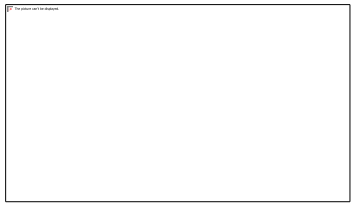
BIO-INSPIRED MATERIALS
NATIONAL CENTER OF COMPETENCE IN RESEARCH

Introduction to ImageJ session 1: Basics

Dimitri Vanhecke

March 2024



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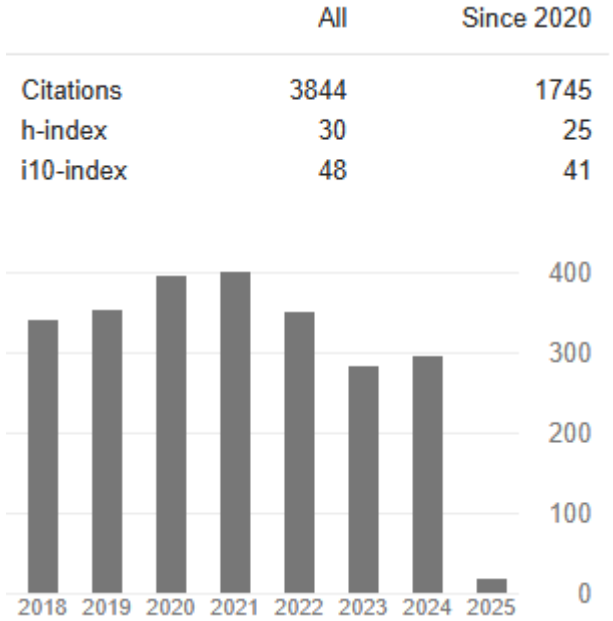
FNSNF
Swiss National Science Foundation

Administration

Who am I?

- PhD in structural biology (University of Bern)
- PostDoc in 3D image quantification
- 2 years at Max Planck Institute of Biochemistry, Munich, Germany
- At AMI since 2012
- Author or co-author on 80+ scientific papers
  - >3500 citations
  - h-index: 30
  - i10-index: 45

- 20 years of experience with scientific images (2D, 3D and 4D: LSM, SEM, TEM, FIB, etc…)
- 20 years of experience with imageJ and Fiji



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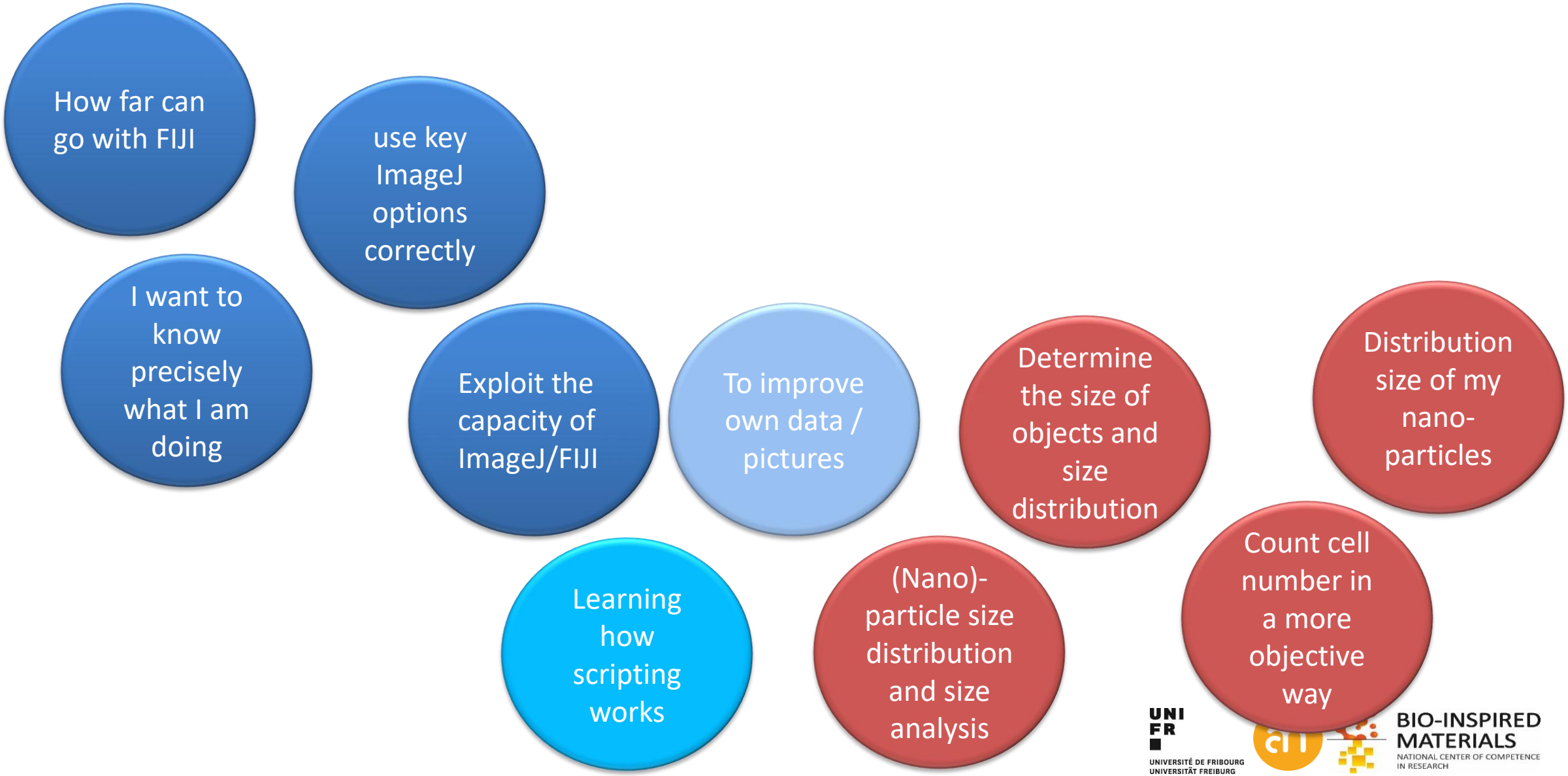
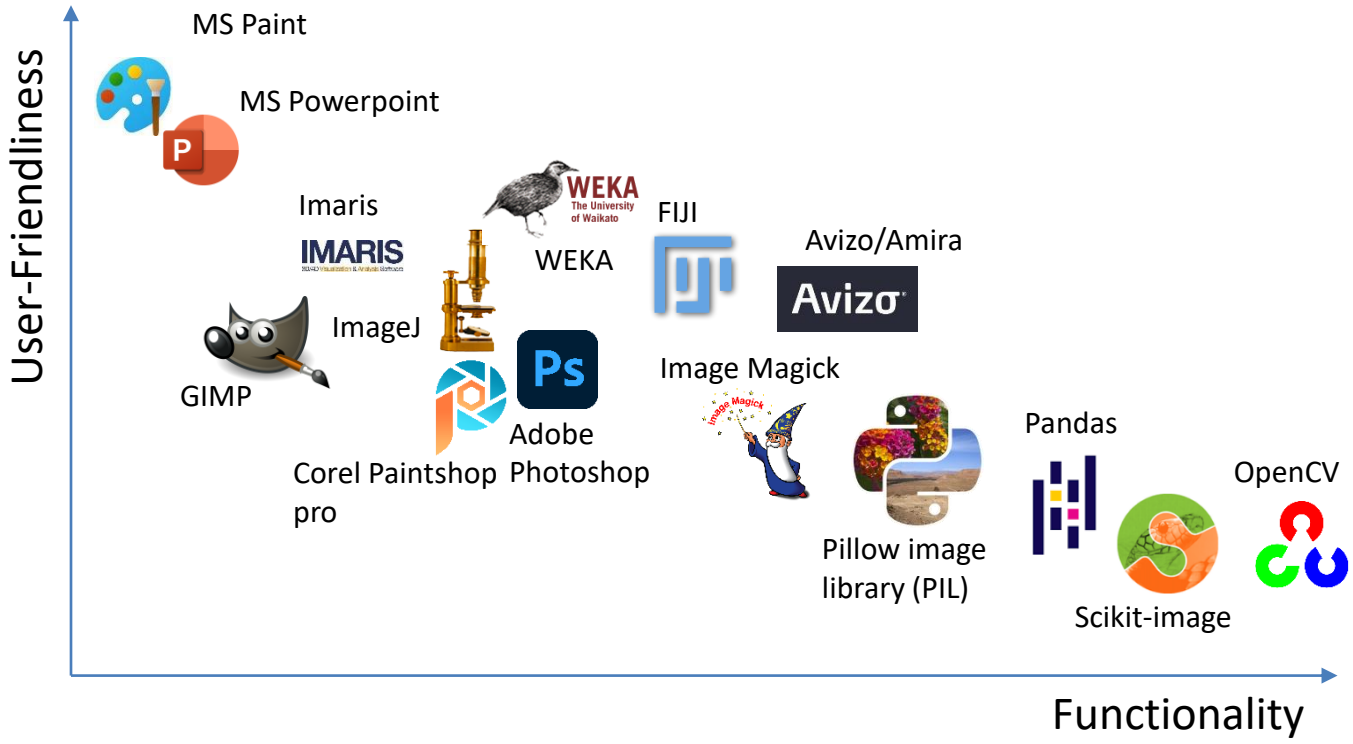


Administration

Why ImageJ (in fact: FIJI)?



FIJI (**F**IJI **i**s **j**ust **i**mageJ)

- Graphical user interface
- Extendable with plugins
- Designed for scientific data
- Broad functionalities
- Scripting possibilities

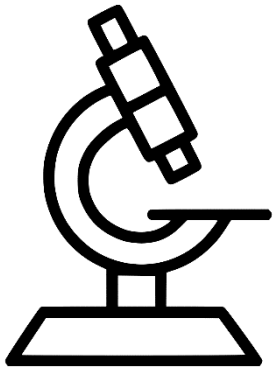
Part I: digital images and pixels

- Analogue to digital
- What is a pixel?
- Noise
- Image file formats
- Bitdepth

Image processing

Image processing starts with recording good images

Sample preparation



Imaging

Image processing

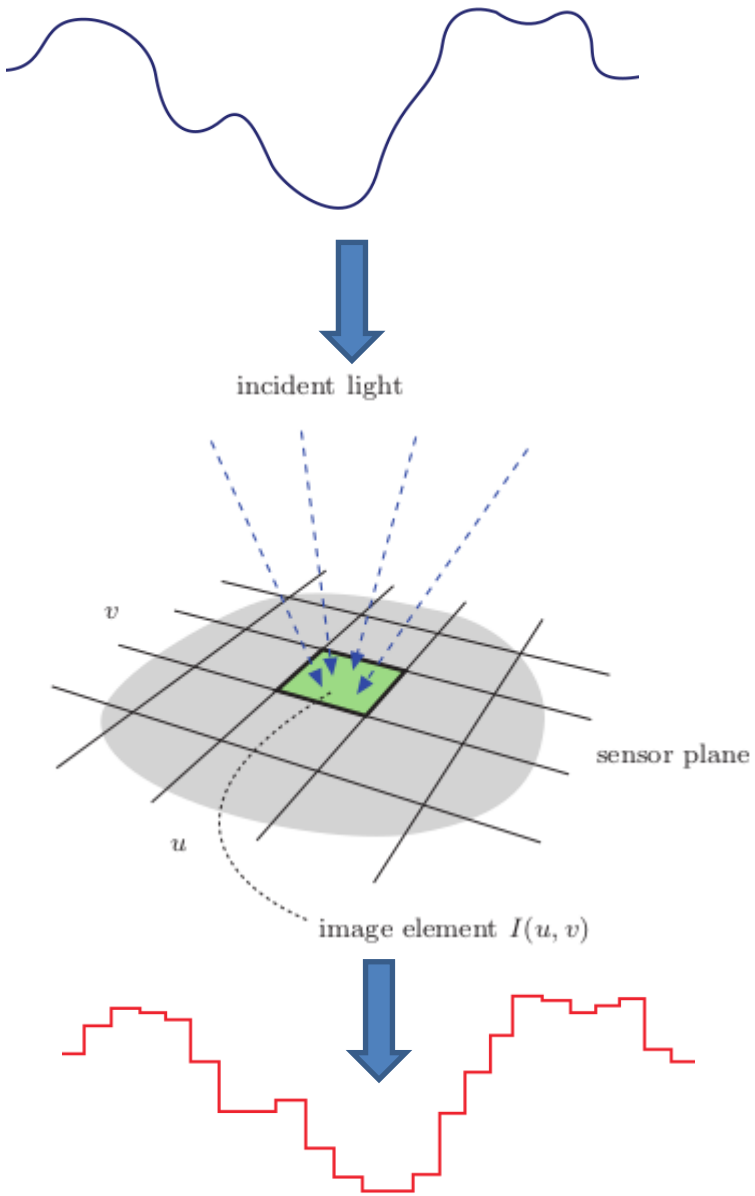
Central idea:

GIGO

Garbage in, garbage out

Good image in ➔ maybe better image out
Bad image in ➔ never a good image out

Going digital: Continuous to discrete function

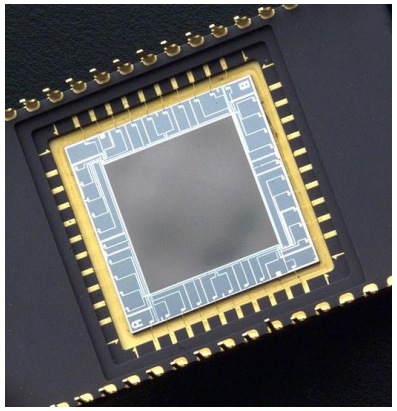


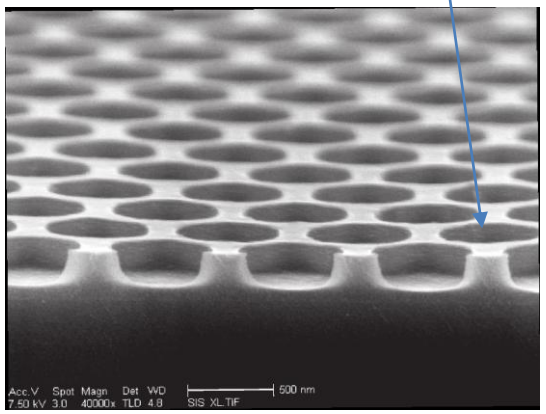
1. Spatial sampling
2. Time sampling
3. Analog to digital conversion

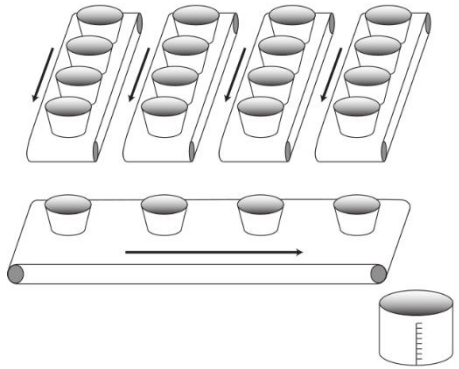
Going digital: Cameras and detectors

Photodiode (or well, or element)

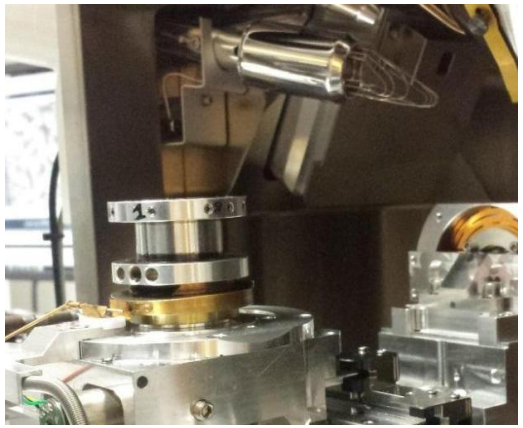
Camera
(e.g LM, TEM)

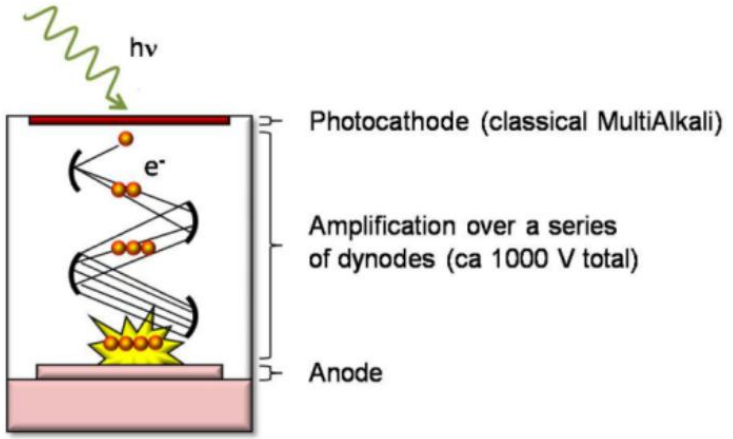






Detector
(e.g LSM, SEM, STEM)

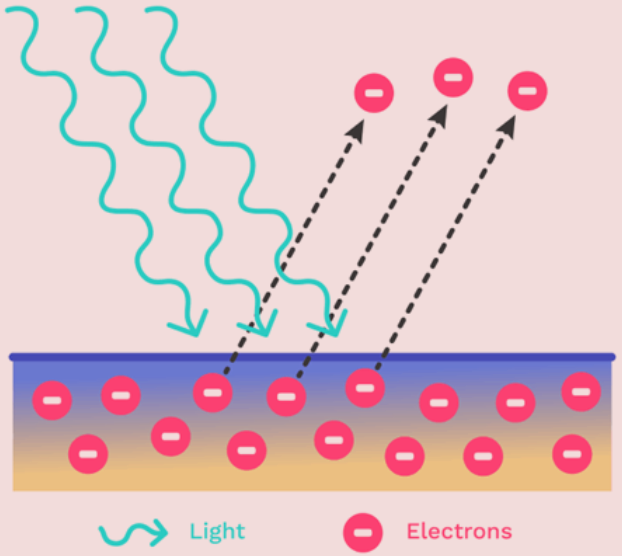




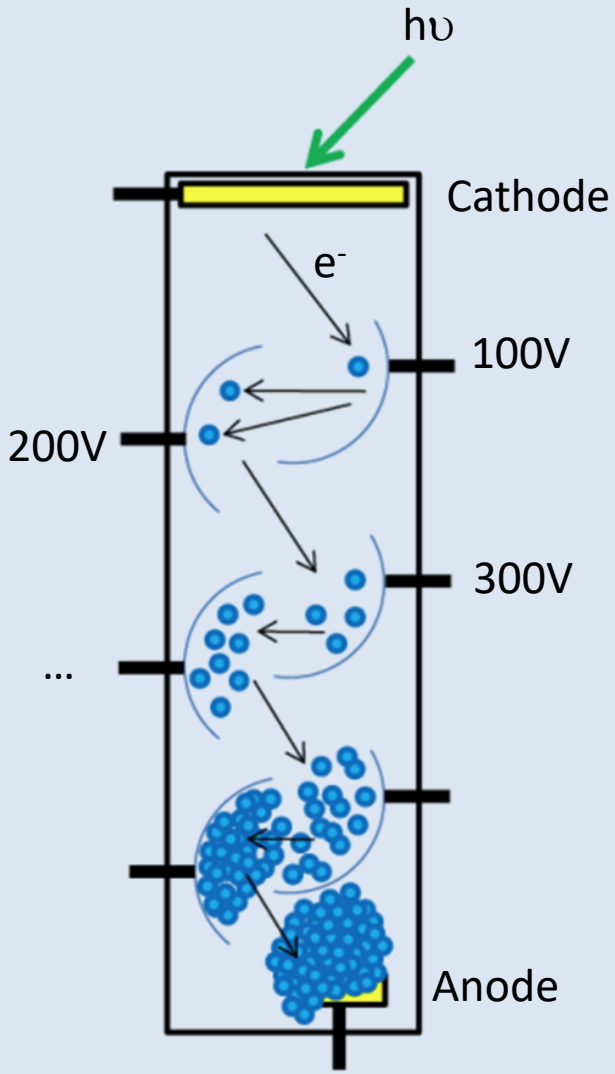
Going digital: from photon to pixel intensity (PMT)

1. Photon to electron conversion

The **outer photoelectric effect: release of** free electrons when light shines on them.

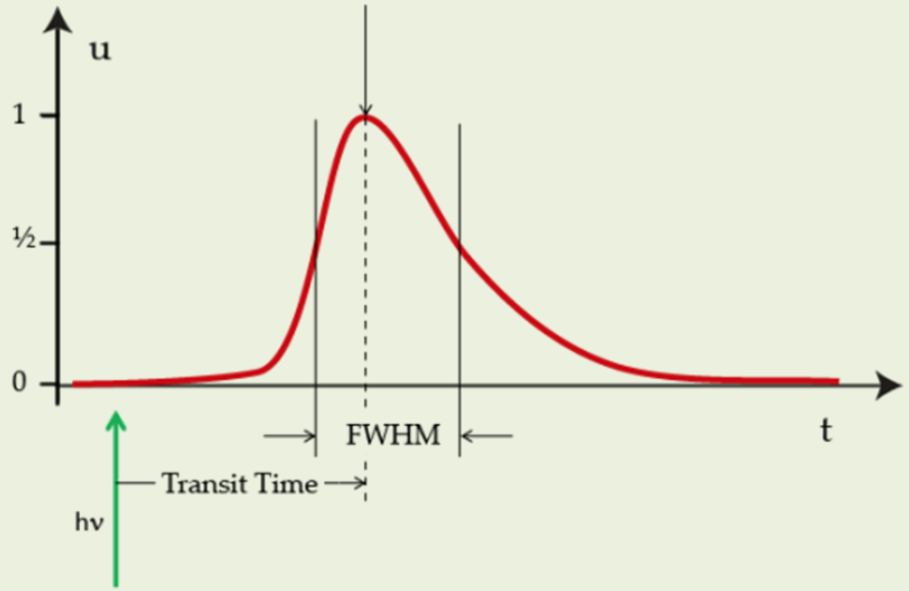


2. Signal multiplier / dynodes



3. Time sampling

The charge is sampled at the anode.
Electrons do not all arrive at the same time
(Because of the different paths can be taken)
➔ **distribution**



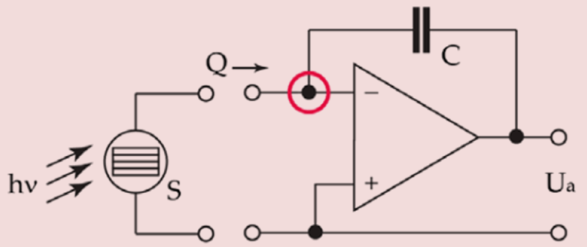
To measure pulse shape:

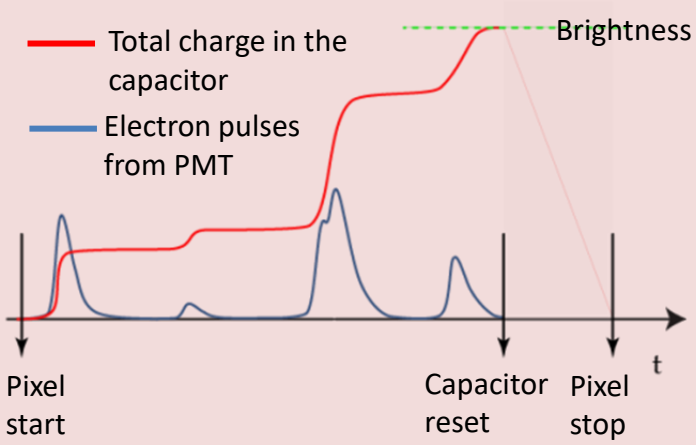
- full width half maximum. Time const.: 20 ns.
- not symmetrical; the rise time is 2-3X times faster than the decay time.

Going digital: from photon to pixel intensity (PMT)

4. The charge amplifier / time sampling

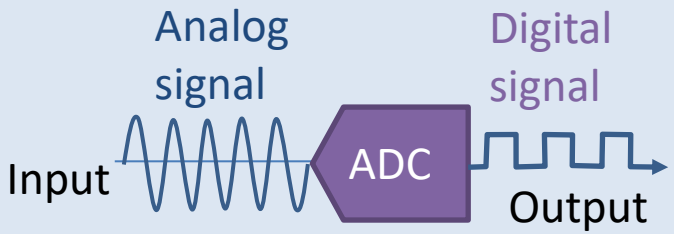
All charges coming from the PMT are stored in a capacitor .

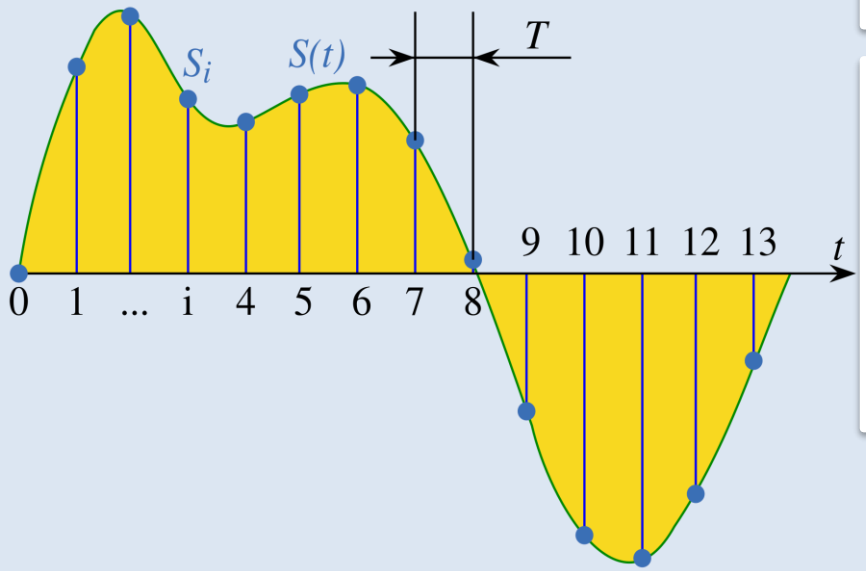




A fraction of the total time is lost for resetting the charge amplifier

5. Analogue-to-digital converter





Shannon Sampling Theorem

If a function S(t) contains no frequencies higher than B hertz, it is completely determined by giving its ordinates at a series of points spaced 1/(2B) seconds apart

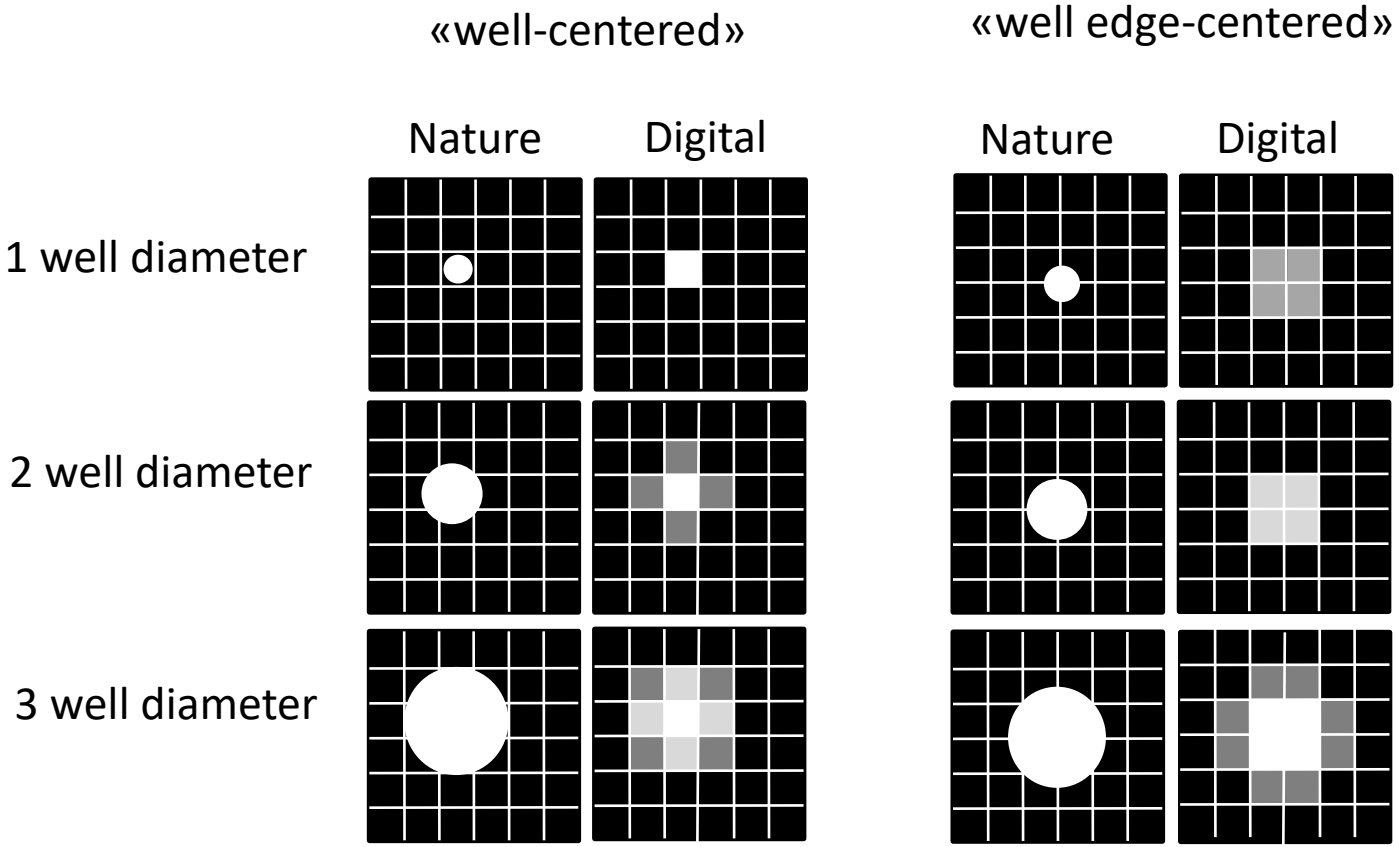
Shannon Sampling Theorem - revisted

If a series of discrete values has been sampled 1/(2B) apart, it does not contain information smaller than B hertz.

