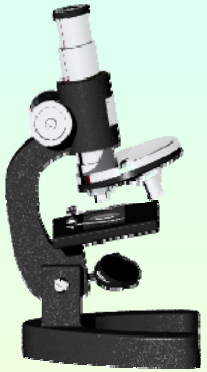
2006 Oct 30, Nov 6, **13**, 20 & 27

Detectors, Sampling & Digital Images

$E = h\nu$



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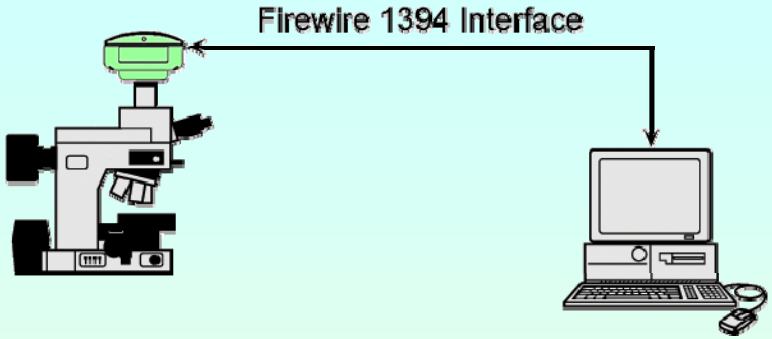
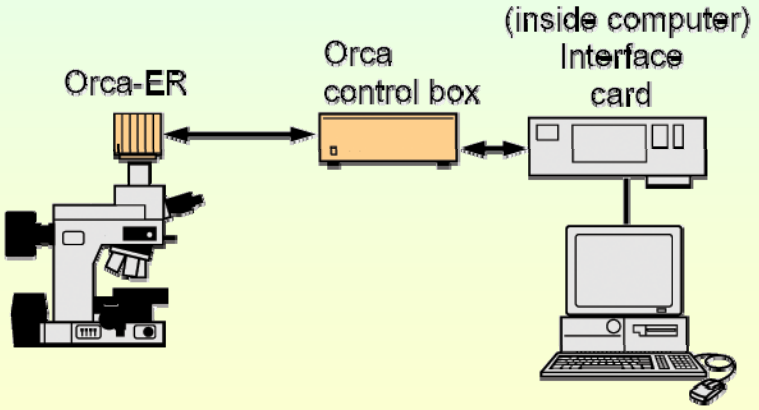
Detectors, Sampling & Digital images

- Image Acquisition
- Digital cameras
- Digital images (8 bit, >8bit, RGB 24 bit)
- File formats (TIFF, GIF, JPEG)
- Image processing (extremely briefly)

Image Acquisition



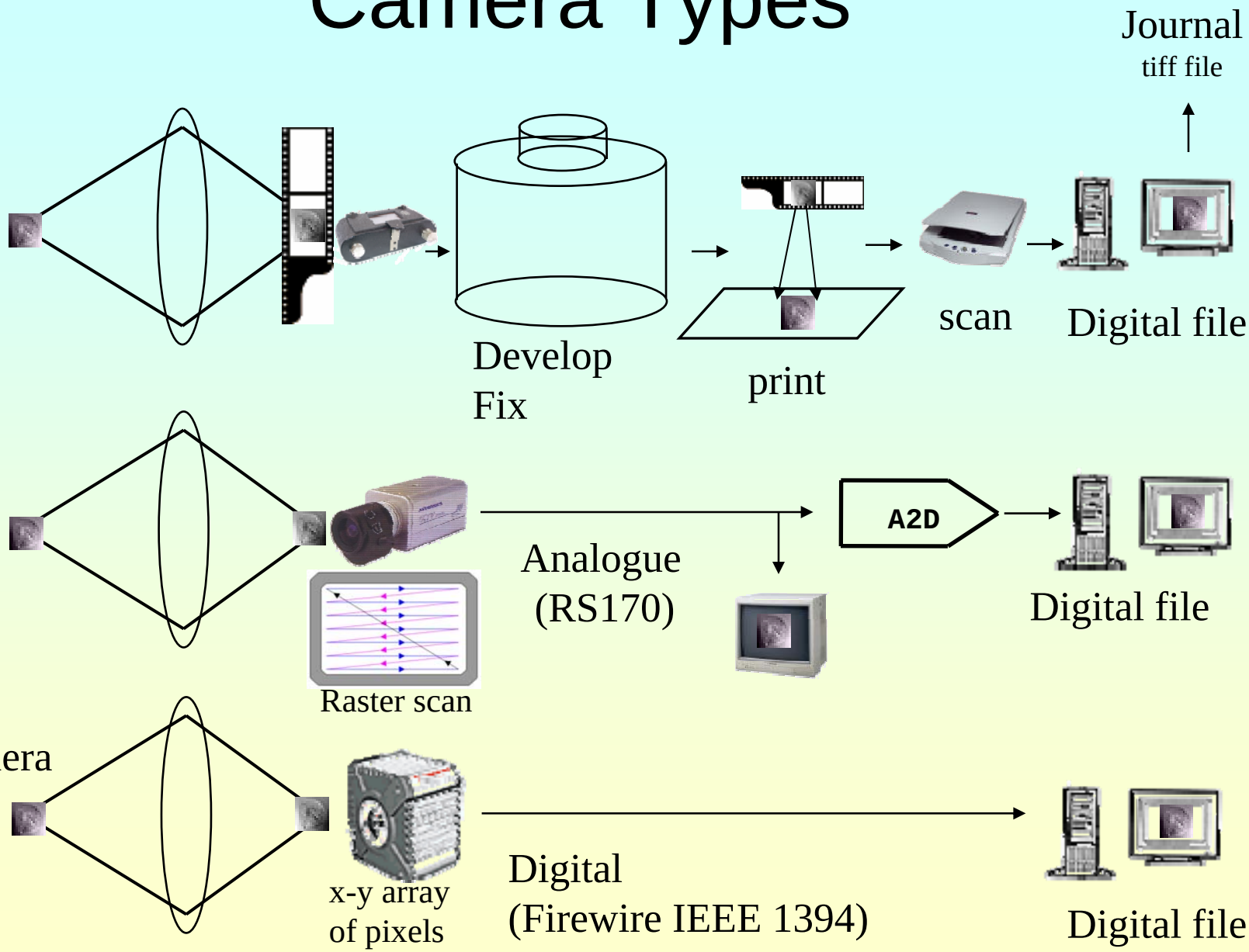
MicroPublisher: Low sensitivity and high resolution color CCD camera.



OrcaER: High sensitivity and precision digital monochrome CCD camera.

Interface options: Firewire (free with computer)
RS422 Interface (~$1000)

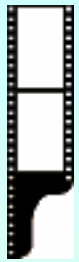
Camera Types



(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)– set frame rate, limited exposure time, poor spatial resolution, poor intensity resolution, poor intensity resolution – noisy (1940's standards based on 1940's capabilities) – requires an expensive A2D (frame grabber) – loose detection time due to raster scan - 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 – but need a computer to see image.

QE = 0.1 to 0.9

QE = Quantum Efficiency – fraction of input photons detected

CCDs for Microscopy – How they Work

Photodiode - Well structure - Charge transfer cycle

Figure 1-1 Photodiode cross section

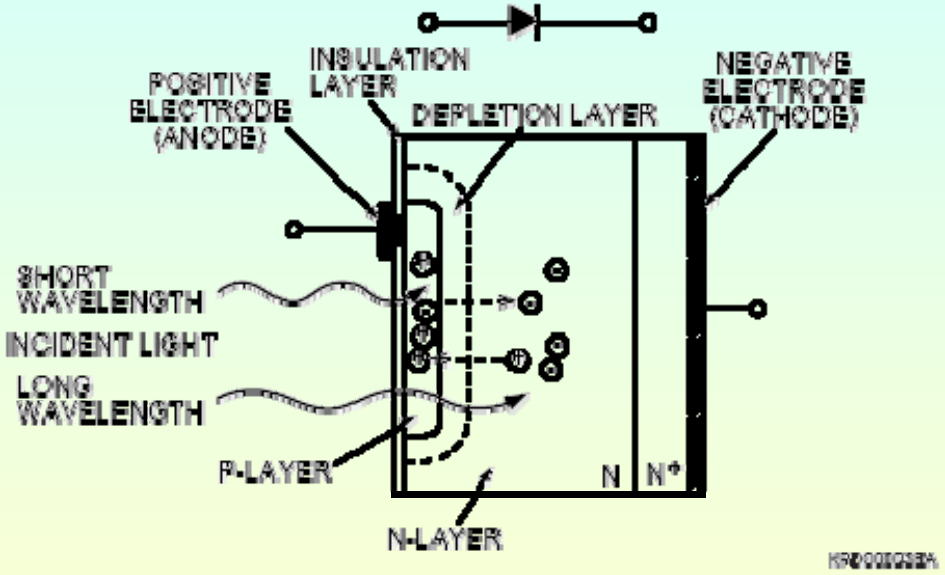
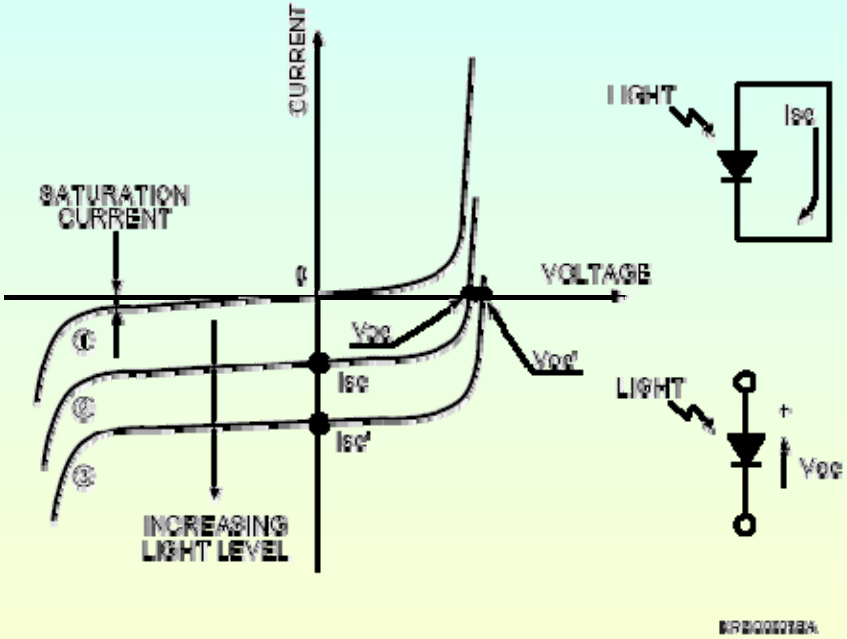


