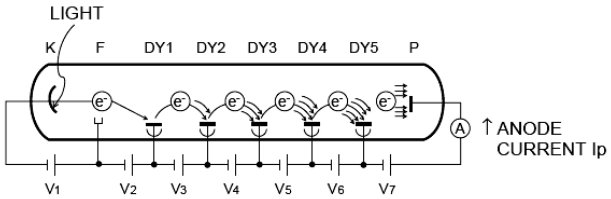
An Introductory Guide to Light Microscopy

April 30 - Detectors, sampling & digital images

16 Apr to 14 May 2007



$E = h\nu$

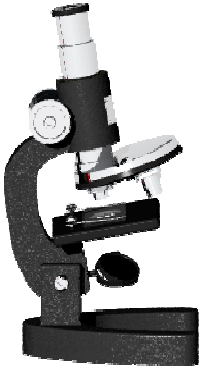


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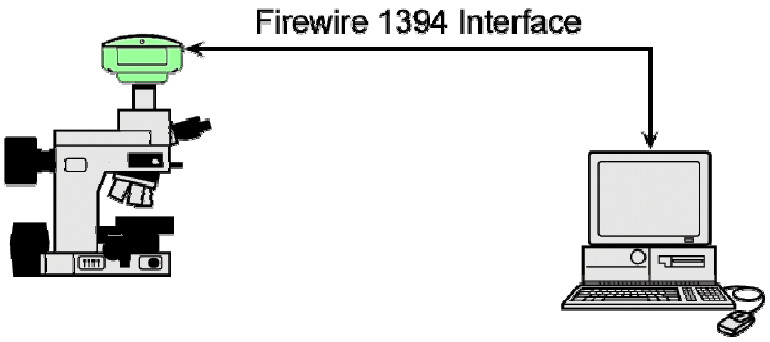
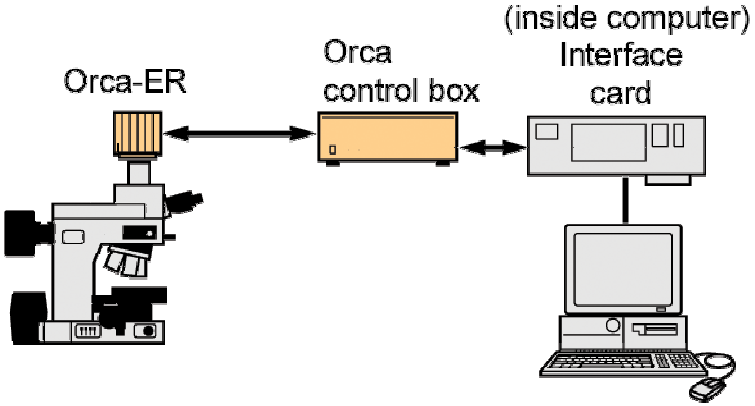
Image Acquisition



MicroPublisher: Low sensitivity and high resolution color CCD camera.

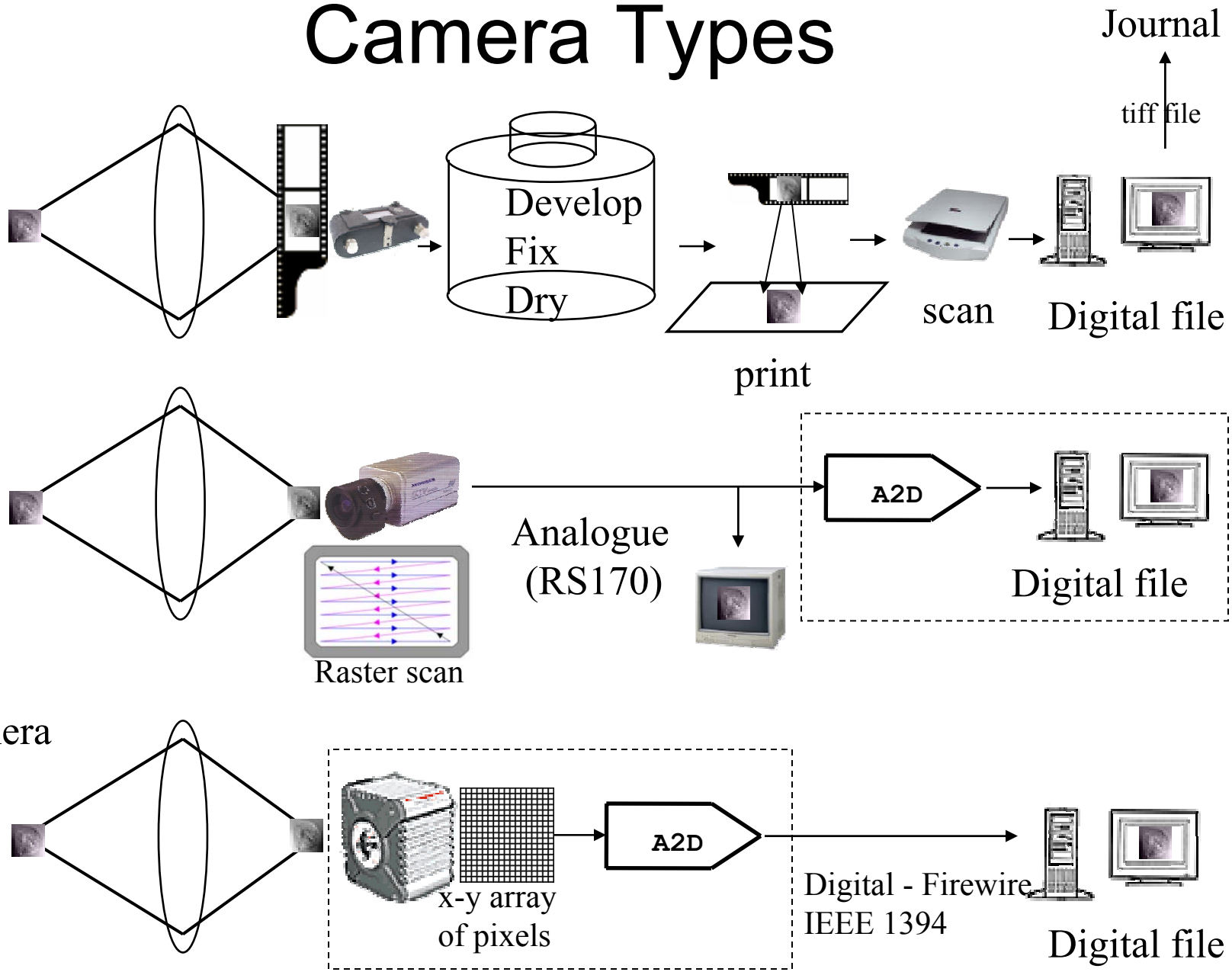
Interface: Firewire (free with computer)



OrcaER: High sensitivity and precision digital monochrome CCD camera.

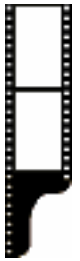
Interface: RS422 Interface

Camera Types



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.

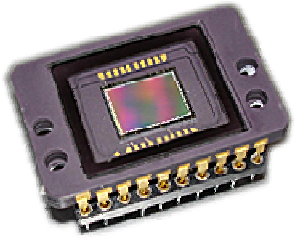
QE = 0.1 to 0.9

QE = Quantum Efficiency – fraction of input photons detected

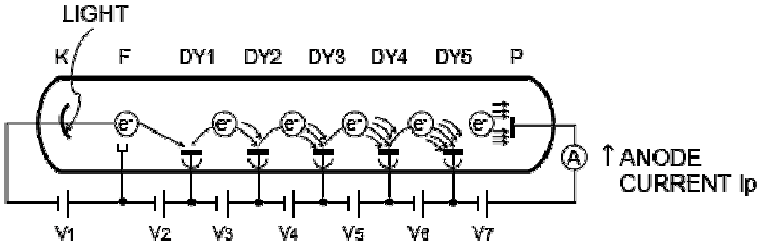
Detectors

- Film - camera

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



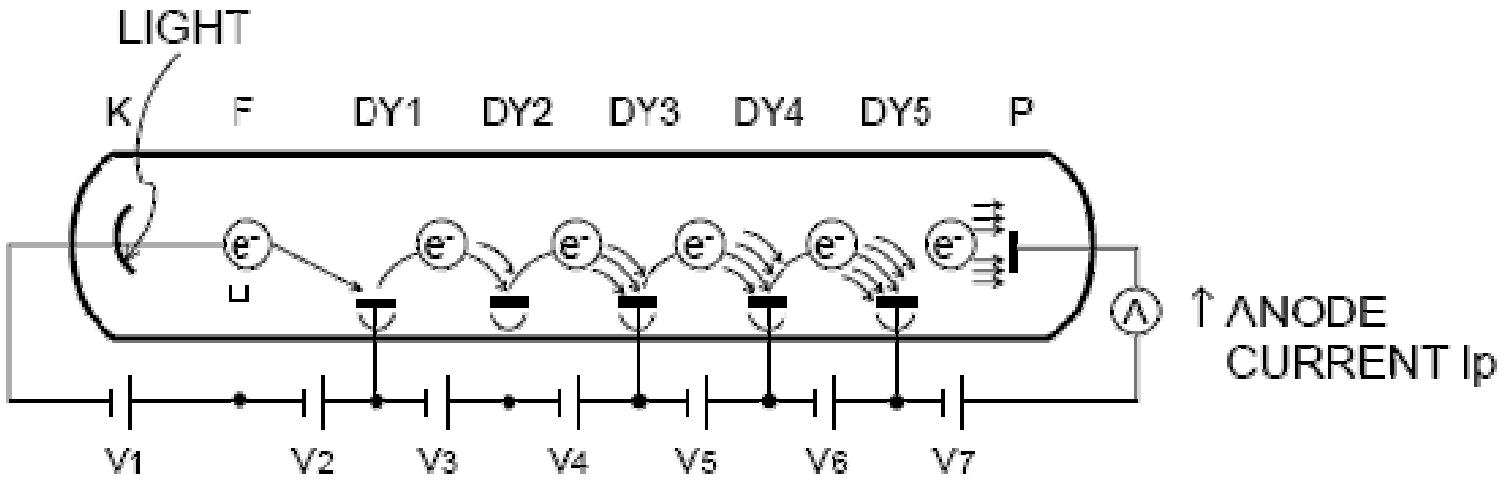
- PMT - confocal scanners – spectrometers
  (Photo Multiplier Tubes)

