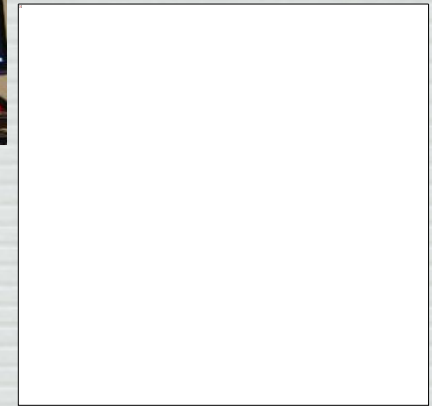
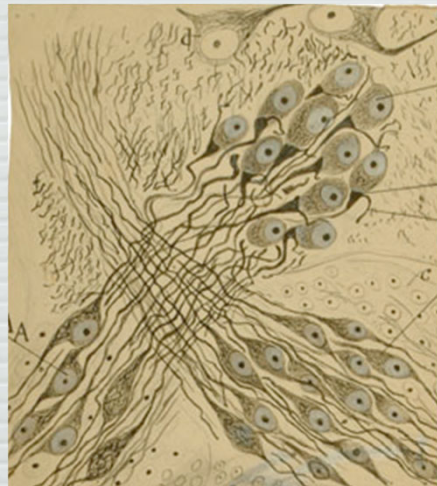


# Digital microscopy: Light Sources and Detectors

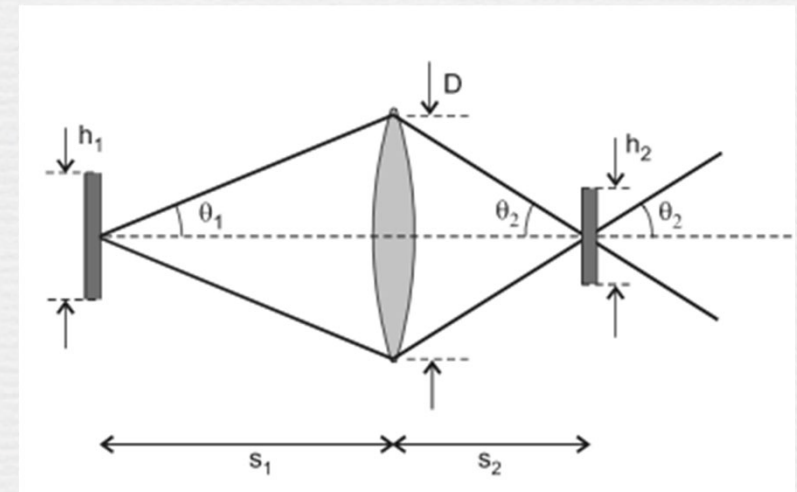


Nico Stuurman, UCSF/HHMI  
UCSF, April 2013

# Light Sources

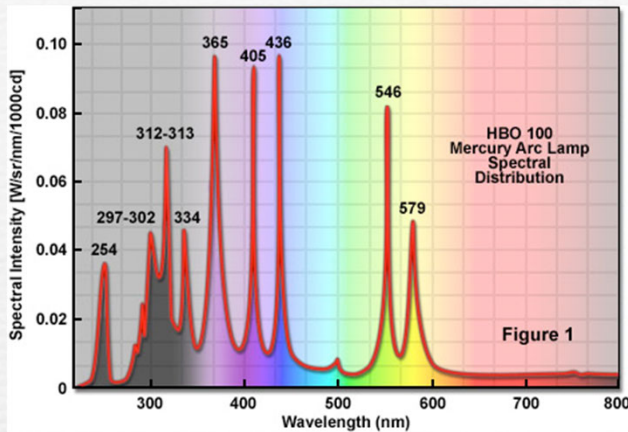
Factors to consider:

- Desired Wavelength (Color)
- Brightness
  - ◆ Inherent Brightness
  - ◆ Angle!!!
  - ◆ Delivery
- Uniformity
- (Computer) Control

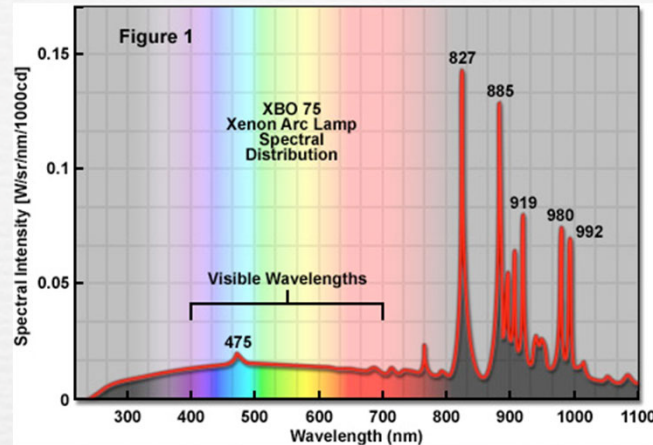


Brightness is determined by size and angle

# Arc Lamps



Mercury Arc



Xenon Arc



## Cons:

- Short Lifetime
- Dangerous (Hg)
- Hot
- Needs mechanical shutter
- Laborious installation



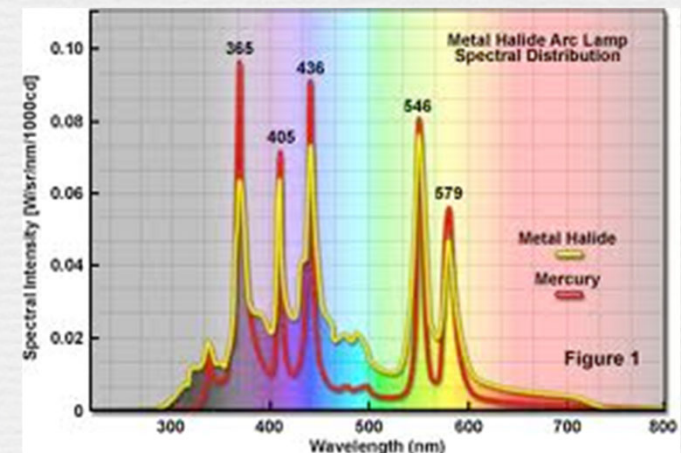
# Metal Halide

Produces light by an [electric arc](#) through a gaseous mixture of vaporized mercury and [metal halides](#) (compounds of metals with bromine or iodine).

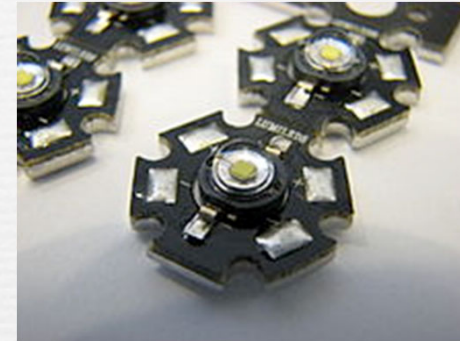
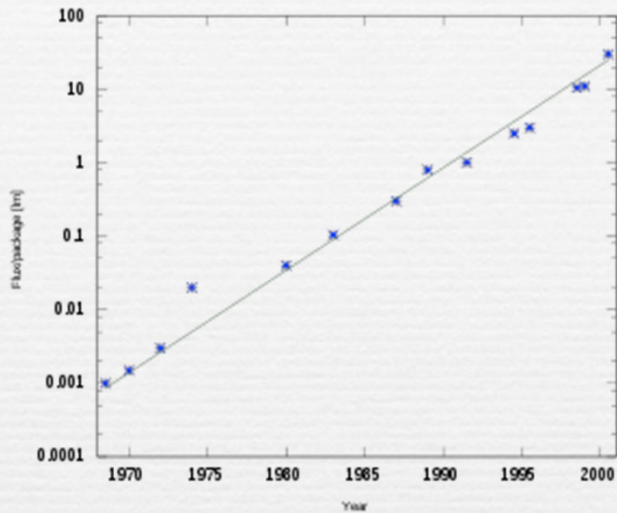


Step up from Arc lamps, still:

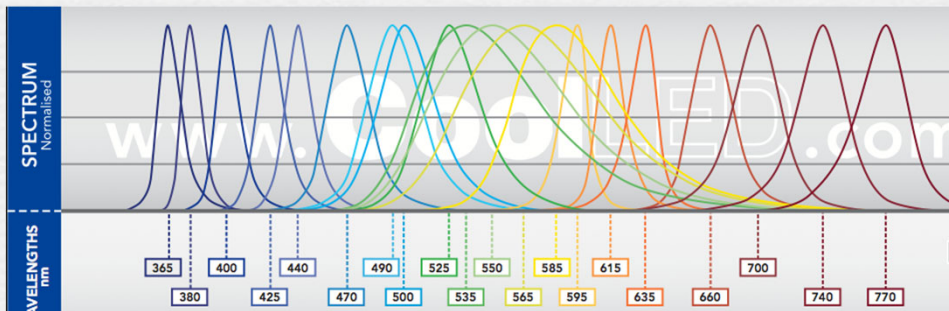
- Hot, loud, lifetime ~1500 hours
- Lamps expensive (\$500-800)
- Needs mechanical shutter



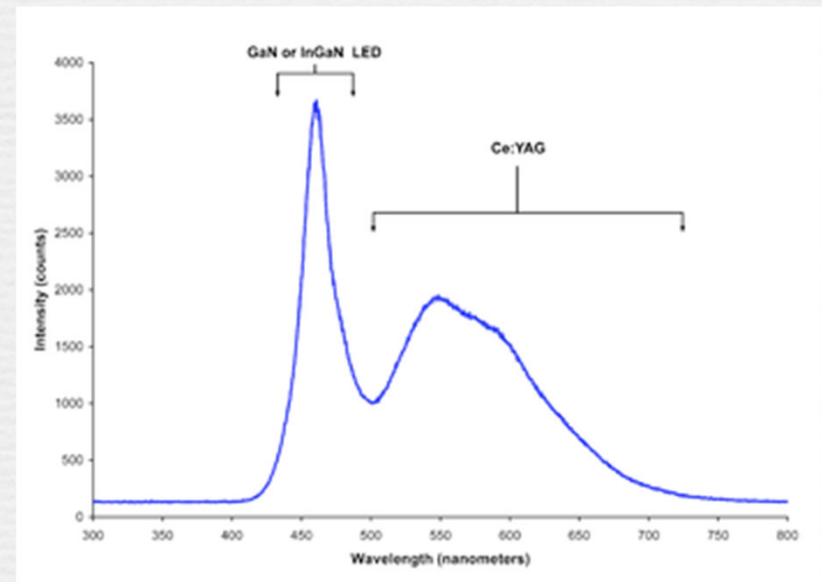
# LEDs



Haitz's law: Every decade the cost per lumen falls a factor of 10, amount of light increases a factor of 20



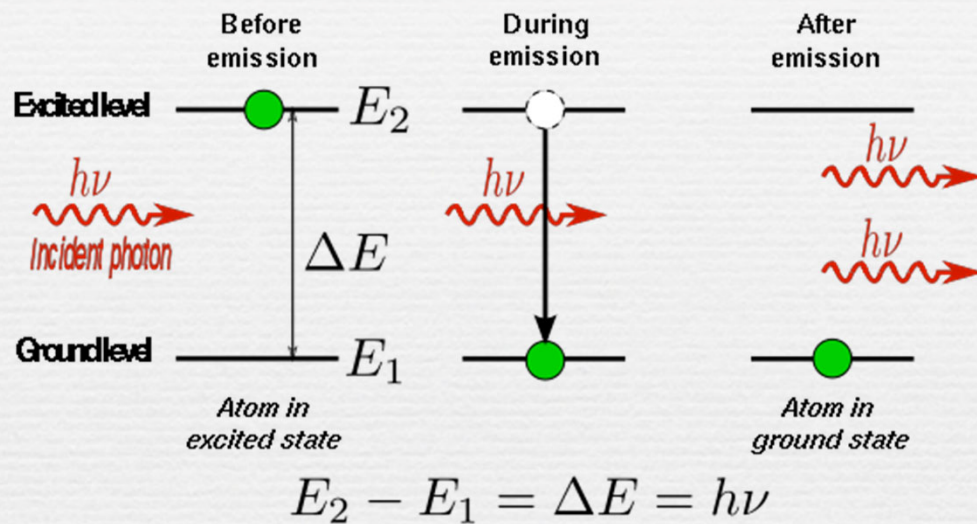
Source: [www.cooled.com](http://www.cooled.com)



White LED with phosphors

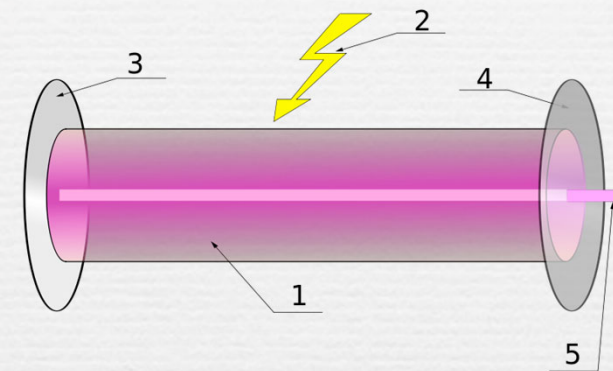
# Lasers

## Stimulated Emission



From Wikipedia

## Principle



1. Gain Medium
2. Laser pumping energy
3. Reflector
4. Output Coupler
5. Laser Beam

From Wikipedia

Coherent (speckles), Collimated  
> Dangerous!

# Lasers

Ion gas Lasers, Argon, Krypton, HeNe



- Inefficient > Hot and Loud
- No modulation
- No longer legal?
- Very nice beam quality

Argon: 476, 488, 514nm

Krypton: 568, 647nm

HeNe: 632nm

pumped by electric discharge

# Lasers

## Solid-state lasers

Optically pumped



Laser Diode

(electrically pumped)

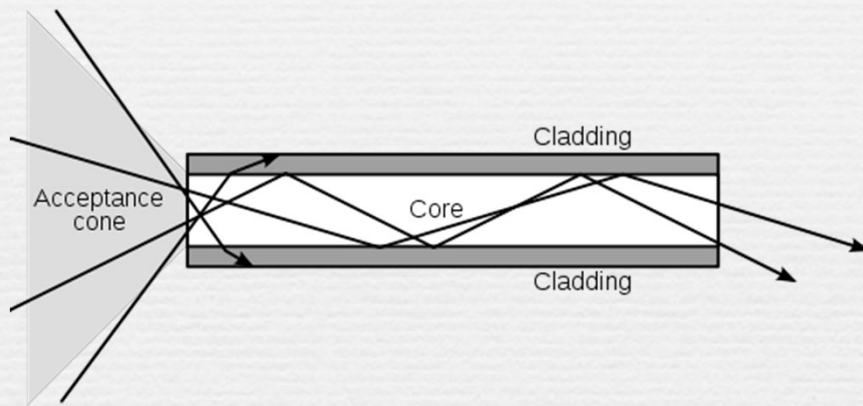
Can be modulated

No good yellow (560nm) line?

