Light microscopy in Cellular Biology

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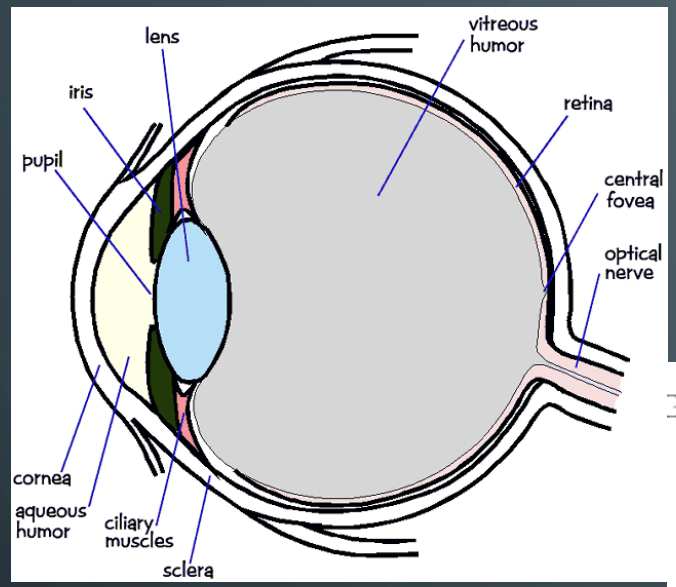
WHAT IS COMPUTER VISION?

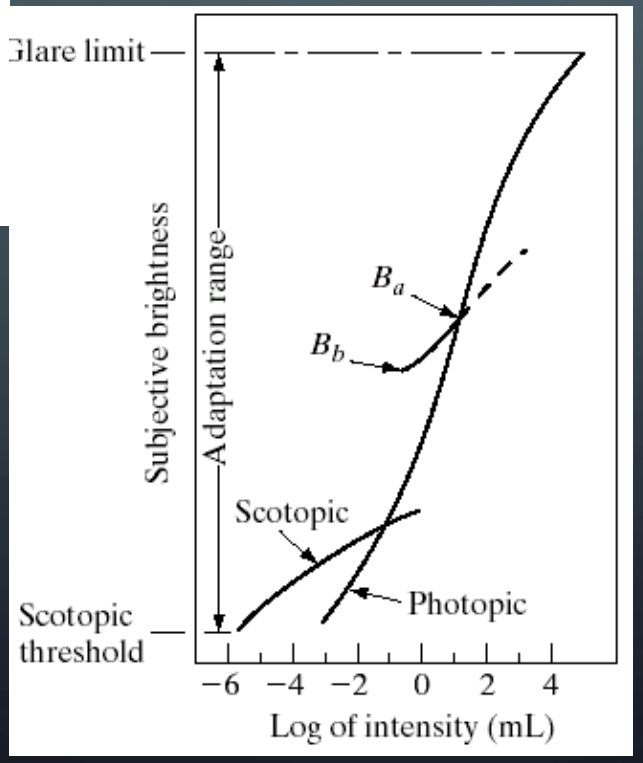
- Inverse Optics
- Intelligent interpretation of Imagery
- Building a Visual Cortex

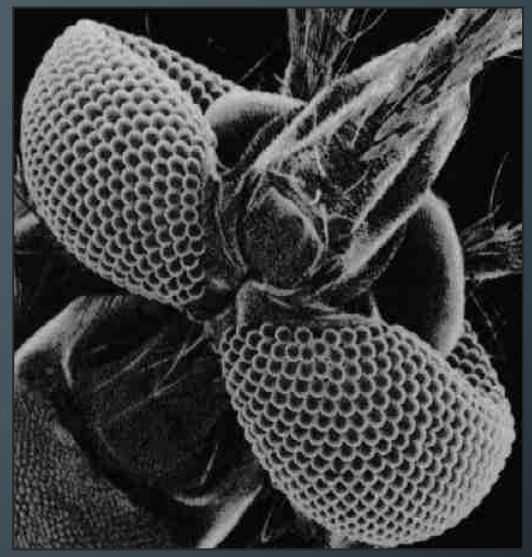
- No matter what your definition is…
  - Vision is hard.
  - But is fun...

BIOLOGICAL CAMERAS



Human Eye

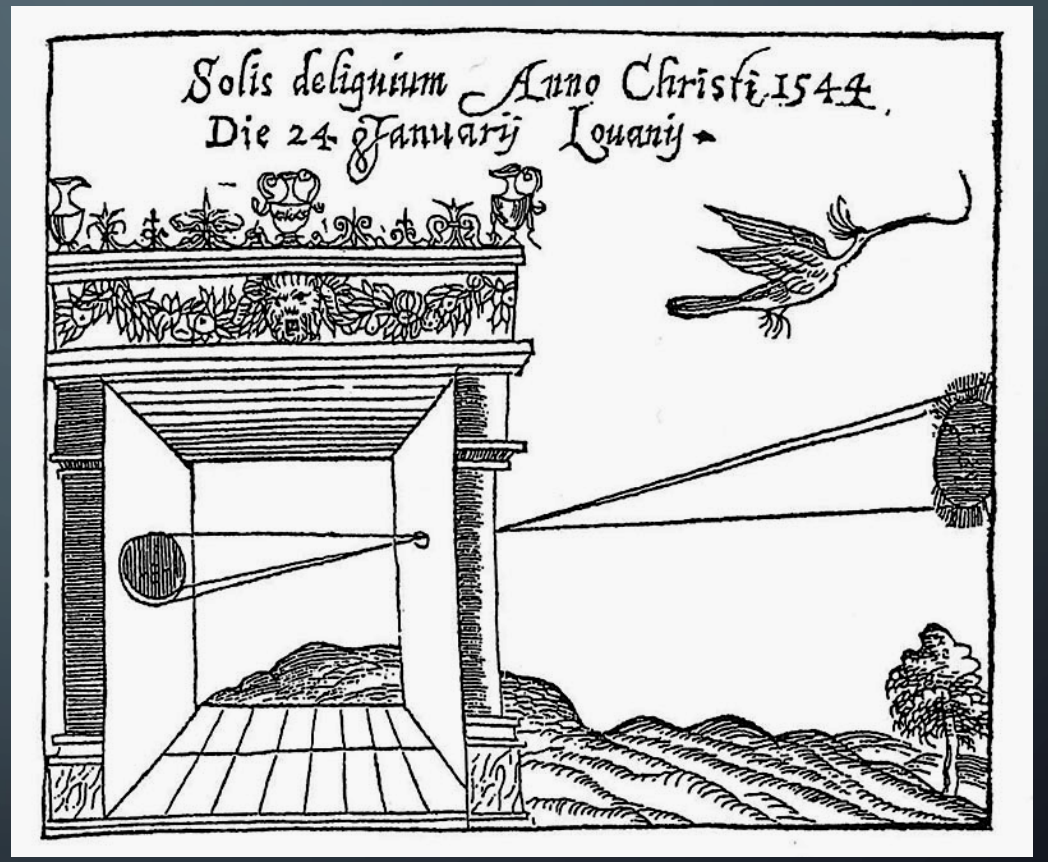




Mosquito Eye

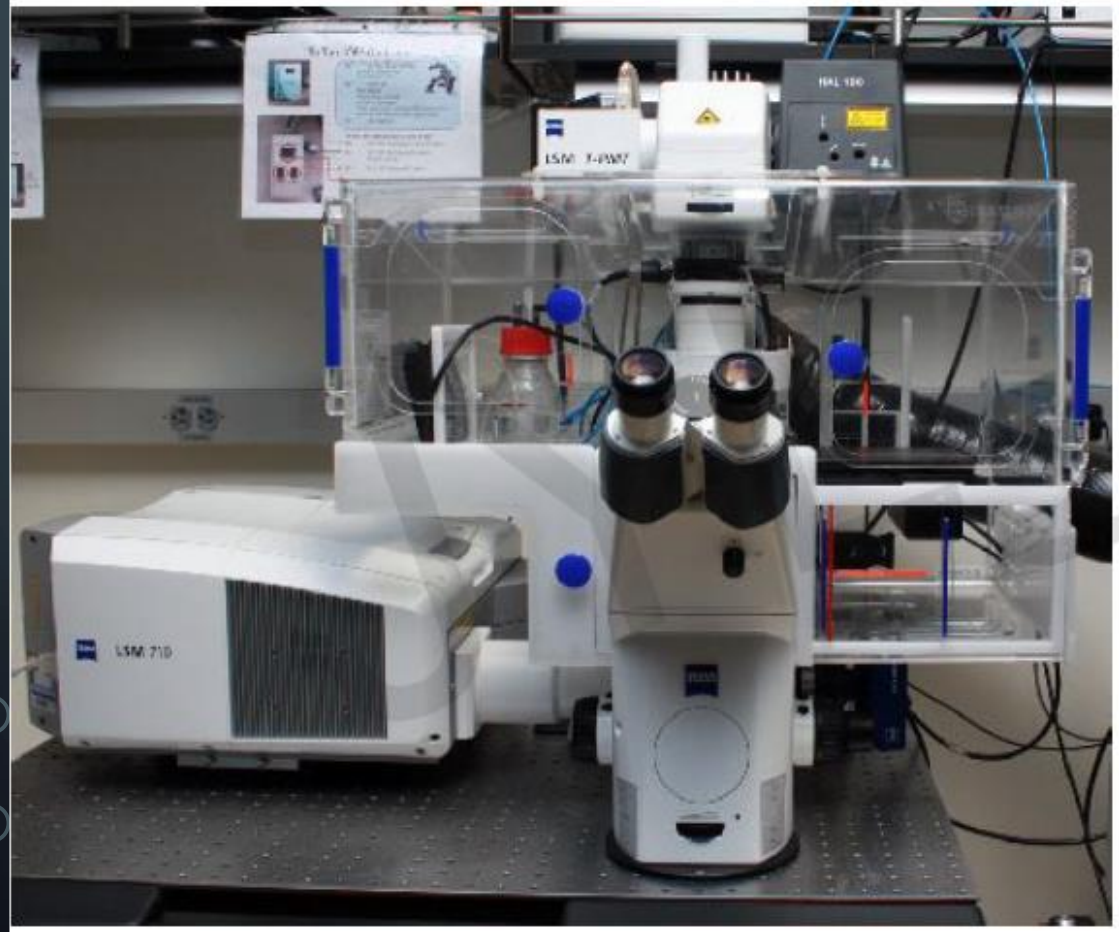
CAMERAS AND THEIR OPTICS

Today's Digital Cameras



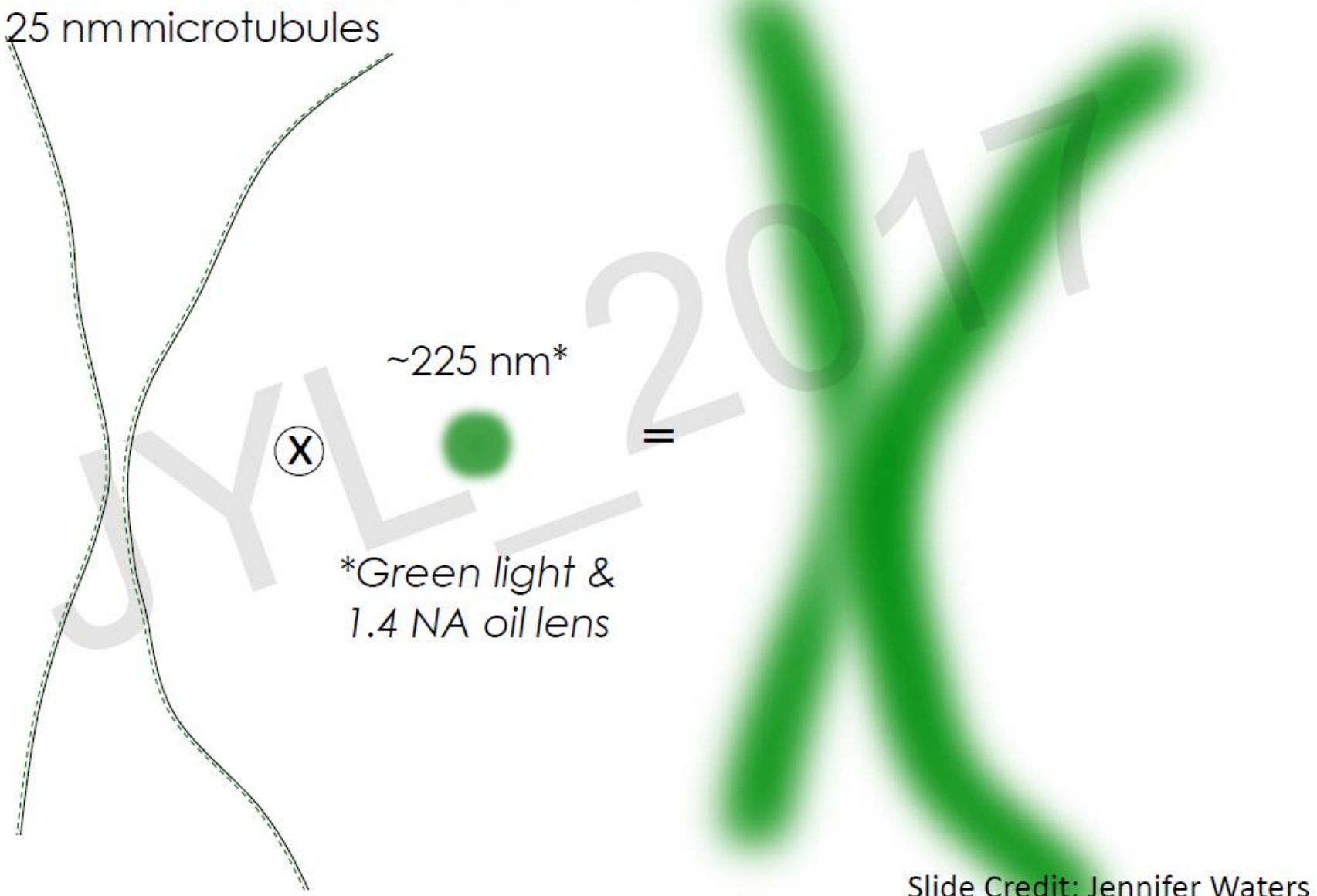
The *Camera Obscura*

Image Acquisition



- Resolution & Sampling
- Digital Image Formation
- Histograms/Saturation
- Calibrating Hardware
- Reproducibility

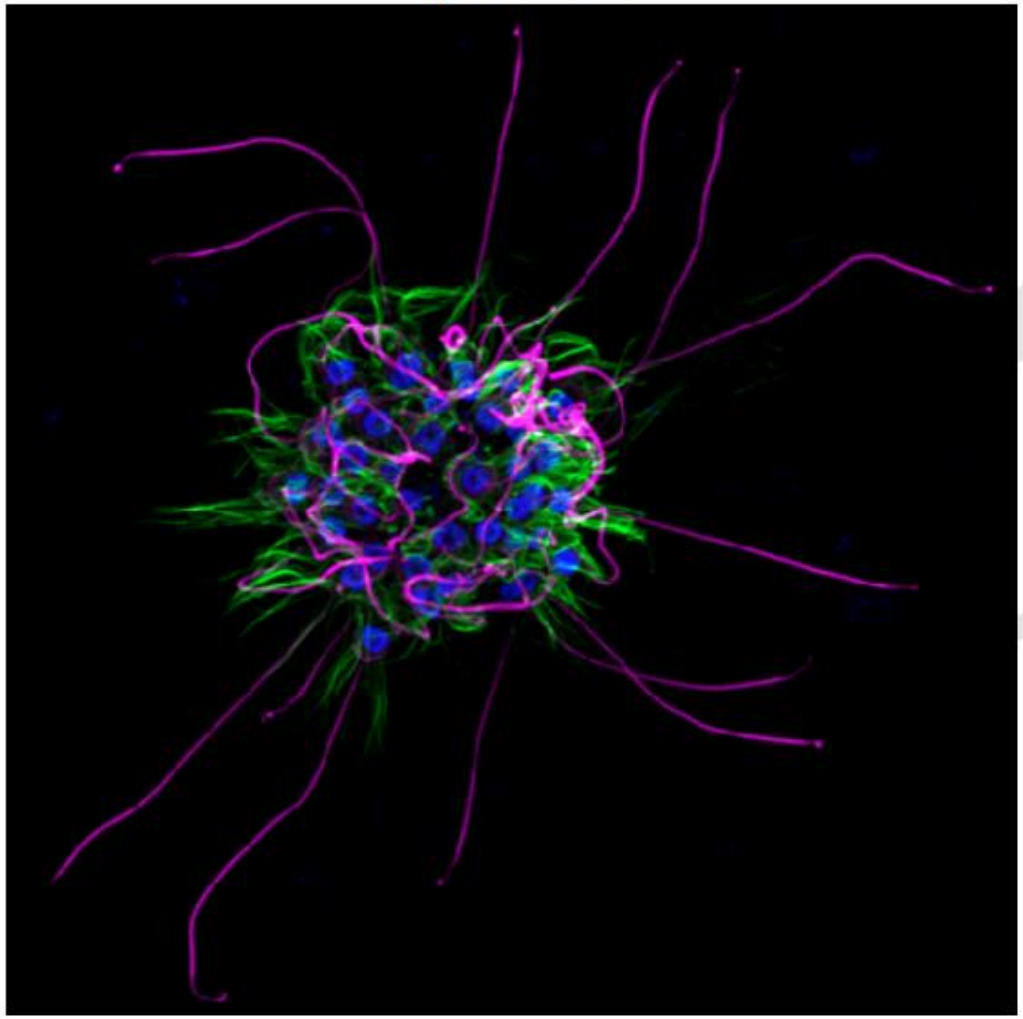
Images from light microscopes are convoluted



Slide Credit: Jennifer Waters

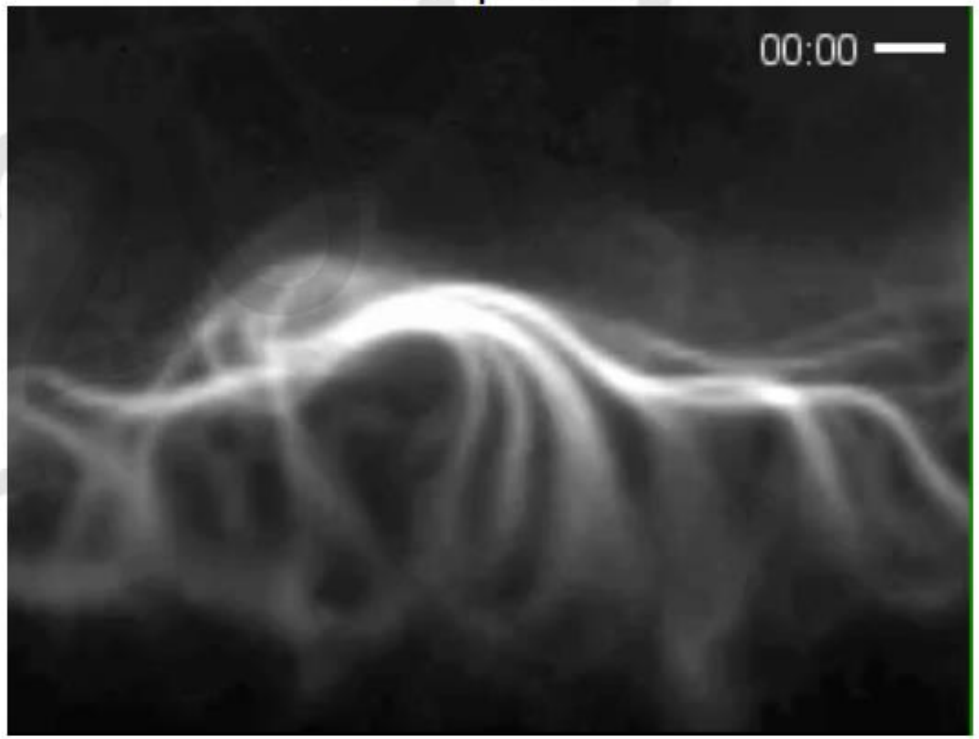
Resolution

Spatial



Kayley Hake, King Lab

Temporal



Sanchez et. al., *Science* 2011

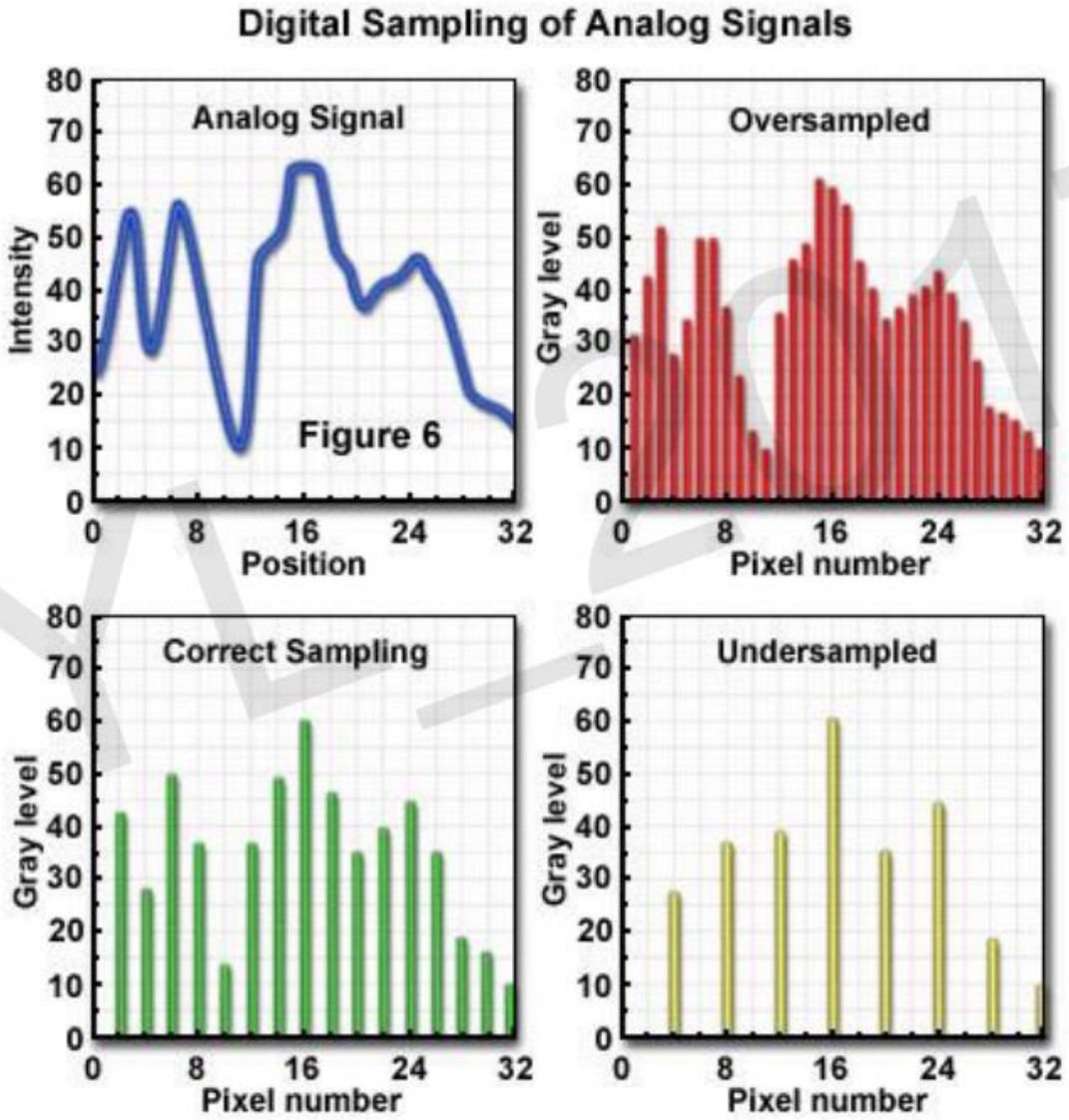
Shannon-Nyquist Sampling Criterion

a.k.a., how to avoid undersampling/aliasing

$$\Delta s \leq \Delta x,y,z,t \,/\, 2$$

Take your limit of resolution & divide it by 2
(Some people divide by 2.3 to be safe)

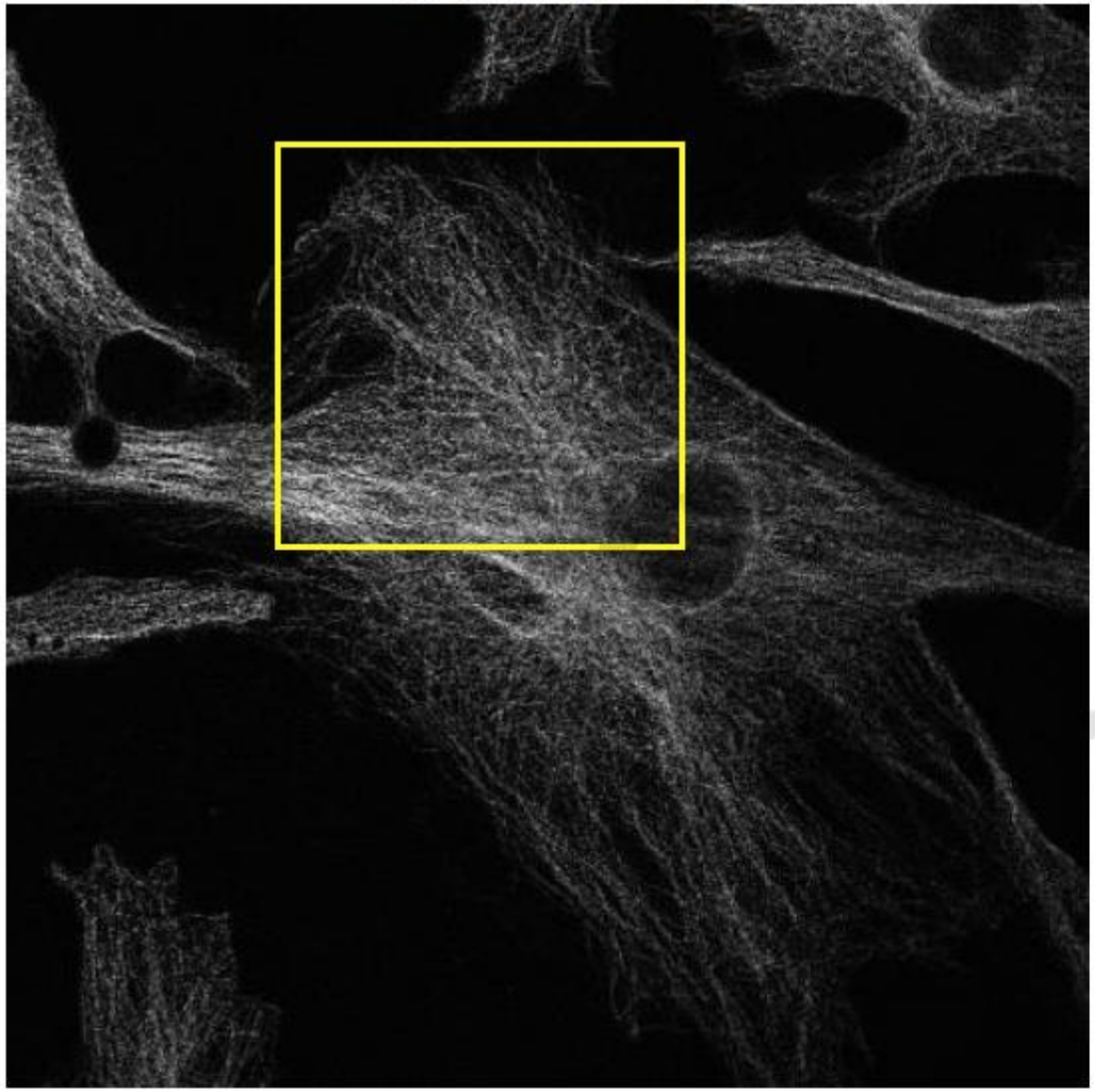
Proper Sampling is Essential in Microscopy