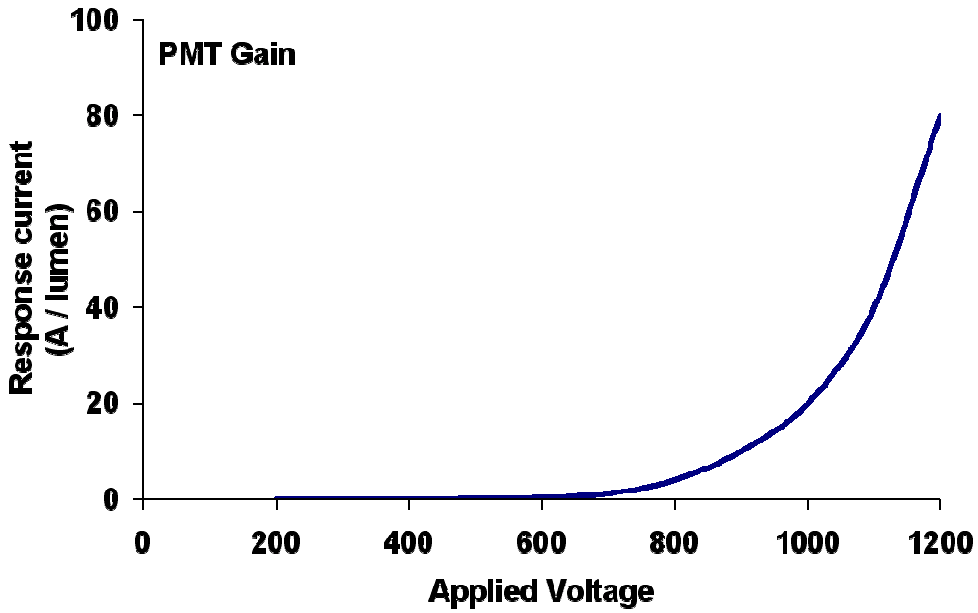


- Other kinds of detectors – but less likely to encounter them

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

CCDs for Microscopy – How they Work

Photodiode - Well structure - Charge transfer cycle

Figure 1-1 Photodiode cross section

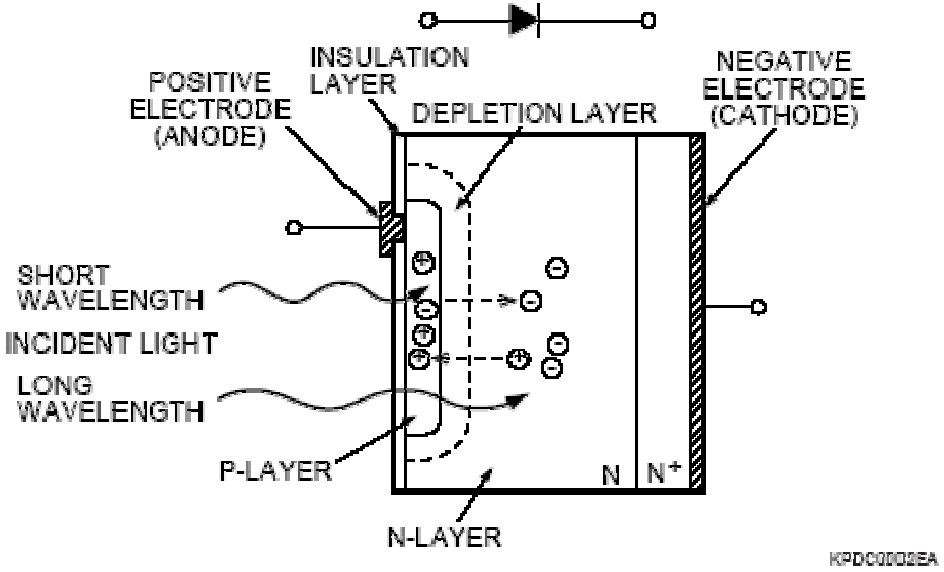
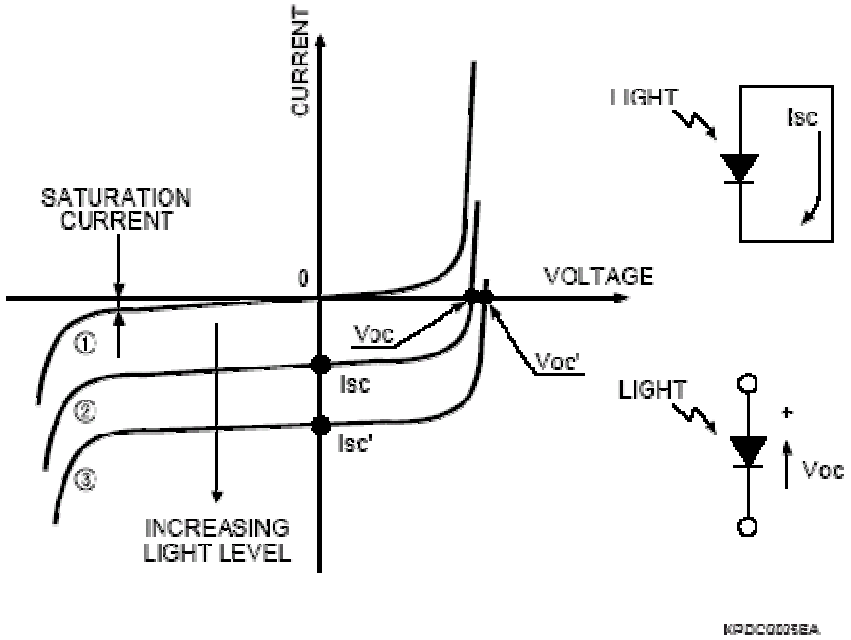


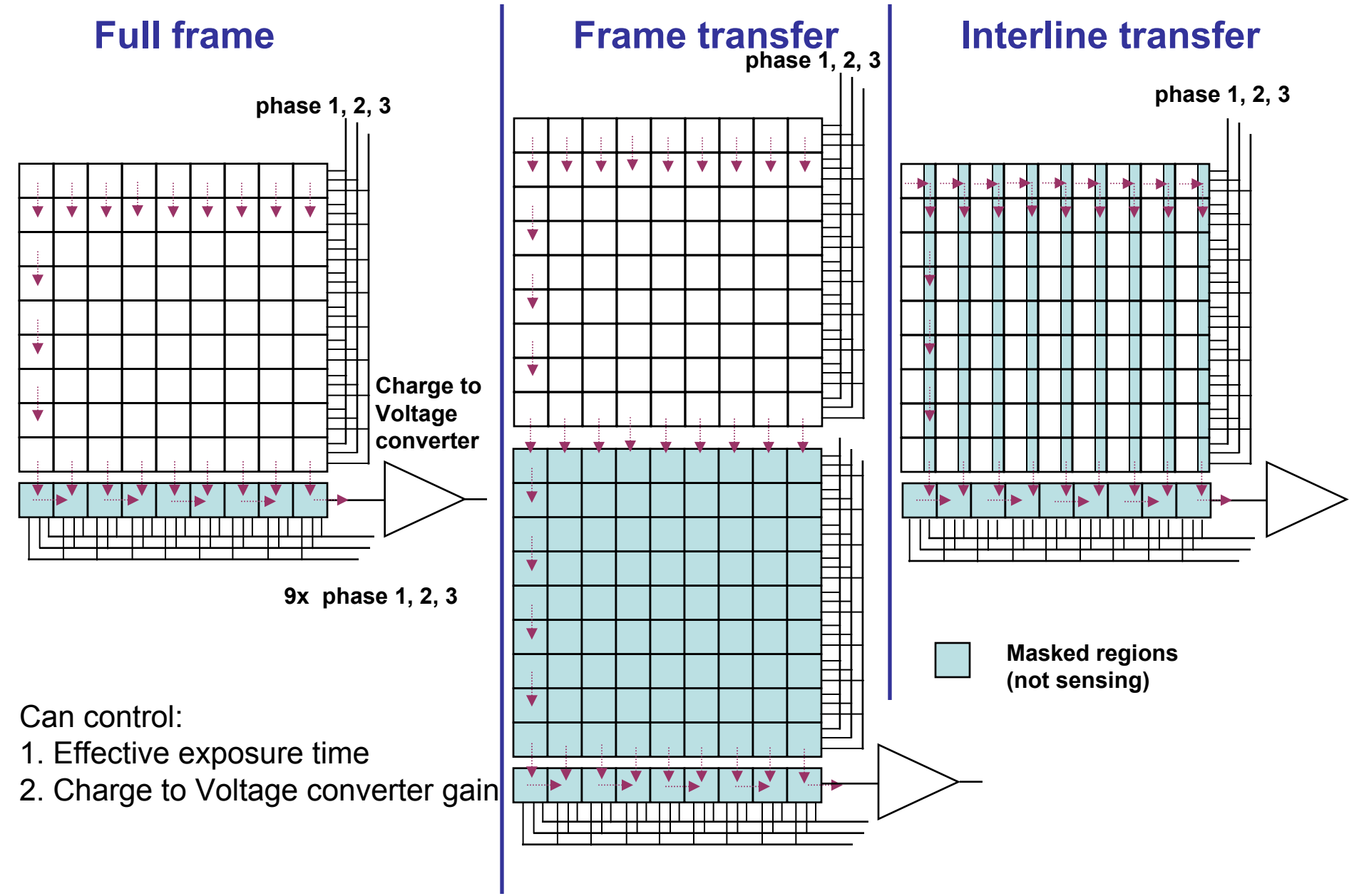
Figure 2-2 Current vs. voltage characteristic



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

From Hamamatsu Photodiode Technical Sheet

CCDs for Microscopy – CCD Formats

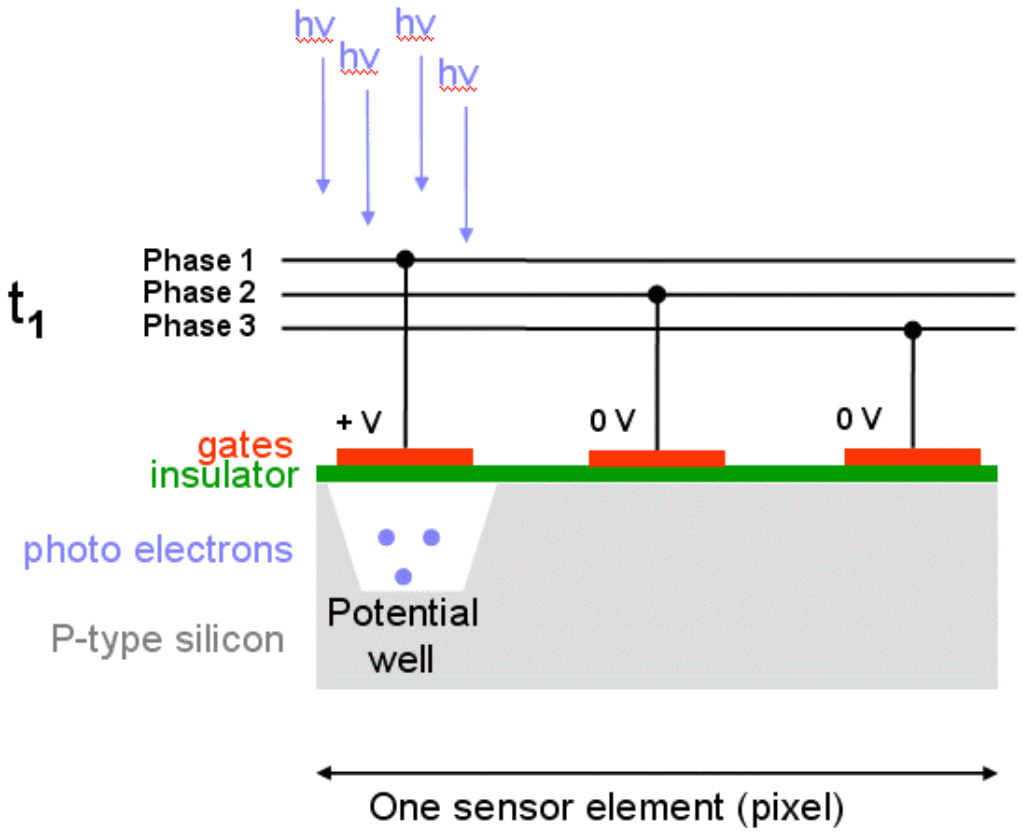


Can control:

1. Effective exposure time
2. Charge to Voltage converter gain

CCDs for Microscopy – How they Work

Photodiode - **Well structure** -
Charge transfer cycle



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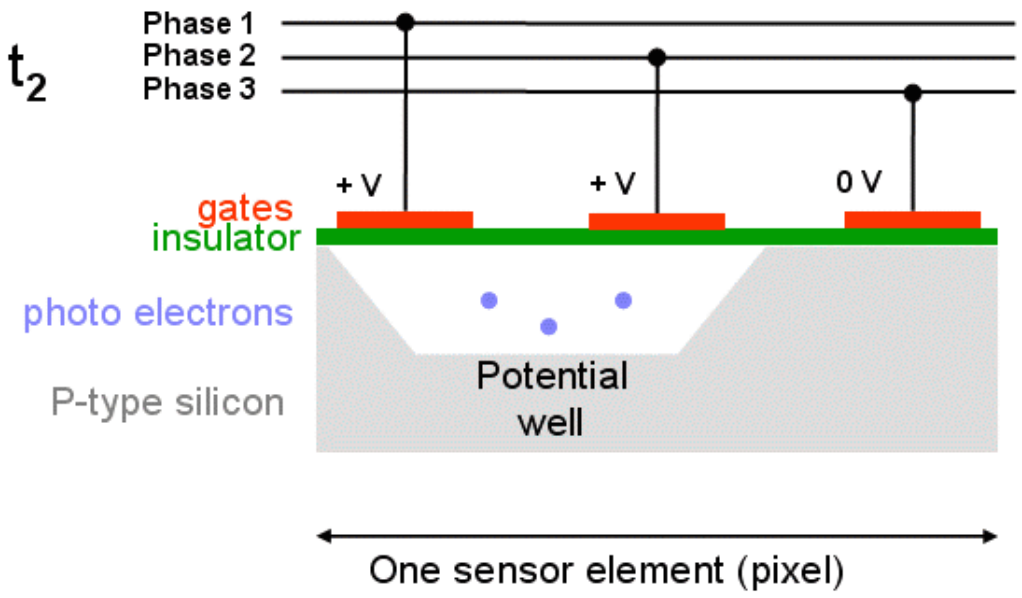

CCDs for Microscopy – How they Work

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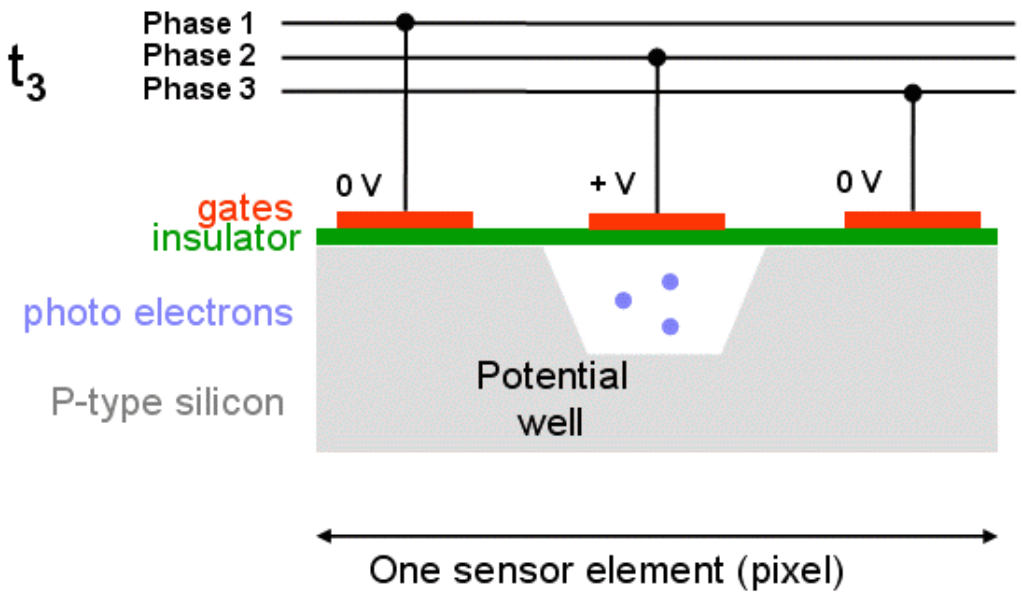
Charge transfer cycle



