Light Microscopy for Biomedical Research

Tuesday 4:30 PM Quantification & Digital Images

$E = h\nu$

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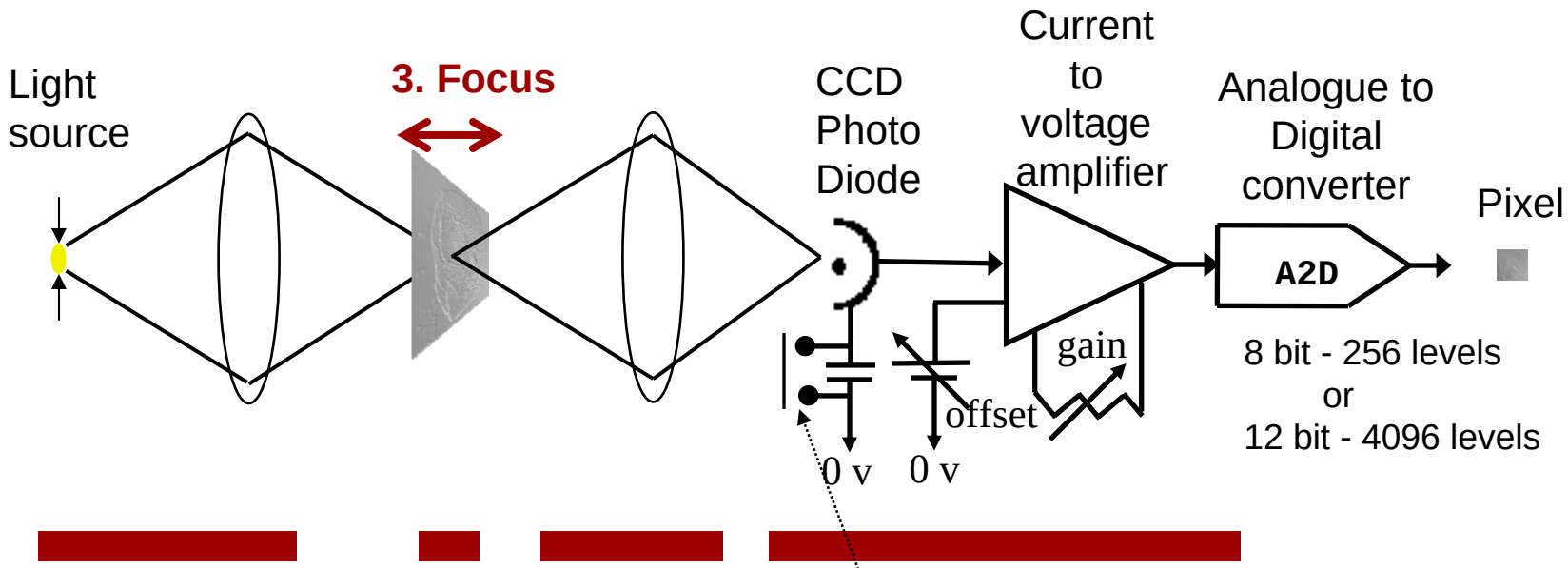
http://microscopy.unc.edu/lmbr

Quantification - intensity



1. Illumination

2. [dye]

4. Optical collection

i.e. objective, lenses, filters, apertures, mirrors, Pin hole size, etc.

5. Detector

charge store reset = exposure time

Pixel value depends on:

1. Illumination intensity
2. Dye concentration
3. Focus
4. Optical collection
5. Detector gain * exposure time

Quantification

Pixel value depends on (broadly):
1. Illumination intensity
2. Dye concentration
3. Focus
4. Optical collection
5. Detector gain

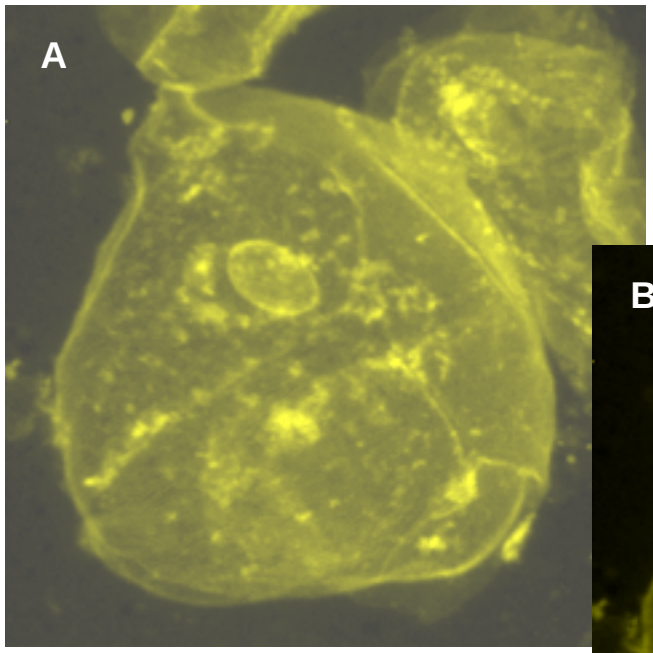
Really a multitude of detailed parameters.

1. Illumination: arc lamp light flicker, laser oscillations, stable control of lamp voltage, long term drift, age of lamp, laser, good Kohler setup, aperture size, coupling lens efficiency, etc, etc, etc.
2. Dye concentration: light absorbance by other material, fluorescent dye not light saturated, photobleaching, etc, etc, etc.
3. Focus: stage does not drift, live cell does not move away, thickness of sample, depth of view, etc, etc, etc.
4. Optical collection: objective NA, objective glass, objective aperture open, confocal pin hole size, etc, etc, etc.
5. Detector gain: exposure time, detector gain, PMT voltage, electrical gain, in linear range of detector, not overloaded A2D converter (saturation), not underloaded A2D converter (black clipping), intensifier gain, etc, etc, etc.

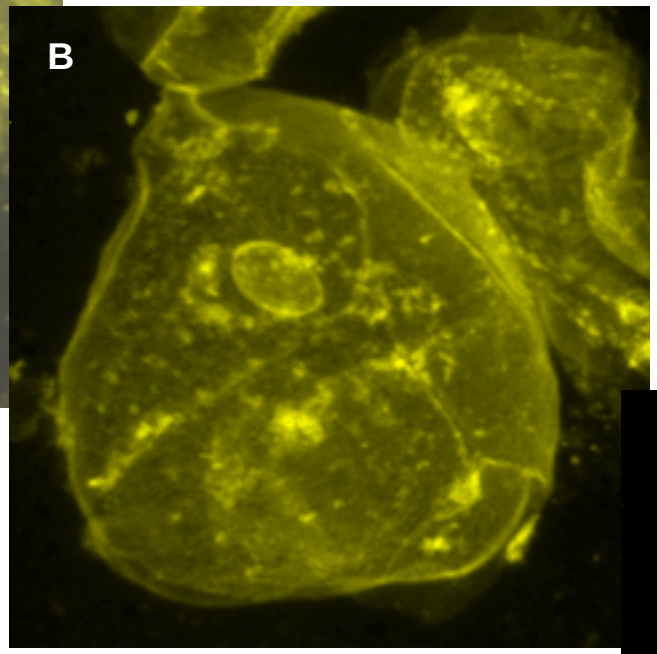
James Pawley published 39 steps: now has even more steps.

Work hard to keep them constant. E.g. parallel processing of samples. E.G. Time lapse can be good control.

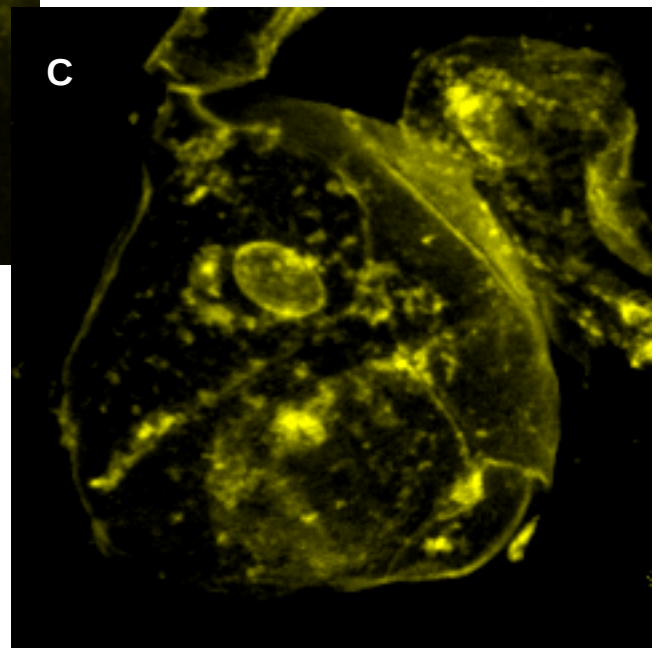
Quantification



Which image looks the best?