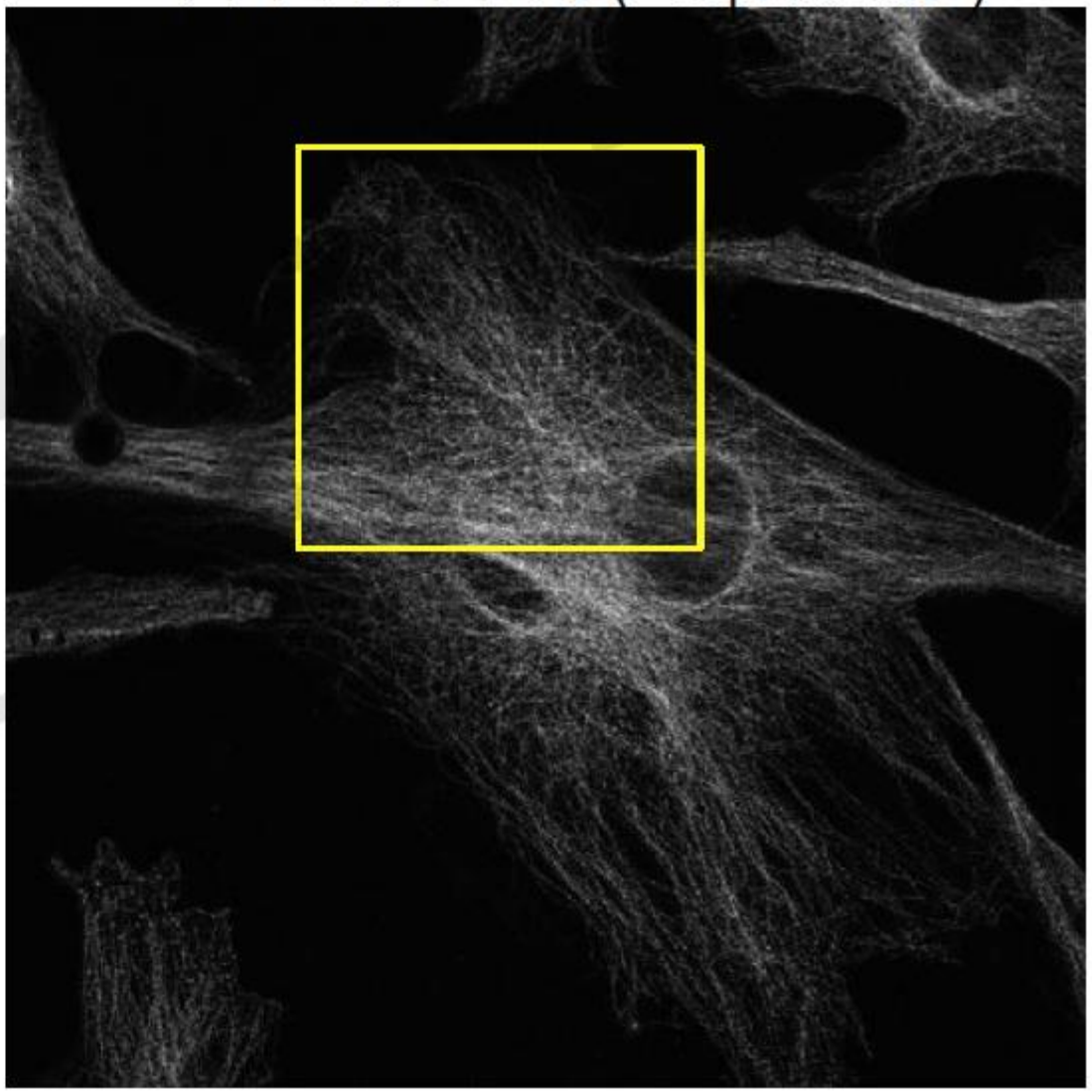
http://www.olympusconfocal.com/theory/resolutionintro.html

Nyquist Sampling - Lateral

512 x 512

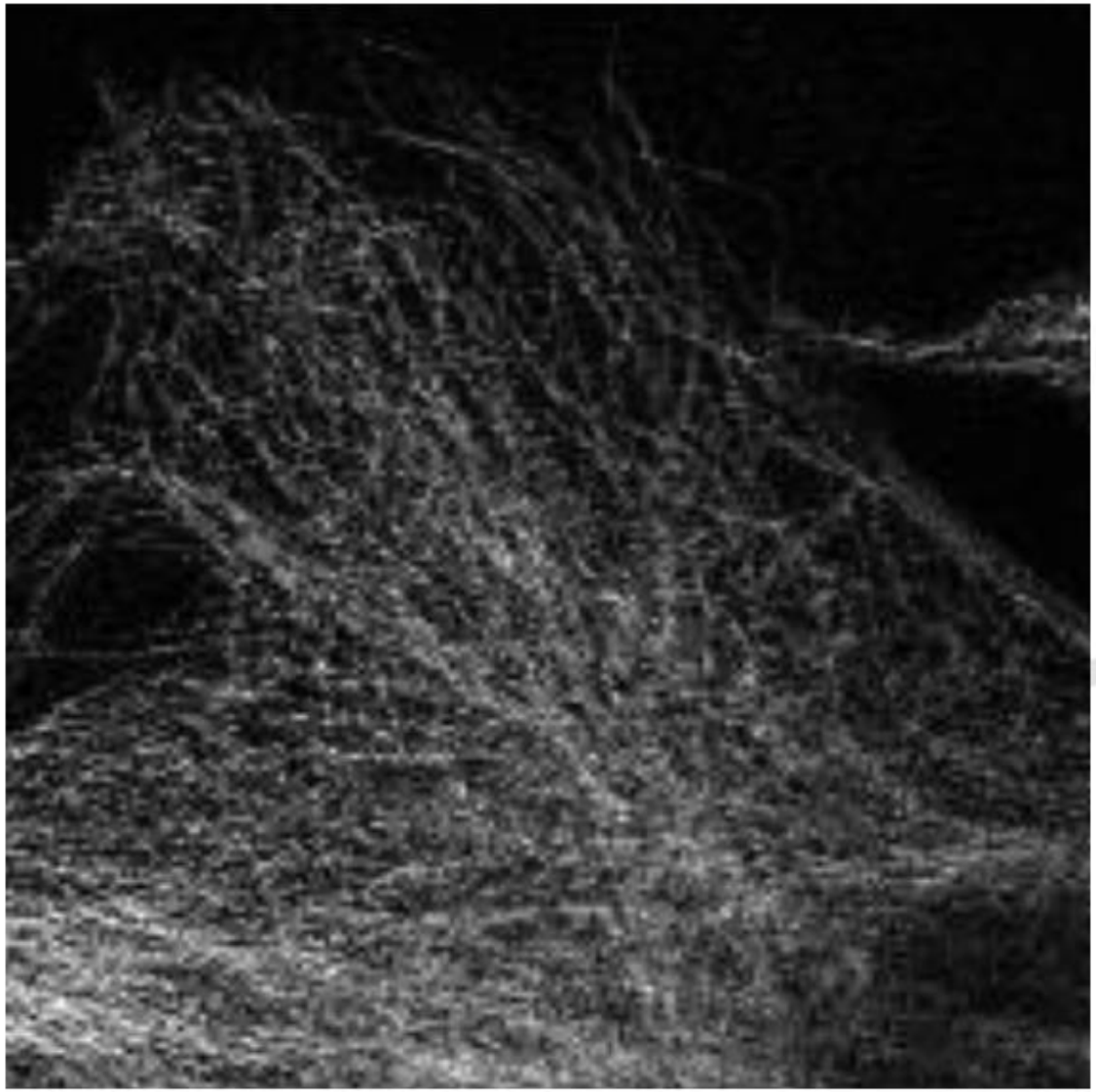


1444 x 1444 (“Optimal”)

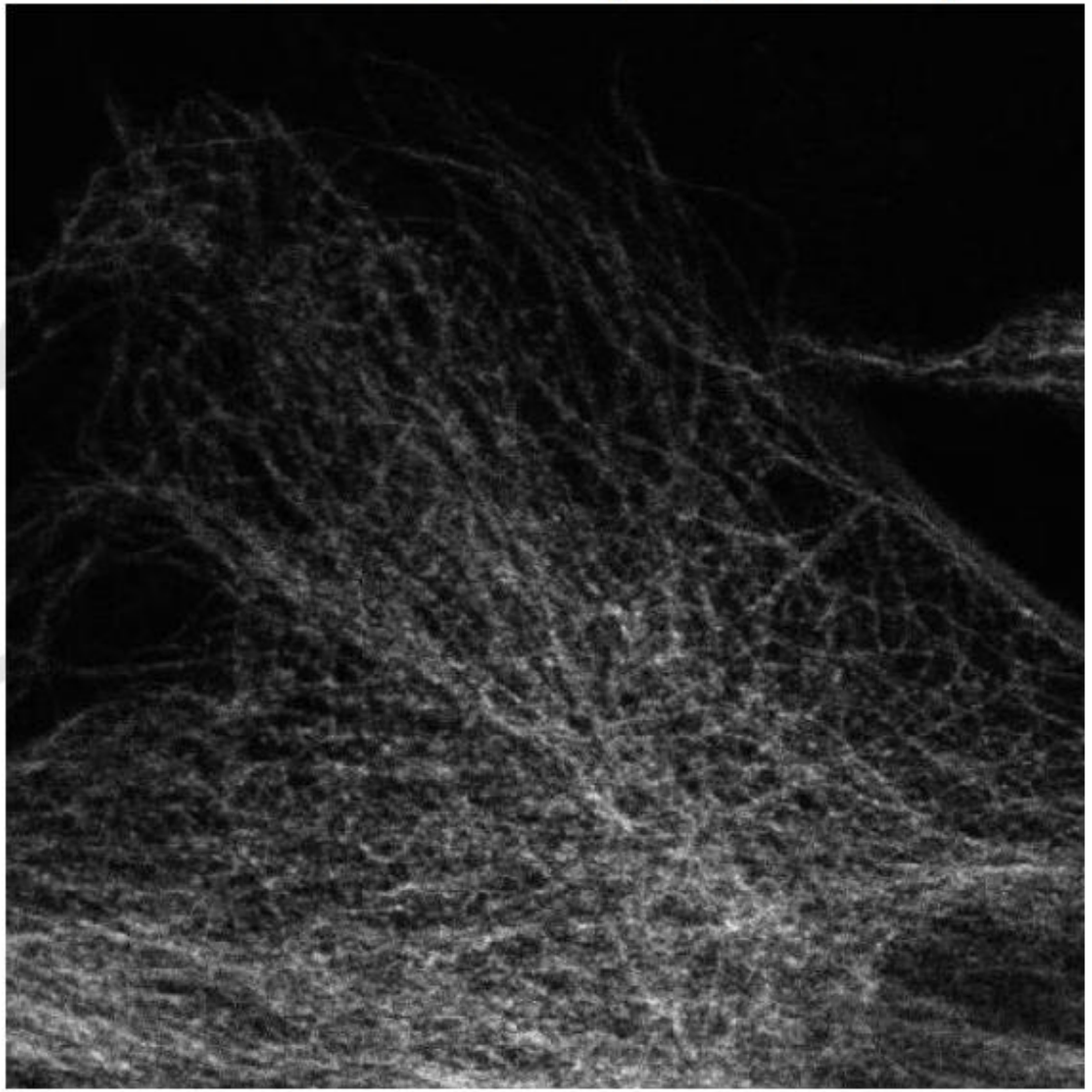


Nyquist Sampling - Lateral

512 x 512



1444 x 1444 (“Optimal”)



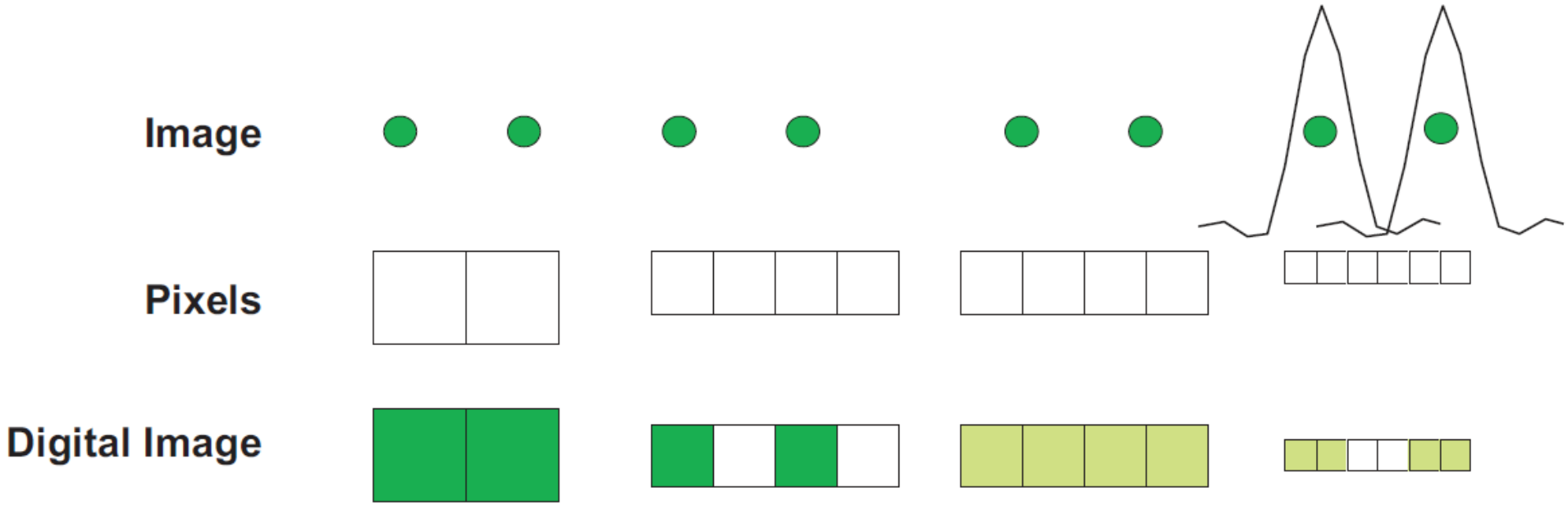
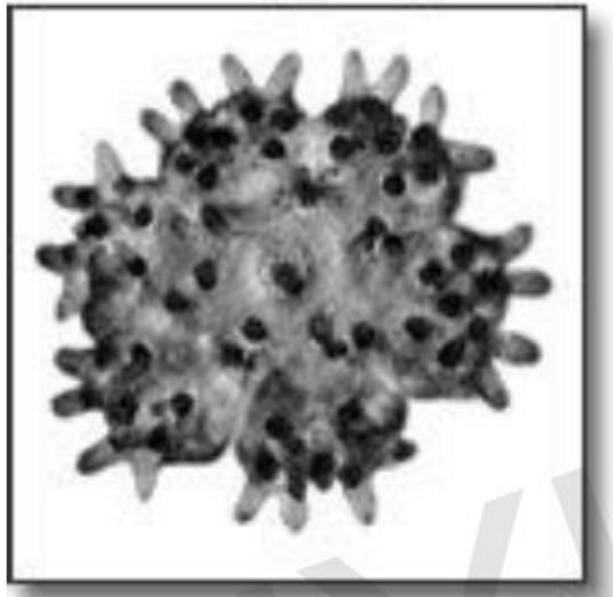


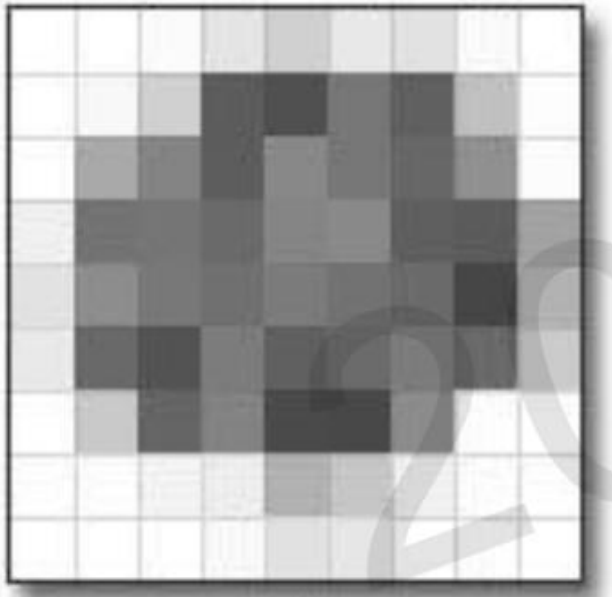
Figure 8: Resolution limitations of a CCD camera (Jennifer Waters, Nikon Imaging Center, Harvard Medical).

Digital Image Formation

Analog Image



Digital Sampling



Pixel Quantization

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

http://hamamatsu.magnet.fsu.edu/articles/digitalimagebasics.html

Bit depth = digital readout of intensity levels

8-bit = 2^8 = 256 levels of gray

12-bit = 2^{12} = 4096 levels of gray

16-bit = 2^{16} = 65,536 levels of gray

The human eye can only detect between 32-64 levels of gray!

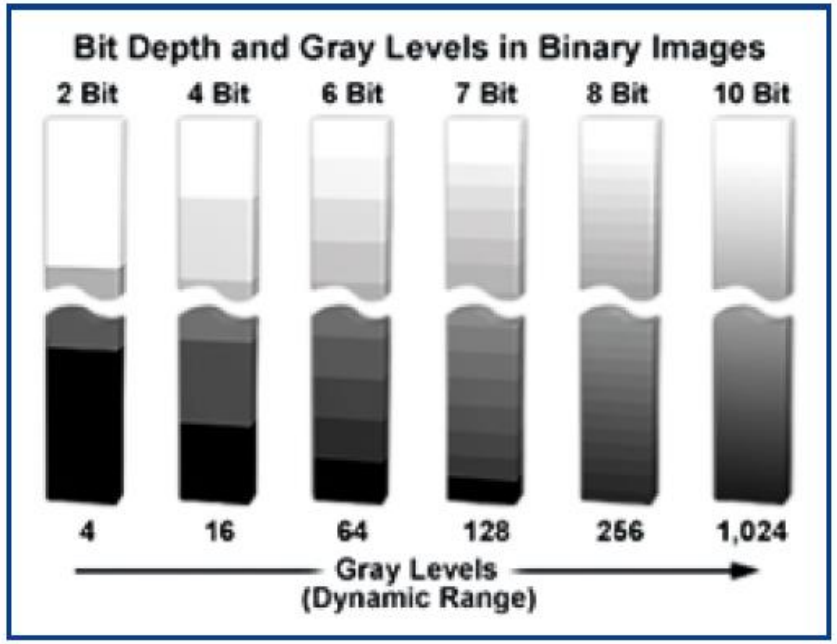


Fig. 9: Bit depth and grey levels in digital images.

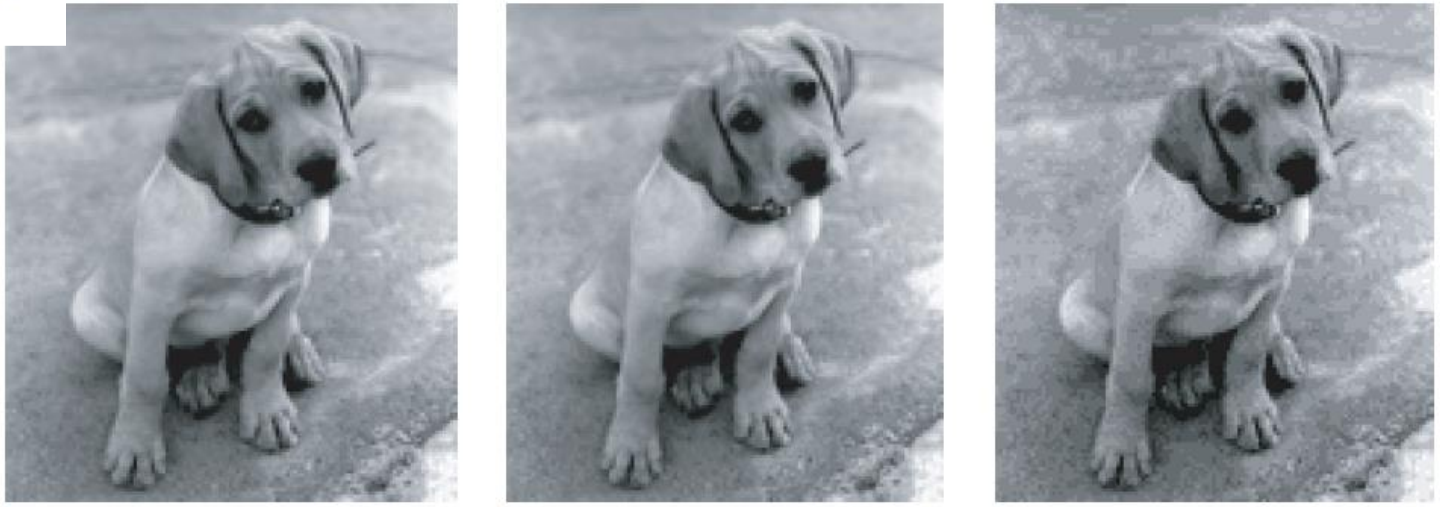
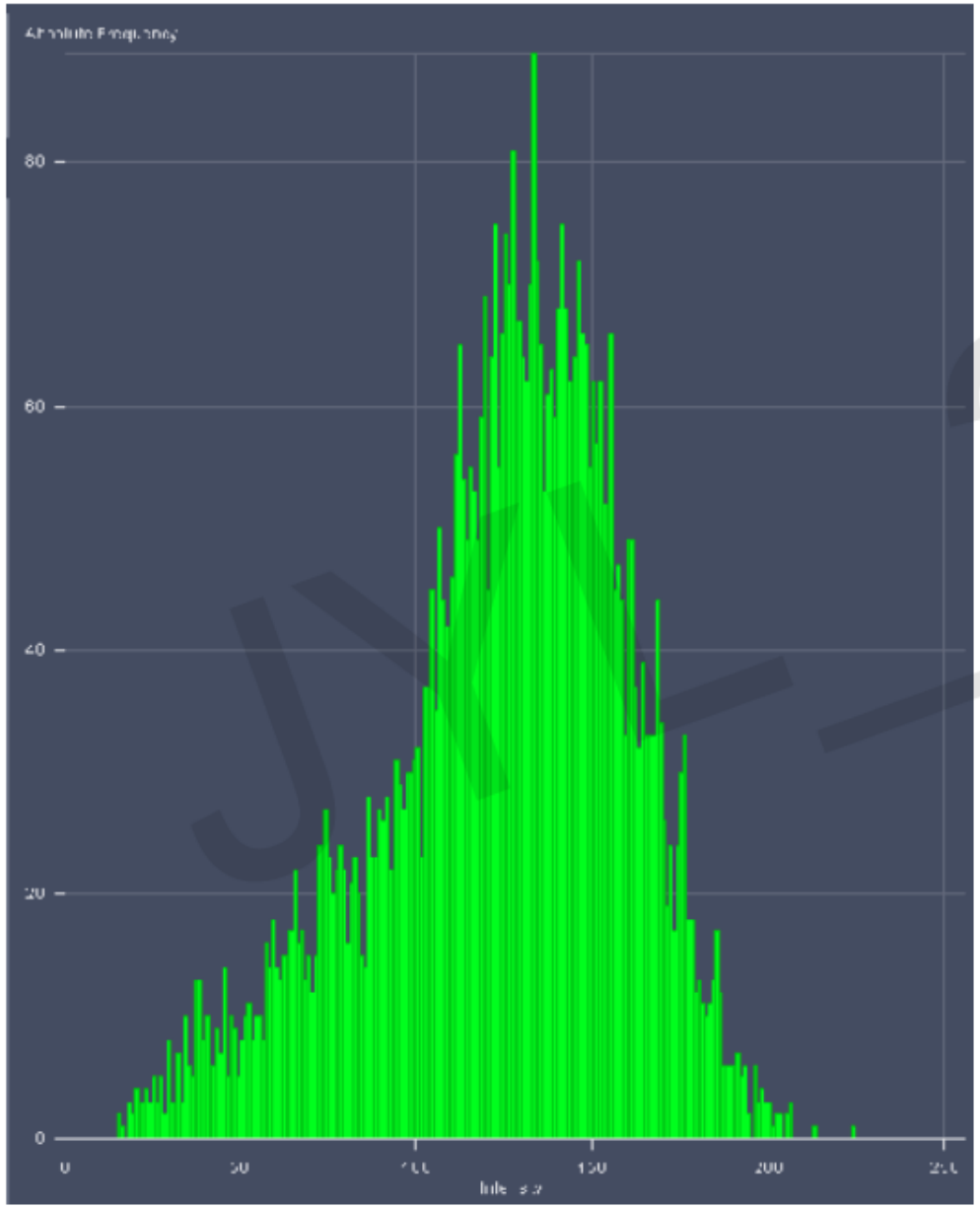


Figure 9: Same picture displayed with different gray levels (Butch Moomaw, Hamamatsu)

Histograms are a Microscopist's Best Friend

Best way to optimize settings for maximum contrast and dynamic range without saturation (i.e., information loss)

What are histograms?