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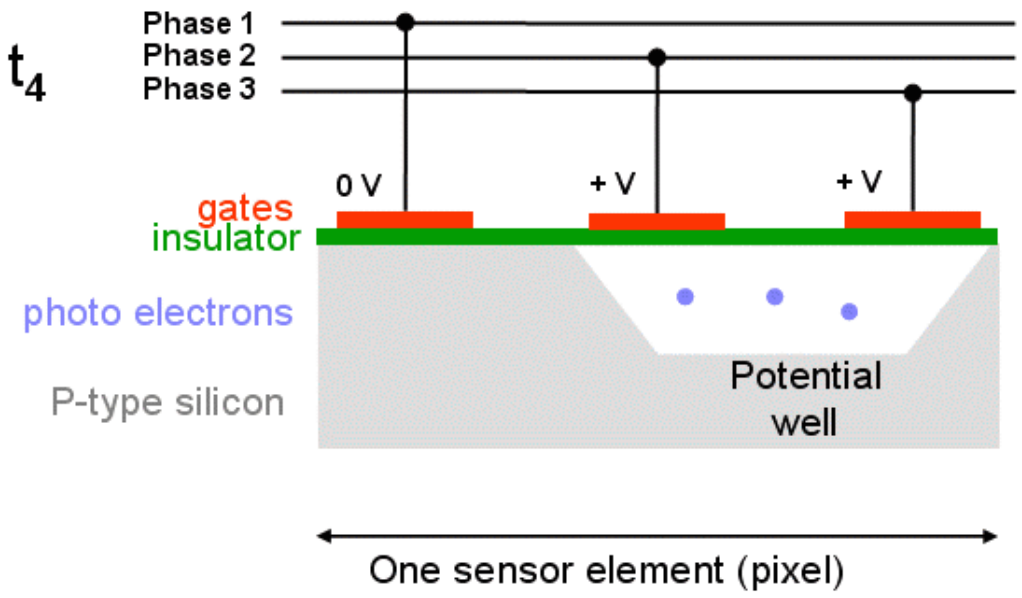

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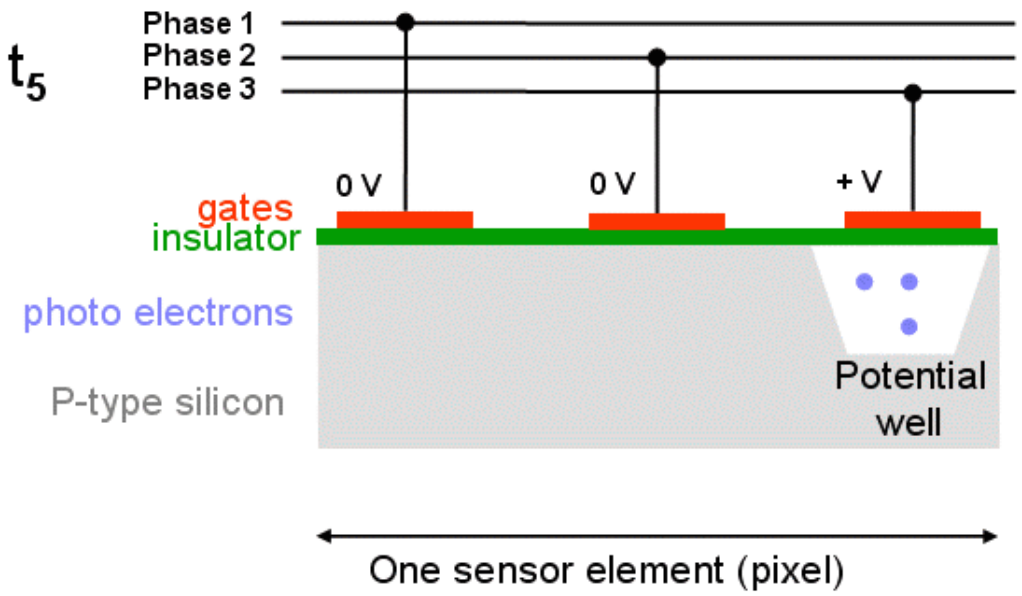

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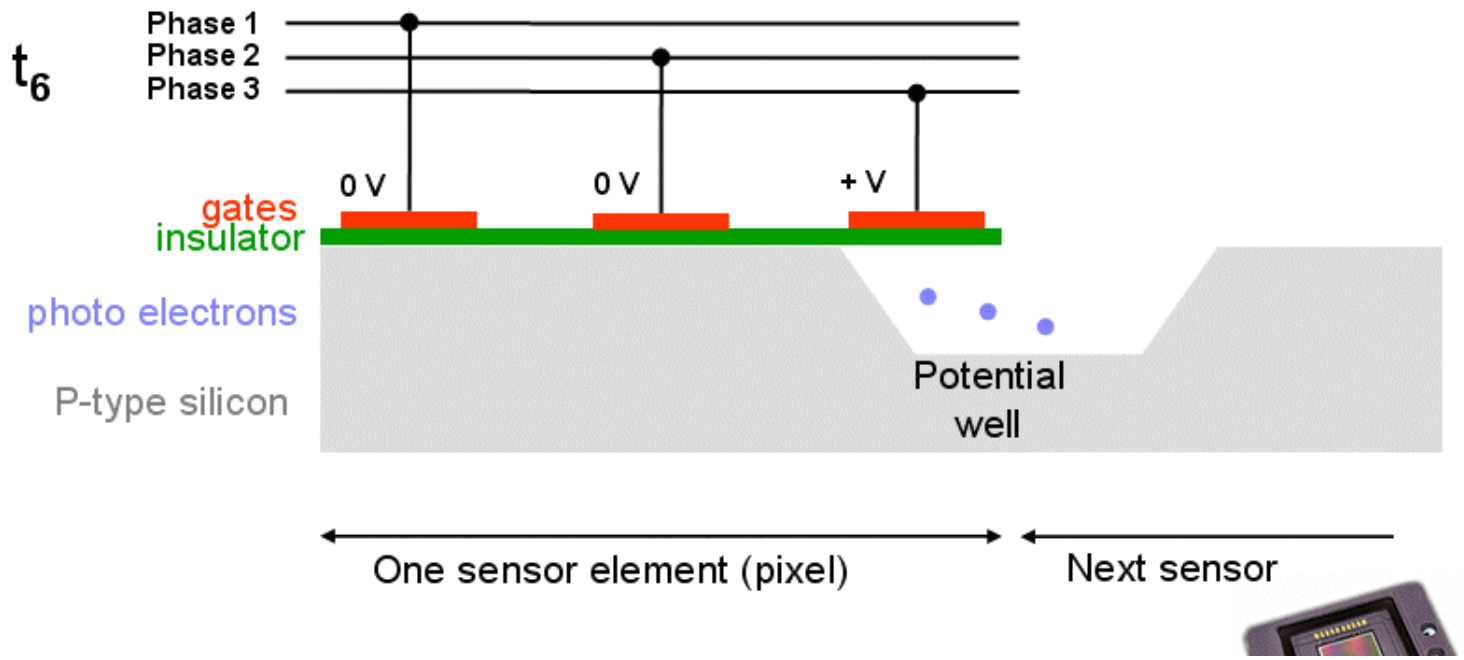

CCDs for Microscopy – How they Work

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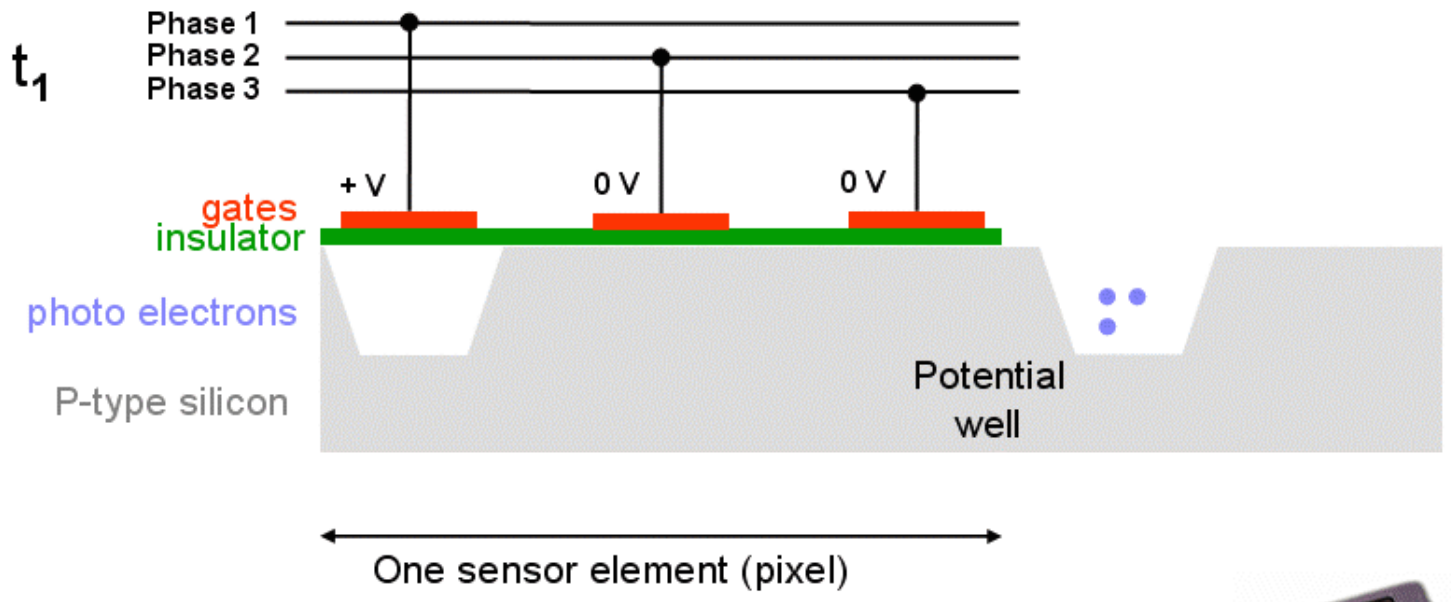
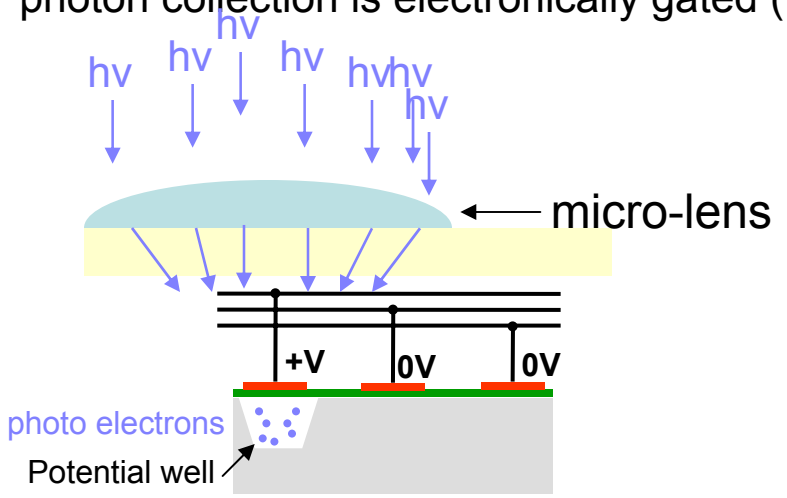


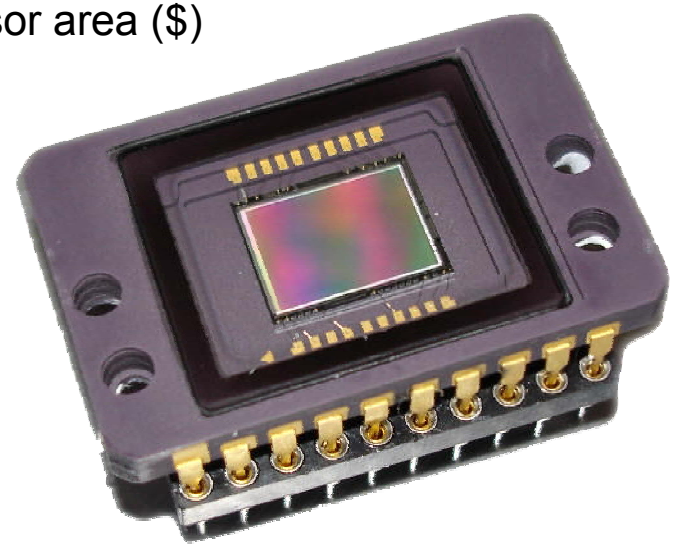
CCDs for Microscopy – CCD Formats

- Full frame
  - need mechanical shutter to stop photon sensing while doing readout (-)
  - read out is relatively slow (-)