Shannon Sampling Theorem - applied

If an image has been recorded at a resolution of e.g. 100 nm, it does not contain information smaller than 200 nm.

What is a pixel: Nyquist theorem

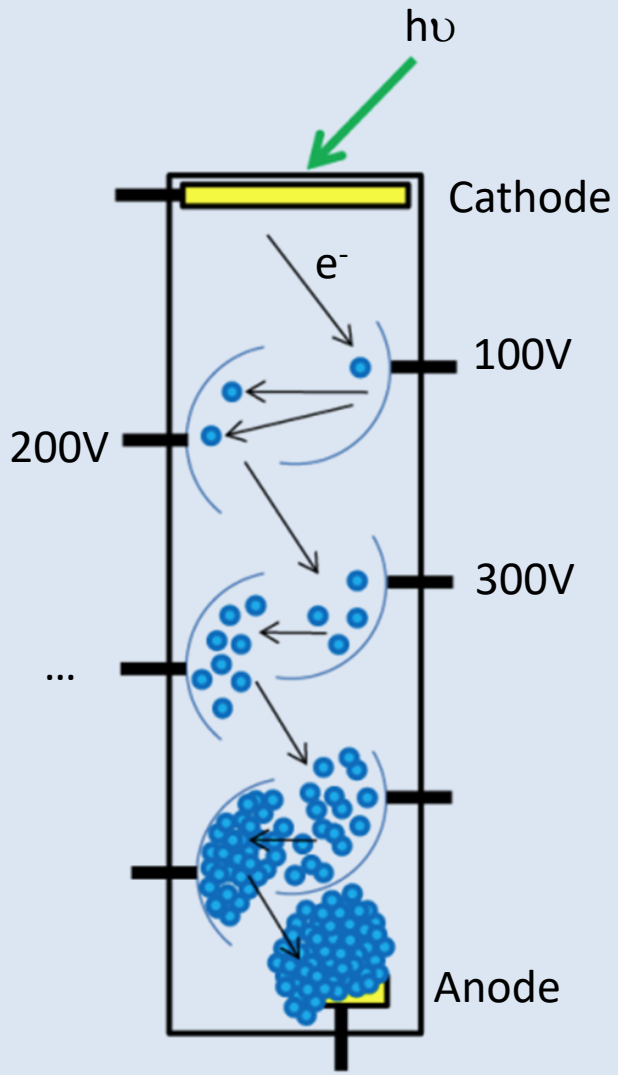


Nyquist's theorem (“howto convert analog to digital”):
the frequency of the digital sample should be twice that of the analog frequency

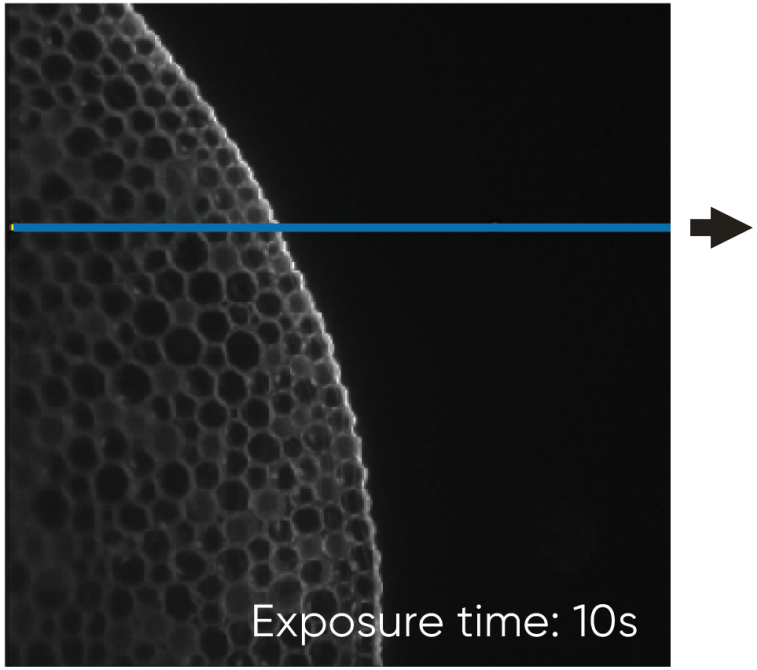
For digital images:
= a "sampling rate" of 2 wells relative to the object image size.

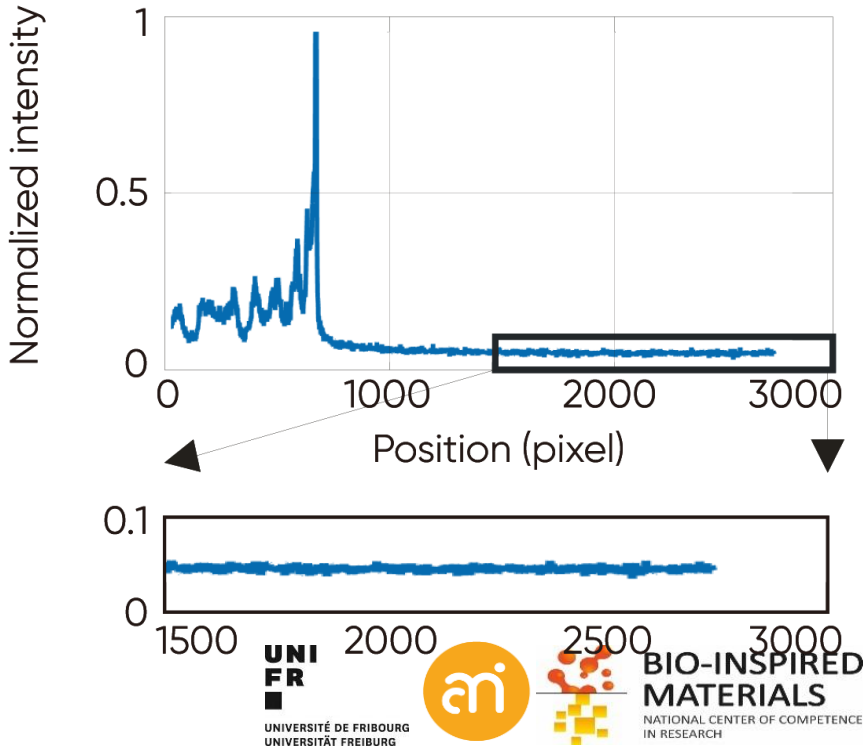
Noise: dark noise (PMT)

Signal multiplier / dynodes



- Even if no photons hit the cathode at all, events will still be measured at the anode
- Triggered by **thermal electrons** emitted by the cathode.
- Amplified by the following stages of dynodes.
- These events are known as 'dark noise'
- For weak signals, they can no longer be differentiated from the background.





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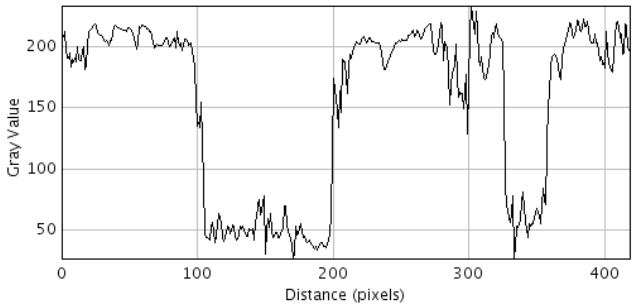
Going digital – what is a digital image?

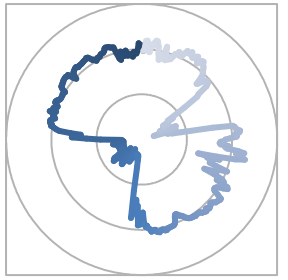
A digital image is an ordered, rectilinear array (or grid) of **numbers (usually integers: 0,1,2,3…)**. Each element (=number) in the grid is also known as a picture element or 'Pixel'

Spectrum

1 dimensional array

208
209
213
198
191
191
195
184
190
188
191
188
200

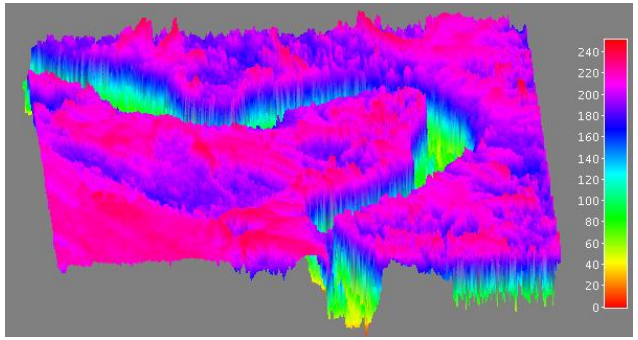




Image

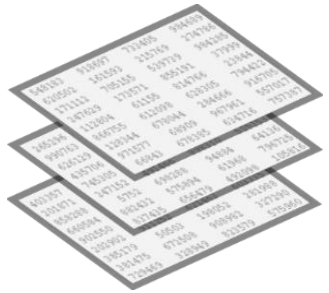
2 dimensional array

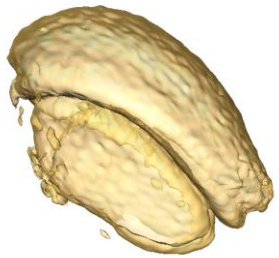
82	72	78	86	65	41
157	144	167	188	201	190
185	191	195	188	188	196
193	195	195	191	189	178
173	170	181	192	194	194
210	214	206	202	203	207
237	224	221	230	232	225
183	180	190	188	192	187
178	170	159	187	195	189
167	164	170	186	192	182
159	162	164	184	170	160
180	172	165	172	185	175
193	180	196	195	185	171
167	184	182	183	180	175
195	191	182	189	195	185
183	188	184	183	174	169
191	196	185	179	188	191



Stacks

3D array
(= volume stack or video





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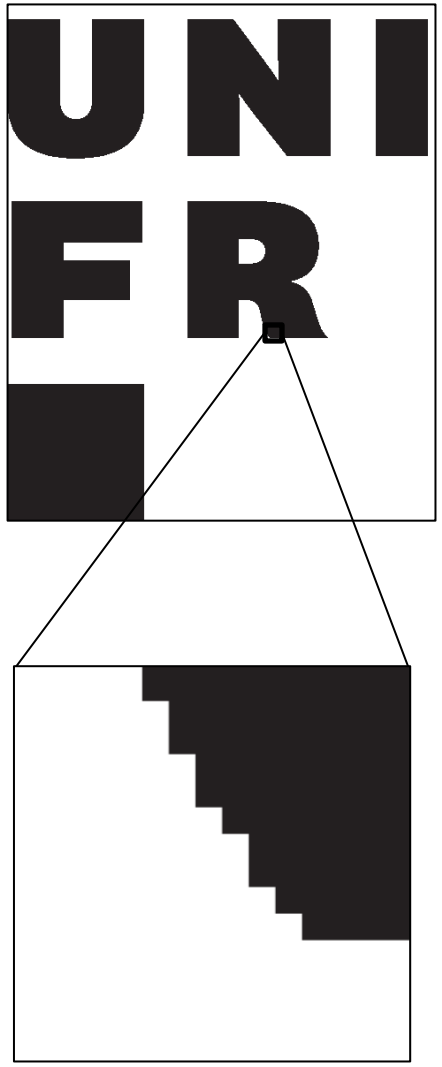
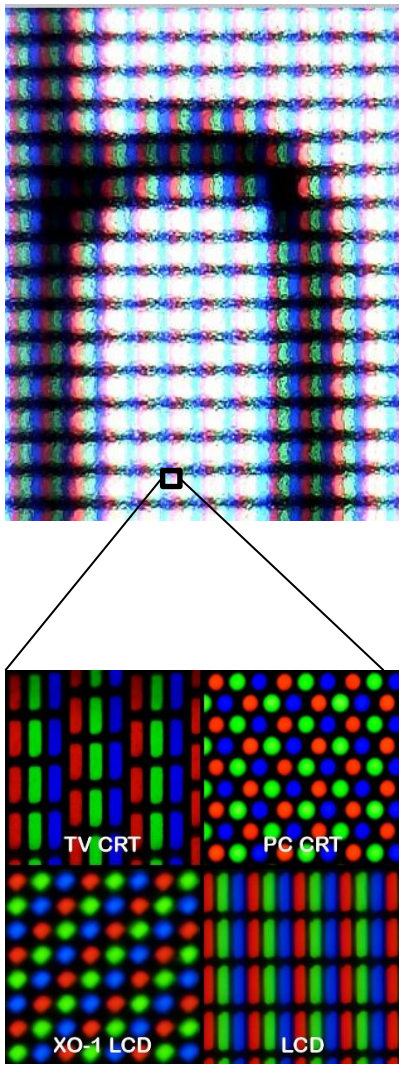
What is a pixel? – it depends on the context

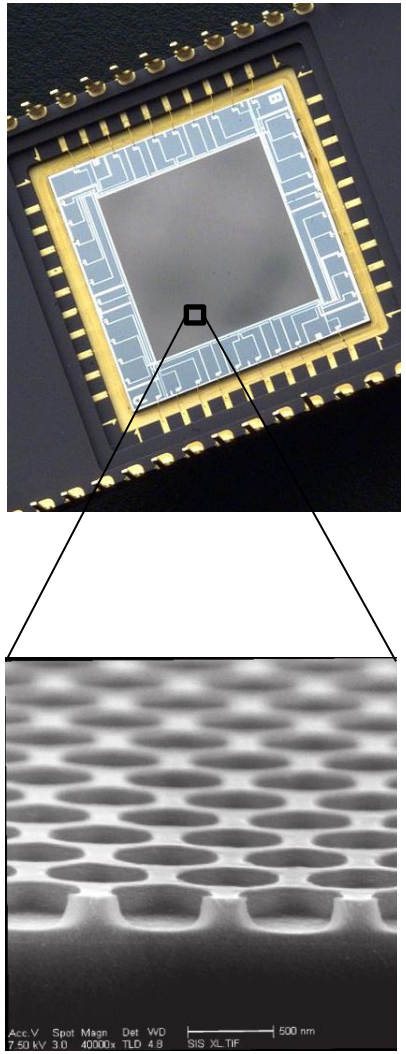
Digital image
Pixels



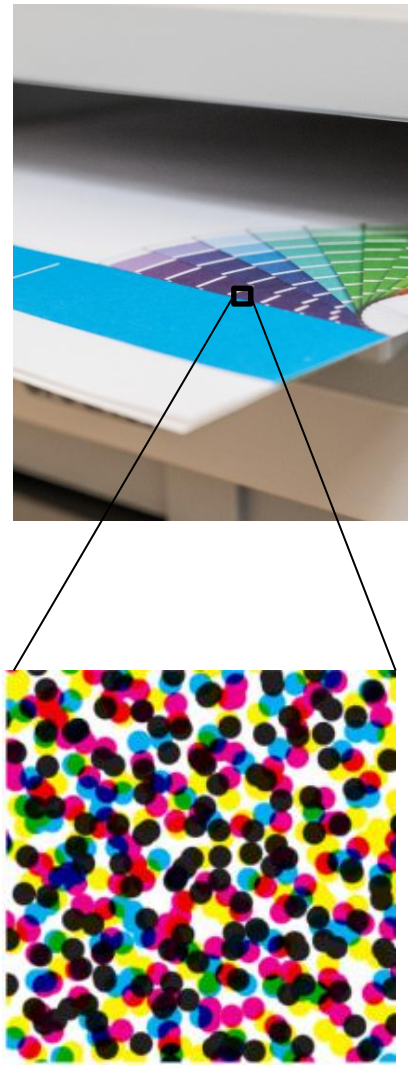
Display
Triades



Camera chip
Wells



Printed output
Dots



Pixel =
'Pic(ture) +
el(ement)'

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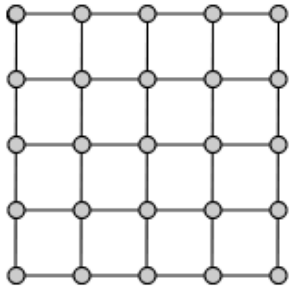
What is a pixel – the little square model

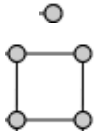
Problem: how to convert a continuous spectrum (nature) to a discrete set of intensities (digital)

Thought experiment

Consider a grid:



What are the pixels?



The corners?

The squares?

5x5 grid?

4x4 grid?

Where is the center of a pixel?

'Integerists'

Pixel (i, j) corresponding to square $(\mathrm{x}, \mathrm{y}) \rightarrow i - 0.5 \leq x \leq i + 0.5, j - 0.5 \leq y \leq j + 0.5$

A pixel is a point sample
(it exists only at **one point***)

e $(\mathrm{x}, \mathrm{y}) \rightarrow i - 1 \leq x \leq i + 1, j - 1 \leq y \leq j + 1$

*For color pixels, there might be 3 or even 4 values at that point

An image = an array on point samples (Shannon sampling theorem!)

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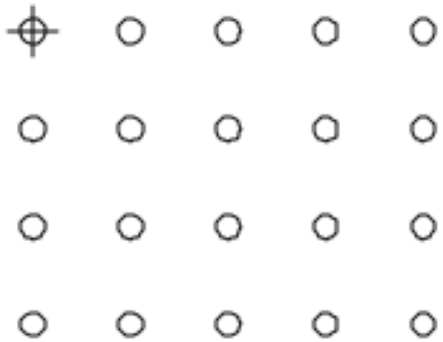
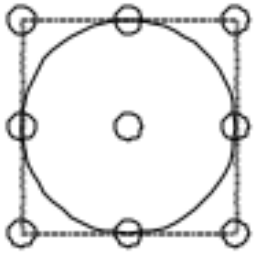
What is a pixel – the reconstruction filter

Problem: how to convert a continuous spectrum (nature) to a discrete set of intensities (digital)



A 5x4 image



The footprint of a
”reconstruction filter”
(e.g. beam)

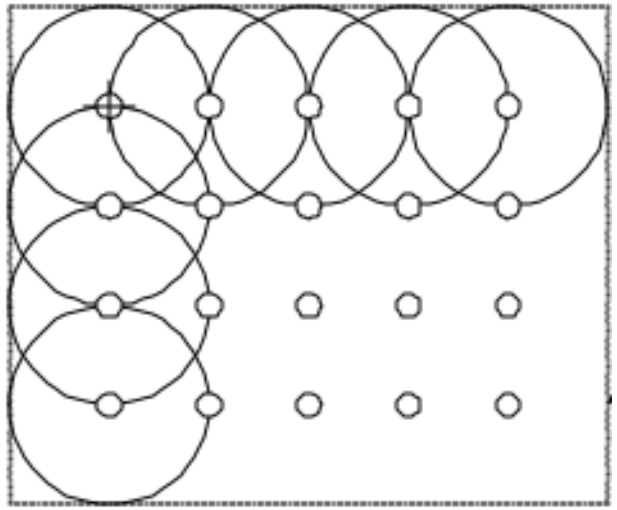
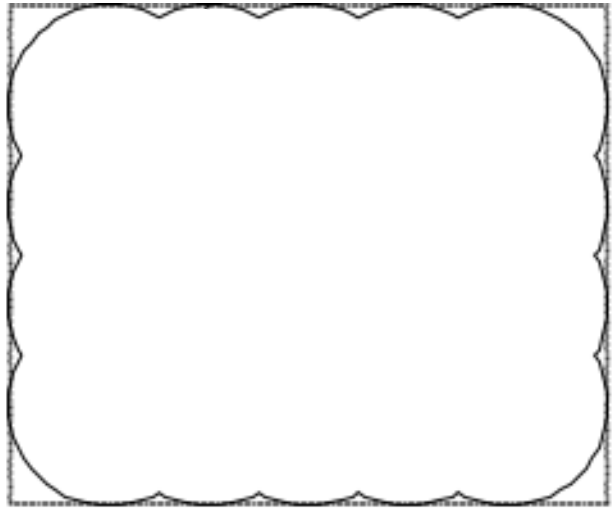


Image under construction

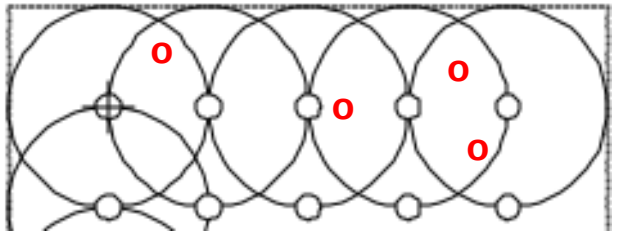


Recorded image

At no point, a square is involved!

What is a pixel – the point sample model

Q: What about the information (O) below the Shannon limit?



A: we simply do not know!
But you could try to guess. This is called **Reconstruction or interpolation**

Nature is continuous (has a value at every position), a digital image is a finite set of measurements: information will be lost.

Using the point sample model, we include the notion that information has been lost, and can talk about the best ways to measure that loss

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)[1]

Technical Memo 6

Alvy Ray Smith
July 17, 1995

What is a resolution? And what is magnification?

Assessment

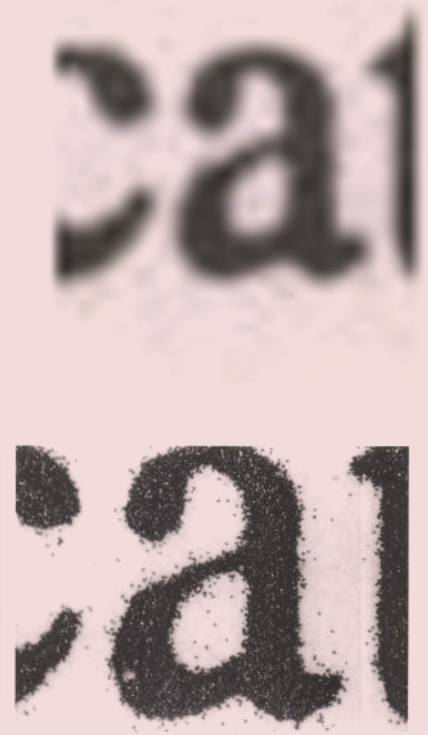
High

Low

Magnification

Easy

Resolution



Very difficult

Resolving power

Noise

Additive noise

Gaussian noise

Sum of all natural sources forming a normal distribution (=Gaussian distribution).

- **Johnson–Nyquist noise** (thermal vibrations of atoms in conductors)
- **Black-body radiation** (radiation from the earth or other objects)
- **Sensor noise** (when not recording at 0 K)
- **Electronic circuit noise** (impedance in electronic cables)
- **kTC noise** (Effects of capacitors)

FFT filtering, Gaussian smoothing.

Poisson (shot) noise

Caused by quantum effects due to the movement of discrete, quantized, packets

- In the source (light)
- In the electronics (electric current).
- **Dark shot** noise: shot noise from the dark leakage current in the image sensor

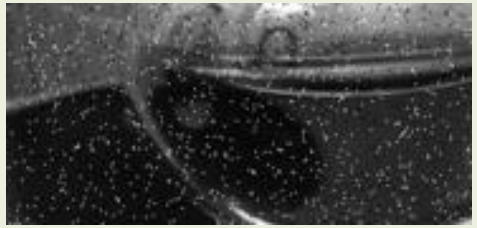
high intensity, exposure times
Correct with bias images

Multiplicative noise

Salt and pepper noise

Caused by errors. Typically B/W distribution

- During data transmission
- Failure in a memory cell
- Analog-to-digital converter errors
- Occasionally in TEM (X-rays)
- In camera-based systems: dead pixels



Median filtering

Repetitive noise

caused by electrical interference during the image capturing process.

Signal deconvolution in reciprocal space

Intermezzo - how does a computer work?

Examples of decimal (=10 digits) counting
- begins with 1 digit (rightmost digit or first digit)
- When all available symbols are exhausted:
  - the least significant digit is reset to *0*,
  - the next digit (one position to the left) is incremented (=overflow*)*

0 1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18 19

Examples of binary (=2 digits) counting
- begins with 1 digit (rightmost digit or first digit)
- When all available symbols are exhausted:
  - the least significant digit is reset to *0*,
  - the next digit (one position to the left) is incremented (=overflow*)*

0 1 10 11 100 101 110 111
(0) (1) (2) (3) (4) (5) (6) (7)

Computer = on/off
= binary digits ('bit')

1 Bit

8 Bits = 1 byte

1024 bytes	= 1 KB	KB	= Kilobyte
1024 KB	= 1 MB	MB	= Megabyte
1024 MB	= 1 GB	GB	= Gigabyte
1024 GB	= 1 TB	TB	= Terabyte
1024 TB	= 1 PB	PB	= Petabyte

From numbers to text
- ASCII: American Standard Code for Information Interchange
- List of numbers with defined characters

Dec	Char	Dec	Char	Dec	Char
32	SPACE	64	@	96	`
33	!	65	A	97	a
34	"	66	B	98	b
35	#	67	C	99	c
36	$	68	D	100	d
37	%	69	E	101	e
38	&	70	F	102	f
39	'	71	G	103	g
40	(	72	H	104	h
41	)	73	I	105	i
42	*	74	J	106	j
43	+	75	K	107	k
44	,	76	L	108	l
45	-	77	M	109	m
46	.	78	N	110	n
47	/	79	O	111	o
48	0	80	P	112	p
49	1	81	Q	113	q
50	2	82	R	114	r
51	3				
52	4				
53	5				
54	6				
55	7				
56	8				
57	9				
58	:				
59	;				
60	<	92	\	124	|
61	=	93	]	125	}
62	>	94	^	126	~
63	?	95	_	127	DEL

'AMI'
(dec): 65 77 73
(bin): 1000001 1001101 1001001

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File formats

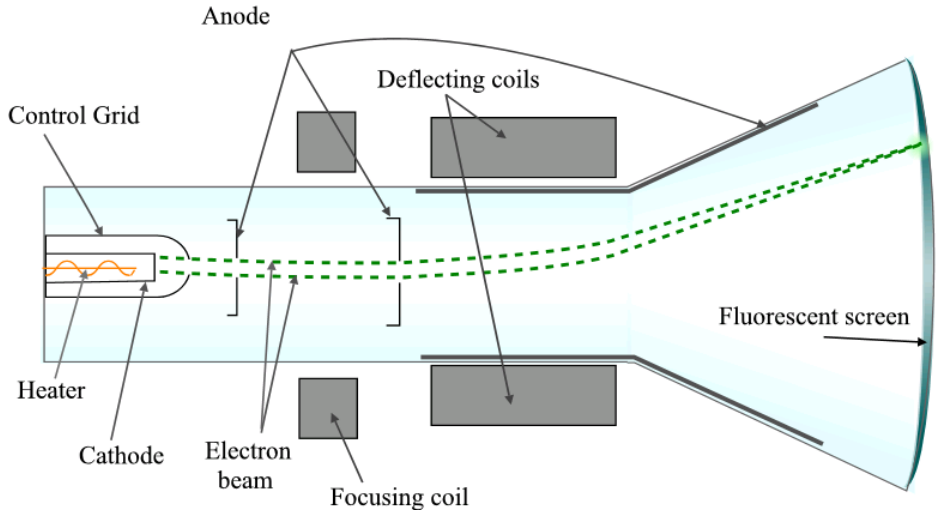
From concept: 3 different approaches:

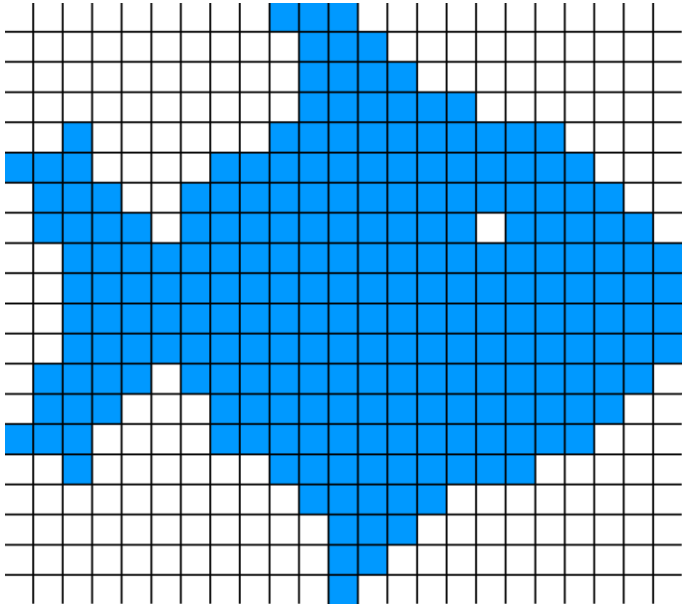
1. Vector graphics formats
2. Raster graphics formats
3. Hierarchical file formats