- Graph of intensity vs. frequency
- Quick way to assess contrast, saturation

Histograms are a Microscopist's Best Friend

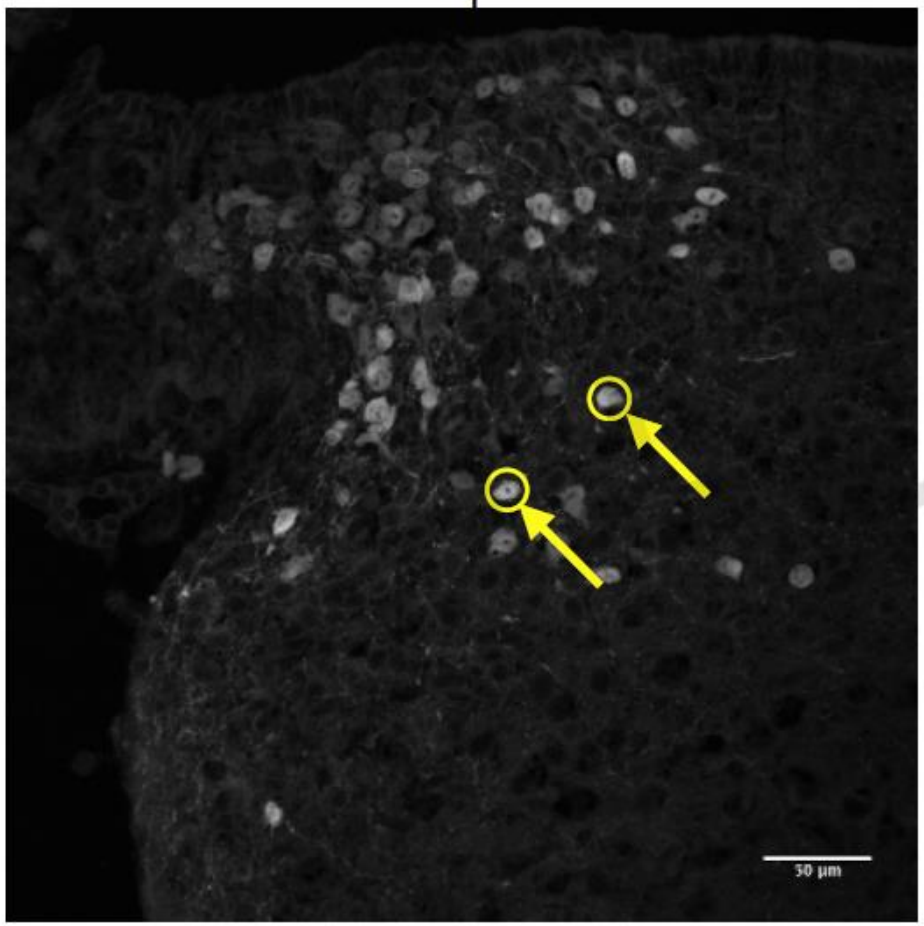
Larger dynamic range = higher signal-to-noise ratio

However, you want to avoid saturation because saturation = data loss

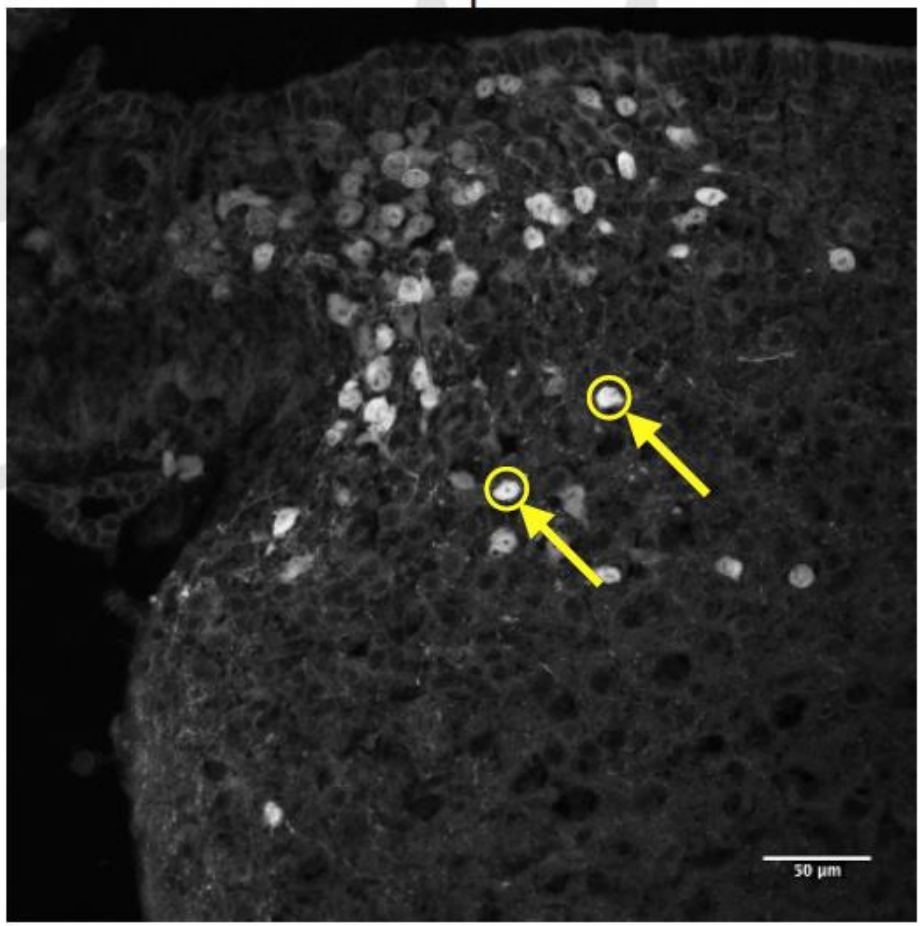
Avoiding Saturation

Mouse brain section, imaged on the LSM 880 (“Trinity”)
20X W/1.0 NA, 1 AU

Sample A

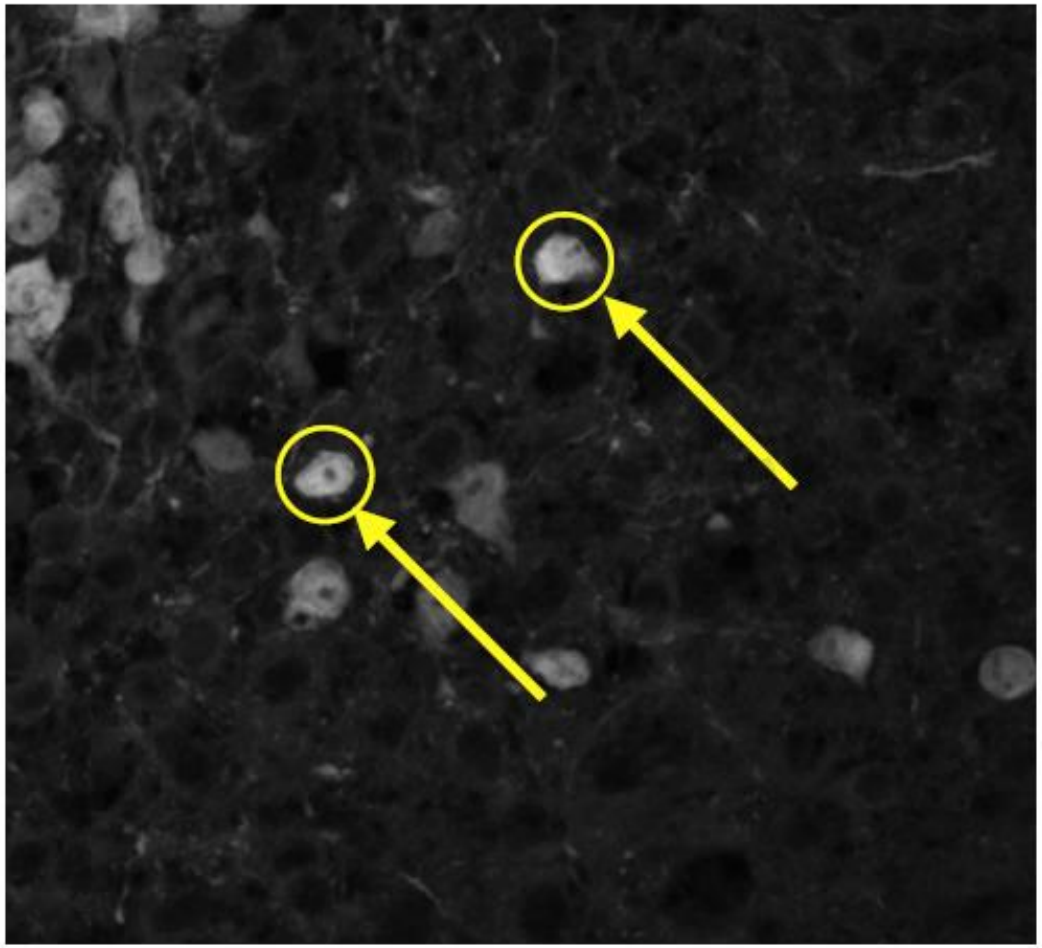


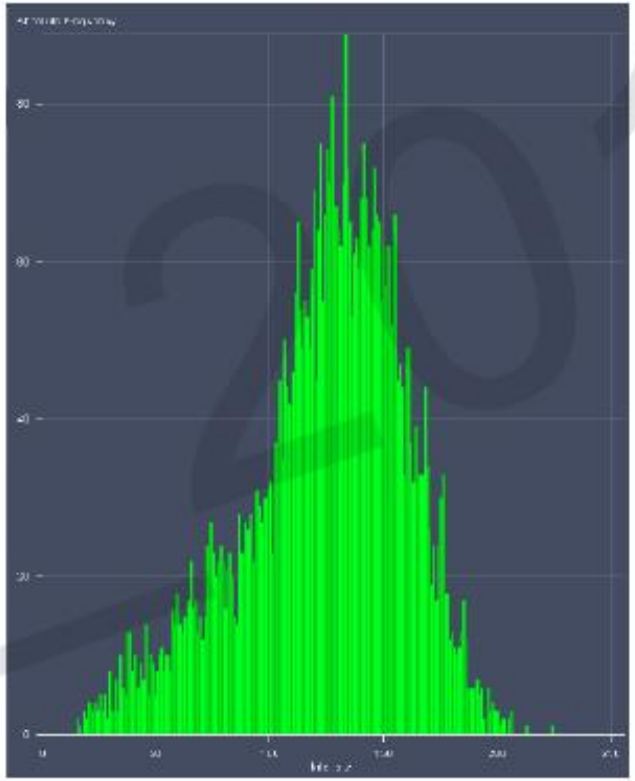
Sample B

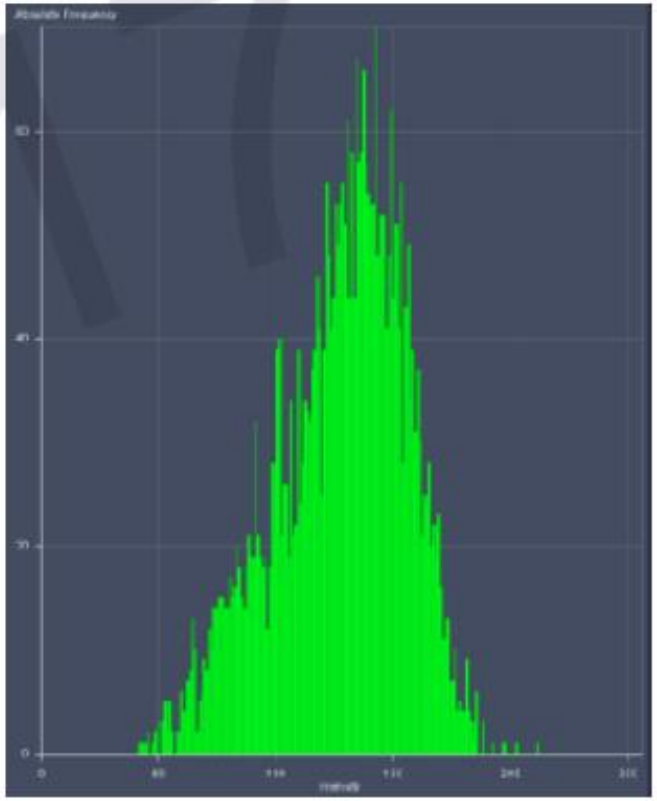


Sample courtesy of George Stratigopoulos, Columbia University

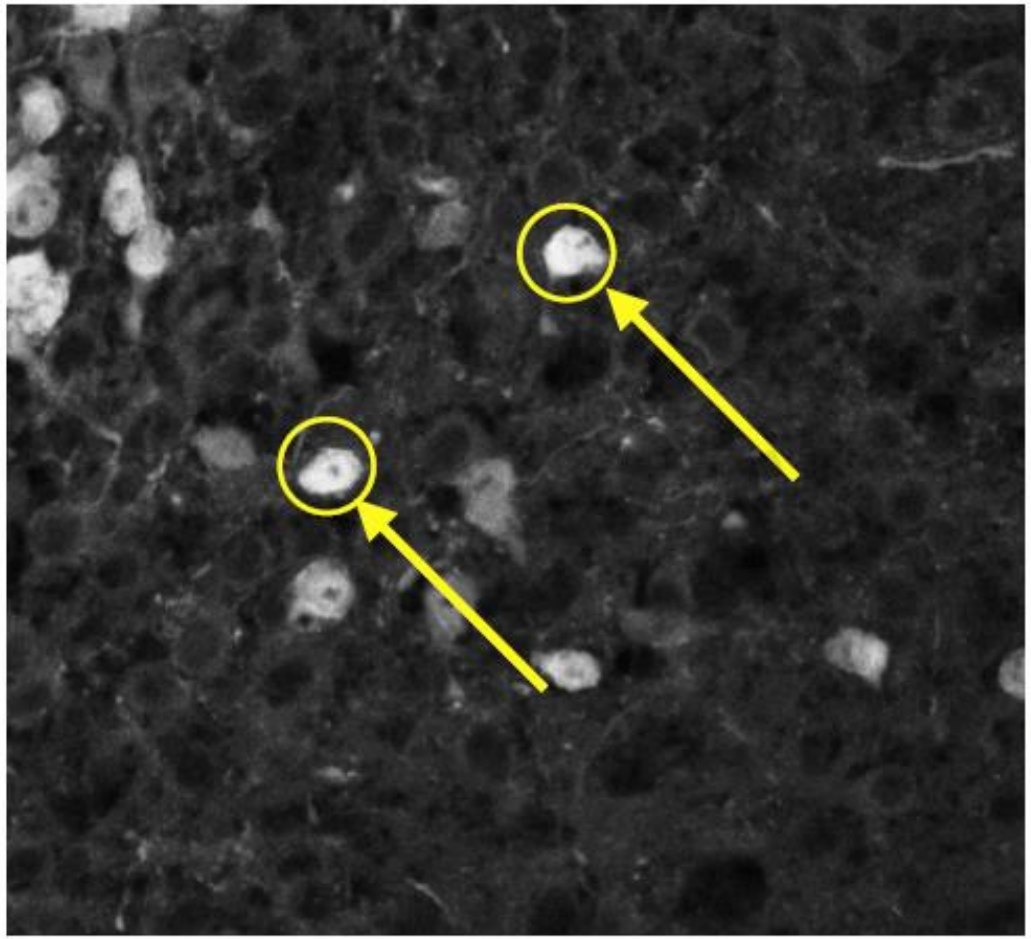
Sample A

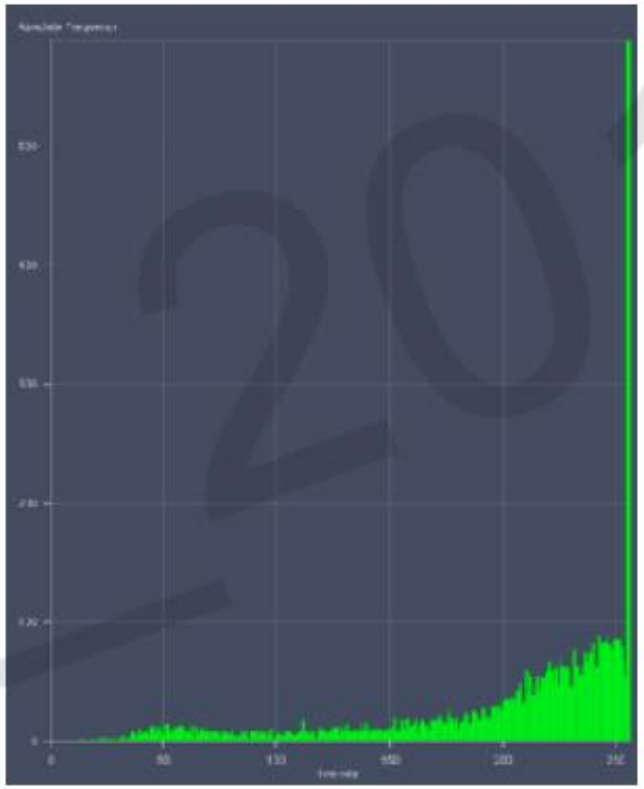


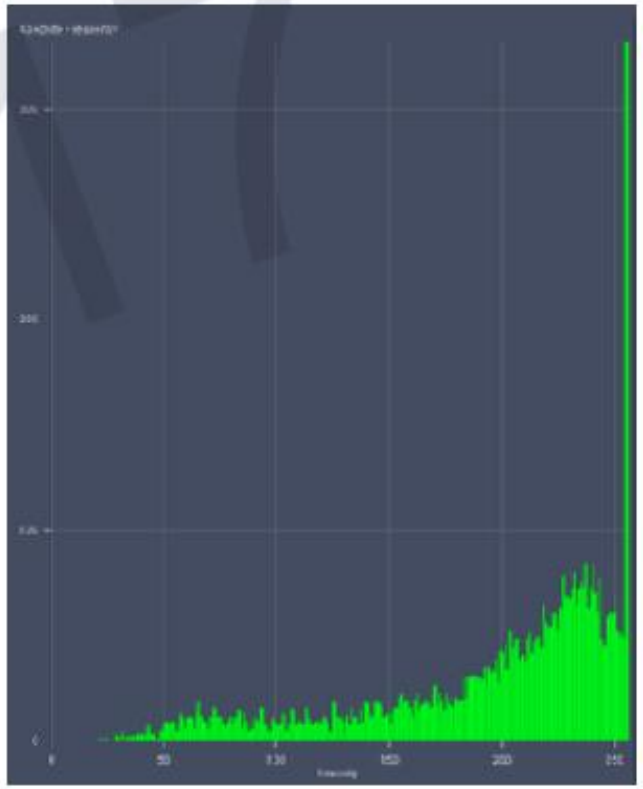




Sample B





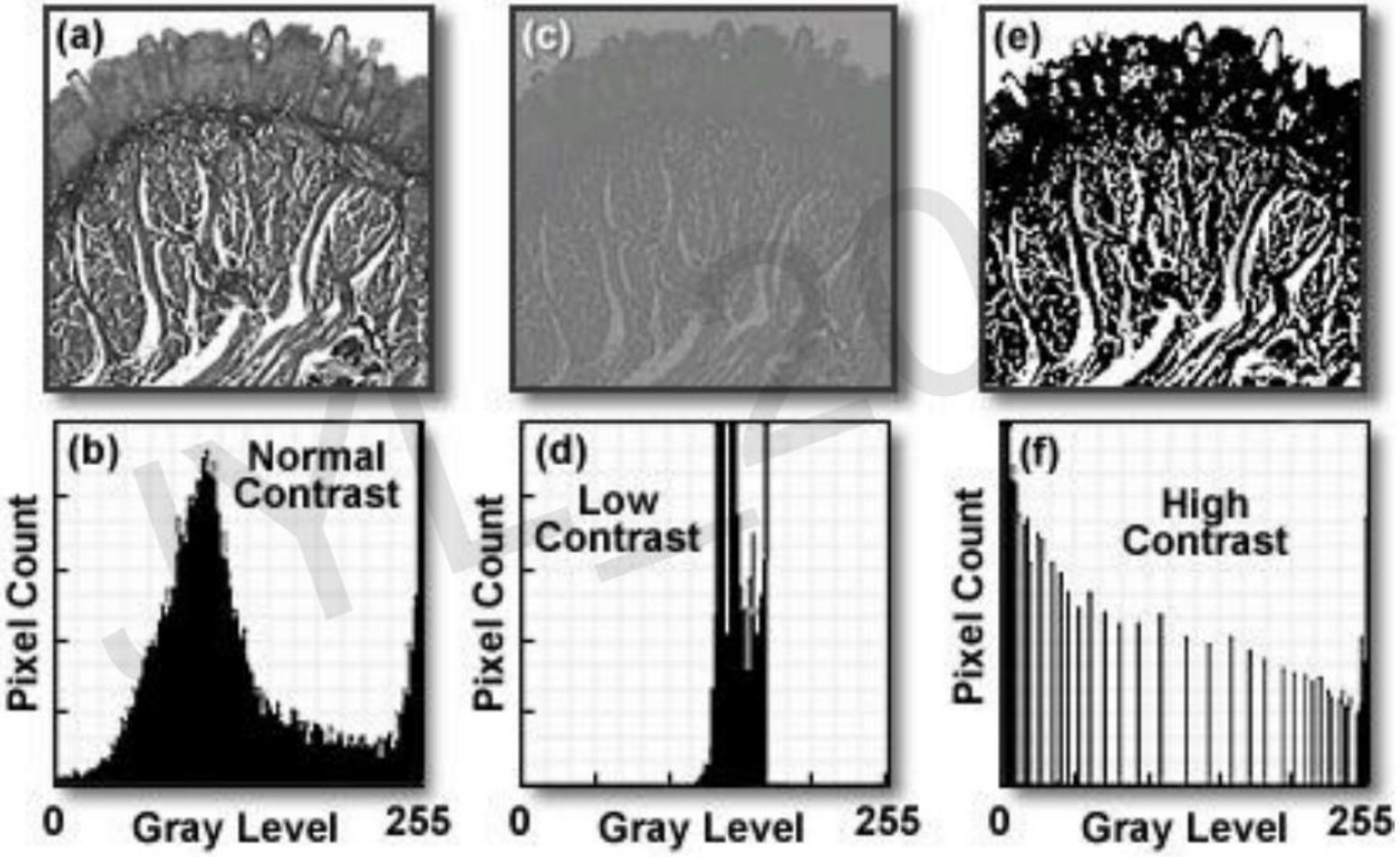


Saturation = Potential Data Loss, Skewed Distribution

	Sample A (Not Saturated)	Sample B (Saturated)
Cell 1- mean intensity	124 (+/- 36)	202 (+/- 57)
Cell 2- mean intensity	127 (+/- 27)	195 (+/- 55)
Cell 1 - saturated pixels	0	590
Cell 2- saturated pixels	0	332

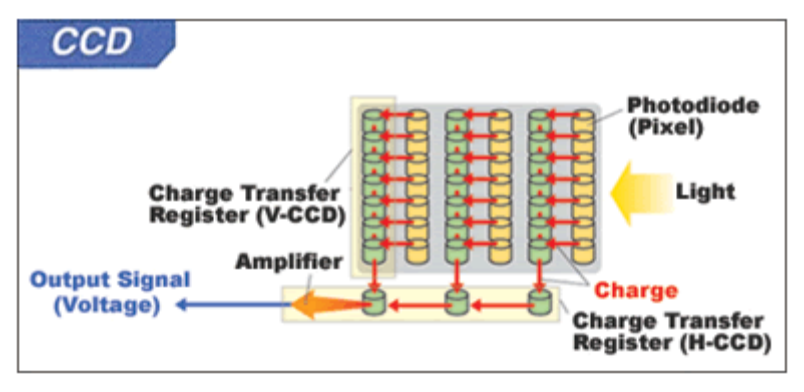
Remember you can always change contrast/levels for presentations/figures.

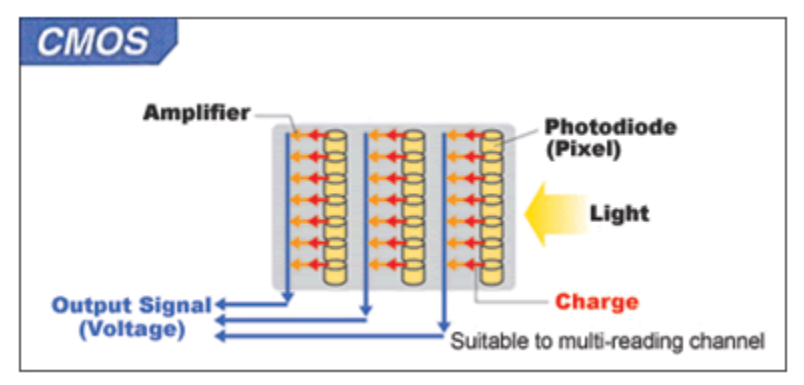
Dynamic Range = Contrast



http://hamamatsu.magnet.fsu.edu/articles/digitalimagebasics.html

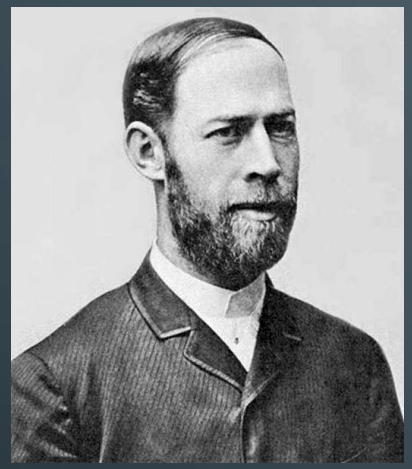
CCD VERSUS CMOS





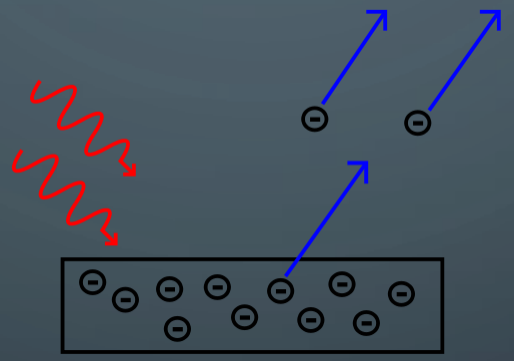
- Both convert photons of light into electric charge and process it into electric signals
- Charge Coupled Devices (CCDs) every pixels charge is transferred through a limited number of output nodes and sent off as an analog signal
- Complementary Metal Oxide Semiconductors (CMOS) is designed to have every pixel to have its own charge to voltage conversion and includes amplifiers, noise correction, and digitization circuits where the chips output is in digital bits
- Generally, CCDs have lower noise and more uniformity with better signal
- CMOS cameras have very high frame rates

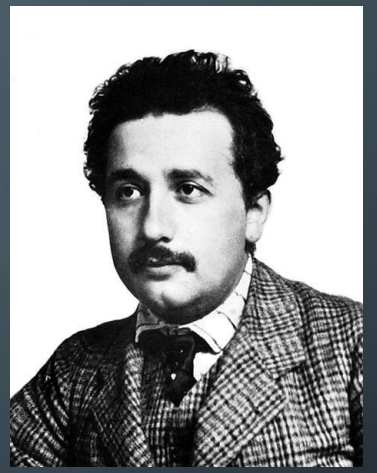
HOW IMAGE SENSORS SENSE AN IMAGE
1. Need way to detect photons



Hertz: observed in 1887

Photoelectric Effect





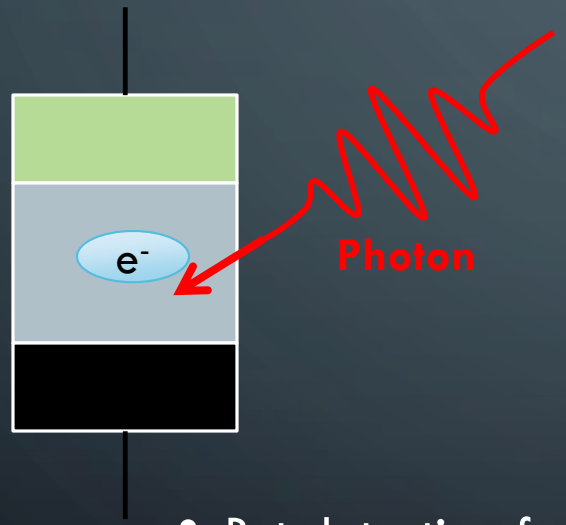
Einstein

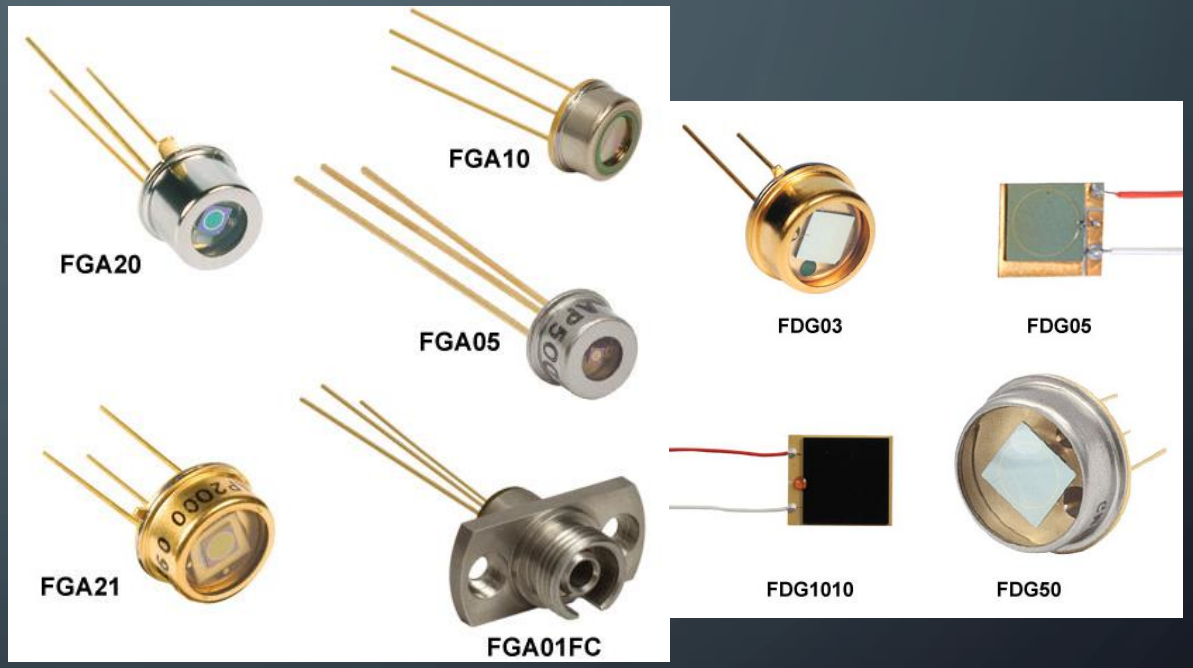
$E = \frac{hc}{\lambda}$

- For silicon, need ≈ 1.14 eV to release an electron
  - So need λ < 1100 nm

POINT DETECTORS

- Photodiodes
  - Photons will generate electron (photoelectron)





- But detecting few electrons is hard
- Need way to amplify the signal

POINT DETECTORS

- Avalanche Photodiode (APD)
  - Initial e^- becomes amplified through *impact ionization*
  - Gains of 100 to 1000