- Highly efficient, small form factor
- Some can be electrically modulated
- Beam quality good enough

# Lasers, coupling

## Optical Fibers

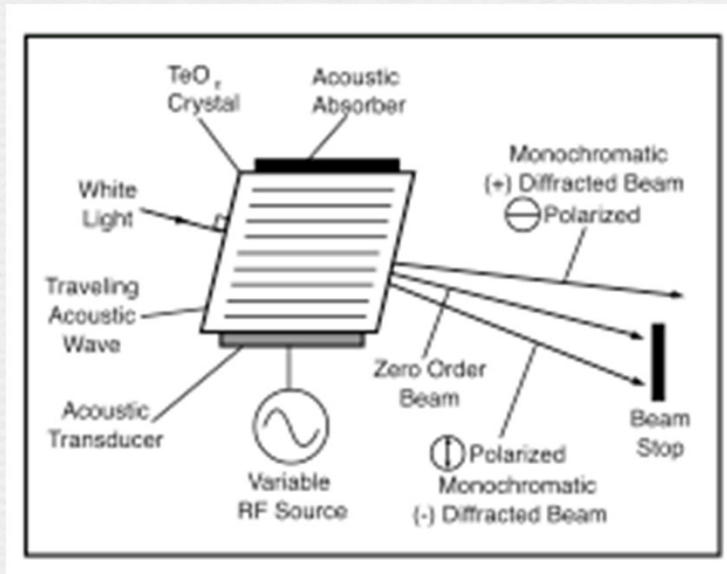


Multi Mode - core  $> 10$  microns

Single Mode - core 3-6 micron (visible)

# Lasers, modulation

- Direct (if possible)
- Mechanical shutter
- AOM or AOTF

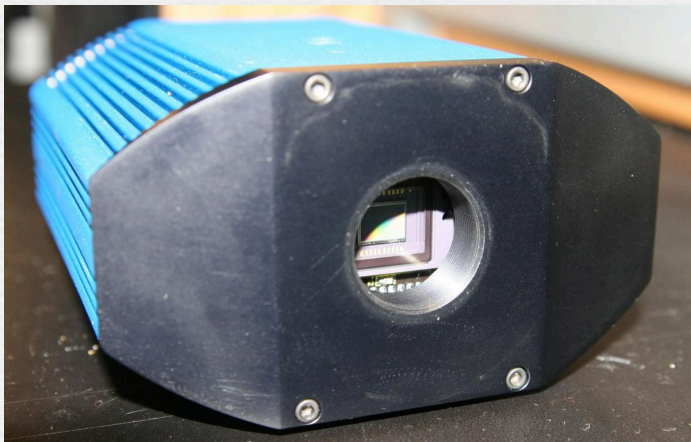


Acousto Optical Tunable Filter

- Piezoelectric Optical Device
- Switches and modulates intensity
- Fast! (sub-microseconds)
- Mainly used for excitation laser light
- Polarization depended

# Imaging Detectors

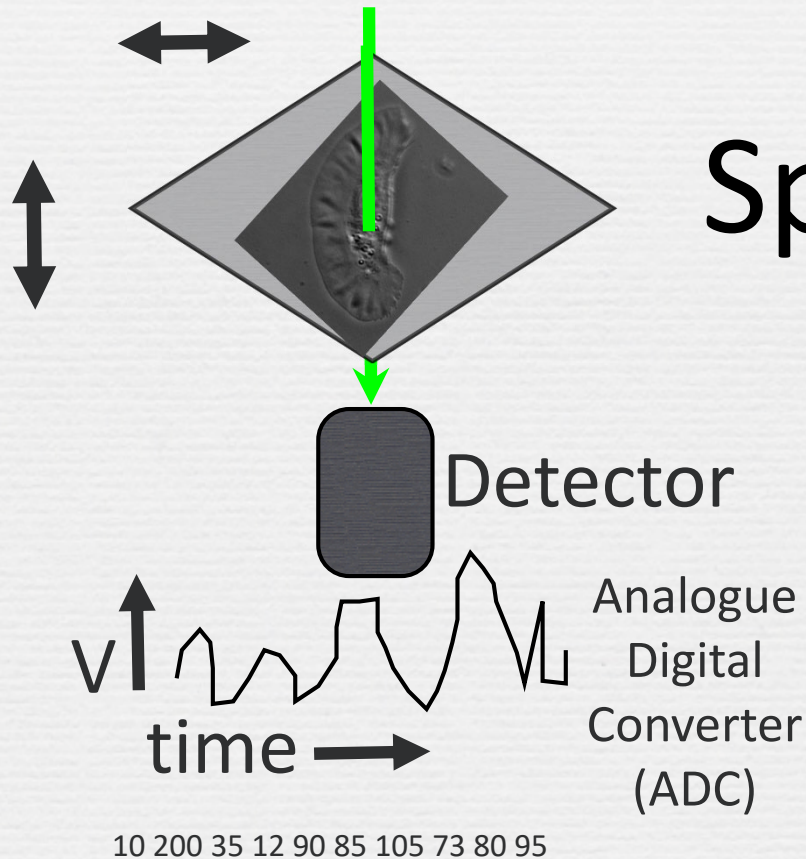
- n Detectors:
  - n 'Single point' detectors
  - n 'Multiple point' detector (cameras)



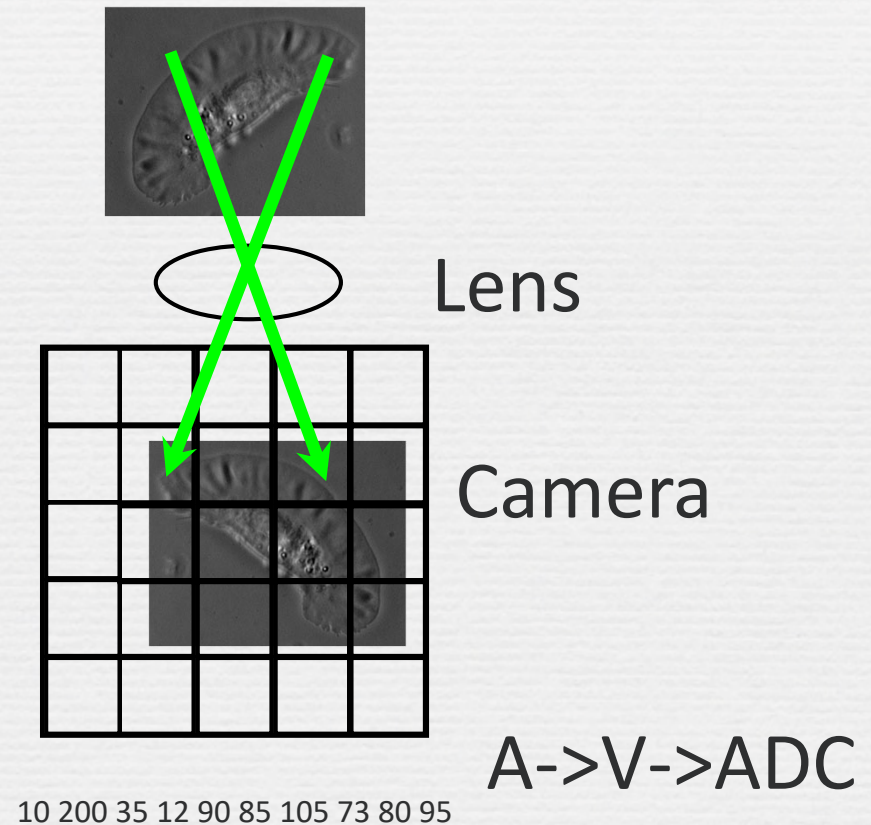
# Imaging Detectors

Photon  $\rightarrow$  Electrons  $\rightarrow$  Voltage  $\rightarrow$  Digital Number

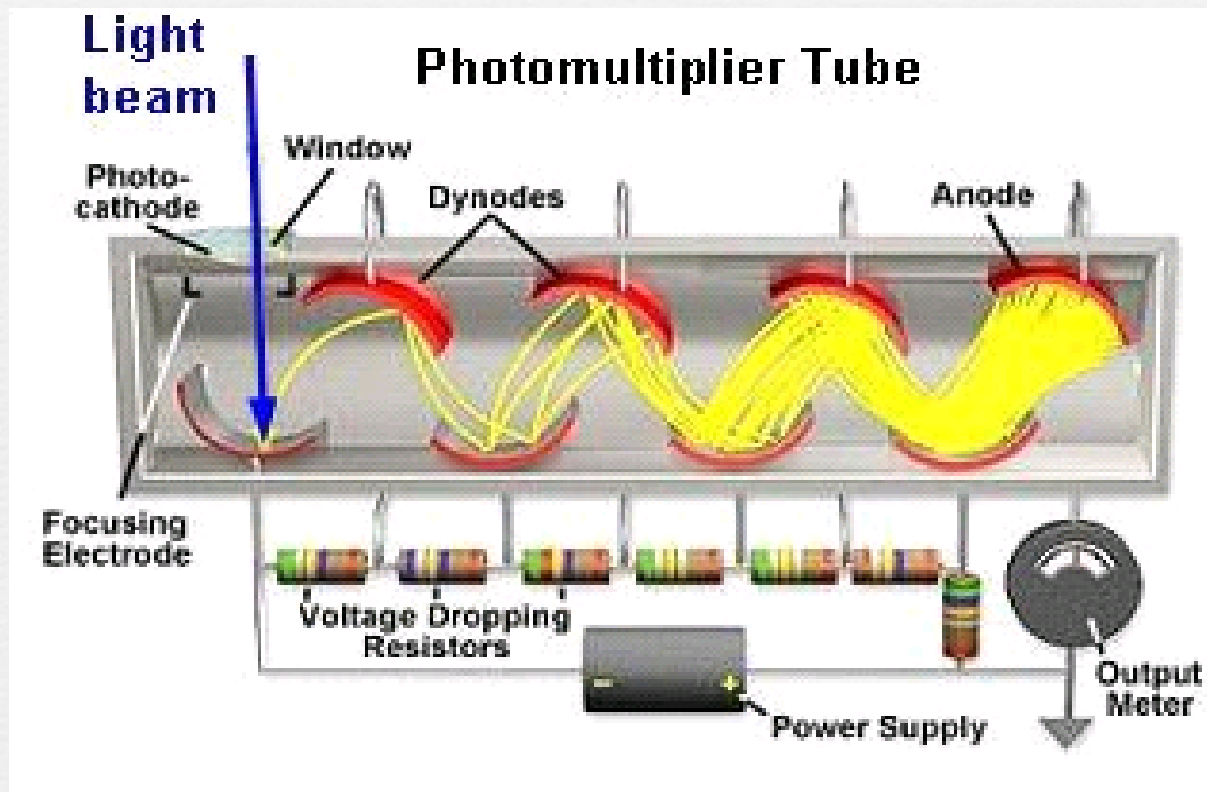
Single point detector



Multi point detector (camera)

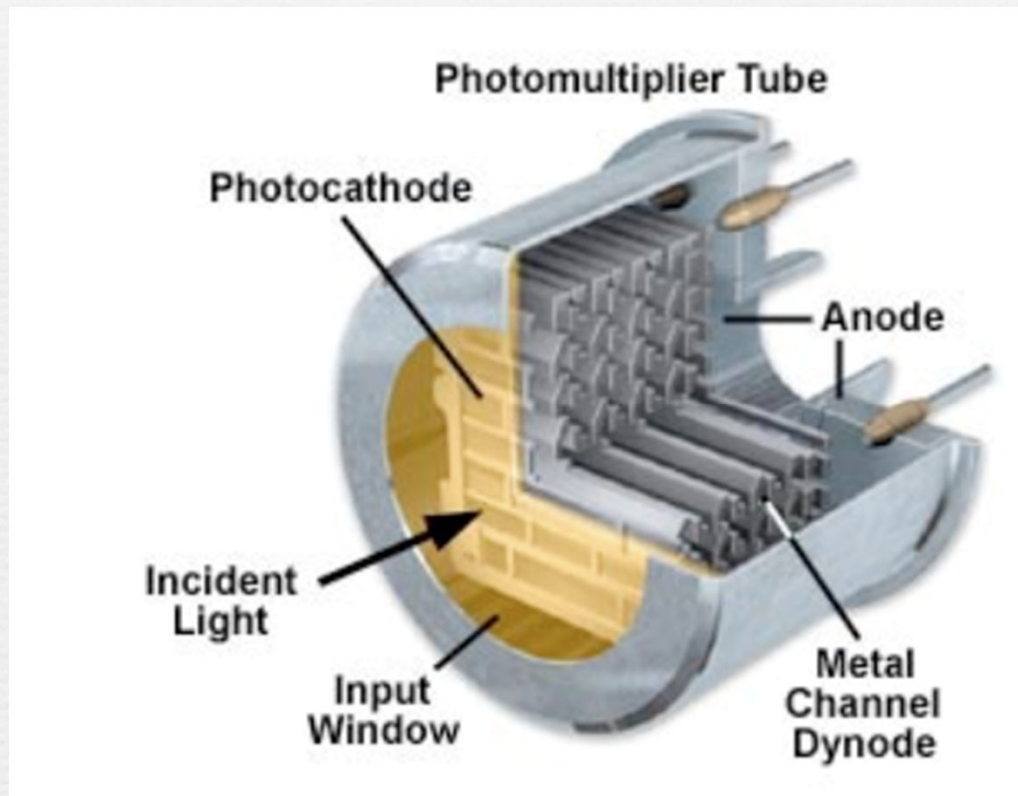


# Photo-Multiplier Tube (PMT)



- Very linear
- Very High Gain
- Fast response
- Poor Quantum efficiency (~25%)