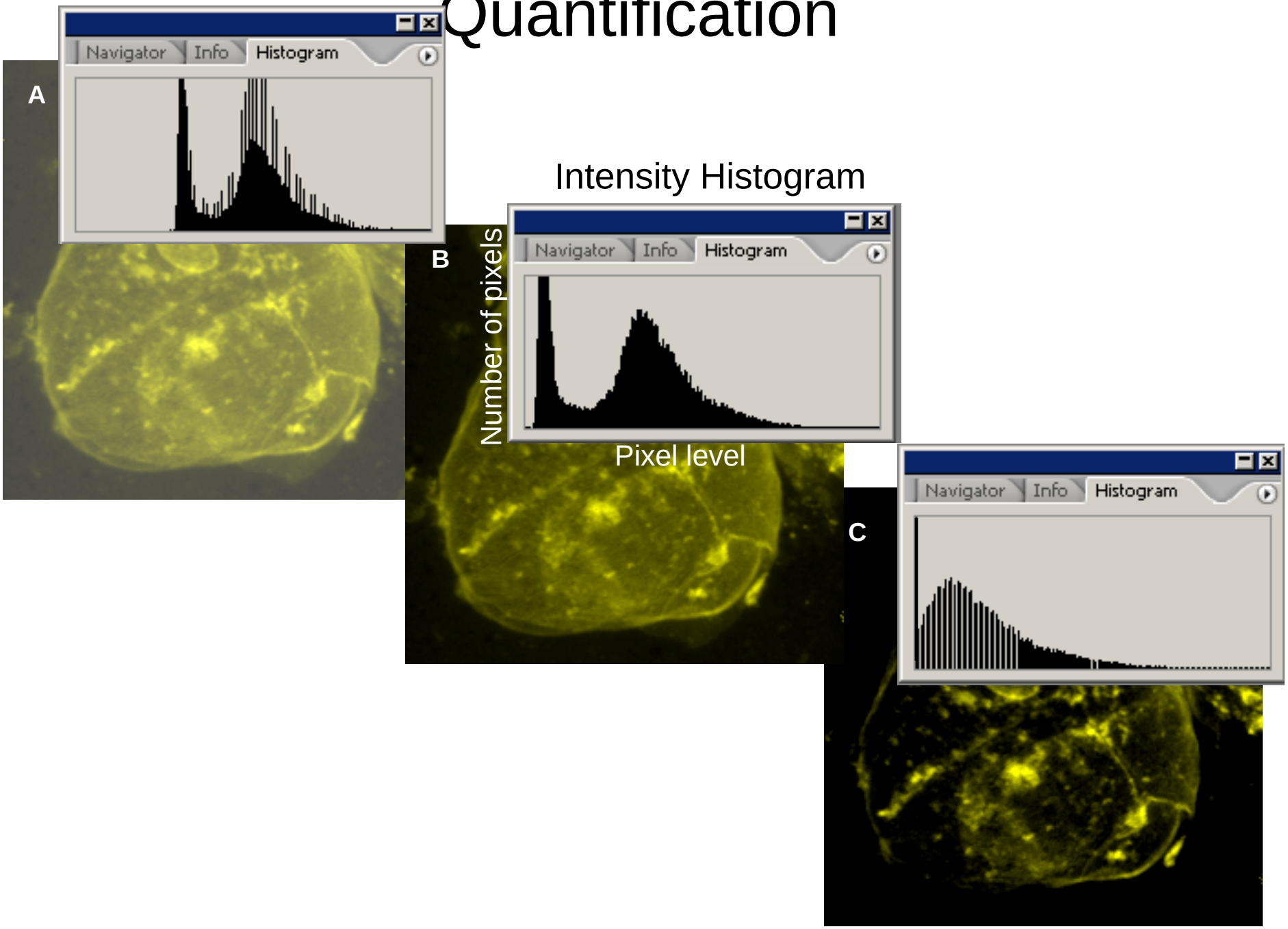


FM 1-43 Intensity is proportional to lipid content



Which image is the best?

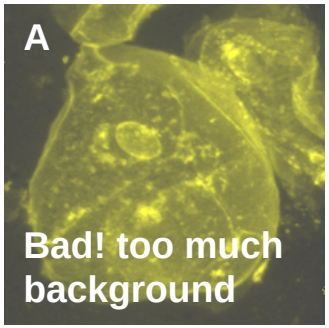
Quantification

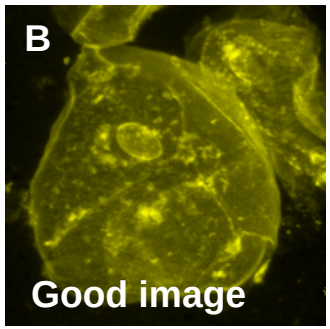


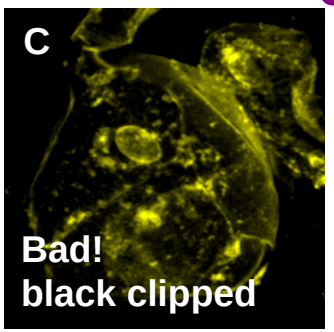
Quantification

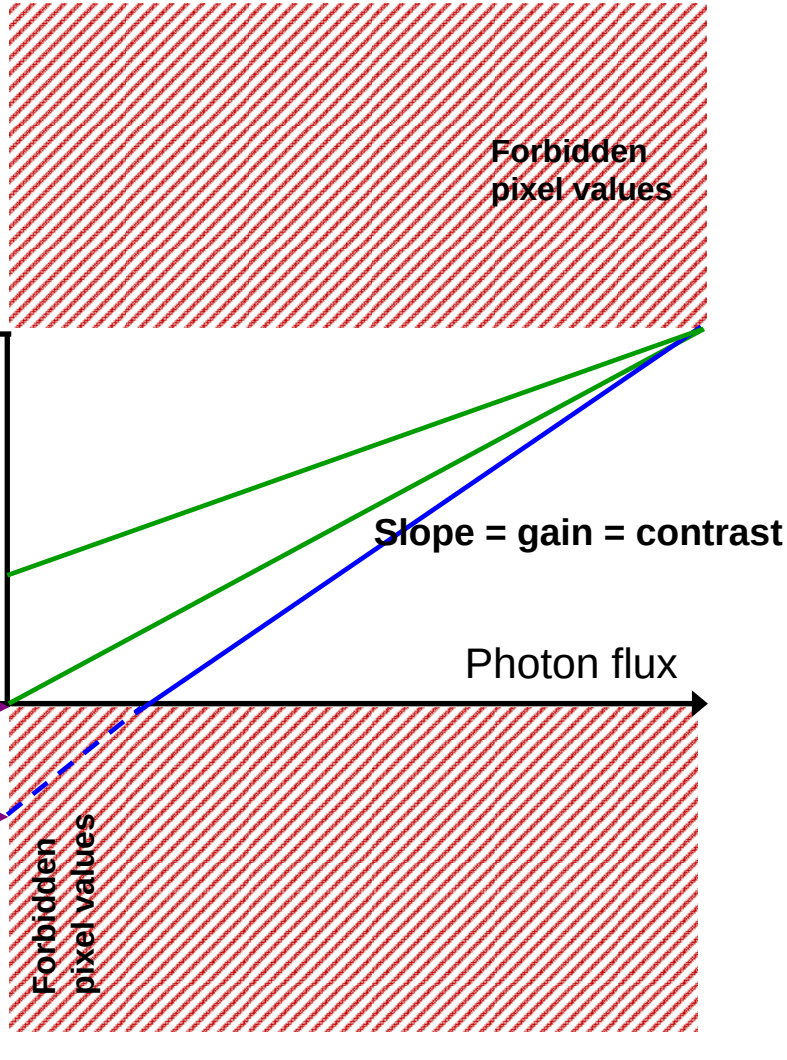
In a well designed system
the A2D converter sets the minimum
and maximum value which can be
digitized.