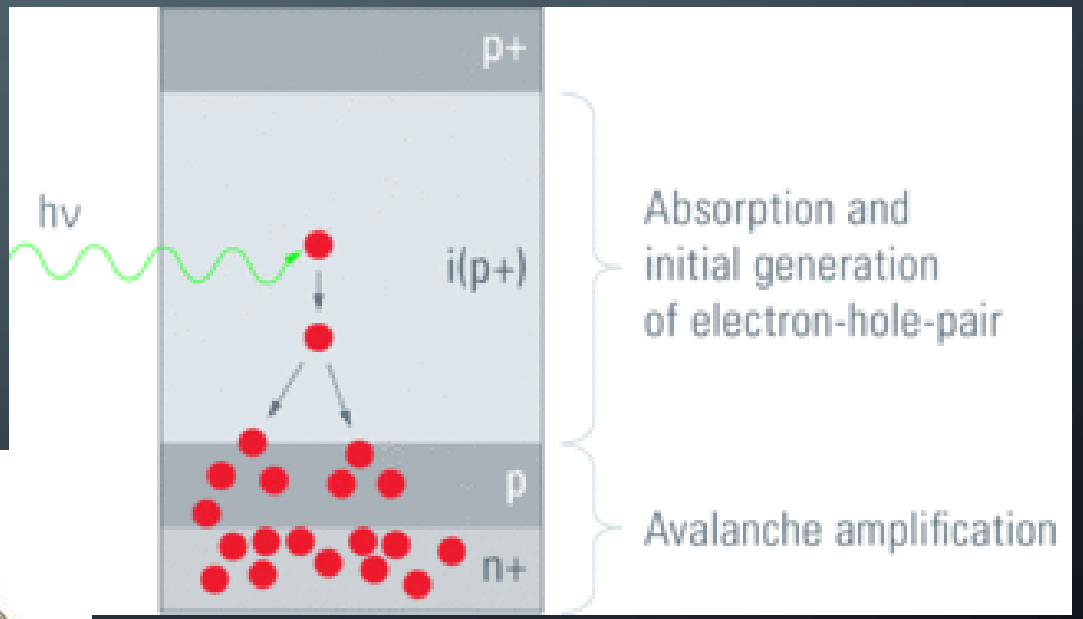
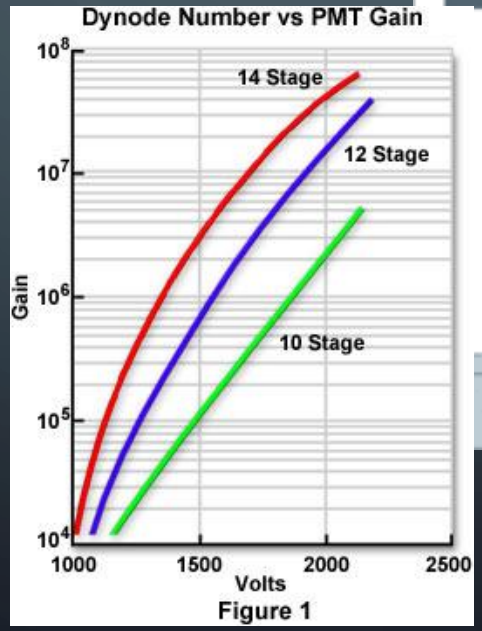


Image from http://www.leica-microsystems.com/science-lab/sensors-for-true-confocal-scanning/

POINT DETECTORS

- Photomultiplier Tube (PMT)
  - Used widely in confocals
  - High linearity
  - Fast response
  - High gain



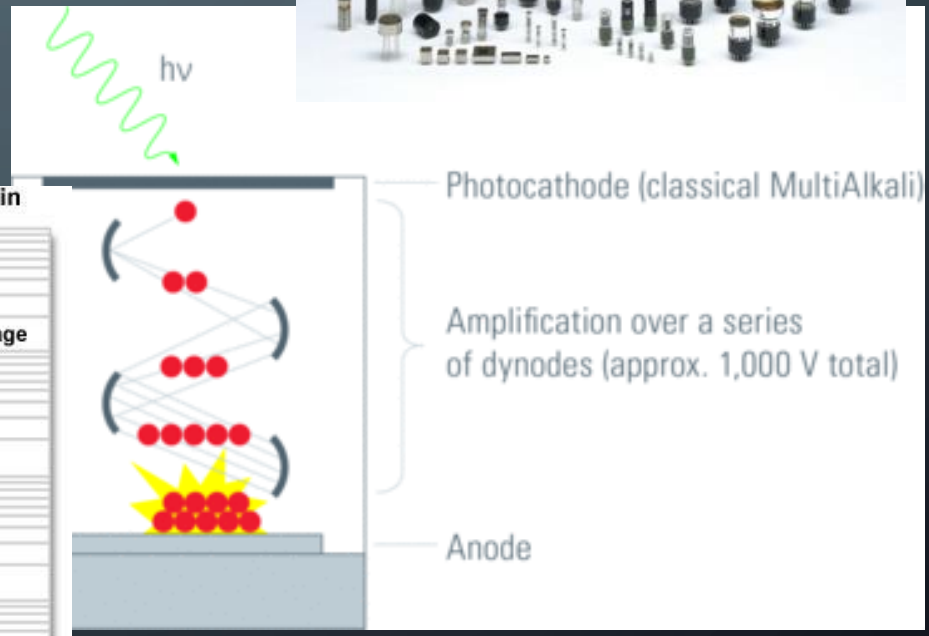
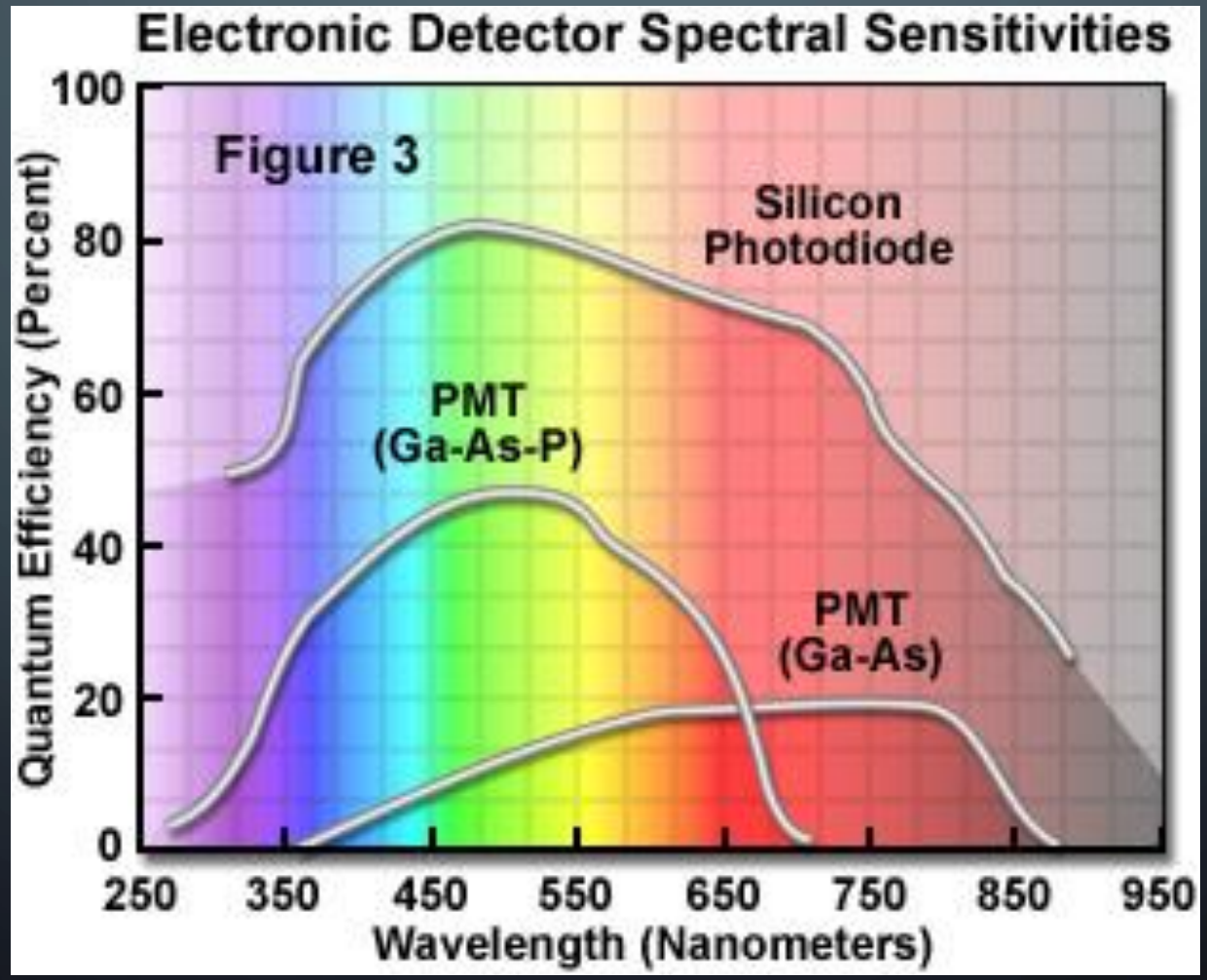


Image from http://www.leica-microsystems.com/science-lab/sensors-for-true-confocal-scanning/

HOW WELL ARE PHOTONS DETECTED?

Quantum Efficiency

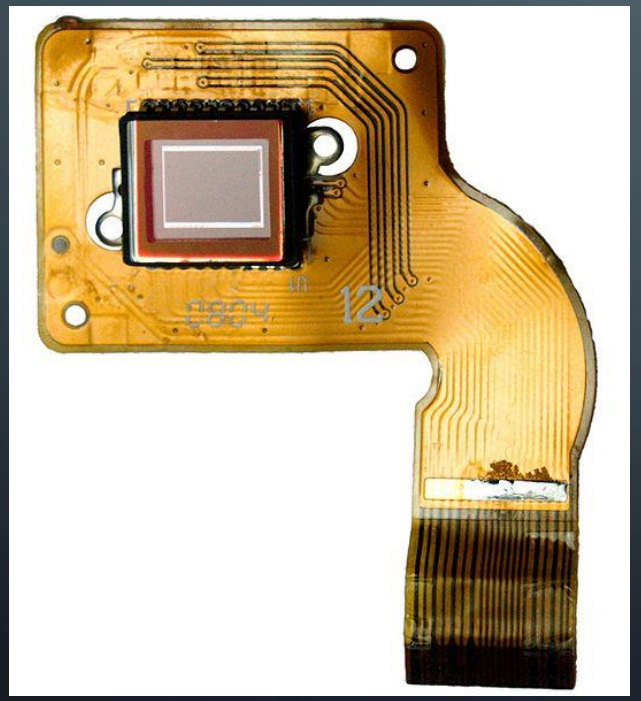


Additional INFO

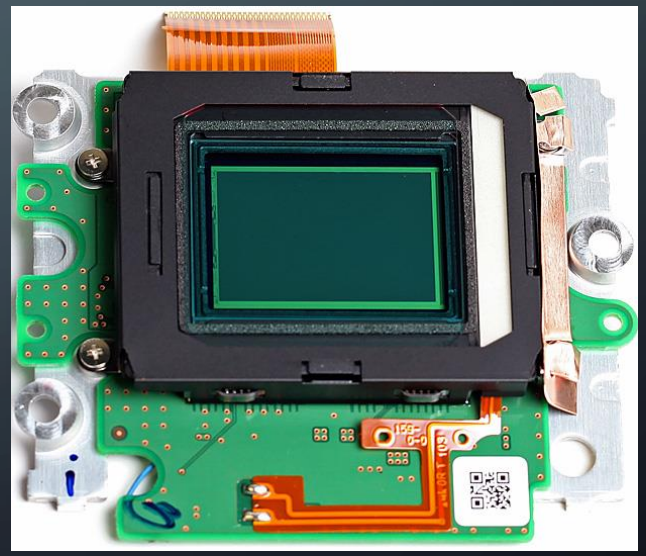
HOW IMAGE SENSORS SENSE AN IMAGE

2. Must detect photons over an array of pixels

CCD



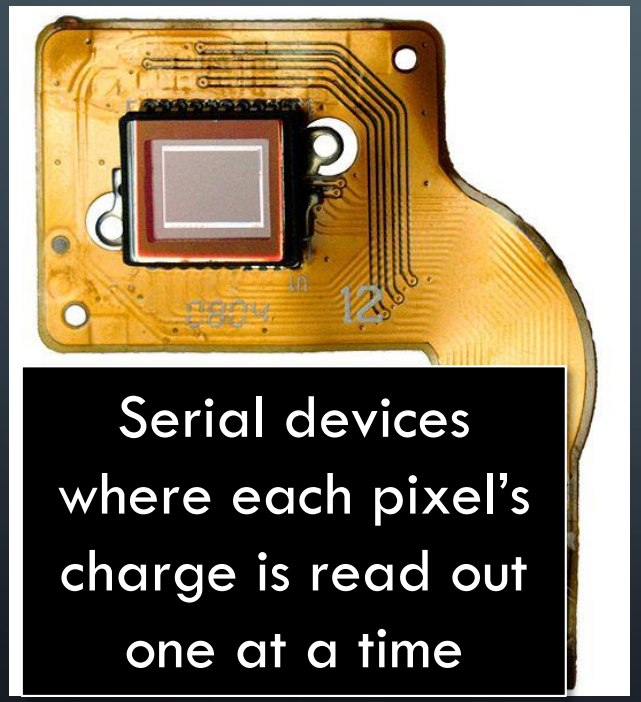
CMOS



HOW IMAGE SENSORS SENSE AN IMAGE

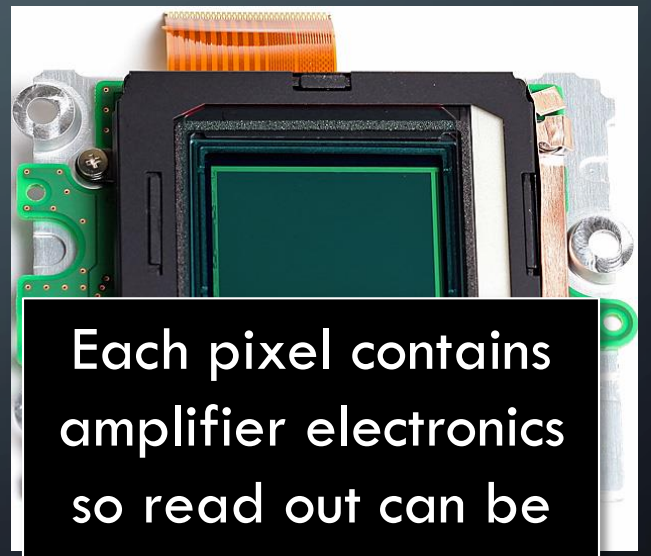
3. Electrons generated in each pixel must be read out

CCD



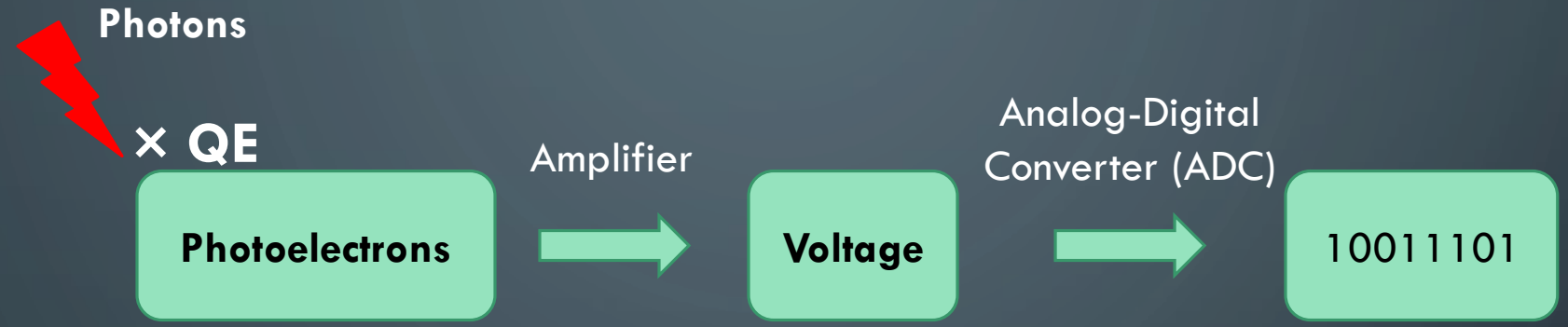
Serial devices where each pixel's charge is read out one at a time

CMOS



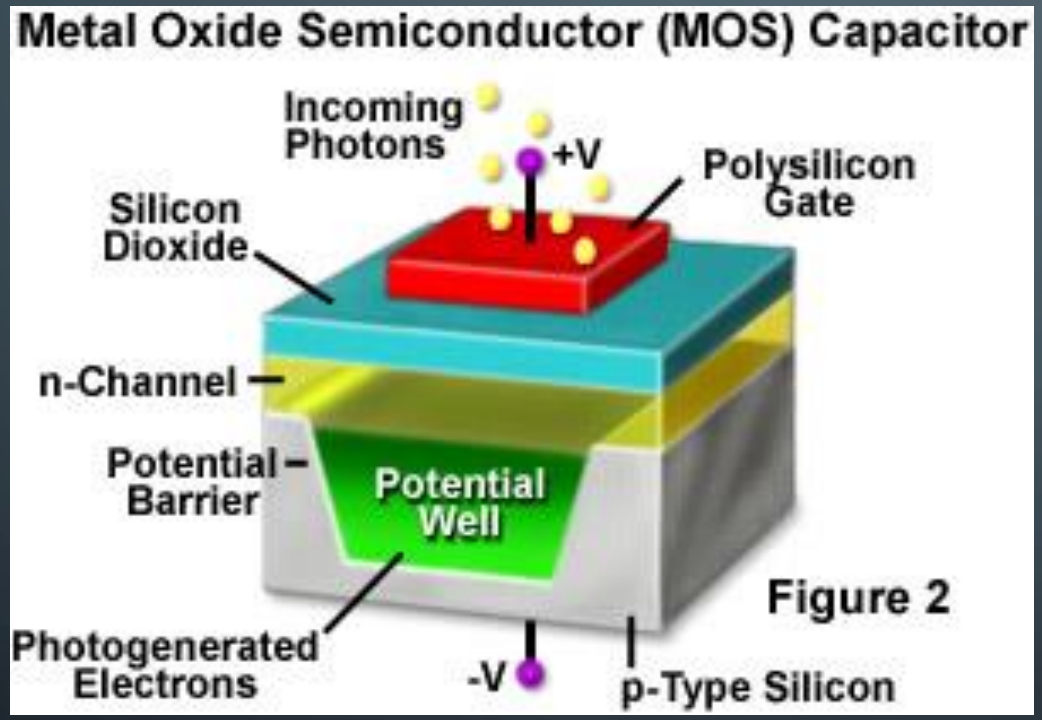
Each pixel contains amplifier electronics so read out can be much faster

WHAT IS MEANT BY "READ OUT"?



Bit Depth	Number of gray levels
8	2^8 = **256**
10	2^{10} = **1024**
12	2^{12} = **4096**
14	2^{14} = **16384**
16	2^{16} = **65536**

CCD DETECTORS

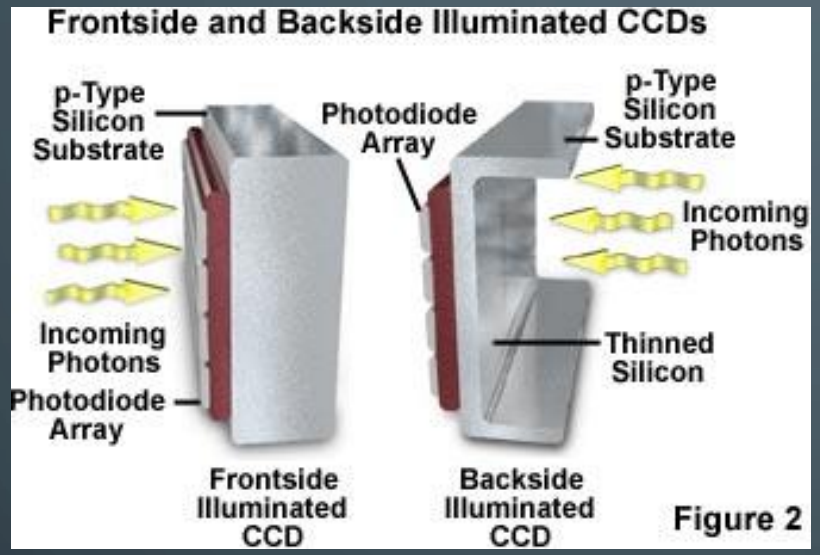


Charge-Coupled Device

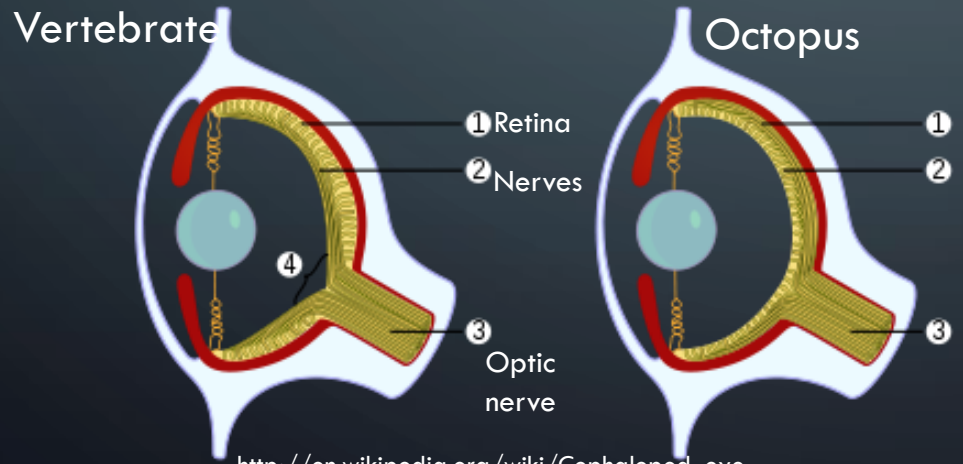
- Invented at Bell Labs in 1970
- 2009 Nobel Prize in Physics
- Widely used in TV, medical, astronomy cameras
- Array of light sensitive MOS capacitors (pixels)
- Incoming light generates electrons which are captured in a potential well
- Electrodes, or gates, move the charge

Image from http://www.microscopyu.com/articles/digitalimaging/ccdintro.html

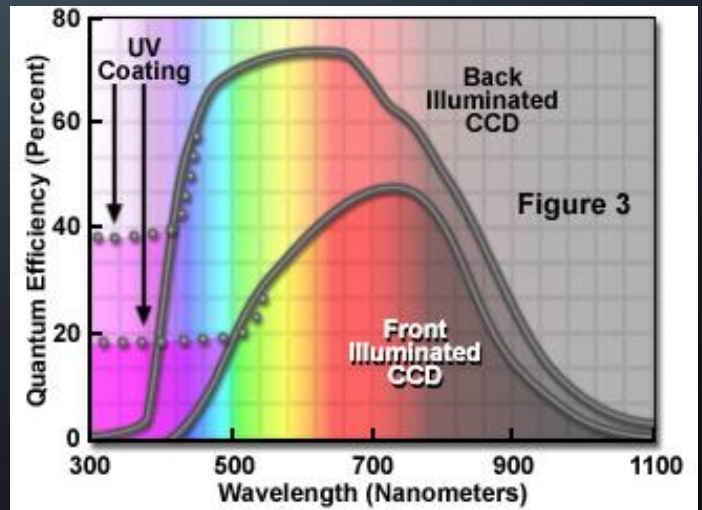
FRONT OR BACK ILLUMINATED



- Traditional, front-illuminated have **wiring in front** of photosensitive region
  - This blocks some light, reducing QE
- Back-illuminated CCDs:
  - Back side of the CCD is etched to 10-15 microns
  - More fragile and costly, but higher QE

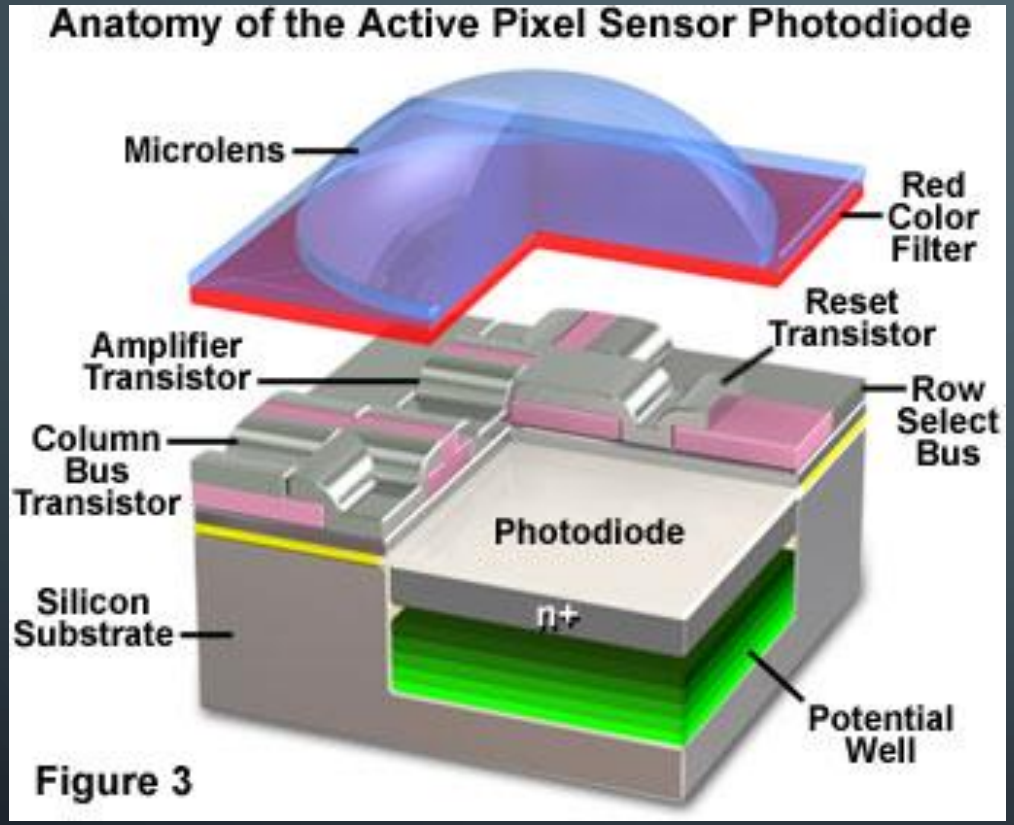


http://en.wikipedia.org/wiki/Cephalopod_eye



From http://learn.hamamatsu.com/articles/quantumefficiency.html

CMOS DETECTORS



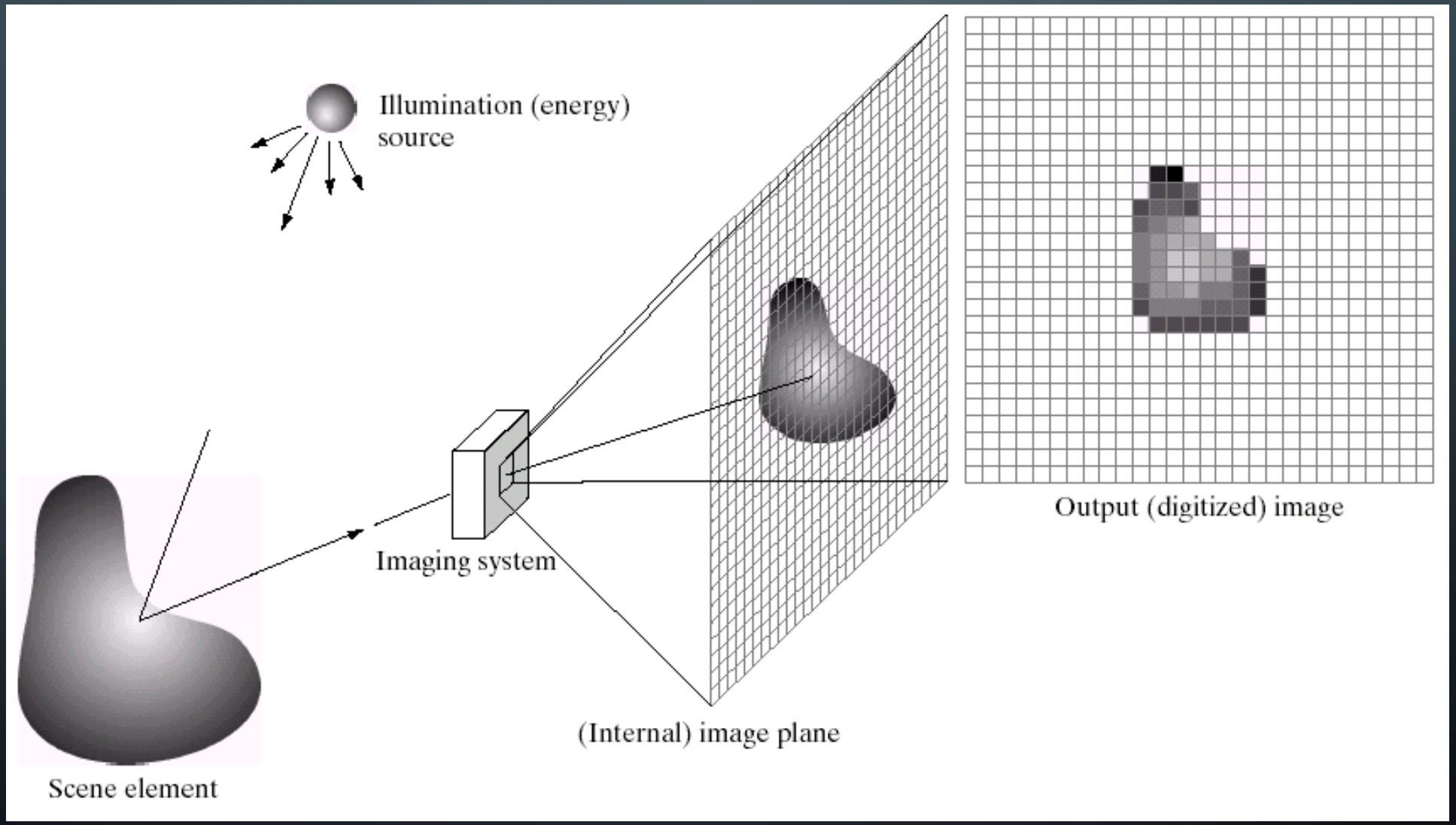
Complementary Metal Oxide Semiconductor

- Transistors in **each pixel** convert charge to voltage
- More can be done within a pixel meaning **frame read out can be faster**
- Fabricated much like microprocessors and RAM so are **cheaper to make**
- Used in webcams, phone cameras since they use **less power**