# PMT modes



n Photon counting mode:

n Count pulses

n Zero background

n Slow

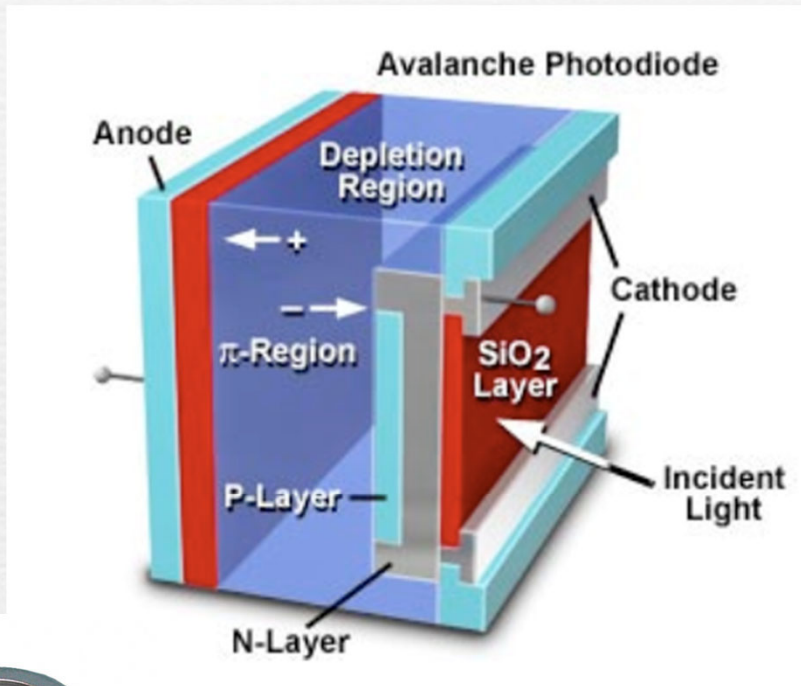
n Linear mode

n Measure current

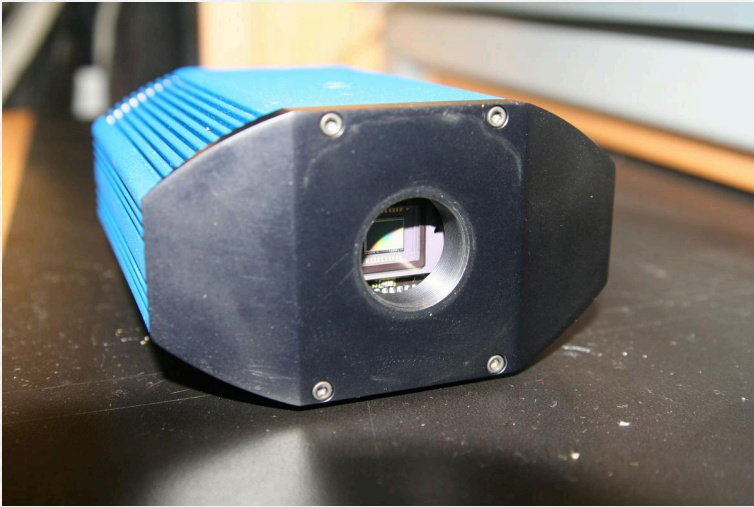
n Fast but noisy

# Avalanche Photo Diode

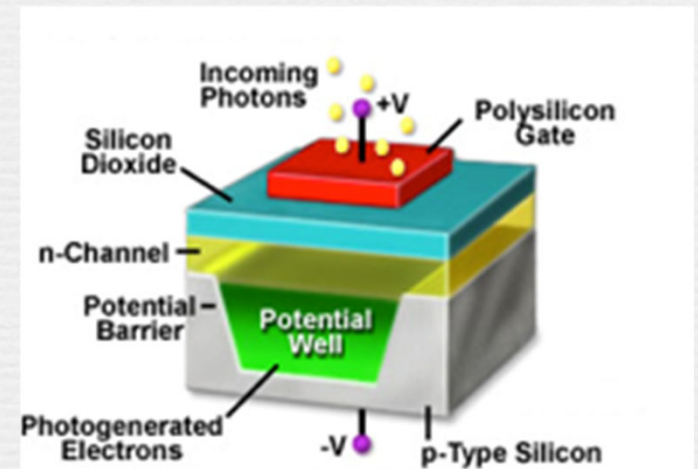
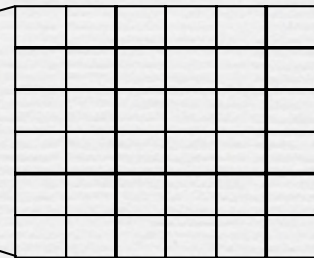
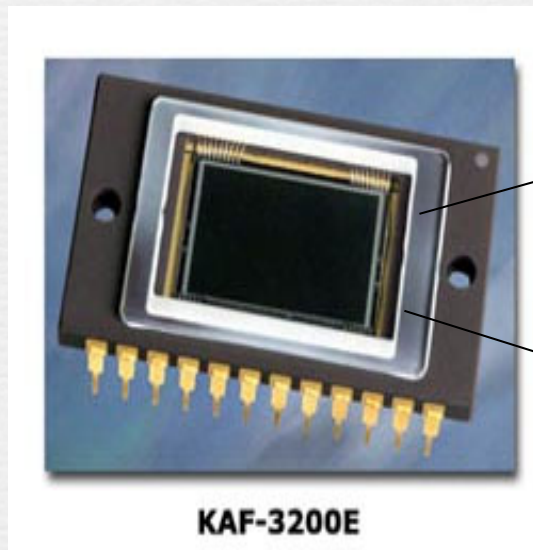
- n Absorbed photons->electron
- n Electrons amplified by high voltage and 'impact ionization'
- n High QE (~90%)
- n Photon-counting ability (different design)
- n Overheats if run too fast



# Cameras in Microscopy

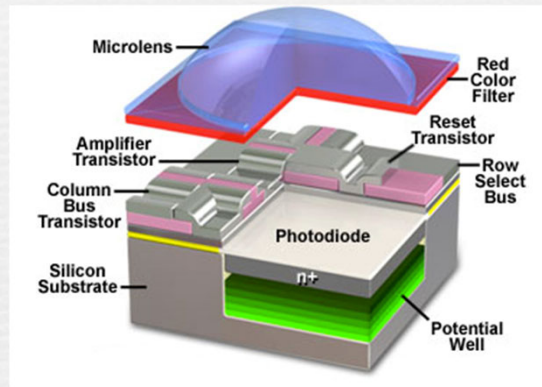


# Arrays of photo-sensitive elements



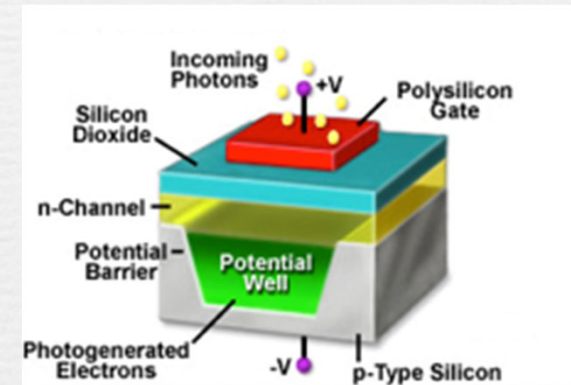
# Two Architectures:

## Complementary Metal Oxide Semiconductor (CMOS)



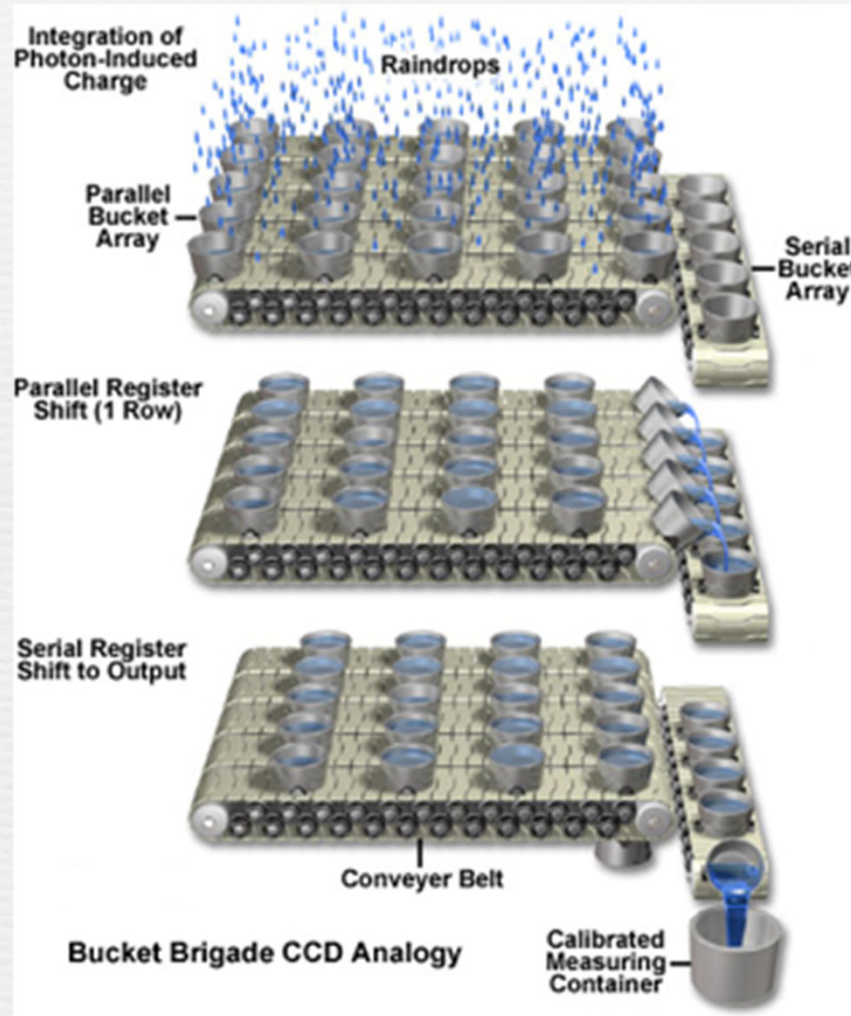
Each pixel has an amplifier  
Transfers voltage  
Fast  
Noisy