Minimum is 0
Maximum set by number of levels





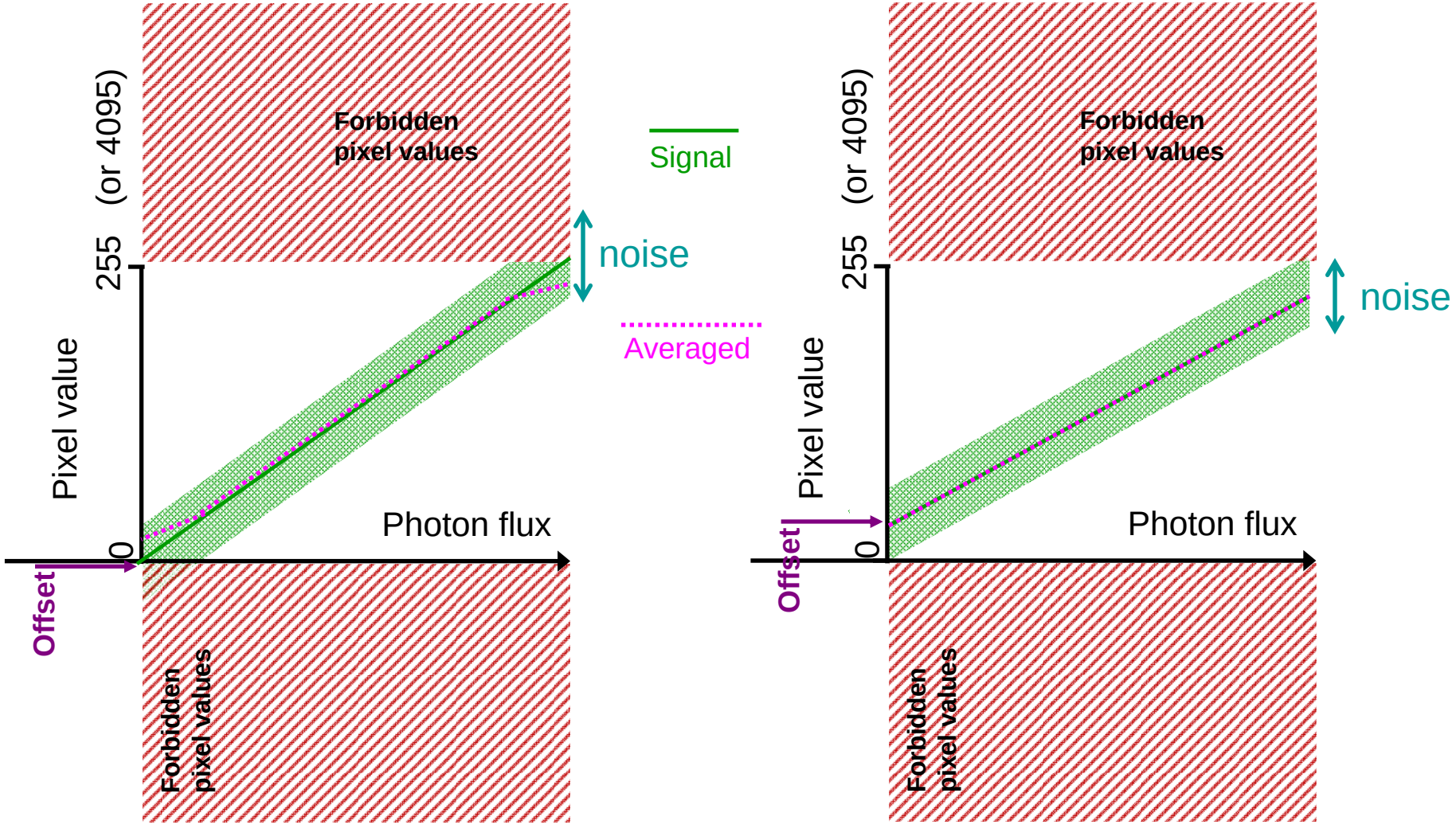




Quantification

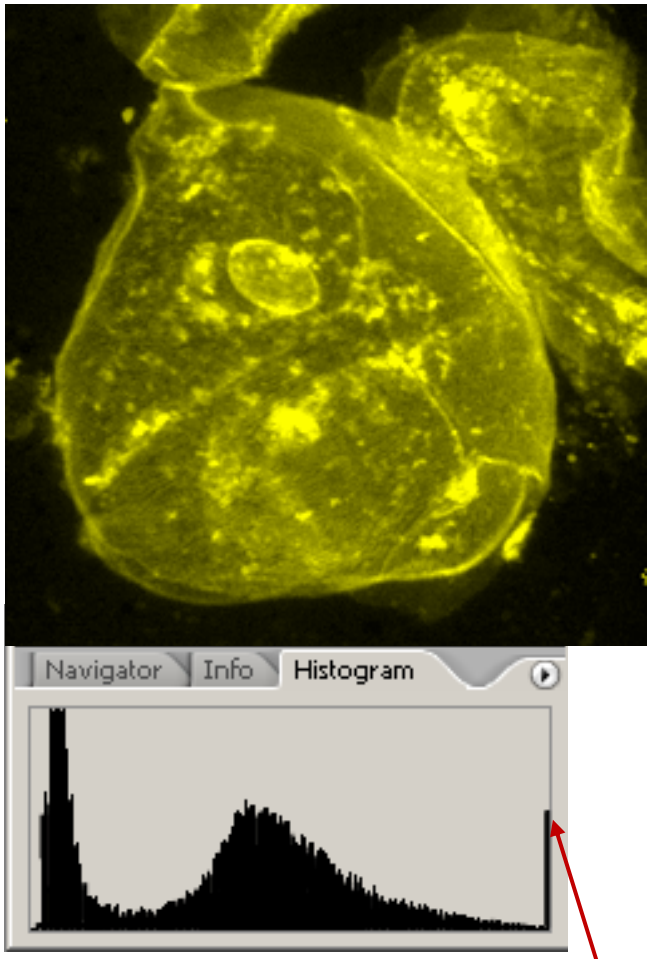
Noise adds linearly to photon signal
Noise will average to zero if sampled without clipping

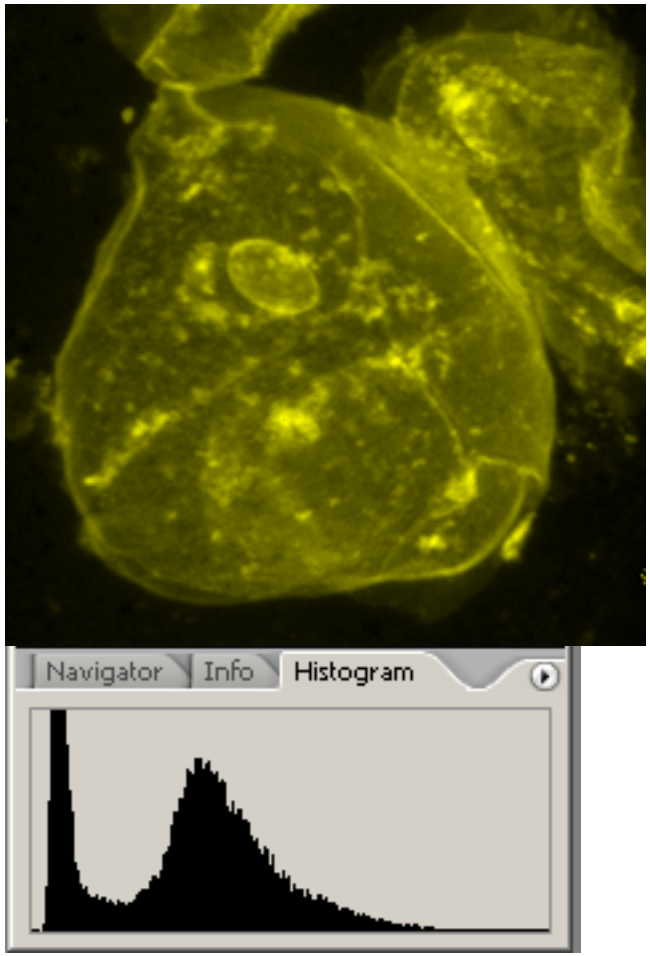
Reduced range – restore contrast after averaging



Quantification

Over exposed image





Saturated Pixels
(Information loss)

Quantification

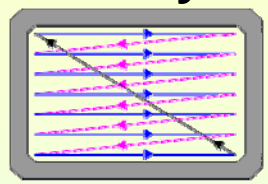
- Overload or underload leads to loss of information
- Allow room for noise (noise contains information)
- Recover contrast after acquisition
- Save data uncompressed or with lossless compression (not jpeg or gif for color images)

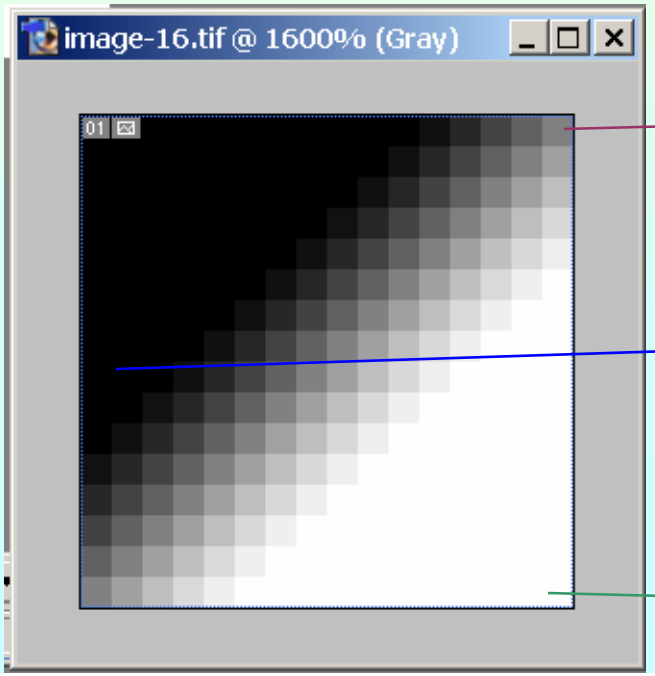
Pixel value depends on:

1. Illumination intensity
2. Dye concentration
3. Focus
4. Optical collection
5. Detector gain

Digital Image Representation

- Intensity values of pixels (picture elements) in a 2-D array for monochrome – f(x,y)
- Rasterised – left to right then top to bottom
- Numbers typically 8 bit binary (intensity values 0 to 255) – good for confocal with only a few dozen photons per pixel or 12 bit (0 to 4095 intensity levels for CCD cameras)