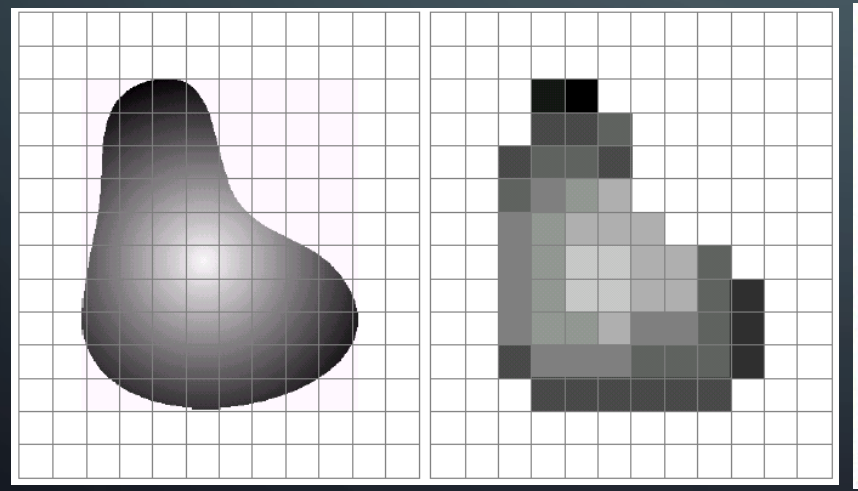
Image from http://www.olympusmicro.com/primer/digitalimaging/cmosimagesensors.html

THE IDEAL CAMERA

- High resolution
  - Optically resolvable features span a couple pixels
- Large area
  - Covers field of view
- Linear response
  - Twice the photons gives twice the output
- Uniform response
  - Same response no matter where photon lands
- Fast
  - Can capture dynamic processes
- High sensitivity
  - All photons get measured


Illumination (energy)
source
Imaging system
(Internal) image plane
Output (digitized) image
Scene element

- Pixel values typically represent gray levels, colours, heights, opacities etc
- Remember *digitization* implies that a digital image is an *approximation* of a real scene



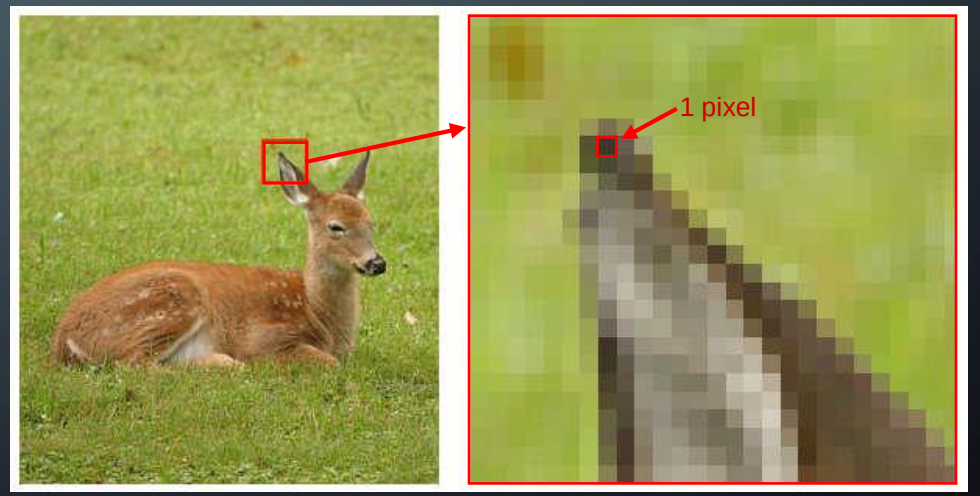
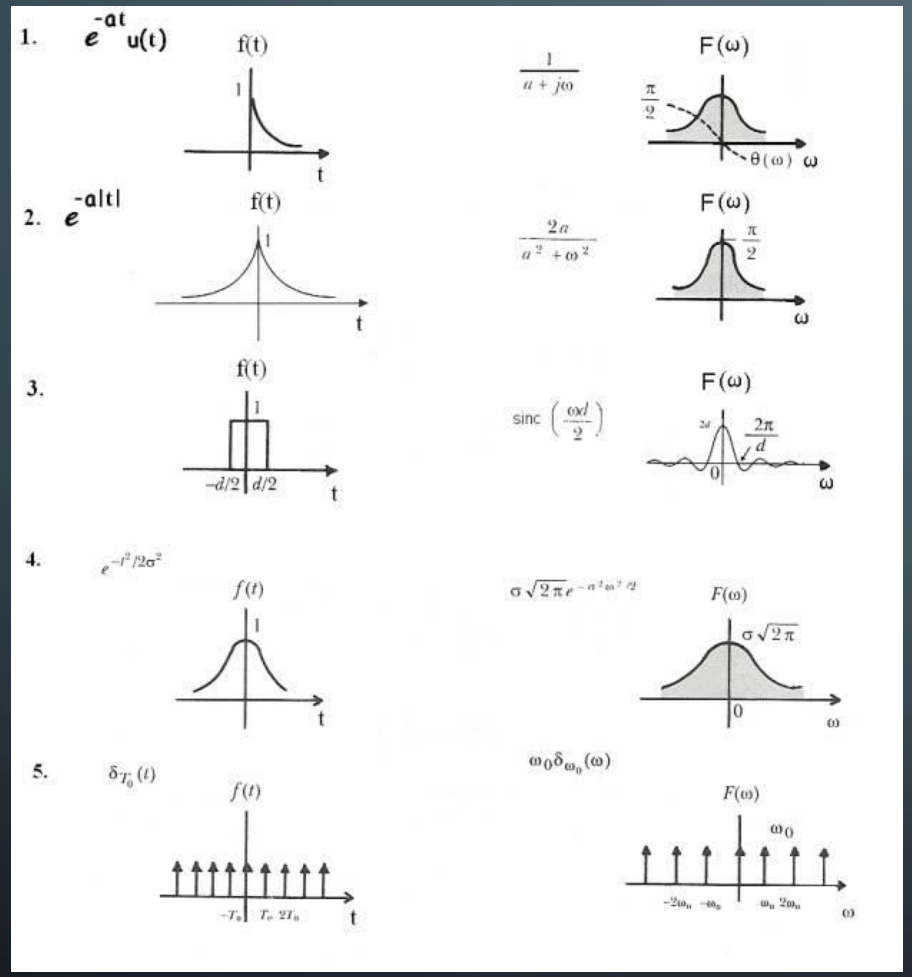


IMAGE PROCESSING



Fourier Transform
Sampling, Convolution

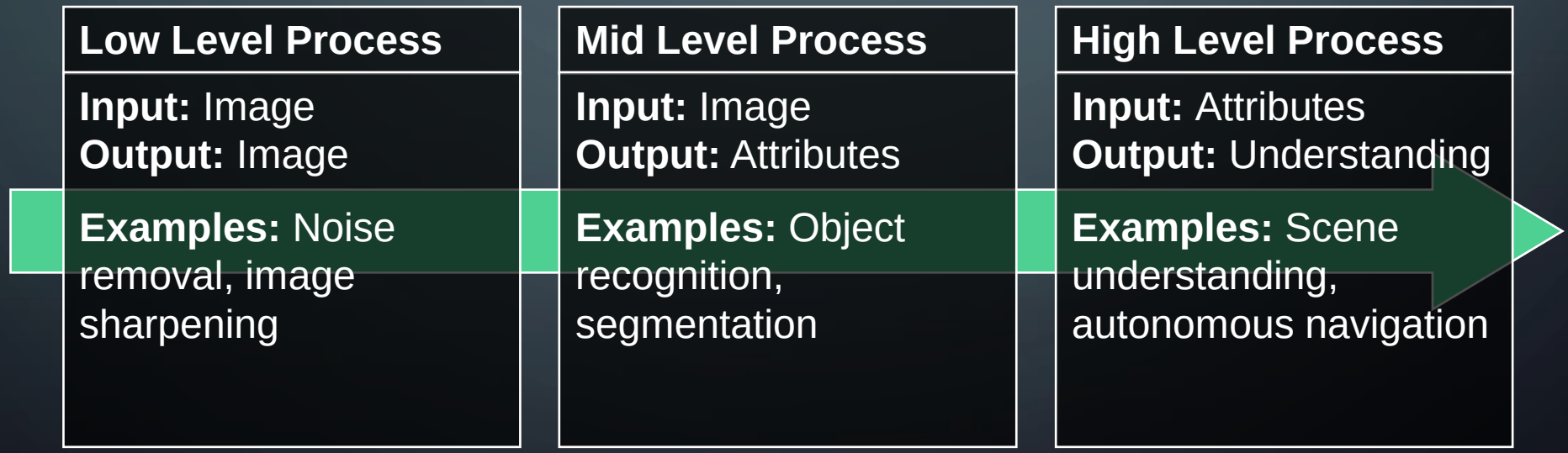
Image enhancement
Feature detection

- Common image formats include:
- 1 sample per point (B&W or Grayscale)
- 3 samples per point (Red, Green, and Blue)

WHAT IS DIGITAL IMAGE PROCESSING?

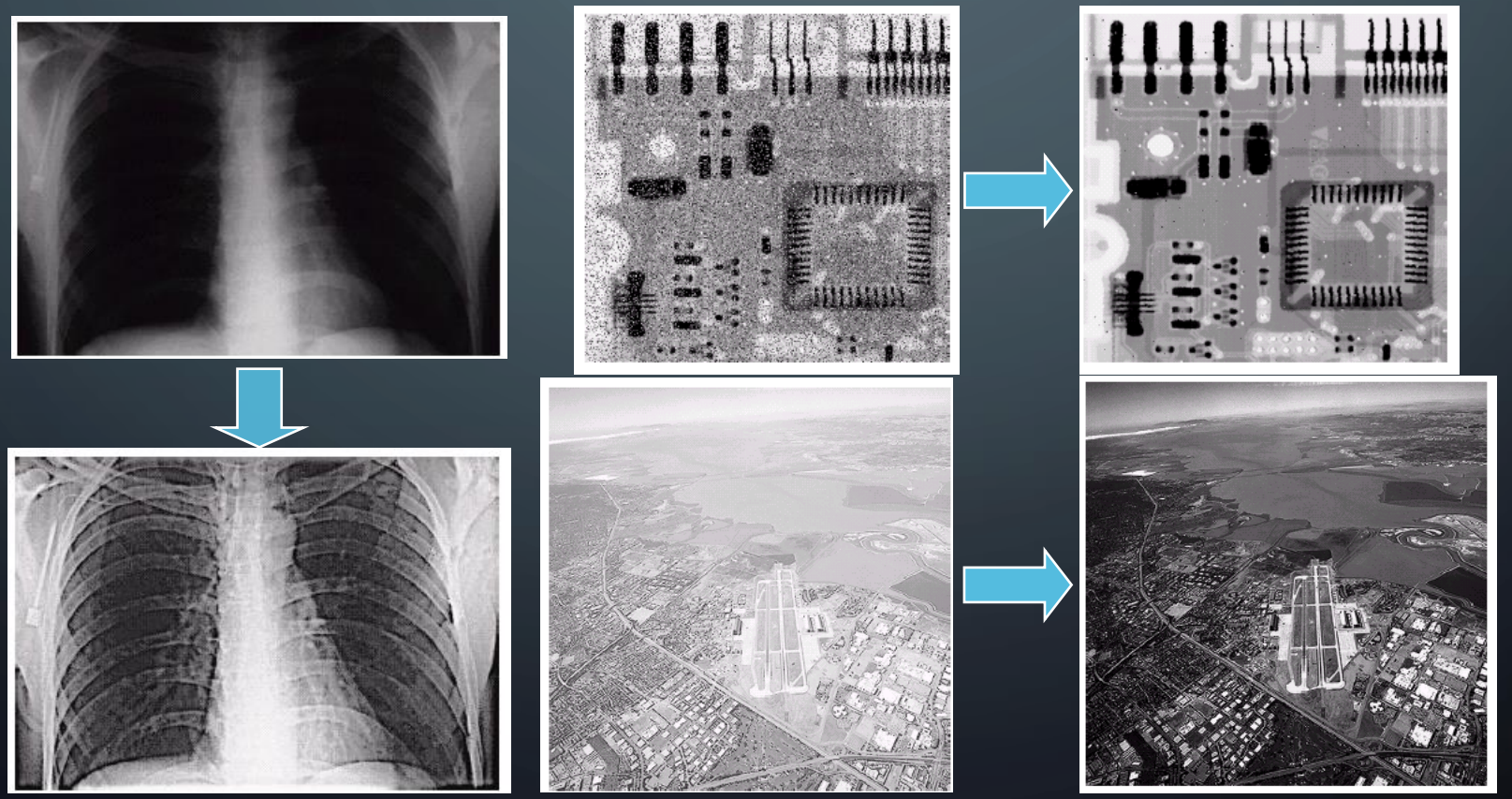
- Digital image processing focuses on two major tasks
  - Improvement of pictorial information for human interpretation
  - Processing of image data for storage, transmission and representation for autonomous machine perception
- Some argument about where image processing ends and fields such as image analysis and computer vision start

- The continuum from image processing to computer vision can be broken up into low-, mid- and high-level processes

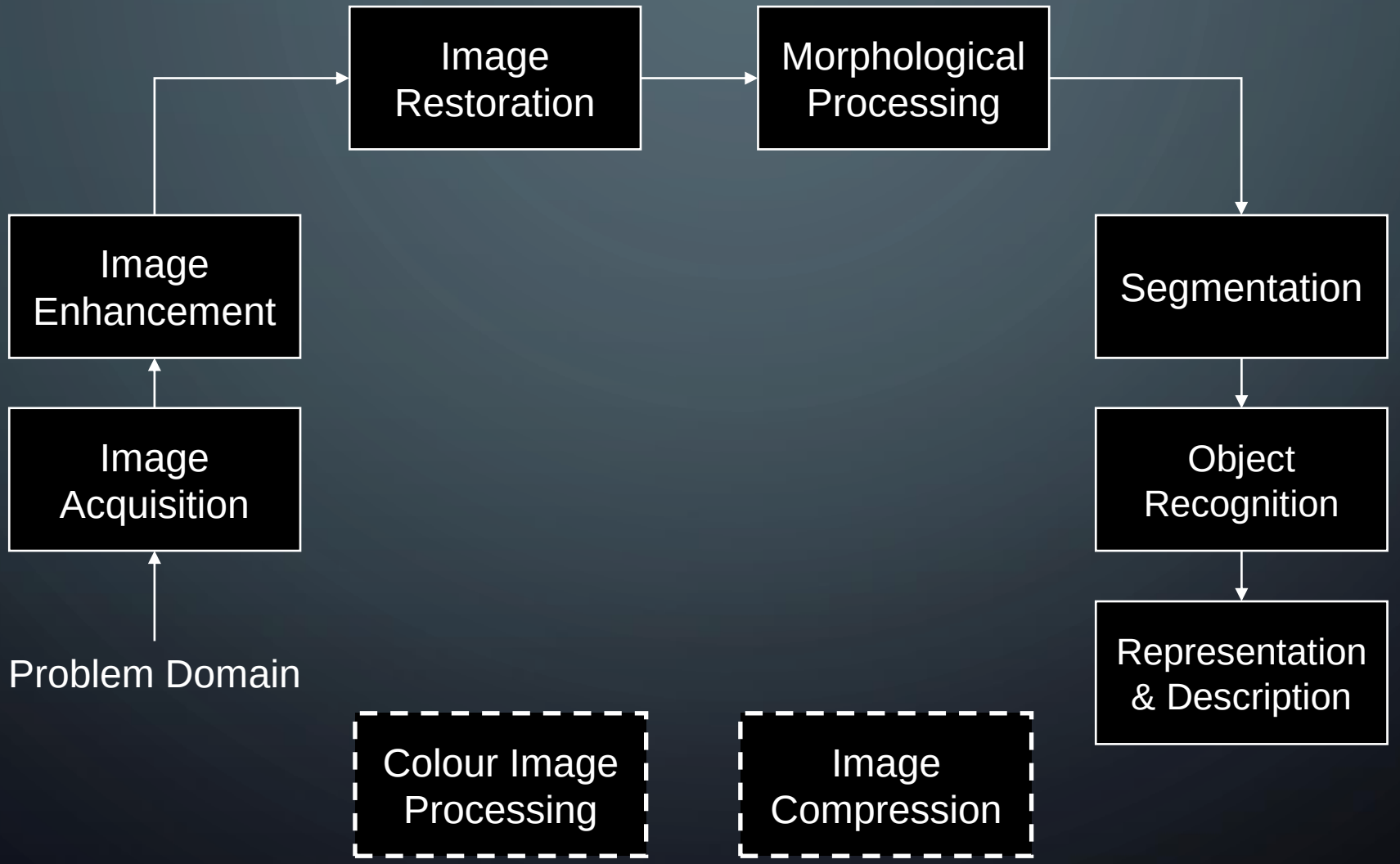


EXAMPLES: IMAGE ENHANCEMENT

- One of the most common uses of DIP techniques: improve quality, remove noise etc