## Charged Coupled Devices (CCD)

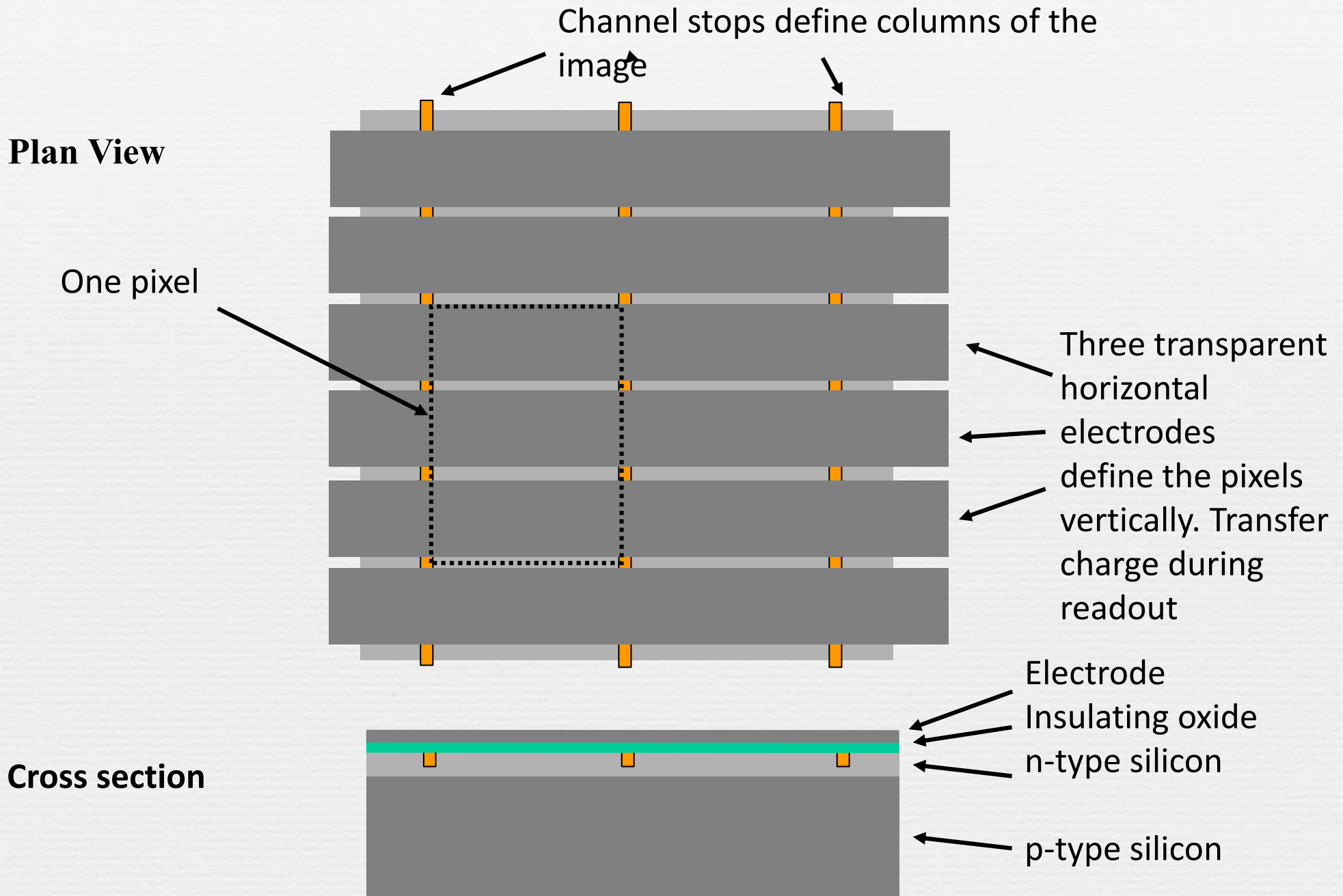


Single read-out amplifier  
Transfers charge  
Slow  
Precise

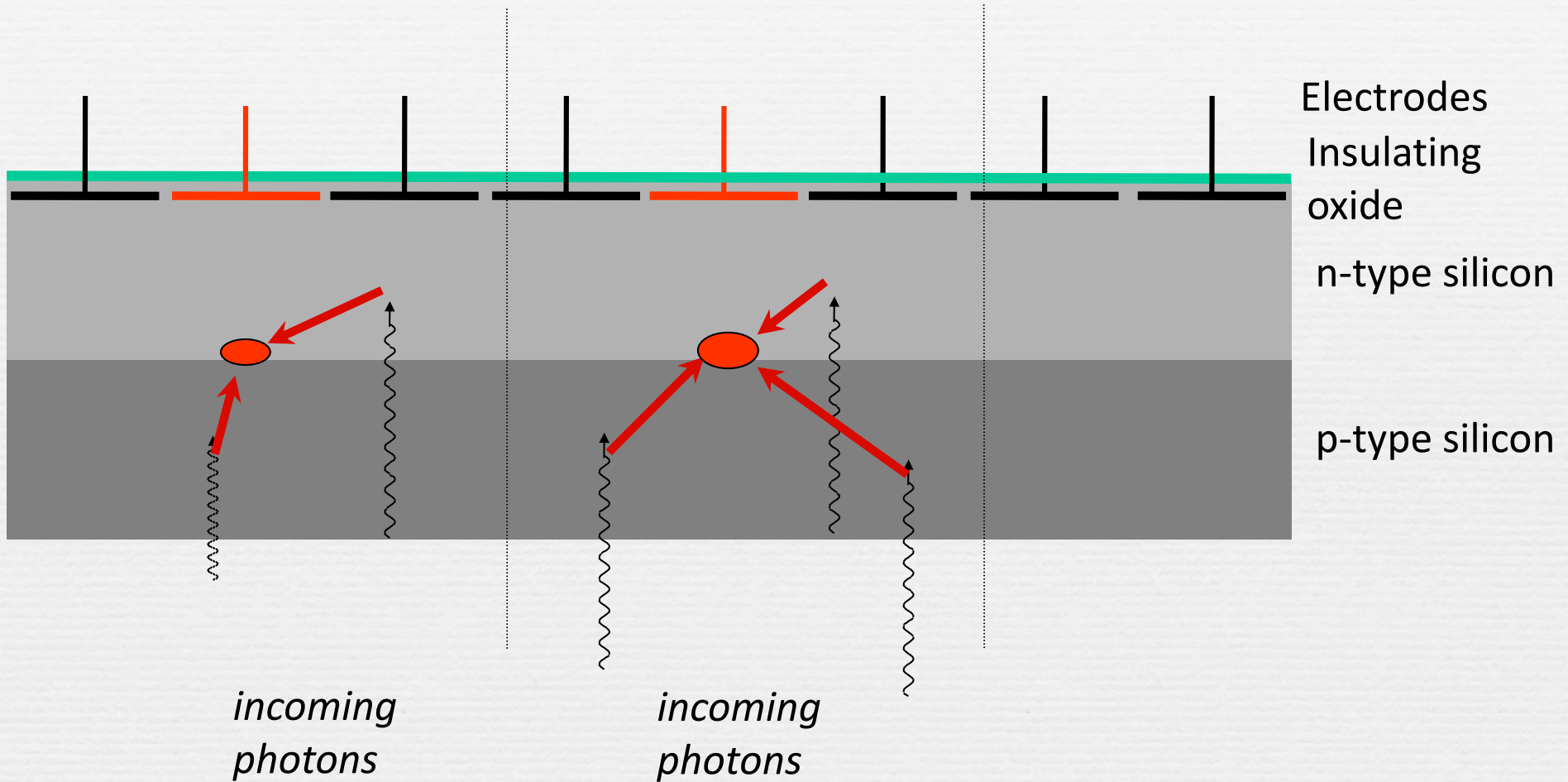
# CCD readout “bucket-brigade” analogy



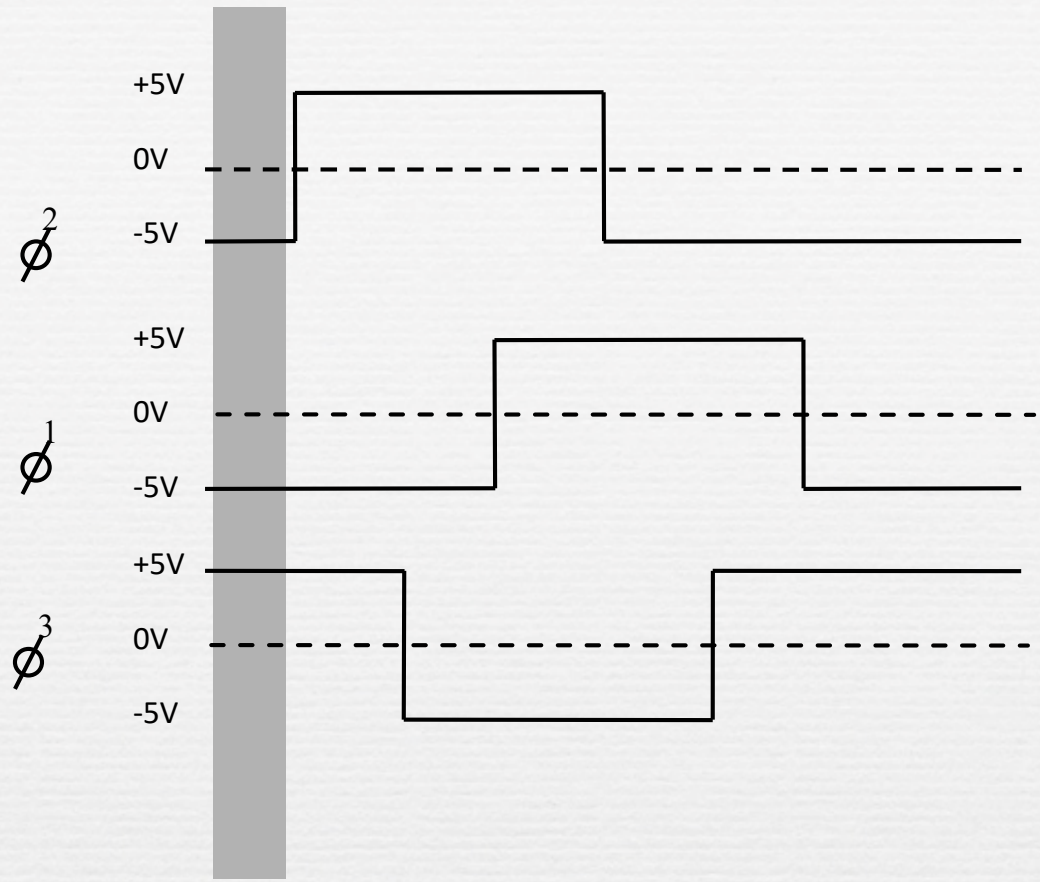
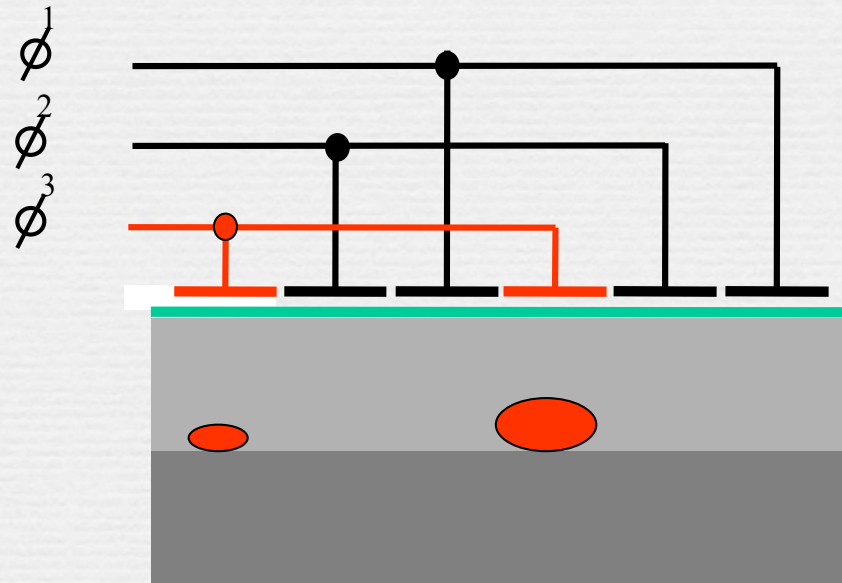
# CCD Architecture



# CCD Architecture

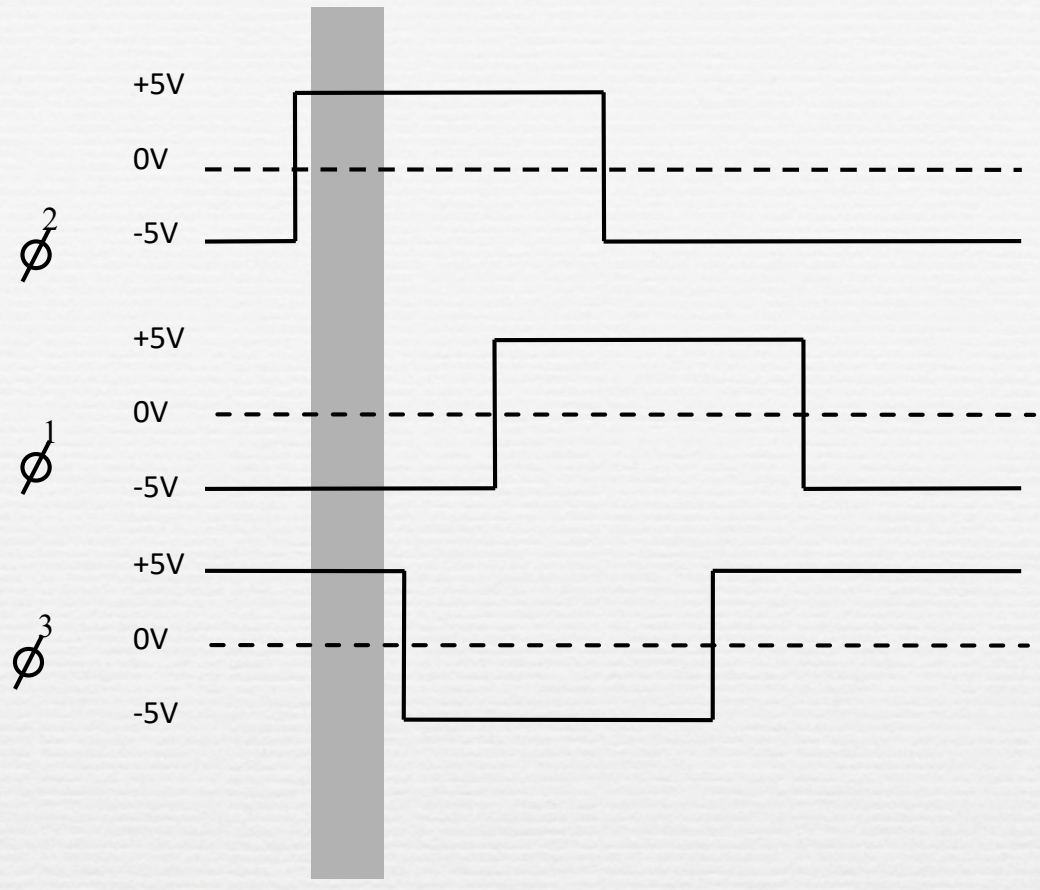
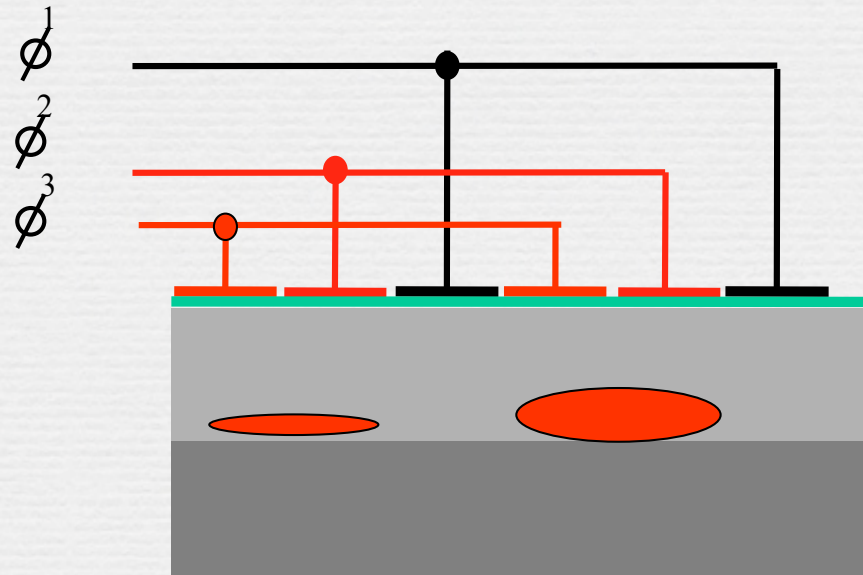


# Charge Transfer in a CCD



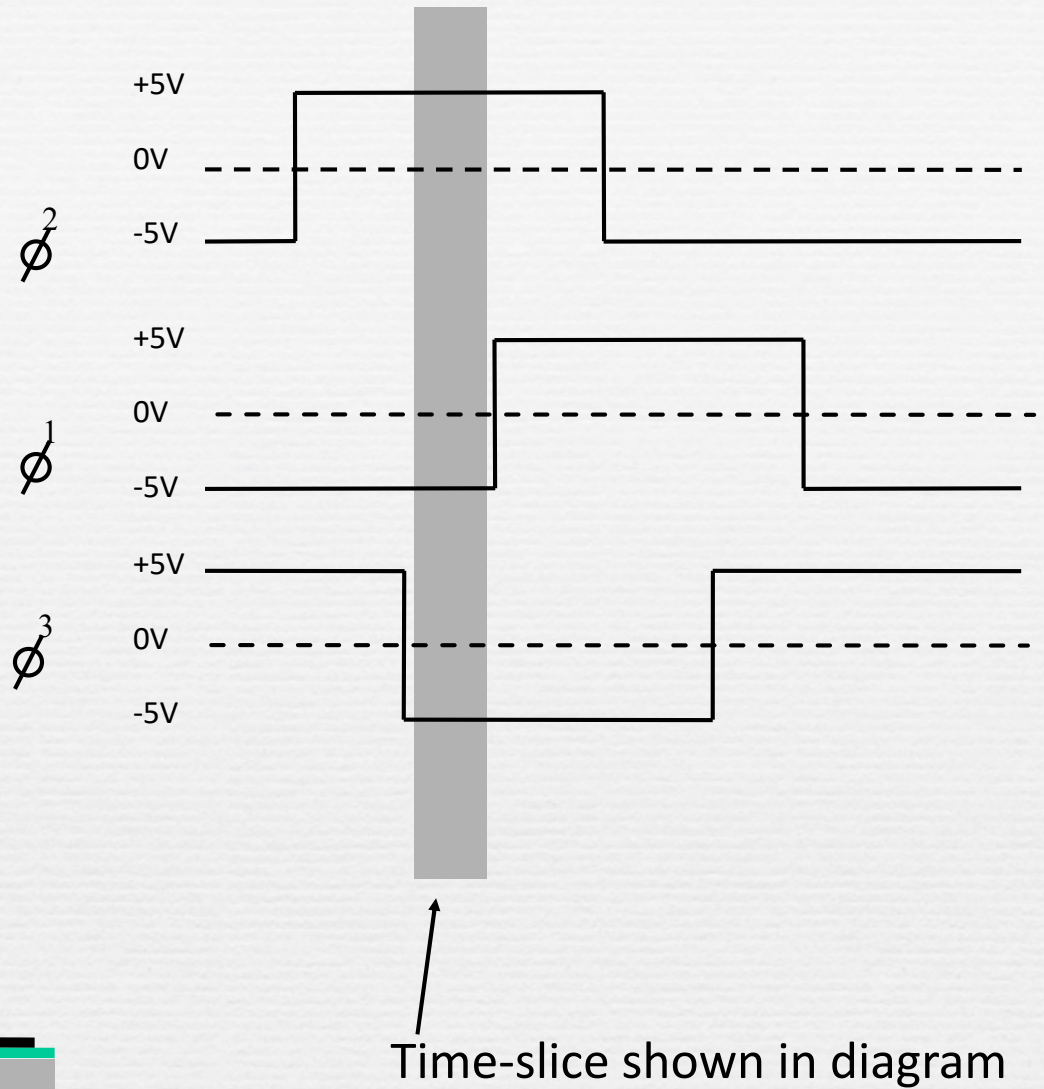
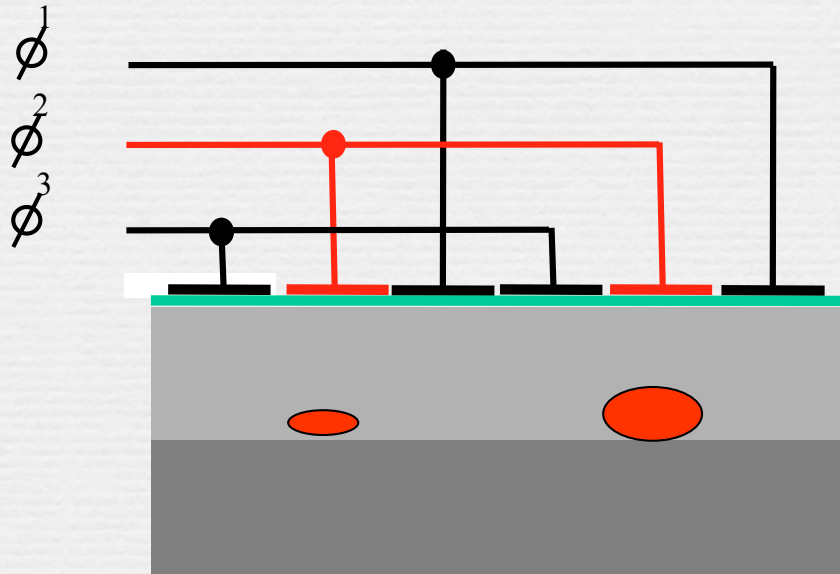
Time-slice shown in diagram