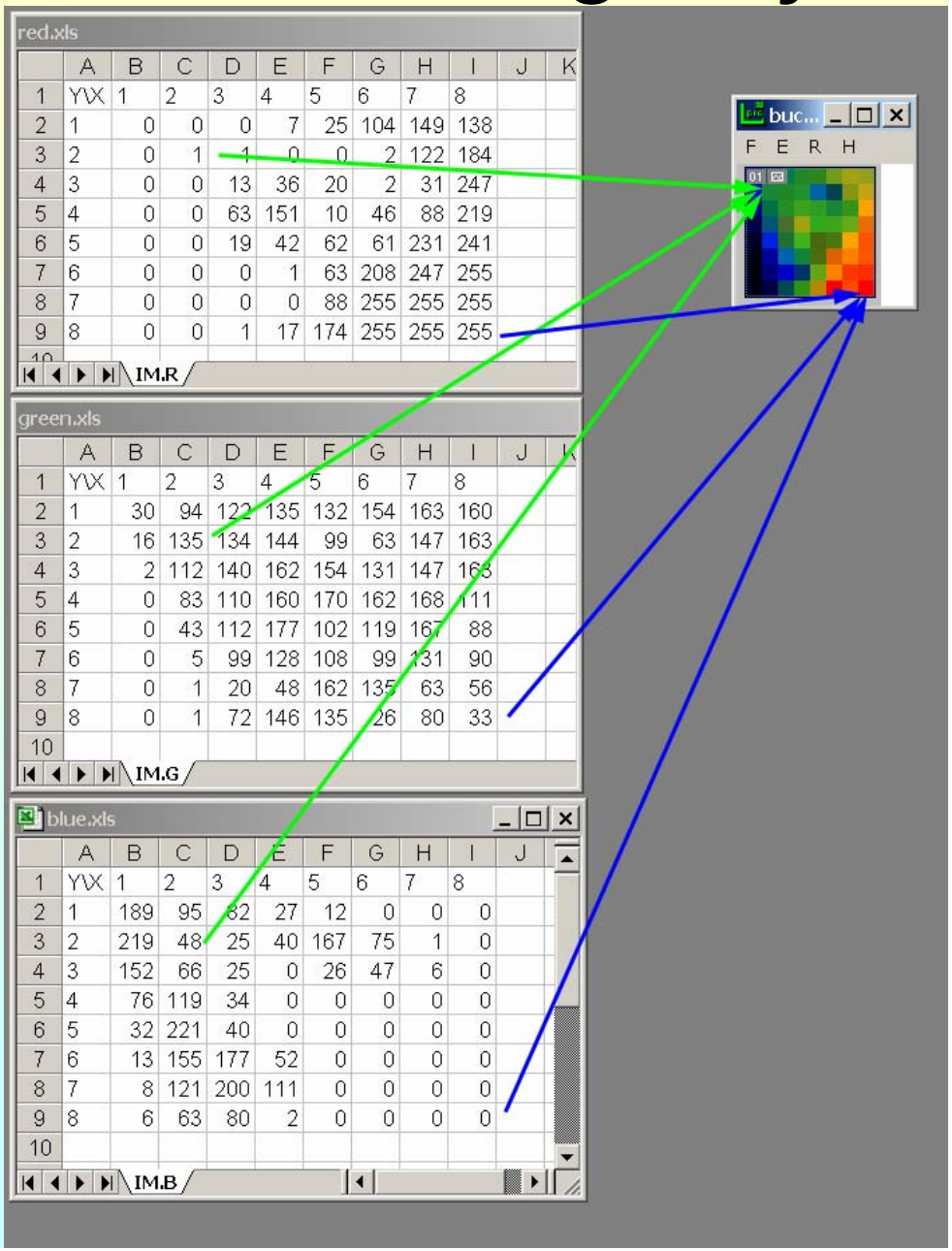
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	YX	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	1	0	0	0	0	0	0	0	0	0	0	0	16	39	66	96	127
3	2	0	0	0	0	0	0	0	0	0	0	16	38	66	95	127	159
4	3	0	0	0	0	0	0	0	0	0	16	38	65	96	128	159	189
5	4	0	0	0	0	0	0	0	0	17	38	66	96	127	159	189	216
6	5	0	0	0	0	0	0	0	16	39	66	96	127	159	190	216	238
7	6	0	0	0	0	0	0	16	38	66	96	128	159	190	217	239	255
8	7	0	0	0	0	0	16	39	65	96	128	159	189	217	239	255	255
9	8	0	0	0	0	16	38	66	96	128	159	189	216	238	255	255	255
10	9	0	0	0	16	39	66	96	128	159	189	216	238	255	255	255	255
11	10	0	0	17	38	66	96	128	159	190	216	239	255	255	255	255	255
12	11	0	16	39	66	96	128	160	189	216	238	255	255	255	255	255	255
13	12	17	39	66	96	127	159	189	216	239	255	255	255	255	255	255	255
14	13	38	66	96	127	159	189	216	238	255	255	255	255	255	255	255	255
15	14	65	96	128	159	189	216	239	255	255	255	255	255	255	255	255	255
16	15	96	127	159	189	217	239	255	255	255	255	255	255	255	255	255	255
17	16	128	159	189	217	239	255	255	255	255	255	255	255	255	255	255	255

Color Image Representation Digitally

Three intensity values, red, green & blue for each pixel in a 2 D array f(x,y,r), f(x,y,g), f(x,y,b)