File formats: Raster graphics

Formats tif, jpg, png, bmp, webP, psb, …

Origin raster scan of cathode ray tubes (i.e. early TV screens)
‘Raster’ comes from rastrum (Lat.) = to scrape





Concept Tesselation (tiling) of a 2D plane with a value for every cell (‘pixel’)

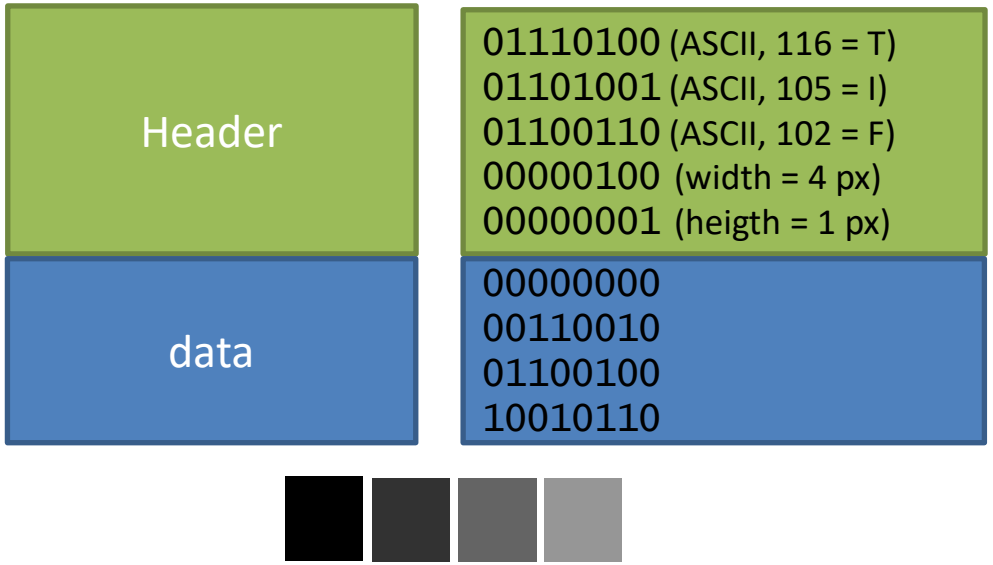
File formats: Raster graphics – Headers

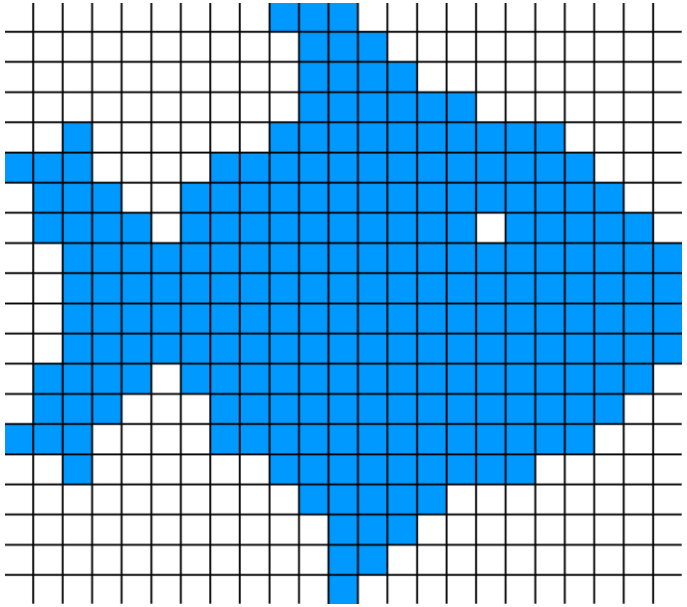
Concept Tesselation (tiling) of a 2D plane with a value for every cell (‘pixel’)

Problem Metadata: How large is the image? When was it made? But also:
e.g. medical data (patient information)
e.g. scientific data (experiment information)
e.g. consumer data (GPS coordinates, time and date)

Solution Header
contains height and width of the raster (=image) +lots of other data

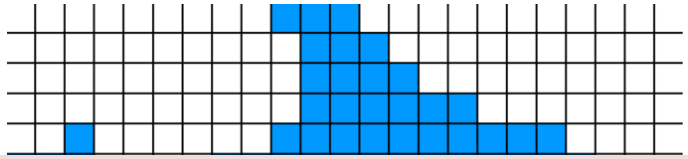
Example





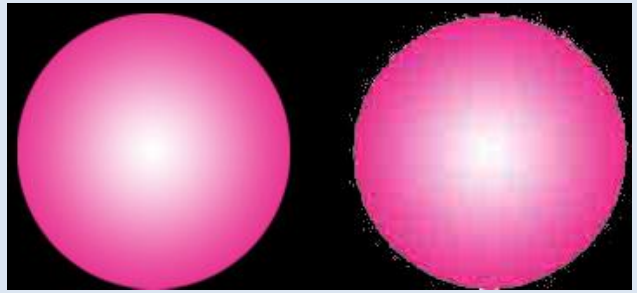
File formats: Raster graphics – Compression

Concept Tesselation (tiling) of a 2D plane with a value for every cell (‘pixel’)
Problem Data gets really big
Solution Compression. There are 2 compression concepts:



Lossy compression

The original data is approximated, but not restored
Example: JPG
Note: compression artefacts!



Lossless compression

the original data can be restored
Example: TIFF, PNG
Note: No artefacts!

Algorithms:
Run length encoding (RLE)
Lempel Ziv (LZx, uses Lookups)

Original data 32 bits

```
00000000
00110010
01100100
10010110
```

RLE compressed 29 bits

```
8x0
2x0 2x1 2x0 10
0 2x1 2x0 1 2x0
1 2x0 10 2x1 0
```

LZ compressed 38 bits

```
A = 00
B = 11
C = 10
4XA
A B A C
0 B A 1 A
1 A C B 0
```

```
Repeated to get 1024x1024:
Original 8 Mb
RLE      7.25 Mb (-10%)
LZ       7 bytes (!)
         (-99.99999%)
```

File formats: Hierarchical data formats (HDF)

Formats	h5
Origin	1987 by the Graphics Foundations Task Force. Around 1992, NASA investigated 15 different file formats for use in the Earth Observing System (EOS) project and settled for HDF.
Concept	Container of multiple, heterogenous data (including really big datasets), with metadata
Some advantages	- really really big datasets possible (e.g. tomography data) - hierarchical data (e.g. google maps) - heterogenous data (e.g. EDX spectra with images) - slices: only part of an image can be read (e.g. google maps) - embedded coding (allows advanced compression techniques)

File formats: raster graphics: bit depth

The range of the values a pixel can take is called the bit depth

2^n = number of shades
n = bitdepth

1 bit (=black and white, =binary image) 2 shades

Binary (e.g. Thresholding!)

8 bit (=common consumer cameras) 256 shades

~9 bit (=human eye) ~500 shades

12 bit (=middle range scientific cameras) 4096 shades

16 bit (=high range scientific cameras) 65536 shades

32 bit (=only computational) 4 294 967 296 shades

Grayscale

Signed (e.g. Signed 16bit). -32768 → +32768

Special

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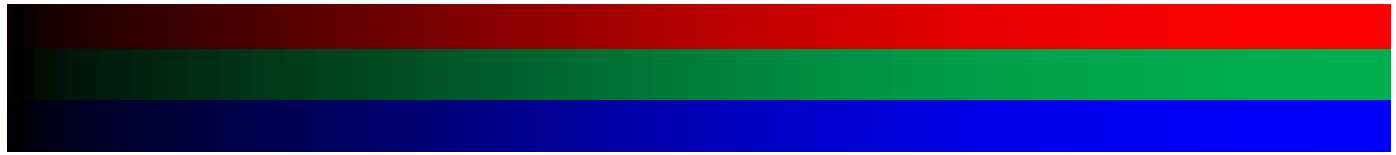
File formats: raster graphics - bit depth & colour

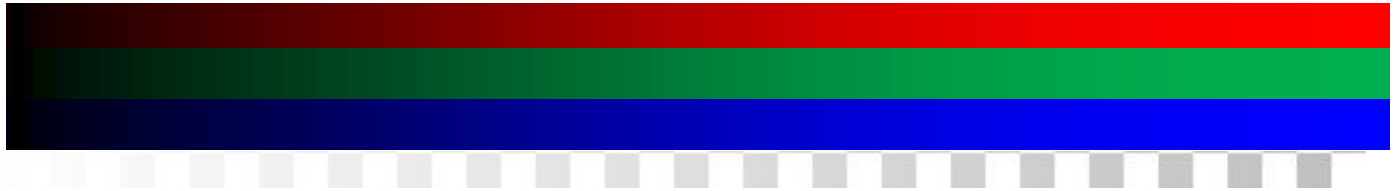
The range of the values a Pixel can take is called the bit depth

2^n = number of shades
n = bitdepth

RGB (=3 channels of each 8 bit = 24 bit) 256 x 256 x 256 colours



RGBA (=4 channels of each 8 bit = 32 bit) 256 x 256 x 256 colours x 256 Alpha



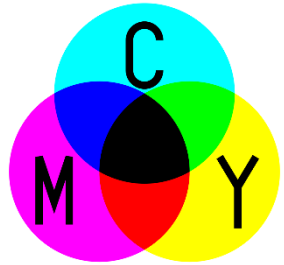
Additive color mixing (Displays)

48 bit RGB (= 3 channels of each 16 bit = 48 bit)

Computational

CMYK (=4 channels of each 8 bit = 32 bit) 256 x 256x 256x 256 colours

Subtractive color mixing (pigments, Printers)



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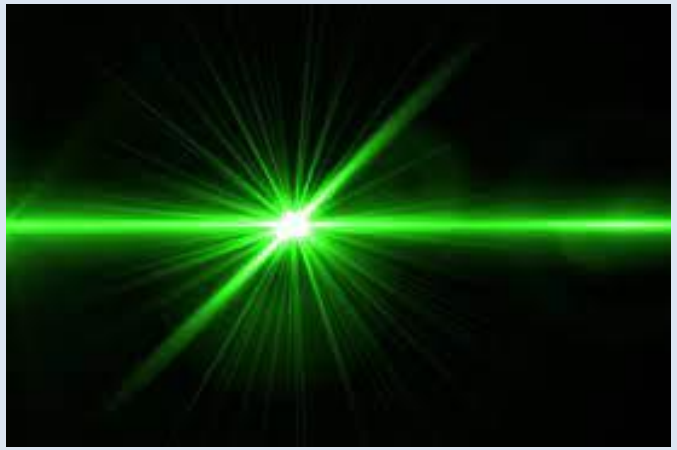
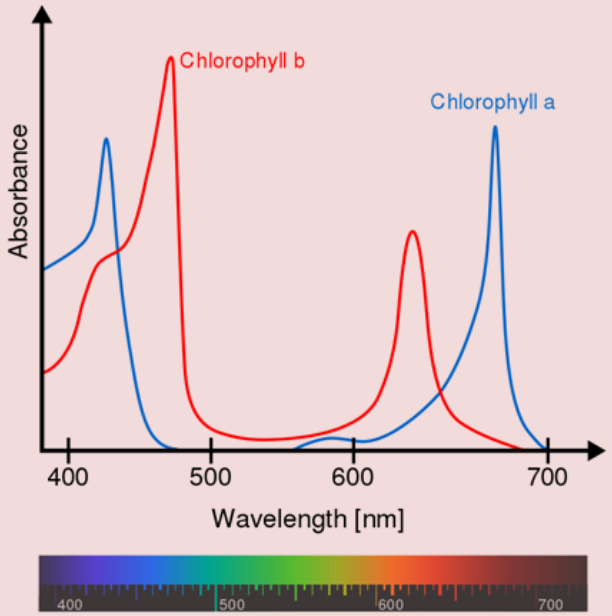
Note: light sources and dyes/pigments

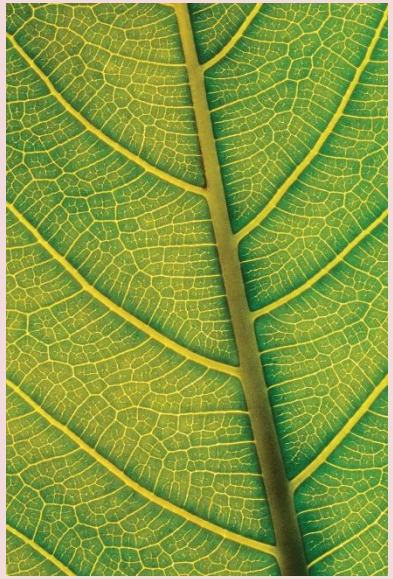
Additive color mixing
Light sources



A green laser is green because it emits green photons

Subtractive color mixing
Dyes and pigments

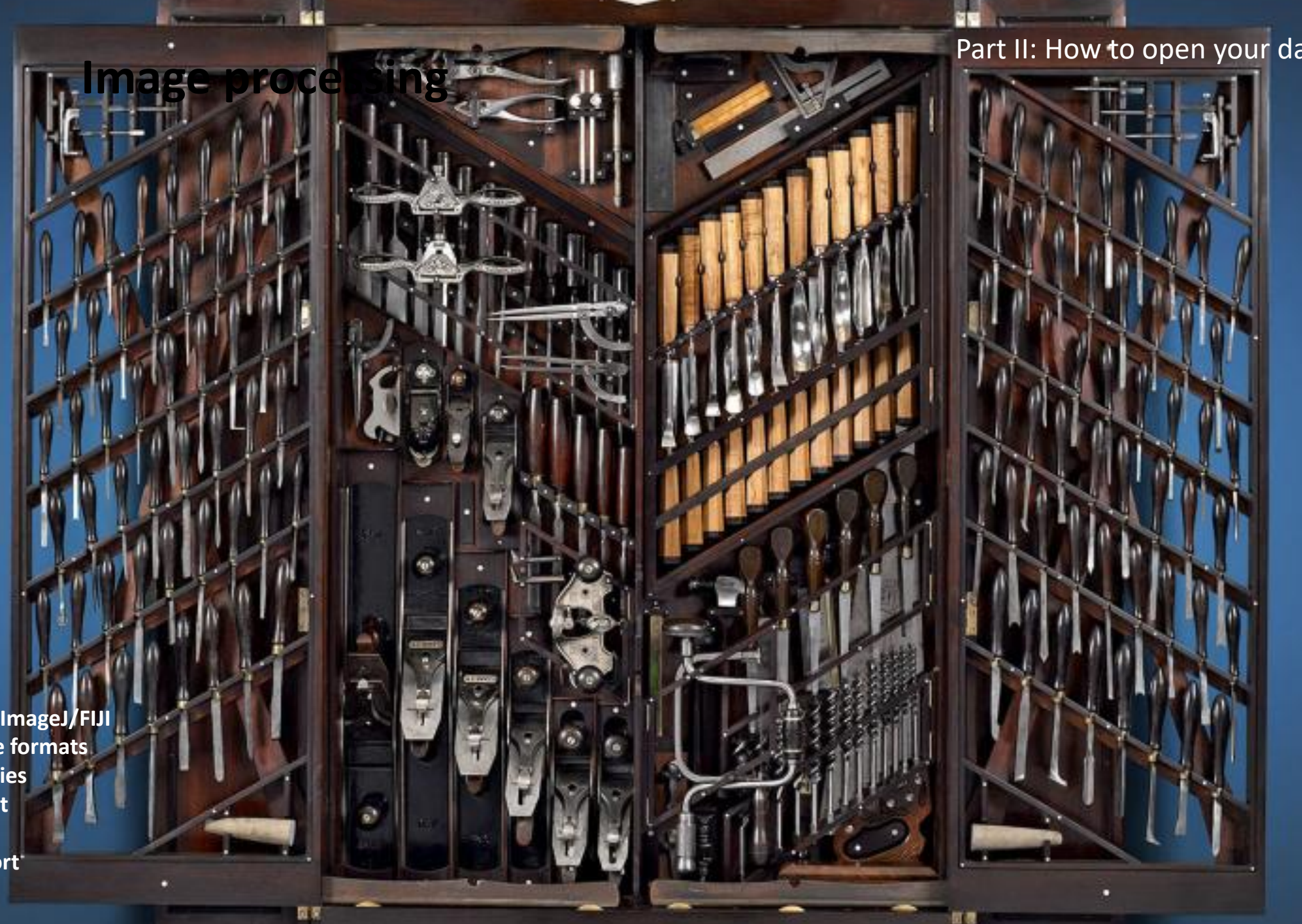




A leaf is green because it absorbs all white light except the green photons (which it reflects)

Image processing

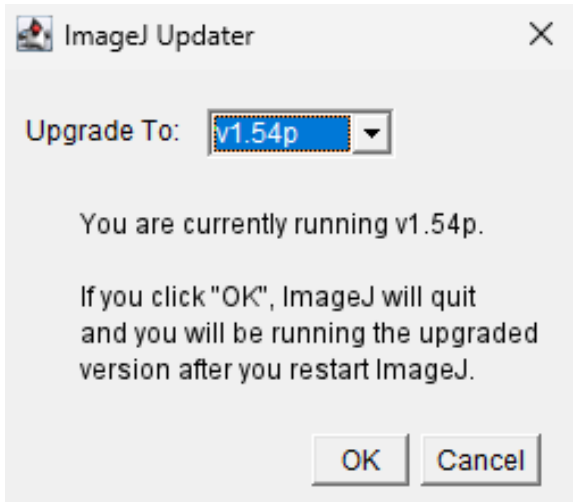
Part II: How to open your data?



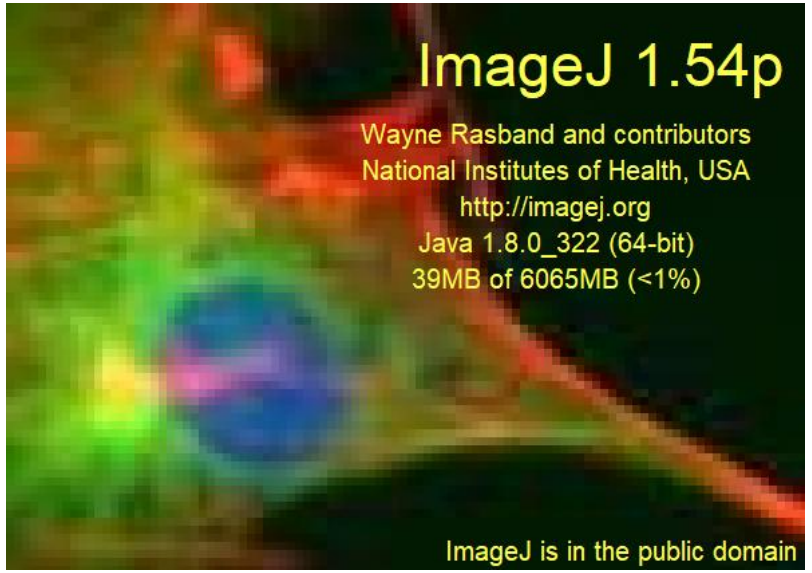
- Updating ImageJ/FIJI
- Native file formats
- Repositories
- FIJI import
- Plugins
- Raw import

0. Update FIJI

Help > update ImageJ…



Start ImageJ, then Help > About ImageJ…



1. How to open your data in FIJI?

5 Possibilities:

- Native file formats
- Use a repository
- Use Import (Fiji only!)
- Use a plugin
- Through RAW import (raster graphics only)

1. Native file formats

5 Possibilities:

- Native file formats
- Use Import (Fiji only!)
- Use a repository
- Use a plugin
- Through RAW import (raster graphics only)

Format	1 bit	8 bit	16 bit	32 bit	RGB	stacks	Hyper-stacks	Compression
TIFF	✔	✔	✔	✔	✔	✔	✔	Lossless
GIF	✔	✔			✔	✔		Lossless
JPEG		✔			✔			lossy
PNG	✔	✔	✔					Lossless
DICOM	✔	✔	✔	✔	✔	✔	✔	Lossless
BMP	✔	✔			✔			Lossless
PGM	✔	✔	✔		✔			Lossless
FITS	✔	✔	✔		✔			Lossless
AVI	✔	✔			✔	✔		Lossless

TIFF is the 'default' format of ImageJ (OME-TIFF)
DICOM is a standard popular in the medical imaging community
FITS Flexible Image Transport System: adopted by the astronomical community
PGM: Portable GrayMap
AVI container format, only uncompressed AVIs supported
Red: no header information

Native and non-native file formats

EXERCISE 1
Try to open example 1 A, B, C or D

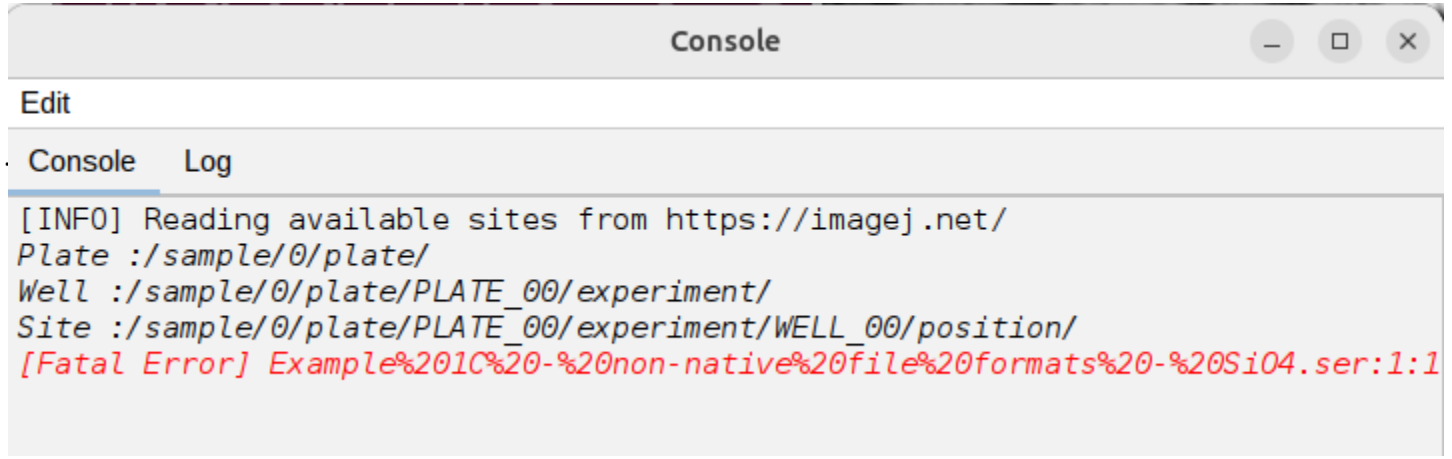
File > open…
Or drag and drop the icon from the folder onto ImageJ/FIJI

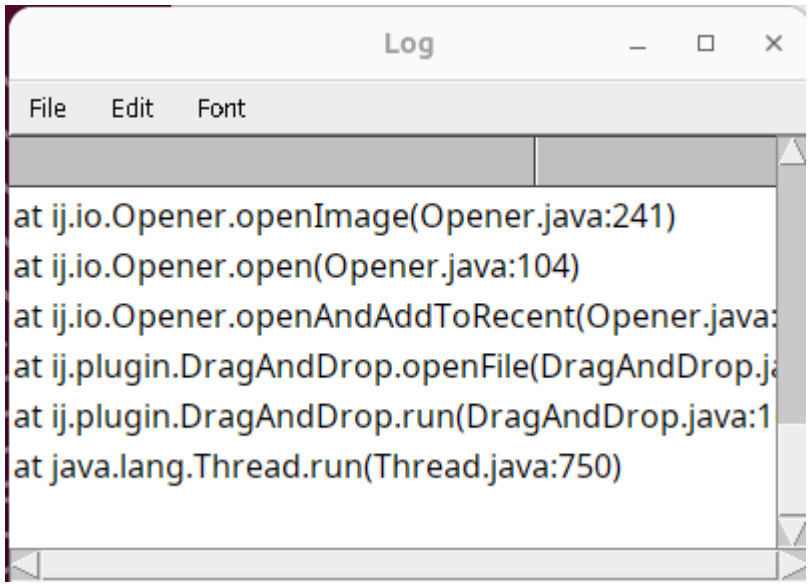
Does it work?

Native and non-native file formats

EXERCISE 1
Try to open example 1 A, B, C and D

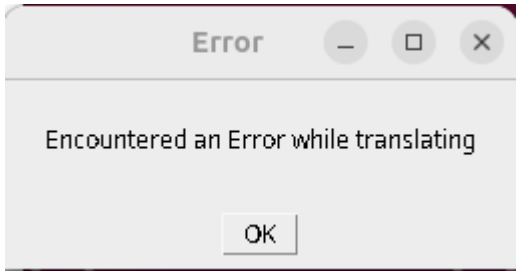
File > open…
Or drag and drop the icon from





Does it work?