# Charge Transfer in a CCD

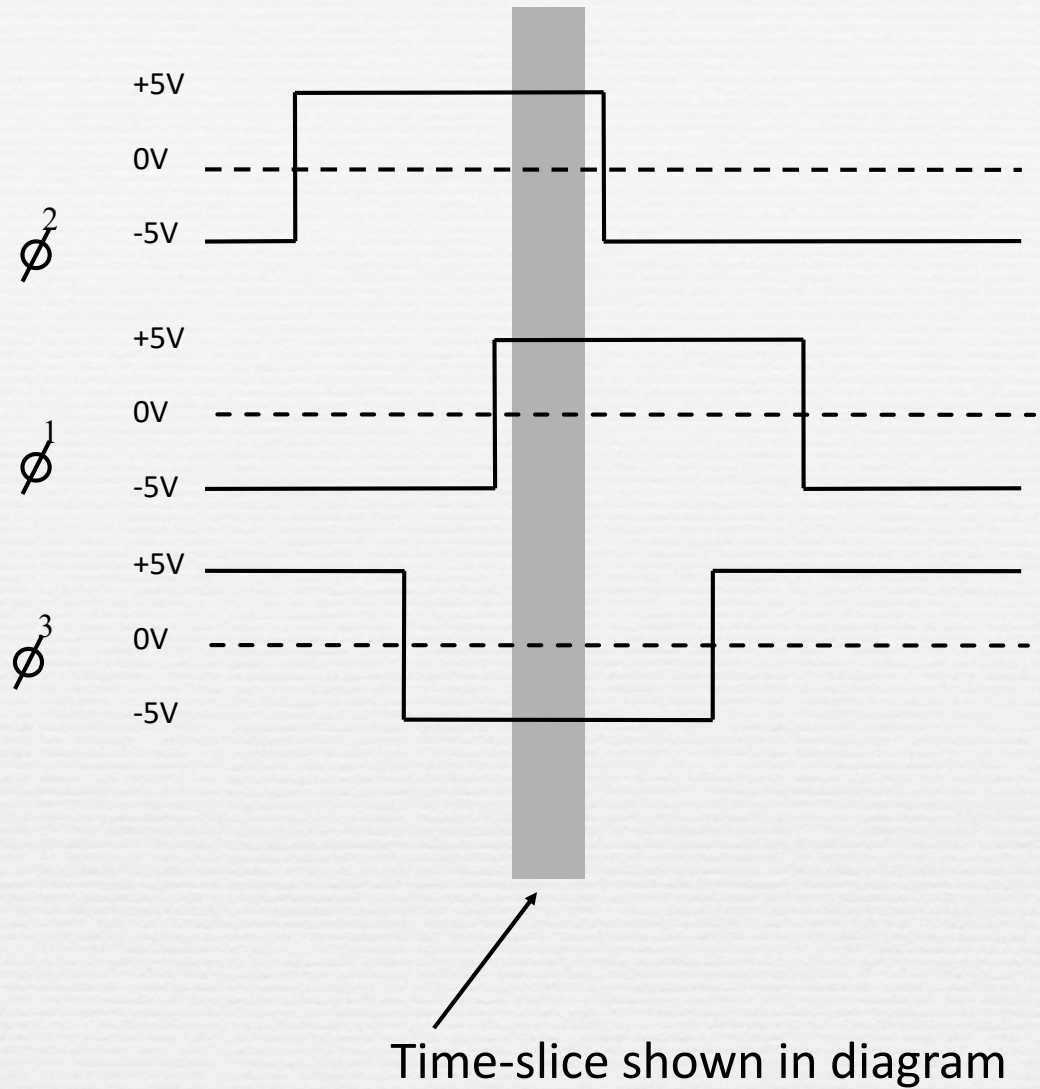
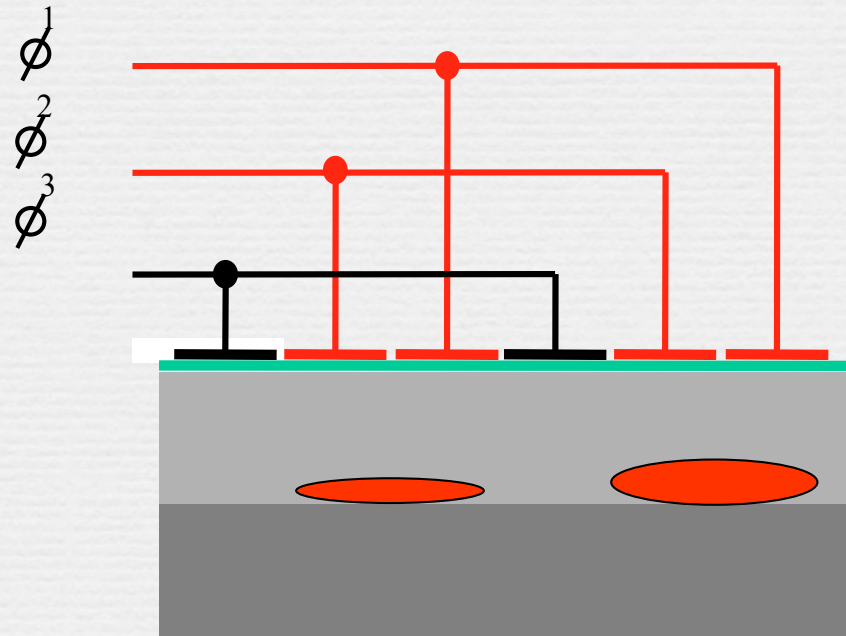


Time-slice shown in diagram

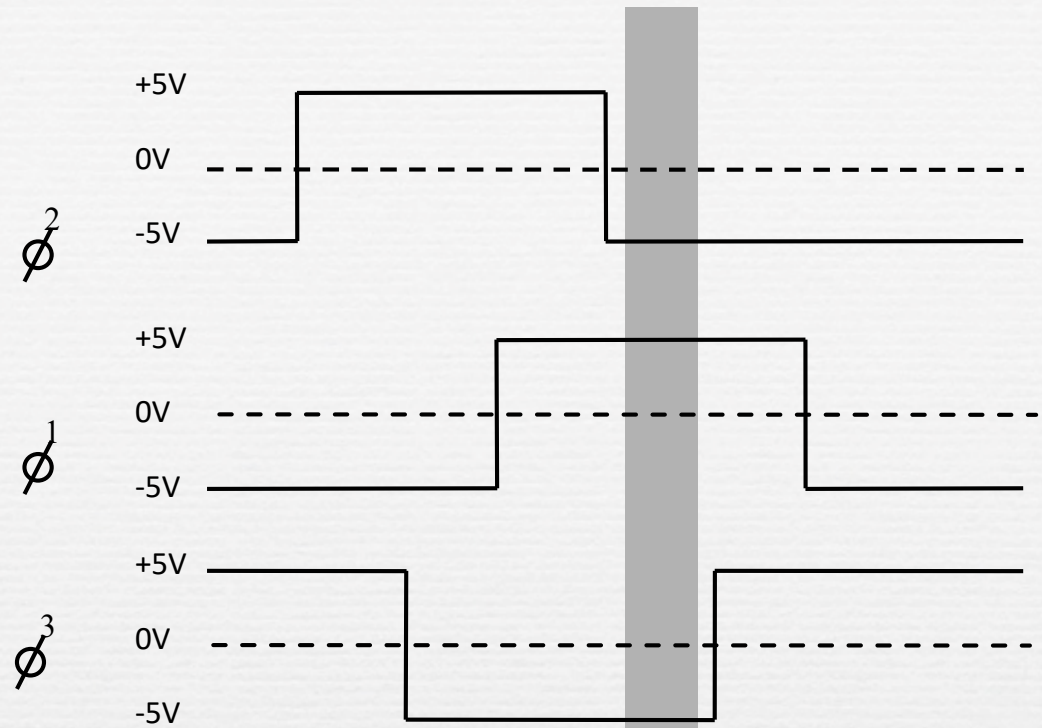
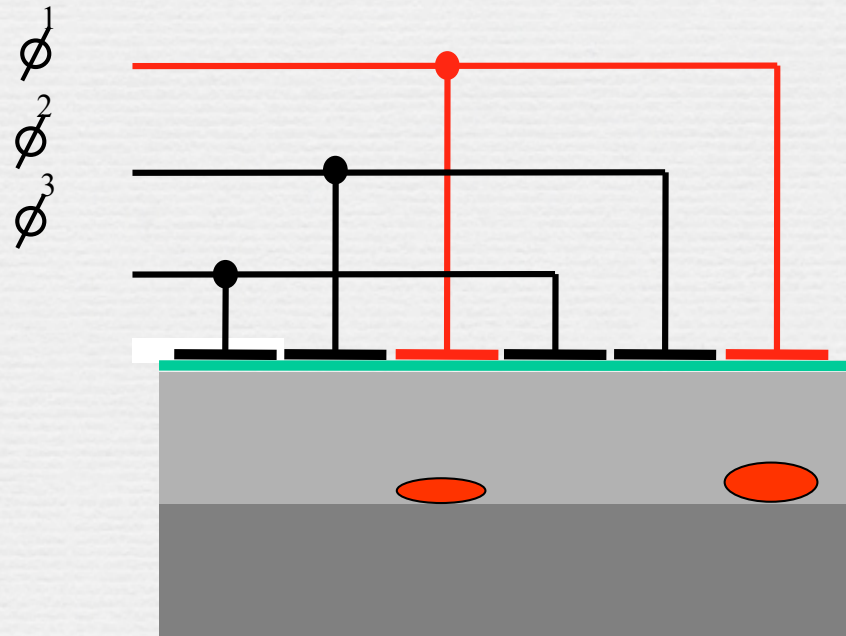
# Charge Transfer in a CCD



# Charge Transfer in a CCD



# Charge Transfer in a CCD



Time-slice shown in diagram

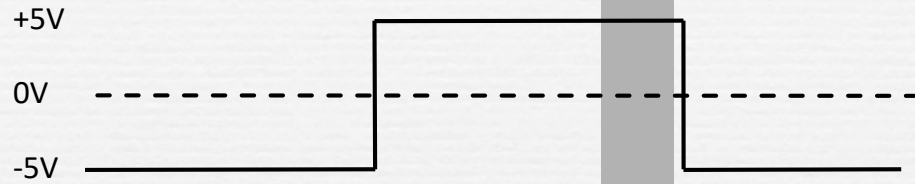
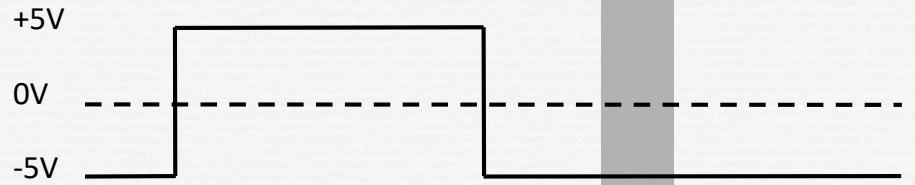
# Charge Transfer in a CCD