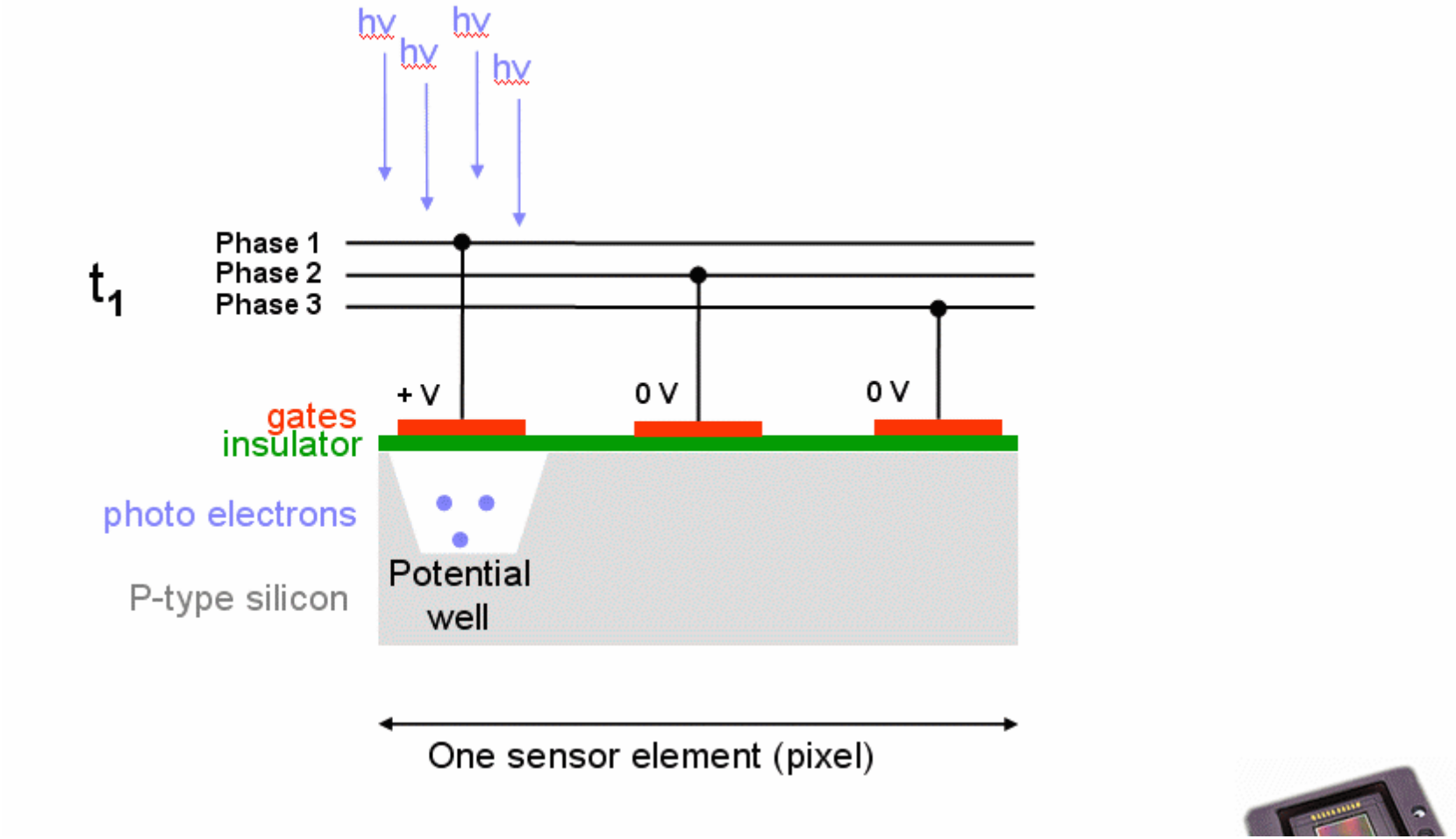
Figure 2-2 Current vs. voltage characteristic



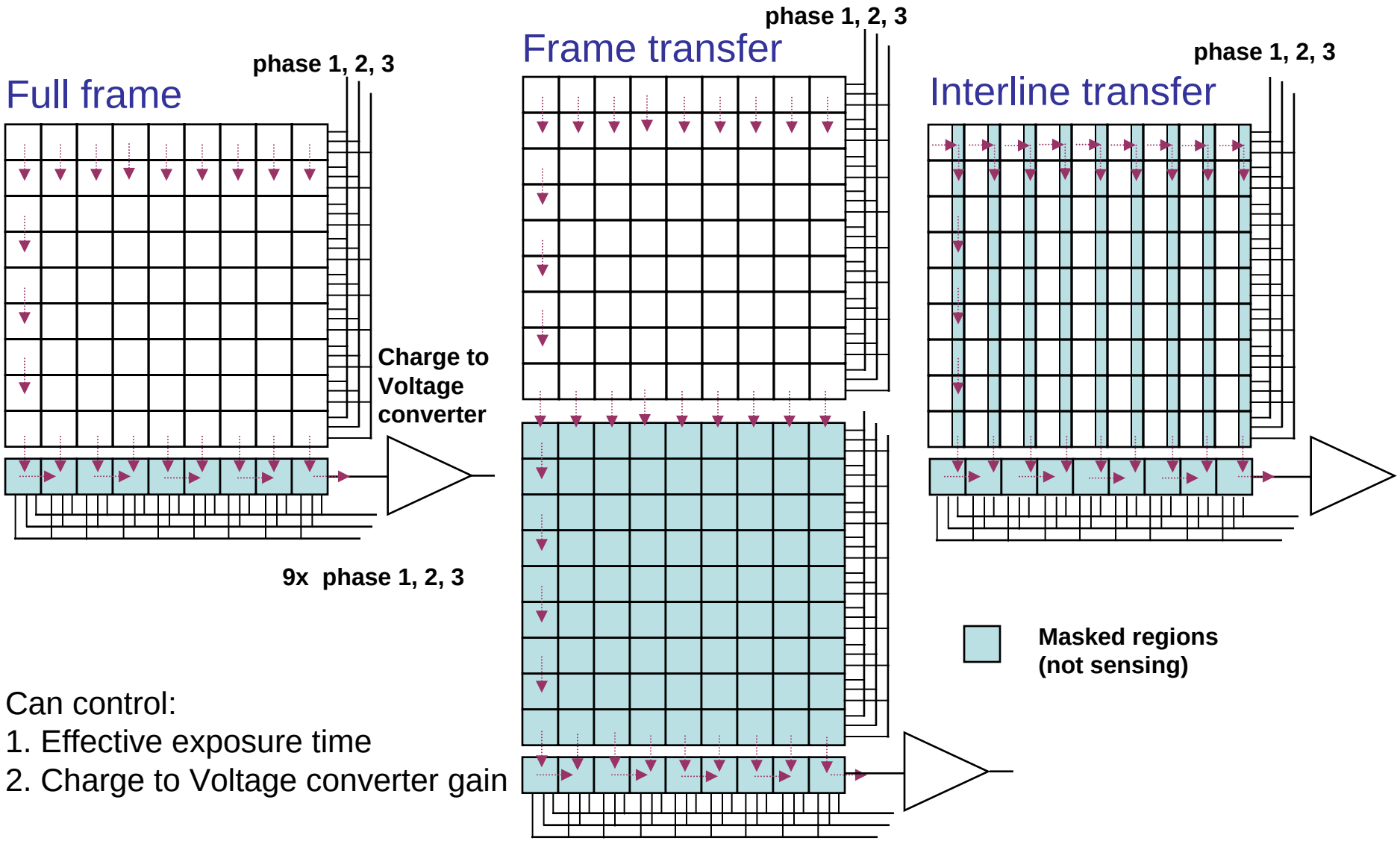
From Hamamatsu Photodiode Technical Sheet