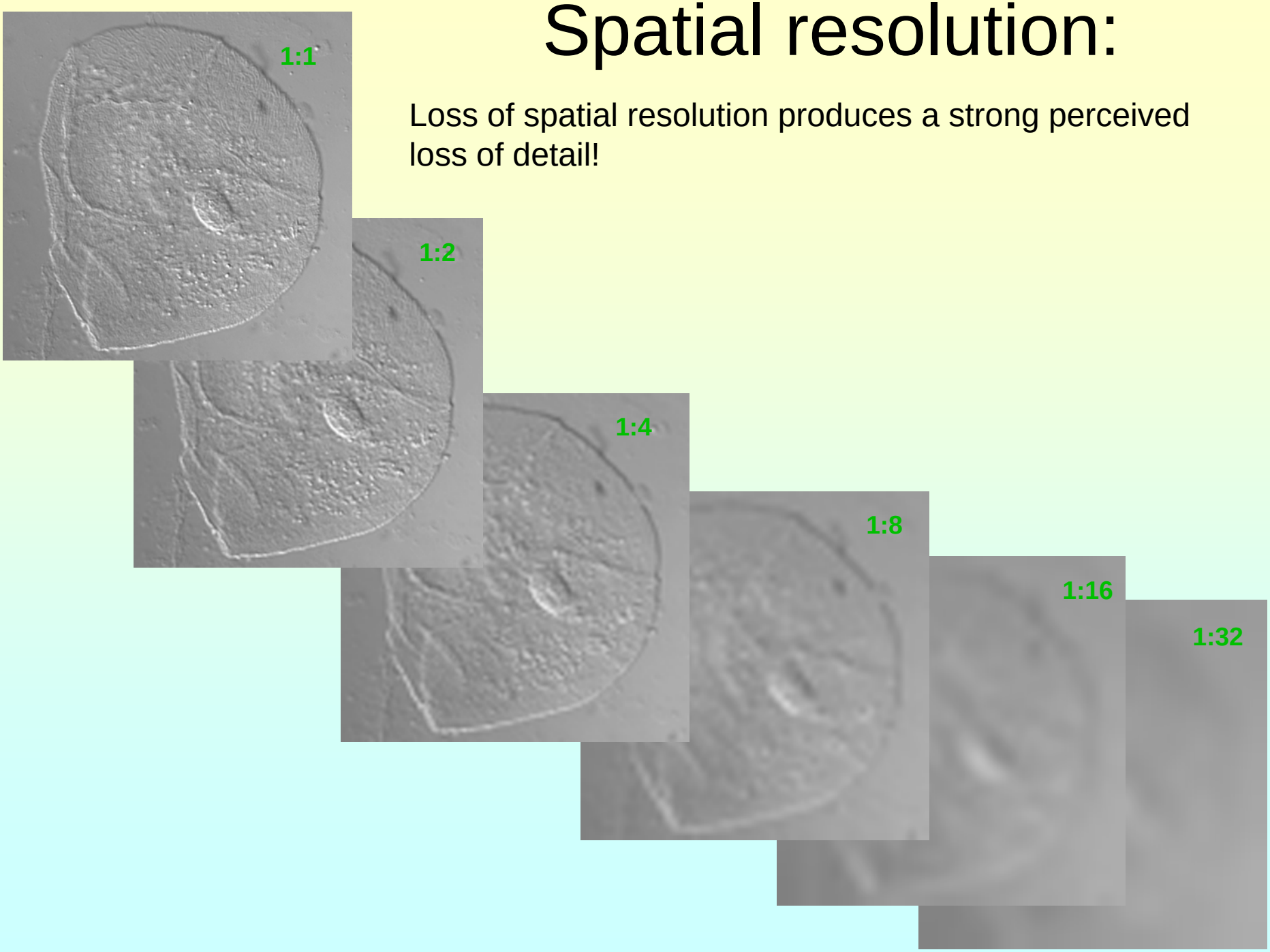
RGB image

- 8 bits red
- 8 bits green
- 8 bits blue
- Referred to as an RGB 24 bit image

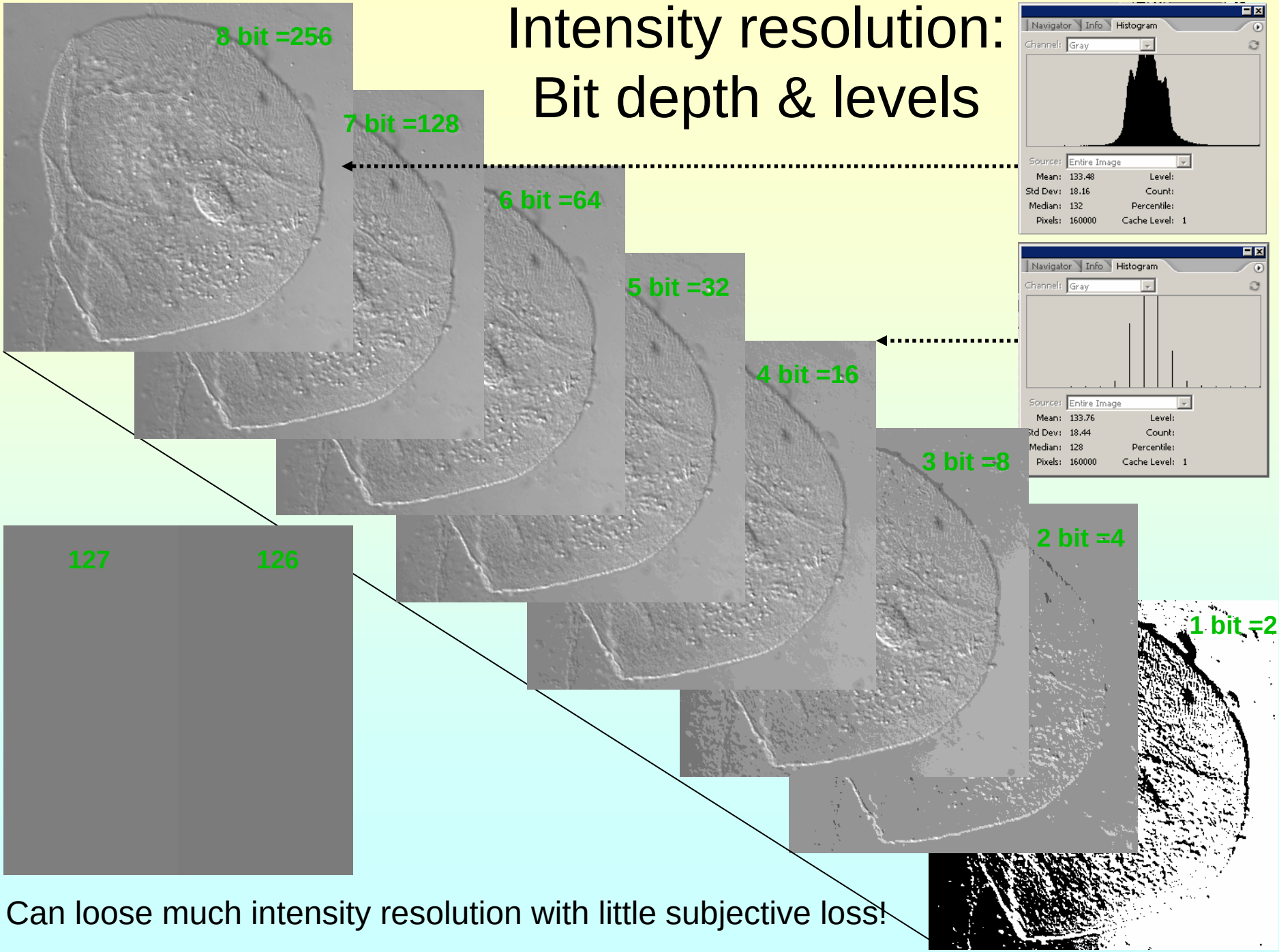


Spatial resolution:

Loss of spatial resolution produces a strong perceived loss of detail!



Intensity resolution: Bit depth & levels



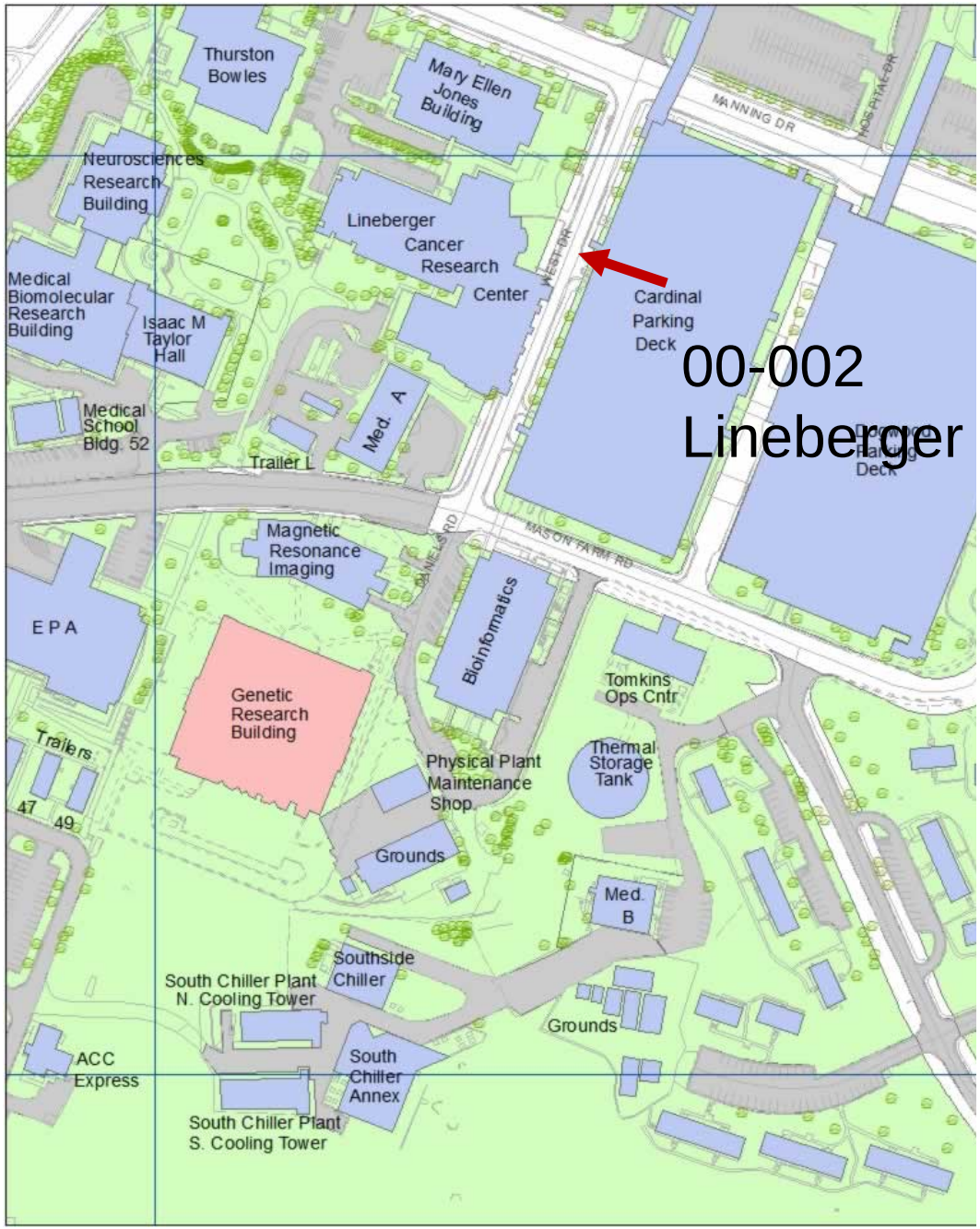
Can loose much intensity resolution with little subjective loss!

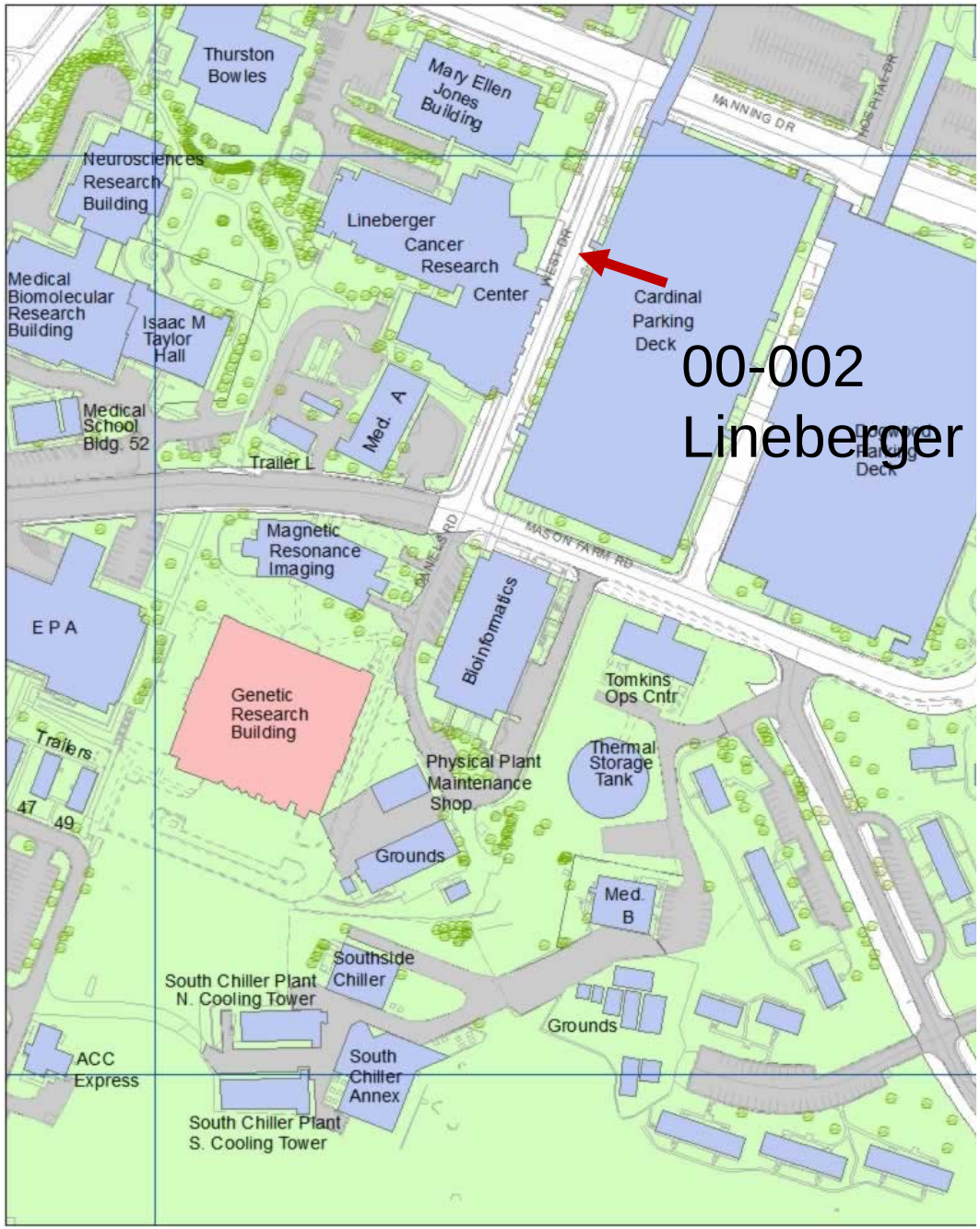
Digital Image

- **Summary:** majority of images are 2-D arrays of 8 bit monochrome, 24 bit RBG color