Transfer charge to the next pixel

$\phi^2$

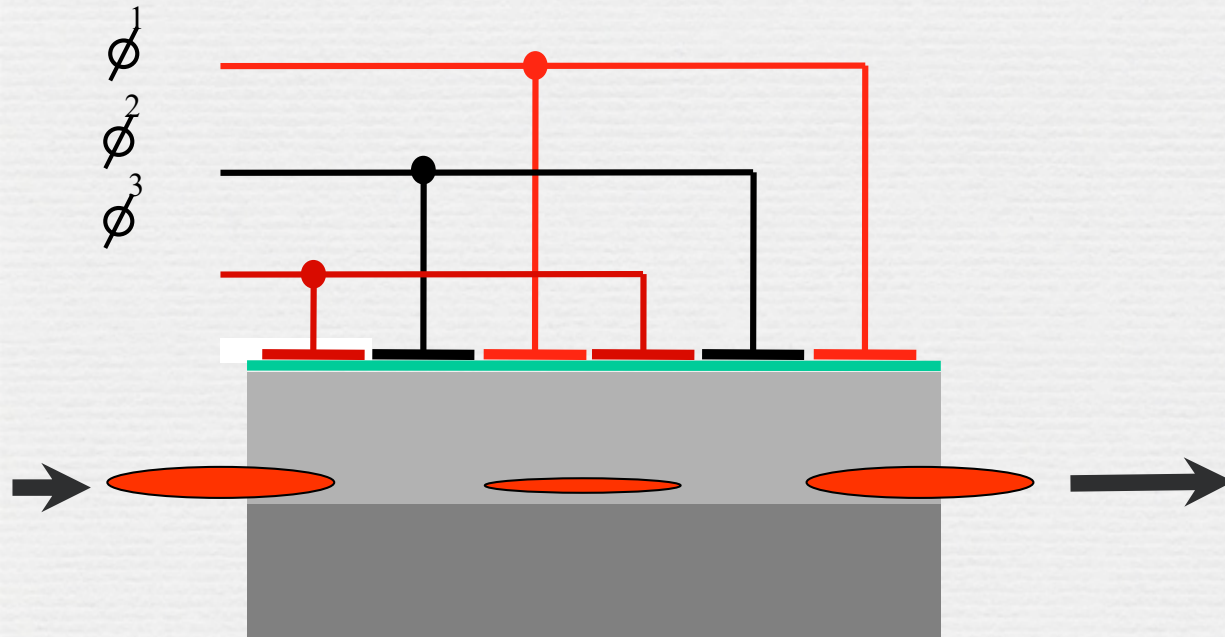
$\phi^1$

$\phi^3$

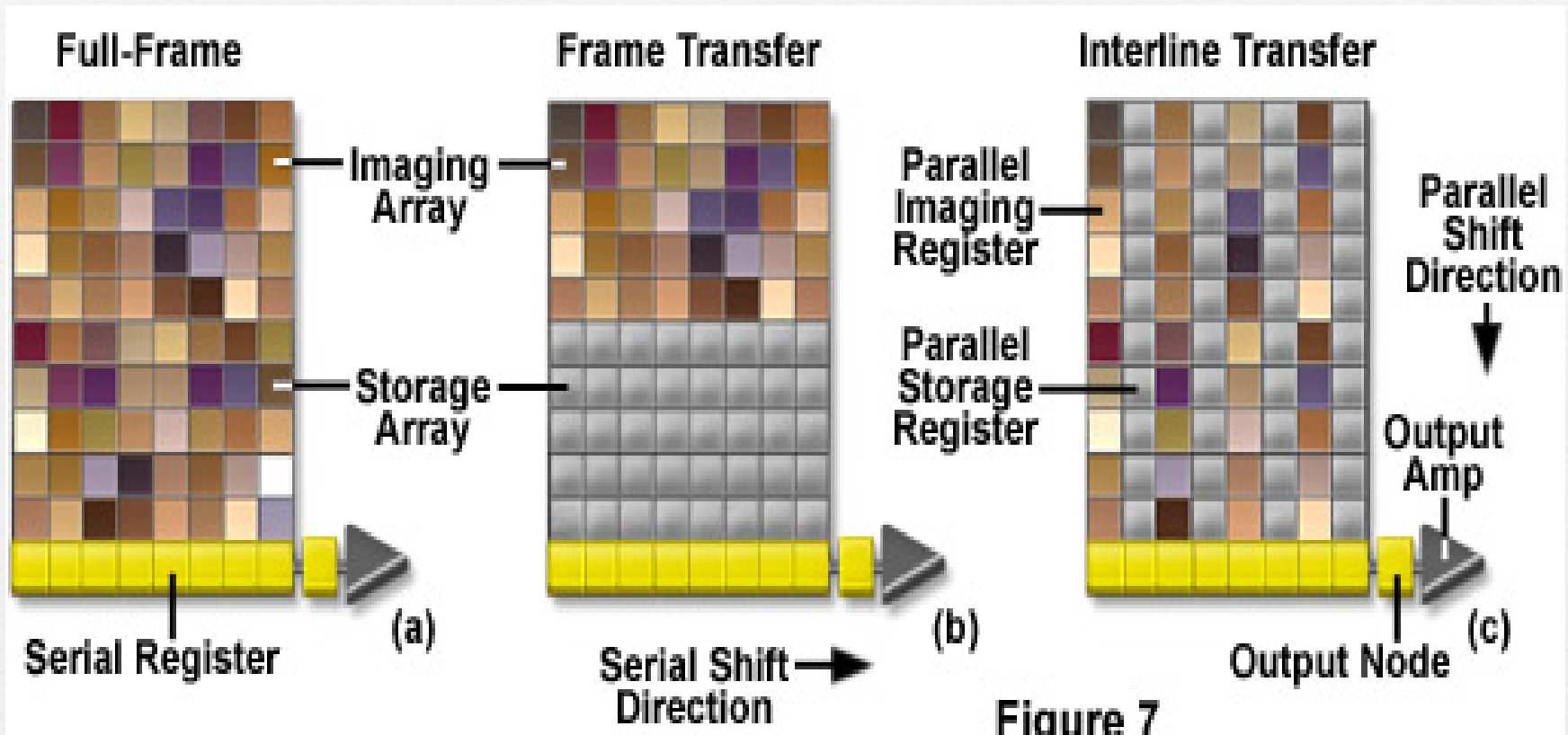


Time-slice shown in diagram

$\phi^1$   
 $\phi^2$   
 $\phi^3$



# CCD Architectures



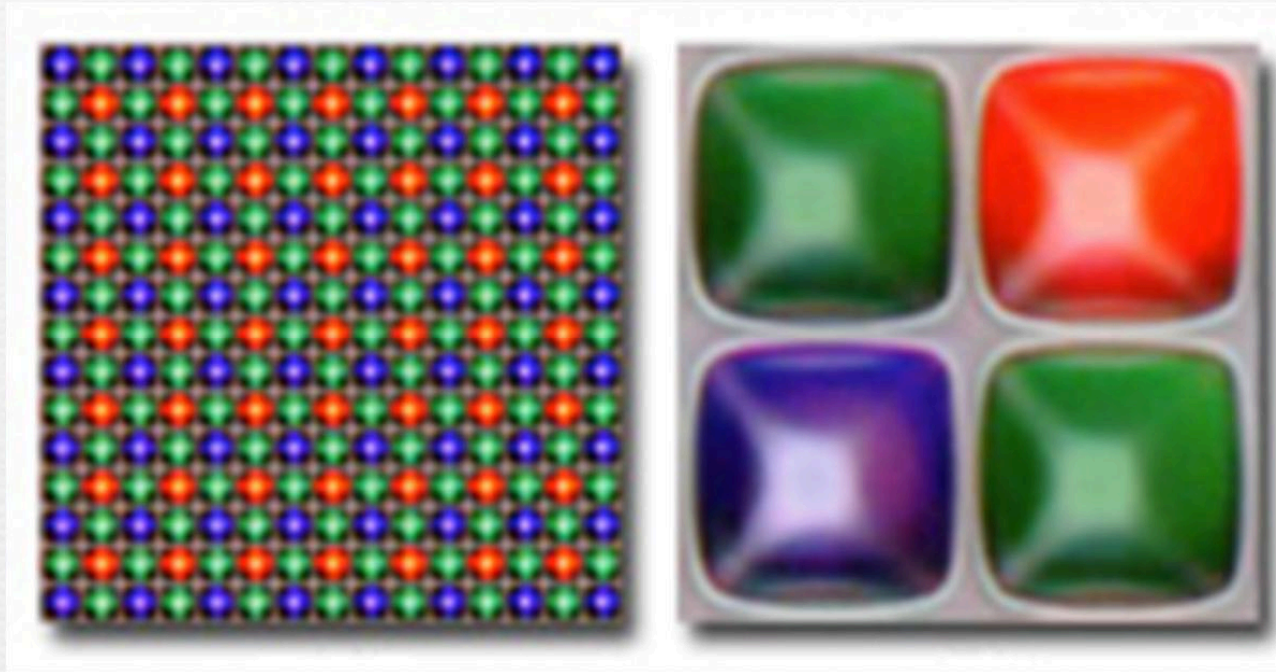
Rare

Mostly EMCCDs

Common

Full frame CCDs cannot acquire while being read out; They also require a mechanical shutter to prevent smearing during readout.

# Why don't we use color CCDs?



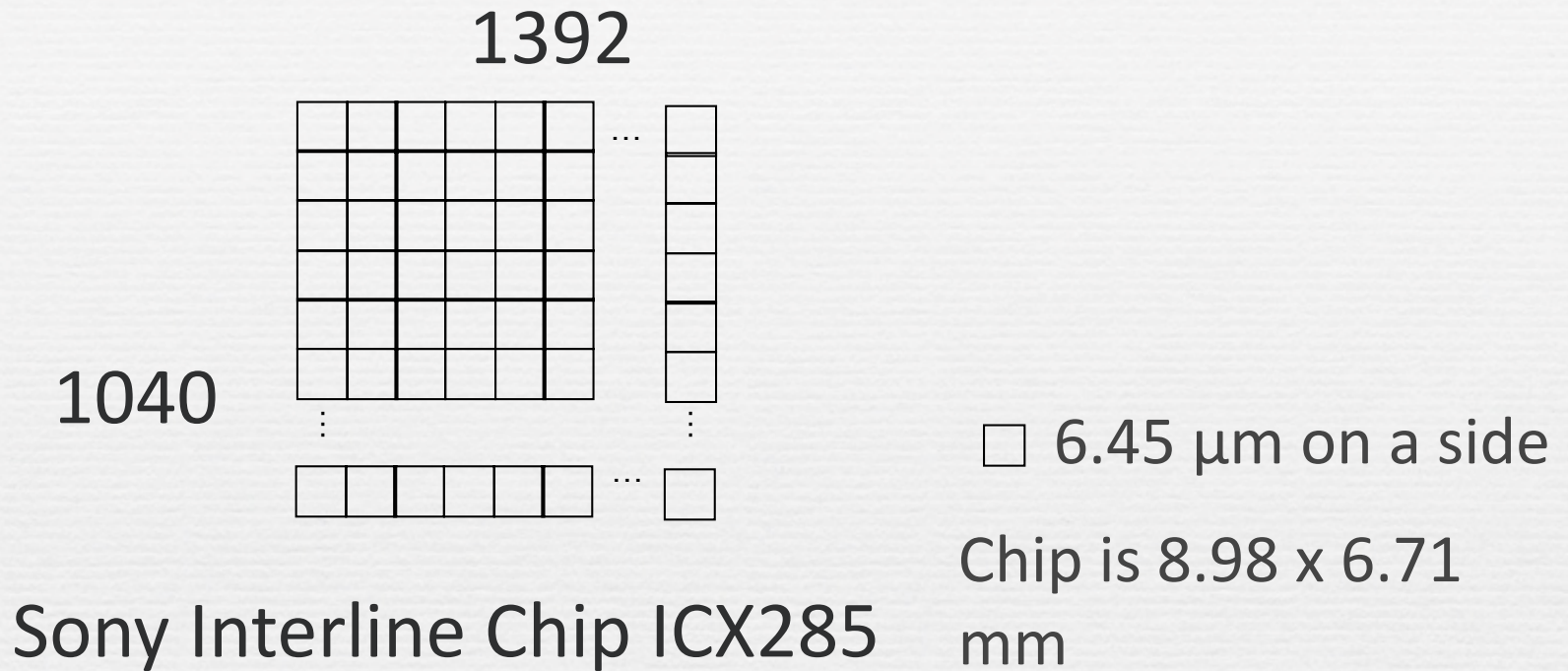
- Four monochrome pixels are required to measure one color pixel
- Your 5MP digital camera really acquires a 1.25 MP red and blue image and a 2.5 MP green image and uses image processing to reconstruct the true color image at 5 MP

# Vital Statistics for CCDs

- Pixel size and number
- Quantum efficiency: fraction of photons hitting the CCD that are converted to photo-electrons
- Full well depth: total number of photo-electrons that can be recorded per pixel
- Read noise
- Dark current (negligible for most biological applications)
- Readout time (calculate from clock rate and array size)
- Electron conversion factor (relate digital

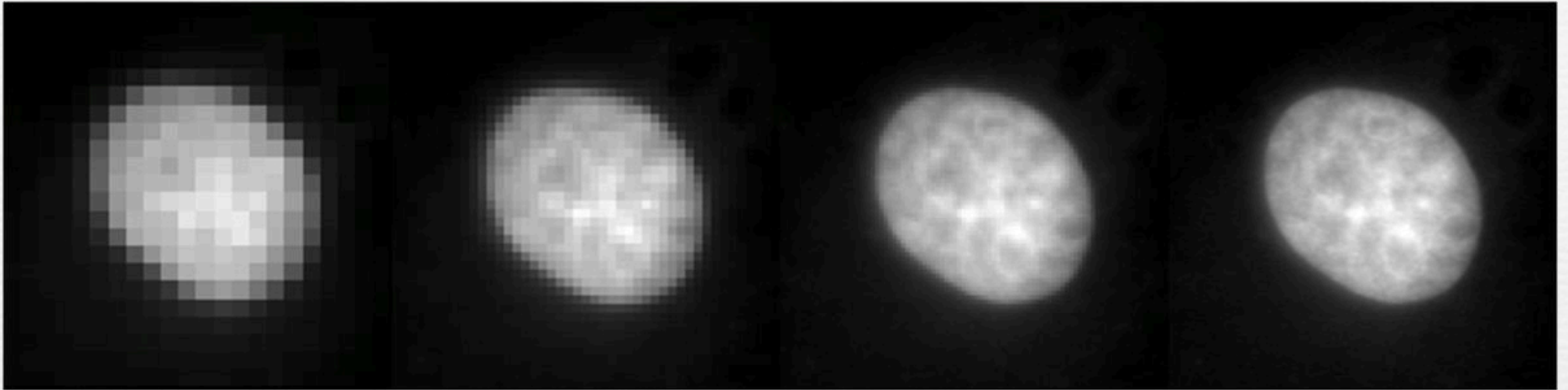
SPECIFICATIONS			
Type number	ORCA-R2 (C10600-10B)		
Camera head type	Hermetic vacuum-sealed head		
Dual cooling	Air cooling / water cooling		
Imaging device	ER-150 progressive scan interline CCD		
Effective number of pixels	1344 (H) × 1024 (V)		
Cell size	6.45 μm (H) × 6.45 μm (V)		
Effective area	8.67 mm (H) × 6.60 mm (V)		
Dual scan mode	Normal scan / Fast scan		
Pixel clock rate	Normal scan	14.00 MHz/pixel	
	Fast scan	28.00 MHz/pixel	
Readout noise (r.m.s.) typ.	Normal scan	6 electrons	
	Fast scan	10 electrons	
Full well capacity typ.	High dynamic range mode ①	OFF	18 000 electrons
		ON	36 000 electrons
Dynamic range typ. ②	3 000 : 1 (at Normal scan / 1X1)		
Cooling method / temperature	Forced-air cooled	- 35 °C	
	Water cooled	- 40 °C (Water temperature : +20 °C)	
Dark current	0.0005 electrons/pixel/s (at - 40 °C)		
Dual A/D converter	12 bit or 16 bit		
Exposure time	10 μs to 4200 s		
Binning	2 × 2, 4 × 4, 8 × 8		

# Pixel size and Resolution



Typical magnification from sample to camera is roughly objective magnification, so 100x objective -> 65nm per pixel

# Resolution and magnification

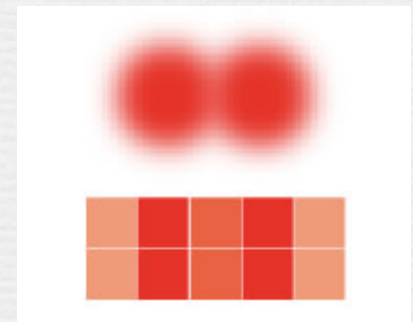


More pixels / resolution element

Where is optimum?