KEY STAGES IN DIGITAL IMAGE PROCESSING



KEY STAGES IN DIGITAL IMAGE PROCESSING: IMAGE AQUISITION

Problem Domain

Image Acquisition

Image Enhancement

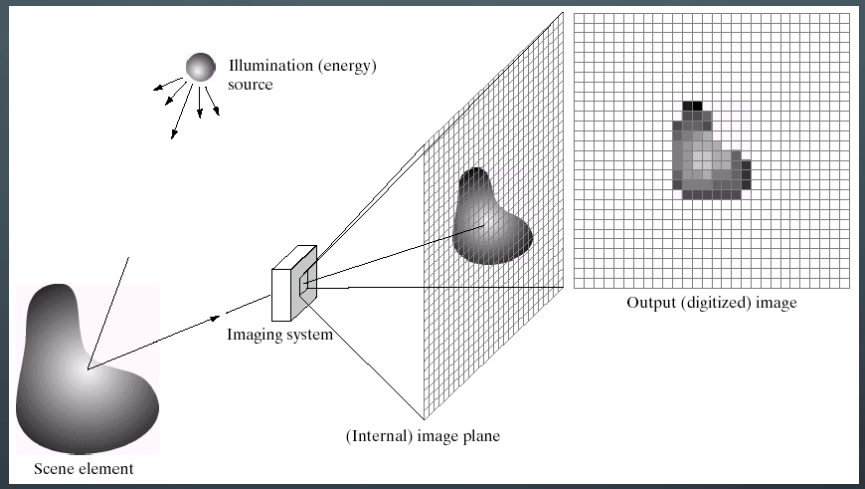
Image Restoration

Morphological Processing

Segmentation

Object Recognition

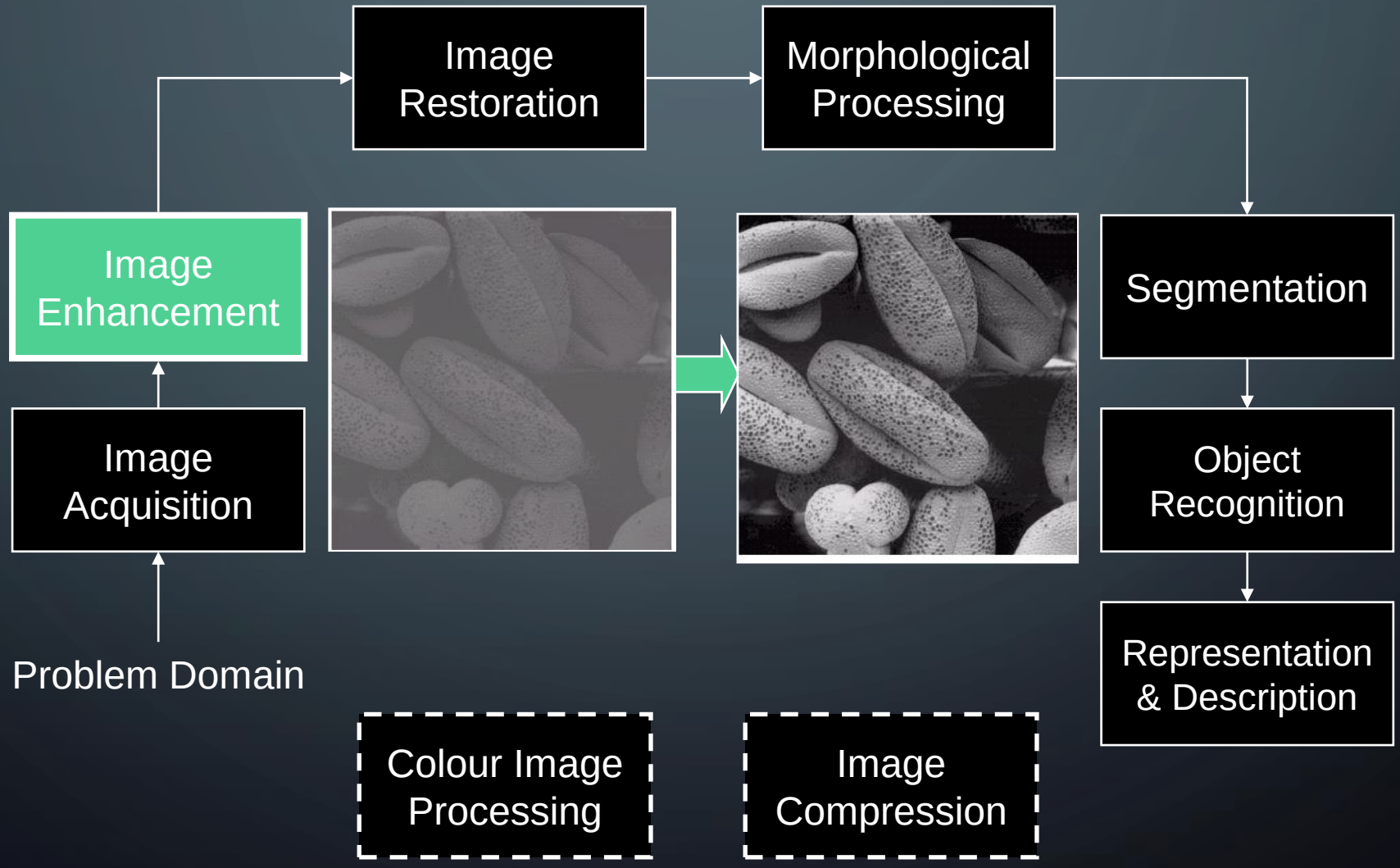
Representation & Description



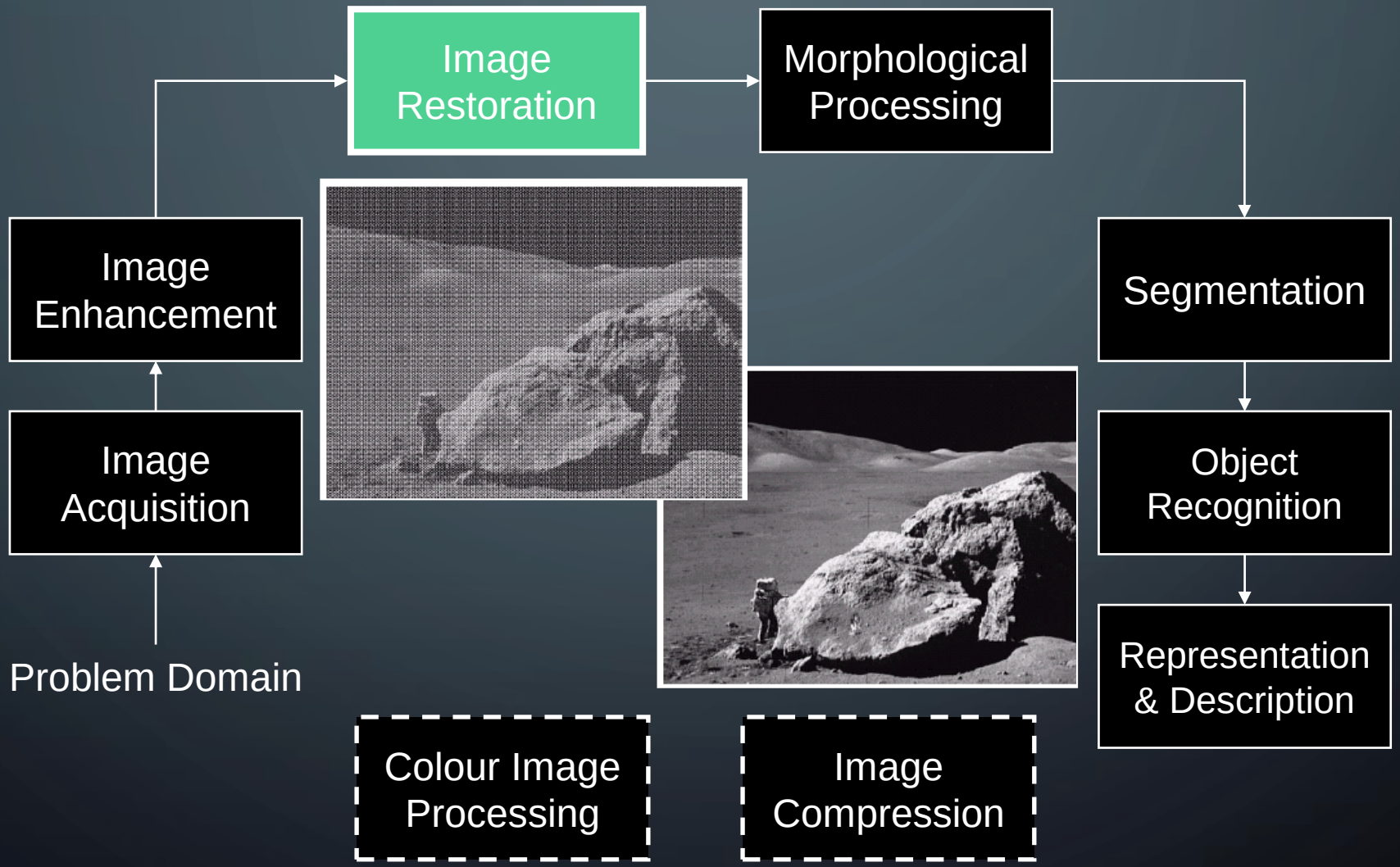
Colour Image Processing

Image Compression

KEY STAGES IN DIGITAL IMAGE PROCESSING: IMAGE ENHANCEMENT



KEY STAGES IN DIGITAL IMAGE PROCESSING: IMAGE RESTORATION



KEY STAGES IN DIGITAL IMAGE PROCESSING: MORPHOLOGICAL PROCESSING

Image Restoration

Morphological Processing

Segmentation

Object Recognition

Representation & Description

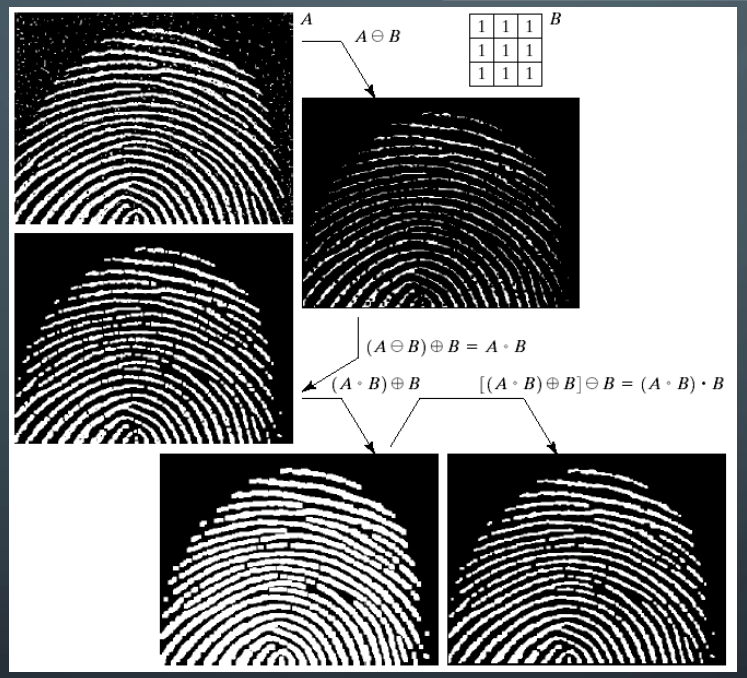
Image Compression

Colour Image Processing

Image Enhancement

Image Acquisition

Problem Domain



KEY STAGES IN DIGITAL IMAGE PROCESSING: SEGMENTATION

Problem Domain

Image Acquisition

Image Enhancement

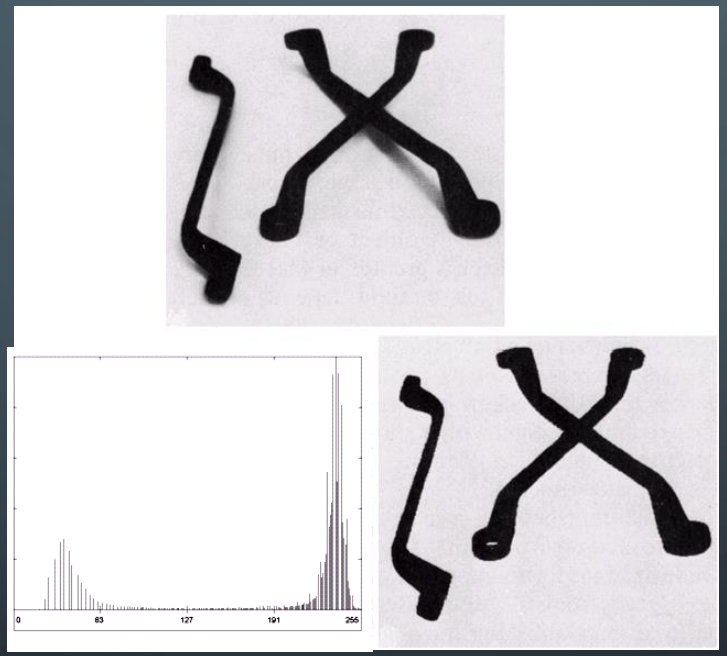
Image Restoration

Morphological Processing

Segmentation

Object Recognition

Representation & Description



Colour Image Processing

Image Compression

KEY STAGES IN DIGITAL IMAGE PROCESSING: OBJECT RECOGNITION

Problem Domain

Image Acquisition

Image Enhancement

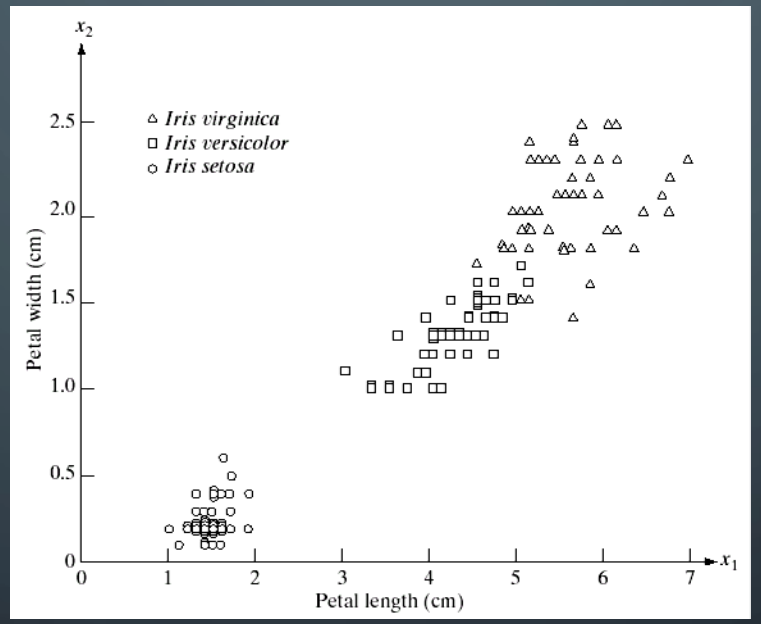
Image Restoration

Morphological Processing

Segmentation

Object Recognition

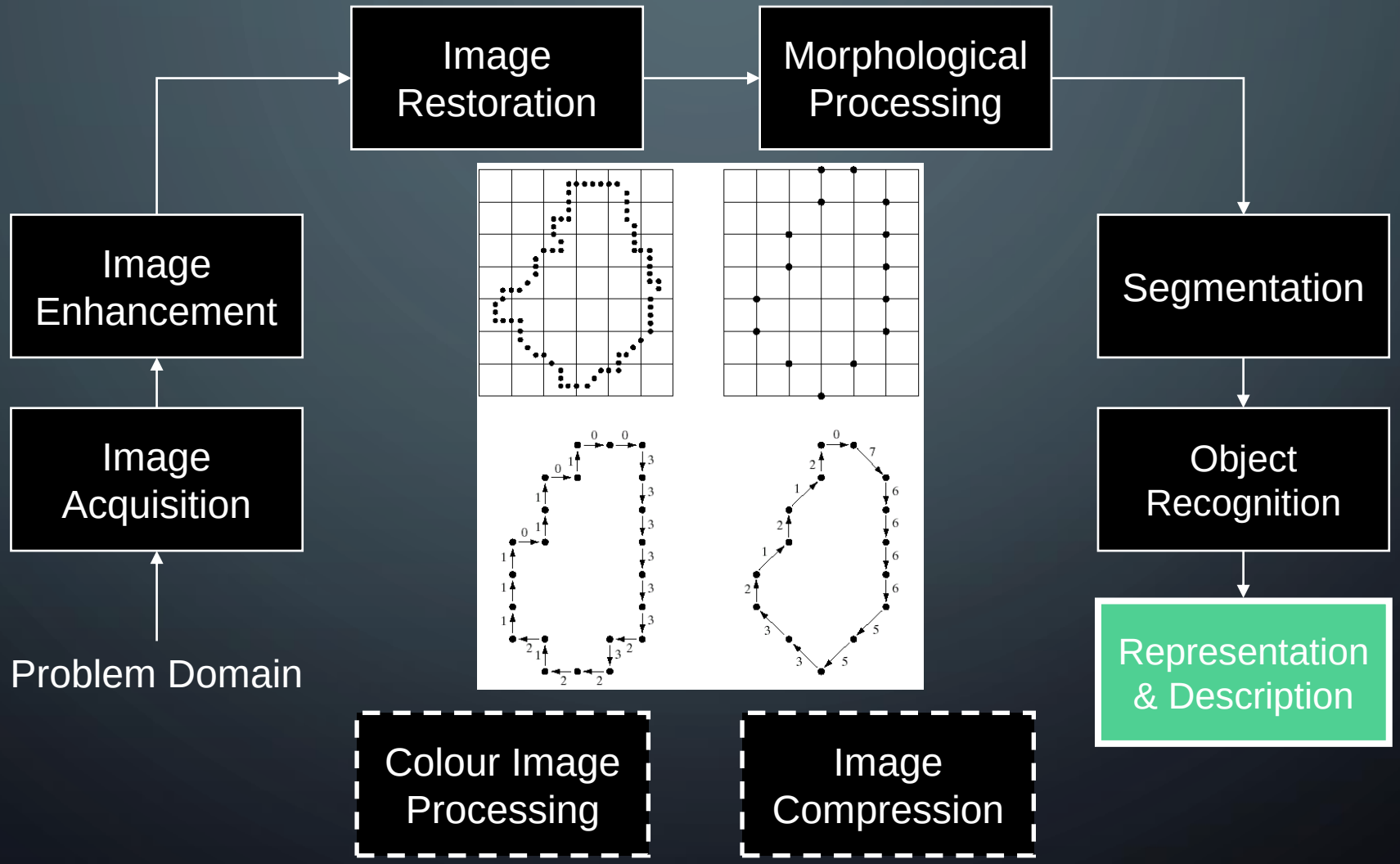
Representation & Description



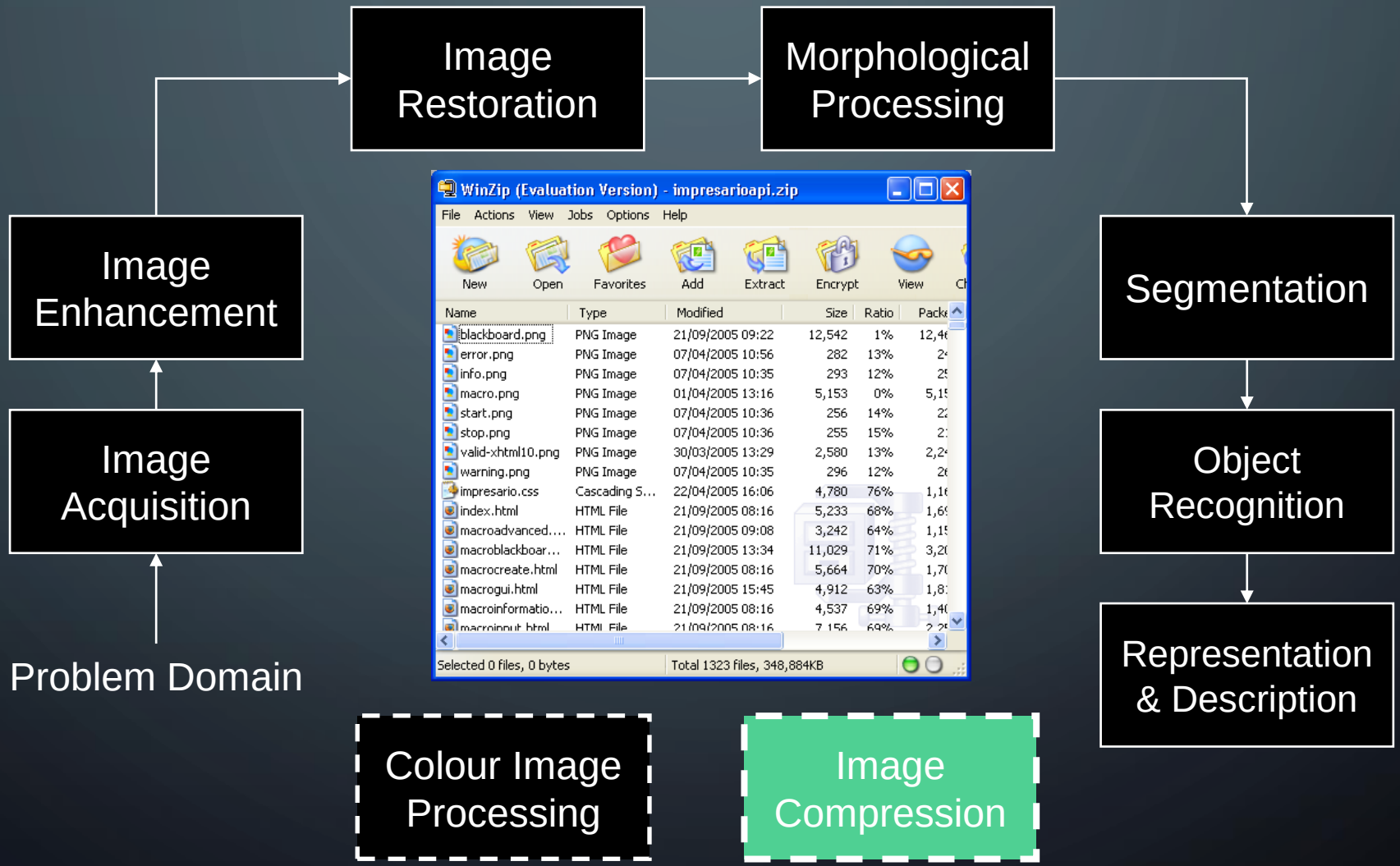
Colour Image Processing

Image Compression

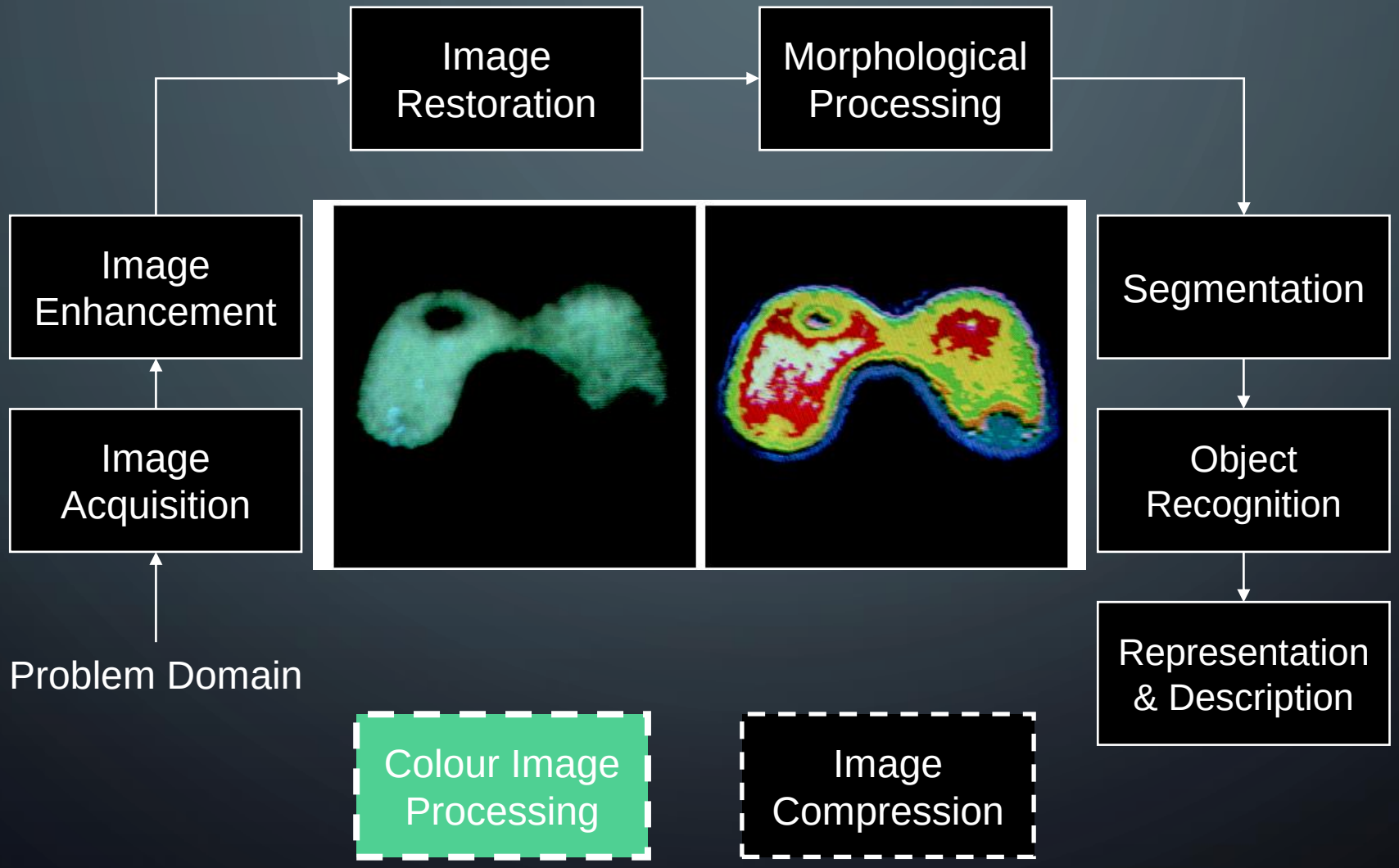
KEY STAGES IN DIGITAL IMAGE PROCESSING: REPRESENTATION & DESCRIPTION



KEY STAGES IN DIGITAL IMAGE PROCESSING: IMAGE COMPRESSION



KEY STAGES IN DIGITAL IMAGE PROCESSING: COLOUR IMAGE PROCESSING



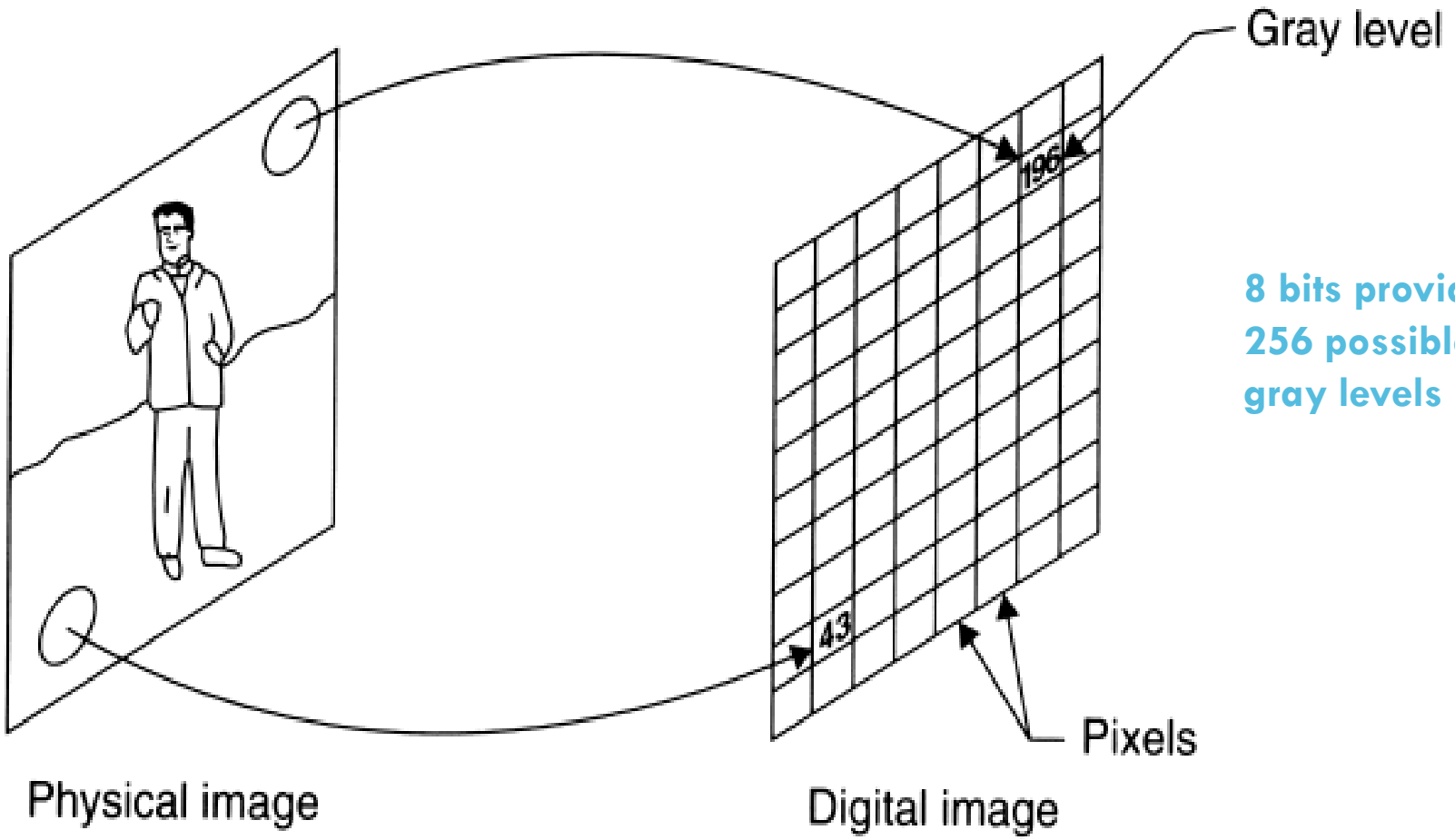
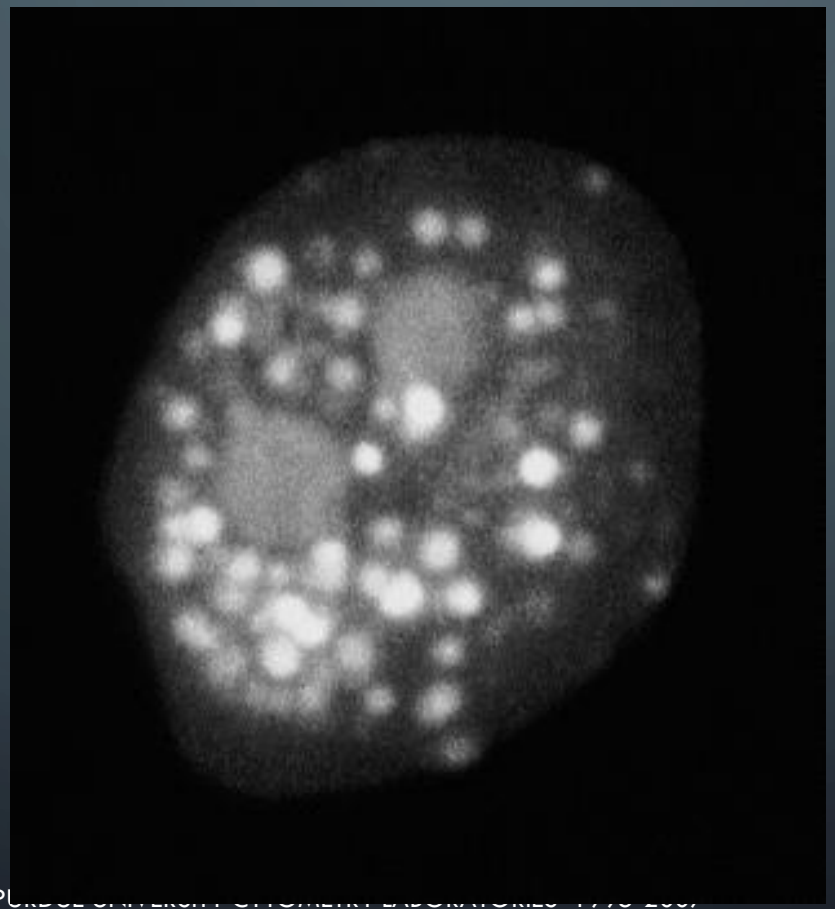


Figure 1–1 A physical image and a corresponding digital image

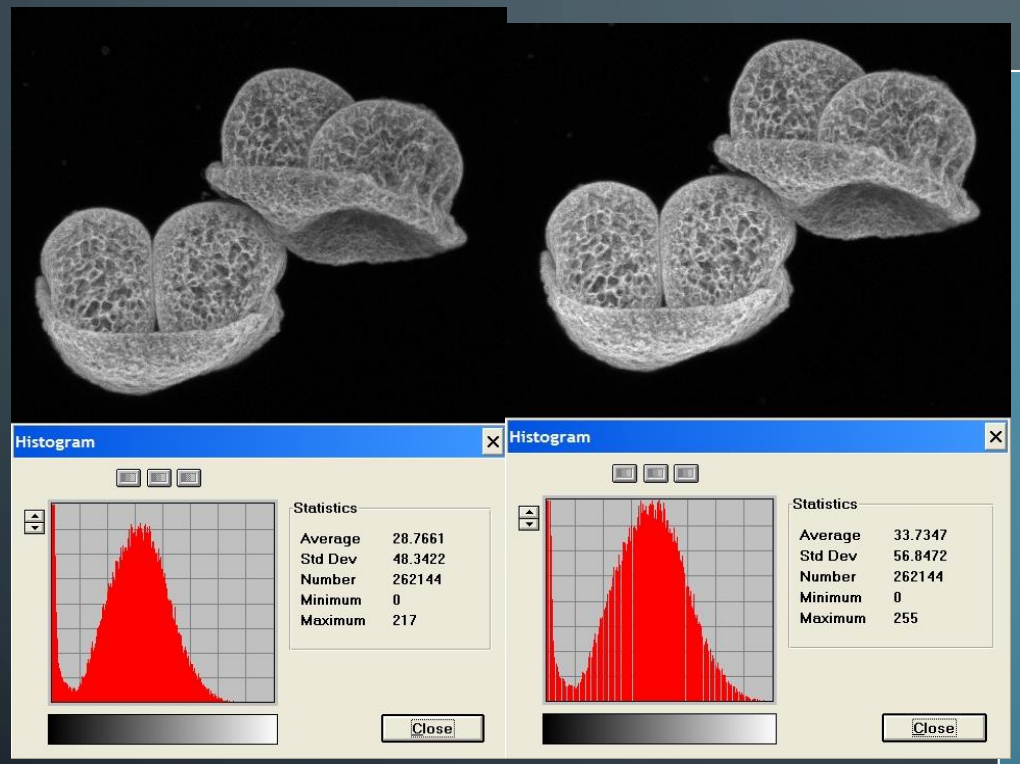
8 bits provide 256 possible gray levels

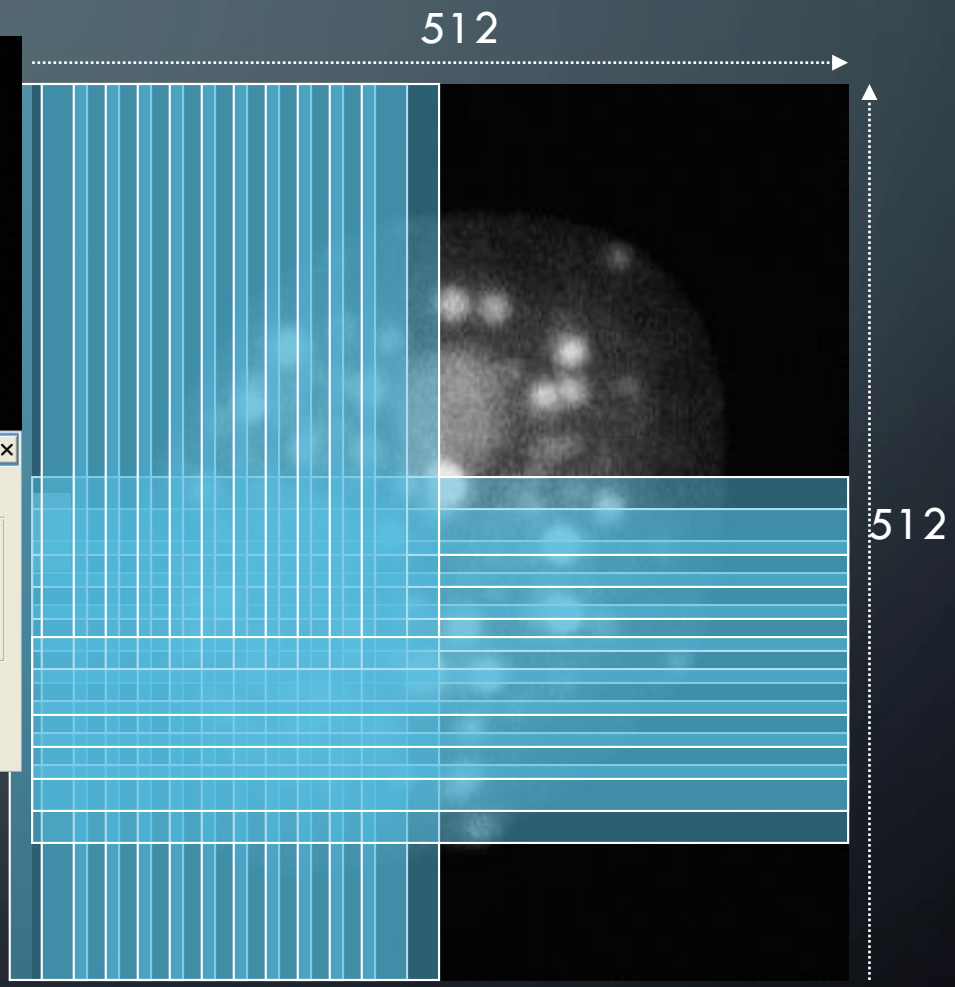
How does this translate from an image into an image map?