No… Example 1D does not open
(maybe even 1C and 1B also not)

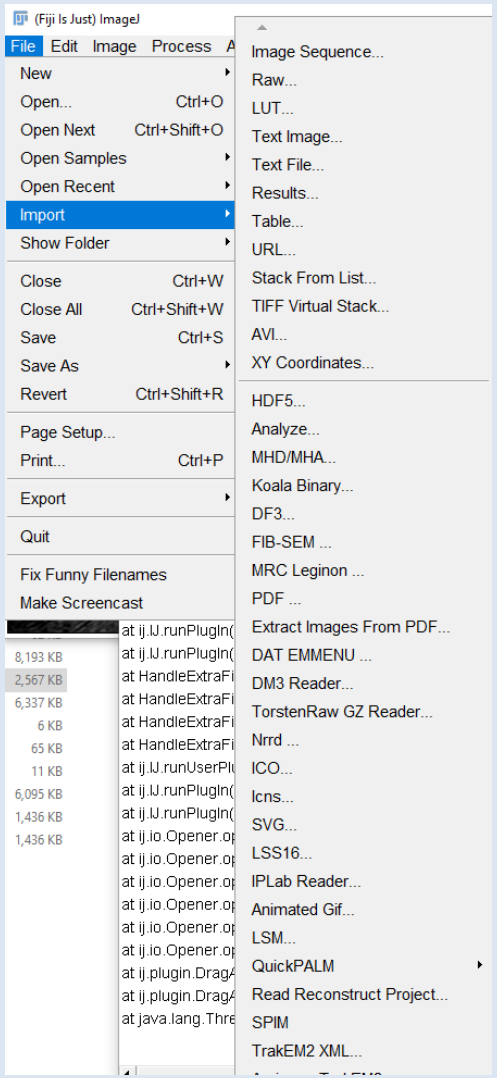


2. Using a Fiji resident plugin (Import)

5 Possibilities:

- Native file formats
- Use Import (Fiji only!)
- Use a repository
- Use a plugin
- Through RAW import (raster graphics only)

File > Import >

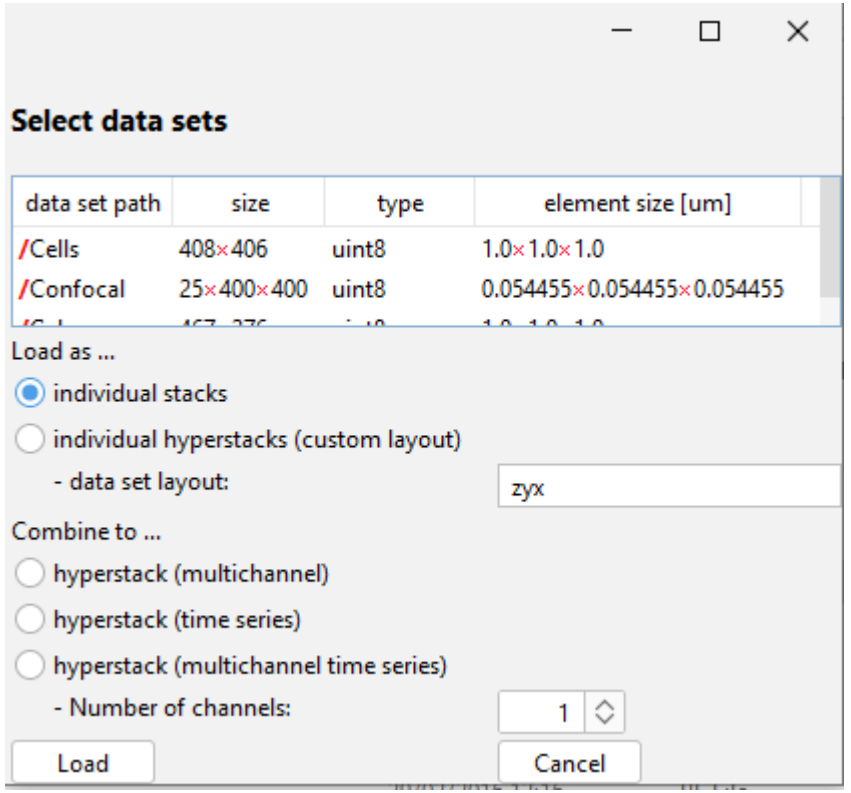


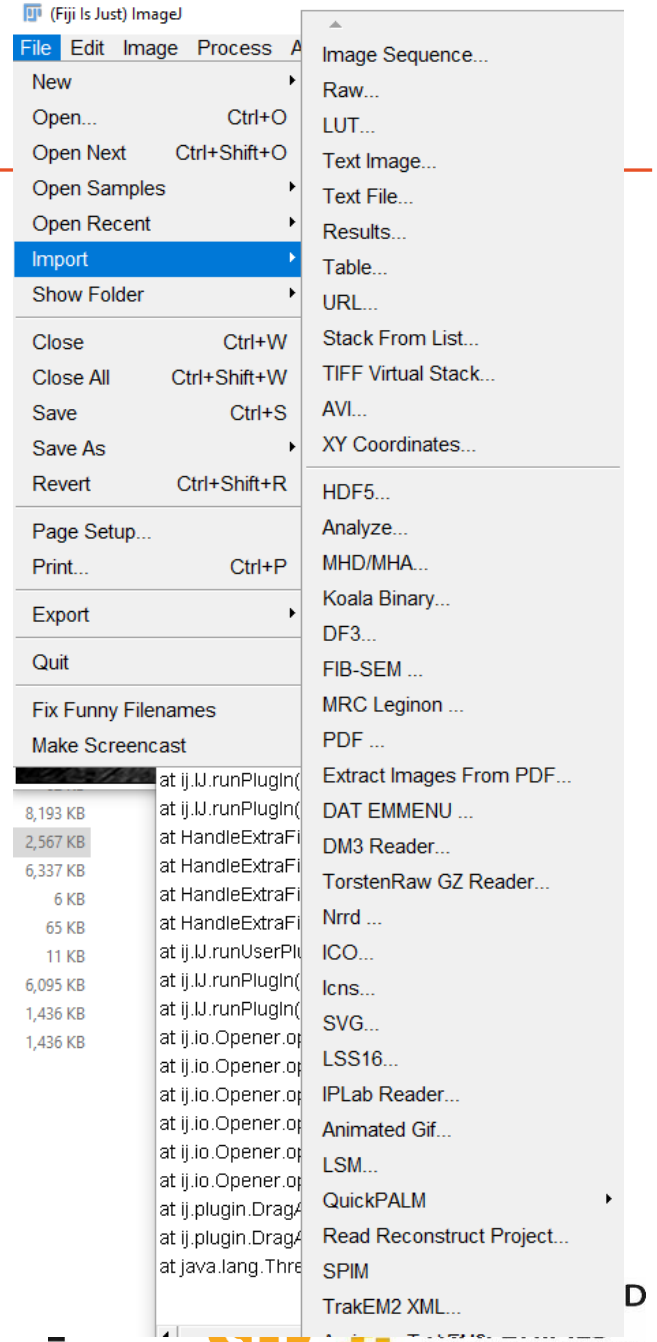
2. Using a Fiji resident plugin (Import)

EXERCISE 1
Try to open example 1C

File > Import > HDF5
Point to the file example 1C

The file contains 3 datasets (!)
- Select /Cells in data set path
- load as ‘Individual stacks’
- Click 'Load'





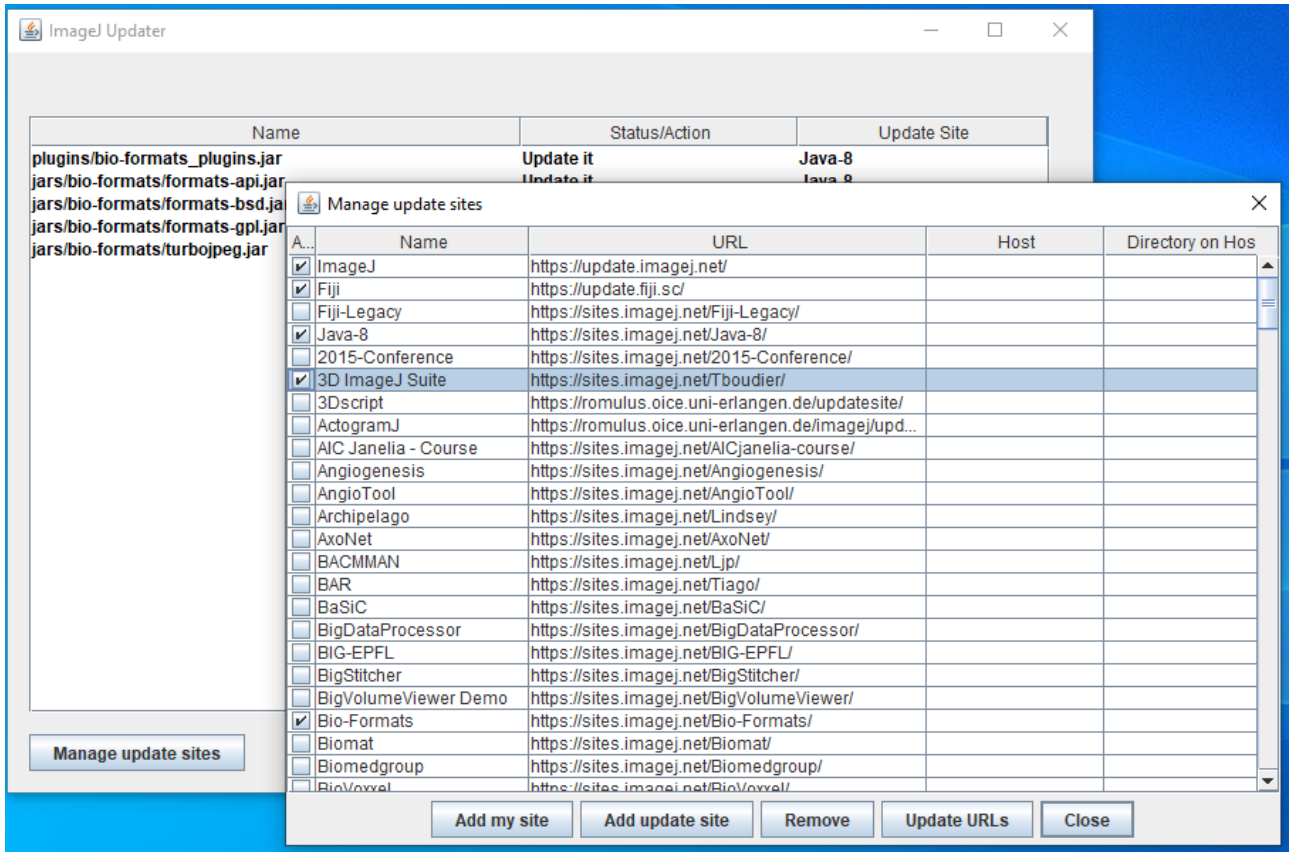
3. Using a plugin from the repository

5 Possibilities:

- Native file formats
- Use Import (Fiji only!)
- Use a repository
- Use a plugin
- Through RAW import (raster graphics only)

From the repositories

- Help > Update…



1 Choose Manage update sites

2. Select the repositories you wish to add
In this case: **Bio-Formats**

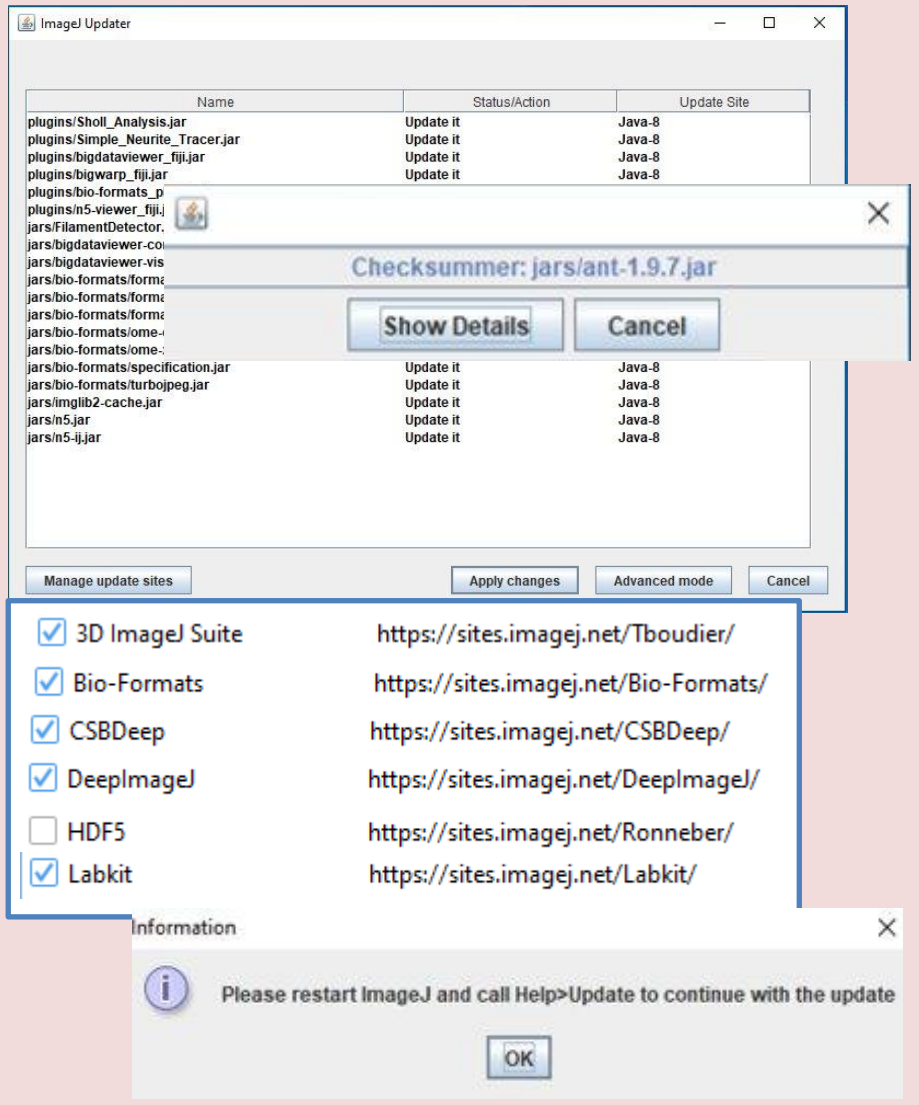
3. Close and
4. Apply changes

3. Using a plugin from the repository

5 Possibilities:

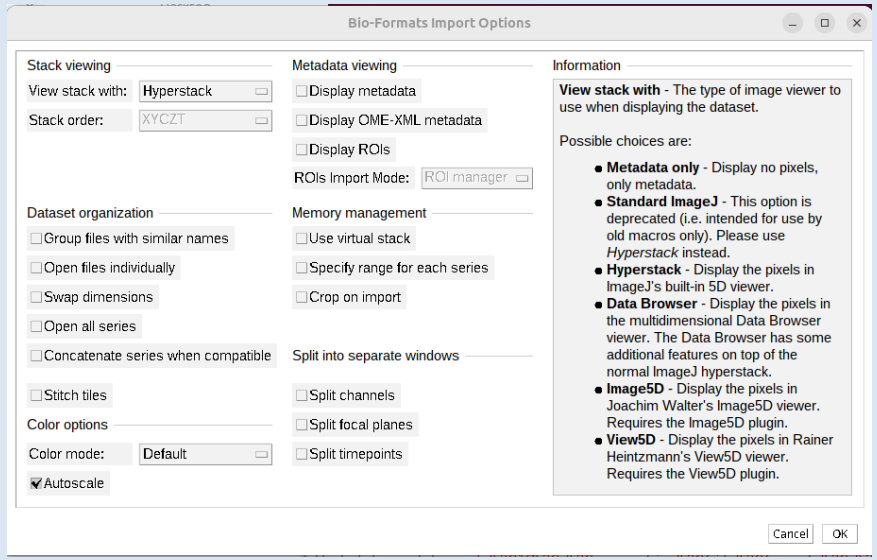
- Native file formats
- Use Import (Fiji only!)
- Use a repository
- Use a plugin
- Through RAW import (raster graphics only)

Help > update…



File > import > Bio-Formats
or
Plugins > Import > Bio-Formats > Bio-Formats Importer

EXERCISE 1
Try to open example 1B with the Bio-Formats plugin



3. Using a plugin from the repository

5 Possibilities:

- Native file formats
- Use Import (Fiji only!)
- Use a repository
- Use a plugin
- Through RAW import (raster graphics only)

Update lists / repositories

ImageJ
Fiji
3D ImageJ Suite
3Dscript
ActogramJ
AIC Janelia
Angiogenesis
AngioTool
Archipelago
AxoNet
BACMMAN
BAR
BaSiC
BigDataProcessor
BIG-EPFL
BigStitcher
BigVolumeViewer Demo
Bio-Formats
Biomat
Biomedgroup
BioVoxxel
Blind Analysis Tools
BoneJ
CALM
CAMDU
CATS
CellTrackingChallenge
CIP
CircleSkinner
clij
clij2
clijx-assistant
clijx-assistant-extensions
ClearVolume
CMCI-EMBL
CMP-BIA tools
CMTK Registration
Colocalization by Cross Correlation
Colour Deconvolution2

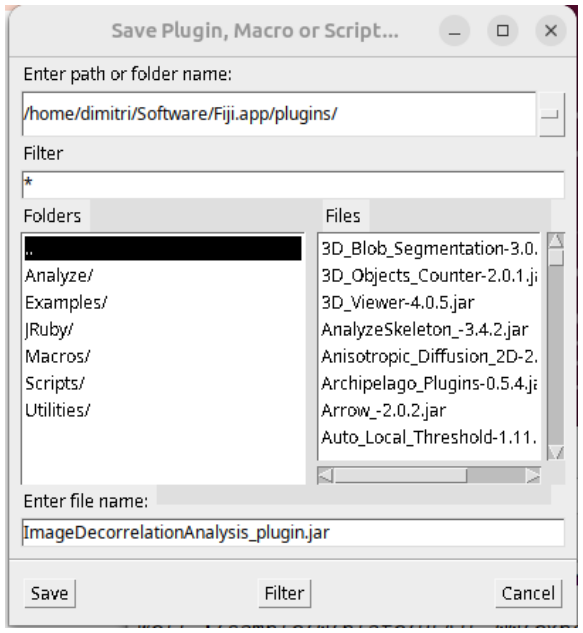
Bio-formats repository

3i SlideBook
AIM
AVI (Audio Video Interleave)
Adobe Photoshop PSD
Alicona 3D
Amersham Biosciences Gel
Amira Mesh
Amnis FlowSight
Analyze 7.5
Andor Bio-Imaging Division (ABD) TIFF
Animated PNG
Aperio AFI
Aperio SVS TIFF
Applied Precision CellWorX
Axon Raw Format
BD Pathway
Becker & Hickl SPCImage
Bio-Rad Gel
Bio-Rad PIC
Bio-Rad SCN
Bitplane Imaris
Bruker MRI
Burleigh
Canon DNG
CellH5
CellVoyager
Cellomics
DICOM
DeltaVision
ECAT7
EPS (Encapsulated PostScript)
Evotec/PerkinElmer Opera Flex
FEI
FEI TIFF
Gatan Digital Micrograph
Gatan Digital Micrograph 2
Hamamatsu Aquacosmos NAF
Hamamatsu HIS
Hamamatsu VMS

4. Using a plugin from the internet

5 Possibilities:

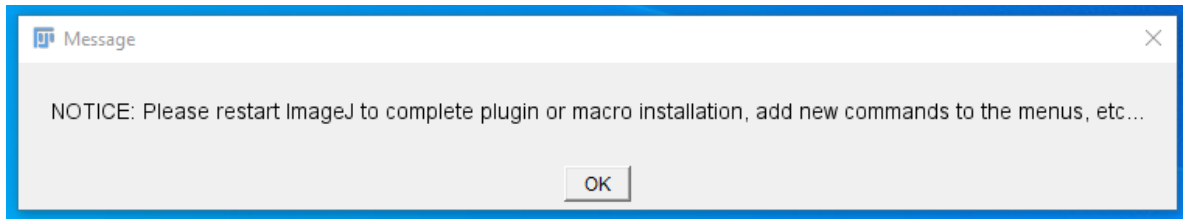
- Native file formats
- Use a repository
- Use Import (Fiji only!)
- Use a plugin
- Through RAW import (raster graphics only)



Installing a plugin from a downloaded .jar file (https://imagej.nih.gov/ij//plugins/tia-reader.html)

3 ways of installing the jar file (it is also provided):

1. Drag and drop the file onto Imagej (and save in the plugin folder)
2. Plugins > install plugin… and point to the .jar file
3. Copy the .jar file into the plugins folder of your FIJI folder



In any case: **Restart FIJI** (close it and start it again)

Find the new functionality under

Plugins > Input / output > Tia Reader

Provided on the ImageJ website

ASCII header format
SPE Images
PICT, Targa using Jimi
Biorad Z-Series
Leica SP multi-channel stacks
QuickTime
Jimi Stack Writer
AVI Writer
JMF Reader
Animated Gif
PDS Images
AVI Reader
LSM Reader (Zeiss LSM confocal microscopes)
Quess RAW
IPLab Reader
Excel Writer
Multi FDF
VFF Opener
OpenSIF (opens Andor SIF files)
EXIF Reader
Bruker NMR
Zeiss ZVI Reader
ISAC images (e.g., Fuji BAS scanners)
Gatan DM3 Reader
Deltavision Opener
Nanoscope AFM files
NIfTI Input/Output
UNC format images
PDF Writer
Leica SP2 TIFF Sequence
EPS (Encapsulated PostScript) Writer
PerkinElmer Reader
Nikon ND2 Reader (Windows only)
TIA Reader (FEI/Emispec .ser files)
…

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4. Using a plugin from the internet

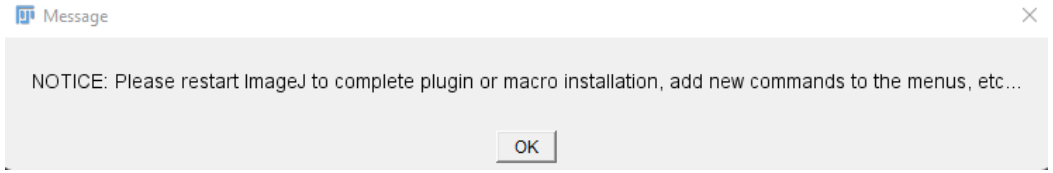
EXERCISE 1

Install the TIA reader plugin (TIA_Reader.jar) if you have not done so

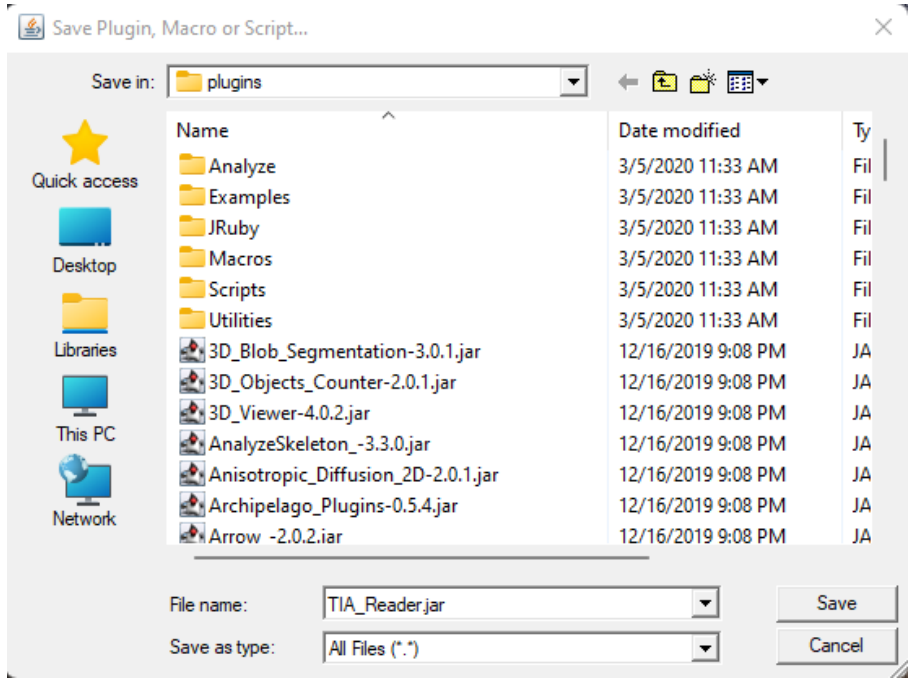
Then try to open Example 1D, the .ser file, using plugins > input/output > TIA reader

Installing a plugin from a downloaded .jar file

- drag and drop the .jar file onto Imagej or click Plugins > install plugin…
- Save it in the plugins folder of your FIJI folder



- Restart FIJI
- Then find the new functionality under
  Plugins > **Input / output** > Tia Reader
- Plugins > Input / output > Tia Reader
- Point to the .ser file
- Click ‘Open’



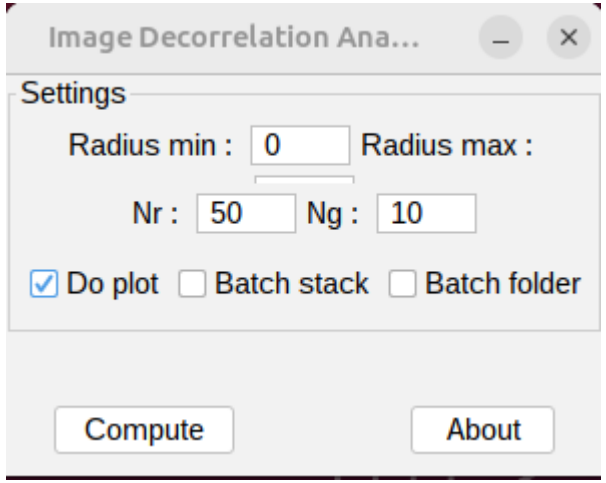
Does it work?

https://imagej.nih.gov/ij//plugins/tia-reader.html

4. Using a plugin from the internet (short resolution intermezzo)

EXERCISE 1
Install the image decorrelation plugin as well (ImageDecorrelationAnalysis_plugin.jar).

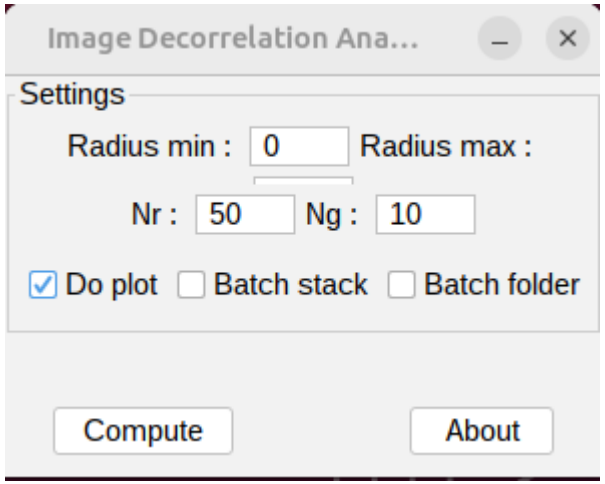
- Open Example 1B – Header – Cells.lsm with the Bio-Formats plugin (Plugins > Bio-Formats > Bio-Formats Importer)
- Run the Image Decorrelation plugin on the blue channel (Plugins > Image Decorrelation Analysis)



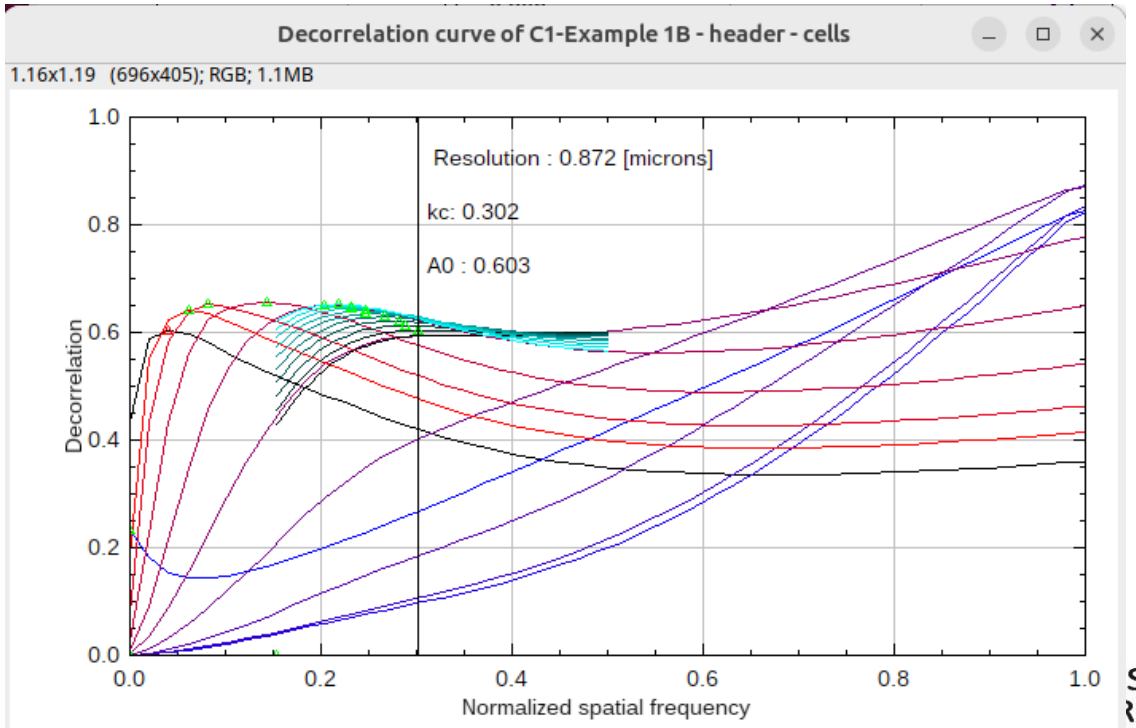
4. Using a plugin from the internet (short resolution intermezzo)

EXERCISE 1
Install the image decorrelation plugin as well (ImageDecorrelationAnalysis_plugin.jar).