# Digital Sampling

- How many CCD pixels are needed to accurately reproduce the smallest object that can be resolved by the scope?
- Nyquist-Shannon Sampling theorem:  
Must have at least two pixels per resolvable element  
2.5 – 3 is preferable

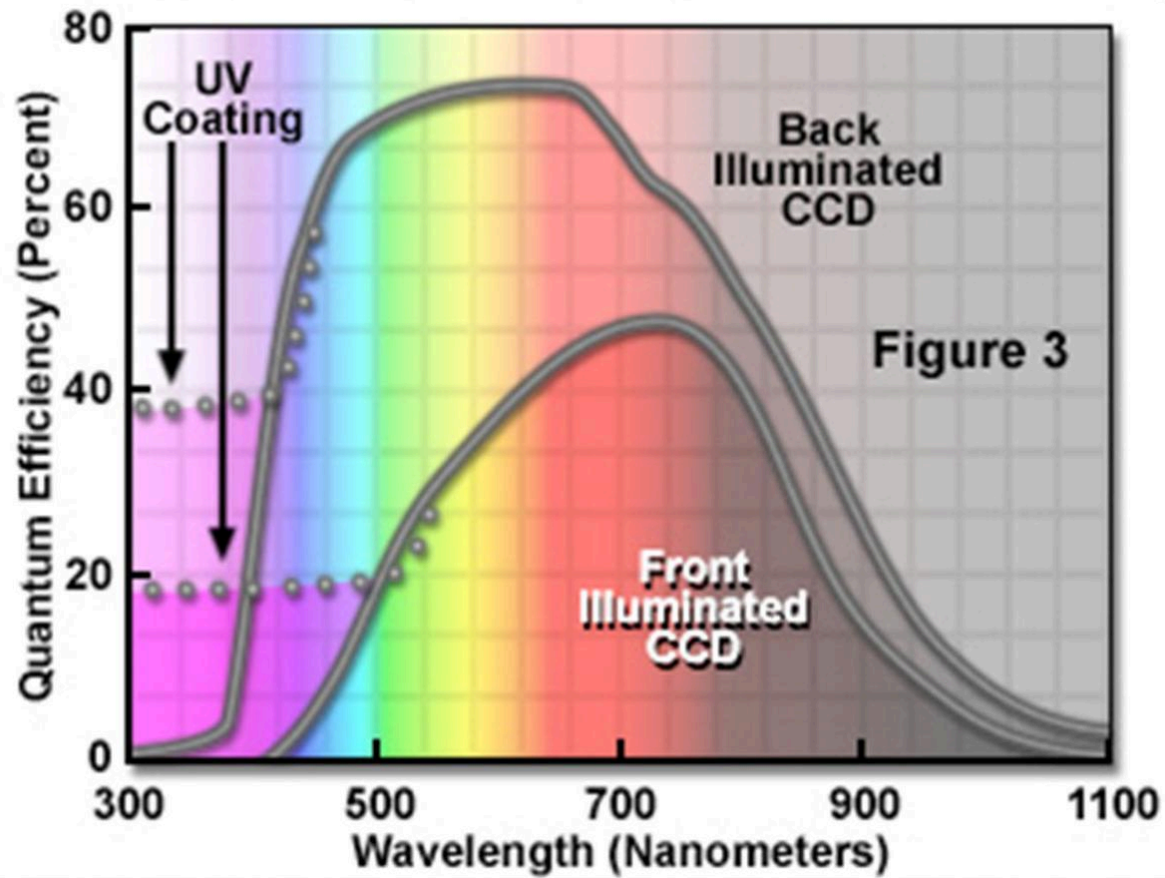


# A resolution-centric view of imaging

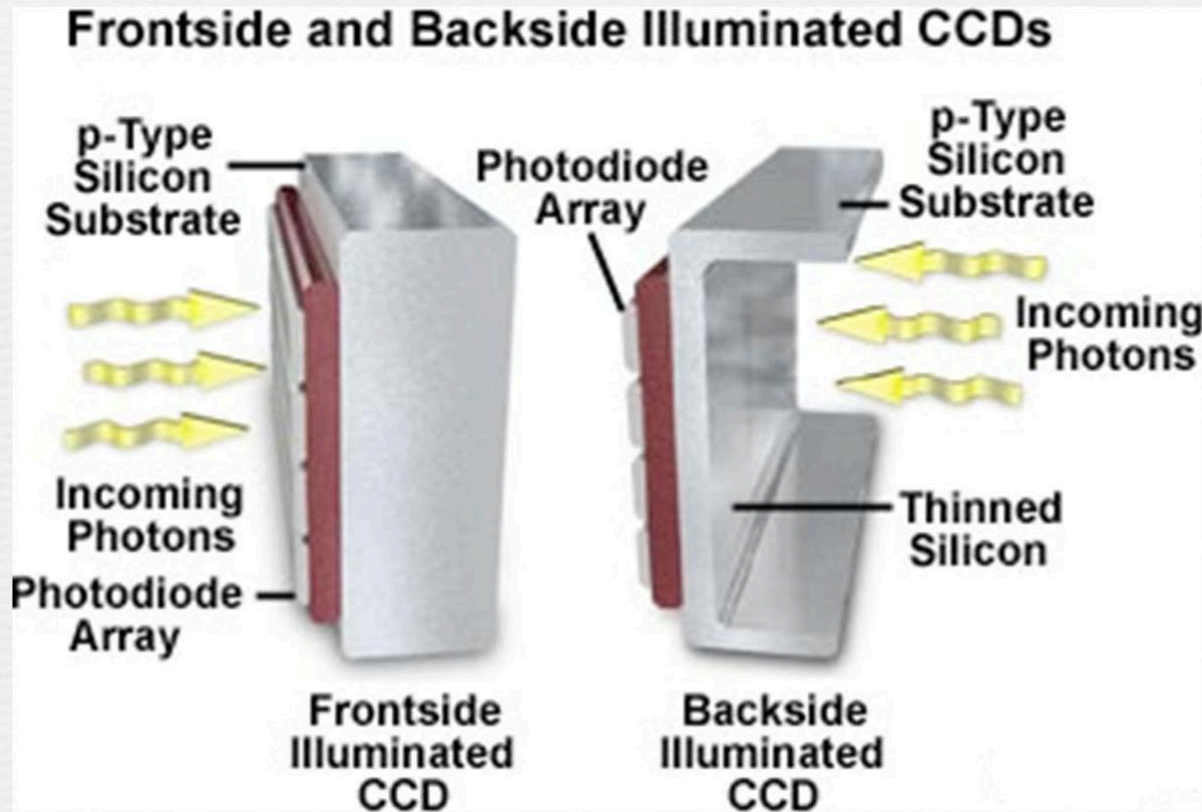
- Resolution is a function of the objective NA and wavelength (e.g. 1.4 NA with 500 nm light  $\rightarrow$   $\sim$  220 nm resolution)
- To achieve this resolution, 220 nm in your image must cover 2 pixels
- Choose your magnification to achieve this
- For 6.45  $\mu\text{m}$  pixels, we need a total magnification of  $6450/110 = 58.6$
- So for 1.4 NA, a 40x lens would be under-sampled, a 60x would be just at the Nyquist limit, and a 100x lens would oversample

# Quantum Efficiency

Frontside and Backside CCD Quantum Efficiency

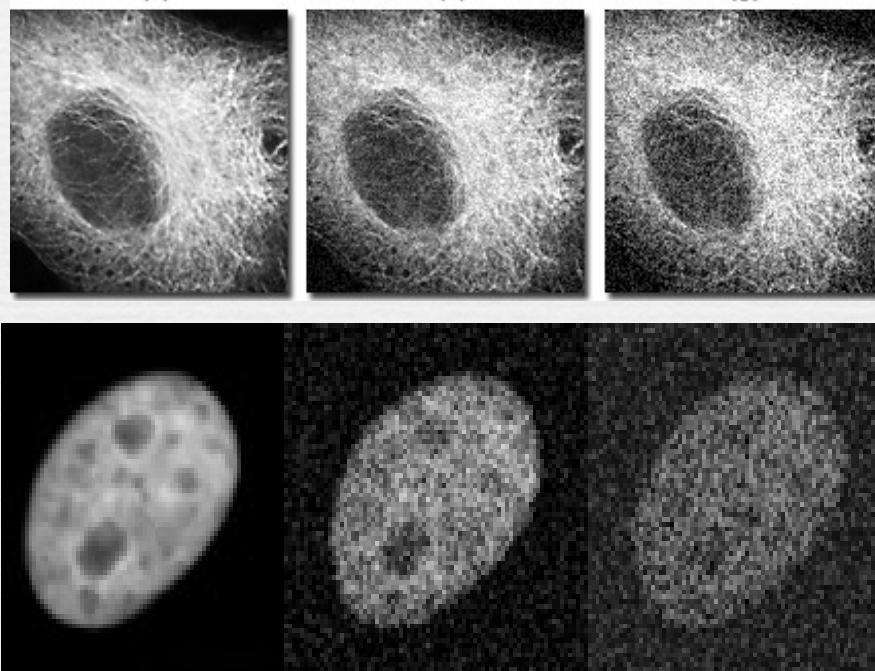


# Back-thinning increases QE



# Noise

n Longer exposure times are better – why?



Decreasing exposure time →

# Noise

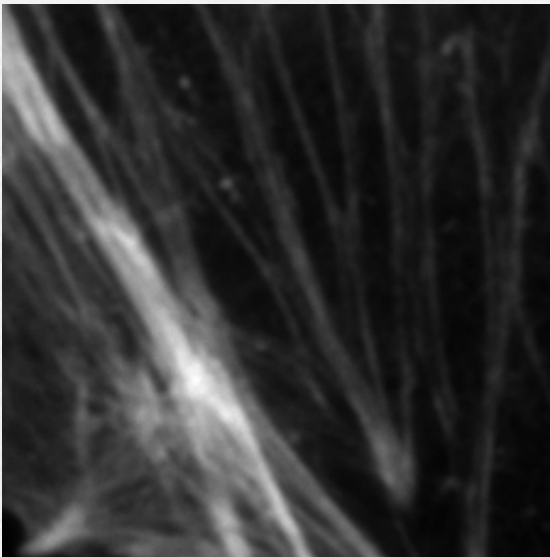
- Photon Shot Noise: Due to the fact that photons are particles and collected in integer numbers. Unavoidable!
  - Scales with  $\sqrt{}$  of the number of photons
- Read noise - inherent in reading out CCD
  - Faster -> Noisier
  - Independent of number of photons
- Fixed Pattern Noise - Not all pixels respond equally!
  - Scales linearly with signal
  - Fix by flat-fielding
- Dark current – thermal accumulation of electrons
  - Cooling helps, so negligible for most applications

# Signal/Noise Ratio (SNR)

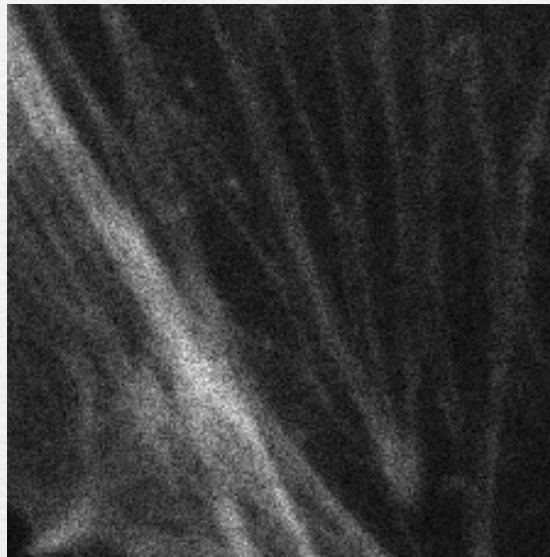
- Signal = # of photons =  $N$
- Noise =  $\sqrt{\text{read noise}^2 + N}$
- When # of photons  $\ll$  read noise<sup>2</sup>  $\rightarrow$  Read noise dominates
- When # of photons  $\gg$  read noise<sup>2</sup>  $\rightarrow$  Shot noise dominates
- When shot noise dominates (Signal/Noise =  $N/\sqrt{N}$ ), to double your SNR, you need to acquire four times as long (or 2x2 bin)

# Often, read-noise dominates

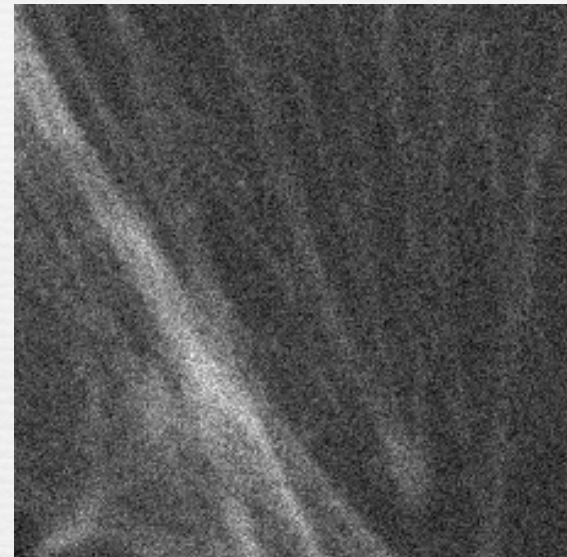
10 photons / pixel on average;  $\sim 50$  in brightest areas