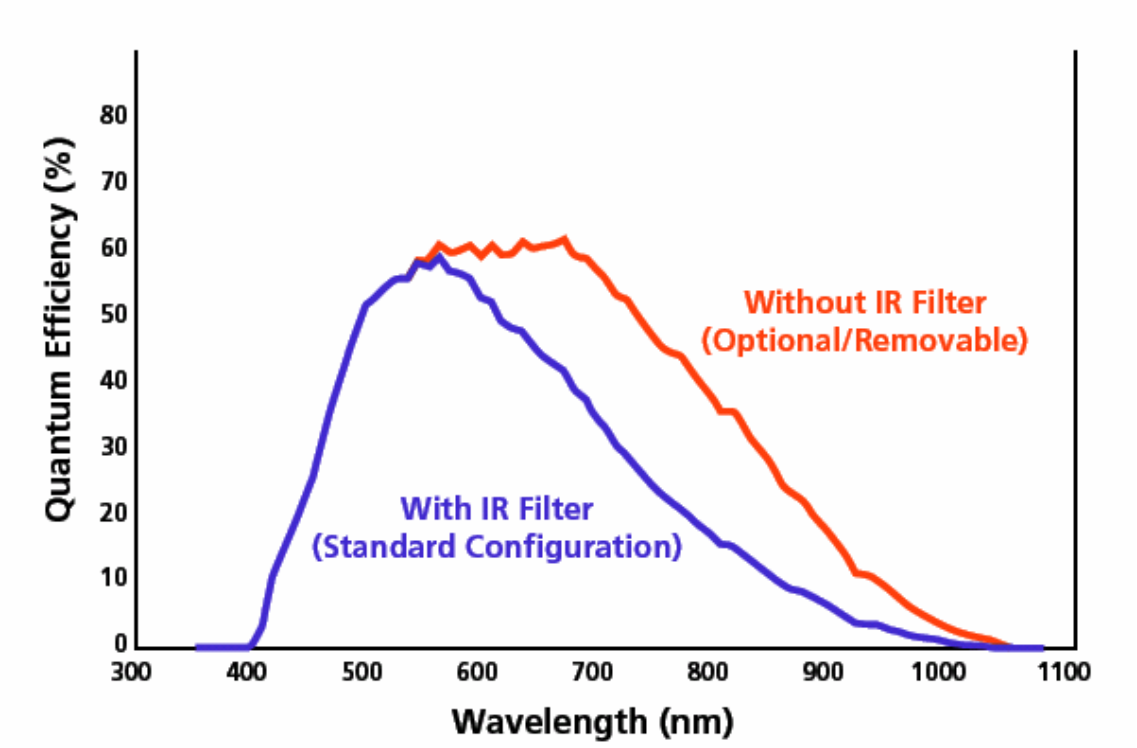
- Frame transfer ($$)
  - photon collection is minimal when charges are rapidly transferred to shielded part of chip
  - charge wells are deep (+)
  - device has most accurate transfer of charges (+)

- Interline transfer
  - fast read out (+)
  - large dead area (-)
  - need micro-lenses to direct photons to active sensor area ($)
  - photon collection is electronically gated (+)





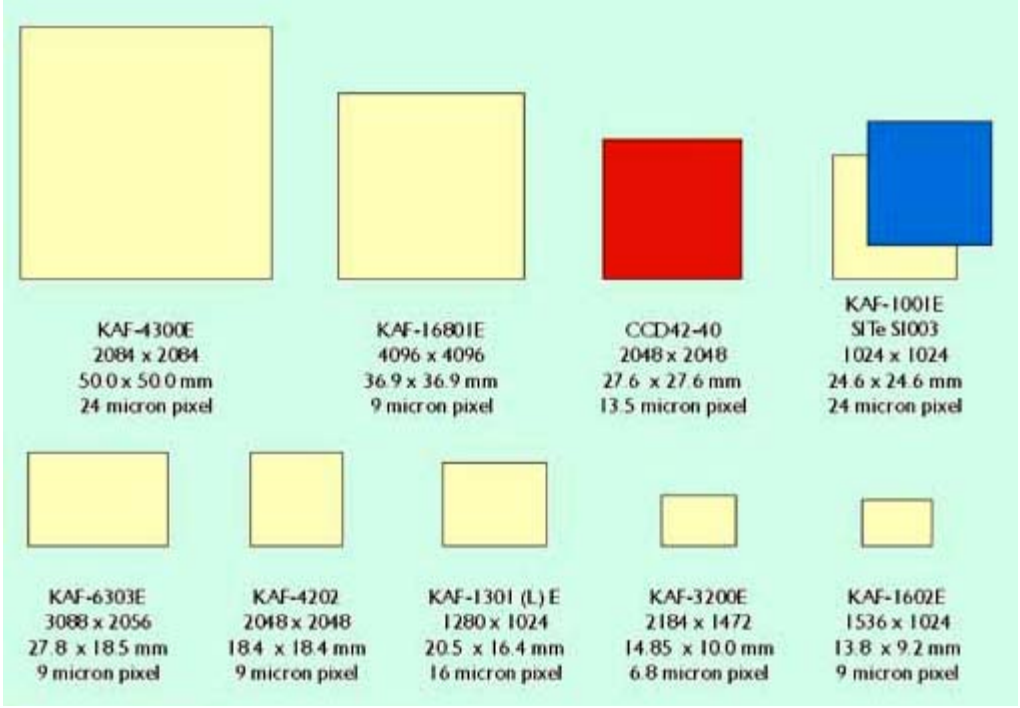
CCDs for Microscopy – Spectral Response



Infra Red (IR) filter usually added to photographic and many scientific ccd cameras in order to make imaged picture look like what the human eye would see – therefore loose some red and beyond sensitivity

Optical projection onto sensory array

- Match image field size to sensor array size
  - Use Optivar / zoom or change coupling lens (0.63x – 2.0x)
  - Or position of camera from tube lens
- Match optical image resolution to individual sensor element:
  - E.g. a 0.22 um structure through a 60x objective = 13.2 um on sensor. 0.22 is resolved with an NA=1.4
  - Nyquist criterion states sample at ~double the frequency i.e. 6.6 um
  - Use sensor with greater pixel element density
  - Have to compromise, since will be changing objective magnification and resolution



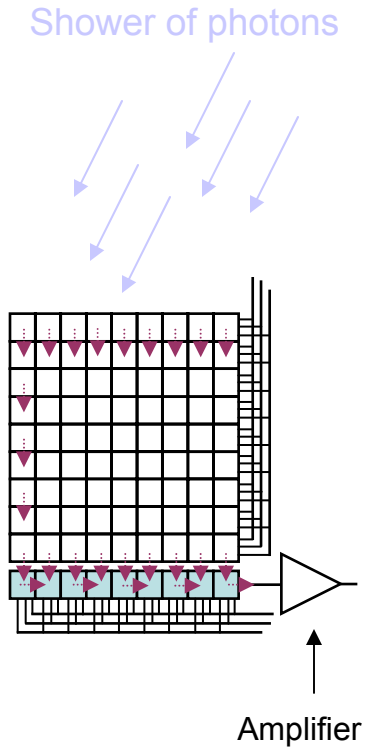
CCDs for Microscopy – Noise

- Dark noise δ_{dark}
  - Present in the absence of illumination
  - Random thermal fluctuation of charges in the sensor material
  - Problem with longer exposures, since wells get filled
  - Reduce with cooling sensor

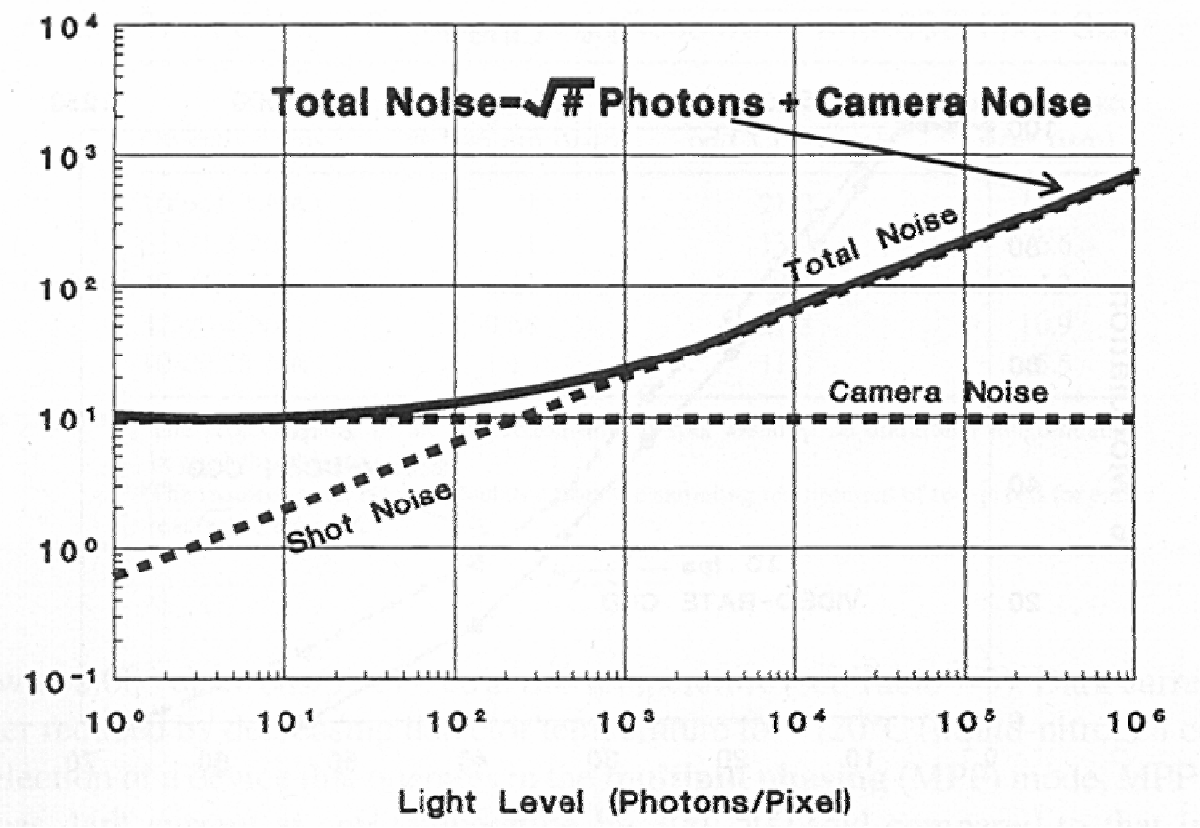
- Read out noise $\delta_{readout}$
  - Amplifier noise mostly
  - Reduce with photon amplification (intensifiers) before sensor or electron multiplication before readout amplifier
  - Also switching transients associated with read out clocks
  - Reduce with careful electronic design and slower readout speeds

- Shot noise δ_{signal}
  - Due to stochastic variation in photon flux (shower of photons)
  - Proportional to square root of mean signal
  - Overcome with longer exposures or brighter illumination

- $\delta_{total} = (\delta^2_{dark} + \delta^2_{readout} + \delta^2_{signal})^{1/2}$

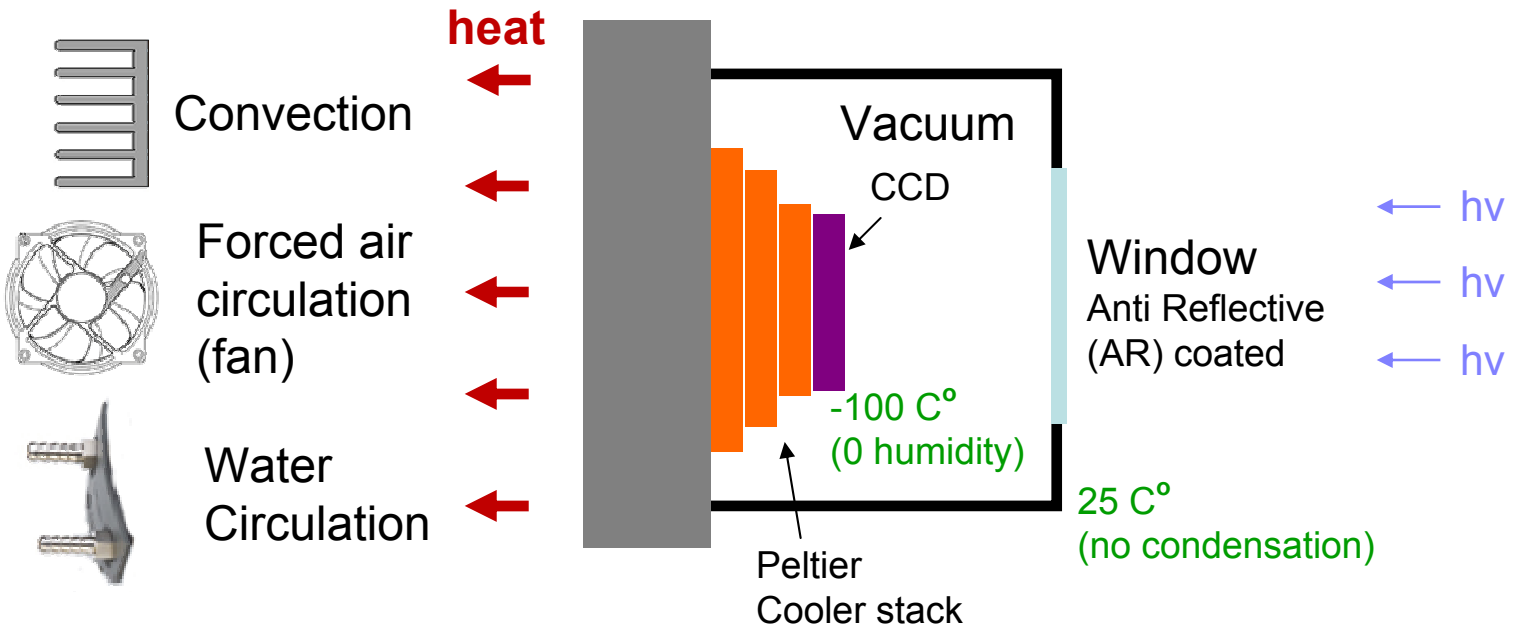


CCDs for Microscopy - Noise factor



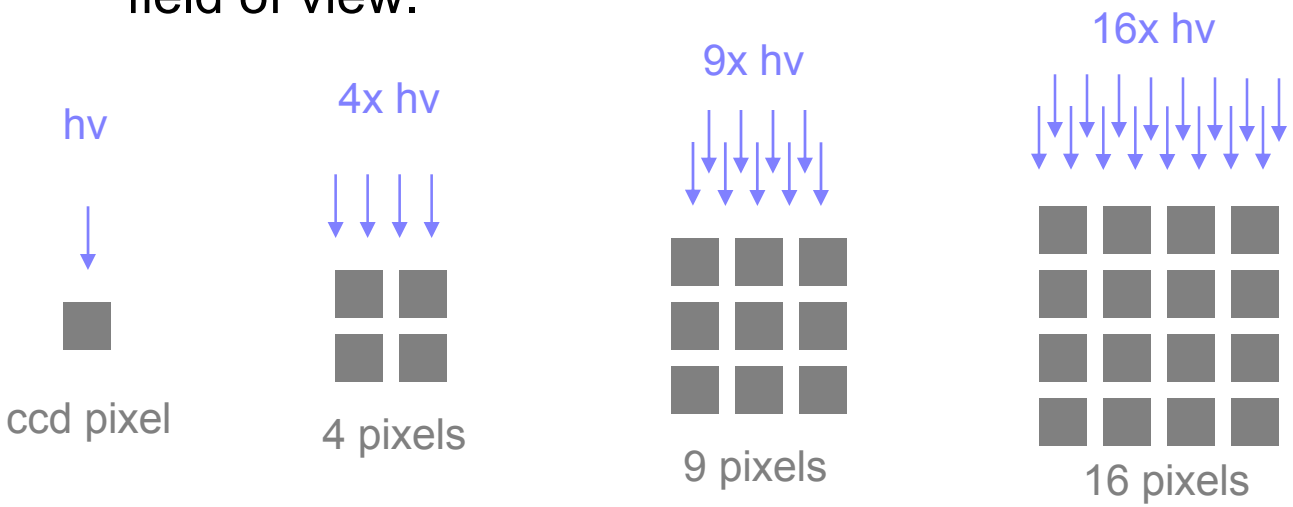
CCDs for Microscopy – Cooling

- Cooling reduces dark noise
- Heat removal – convection or fan or water flow



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.