- Open Example 1B – Header – Cells.lsm with the Bio-Formats plugin (Plugins > Bio-Formats > Bio-Formats Importer)
- Run the Image Decorrelation plugin on the blue channel (Plugins > Image Decorrelation Analysis)



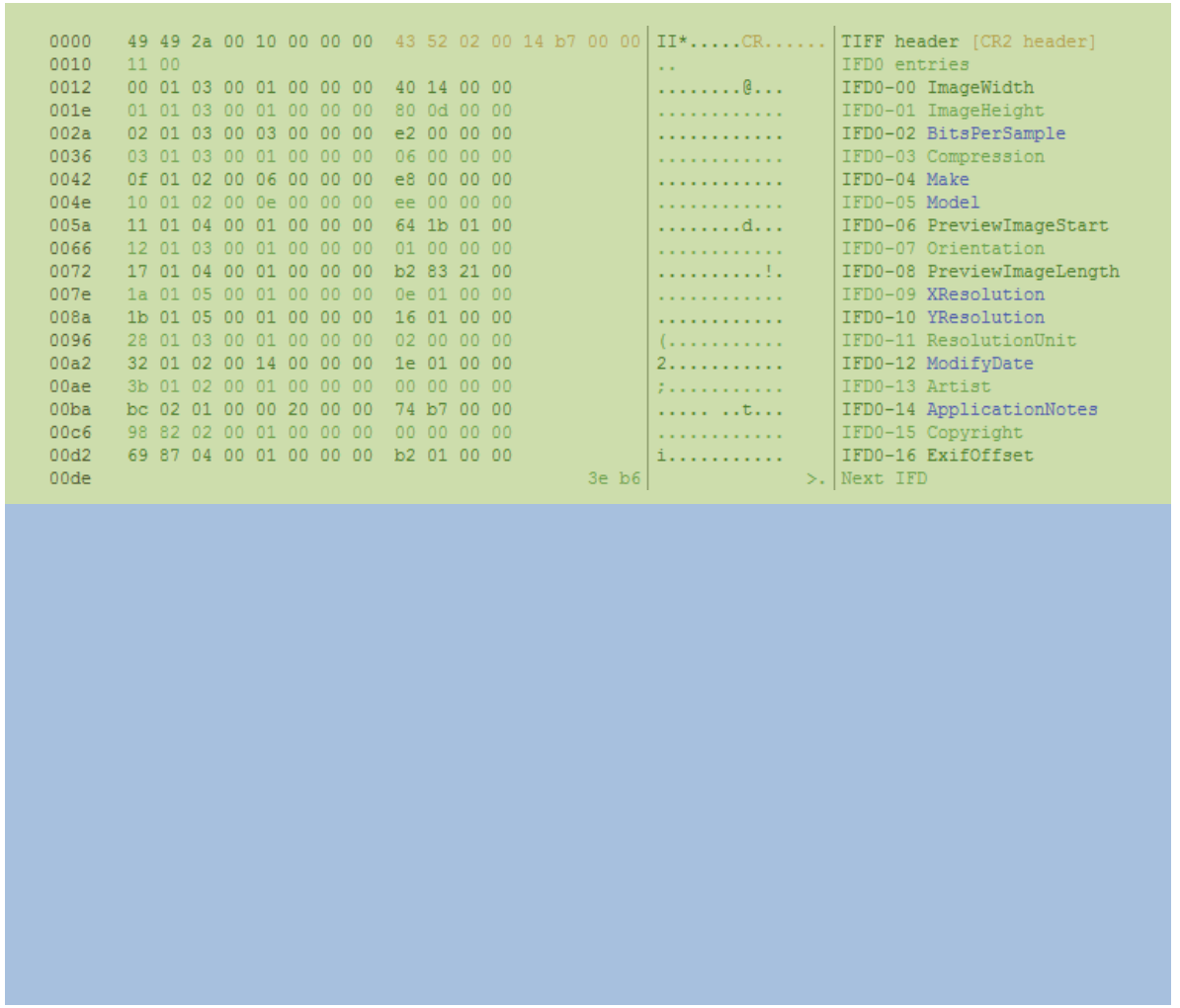
Label	Res.	units
C1-Example 1B - header - cells	0.872	[microns]



5. RAW import

5 Possibilities:

- Native file formats
- Use a repository
- Use Import (Fiji only!)
- Use a plugin
- Through RAW import (raster graphics only)



Total file size (in bytes) = Header size + Image data size

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5. RAW import

3 Possibilities:

- Native file formats
- Use a plugin
- **Through RAW import (raster graphics only)**

01110100 (ASCII, 116 = T)
01101001 (ASCII, 105 = I)
01100110 (ASCII, 102 = F)
00000100 (width = 4 px)
00000001 (height = 1 px)

00000000
00110010
01100100
10010110

Header: 5 bytes (TIF, 4x1 pixel image)

Data: 4 bytes 

Total file size: 9 bytes

Total file size (in bytes) = Header size + Image data size

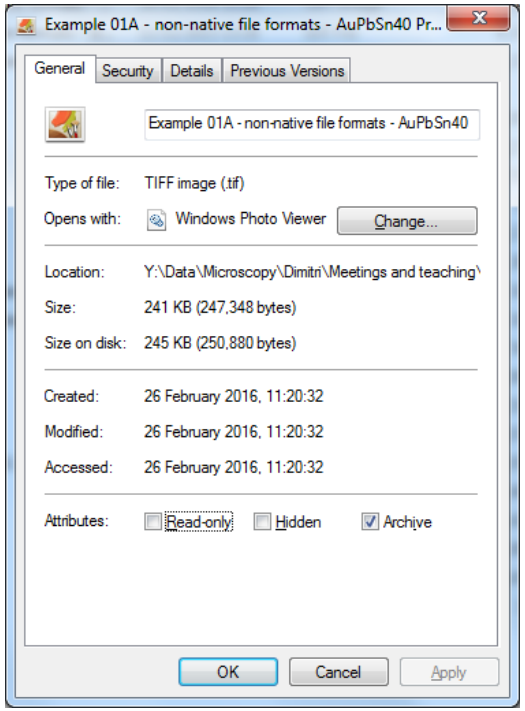
5. RAW import

EXERCISE 1
Import through RAW (file > import > Raw…) of
Example 1A, the .tif file and Example 1D, the .SER file

Idea: we will only read the DATA – and jump over the HEADER

Example 1A

1. A priori information: Camera size = 600x412 px x 8 bit depth
2. Use your operating system to find the file size (in bytes) of Example 1A



Size: 241 KB (247,348 bytes)

Size on disk: 245 KB (250,880 bytes)

Example 1D

Camera size: Veleta at AMI (2048 x 2048, 16 bit)
Use your operating system to find the file size

5. RAW import

EXERCISE 1
Import through RAW (file > import > Raw…)
Example 1A, the .tif file
Example 1D, the .ser file

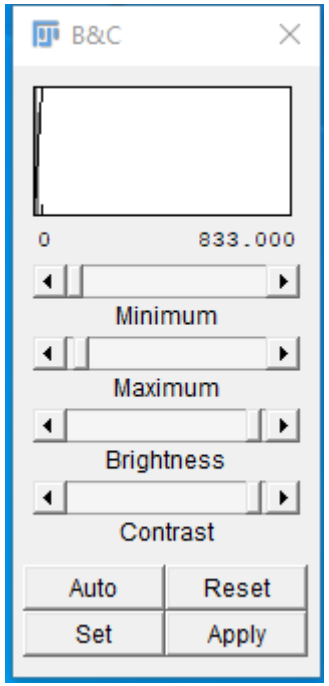
Idea: we will only read the DATA – and jump over the HEADER

Example 1A
1. Camera size: 600x412 px x 8 bit
2. 247 348 bytes

Example 1D
1. Camera size: Veleta at AMI (2048 x 2048, 16 bit)
2. 8 388 754 bytes

File > import > RAW...

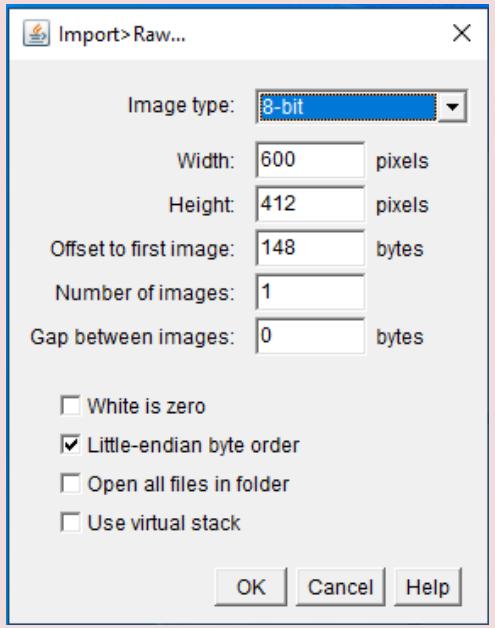
If your image is black, update brightness contrast:
Image > adjust > Brightness / contrast…
And click auto, or set (between 100 and 450)



5. RAW import

1. Data size: 600 x 412 = 247 200 bytes
2. File size : 247 348 bytes

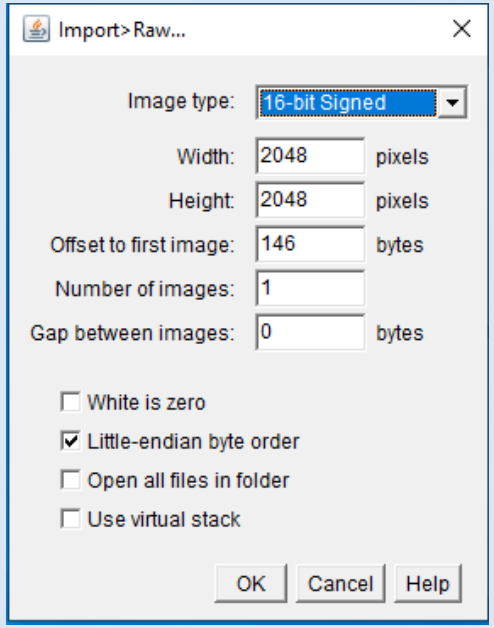
Therefore: header is 247 348 – 247 200 = 148 bytes



Endian: big-endian is often an “Apple thing”

1. Data size: 2048 x 2048 x 2 = 8 388 608 bytes (**why x 2??**)
2. File size : 8 388 754 bytes

Therefore: header is 8 388 754 – 8 388 608 = 146 bytes



Header

Files may (must) contain meta data (additional, useful information about the image)
F.A.I.R. Principles (https://www.go-fair.org/**), open source data**

Open Example 1D using File > **import > RAW...**
Select Image > Show info...

Title: Example 1D - non-native file formats - SiO4.ser
Width: 2048 pixels
Height: 2048 pixels
Size: 8MB
Pixel size: 1x1 pixel^2
ID: -77
Bits per pixel: 16 (signed)
Display range: 100 - 450
No threshold

Open Example 1D **using the TIA Plugin** >
Select Image > Show info...

Title: /home/.../Example 1D - non-native file formats.ser
Width: **8.3029 microns** (2048)
Height: **8.3029 microns** (2048)
Size: 8MB
Resolution: 246.6609 pixels per microns
Pixel size: **0.0041x0.0041** microns^2
ID: -63
Bits per pixel: 16 (unsigned, grayscale LUT)
Display range: 0-922
Pixel value range: 0-922
Image: 1/1 (1) No threshold
...

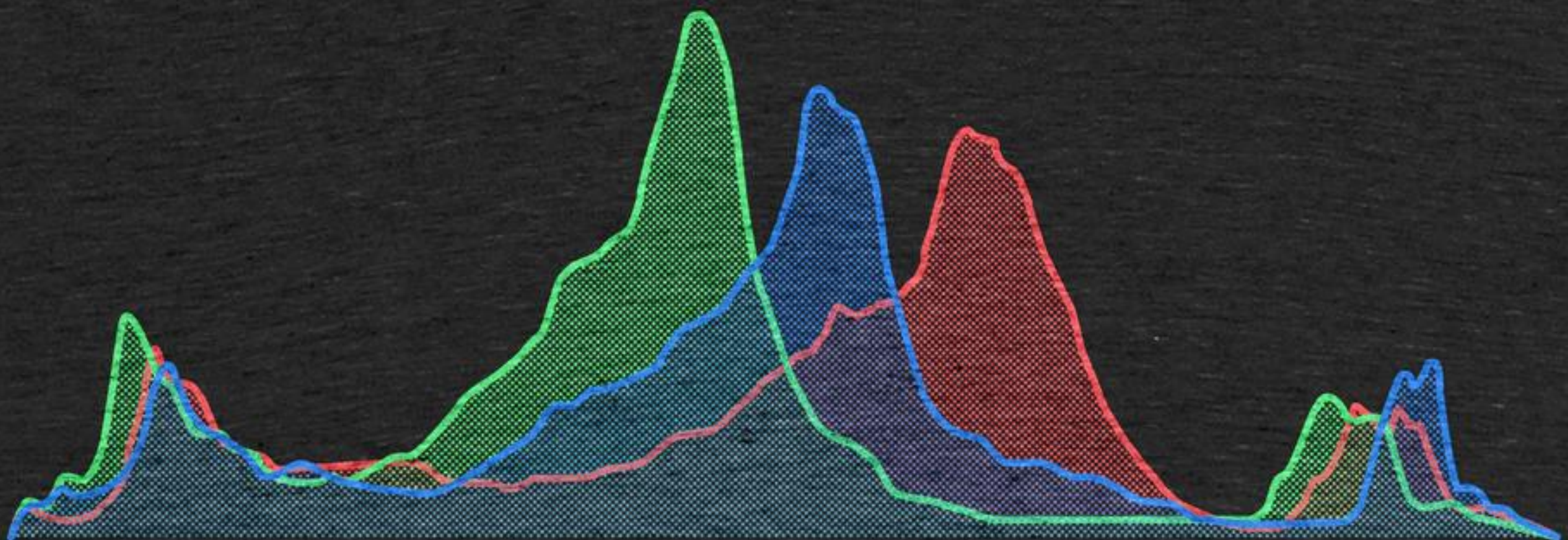
Summary: Howto open your data in FIJI

Native file formats	**FIJI Import**	**Repositories**	**Install a plugin**	**Raw import**
Use TIF whenever possible Forget JPEG (has no header) Metadata properly imported	Using the repositories or from the internet	500+ scientific file formats available through Repositories. Metadata is (very very often) imported E.g. Zeiss, Leica, Olympus, Nikon, FEI, ...	Using the repositories or from the internet	Last resort, if everything else fails Typically: *a priori* information about the file needed (dimension, bit depth) Opens only the image data. No metadata.

Metadata is a love note to the future

Part III: Histograms

- Histograms of grayscale image
- Color images
- Histogram normalization and histogram equalization

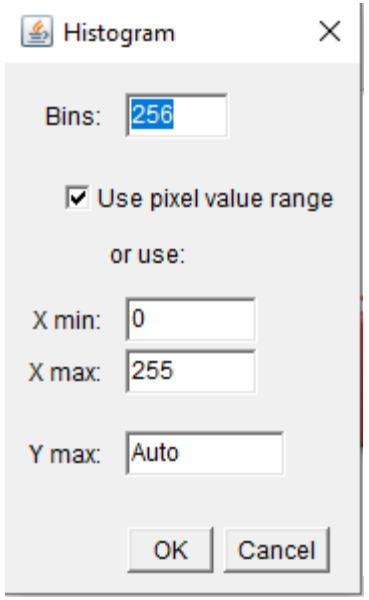


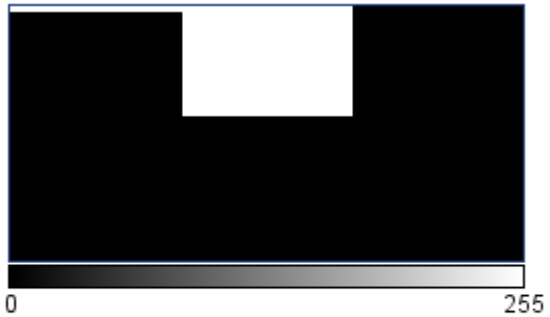
Histograms

EXCERCISE 2
Open Example 1A and produce a histogram

Analyze > histogram (or CTRL+H)

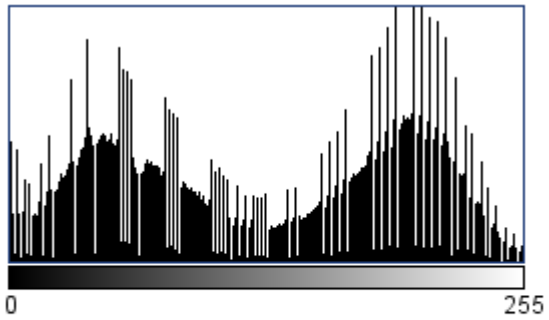
- What can you deduce from the histogram?
- Try: List, Copy, Log, Live
- Save the histogram itself to a TIFF?
- Also try CTRL+ALT+H





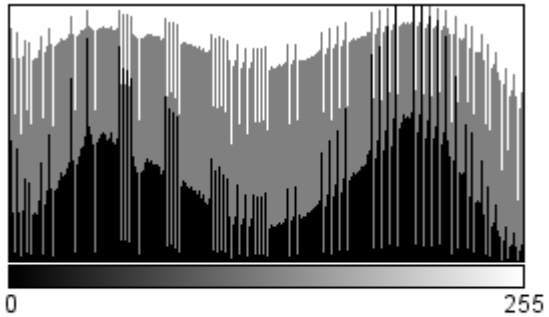
N: 247200
Mean: 125.901
StdDev: 73.247
Bins: 3
Value: 85

Min: 0
Max: 255
Mode: 170 (97279)
Bin Width: 85.333
Count: 55188



N: 247200
Mean: 125.901
StdDev: 73.247
Value: 101

Min: 0
Max: 255
Mode: 201 (3164)
Count: 129



N: 247200
Mean: 125.901
StdDev: 73.247
Value: 80

Min: 0
Max: 255
Mode: 201 (3164)
Count: 211

Histogram
a representation of the distribution of numerical data.
Pearson, K. (1895)

The intensity distribution of the image (= it plots the number of pixel for each intensity or tonal value)

Histograms

EXCERCISE 2

Open Example 2A and 2B and look at the image

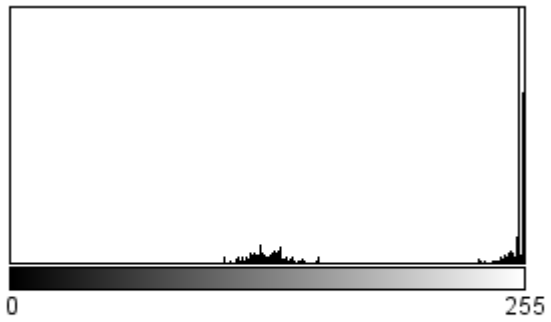
- Do you see a difference between the images?
- Check the histograms
- Do you see a difference between the histograms?

Histograms

EXCERCISE 2
Open Example 2A and 2B and look at the image

N: 65536
Mean: 247.269
StdDev: 30.036
Value: 23

Min: 128
Max: 255
Mode: 255 (61272)
Count: 0



N: 65536
Mean: 245.270
StdDev: 29.543
Value: 134

Min: 88
Max: 255
Mode: 253 (57340)
Count: 219

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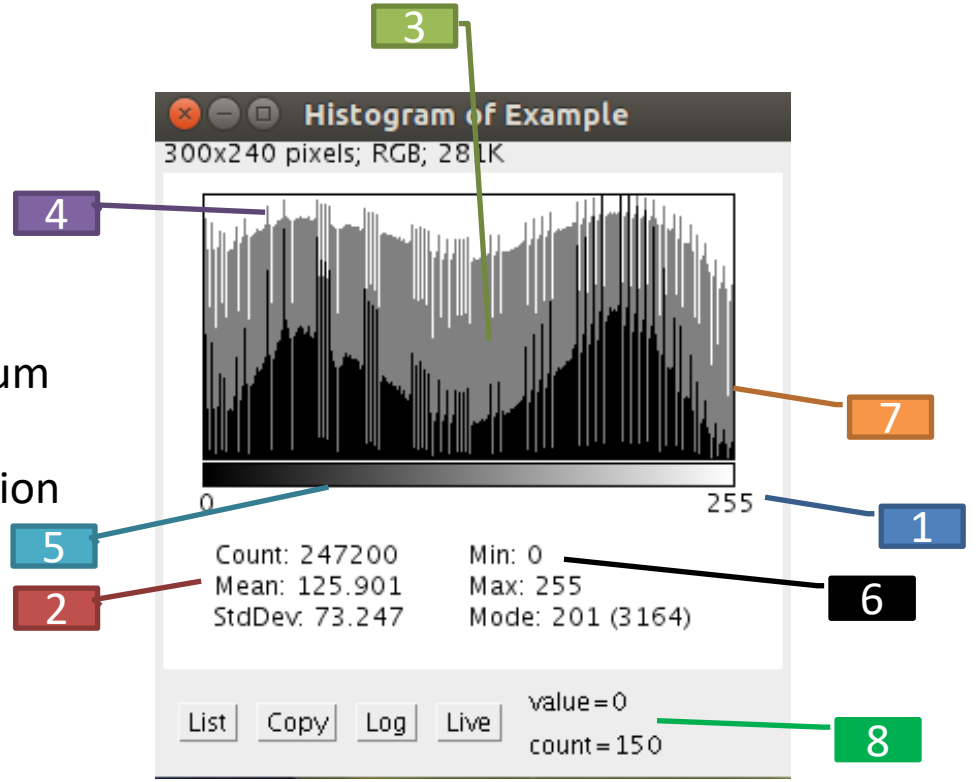
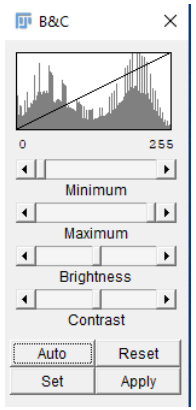
Histograms

What can you deduce from the histogram?

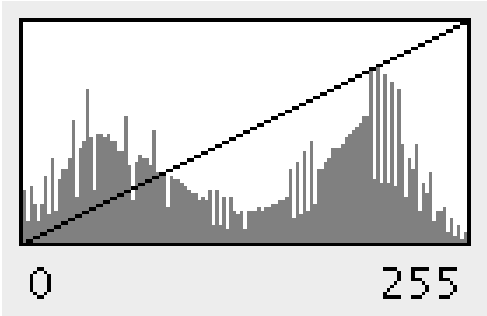
- 1. Bitdepth: 8 bit image (ranging from 0 to 255)
- 2. Pixel value distribution:
  Mean pixel value, variance of the intensity,
  Min, Max & modal
- 3. Type of distribution: e.g. bimodal, exponential, …
- 4. Spikes: image normalization or equalization occurred (see later)
- 5. Contrast and Lookup table (see later): the range between maximum and minimum (in this case: 255)
- 6. Dynamic range: the number of distinct pixel values. Eg. Compression will affect the dynamic range
- 7. Overillumination effects
- 8. The intensity at each grayscale value



Histograms → Linear transfer function



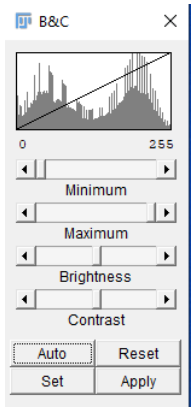
Find the contrast brightness window (Image > adjust > brightness/contrast)



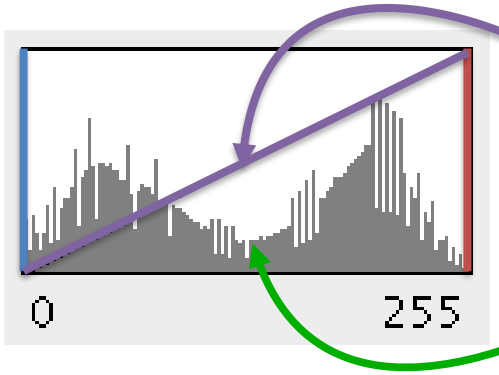
Each image intensity value is mapped to its corresponding screen value:
The linear transfer function is running from bottom left to top right

The linear transfer function
assigns every original pixel value a new value on a linear scale. The endpoints of the function determine what value is white, and what black

Histograms → Linear transfer function



Find the contrast brightness window (Image > adjust > brightness/contrast)



Transfer function: Attribute of visual perception

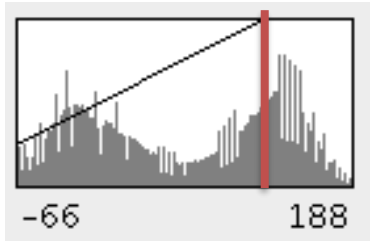
Histogram: Attribute of the image

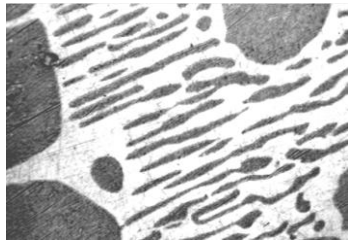
Pixels to the left of TF_{min} = black
Pixels to the right of TF_{max} = white
Pixels > TF_{min} and < TF_{max} = gray (linearly)

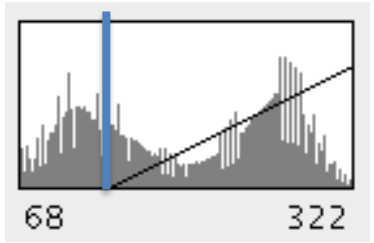
KEY: Changing the transfer function does not alter your data, only the way it is depicted

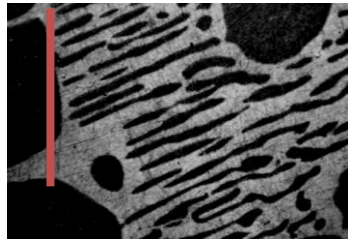
Brightness

The transfer function is shifted



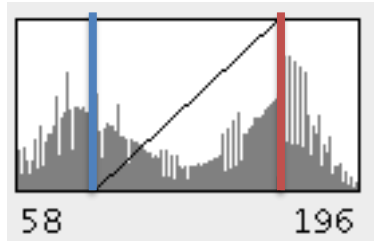


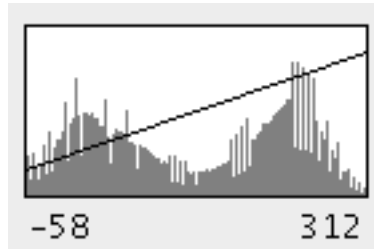


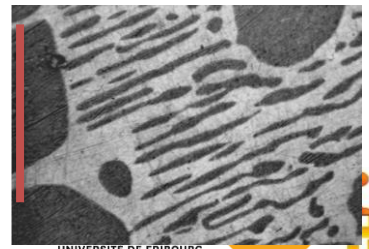


Contrast

The transfer function is tilted



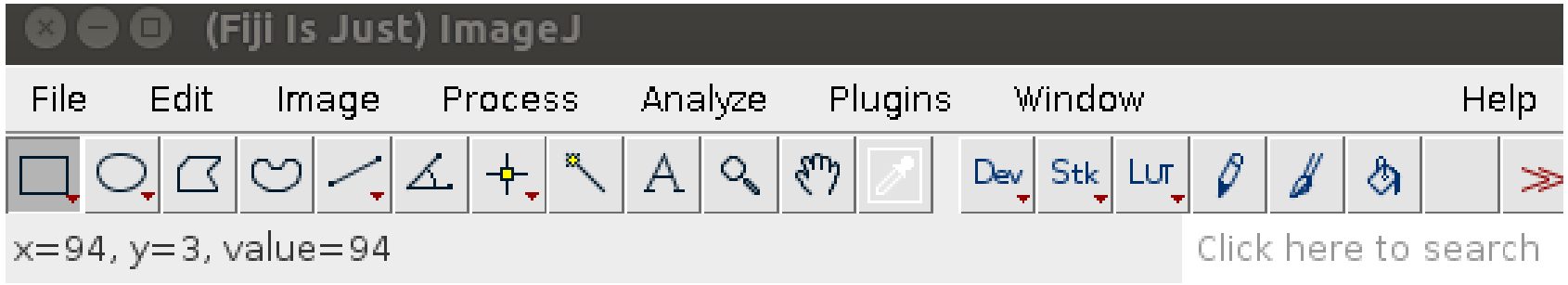




Histograms

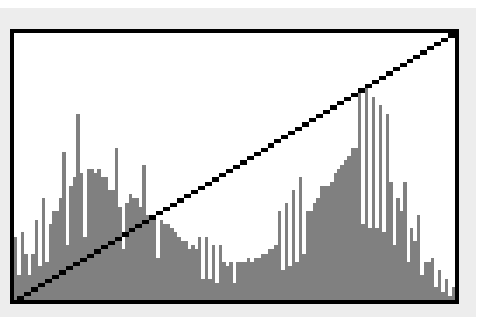
EXCERCISE 3
Load Example 3 – GrayscaleLUT, and read out the grayscale values in the ImageJ statusbar.

- Use (+ to zoom in, - to zoom out)
- Hoover with the mouse over the image and check the status bar in ImageJ
- Image > adjust > Brightness/Contrast (CTRl+Shift+C)
- Make a histogram of this image. How do you interpret the histogram?
- Play around with the Minimum and Maximum in the transfer function (Image > Adjust> Brightness/Constrast). Check the effect on the values.
- Do you delete information?