CCDs for Microscopy – How they Work

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



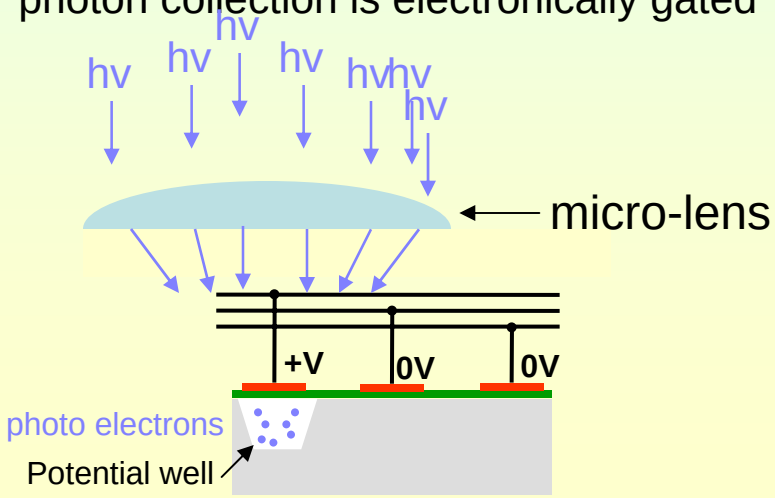
CCDs for Microscopy – CCD Formats

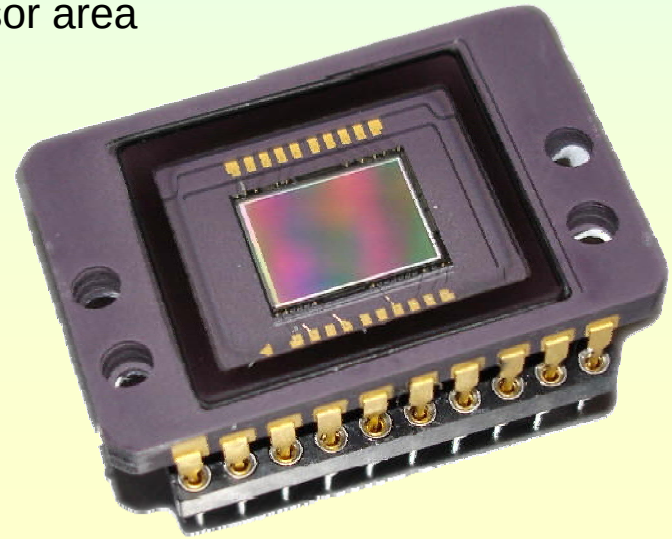


Can control:
1. Effective exposure time
2. Charge to Voltage converter gain

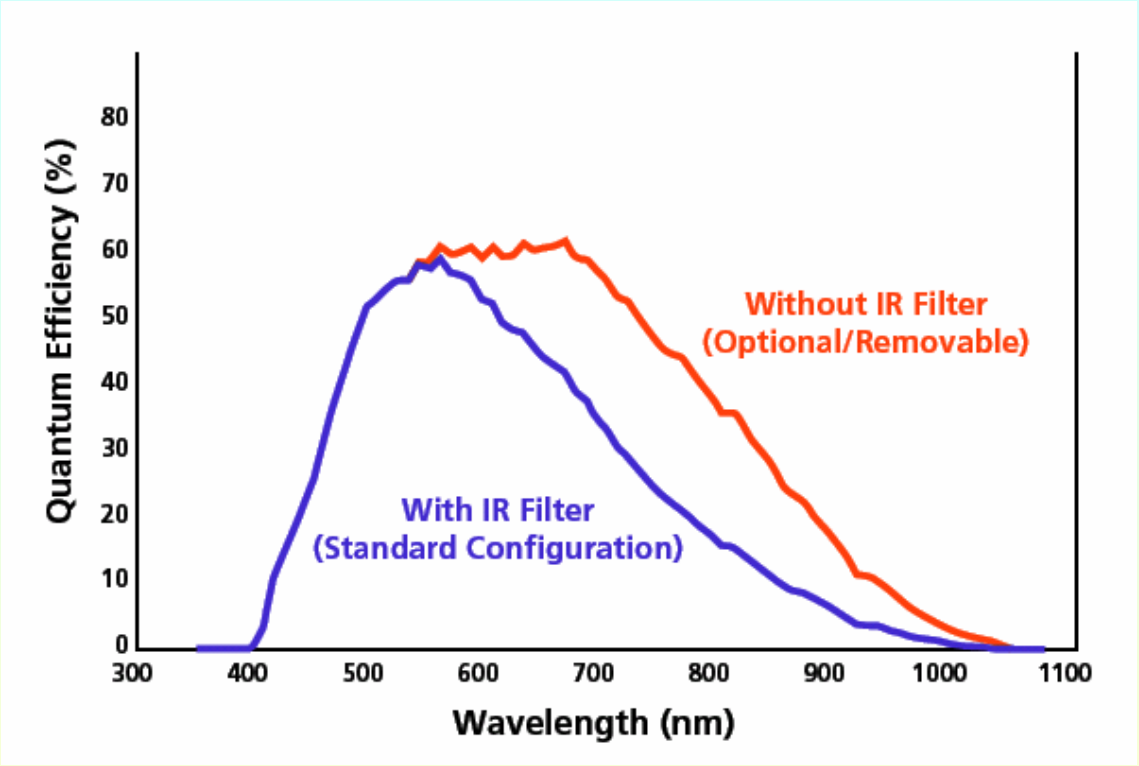
CCDs for Microscopy – CCD Formats

- 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

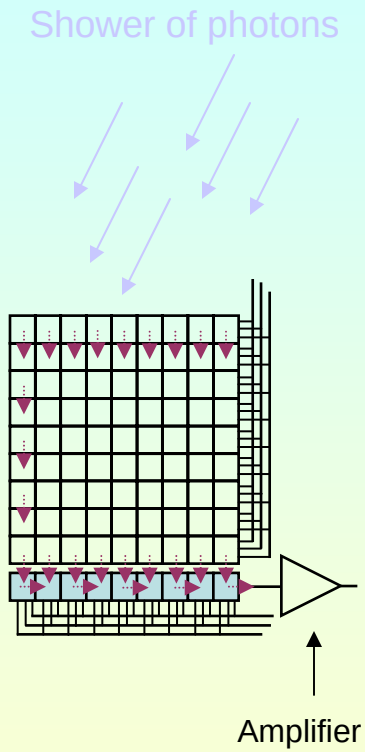
Optical projection onto sensory array

- 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

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

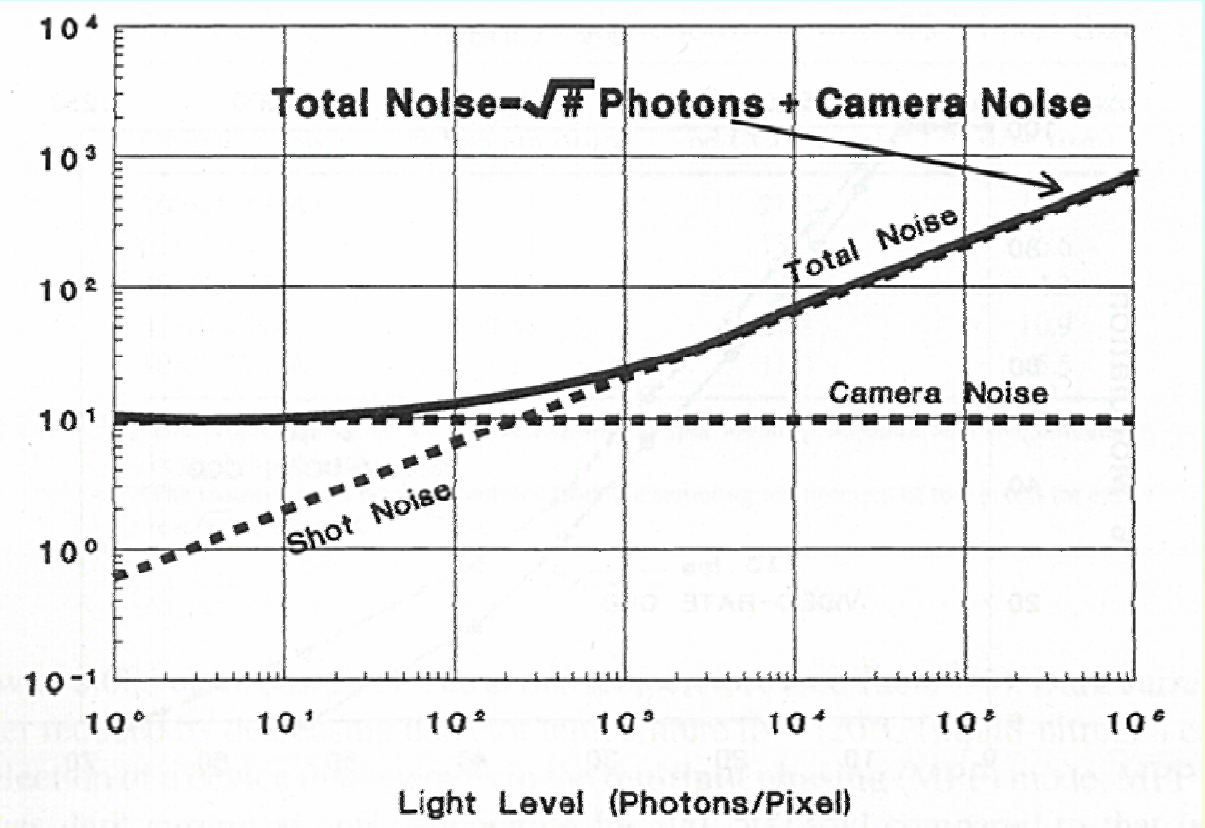
- Read out noise $\delta_{readout}$
  - Amplifier noise mostly
  - Reduce with photon amplification (intensifiers) before sensor or electron multiplication before readout amplifier
  - Also switching transients associated with read out clocks
  - Reduce with careful electronic design and slower readout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