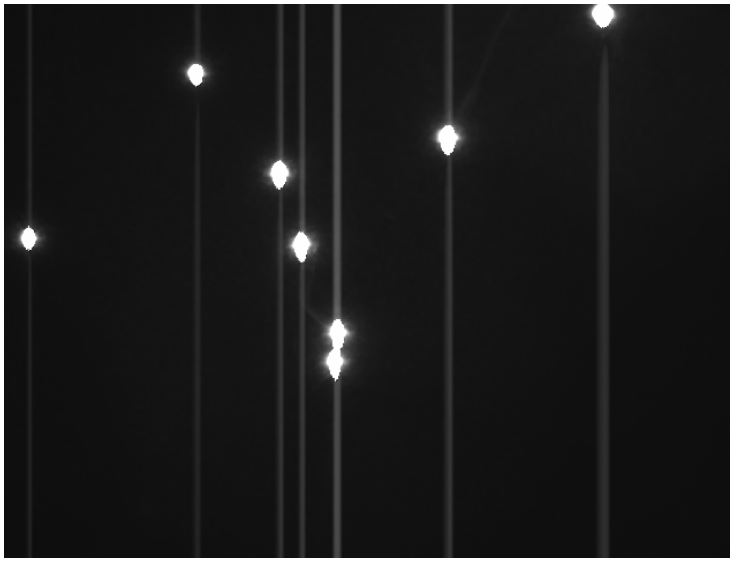


CCD – Sensitivity & Dynamic Range

- **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

CCDs for Microscopy – Blooming

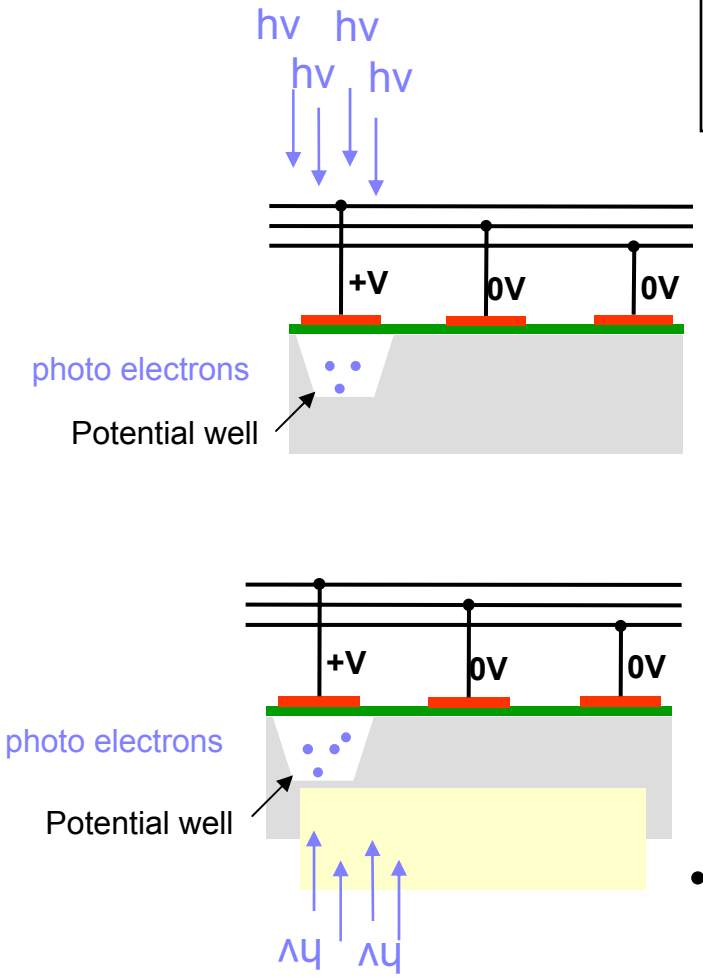


Blooming: In bright light conditions photoelectron charge can fill a well and spill over to adjacent wells. Charge transfer is preferred in the vertical direction, so vertical streaks result in the image.

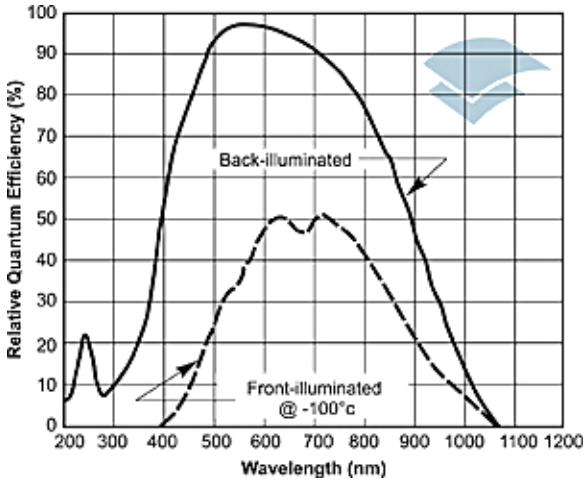
Anti-blooming: Circuitry can be enabled, in many high end CCDs, which drains excess photoelectrons to the substrate. Sensitivity is reduced (not a problem when there are an excess of photons anyway)

CCDs for Microscopy – Quantum Efficiency

QE = quantum efficiency is the fraction of incoming photons converted into photoelectrons



- Front illumination: – light passes through chip wiring/gates which results in losses. max QE = ~0.6

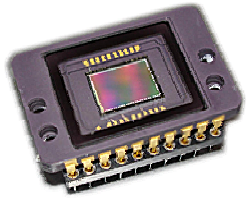


- Back illumination: avoids wiring, but chip has to be thinned to avoid absorption
- Chip is fragile (~10 um thick) and expensive, but max QE = ~0.9

CCDs for Microscopy – Intensification

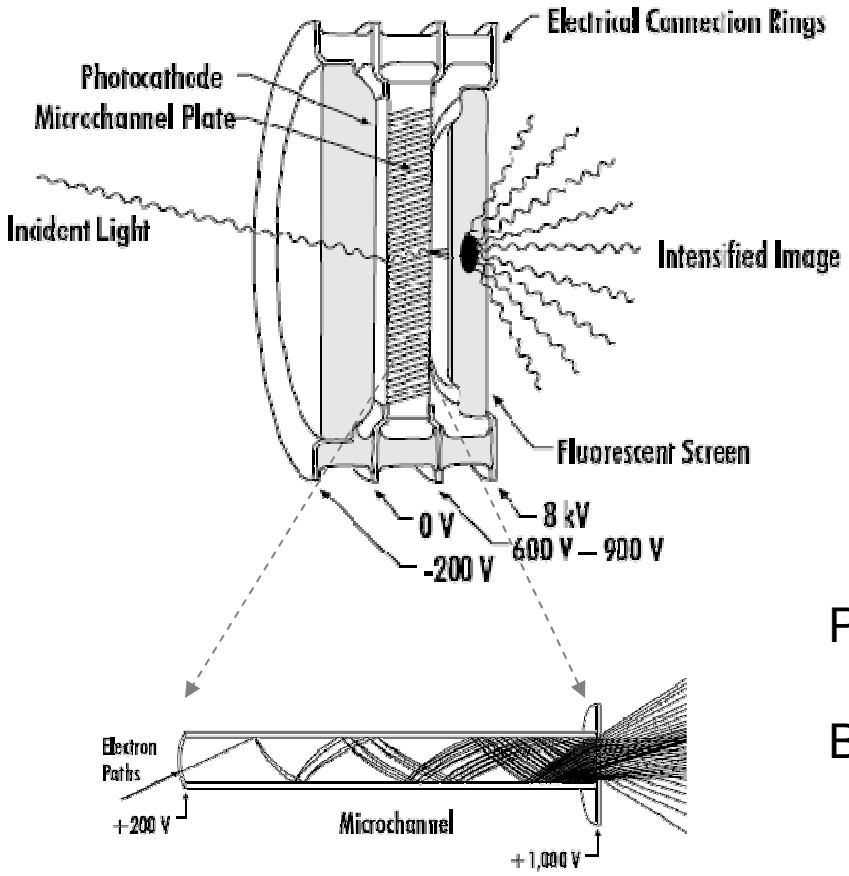
- Overcome readout noise – detect very low light levels
- Get image which exceeds threshold of detector. e.g. night vision goggles for human eye or fast acquisition of dim image
- **Penalty is increased noise due to fluctuations in the intensifier device.**

- Common current intensifier technologies:
  - Micro-channel plate intensifier before the CCD. Improves CCD sensitivity and overcomes readout noise.
  - Electron multiplication after CCD readout and before readout amplifier c.f. avalanche photodiode (APD). Improves readout noise.

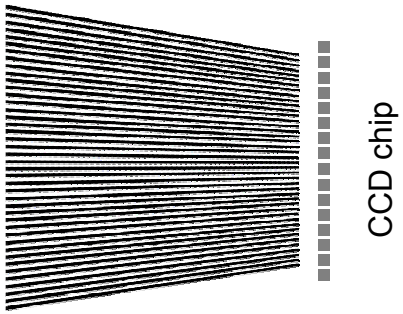


CCDs for Microscopy – Intensification

Micro-channel intensifier (MCI) before the CCD



Similar to a photomultiplier tube (PMT)



Fiber optic coupler
(Must be close to
fluorescent screen)

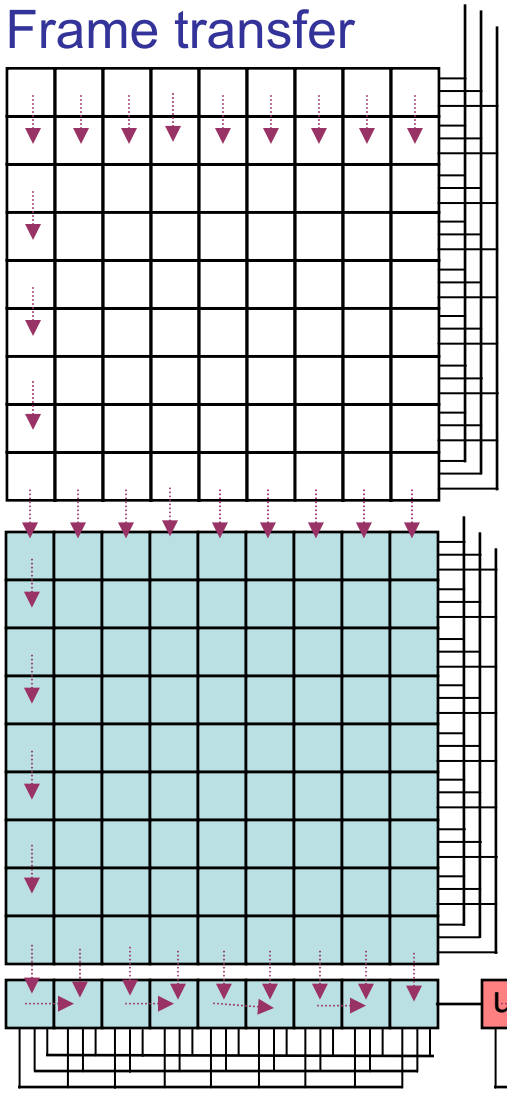
Possible gain beyond ~ 1 000 000

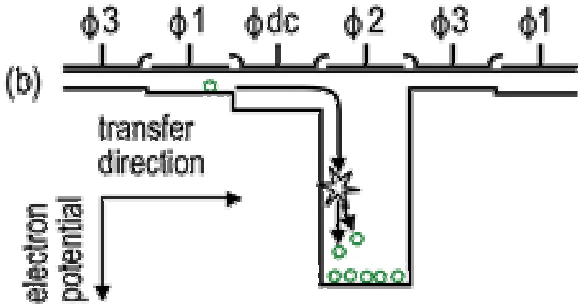
Bright light will cause MCI burnout

CCDs for Microscopy – Intensification

Electron Multiplication CCD

Frame transfer





High well potential
Impact ionization
c.f. Avalanche Photodiode or Zener Diode
Maximum gain ~1000