Histograms: other lookup tables (LUTs)

82	72	78	86	65	41
157	144	167	188	201	198
185	191	195	188	188	196
193	195	195	191	189	178
173	170	181	192	194	194
210	214	206	202	203	207
237	224	221	230	232	225
183	180	190	188	192	187
178	170	159	187	195	189
167	164	170	186	192	182
159	162				
180	172				
193	180				
167	184				
195	191				
183	188				
191	196				



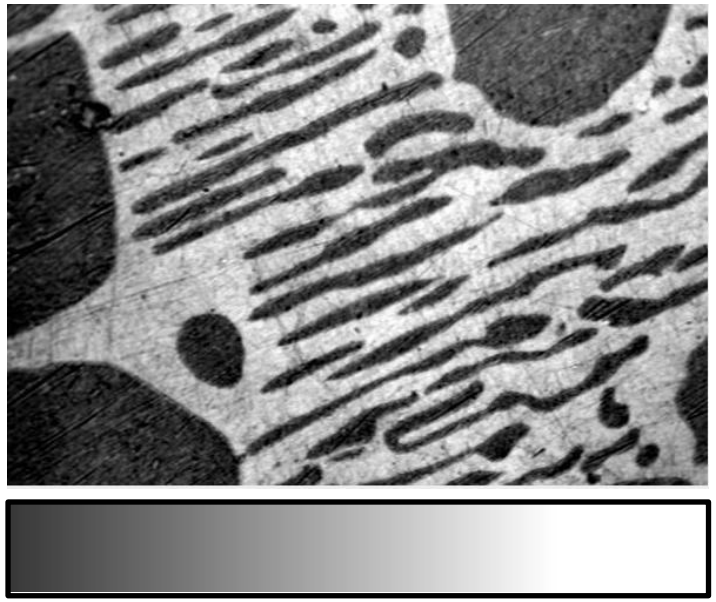
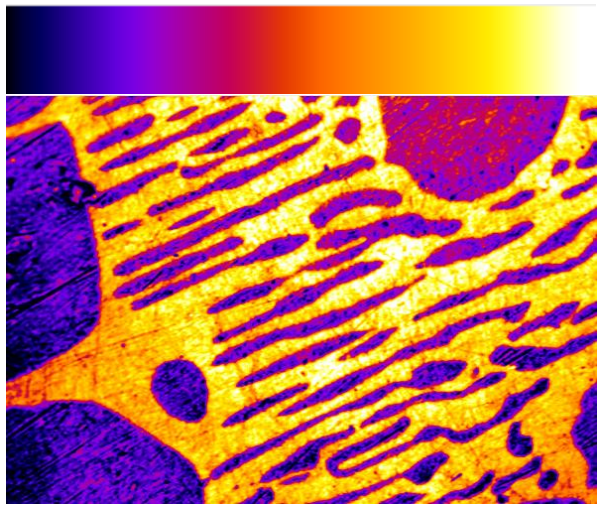
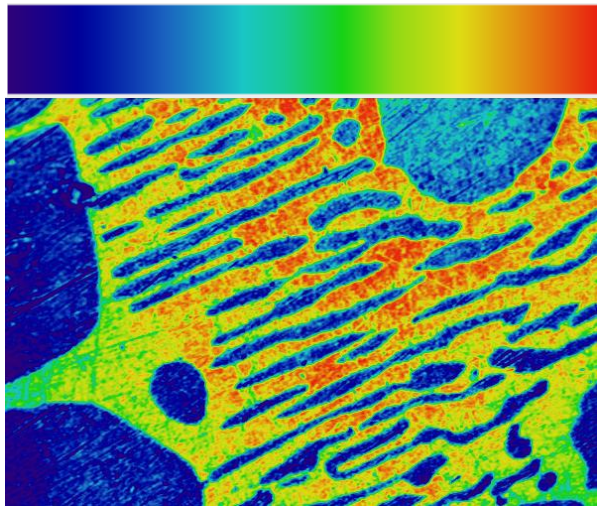
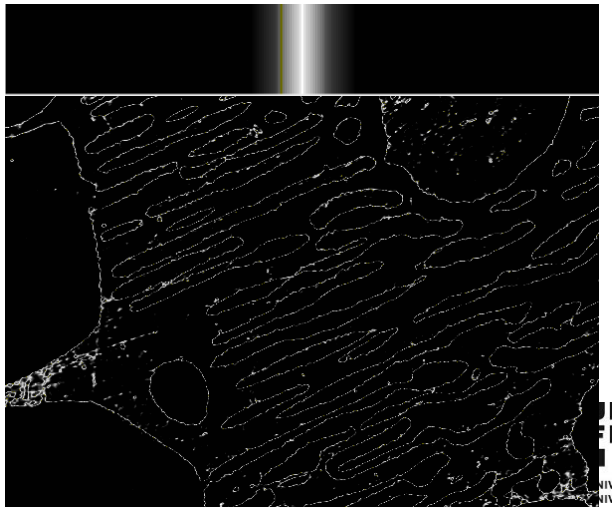


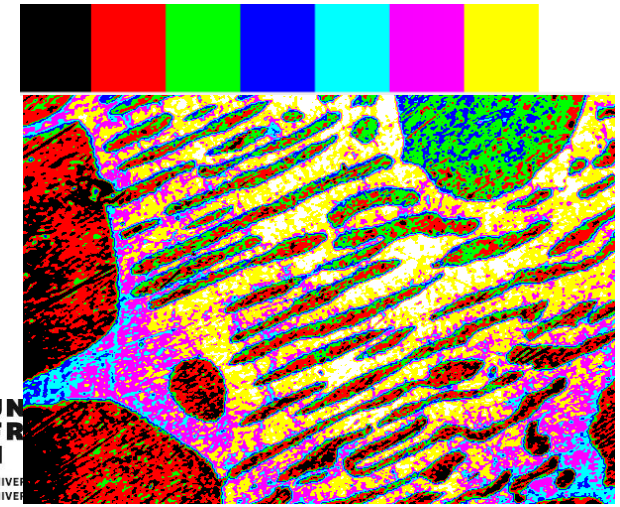
Image = 2D array of numbers

Grayscale = giving a graphical meaning to these Look-Up Tables.
But Grayscale is just one of these Look-Up tables!









Histograms: other lookup tables (LUTs)

EXCERCISE 3
Load Example 3 or Example 1A and try different LUTs

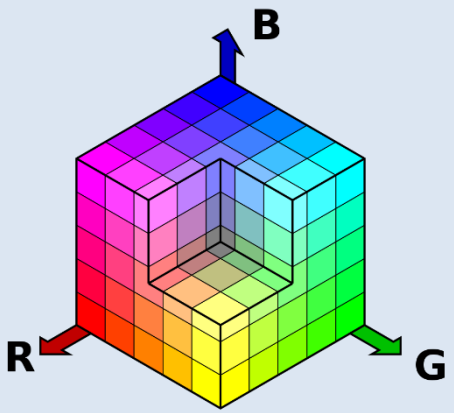
- Image > Lookup Tables > ...
- Image > Color > Show LUT
- Change brightness and contrast with some exotic LUTs

- You can make your own LUT using Image > color > Edit LUT

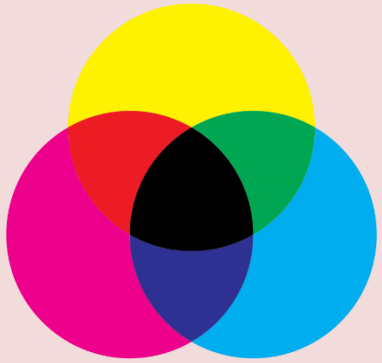
Color: the color model

A color model
is a method of describing a color. Color models can be represented as tuples of numbers, typically as three or four values or color components.

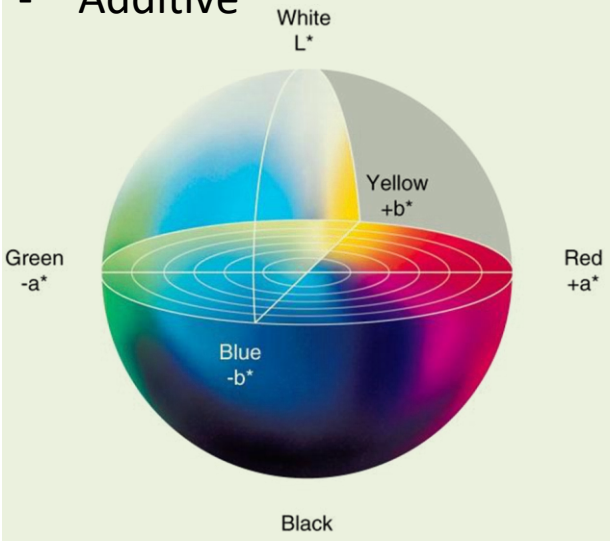
RGB
- 3 values: Red, green & blue
- Additive



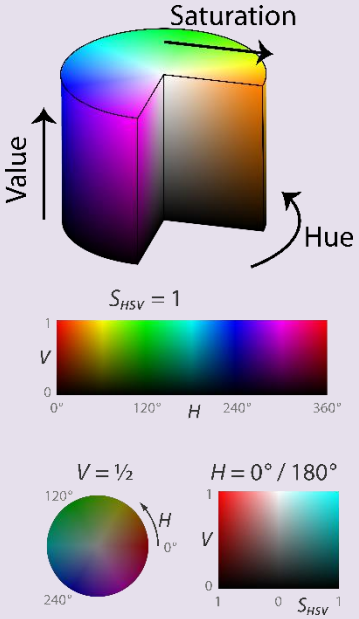
CMYK
- 4 values: Cyan, Mangenta, Yellow and black
- Subtractive



L*a*b (CIELAB)
- 3 values: Lightness, a (green to red) and b (blue to yellow). Human vison based.
- Additive

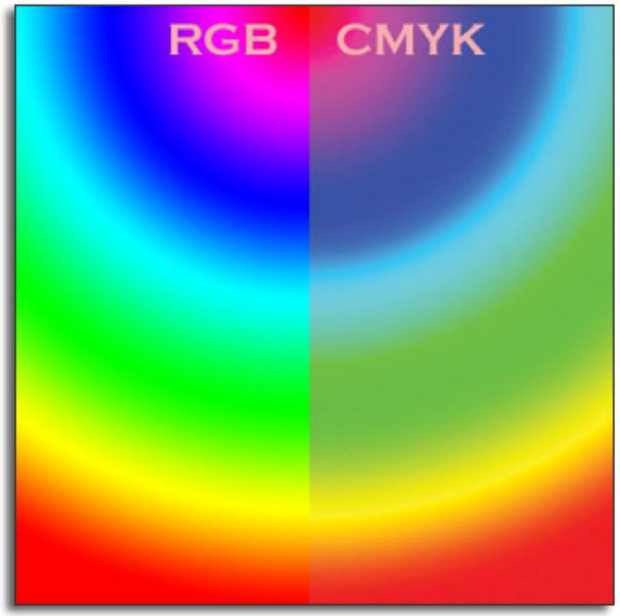


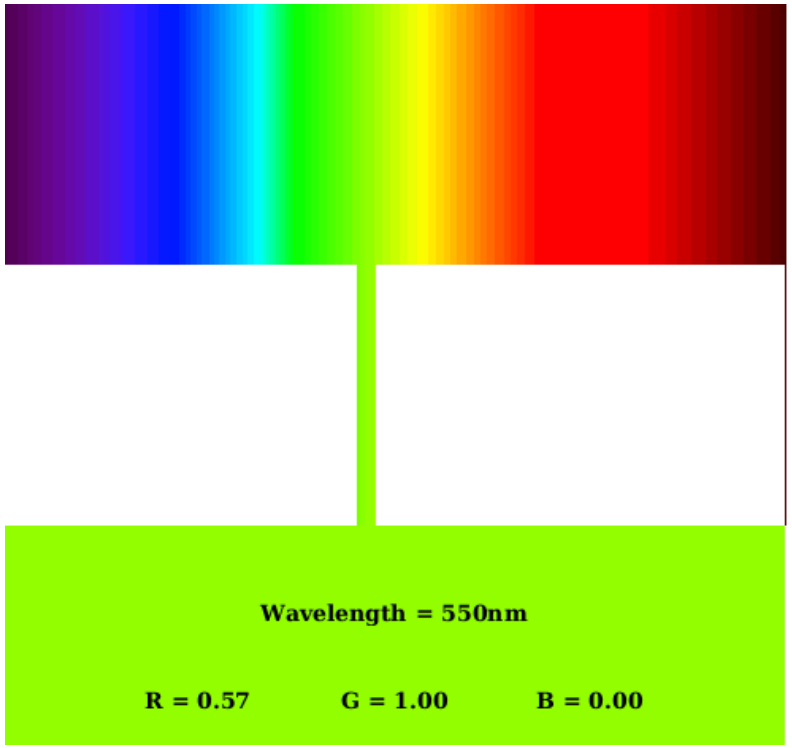
HSV
- 3 values: Hue, Saturation and Value
- Additive



Color: the color space

A color space
is a way of mapping real colors to the color model's particular values. the goal having reproducible, unambiguous representations of color – whether such representation entails an analog or a digital representation.





Based on human perception (uniform color spaces)
CIE 1931 XY
CIEUVW
HSLuv

Non-uniform color spaces
sRGB (Adobe)
REC 2020 (UHDTV, covers about 75% of the visible colors)

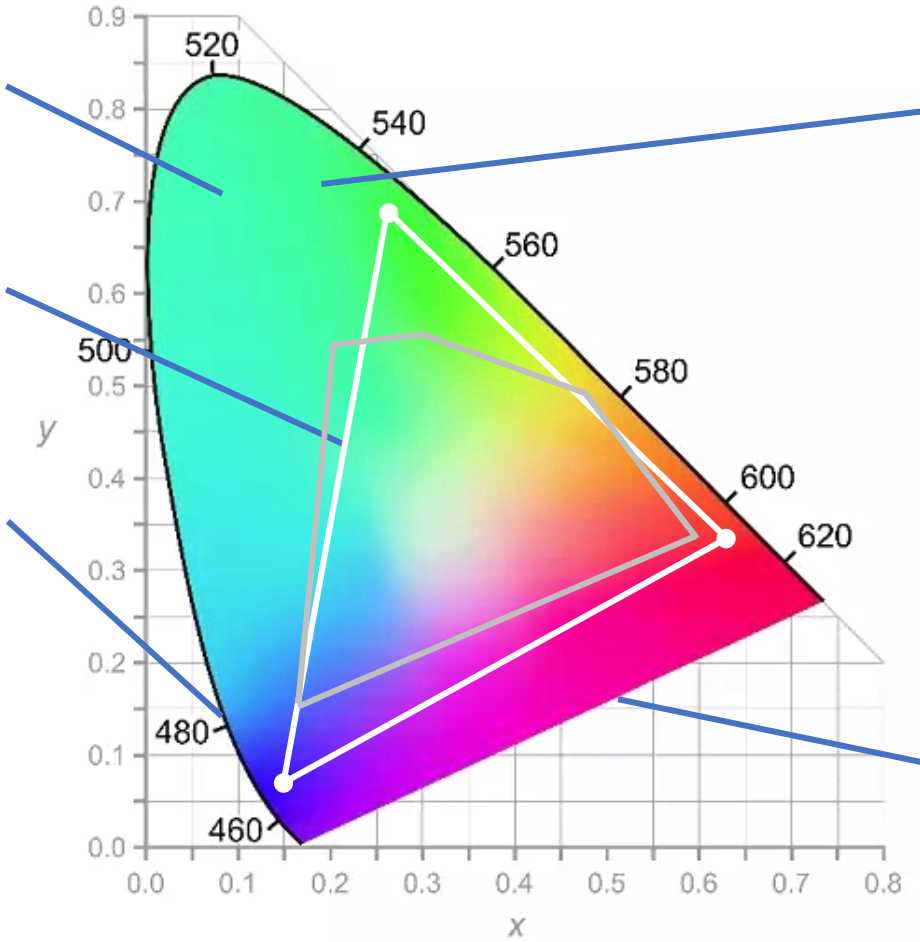
Color: the color gamut

The color gamut
The entire range of colors and tones achievable by an imaging system (eyes, printer, display)

Inside the diagram:
All colors visible to the average human eye (= CIELAB color space)

Color mixing:
The colors along any line between two points can be made by mixing the colors at the end points

Spectral locus:
The edge of the diagram represents pure monochromatic light (single wavelength, in nm)



Color gamut:
Subset of colors that can be represented by mixing the colors at it's corners

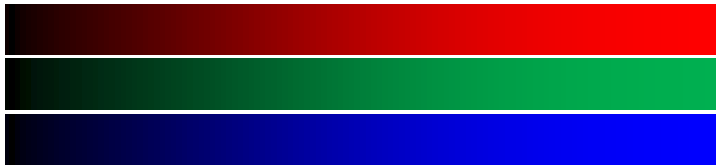
White: sRGB color space
Gray: color space defined by CMYK

Line of purples:
Non-monochromatic saturated colors (cannot be represented by a single wavelength)

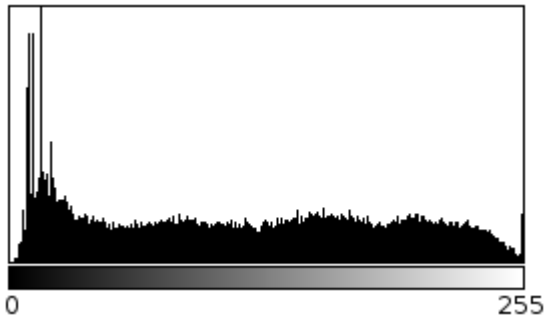
CIE 1931 XY colorogram

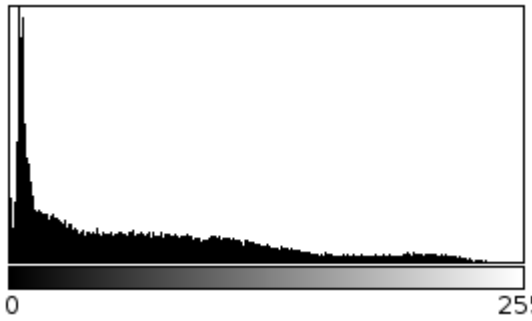
Color images

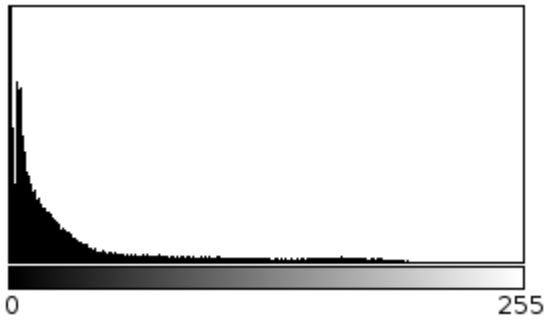
- Color image = 3 grayscale images combined (=composite).
- with a red LUT
- With a green LUT
- With a blue LUT



They are also called RGB images, or 24 bit images (=3x8 bit)







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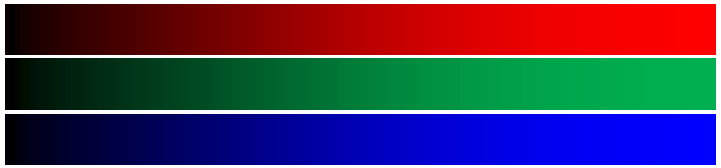
Color images

- Color image = 3 grayscale images combined (composite).
- with a red LUT
- With a green LUT
- With a blue LUT



They are also called RGB images, or 24 bit images (=3x8 bit)

Header

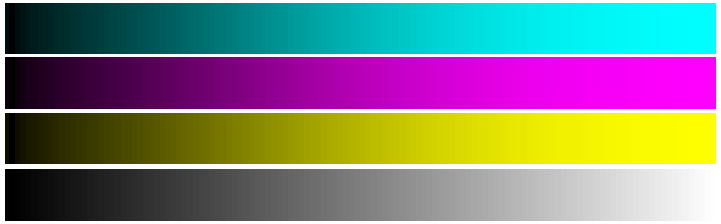
Image – red data

Image – green data

Image – blue data

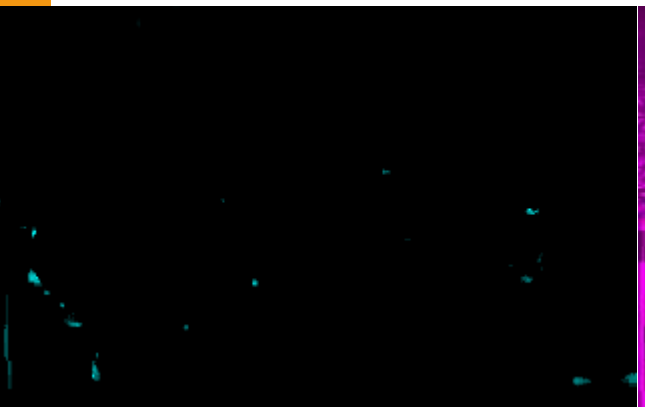
Color images

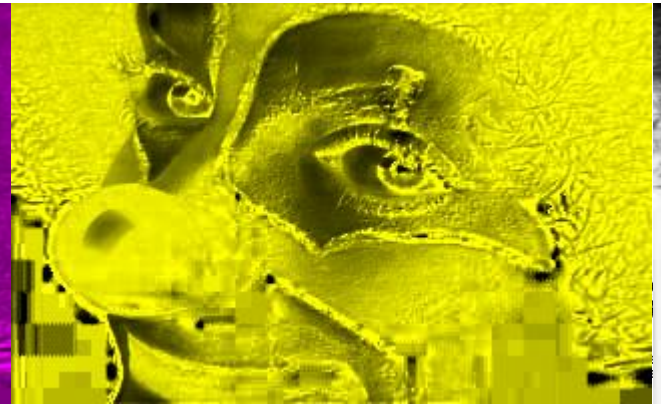
- Color image = 4 grayscale images combined (composite).
- with a cyan LUT
- With a magenta LUT
- With a yellow LUT
- With a black LUT



They are also called CMYK images, or 32 bit color images (=4x8 bit)

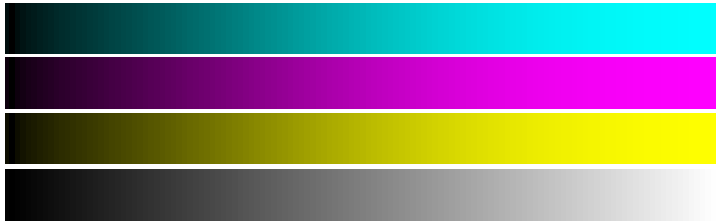
Header
Image – cyan data
Image – Magenta
Image – blue data
Image – blue data





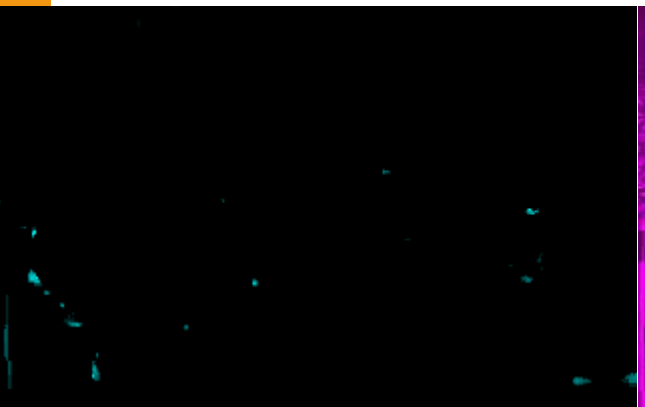
Color images

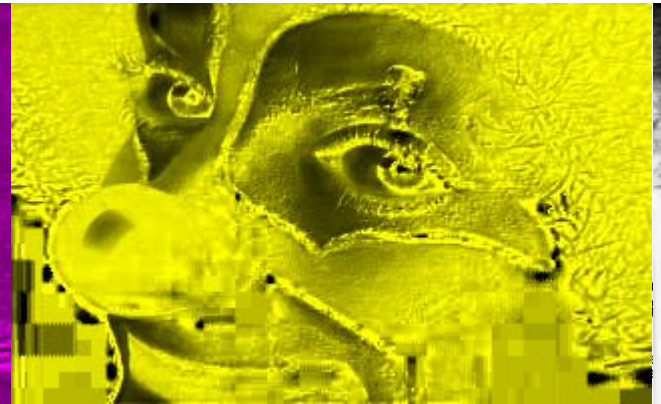
- Color image = 4 grayscale images combined (composite).
- with a cyan LUT
- With a magenta LUT
- With a yellow LUT
- With a black LUT



They are also called CMYK images, or 32 bit color images (=4x8 bit)

Header
Image – cyan data
Image – Magenta
Image – blue data
Image – blue data

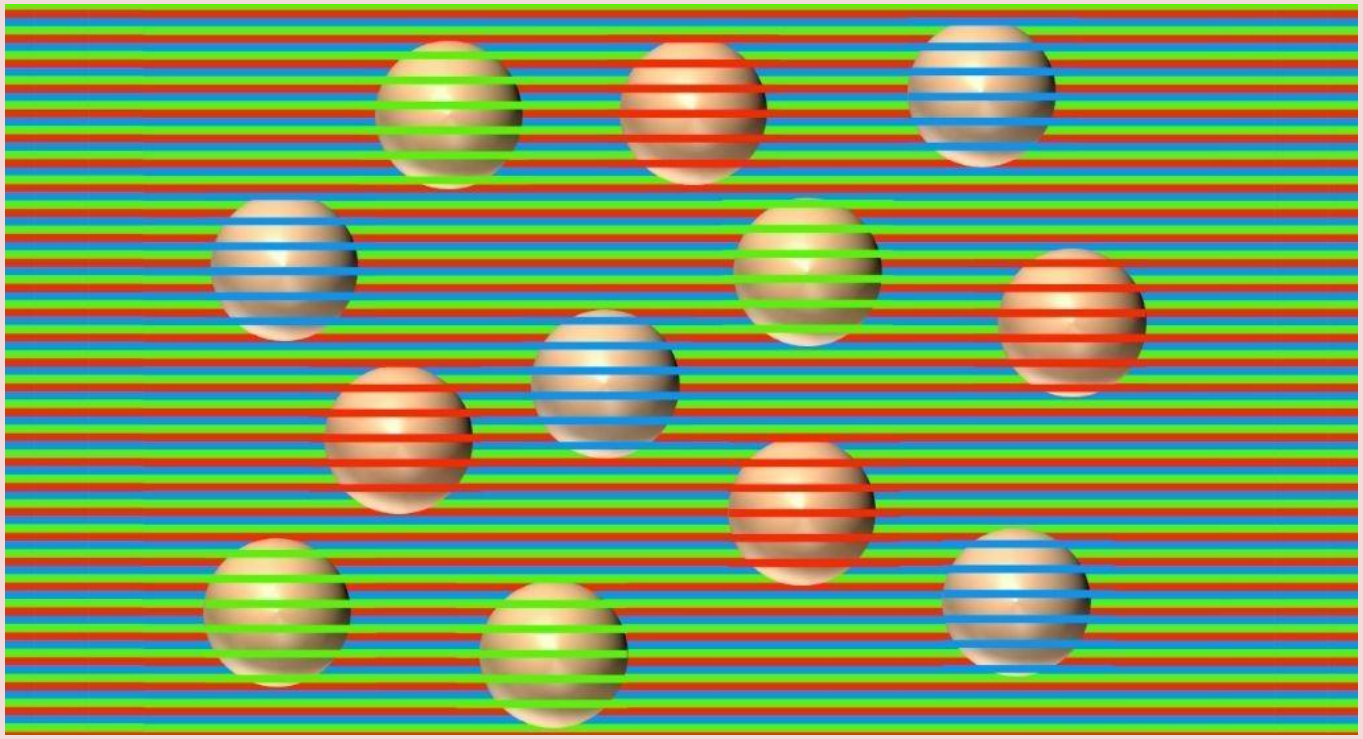




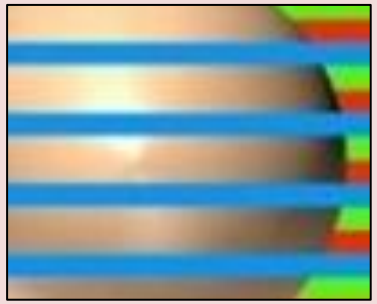
Measuring color

Natural: by eyes

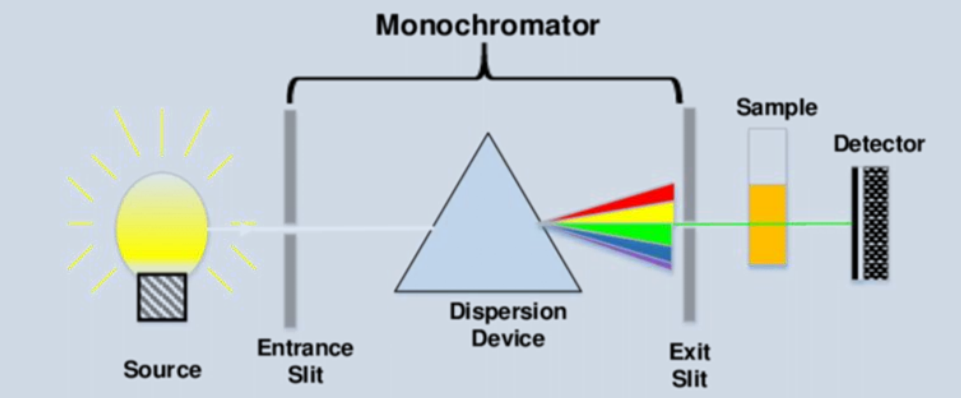
(generally a very bad idea)



“green” “blue” “red”