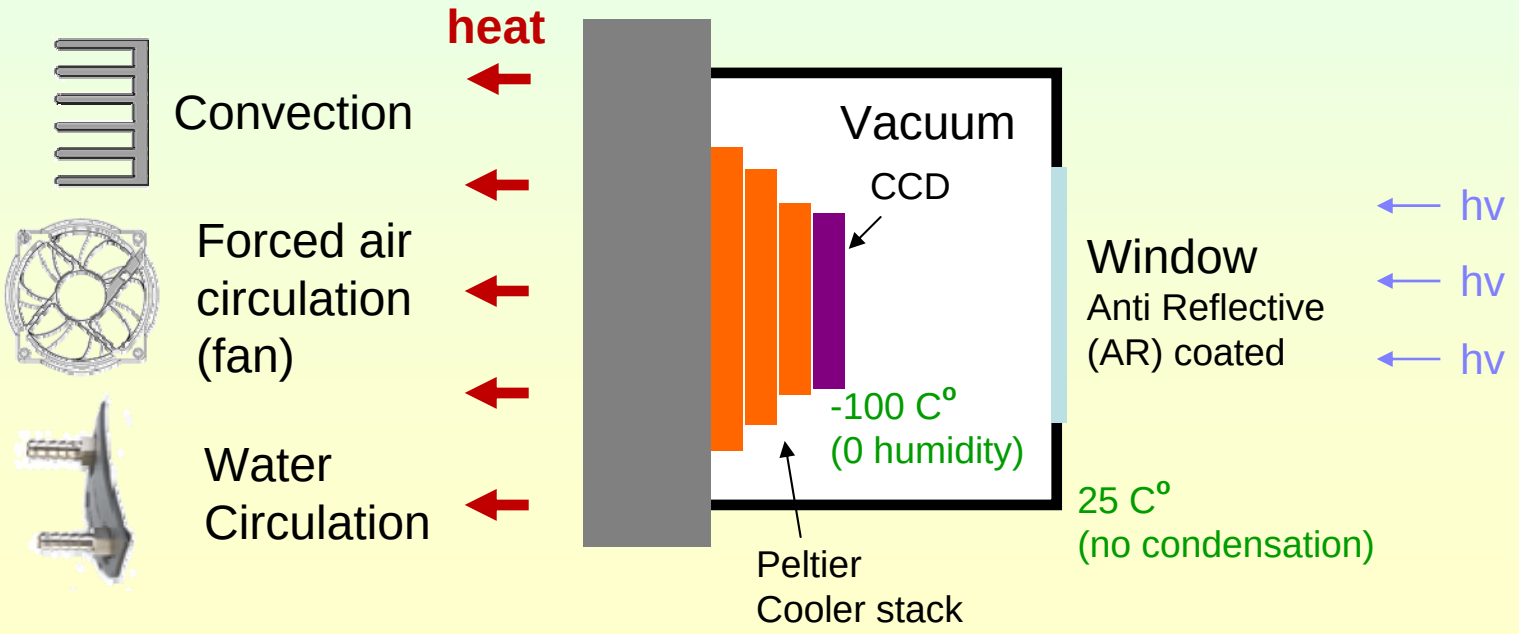
- $\delta_{total} = (\delta^2_{dark} + \delta^2_{readout} + \delta^2_{signal})^{1/2}$

Noise - factor



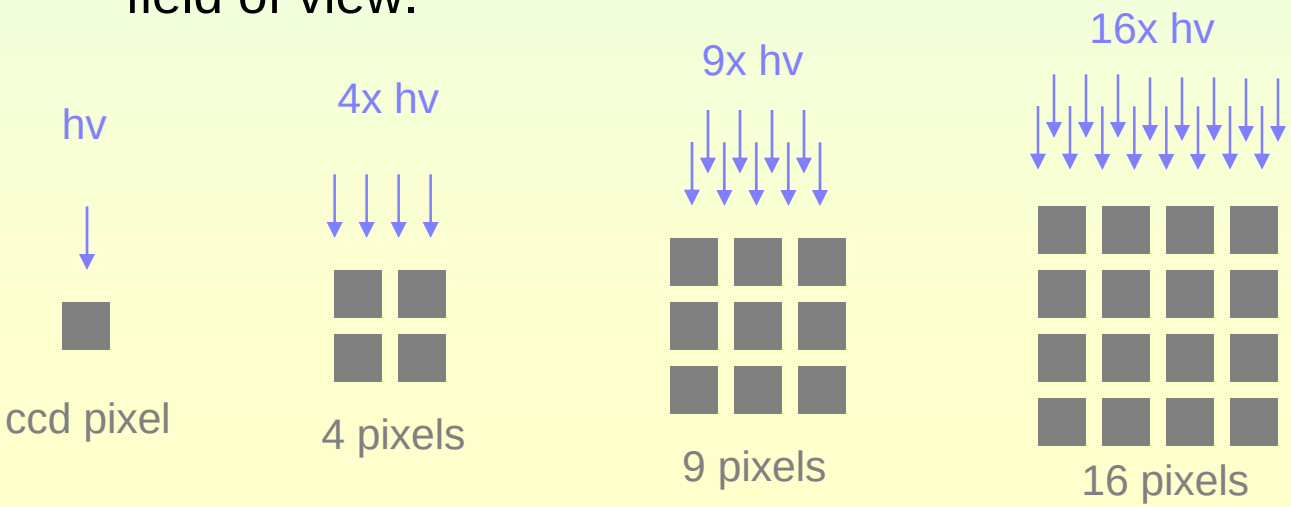
CCDs for Microscopy – Cooling

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



CCDs for Microscopy – Binning

- Pixel binning: merge adjacent pixels together electronically.
  - 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.

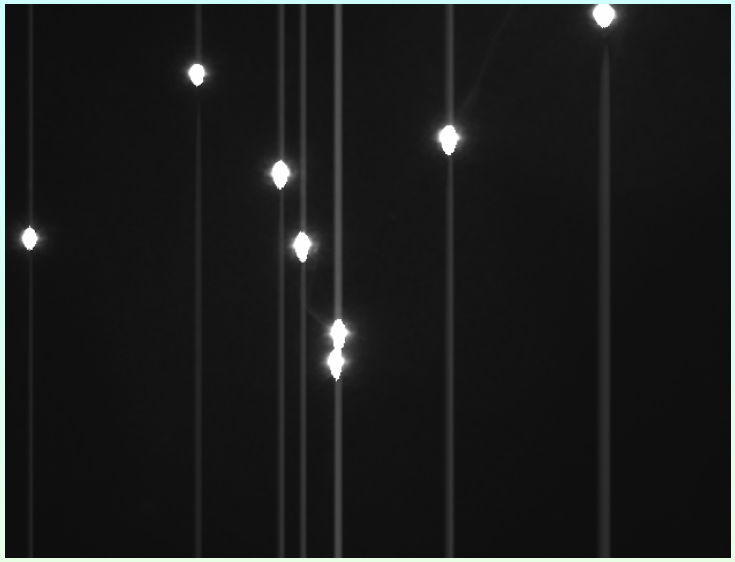


CCD – Sensitivity & Dynamic Range

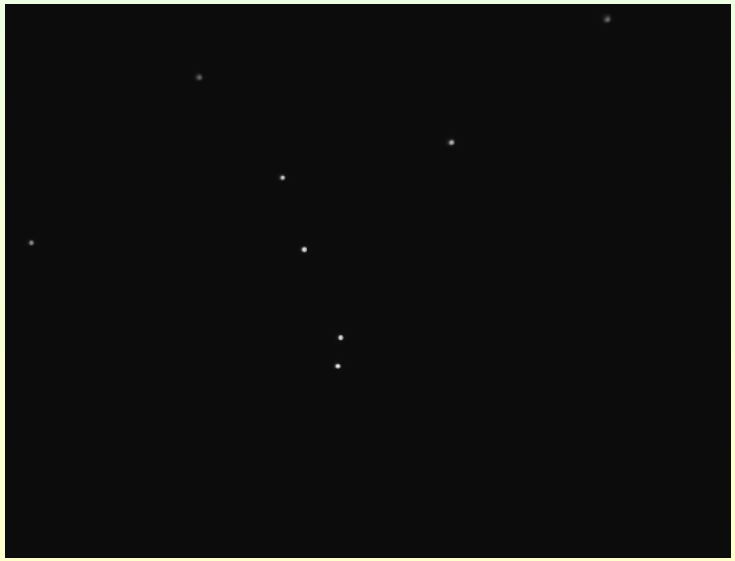
- Sensitivity: minimum light signal which can be detected. Limits set by noise floor.
  - With short exposures shot noise increases and signal amplitude can approach read out noise level
  - Long exposures - shot noise integrates (averages) out and the large signal offset caused by dark current is mitigated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