$$\text{Gain} = \prod_{i=1}^{i=n} u_i$$

u approx 1.02

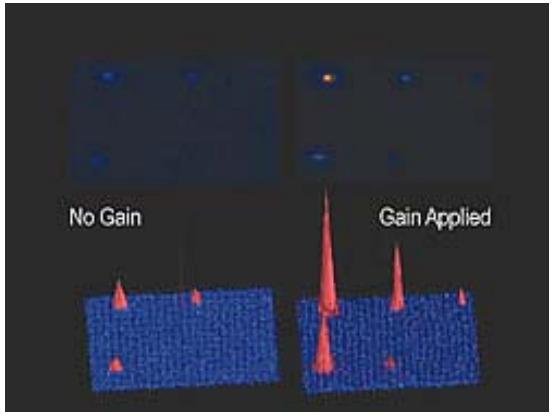
masked wells

u_1 u_2 ... u_n

Readout amplifier

Readout registers

Multiplication registers

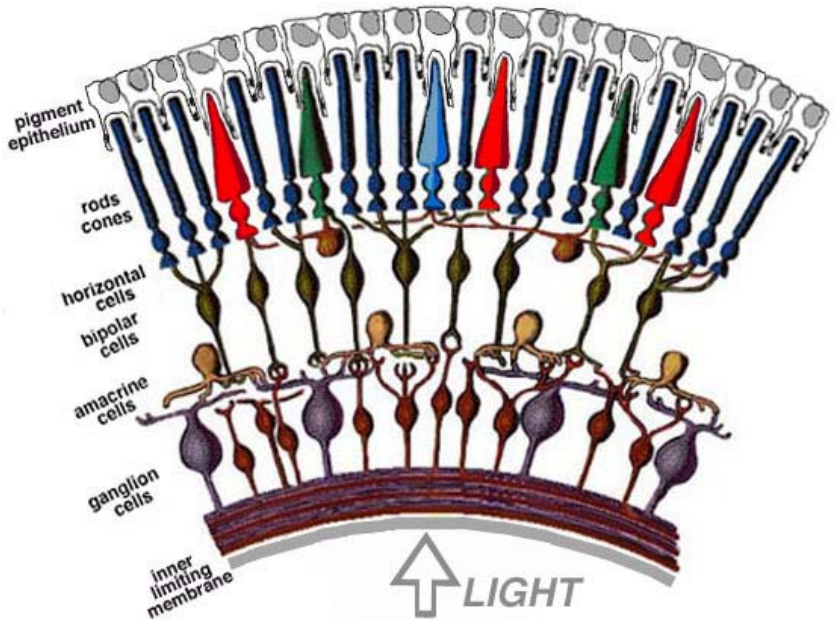


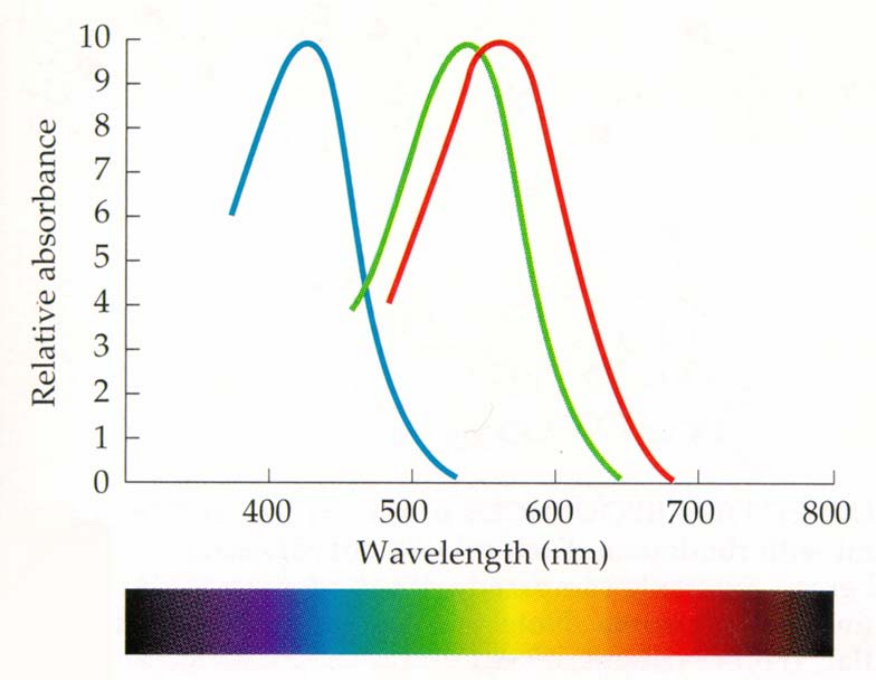
CCD Noise - mitigation

- Cooling reduces dark current to almost 0 (in liquid nitrogen)
- Reading out CCD slower reduces switching noise but compromises frame rate and makes blooming more likely
- A2D noise reduced by using expensive version
- Reduce shot noise by increasing excitation light until get bleaching
- Reduce instrument or shot noise by averaging over time or taking a longer exposure
- Bin pixels – i.e. averaging over (more) space

Color for Humans - Spectral Separation

- Cones - red green blue color vision in bright light
- Rods - monochrome for night vision – good sensitivity (less resolution)



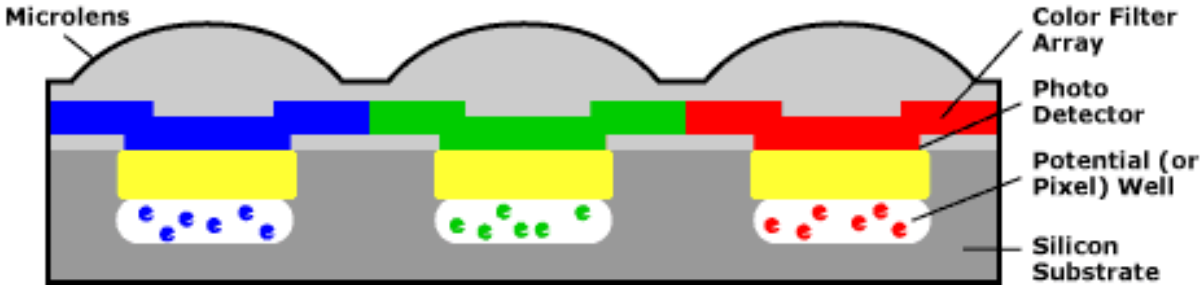


Spectral sensitivity of cone receptors

Color for Cameras

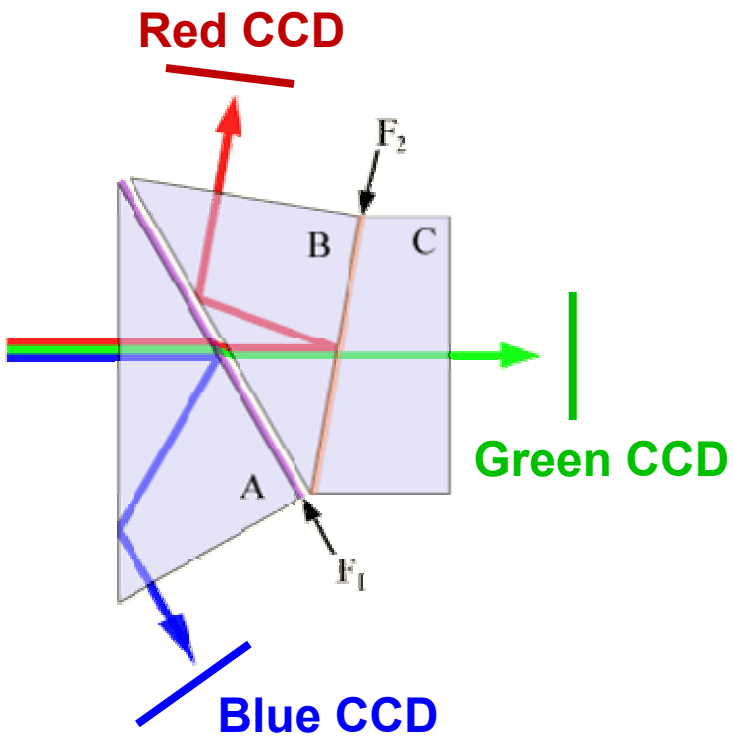
- Absorptive filters in front of CCD pixel elements – even lose light close to desired wavelength –
- Can not pack 3 colors into rectangular array
- Therefore Bayer Pattern - loss of intensity especially in blue and red & loss of spatial resolution

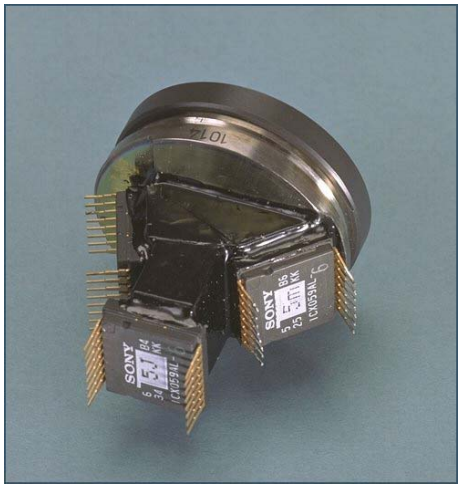
G	R	G	R
B	G	B	G
G	R	G	R
B	G	B	G



Color for Cameras

- Dichroic prisms split light into red, green & blue wavelengths
- Need 3 CCD sensor chips
- CCDs must be carefully aligned
- 3x A2D converters
- Expensive $$$!



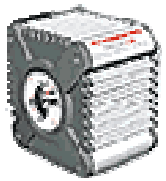


CCDs for Microscopy – Camera Specs

- Number of pixels 0.5M to 12 Mpixels
- Pixel cell size 4 to 30 um^2
- Pixel clock 10 KHz to 40 MHz
- Frame rate 0.01 to 240 fps
- Binning & sub arrays 4, 9, 16
- Interface – e.g. RS422, USB, Firewire, PCI dedicated card
- Readout noise - electrons (RMS) 2 $electrons_{RMS}$
- Full well capacity 5000 to 100000
- Cooling room, -10 to -100 C^o or more
- Dark current 0.01 e^-/pixel/second
- A/D converter – 8, 10, 12, 14, 16 bits
- Signal to noise ratio up to 1000
- Chip grade – no. of defects

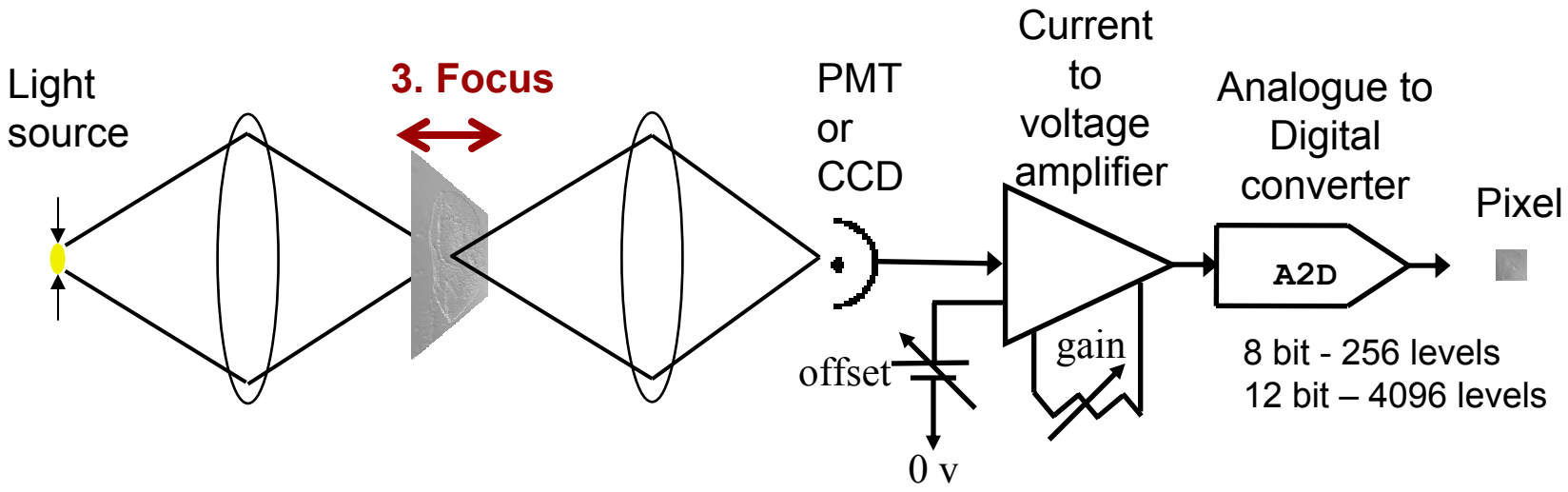
Typical CCD Cameras

- OrcaER (extended red)
- 1024 by 1024 pixels resolution
- pixel size=6.4 square um
- 8 bit or 12 bit
- spectral sensitivity 350 nm to 750 nm
- Quantum Efficiency of 0.7
- 18000 electrons per well
- Noise 8 electrons / second
- Good for low and high light imaging
- Good for fluorescence!



