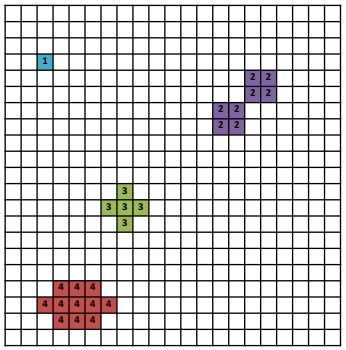
Low threshold



Tagged map



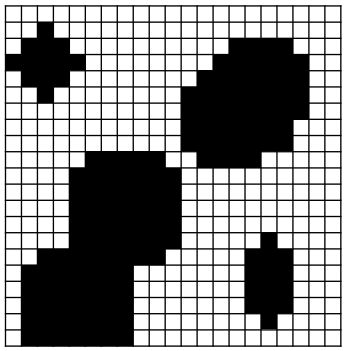
High threshold



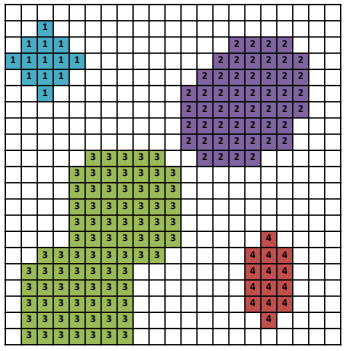
Tagged map

Image as a collection of objects

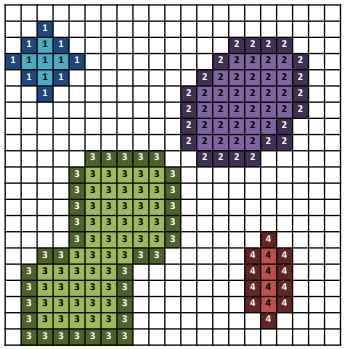
Connexity analysis: extracting data



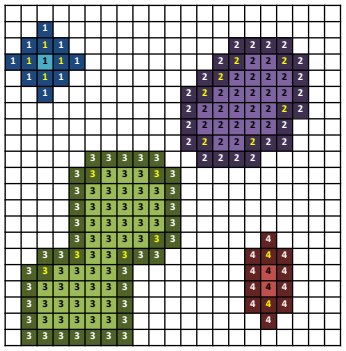
Low threshold



Tagged map



4-connected



8-connected

Example parameters:

Area: number of pixels carrying the same tag
Perimeter: number of pixels carrying the same tag, and lacking at least one neighbour

Tutorial 7

Grouping pixels into objects and extracting outlines

- Activate the thresholded, watershed processed image

- Group pixels into objects

  Analyze/Analyze particles

  (Check « Add to manager »)

- Display the detected ROIs over the image

  ROI Manager window>Check « Show all »

Tutorial 8

Extracting intensities for all channels

- Set the parameter to extract

  Analyze/Set Measurements

  Check: mean

- Activate the composite image and duplicate a channel

  Image>Duplicate

- Perform the measurements of all ROIs

  ROI Manager window>More>Multi-Measure

  Check: Nothing

- Get the distribution of the mean intensity, estimate nb of +w cells

  Result Table window>Results>Distribution

- Repeat for all channels

Final results

Nb cells	DAPI *cyan*	p16 *magenta*	Ki67 *yellow*
DMSO			
ATRAC			

Final results

Nb cells	DAPI *cyan*	p16 *magenta*	Ki67 *yellow*
DMSO	716	9 ***1.25%***	7 ***0.97%***
ATRAC	461	8 ***1.73%***	5 ***1.08%***