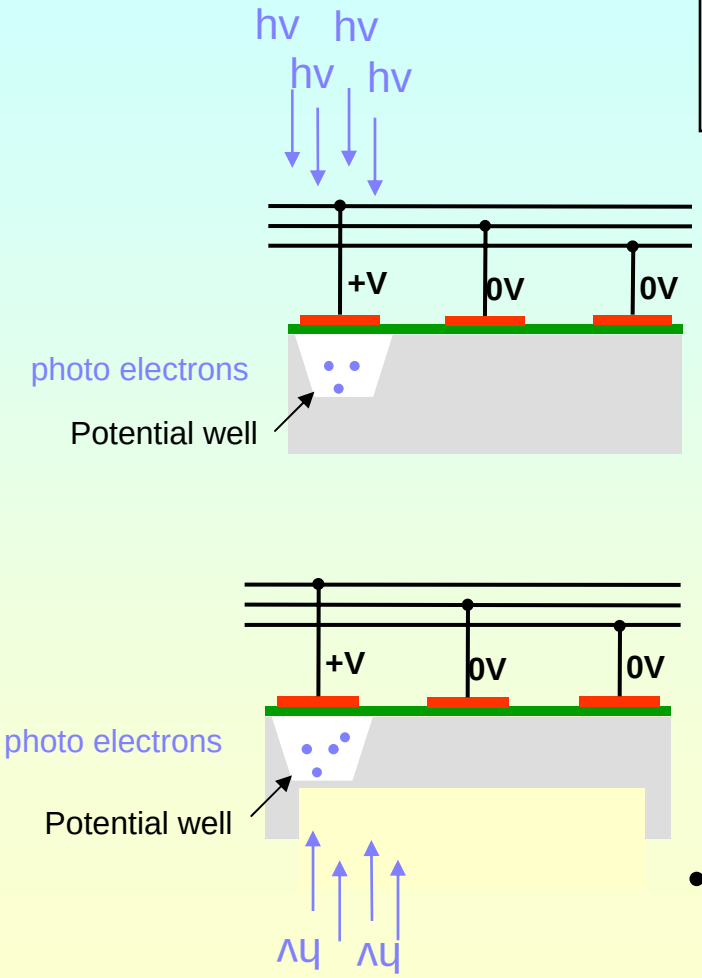
Blooming: In bright light conditions photoelectron charge can fill a well and spill over. Charge transfer is preferred in the vertical direction, so vertical streaks result in the image.



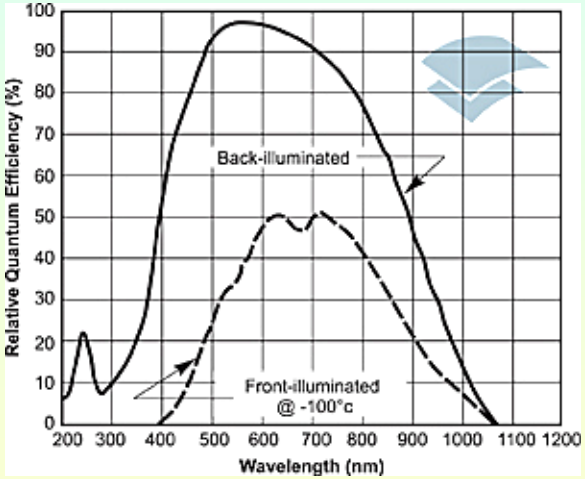
Anti-blooming: Circuitry can be enabled, in many high end CCDs, which drains excess photoelectrons to the substrate. Sensitivity is reduced (not a problem when there are an excess of photons anyway)

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

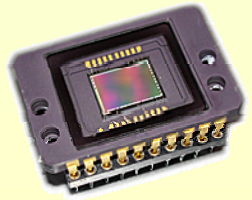


- Back illumination: avoids wiring, but chip has to be thinned to avoid absorption
- Chip is fragile (~10 um thick) and expensive, but max QE = ~0.9

CCDs for Microscopy – Intensification

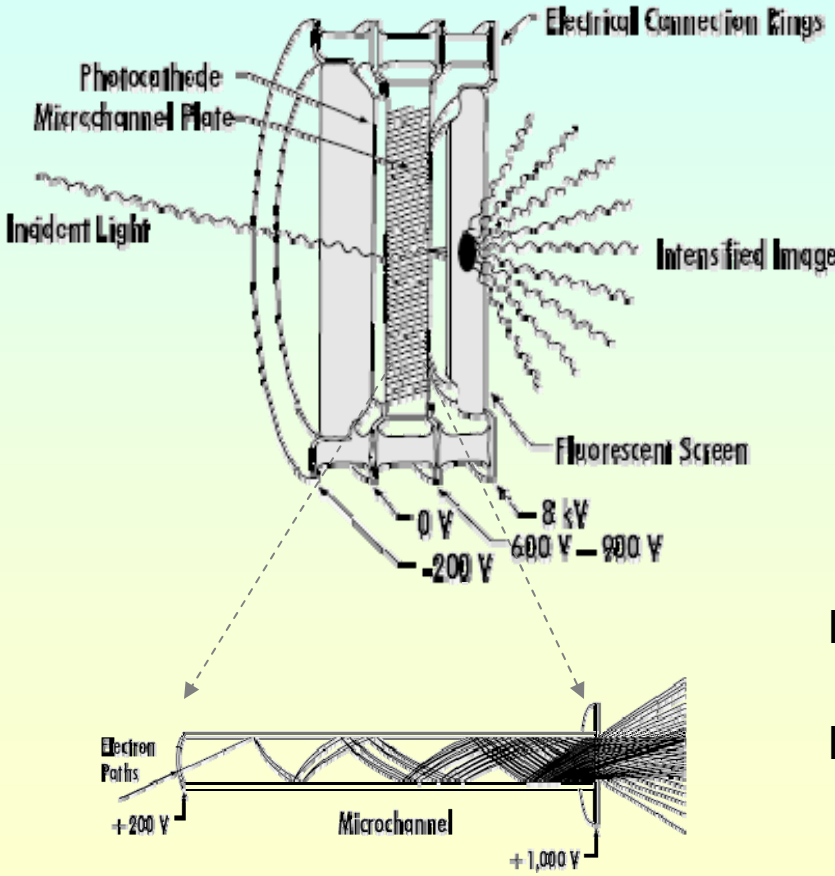
- Overcome readout noise – detect very low levels
- Get image which exceeds threshold of detector. c.f. for night vision goggles for human eye
- Penalty is increased noise due to fluctuations in the intensifier device.

- 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.

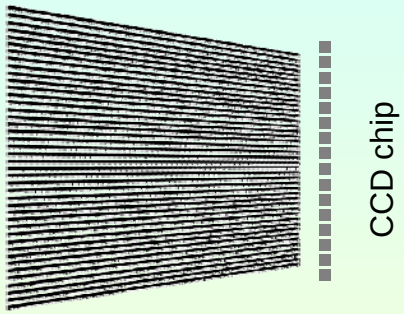


CCDs for Microscopy – Intensification

Micro-channel intensifier (MCI) before the CCD



Similar to a photomultiplier tube (PMT)



Fiber optic coupler
(Must be close to
fluorescent screen)

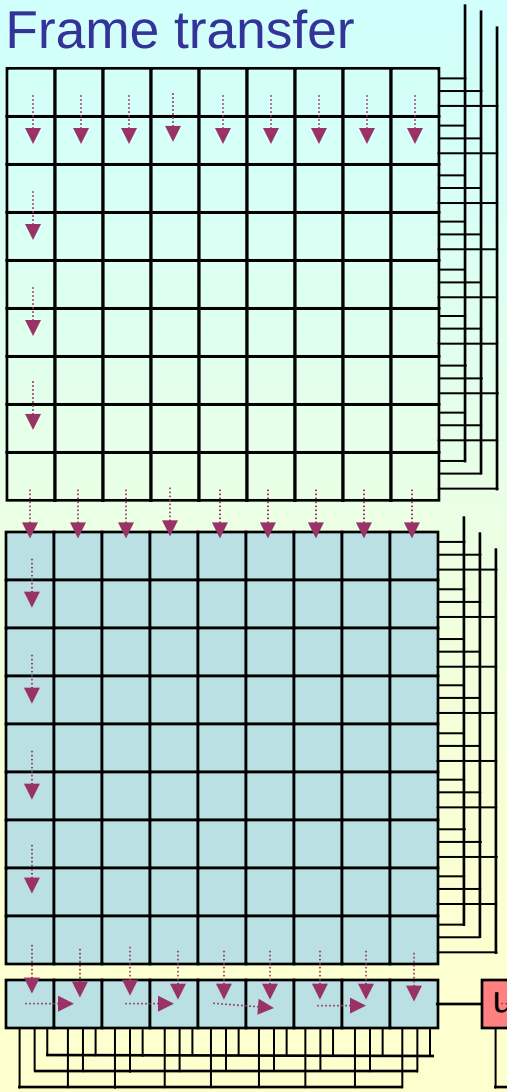
Possible gain beyond ~ 1 000 000

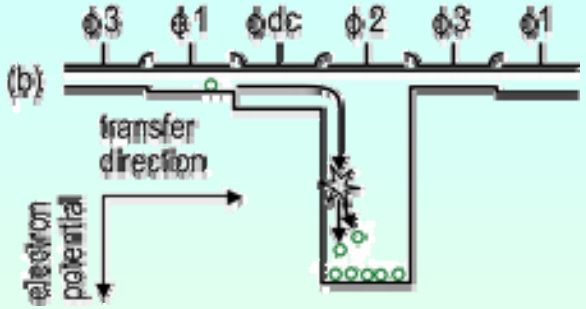
Bright light will cause MCI burnout

CCDs for Microscopy – Intensification

Electron Multiplication CCD

Frame transfer



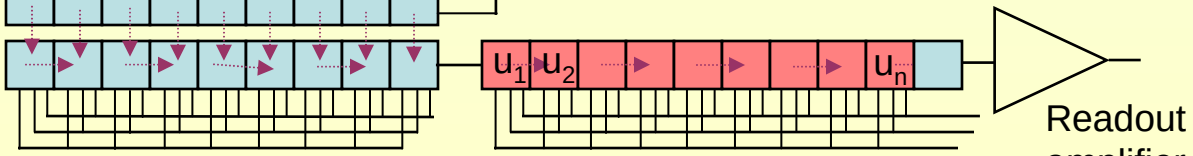


High well potential
Impact ionization
c.f. Avalanche Photodiode or Zener Diode
Maximum gain ~1000