- MicroPublisher (color)
- 2048 by 1536 pixels resolution
- pixel size=7.5 square um
- 8 bit or 10 bit
- spectral sensitivity 420 nm to 650 nm
- Quantum Efficiency of 0.2
- 6000 electrons per well
- Bright light imaging
- Not good for fluorescence!

Quantification - intensity



1. Illumination **2. [dye]** **4. Optical collection** **5. Detector**

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

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

Quantification

Pixel value depends on:
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, 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.

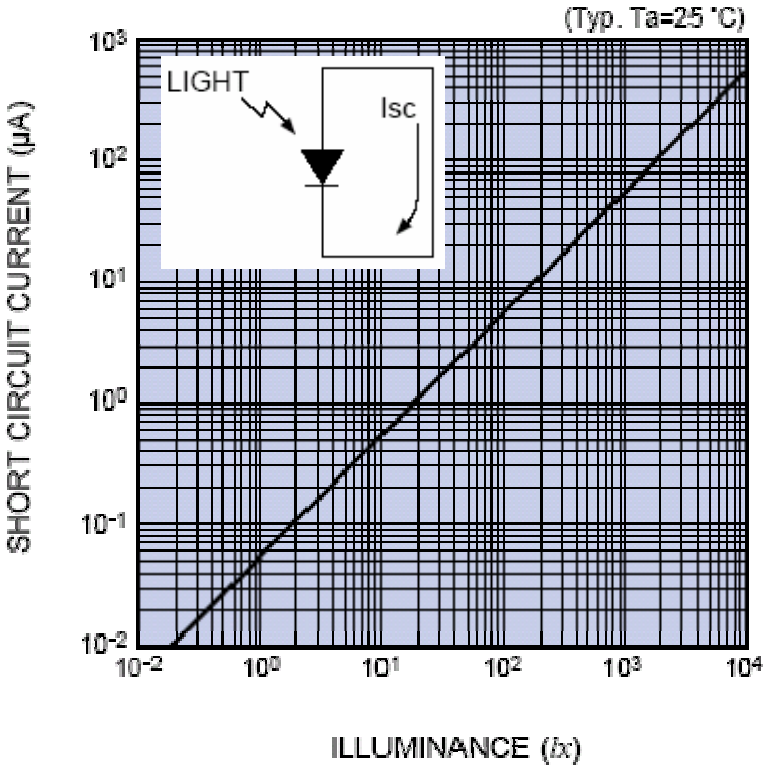
James Pawley published 39 steps: now has even more steps.

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

Quantification

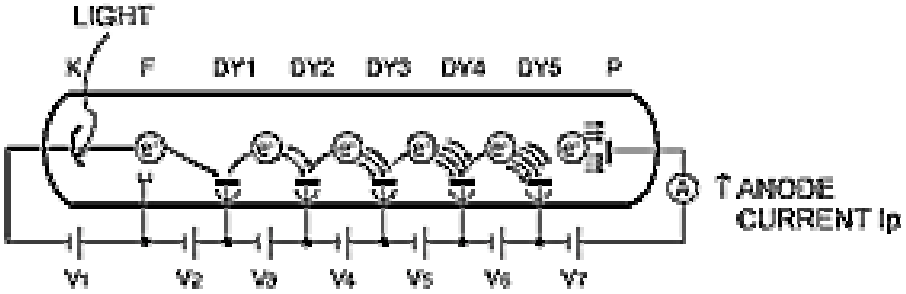
CCD in photoconductive mode

(a) Short circuit current



Current out is proportional to photons/s in.

PMT at fixed anode cathode voltage



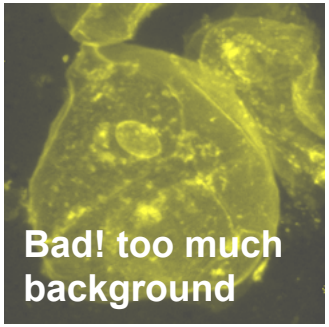
Current out is proportional to photons/s in.

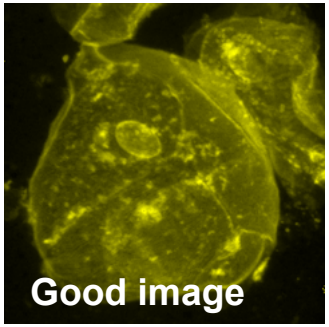
However current out is not linearly proportional to PMT gain (voltage) Therefore use fixed PMT voltage

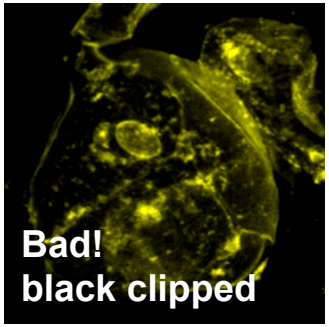
Quantification

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







Forbidden pixel values

255

Pixel value

Slope = gain = contrast

Photon flux

Offset = black level = brightness

0

Offset

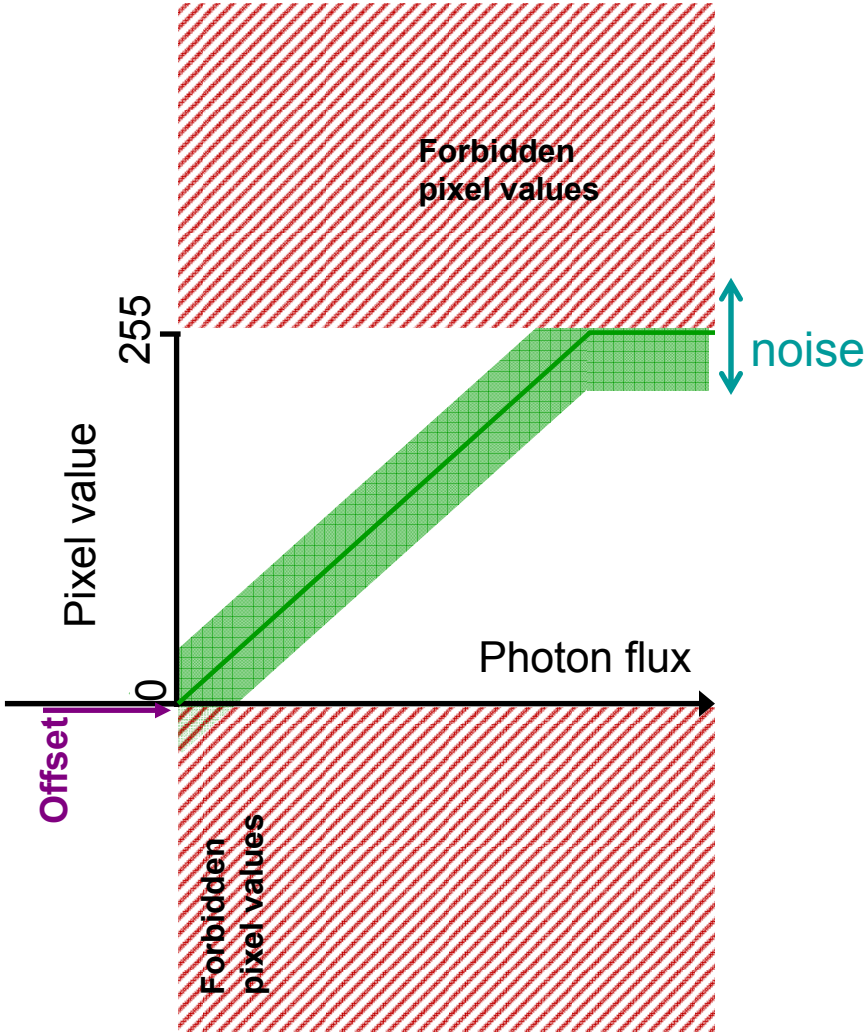
Forbidden pixel values

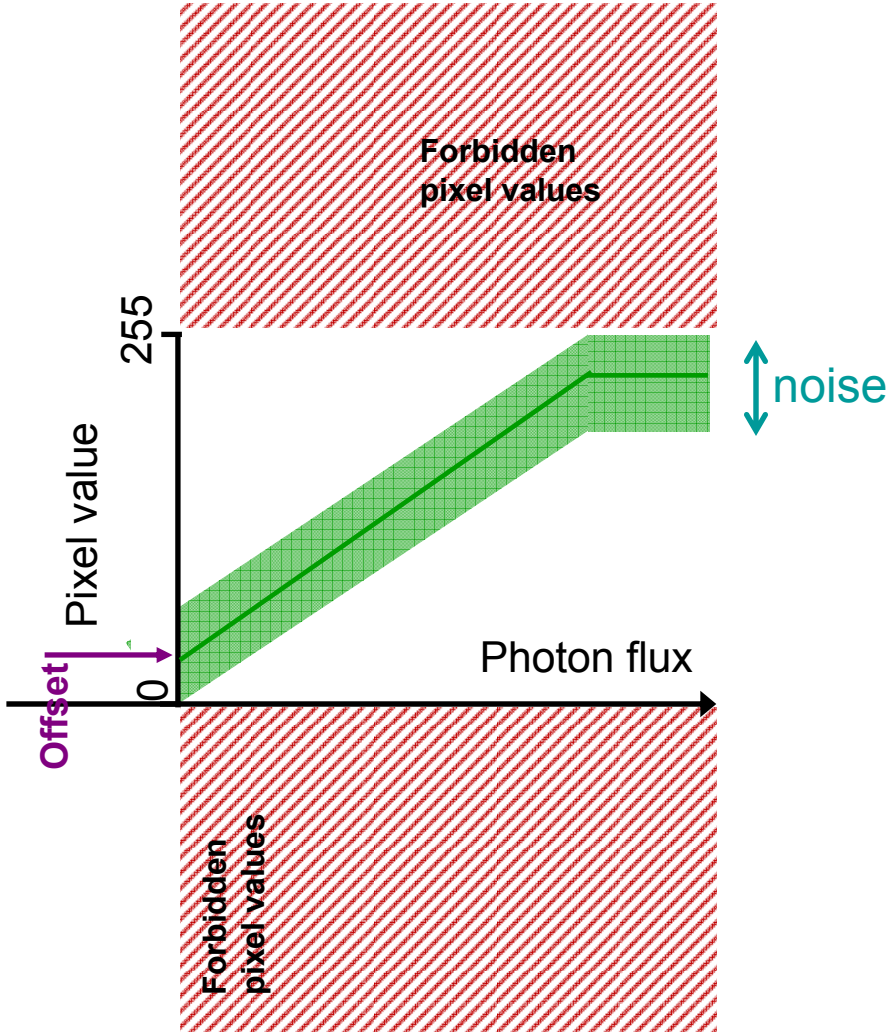
-ve

Quantification

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

Reduced range – restore contrast after averaging





Quantification

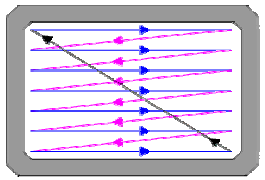
- Overload or underload leads to loss of information
- Allow room for noise (noise contains information)
- Recover contrast after acquisition
- Save data uncompressed or with lossless compression
  (not jpeg or gif for color images)

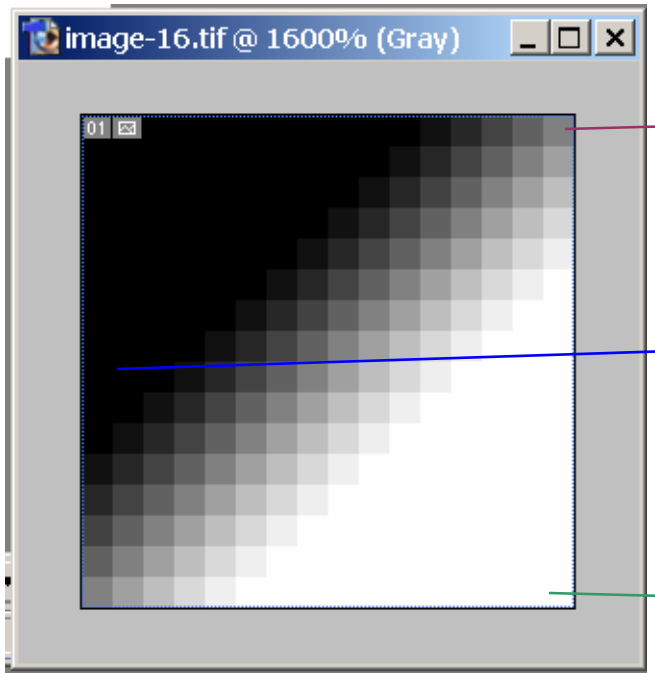
Pixel value depends on:

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

Image Representation Digitally

- 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





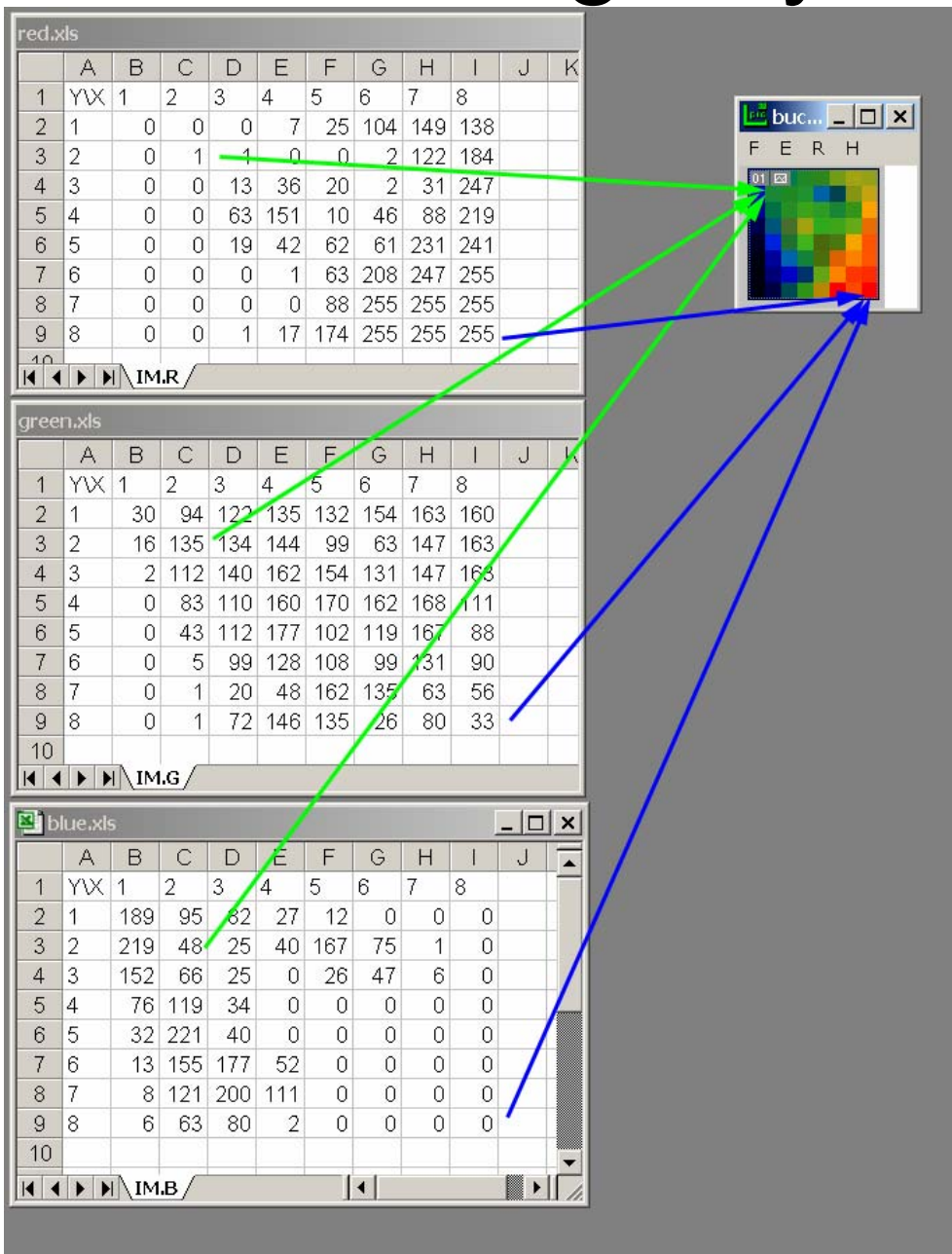
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Y\X	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	1	0	0	0	0	0	0	0	0	0	0	0	16	39	66	96	127
3	2	0	0	0	0	0	0	0	0	0	0	16	38	66	95	127	159
4	3	0	0	0	0	0	0	0	0	0	16	38	65	96	128	159	189
5	4	0	0	0	0	0	0	0	0	17	38	66	96	127	159	189	216
6	5	0	0	0	0	0	0	0	16	39	66	96	127	159	190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

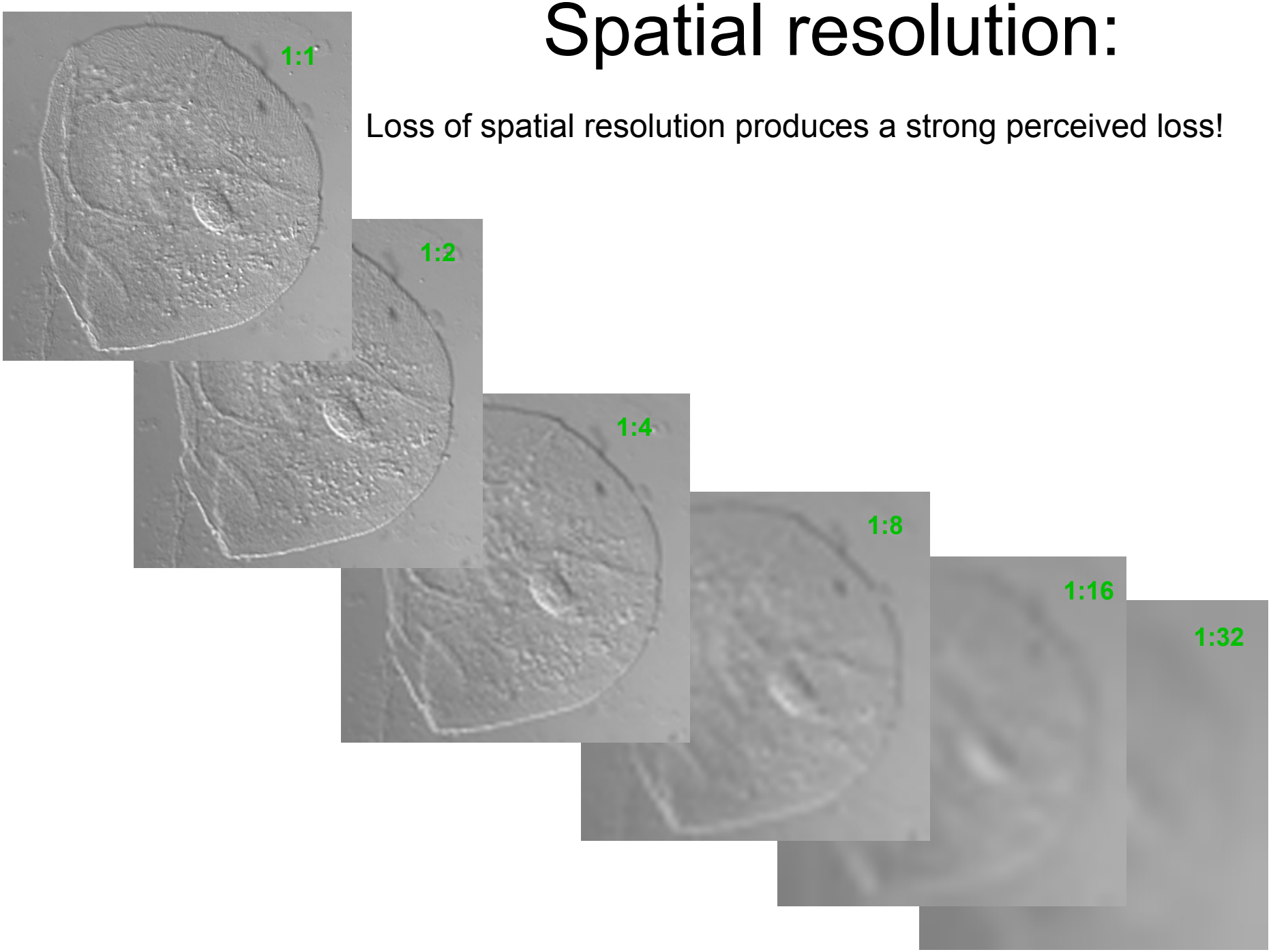
RGB image

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

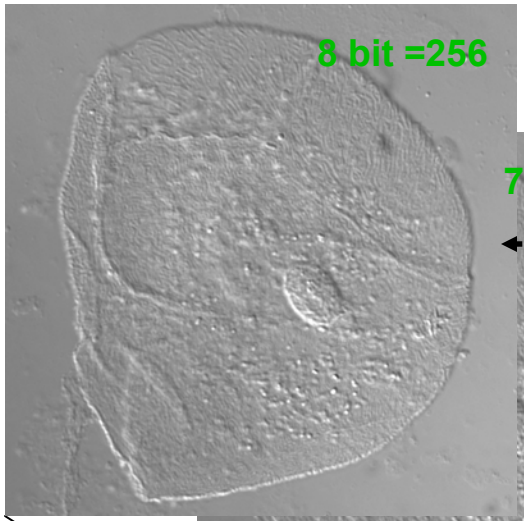


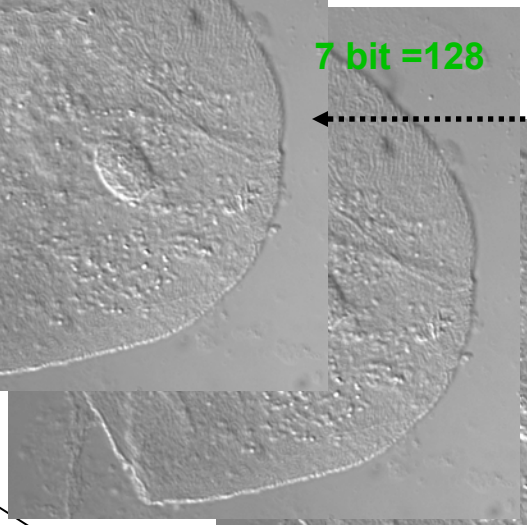
Spatial resolution:

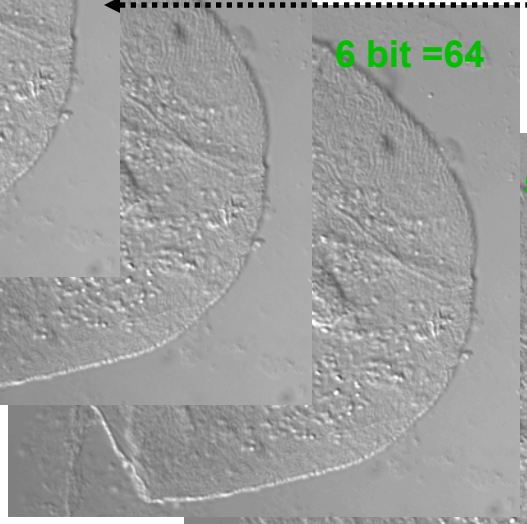
Loss of spatial resolution produces a strong perceived loss!

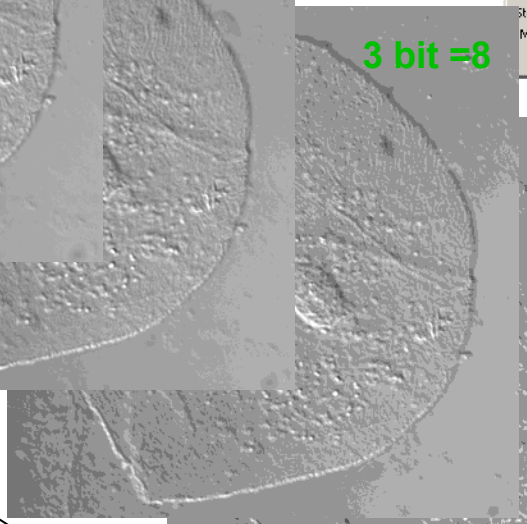


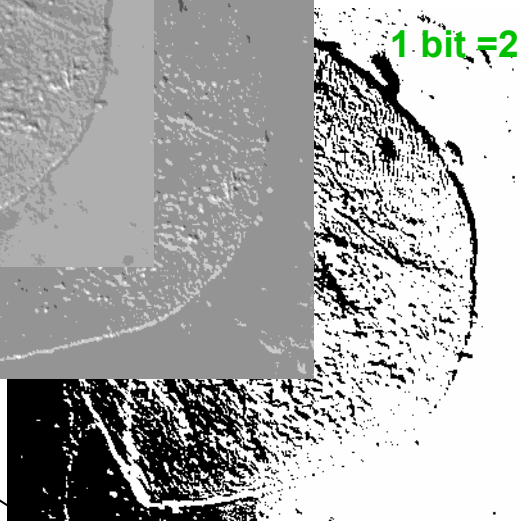
Intensity resolution: Bit depth & levels

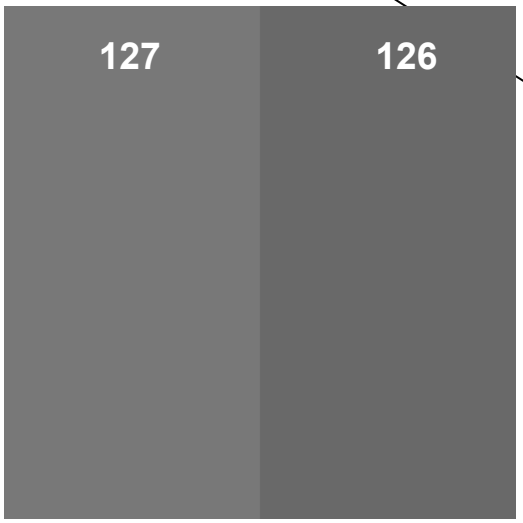


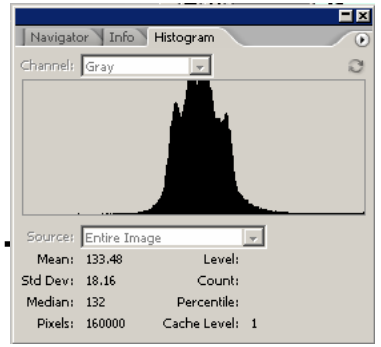


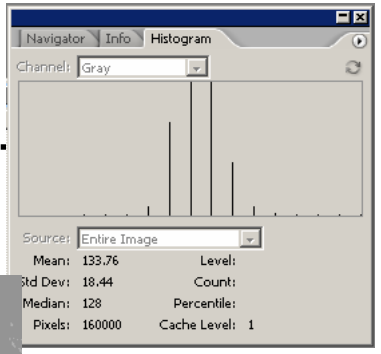












Can loose much intensity resolution with little subjective loss!

Digital Image

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

- 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)

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/