Test image



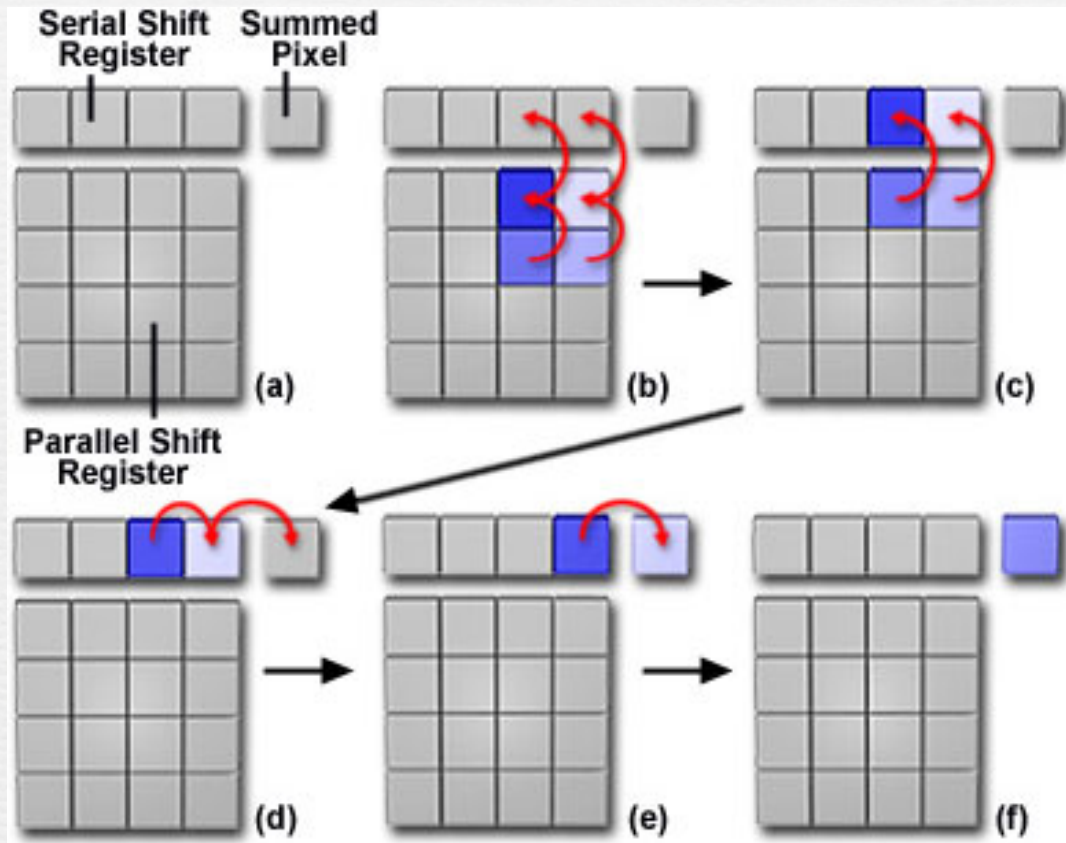
no read noise



5 e<sup>-</sup> read noise

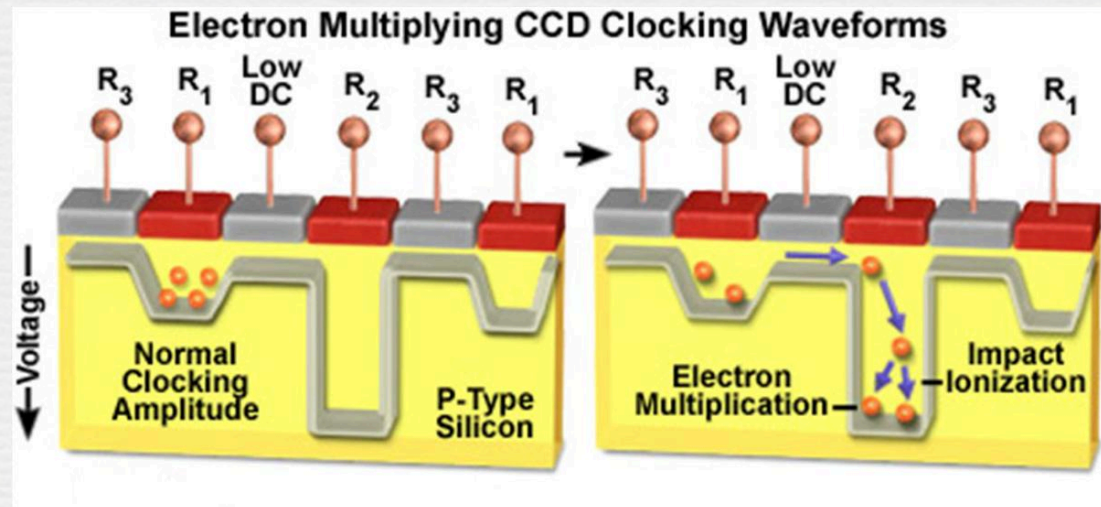
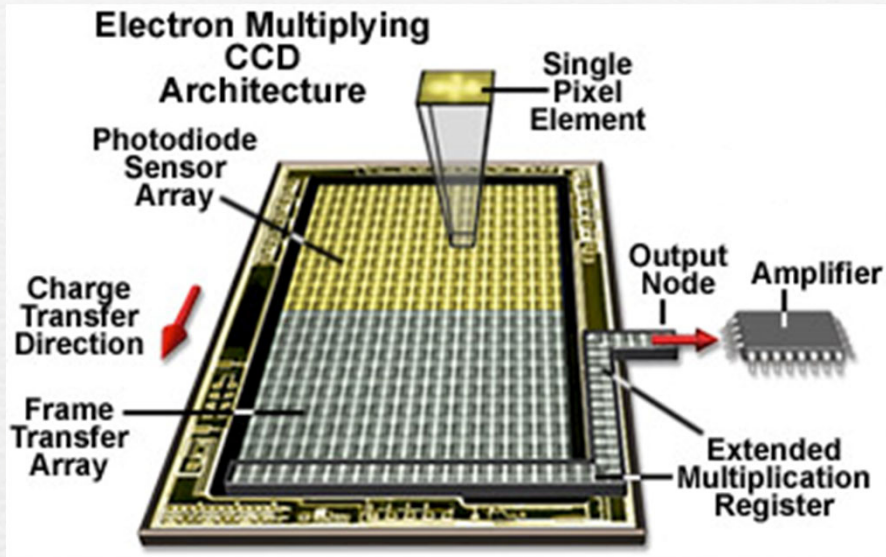
Photon shot noise  $\sim 3/5$  read noise

# Binning



- Read out 4 pixels as one
- Increases SNR by 2x
- Decreases read time by 2 or 4x
- Decreases resolution by 2x

# Beating the read-out noise EMCCD



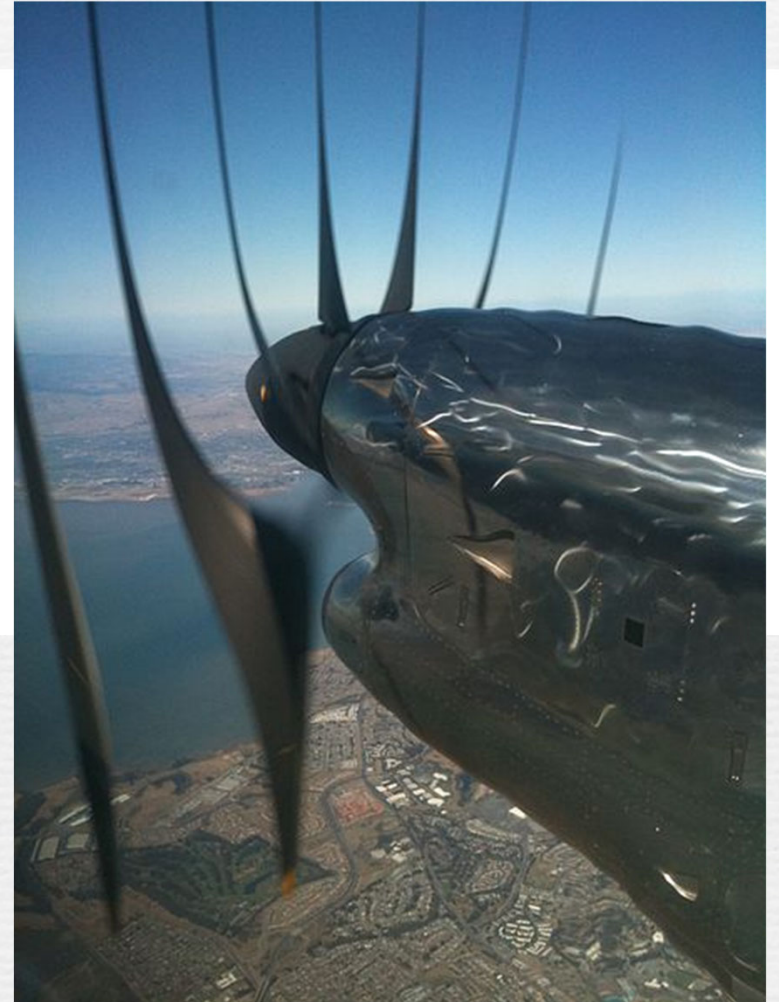
# EMCCD result

- n Fast noisy CCD – runs at 30 fps, but 50 e<sup>-</sup> read noise
- n Multiply signal by 100-fold – now read noise looks like 0.5 e<sup>-</sup>
- n Downside – multiplication process adds additional Poisson noise (looks like QE is halved)
- n Upside – you get to image fast without worrying about read noise

# s(cientific)CMOS

< 1.5 electron read-noise!

- 2,000 x 2,000 pixels, 6.5 micron
- 100 fps full frame, subregions up to 25,000 fps
- fixed pattern noise
- binning does not reduce r.o. noise
- global versus rolling shutter





# Dynamic Range: How many intensity levels can you distinguish?

- Full well capacity (16 000 e<sup>-</sup>)
- Readout noise: 5e<sup>-</sup>
- Dynamic range:
  - FWC/readout noise: 3200
  - $0.9 * \text{FWC} / (3 * \text{readout noise}) = 960$
- (Human eye ~ 100)

# Bitdepth

- n Digital cameras have a specified bitdepth = number of gray levels they can record
- n 8-bit  $\rightarrow 2^8 = 256$  gray levels
- n 10-bit  $\rightarrow 2^{10} = 1024$  gray levels
- n 12-bit  $\rightarrow 2^{12} = 4096$  gray levels
- n 14-bit  $\rightarrow 2^{14} = 16384$  gray levels
- n 16-bit  $\rightarrow 2^{16} = 65536$  gray levels

# Photons and Numbers

- Zero photons collected doesn't result in number zero. Offset can often be changed
- 1 photon does not necessarily equal 1 count in your image – electron conversion factor - depends on camera gain

Measure the electron conversion factor:

*When the dominant noise source is Photon Shot noise:*

$$\sigma(N) = \sqrt{N}$$

$$N = c \cdot DN$$

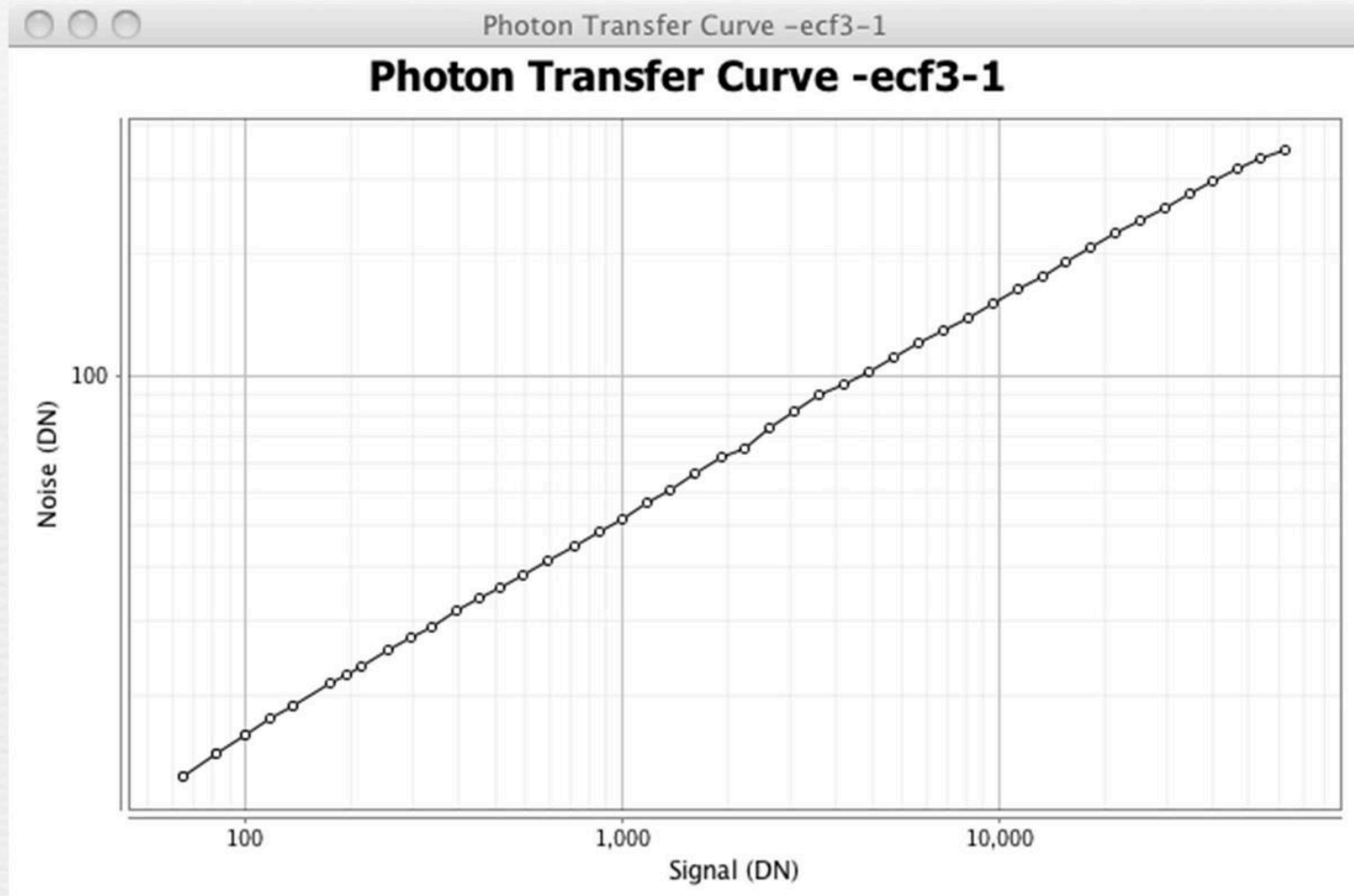
$$\sigma(N) = c \cdot \sigma(DN)$$

$$c = DN / \sigma^2(DN)$$

Photon Shot noise

c = electron conversion  
factor

# Measure Photon Conversion Factor and full well capacity



Photon Transfer Curve from: James R. Janesick, Photon Transfer, DN  $\rightarrow$   $\lambda$ . SPIE Press, 2007

[http://valelab.ucsf.edu/~MM/MMwiki/index.php/Measuring\\_camera\\_specifications](http://valelab.ucsf.edu/~MM/MMwiki/index.php/Measuring_camera_specifications)

# Credits and resources:

- n Kurt Thorn (UCSF Nikon Imaging Center)
- n <http://micro.magnet.fsu.edu>
- n James Pawley, Handbook of Biological Confocal Microscopy
- n James R. Janesick, Photon Transfer, DN  $\rightarrow$   $\lambda$ . SPIE Press, 2007