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

u approx 1.02

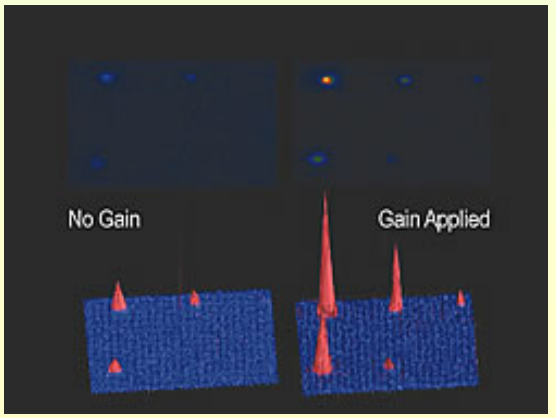
masked wells



Readout amplifier

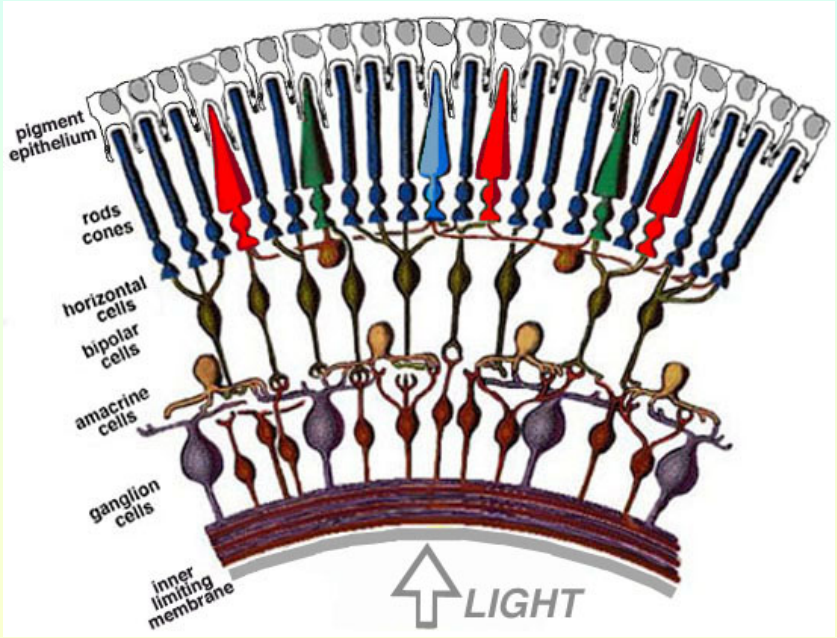
Readout registers

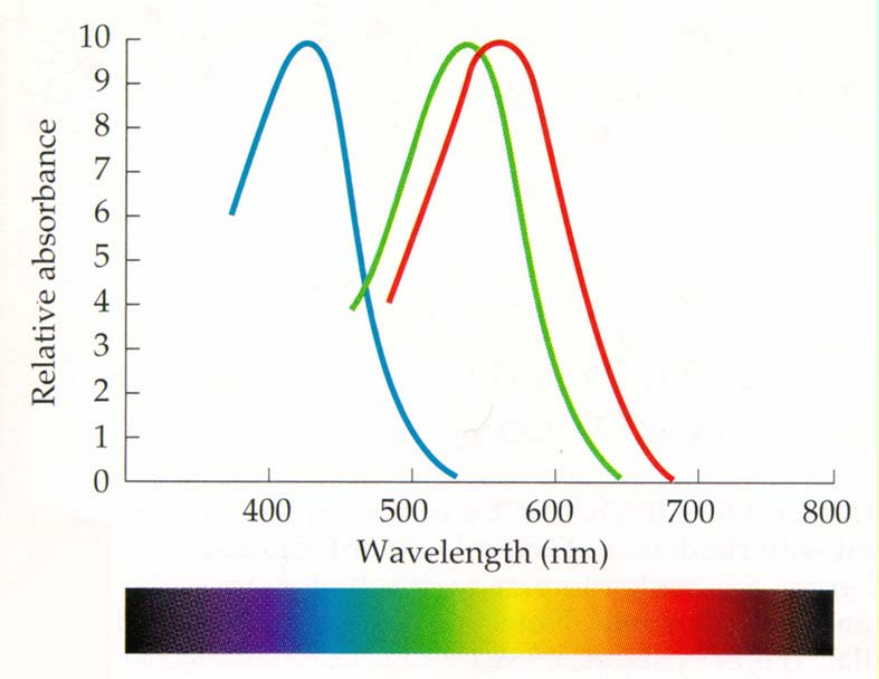
Multiplication registers



Color for Humans - Spectral Separation

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



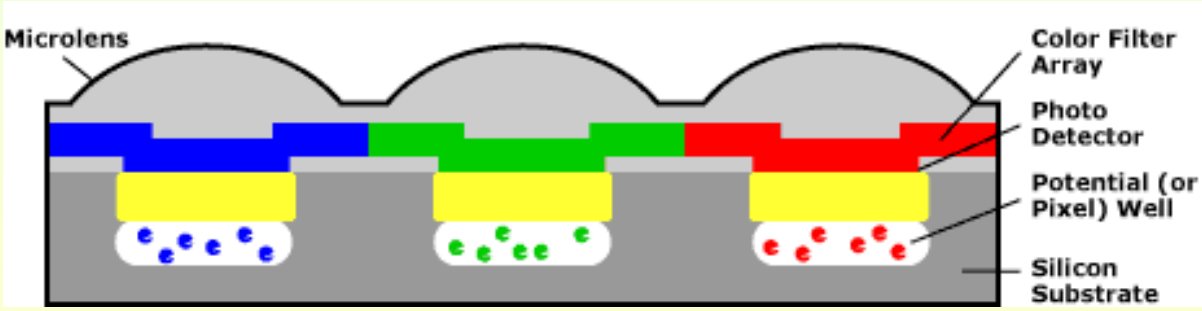


Spectral sensitivity of cone receptors

Color for Cameras

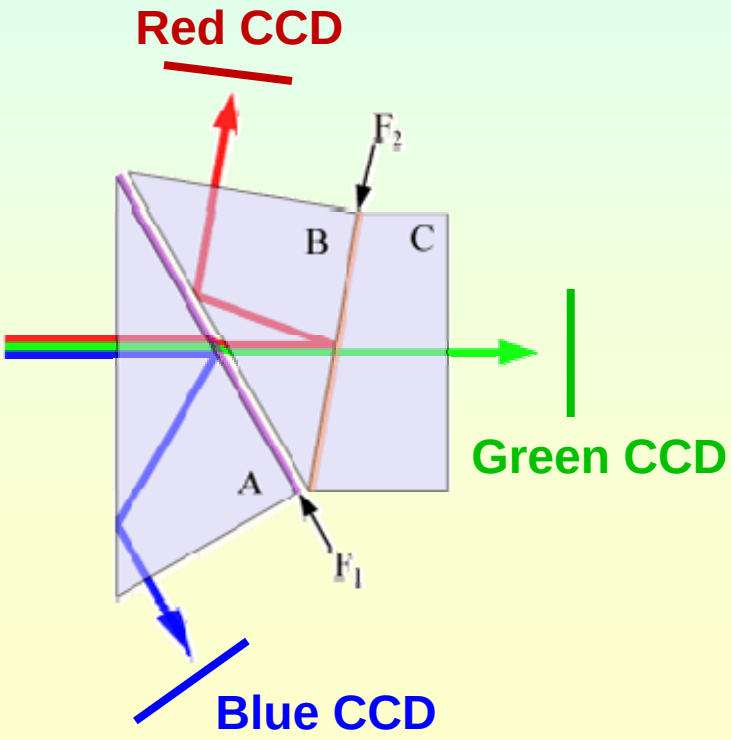
- Absorptive filters in front of CCD pixel elements – even lose light close to desired wavelength –
- Can not pack 3 colors into rectangular array
- Therefore Bayer Pattern - loss of intensity especially in blue and red & loss of spatial resolution

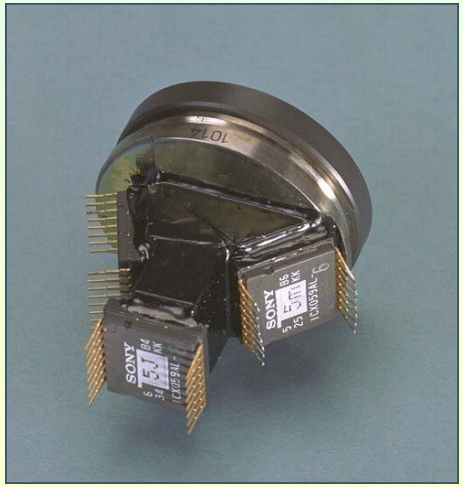
G	R	G	R
B	G	B	G
G	R	G	R
B	G	B	G



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!