- Image processing not easy or meaningful unless image is a linear gray scale or RGB image. (photometrically correct, i.e. intensity corresponds to pixel value)

http://microscopy.unc.edu/lmbr


Thurston Bowles
Mary Ellen Jones Building
MANNING DR
HOSPITAL DR
Neurosciences Research Building
Lineberger Cancer Research Center
WEST DR
Medical Biomolecular Research Building
Isaac M Taylor Hall
Cardinal Parking Deck
00-002
Lineberger
Medical School Bldg. 52
Trailer L
Med. A
Magnetic Resonance Imaging
DANIELS RD
MASON FARM RD
EPA
Bioinformatics
Tomkins Ops Cntr
Genetic Research Building
Trailers
47
49
Physical Plant Maintenance Shop
Thermal Storage Tank
Grounds
Med. B
Southside Chiller
South Chiller Plant N. Cooling Tower
Grounds
ACC Express
South Chiller Annex
South Chiller Plant S. Cooling Tower



CAROLINA BREWERY
Chapel Hill Pittsboro

Dinner 6:30 PM
Carolina Brewery
540 W, Franklin St.
Chapel Hill

An Introductory Guide to Light Microscopy
Five Talk Plan

- Apr 16. A brief perspective of light microscopy - transmitted light, Kohler illumination, the condenser, objectives, Nomarski, phase contrast, resolution

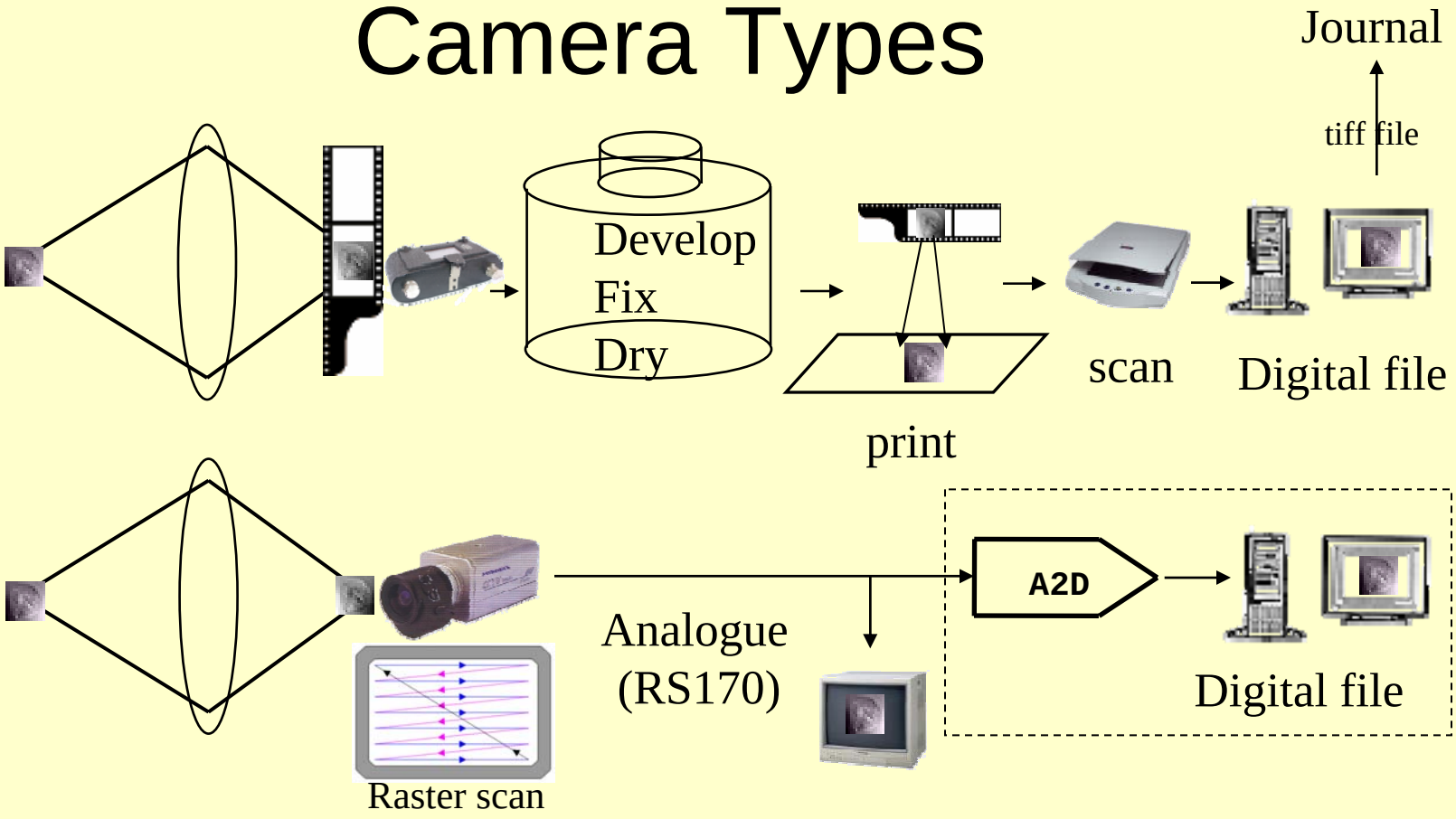
- Apr 23. Fluorescence - contrast, resolution, filters, immuno staining, fluorescent proteins, dyes.

- Apr 30. Detectors, sampling & digital images: Solid state digital cameras, Photomultipliers, noise, image acquisition, Nyquist criterion/resolution, pixel depth, digital image types/color/compression

- **May 07. Confocal Microscopy: Theory, sensitivity, pinhole, filters, 3-D projection/volume renders**

- May 14. Advanced Fluorescence/Confocal: Live cell imaging, co-localization, bleed through/cross talk, FRAP, fluorescence recovery after photobleaching, deconvolution

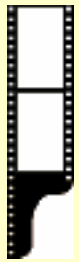
Camera Types



(A2D = analogue to Digital converter)

Camera Types - Comparison

- Film – negative - develop - print - slow, tedious, less sensitive, more expensive, non linear, color not so easy for multiple exposures with different filters e.g. multiple antibodies – no instant gratification!



QE < 0.03

- Video (TV)– 30 Hz set frame rate, exposure time limited by frame rate (16 ms), poor spatial resolution, poor intensity resolution – noisy (1953 standard based on 1940's capabilities) – requires an expensive A2D (frame grabber) – loose detection time due to raster scan – noisy connection to computer/monitor - It's so last century!

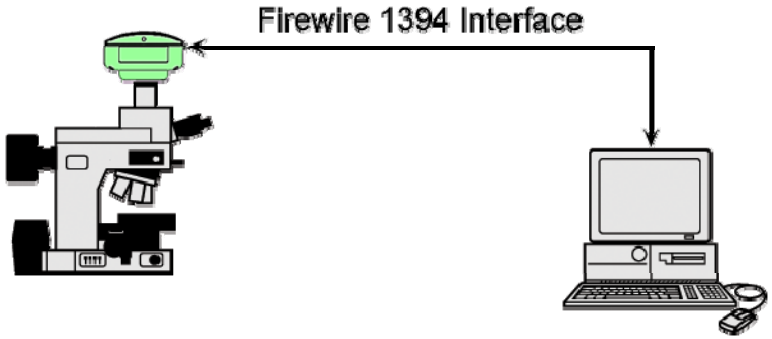
QE = 0.05 to 0.4

- CCD (charge coupled device) frame capture (c.f. domestic digital camera) – low noise, good linearity, good resolution, direct digital input to computer at no loss rate – but need a computer to see image.

QE = 0.1 to 0.9

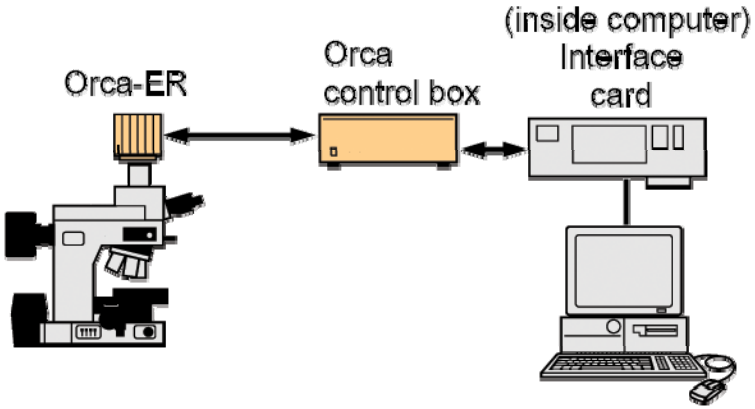
QE = Quantum Efficiency – fraction of input photons detected

Image Acquisition



MicroPublisher: Low sensitivity and high resolution color CCD camera.

Interface: Firewire (free with computer)



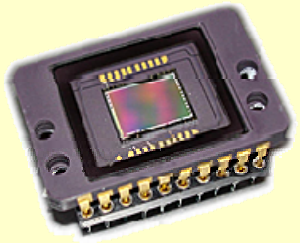
OrcaER: High sensitivity and precision digital monochrome CCD camera.