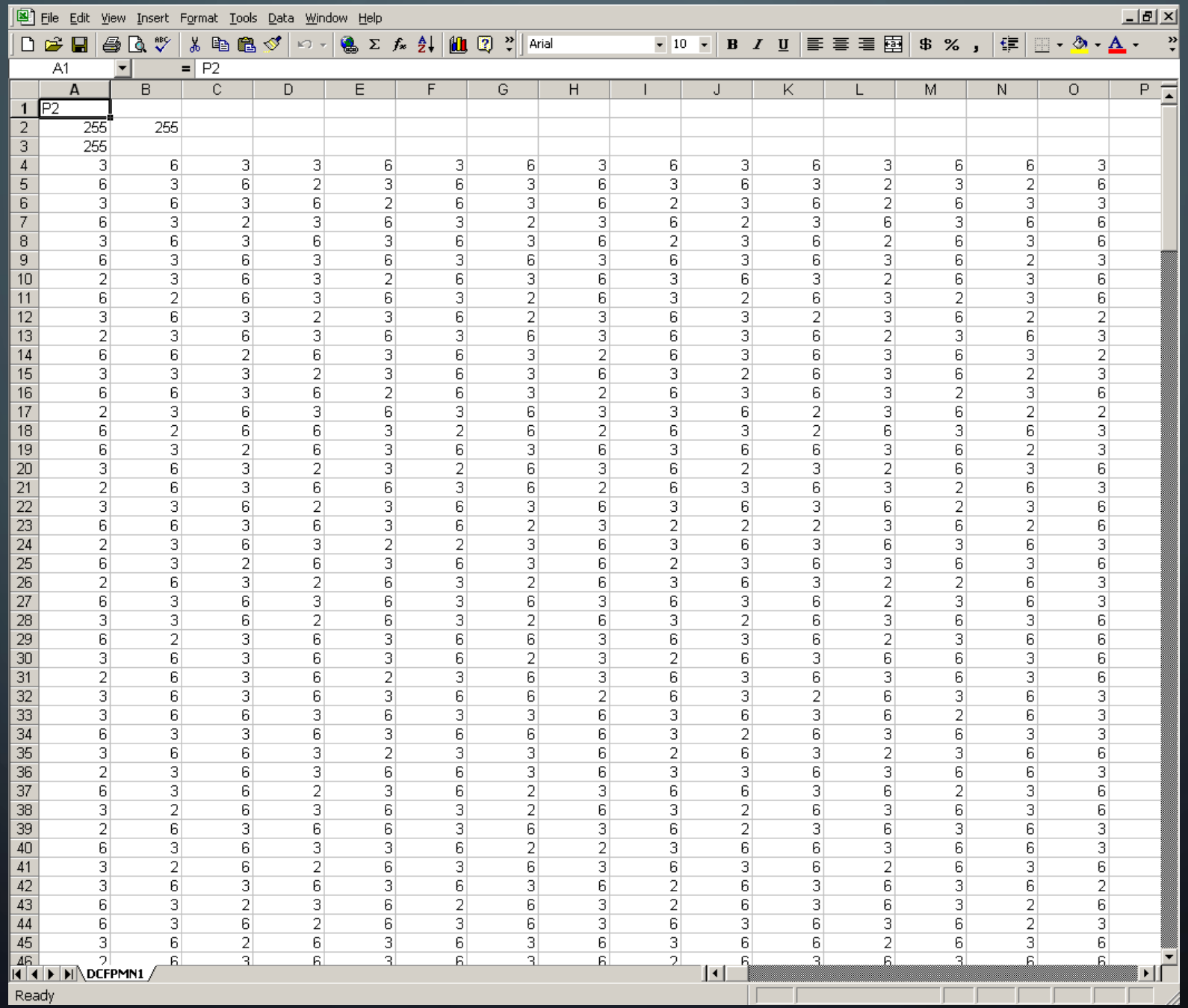


How does this translate from an image into an image map?

How does this translate from an image into an image map?







	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	P2															
2	255	255														
3	255															
4	3	6	3	3	6	3	6	3	6	3	6	3	6	6	3	
5	6	3	6	2	3	6	3	6	3	6	3	2	3	2	6	
6	3	6	3	6	2	6	3	6	2	3	6	2	6	3	3	
7	6	3	2	3	6	3	2	3	6	2	3	6	3	6	6	
8	3	6	3	6	3	6	3	6	2	3	6	2	6	3	6	
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11	6	2	6	3	6	3	2	6	3	2	6	3	2	3	6	
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45	3	6	2	6	3	6	3	6	3	6	6	2	6	3	6	
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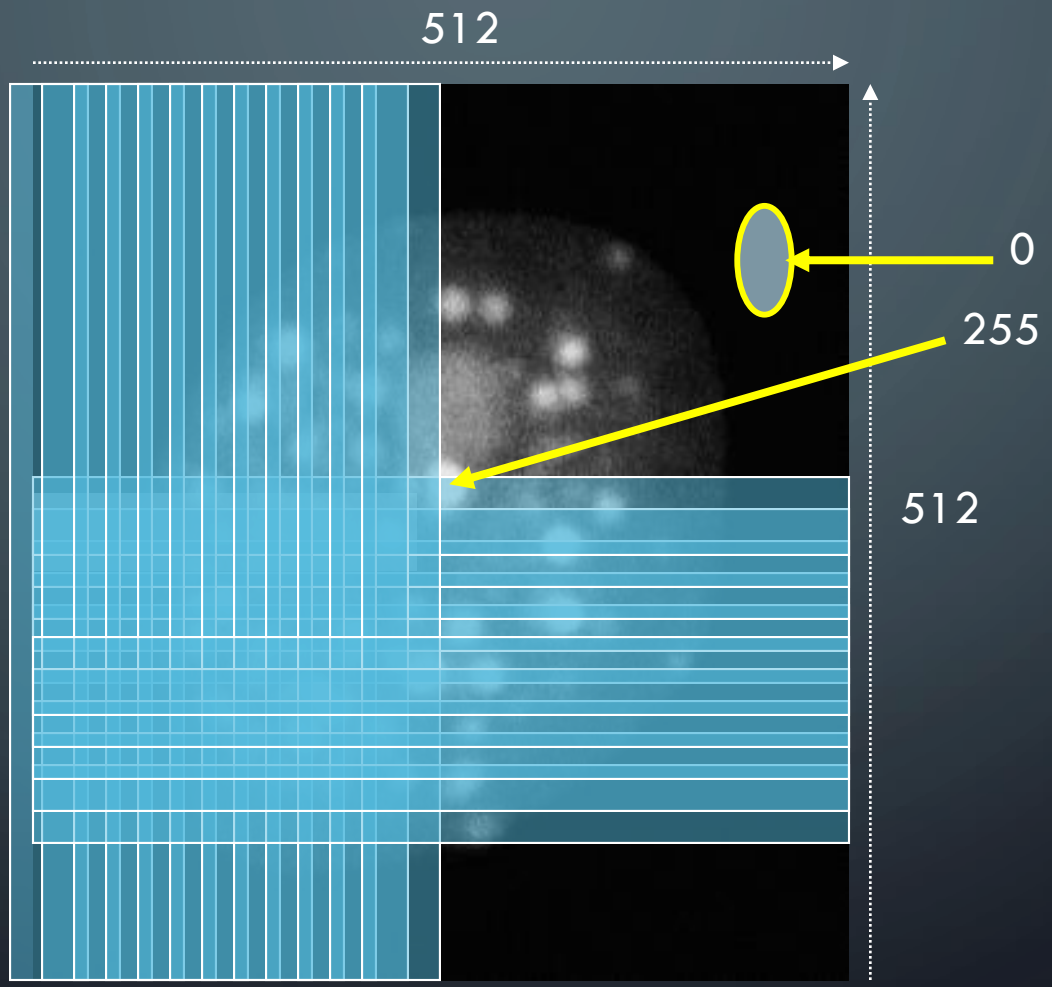


IMAGE SIZE

- The higher the resolution of the image, the more data points there are
- Very high resolution files need to be reduced in size to store the data
- We need to employ compression algorithms to reduce the file size
- But the goal is to maintain the quality of the image

Compression

Run Length Encoding – count the number of identical values, replace the values with a count followed by the value

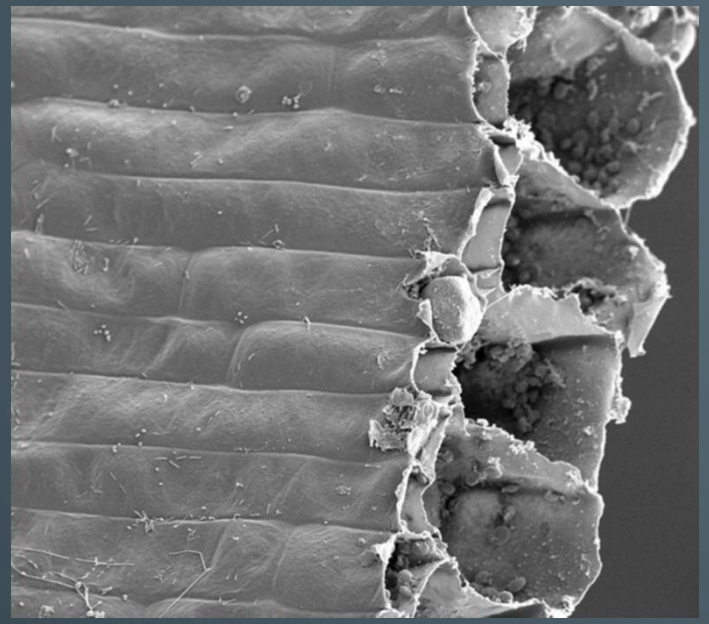
				RUN LENGTH ENCODING										
			Data File											
0	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**					
1	**0**	**0**	**1**	**2**	**0**	**0**	**1**	**1**	**1**					
1	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**					
0	**2**	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**1**					
			Lossless Run-length encoding											
5	**0**	**2**	**1**	**3**	**0**									
1	**1**	**2**	**0**	**1**	**1**	**1**	**2**	**2**	**0**	**3**	**1**			
2	**1**	**4**	**0**	**1**	**1**	**2**	**0**	**1**	**1**					
1	**0**	**1**	**2**	**1**	**0**	**1**	**1**	**0**	**1**	**1**	**2**	**0**	**2**	**1**
			Lossy Run-length encoding											
10	**0**													
10	**0**													
10	**0**													
10	**0**													

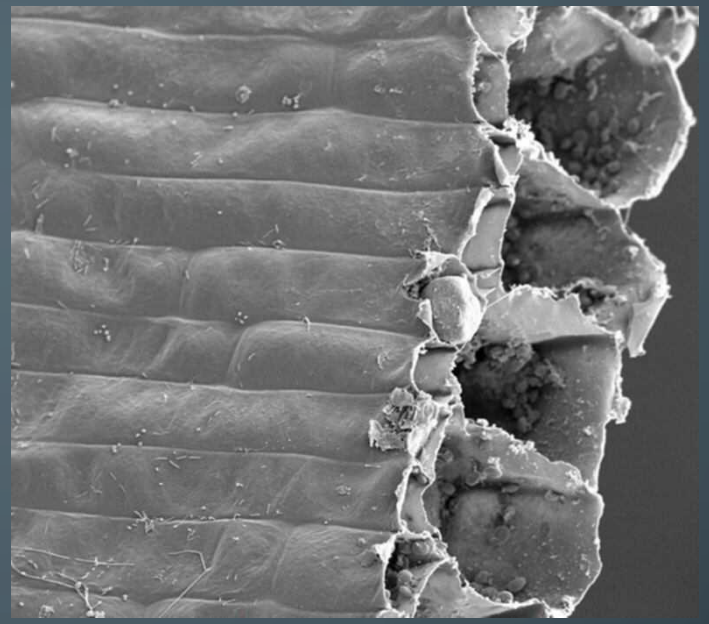
A kind of compression algorithm which replaces sequences ("runs") of consecutive repeated haracters

0% Compression

50% Compression

76k



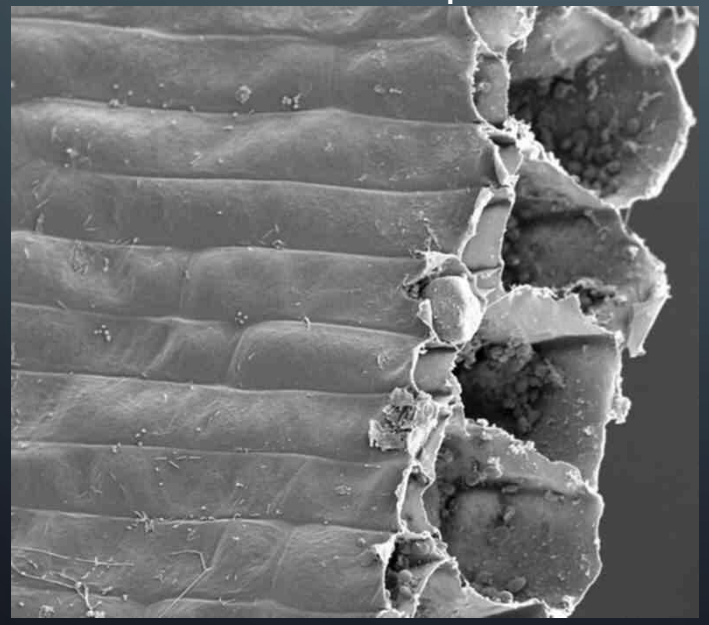


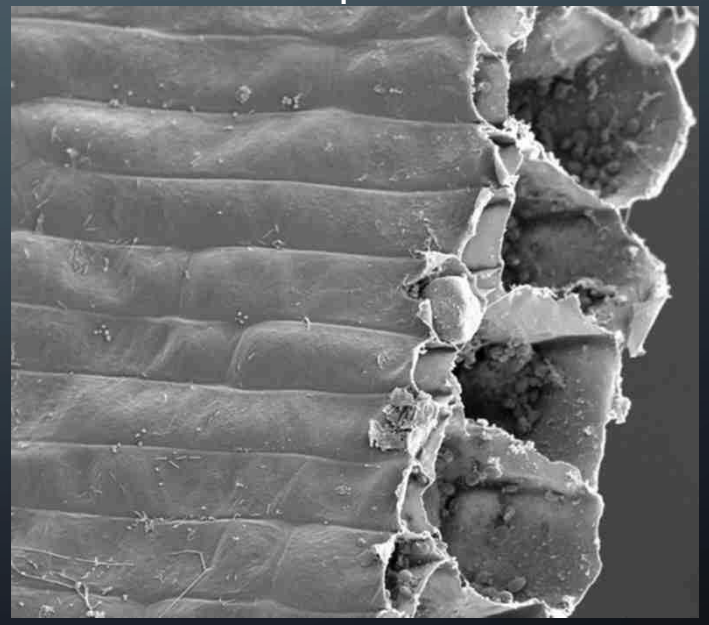
42k

75% Compression

80% Compression

28k





26k

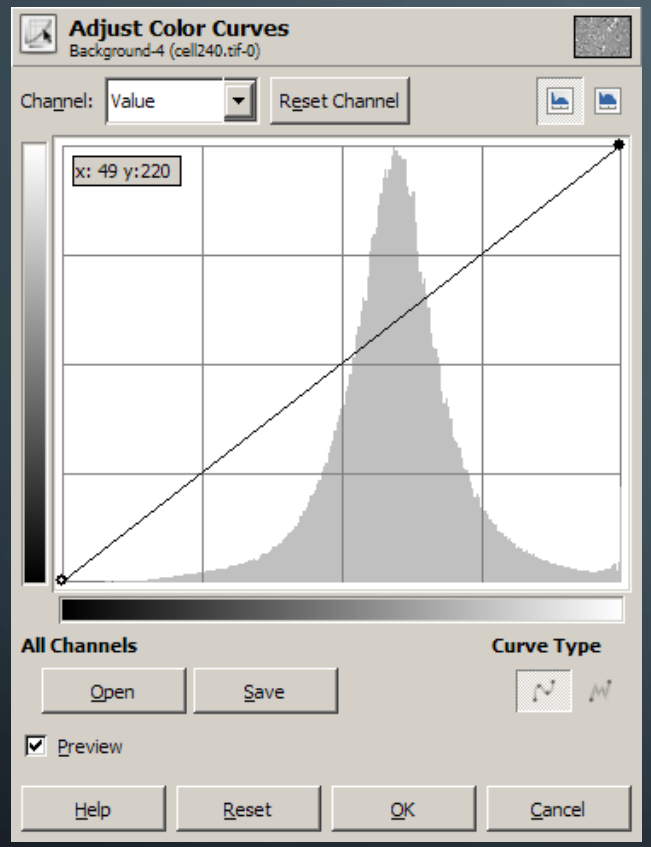
What is image analysis?

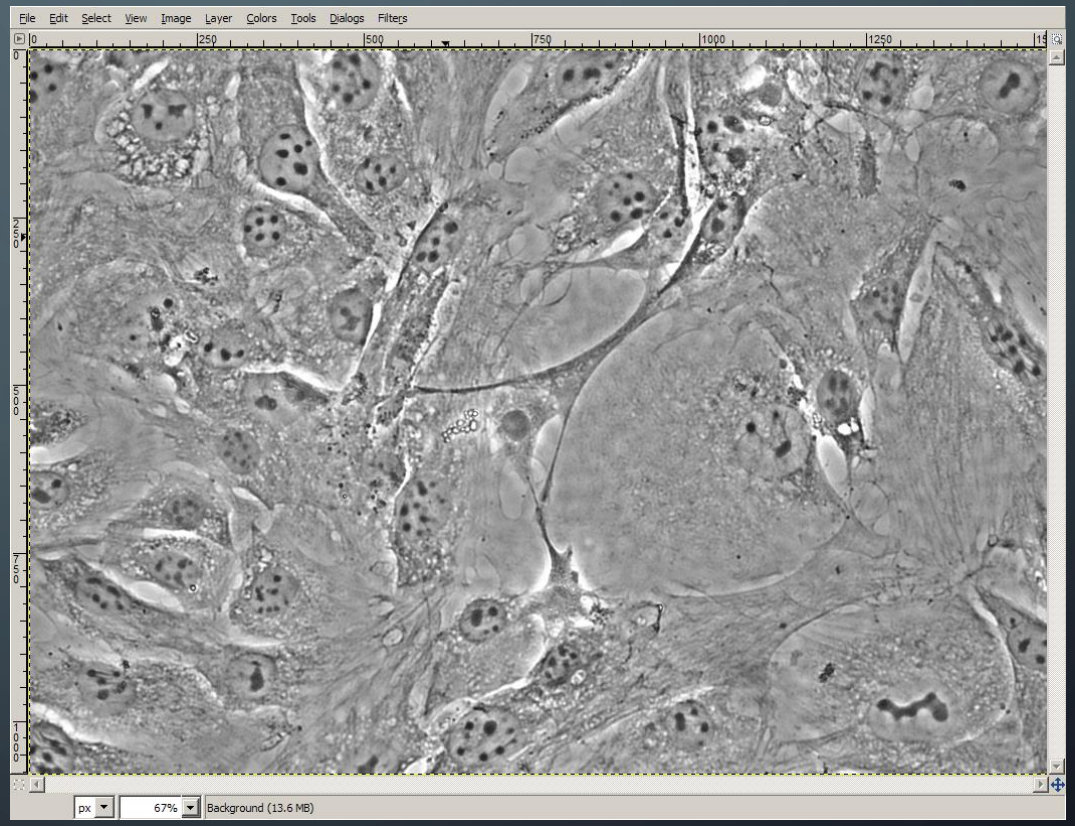
Brightness and contrast variation are controlled by a system input-output curve.

Spatial kernel filtering and median filtering use information local to a particular area of an image to modify that area.

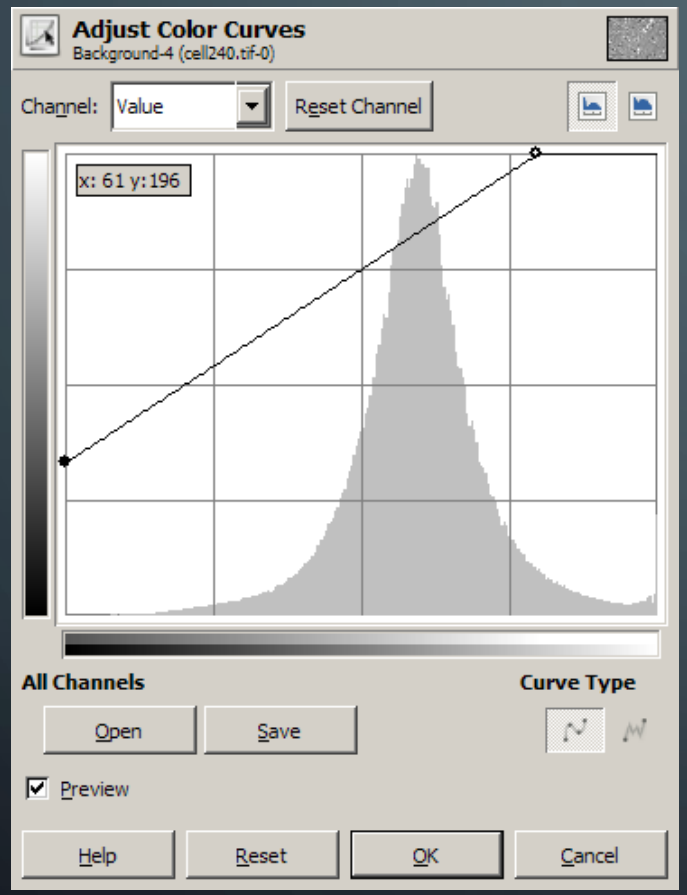
Digital image analysis is “Data Analysis”.