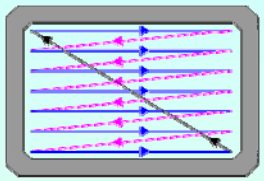
CCDs for Microscopy – Camera Specs

- 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

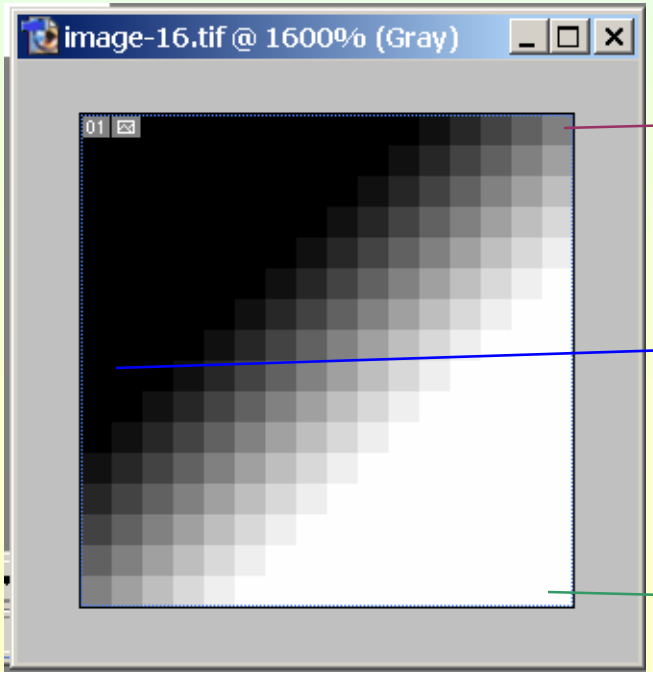
- **Digital Image** - as represented inside the computer
- Image file formats
- Image processing

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



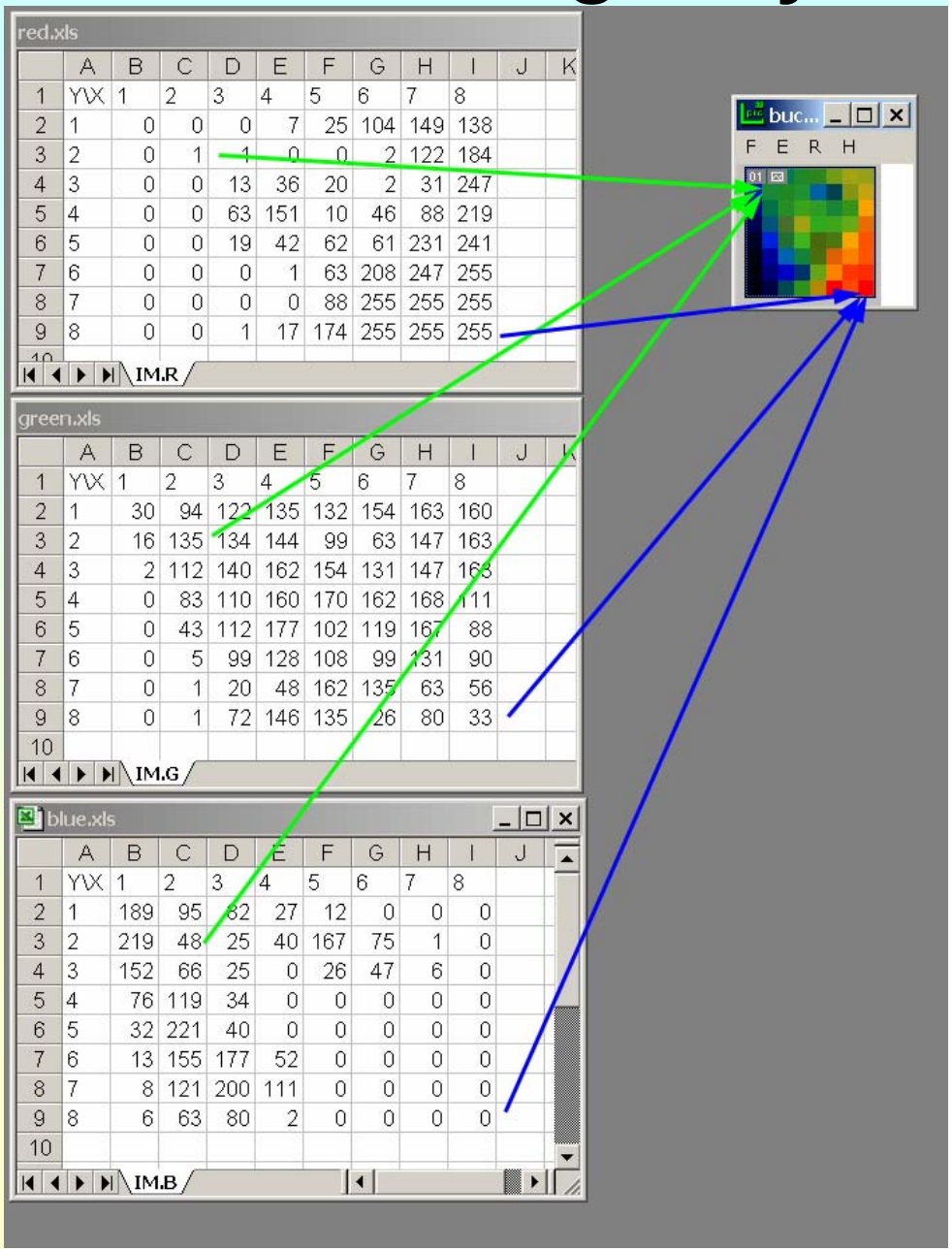
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Y\X	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	1	0	0	0	0	0	0	0	0	0	0	0	16	39	66	96	127
3	2	0	0	0	0	0	0	0	0	0	0	16	38	66	95	127	159
4	3	0	0	0	0	0	0	0	0	0	16	38	65	96	128	159	189
5	4	0	0	0	0	0	0	0	0	17	38	66	96	127	159	189	216
6	5	0	0	0	0	0	0	0	16	39	66	96	127	159	190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