Interface: RS422 Interface

Detectors

- Film - camera

- CCD - cameras – scanners – spectrometers
  (Charge Coupled Devices)



- PMT - confocal scanners – spectrometers
  (Photo Multiplier Tubes)

- Other kinds of detectors – but less likely to encounter them

CCD Photodiode – Linear transducer

Figure 1-1 Photodiode cross section

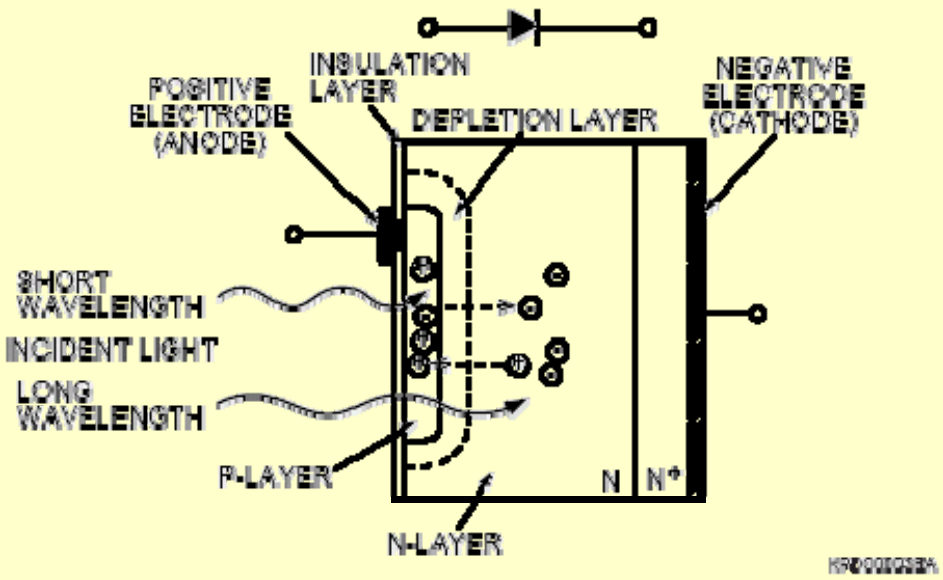
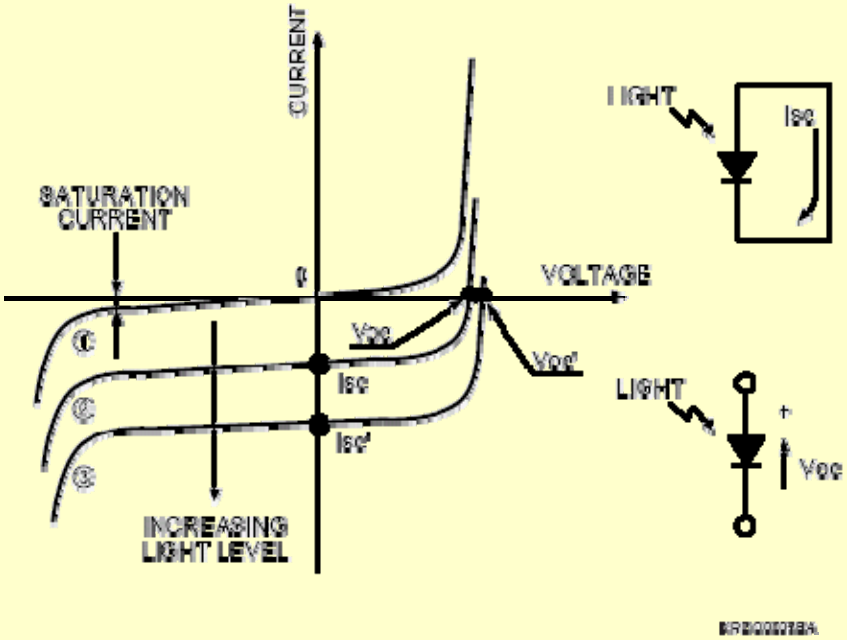


Figure 2-2 Current vs. voltage characteristic

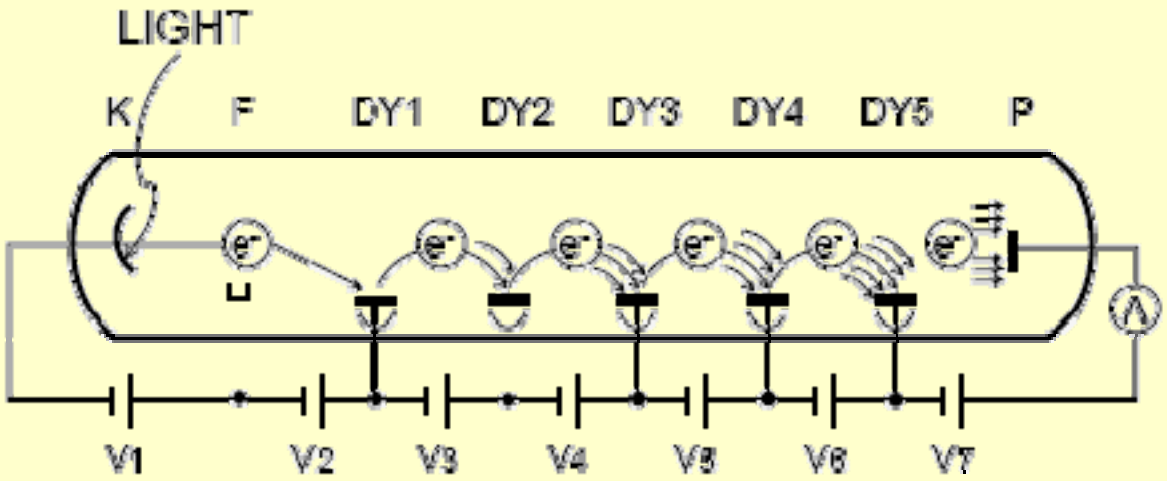


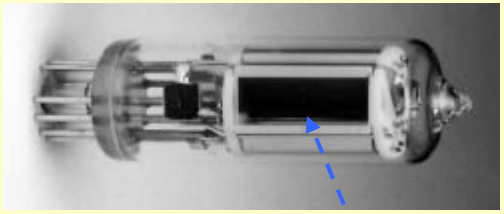
N-MOS substrate – sensitive ($$$)
CMOS substrate – less sensitive ($)

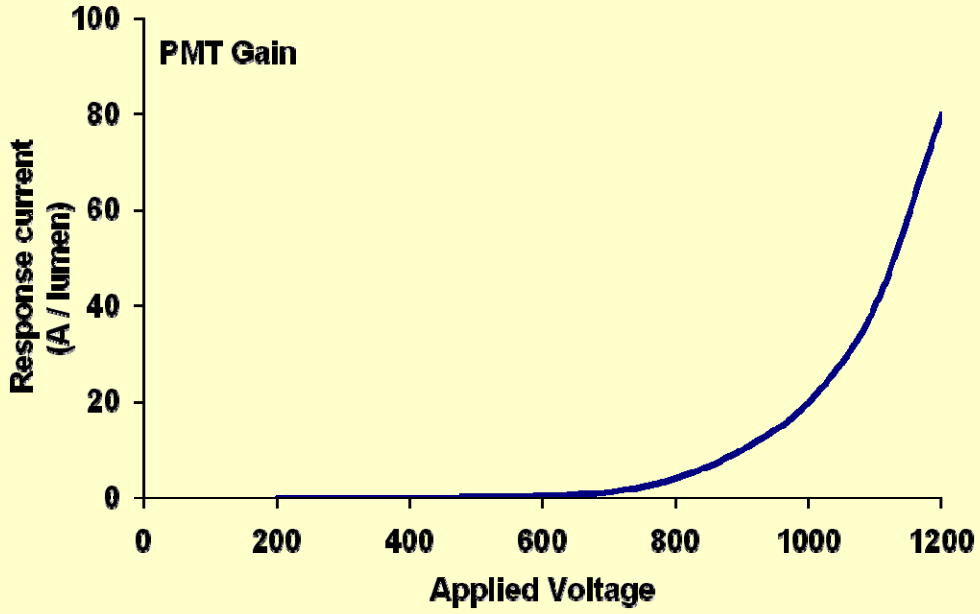
From Hamamatsu Photodiode Technical Sheet

Confocal Laser Scanning Microscope – PMT

Photo Multiplier Tube (PMT)