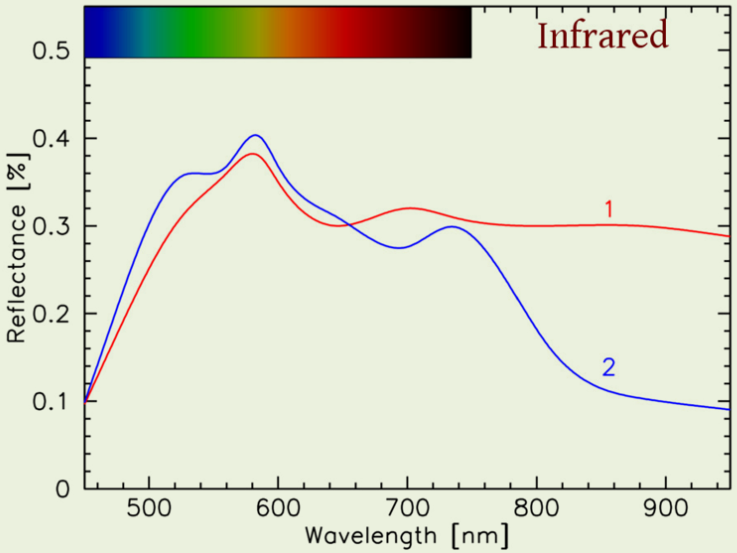
Standard: using a spectrophotometer



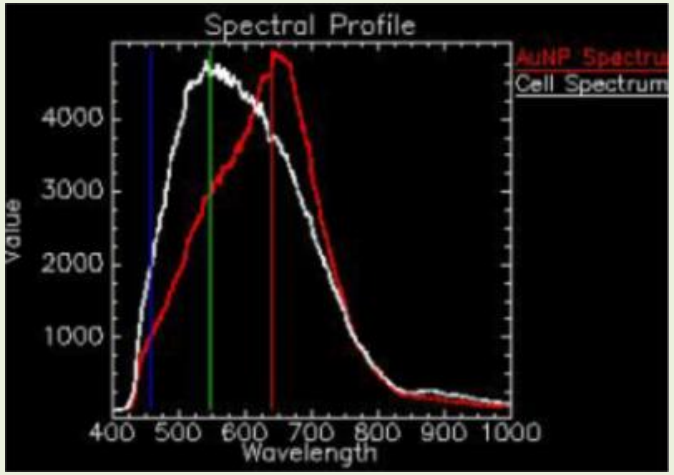
Measuring color

Cytoviva: measuring reflectance spectra

Reflectance photospectrometer output



Cytoviva darkfield microscope output



More tricky: using a commercial camera

→ Need for calibration using a color calibration chart

Note: non-linear shifts in Red, Green and Blue!
Output: RGB color space

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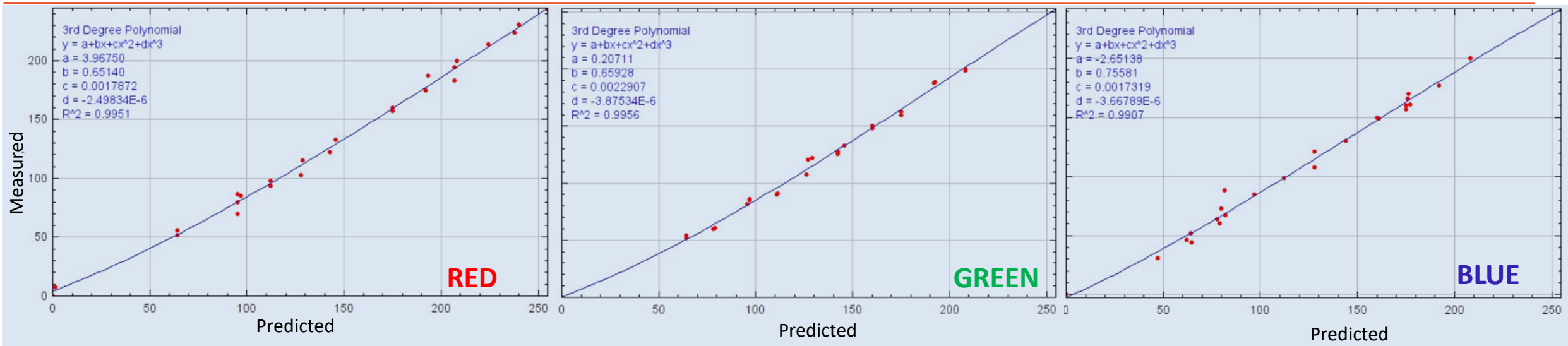
Measuring color



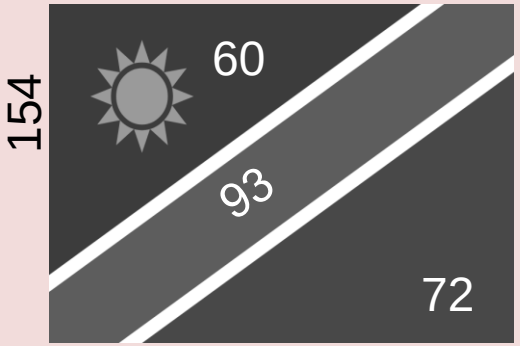
Mismatch:
RED: 15.5%
GREEN: 12.3%
BLUE: 16.1%

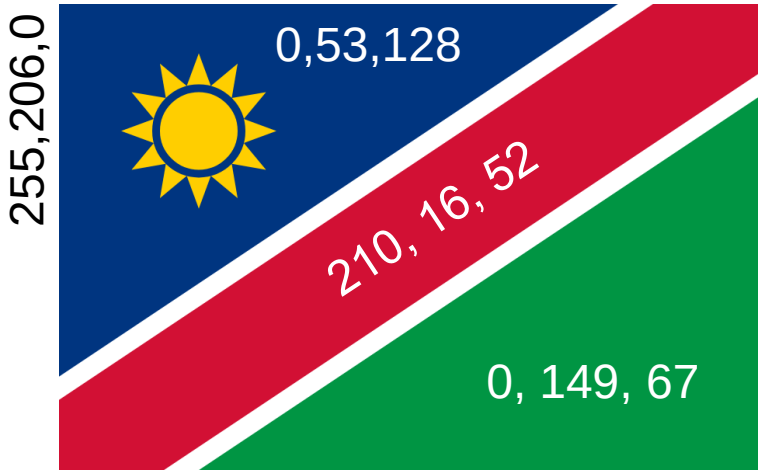
R=129 G=95 B=83	R=207 G=160 B=144	R=112 G=129 B=175	R=95 G=126 B=82	R=146 G=142 B=192	R=128 G=192 B=176
R=225 G=142 B=66	R=95 G=112 B=177	R=207 G=111 B=112	R=112 G=78 B=128	R=175 G=192 B=78	R=242 G=170 B=68
R=64 G=79 B=160	R=95 G=160 B=80	R=192 G=64 B=79	R=240 G=208 B=47	R=193 G=97 B=161	R=1 G=146 B=177
R=255 G=255 B=255	R=208 G=208 B=208	R=175 G=175 B=175	R=144 G=128 B=129	R=97 G=97 B=97	R=64 G=64 B=64

Converting color to grayscale

Direct conversion

$$8bit = \frac{Red + Green + Blue}{3}$$

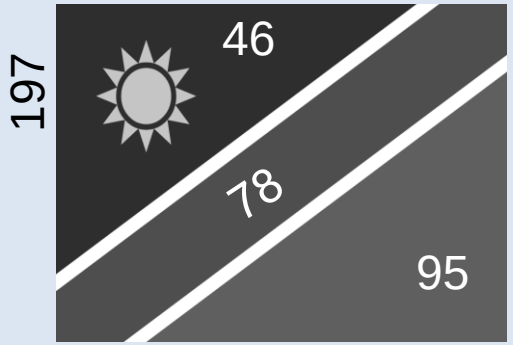




Edit > Options > Conversions…

Weighted conversion

$$8bit = 29.9\%Red + 58.7\%Green + 11.4\%Blue$$



Converting formats, saving data

EXCERCISE 4
Load the clown test image and convert it to another format

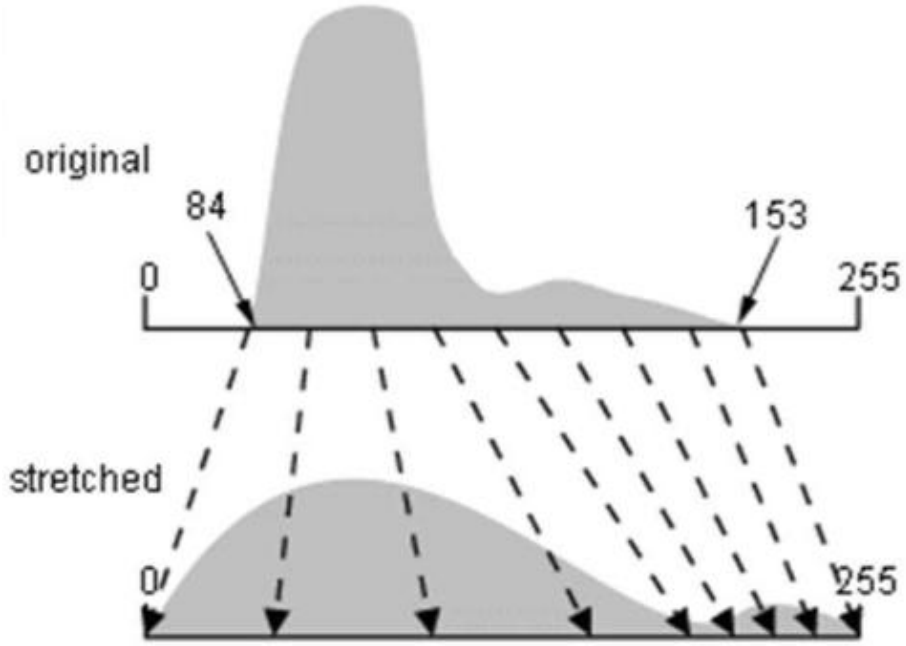
- File > Open Samples > Clown
- Image > Type
- Change the type to e.g. 8-bit and check what happened to the size
- Can you change back to RGB (without Edit > undo, off course)?
- Save as 8-bit, 16-bit and RGB TIFF. Compare file sizes

Bit depth	Channels	Filesize (bytes)	Data	Header
8 bit	1	64 236 bytes	64 000 bytes (=320x200x1)	236
16 bit	2	128 254 bytes	128 000 bytes (=320x200x2)	254
RGB	3	192 166 bytes	192 000 bytes (=320x200x3)	166
32 bit	4	256 266 bytes	256 000 bytes (=320x200x4)	266
L*a*b (3x32 bit)	3x4	768 672 bytes	768 000 bytes (320x200x4x3)	672

- Convert to CMYK: https://imagej.nih.gov/ij/plugins/cmyk/index.html

Histogram normalization, histogram equalization

Goal: to use the entire range of intensities in the histogram



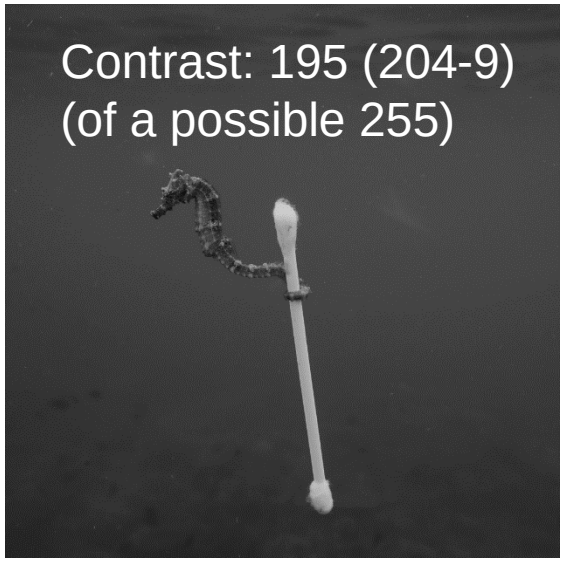
WARNING:

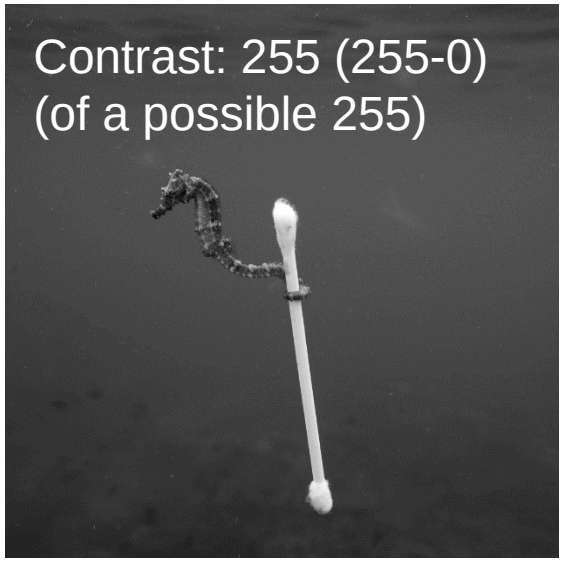
1. GIGO
2. We will now **change the actual data**, not longer only the transfer function.

Solutions:

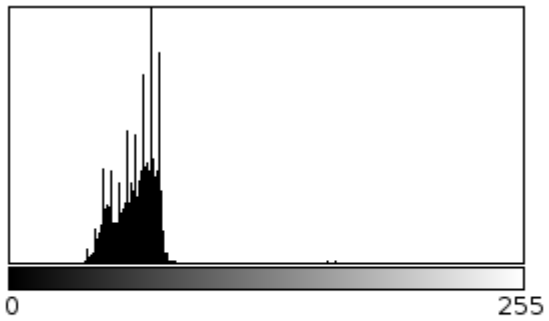
- Histogram normalization (histogram stretching)
- Histogram equalization

Histogram normalization

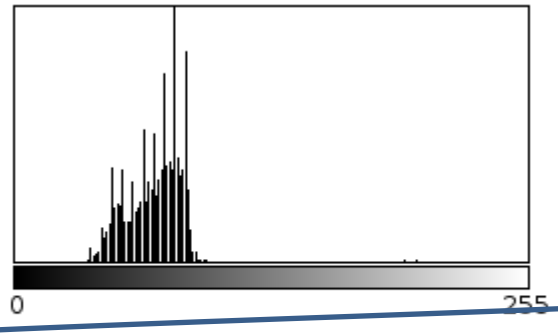




Contrast: the range between the maximum and minimum intensity in the image



Count: 1210000
Mean: 63.619
StdDev: 15.943
Min: 9
Max: 204
Mode: 70 (101022)



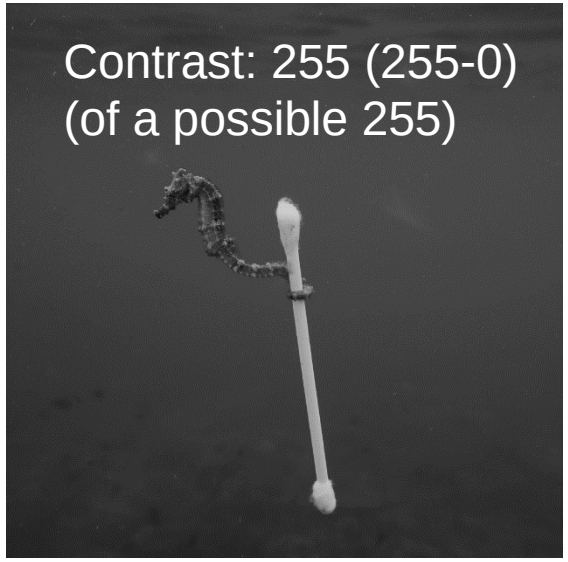
Count: 1210000
Mean: 70.985
StdDev: 20.861
Min: 0
Max: 255
Mode: 79 (101022)

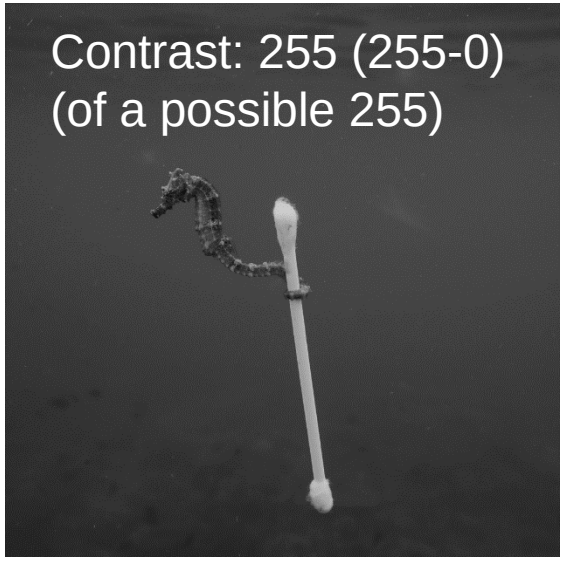
$$g(x,y) = \frac{f(x,y) - I_{min}}{I_{max} - I_{min}} \cdot 2^n$$

$$g(x,y) = \frac{f(x,y) - 9}{204 - 9} \cdot 256$$

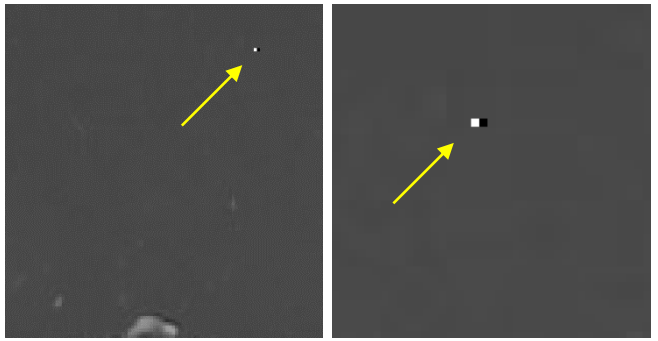
Histogram normalization: attention!

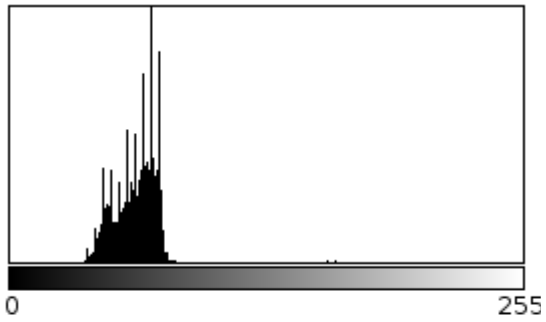
Problem: hot/cold pixels



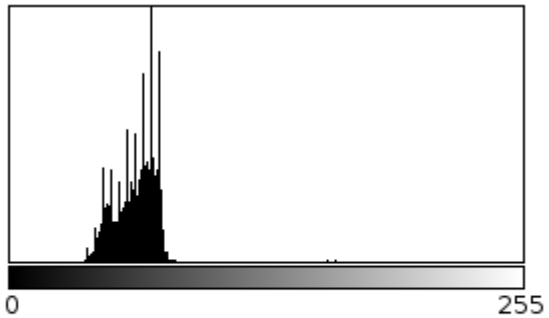


$$g(x,y) = \frac{f(x,y) - I_{min}}{I_{max} - I_{min}} \cdot 2^n$$





Count: 1210000
Mean: 63.619
StdDev: 15.944
Min: 0
Max: 255
Mode: 70 (101022)

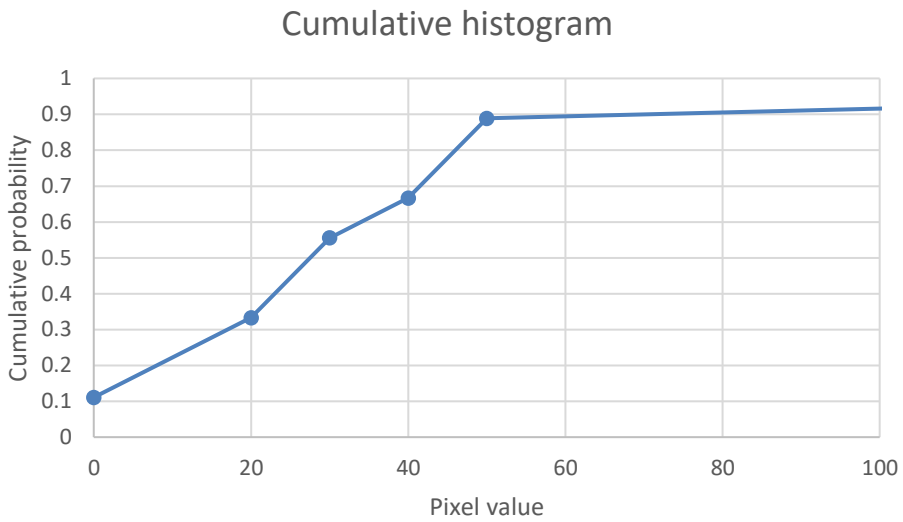


Count: 1210000
Mean: 63.619
StdDev: 15.944
Min: 0
Max: 255
Mode: 70 (101022)

Histogram equalization

5	20	40
20	30	30
50	100	50

Values	Occurrence	PMF*	CDF*
5	1	0.111	0.111
20	2	0.222	0.333
30	2	0.222	0.555
40	1	0.111	0.666
50	2	0.222	0.888
100	1	0.111	1
	Total = 9	Total = 1	



= the normalization of the cumulative histogram of the image

*PMF = Probability mass function
*CDF = Cumulative distribution function = cumulative histogram

Histogram equalization

Note to the not-so-mathematically-inclined:
This is exactly the same formula as 3 slides back, but now we use the newly created cumulative histogram instead of the histogram

5	20	40
20	30	30
50	100	50

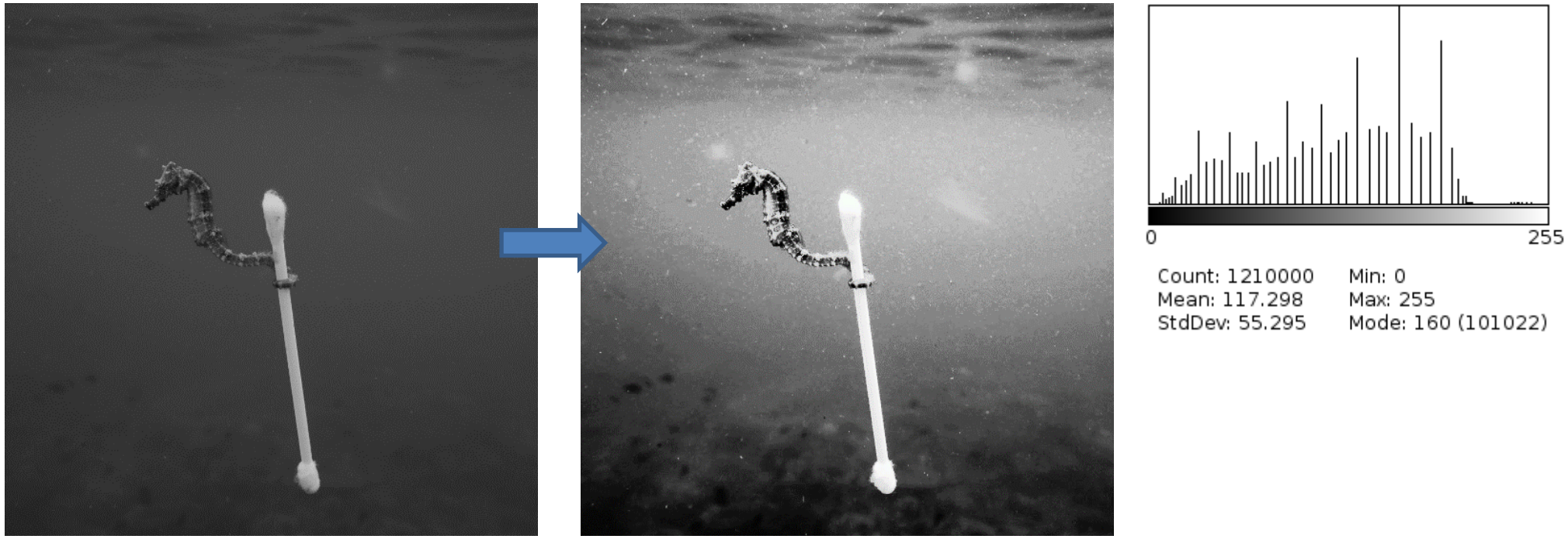
Values	CDF*	CDF norm	8 bit
5	0.111	0	0
20	0.333	0.25	63
30	0.555	0.5	128
40	0.666	0.625	159
50	0.888	0.875	223
100	1	1	255

0	63	159
63	128	128
223	255	223

= the normalization of the cumulative histogram of the image

*PMF = Probability mass function
*CDF = Cumulative distribution function = cumulative histogram

Histogram equalization: attention!



Watch out with Histogram equalization!

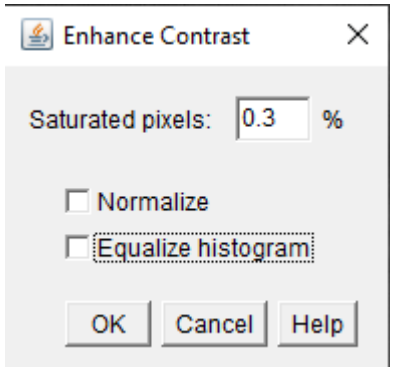
- Unrealistic artefacts in 8 bit (grayscale images)
- Image gradients in images with low depth (it will further reduce dynamic range)
- Undesired effects when histogram is not continuous

Histogram equalization: attention!

EXCERCISE 5

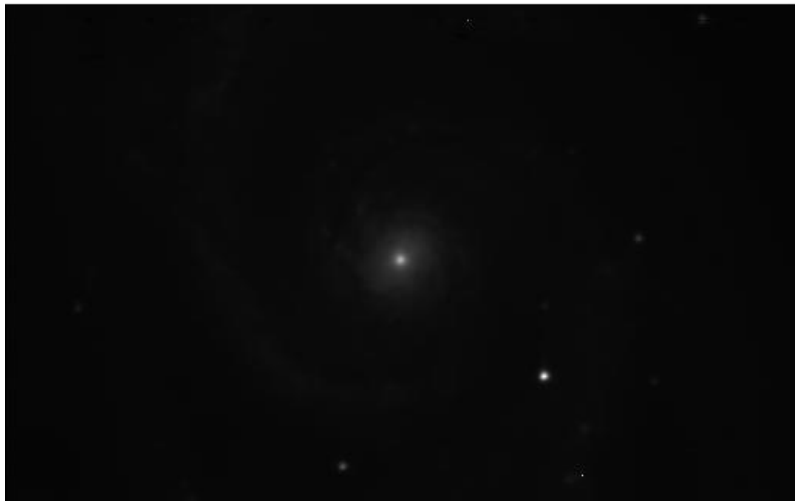
Open Example 5B.tif and try the histogram normalization and histogram equalization

Process > Enhance contrast…
Amount of Saturated pixels: default = 0.3% (but play with it)
(usually, you want this very low)

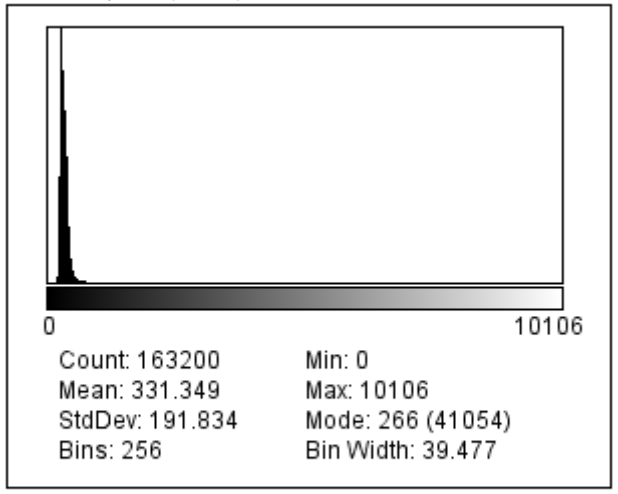


Histogram equalization: attention!

Example (Galaxy M51)

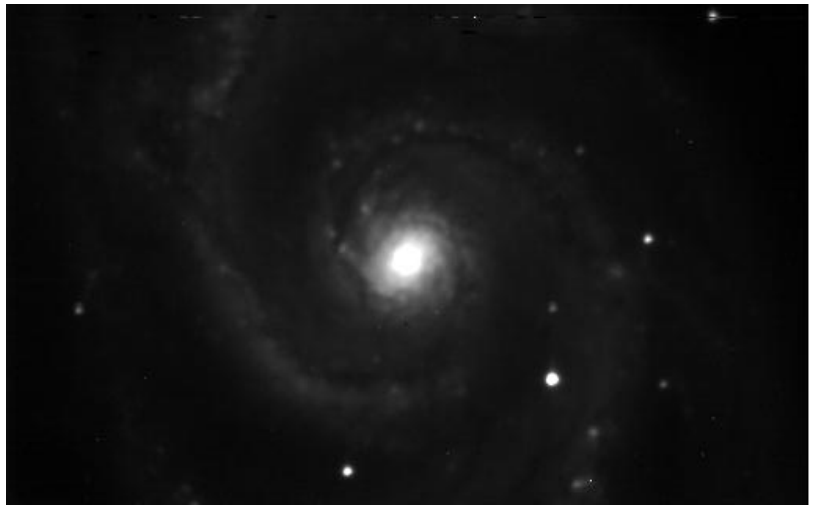


300x240 pixels; RGB; 281K

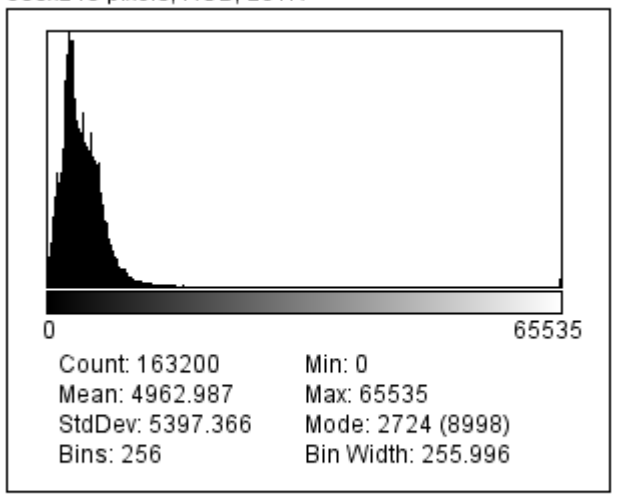


List Copy Log Live value=4342.422 count=0

Histogram normalization

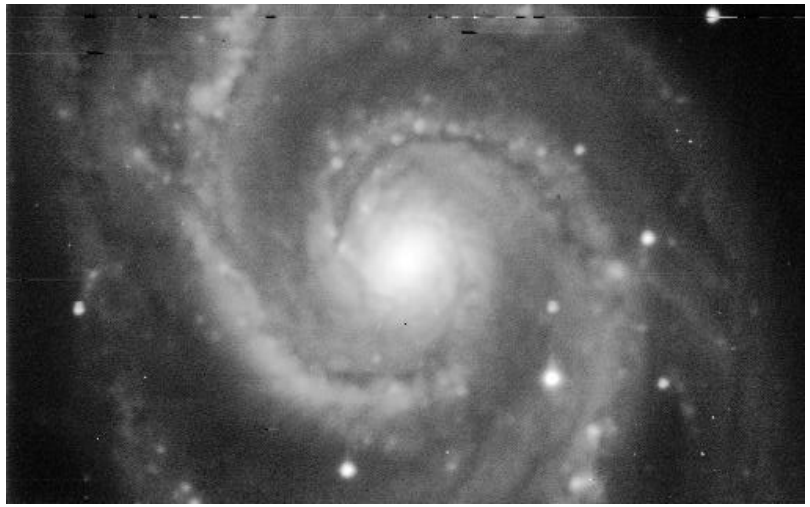


300x240 pixels; RGB; 281K

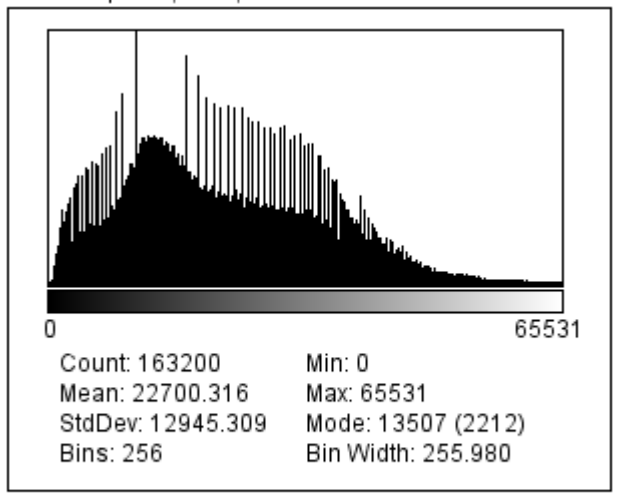


List Copy Log Live

Histogram equalization



300x240 pixels; RGB; 281K



List Copy Log Live value=6655.492 count=1147

Part IV: Overlays and preparation for publication

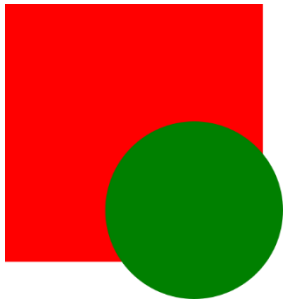
- Overlays
- Annotations and scale bars
- Preparing figures for publications
- Inkscape
- FigureJ

Overlays

Overlays are **vector graphics**: non-active, non-destructive selections displayed ‘over’ the rastered graphics data.