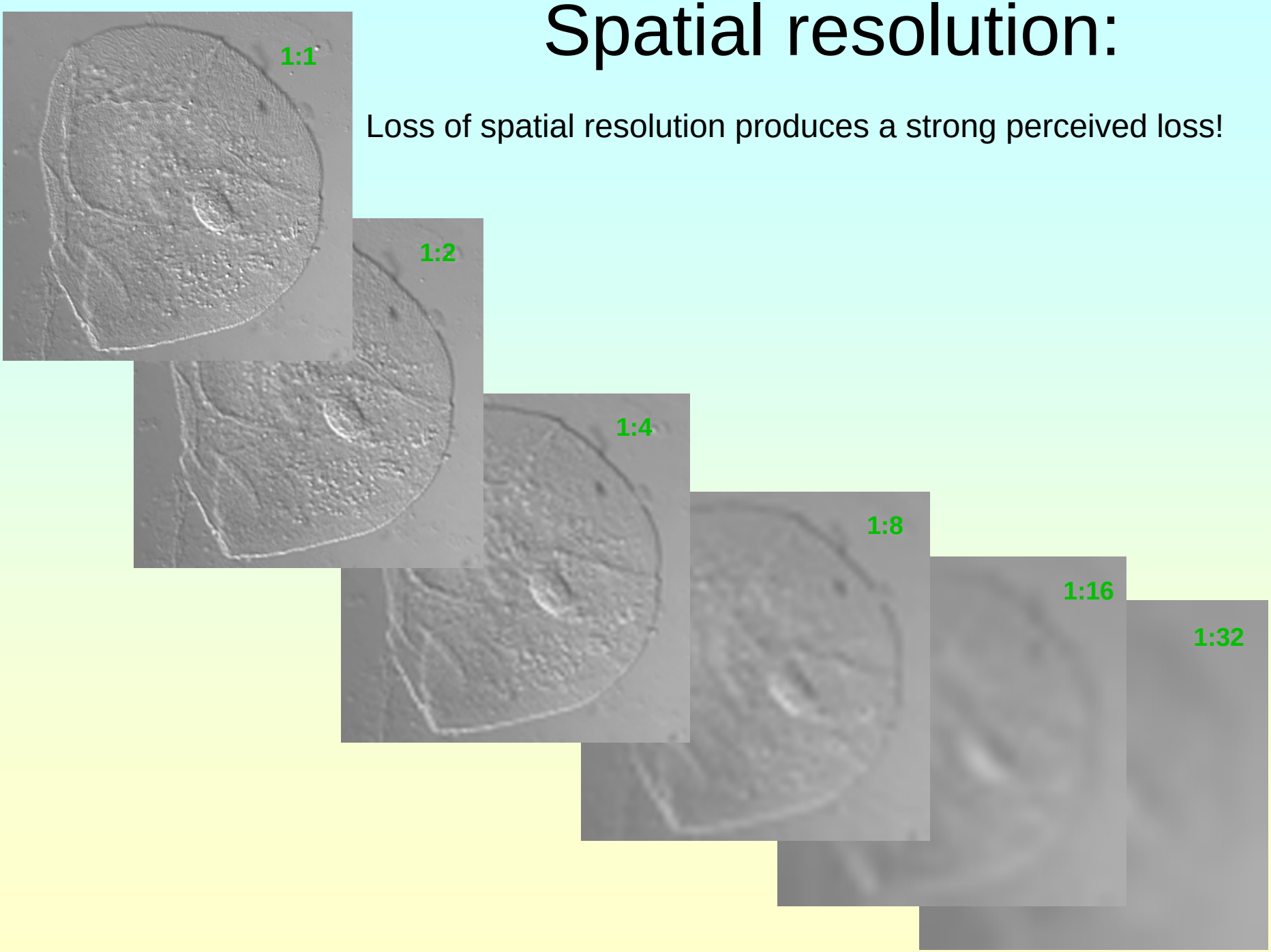
RGB image

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

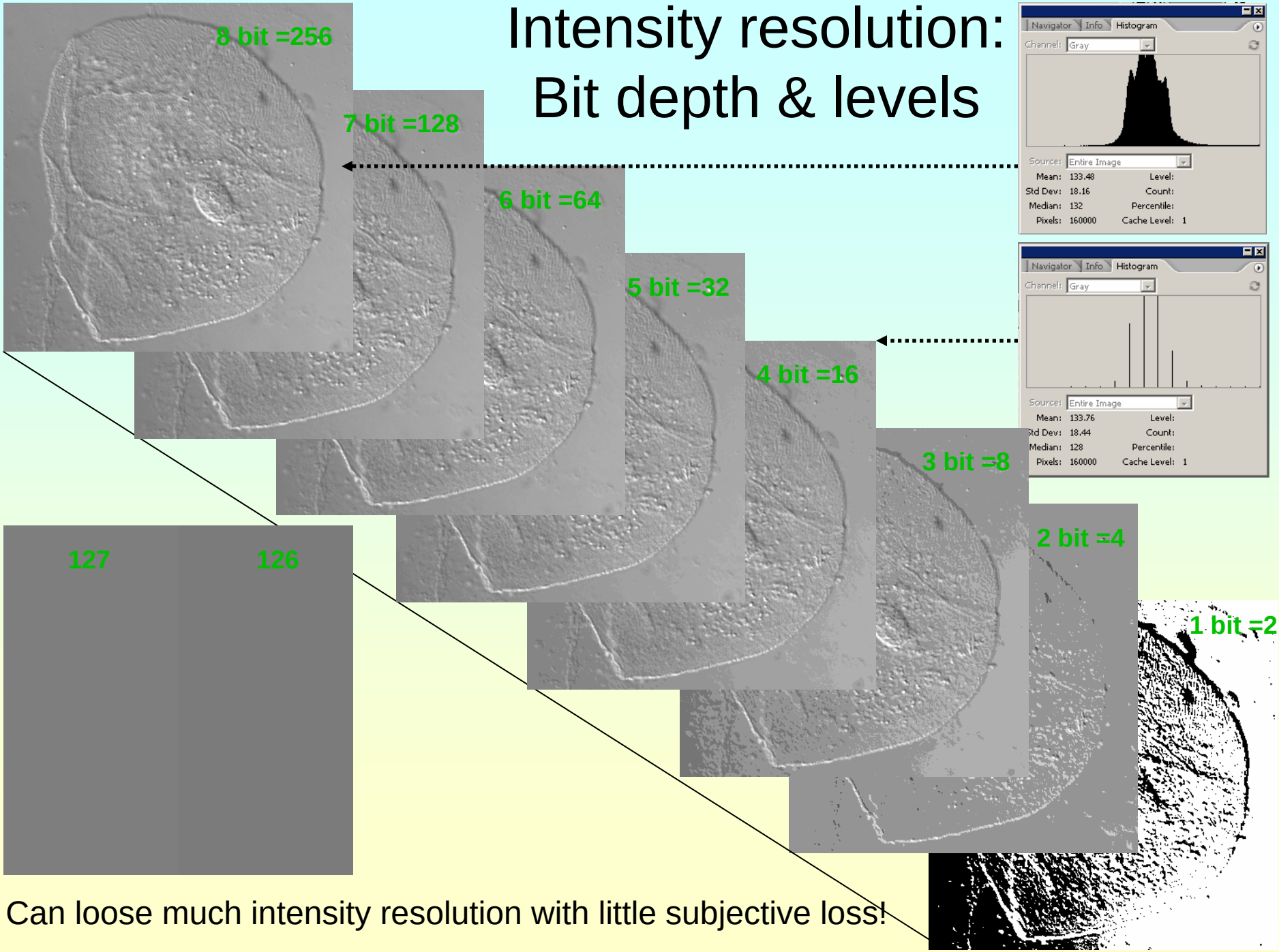


Spatial resolution:

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)

- Digital Image
- **Image file formats**
- Image processing

File Formats

- There are a large number of ways images can be represented and stored in a computer file. All formats have variations, sub-formats. Common formats are TIFF, GIF, JPG. Some software will even use a proprietary format, and force the use of proprietary software or conversion programs.

- Details of formats can be found readily via Google.

Image File Representation - Header

- Header (typical)
  - Code for format type *4949*=TIF or *GIF87a*=GIF or *FFD8FF*=JPG, etc.
  - Size of image file on disk
  - X dimension (horizontal pixels)
  - Y dimension (vertical pixels)
  - Bits per pixel (e.g. 8, 12, 16)
  - Channels per pixel (e.g. 1=monochrome, 3=RGB, 2=indexed)
  - Calibration (e.g. pixels per inch)
  - Other information both useful and redundant

- Number stream saved as a 2-D array – f(x,y)
  - Pixel intensity values

Typical Digital Image

- Grayscale – 8 bit usually. No color information.

- Color – RGB 8 bit red – 8 bit green – 8 bit blue for each pixel. Referred to as 24 bit (handled in most imaging software). Can have more bits per pixel, but rarely so.

- Index Color (can also have index which is gray scale) f(x,y) array– 8 bit photometric correct data passed through a red, green blue look up table (LUT). Can only have 256 different colors per image. But colors can be chosen from 16 million (24 bit RGB). Intensity of translated data may not be related to original intensity of detected pixel. Color is pseudo color.

File Formats - Typical

- TIFF – tagged image file format – blocks of various data are proceeded by a tag (information about what the nature of the block is) – tags describe whether block is text, 8, 12, 16 bit numbers, start of block, length of block, location of next tag. This format is very flexible but can have software which does not know how to handle a specialized TIF file image structure. Compression is generally lossless. However lossy compression can be specified.

- GIF – 8 bit indexed (can not handle RGB color). OK for single channel data. Can colorize using the look up table. Data is compressed. Compression is lossless.

- JPG – RGB or 8 bit. Compression is lossy compression. Not good for data to be analyzed or measured

- Digital Image
- Image file formats
- **Image processing –** see Nov 27

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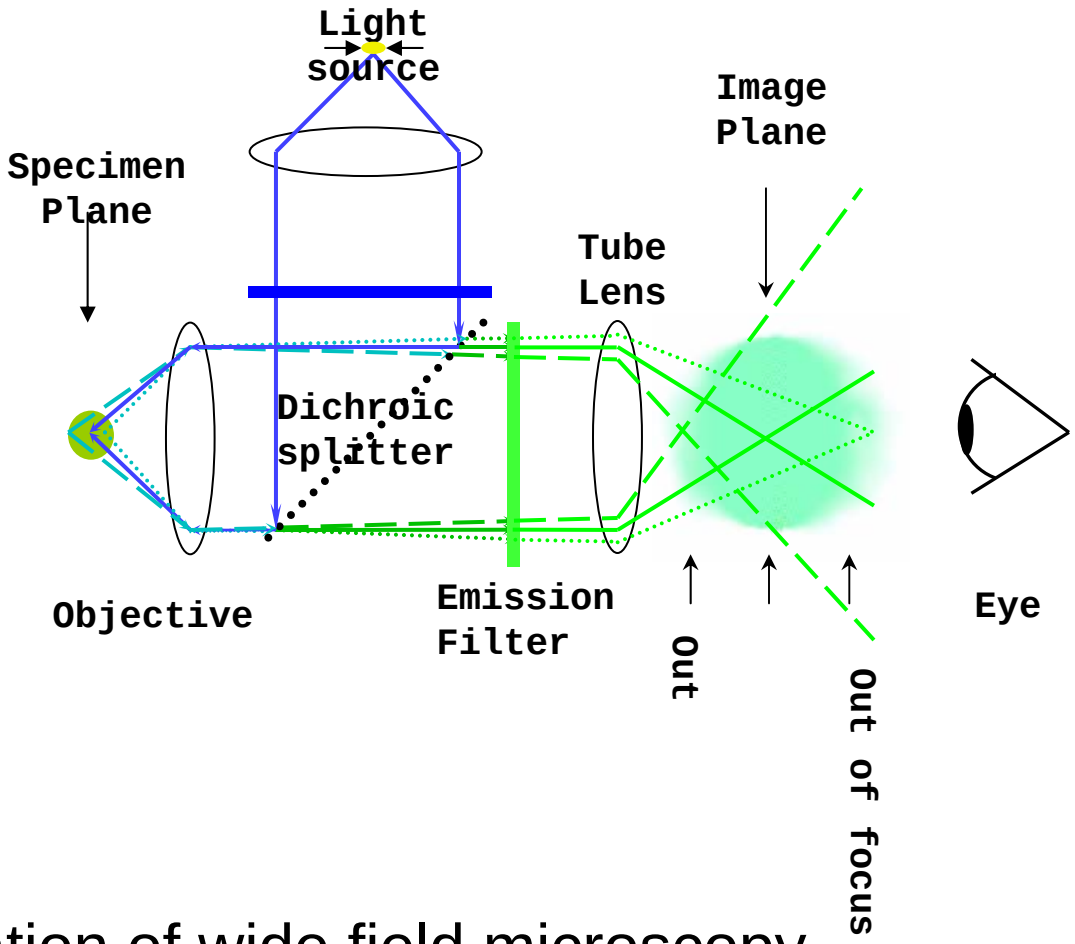
Confocal Laser Scanning Microscopy

- Next Week -



Limitation of wide field microscopy