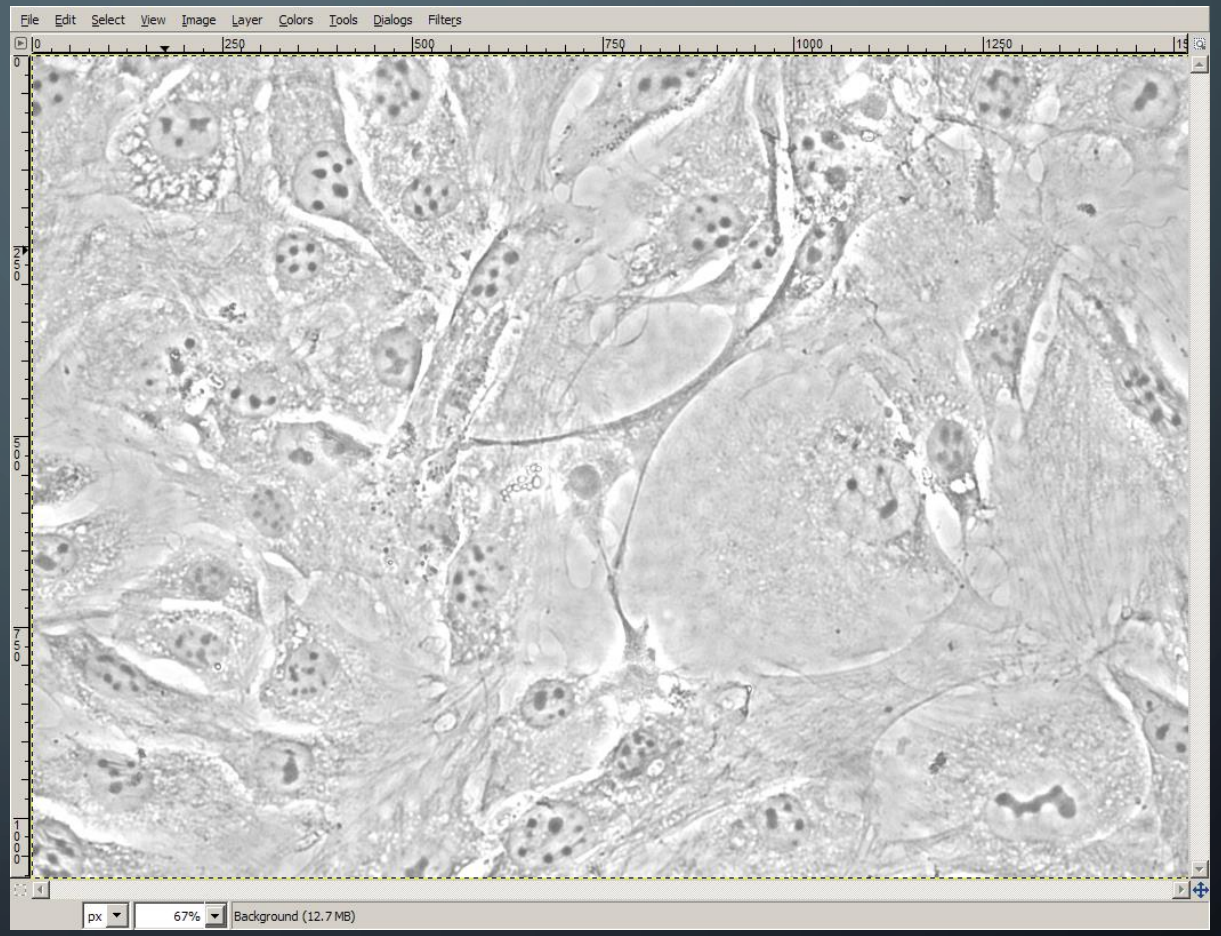
Input-Output Curve



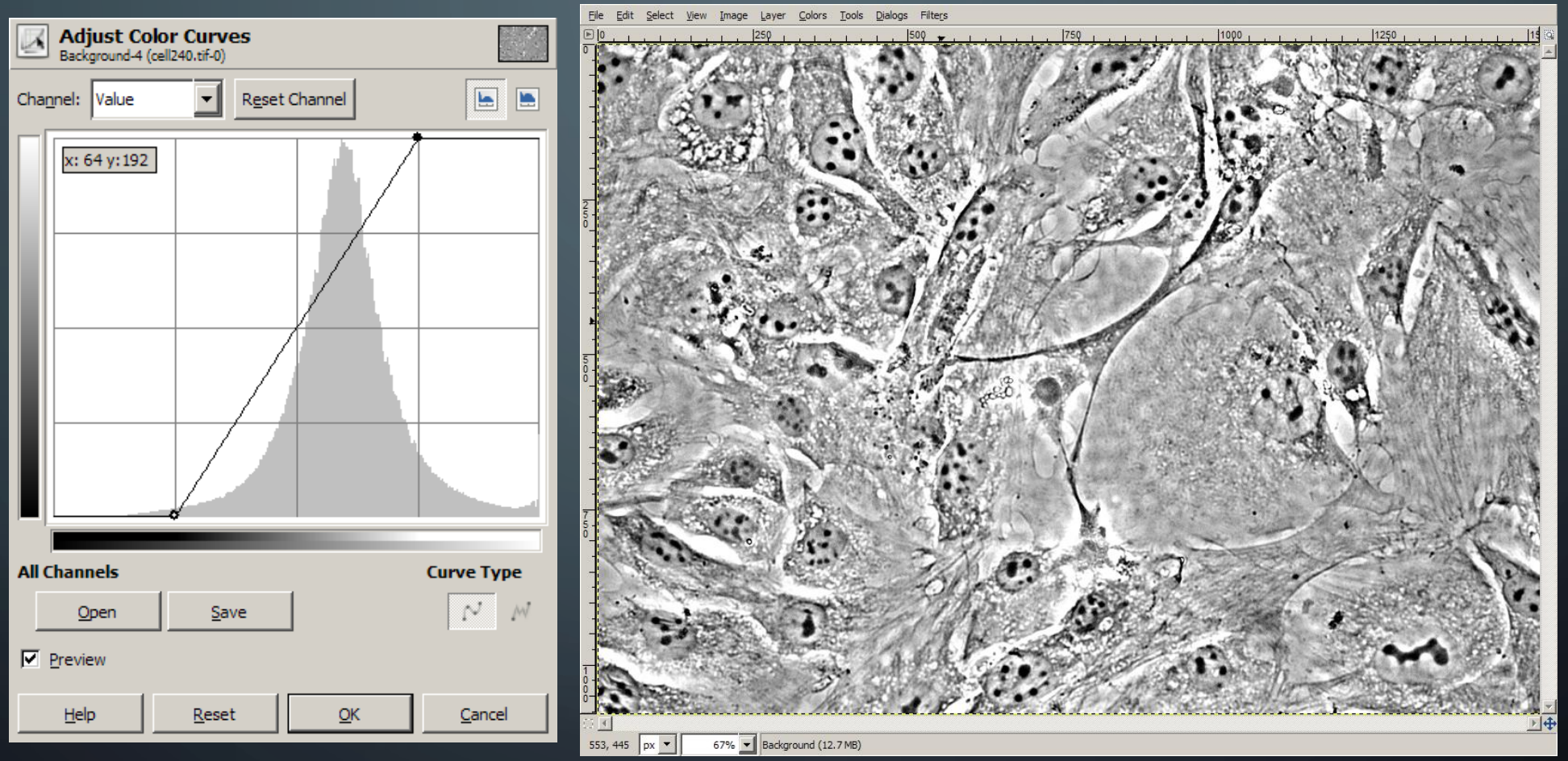


Input-Output Curve





Input-Output Curve



Mechanics of kernel convolution

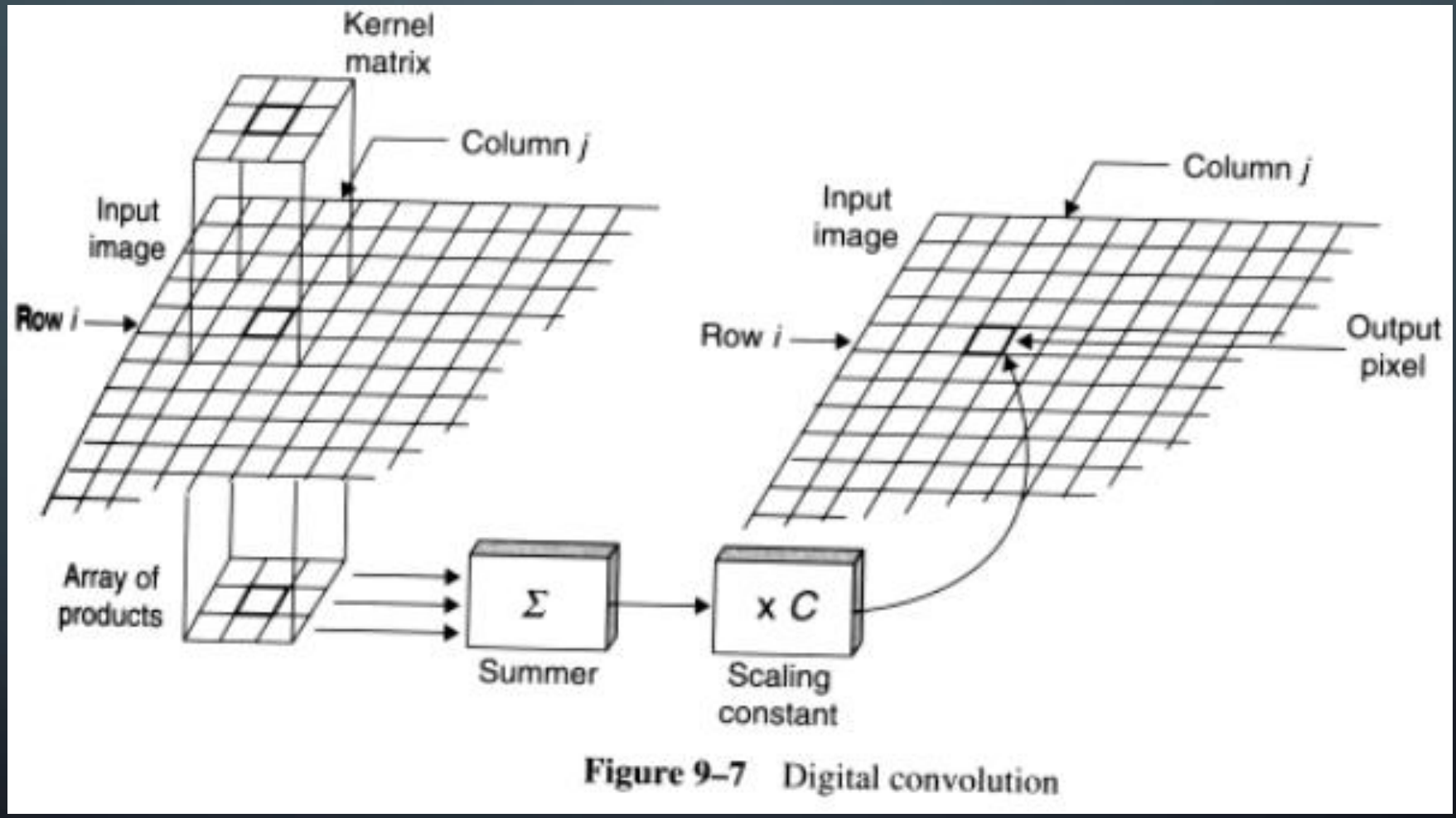


Figure 9–7 Digital convolution

Gaussian kernel filter

1	2	1
2	4	2
1	2	1

Divisor=1+2+1+2+4+2+1+2+1
=16

Section of image file

127	129	123	121	124	130
139	134	133	134	137	137
127	130	133	134	132	131
123	122	131	129	129	125
118	122	122	124	123	122
124	121	117	116	114	118
127	121	114	110	109	114
136	124	122	117	111	109

Smoothing

cells2.pgm

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
2	200	150												
3	255													
4	1 x 127	2 x 129	1 x 123	121	124	130								
5	2 x 131	4 x 134	2 x 133	134	137	137								
6	1 x 127	2 x 130	1 x 133	134	132	131								
7	123	122	131	129	129	125								
8	118	122	122	124	123	122								
9	124	121	117	116	114	118								
10	127	121	114	110	109	114								
11	136	124	122	117	111	109								
12	139	134	123	115	110	106								
13	157	148	136	124	113	105								
14	161	151	140	128	121	116								
15	178	170	162	150	138	122								
16	172	172	162	152	138	123								
17	176	173	163	162	148	134								
18														
19														
20														
21														
22	127	1 x 129	2 x 123	1 x 121	124	130								
23	131	2 x 134	4 x 133	2 x 134	137	137								
24	127	1 x 130	2 x 133	1 x 134	132	131								
25	123	122	131	129	129	125								
26	118	122	122	124	123	122								
27	124	121	117	116	114	118								
28	127	121	114	110	109	114								
29	136	124	122	117	111	109								
30	139	134	123	115	110	106								
31	157	148	136	124	113	105								
32	161	151	140	128	121	116								
33	178	170	162	150	138	122								
34	172	172	162	152	138	123								
35	176	173	163	162	148	134								
36														

										130.75				

										130.75	130.875			

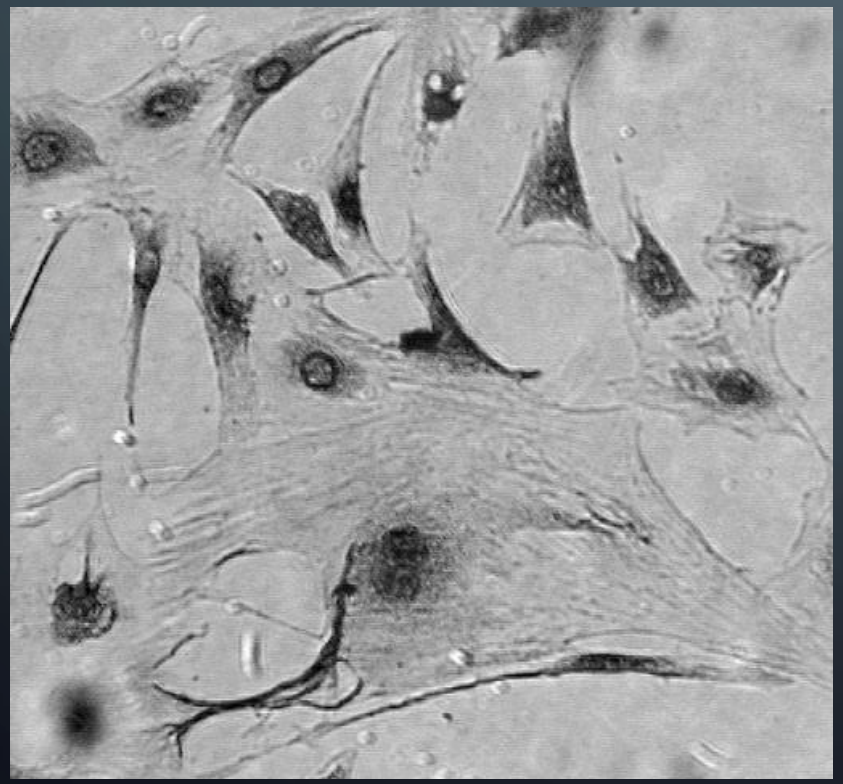
What is image manipulation?

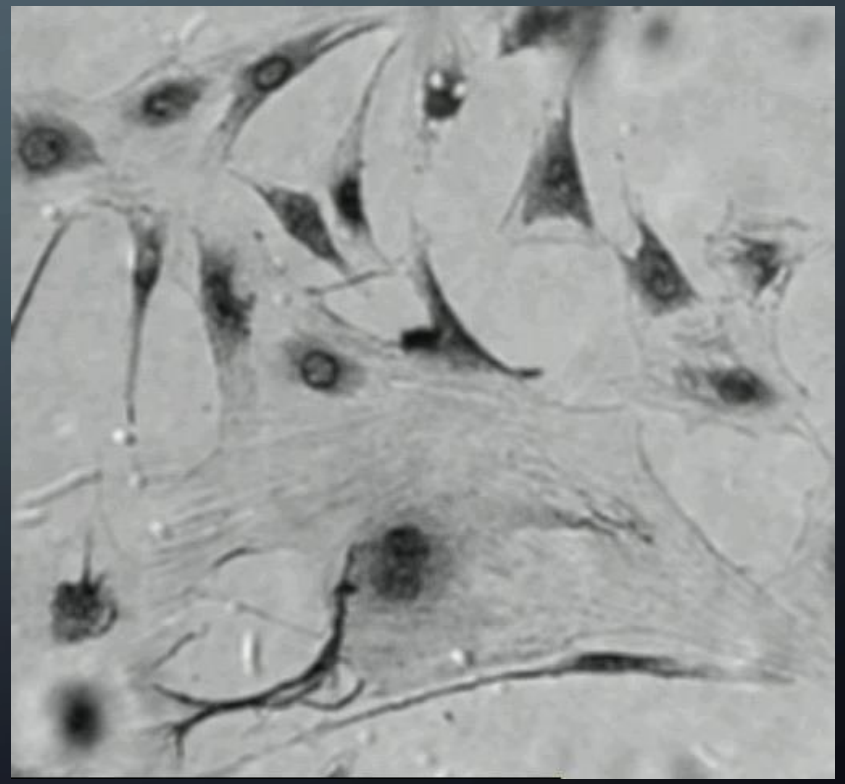
For most purposes, a one-to-one mapping of pixels to data values is most useful, but the internal representation of the data values may be different for different file formats.

Files can be either compressed, or not, and compression can be either lossy or not. For scientific analysis lossy compression is unacceptable; it may be useful for overview presentations.

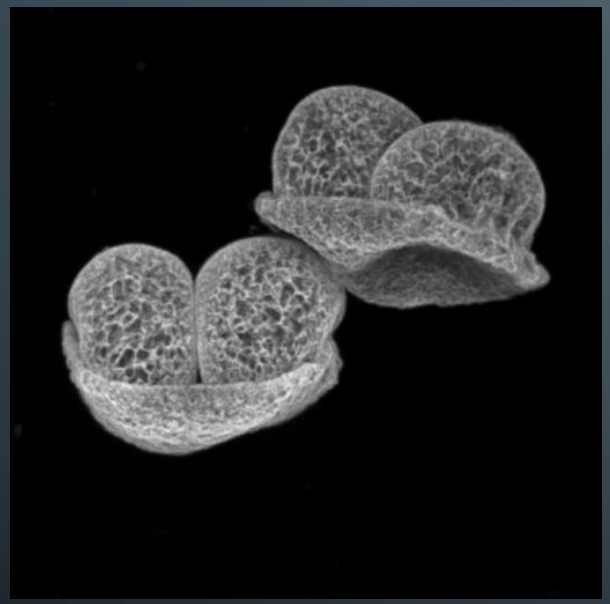
Image manipulation can take place before image acquisition, during image acquisition, on the digital data, or during recreation of an output image.

Smoothing Filter		
1	1	1
1	1	1
1	1	1

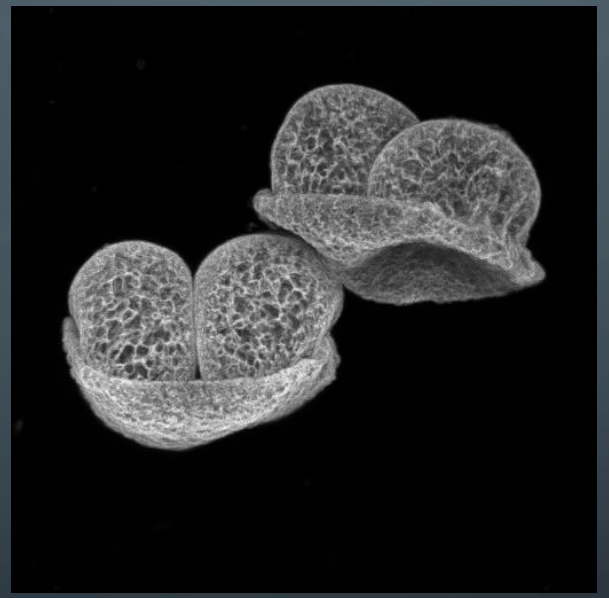




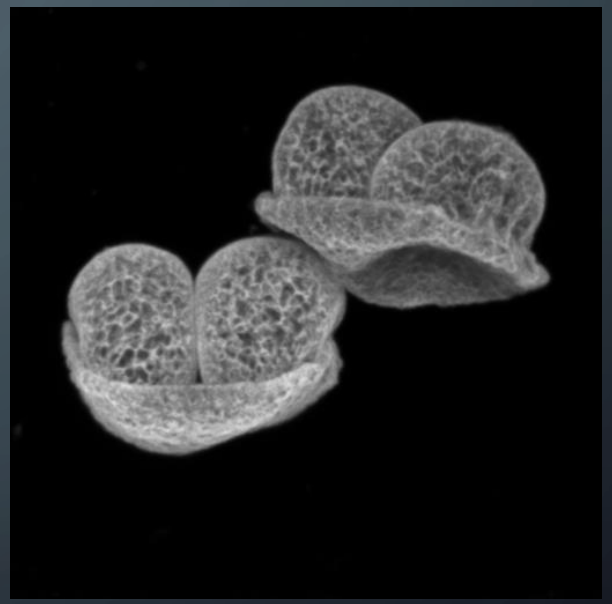
SMOOTHING



3 x 3 Smooth

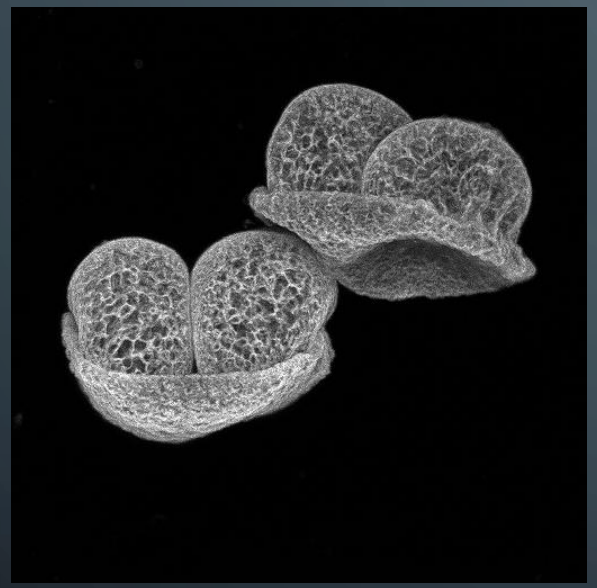


original

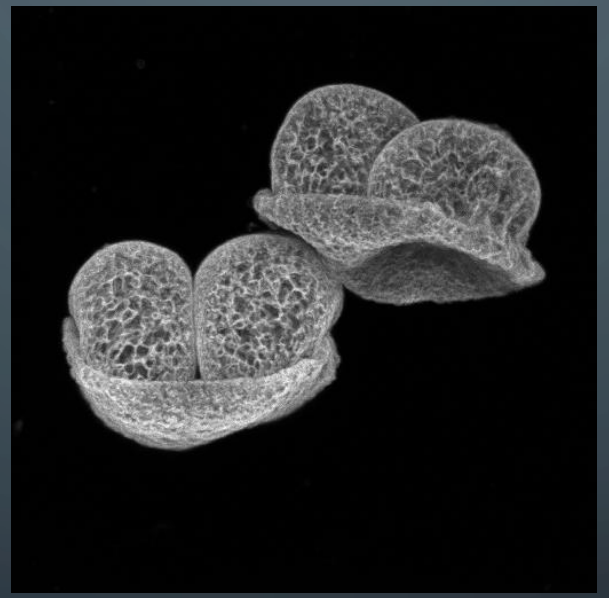


9 x 9 Smooth

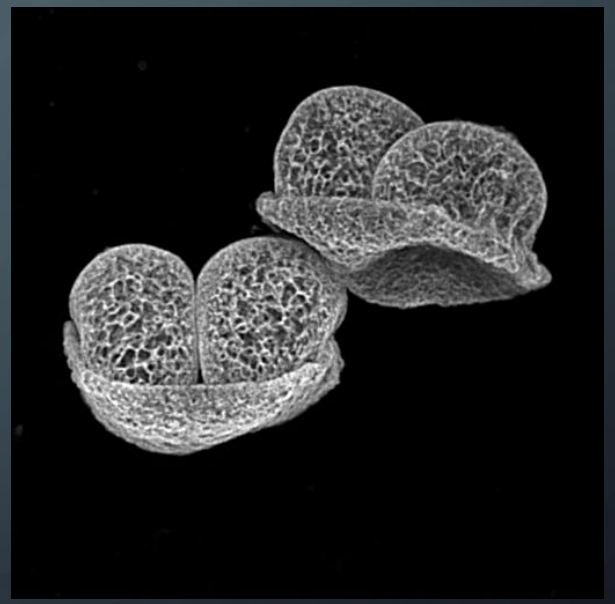
SHARPENING



3 x 3 Sharpen

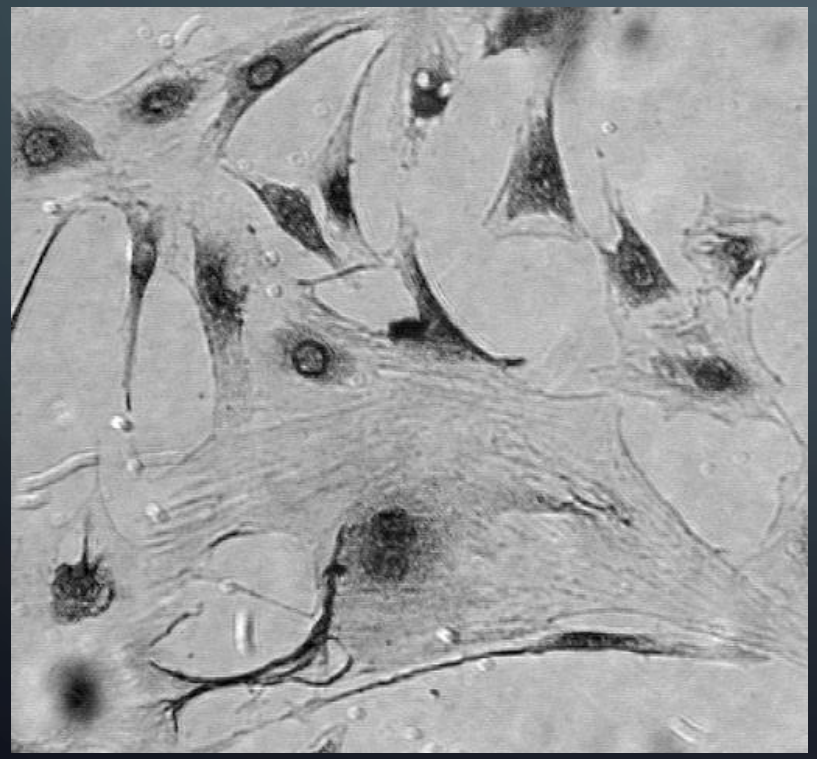


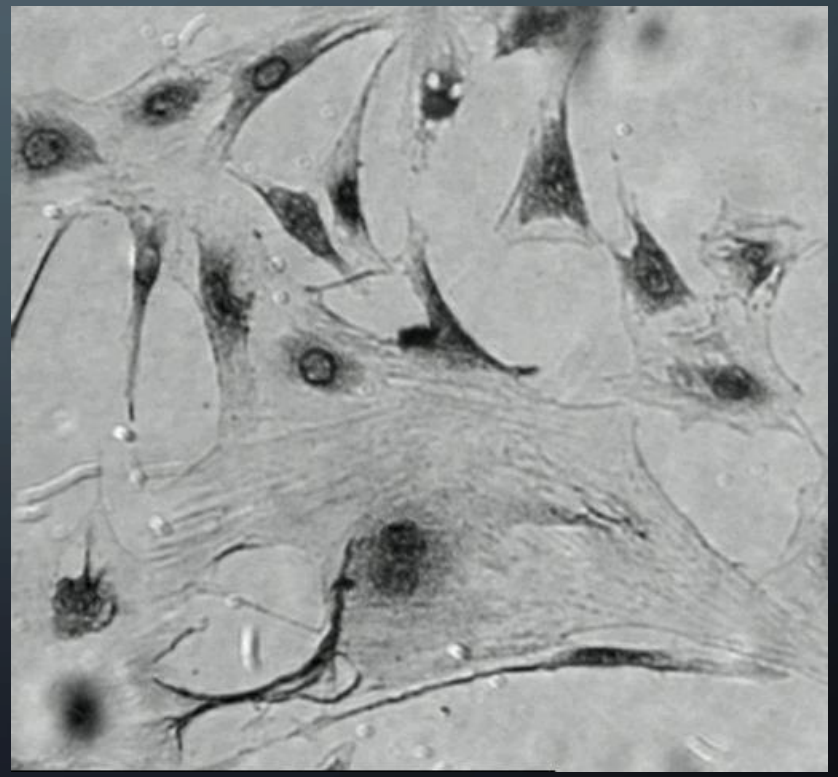
original



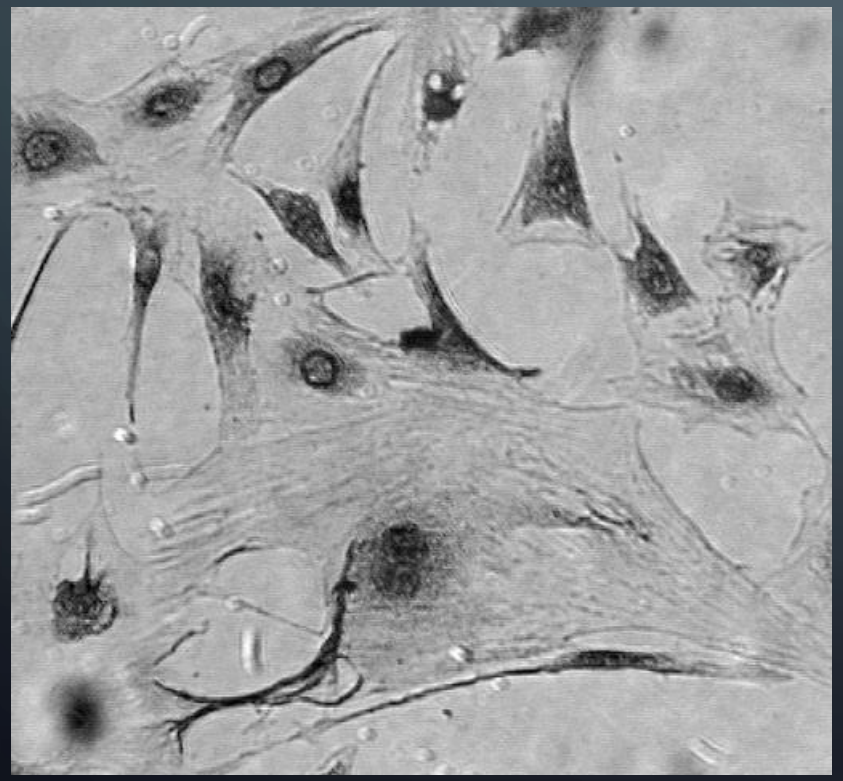
9 x 9 Sharpen

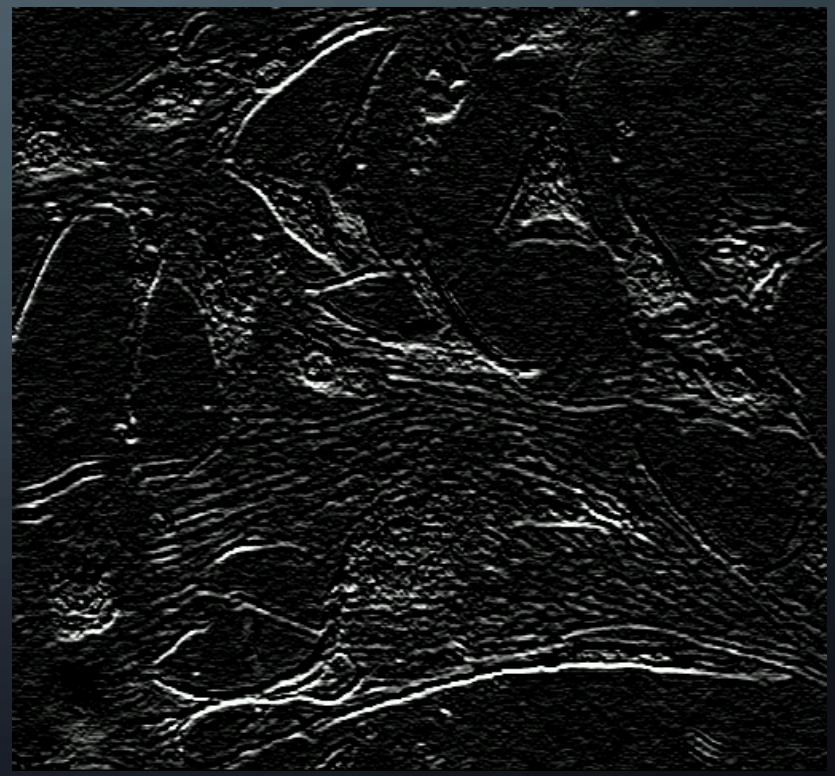
Gaussian Filter		
1	2	1
2	4	2
1	2	1



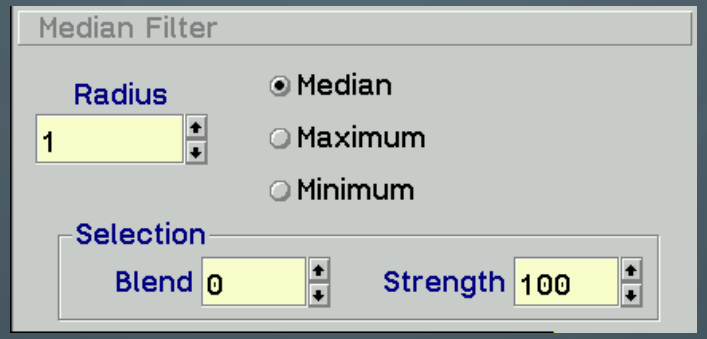


Sobel Filter		
1	2	1
0	0	0
-1	-2	-1





Application of a median filter



LOSSY COMPRESSION

Most filters will result in lossy compression

This means once you compress the image, you can never return the image back to its original self

One needs to be careful with biological data to ensure that you preserve the raw files

Lossless: TIF, BMP, GIF

Lossy: jpg, png

SUMMARY

- Structure of data files
- Image manipulation
- Kernels
- Filters