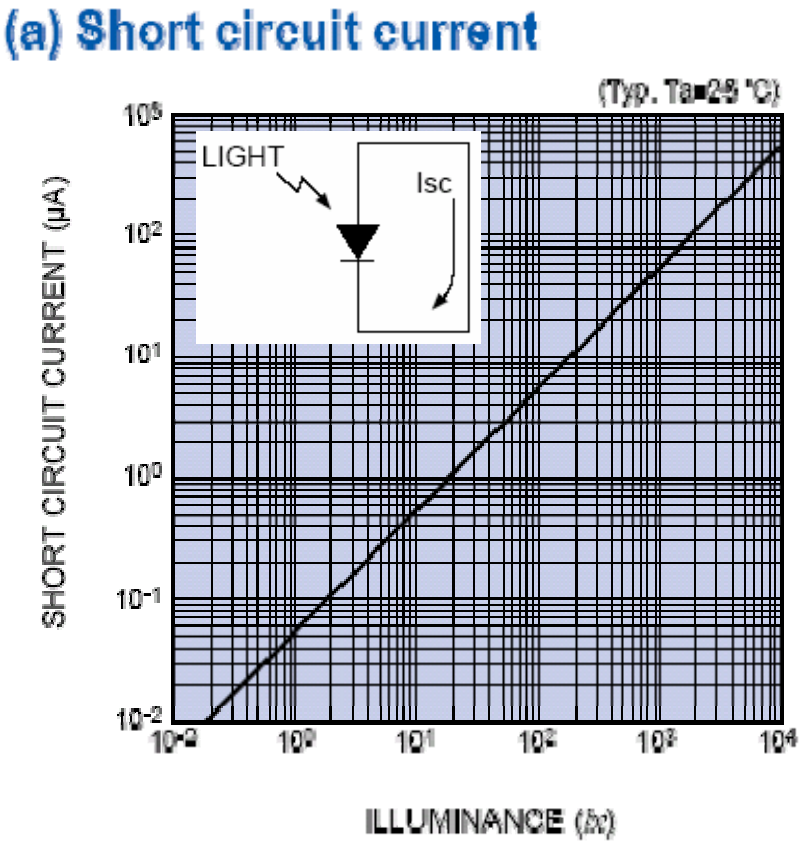




- Maximum Quantum yield ~= 0.3
- Gain can be very large $>10^8$
- Gain is exponential function of applied voltage
- Noise increases disproportionably at high gain
- Large dark current
- Spot detector – need rasterization

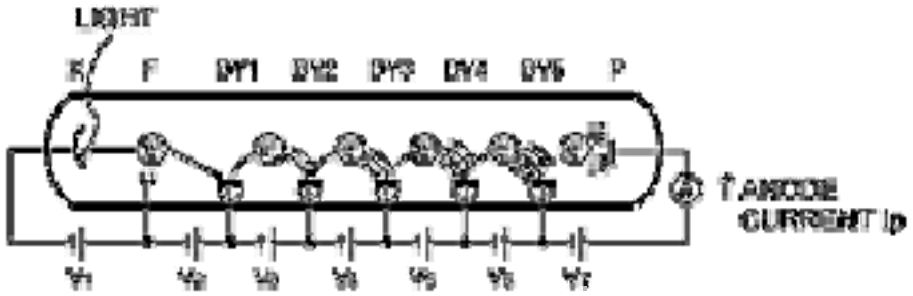
Quantification

CCD in photoconductive mode



Current out is proportional to photons/s in.

PMT at fixed anode cathode voltage



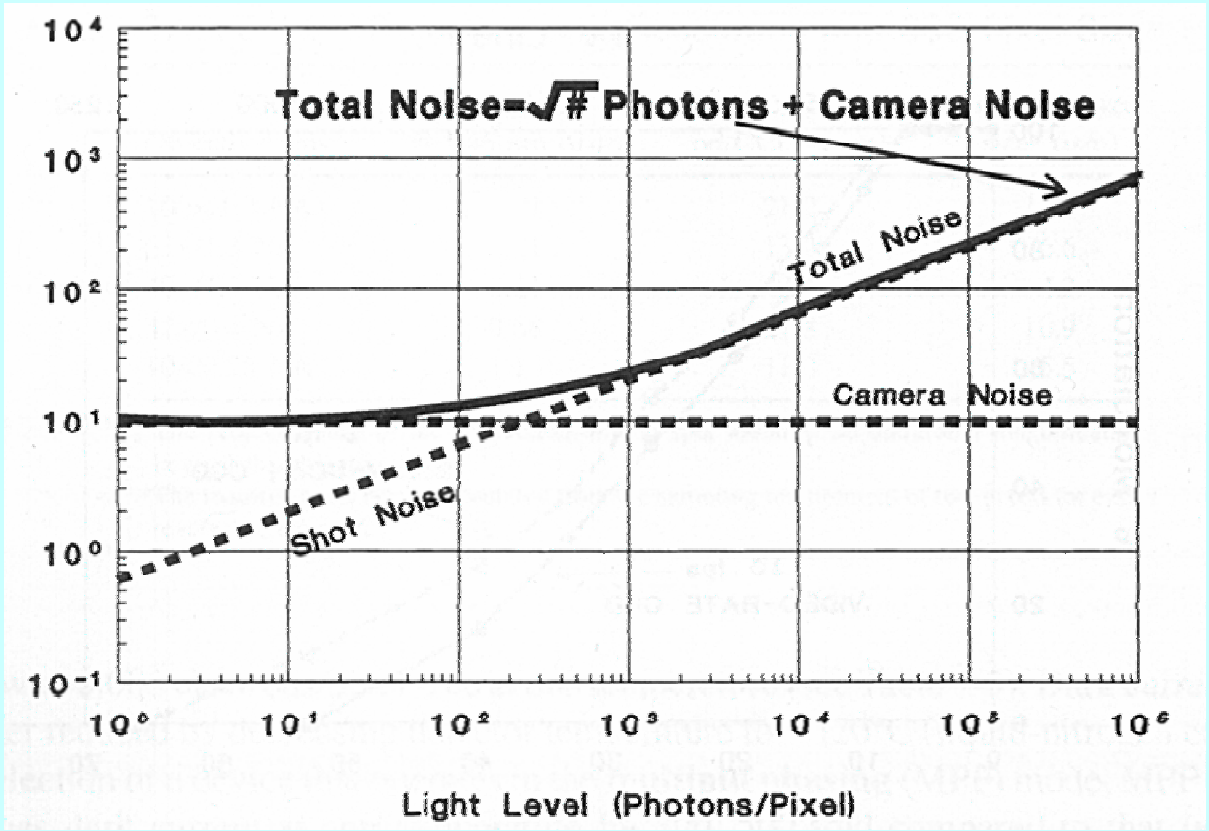
Current out is proportional to photons/s in.

However current out is not linearly proportional to PMT gain (voltage)
Therefore use fixed PMT voltage

CCD Camera technology for Quantitative Microscopy

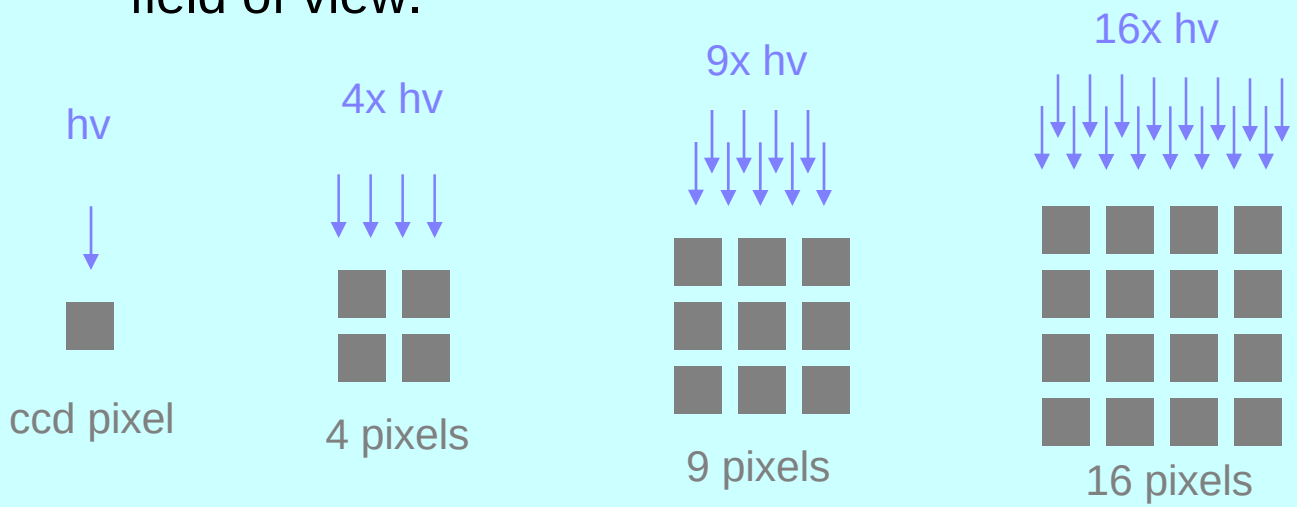
- Scientific Charge-Coupled Devices, James Janesick, 2000 SPIE
- Video Microscopy: the Fundamentals, Inoue, S., Spring, K., 2nd ed., Plenum Press
- http://www.andor.com/
- http://www.cookecorp.com
- http://dvcco.com
- http://hamamatsucameras.com/
- http://roperscientific.com/
    - http://www.qimaging.com/
    - http://www.princetoninstruments.com/
    - http://www.photomet.com/

CCDs for Microscopy - Noise factor



CCDs for Microscopy – Binning

- Pixel binning: merge adjacent pixels together electronically on CCD chip.
  - Many CCD cameras can merge 2 x 2, 3 x 3 or 4 x 4 pixels
  - Gives better sensitivity, e.g. 4, 9 or 16 fold better
  - Decreases amount of data to be read out. Therefore can transfer substantially more frames per second (fps)
  - Decreases shot noise proportionally to the square root of the number of bins merged
  - Down side is loss of resolution. Recover resolution with intermediate magnification in the microscope at the expense of field of view.



CCD – Sensitivity & Dynamic Range

- **Sensitivity:** minimum light signal which can be detected. Limits set by noise floor.
  - With short exposures shot noise increases and signal amplitude can approach read out noise level
  - Long exposures - shot noise integrates (averages) out and the large signal offset caused by dark current is mitigated by cooling the sensor.

- **Dynamic range:** maximum detectable intensity (well depth) relative to minimum detectable intensity (set by the noise floor)
  - Bigger pixels give bigger wells, hence greater maximum detectable signal
  - Anti-blooming reduces well depth and sensitivity
  - Shorter exposure times drain wells sooner so can detect more photons/sec