Overlay selections are

- Mathematically-defined **paths** (=not rastered), not affected by scaling, i.e., do not become pixelated.
- Overlays are **saved in the header** (e.g. of tif images), and do not need to be saved externally.
- Examples: Scalebars, annotations, …

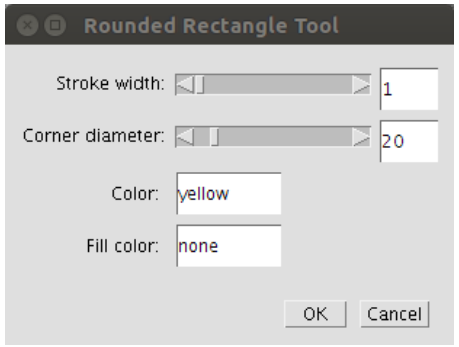


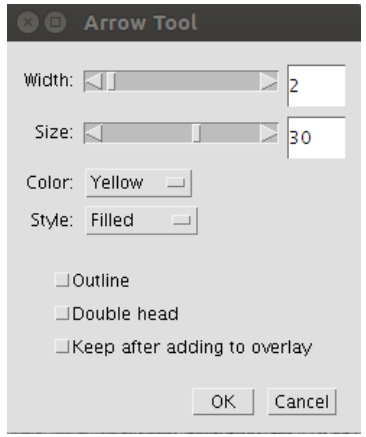
Selections

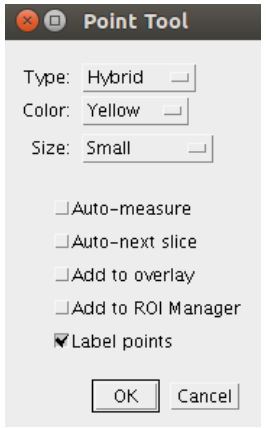
EXCERCISE 6

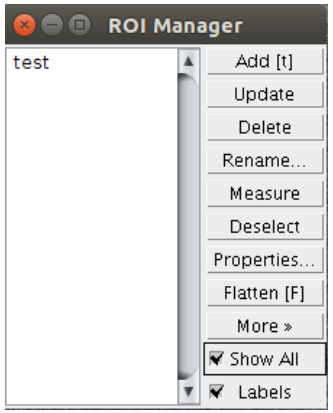
Open Example 4 – Manual scale

- Select the line selection and draw lines on the image. Check the status bar. Length? Unit of length?
- Try out the other selection options
- Right-click the icons with a red triangle at the bottom right.
- Use Analyze > Tools > ROI manager to
  - Add selections, rename them and remove them
  - Save selections to a file and open them again







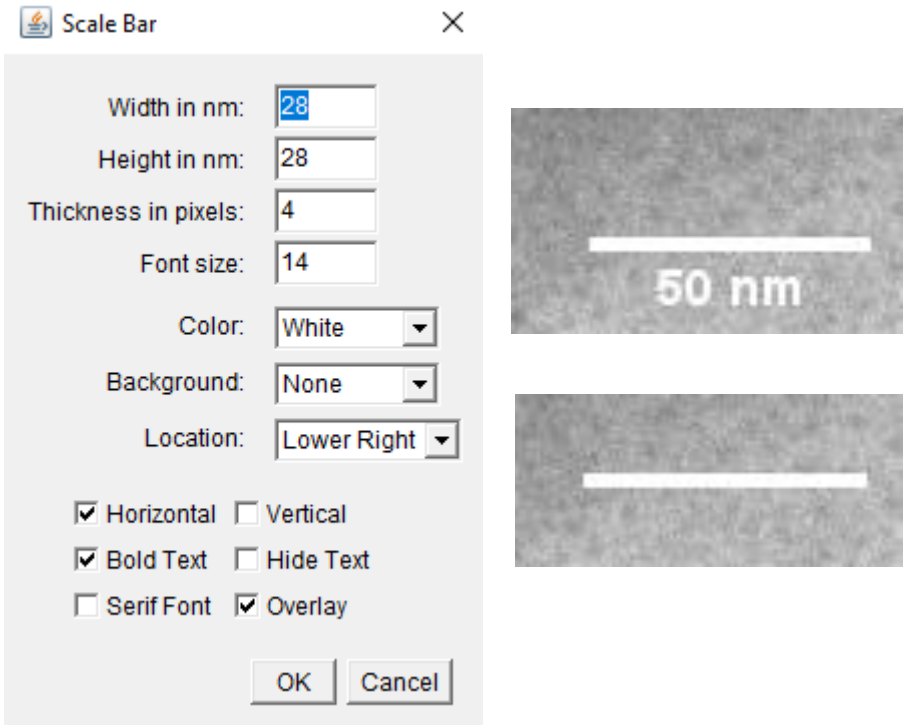


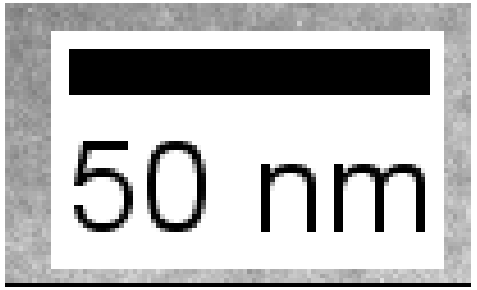
Scale bars

EXCERCISE 6
Add a scale bar to Example 1D (the .ser file)

- Open Example 1D (Plugins > Input-Output > TIA reader)
- Check the info in the header: CTRL + i (or Image > Show info …)
- Analyze > Tools > Scale bar…

Width: 8.3029 microns (2048)
Height: 8.3029 microns (2048)
Size: 8MB
Resolution: 246.6609 pixels per microns
Pixel size: 0.0041x0.0041 microns^2





Scale bars

EXCERCISE 6

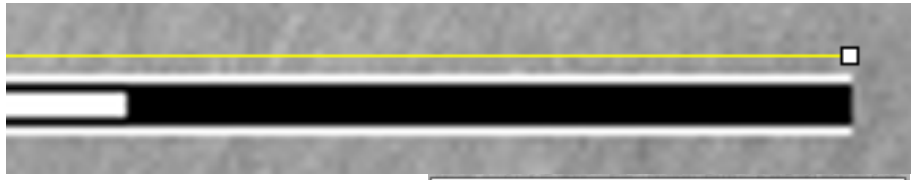
Try to repeat for Example 4 – Manual scale. Retrieve the scale and add a scale bar to a cropped version of Example 4

- Open Example 4
- Image > show info…

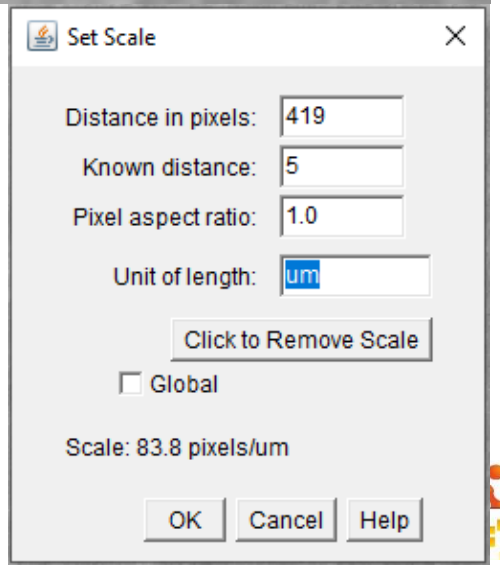
Scale bars

EXCERCISE 6
Try to repeat for Example 4 – Manual scale. Retrieve the scale and add a scale bar to a cropped version of Example 4

- Open Example 4
- Image > show info… The pixel size is NOT calibrated →
- But! There is a scale bar burnt into the rastered image.
- Draw a line exactly the length of the burnt-in scalebar



- Use Analyze > Set Scale
- The length of the line (in pixels) will be automatically filled in
- Enter the known distance (1) and the unit (um, there is no μ)
- (Global will set this scale to all open images)
- Click OK. Your image is now calibrated (Check the info!)



Now add the overlay scalebar

- Analyze > Tools > Scale bar…
- Note: the burnt-in scale cannot be removed, only cropped out

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Annotations

EXCERCISE 7

Open Example 4 – Manual scale and annotate the image

Arrows, lines, text

- Image > annotate > Arrow..

Accepting the annotation

- Press CRTL+B to make the annotation in overlay (recommended)
- Press CTRL+D to **d**raw the annotation in the image (burn in: convert vector to pixels)

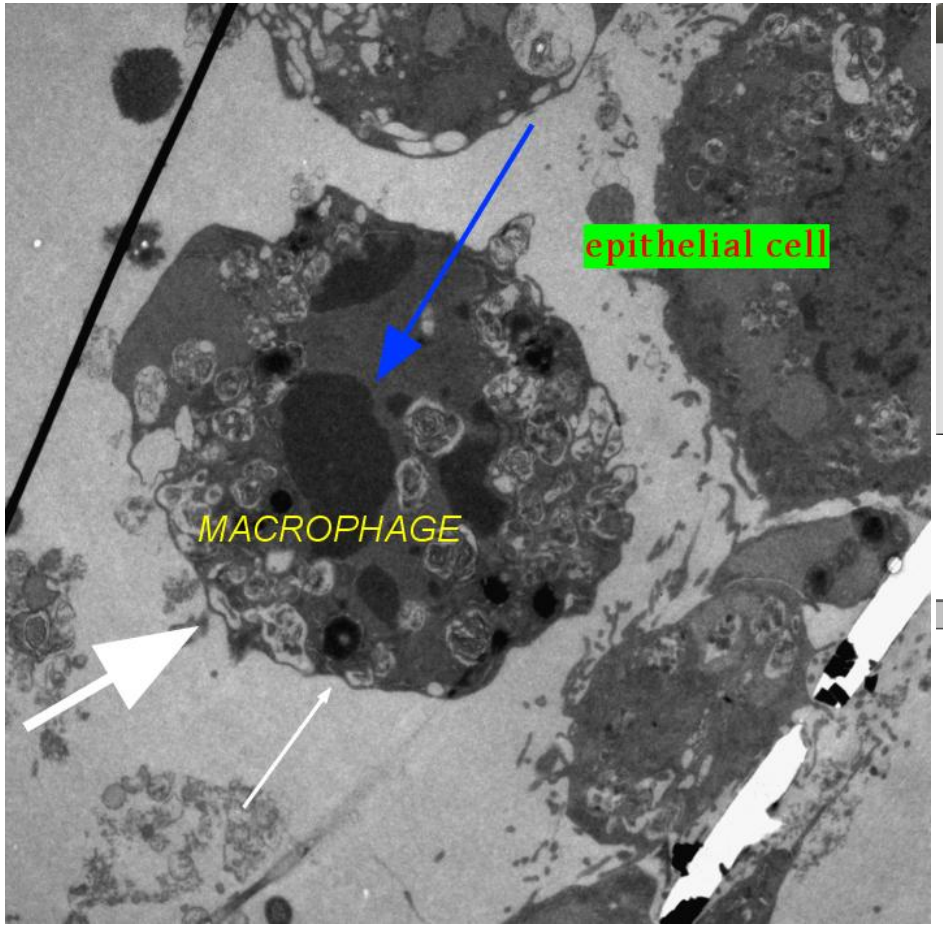
Note: ImageJ/FIJI is not great for annotations

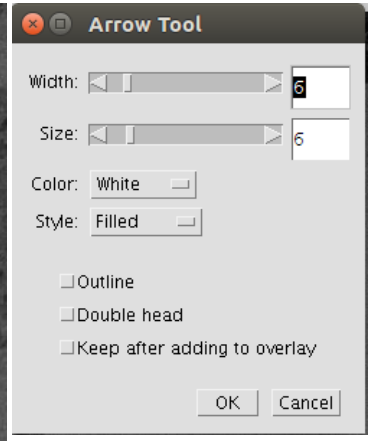
Illustrator, Affinity designer, Inkscape, CorelDraw, … are superior tools for vector graphics design.

Annotations

EXCERCISE 7

Open Example 4- Manual scale and annotate the image





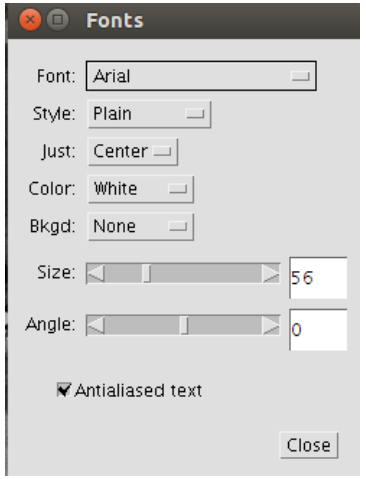


Image > Overlay > List elements

Type	X	Y	Width	Height	Color	Fill	LWidth
Straight Line	396	1070	139	189	white	none	6
Straight Line	58	977	273	164	white	none	16
Straight Line	602	204	245	400	#0036ff	none	8
Text	320	795	422	71	yellow	none	0
Text	923	360	378	68	red	green	0

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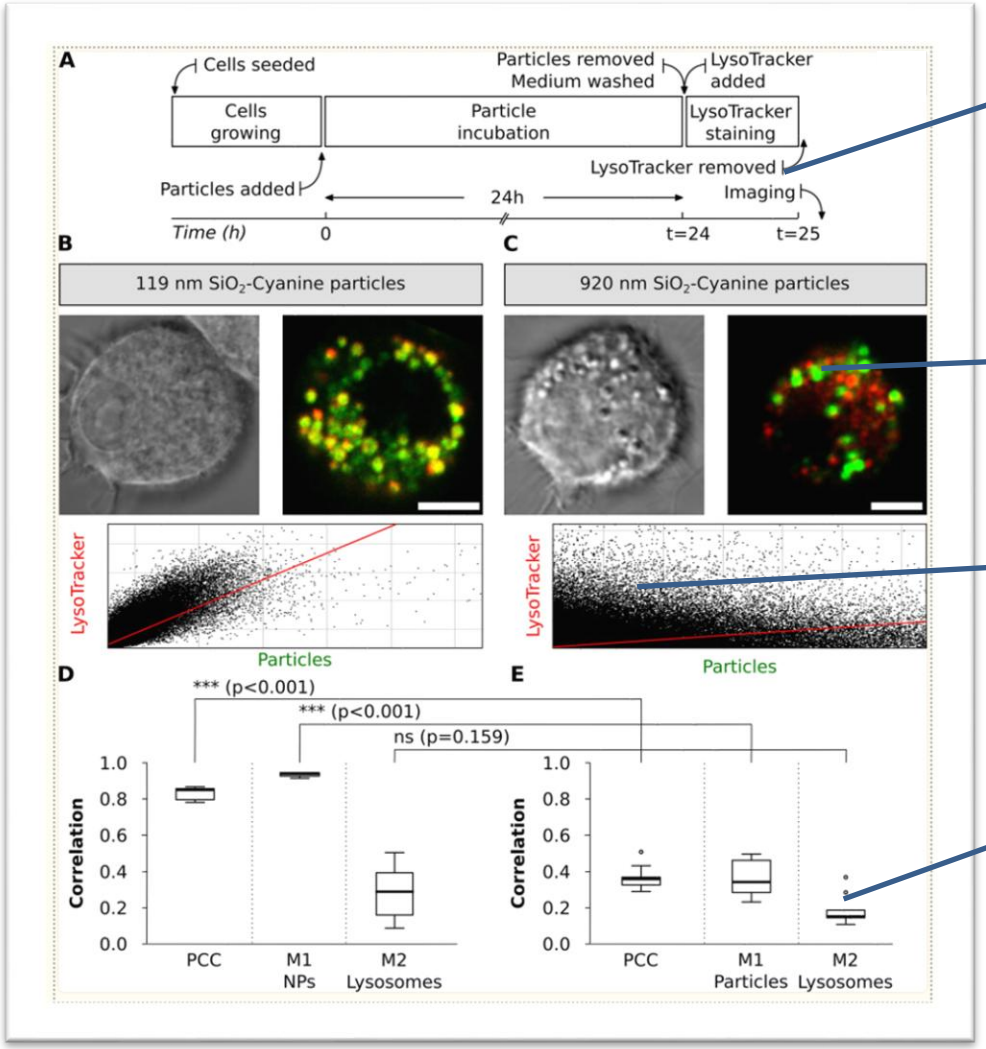
Preparing for publication - semantics

Some semantics:



Line art

Image

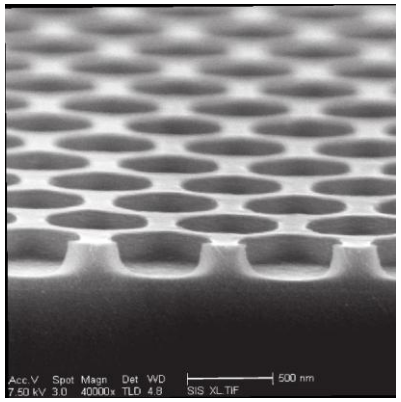
Graph or plot

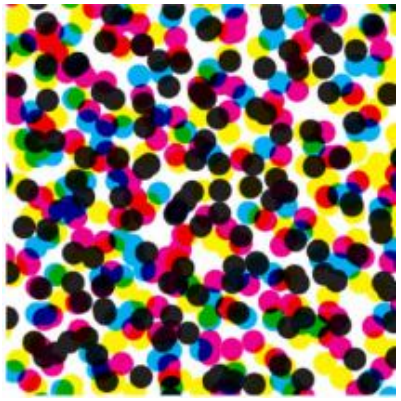
Line art

Figure

Preparing for publication - DPI

Images: from camera to printer: PPI and DPI





Dots per inch (DPI)
is a measure of spatial dot density in printing, in particular the number of individual dots that can be placed in a line within the span of 1 inch (2.54 cm).

Points per inch (PPI)
is the same but with concerning electronic displays. Not relevant here.

Rule of thumb: what is the resolution I need to publish my data?
Images: at least 300 DPI, but 600 DPI has been requested
Line art: higher: 1200 DPI, but I have seen 2400 DPI requests
Graphs: 600 DPI and up

Low resolution

High resolution

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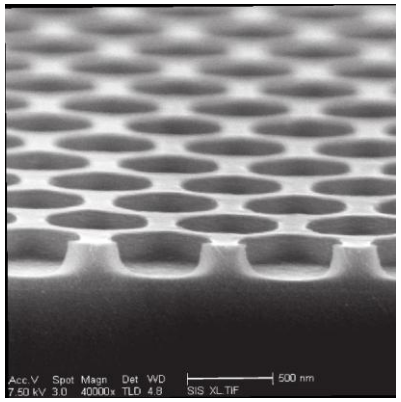
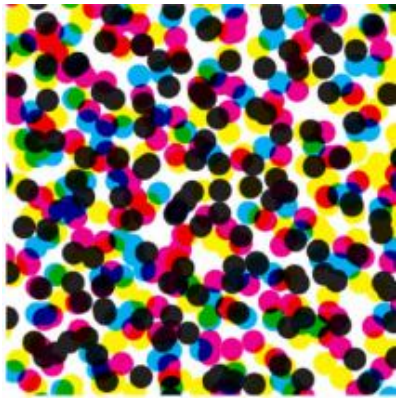
Preparing for publication - Example

Images: from camera to printer: PPI and DPI





Info:
1 inch = 2.54 cm
Expected Image width in print: 10 cm
Expected printed resolution: 300 DPI

Question: how many pixels wide does my digital picture have to be?

Calculation:

Width (printer) = $\frac{10\ cm}{2.54\ \frac{cm}{inch}}$ = 3.937 inch

at 300 dots per inch = 3.9737 inch x 300 $\frac{dots}{inch}$ = 1181 dots

Result:
The image must be 1181 pixels wide to get a 10 cm wide figure at 300 DPI

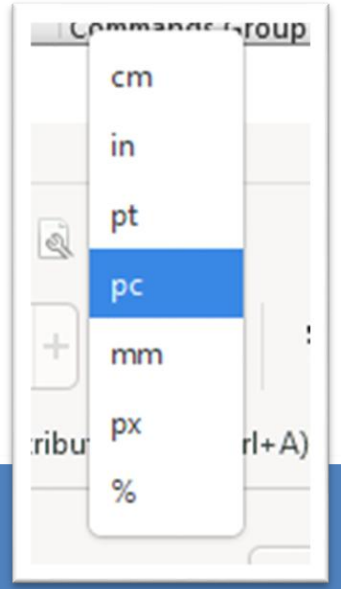
Dots per inch (DPI)
is a measure of spatial dot density in printing, in particular the number of individual dots that can be placed in a line within the span of 1 inch (2.54 cm).

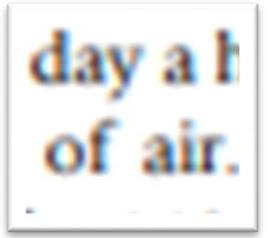
Resizing and cropping
What happens with my image during resizing? Am I allowed to crop an image?
==> See lecture 2 Advanced Image processing

Preparing for publication - pica

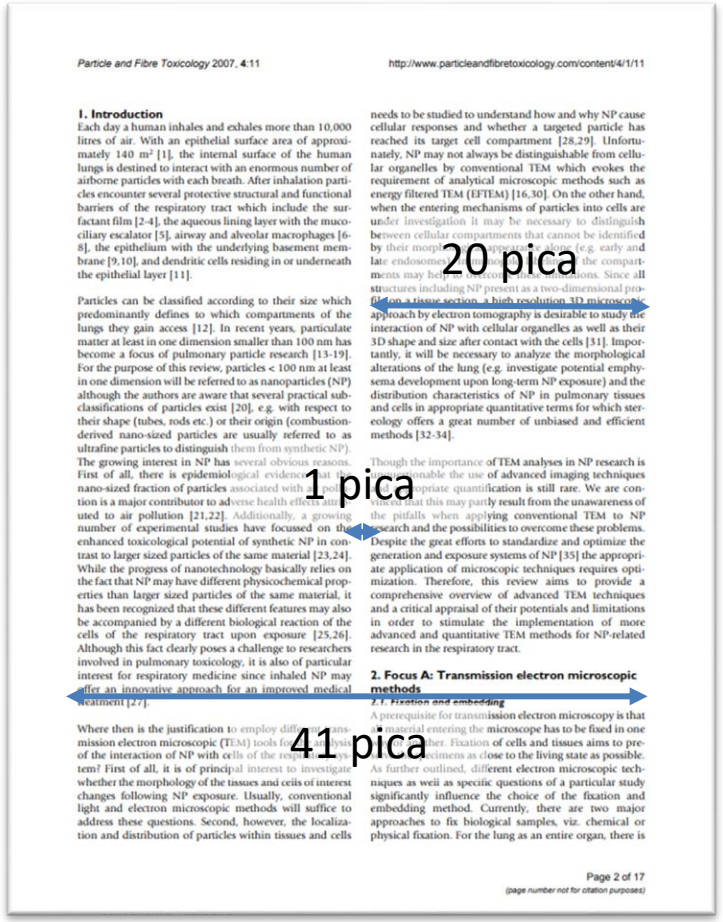
Publishers and printers work often with PICA (pc) and POINTS (pt).

• 12 points (pt)	1 pica (pc)
• 1 pc	1/6 of an inch
• 1 pc	4.23333 mm
• 1 pt	0.35277 mm
• Text height	1 pica (12 points)
• Text width (page)	41 pica
• Column width (2 / page)	20 pica
• Central space	1 pica





1 pica (12 points)



20 pica

1 pica

41 pica

Info

Expected Image width in print: 20 pc

Expected printed resolution: 300 DPI

Question: how many pixels wide does my digital picture have to be?

Calculation:

20 pc = 20/6 inch = 3.333 inch (= 8.333 cm)

Width (printer) = 3.333 inch

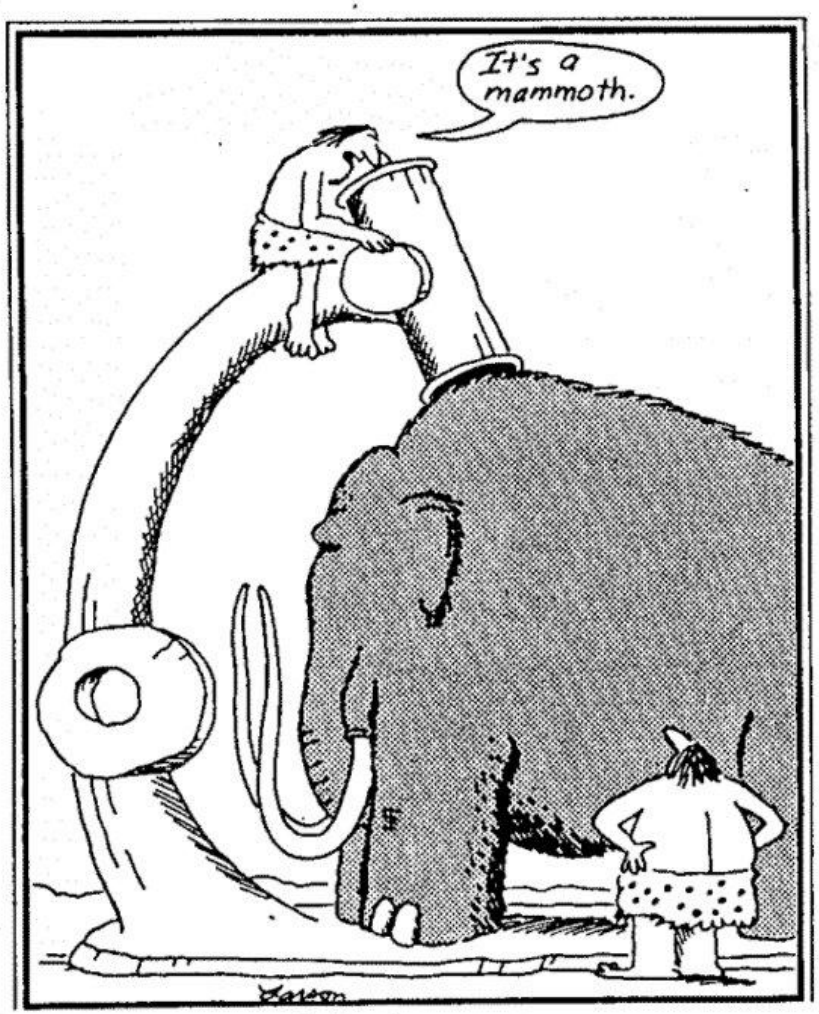
at 300 dots per inch = 3.333 inch x 300 $\frac{dots}{inch}$ = 1000 dots

Result:

The image must be 1000 pixels wide to get a columnwide figure at 300 DPI

Finished!

✓ Congratulations,
You finished Part I, Basics!



Early microscope