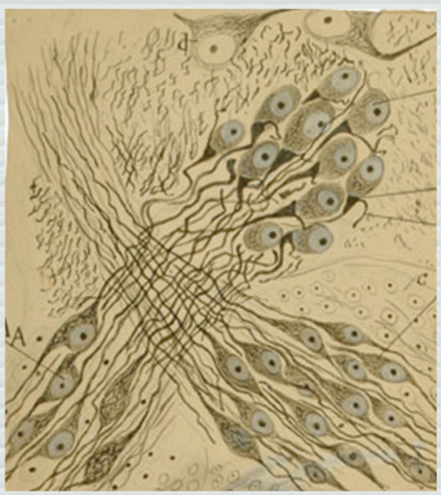
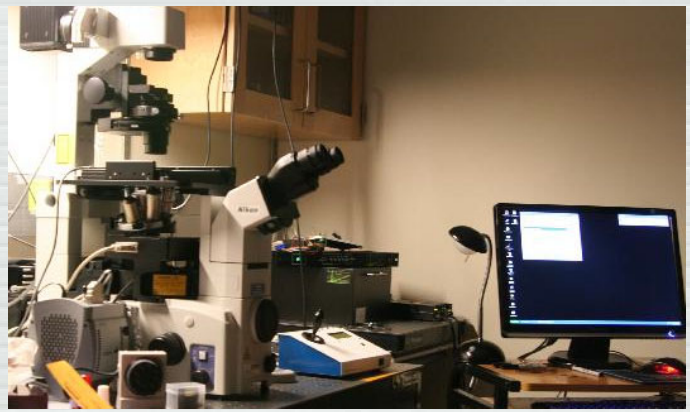
Digital microscopy: Light Sources and Detectors





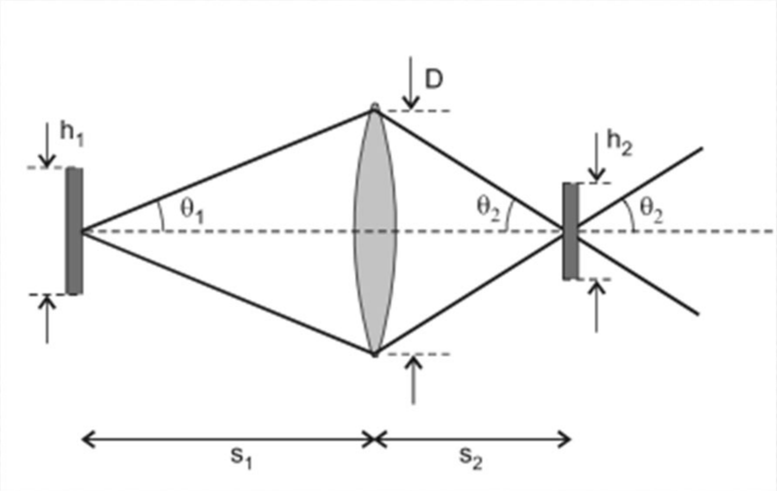
Nico Stuurman, UCSF/HHMI

UCSF, April 2013

Light Sources

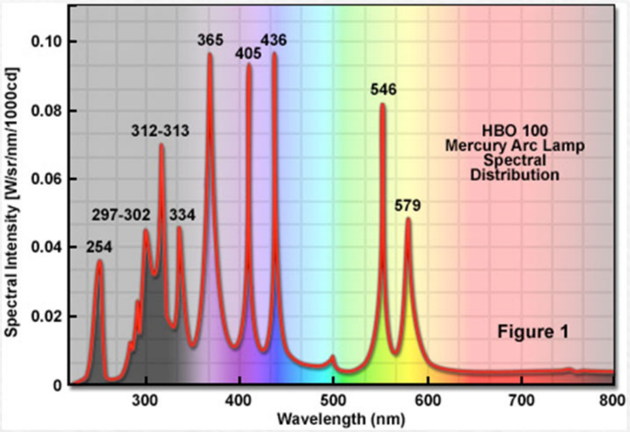
Factors to consider:

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

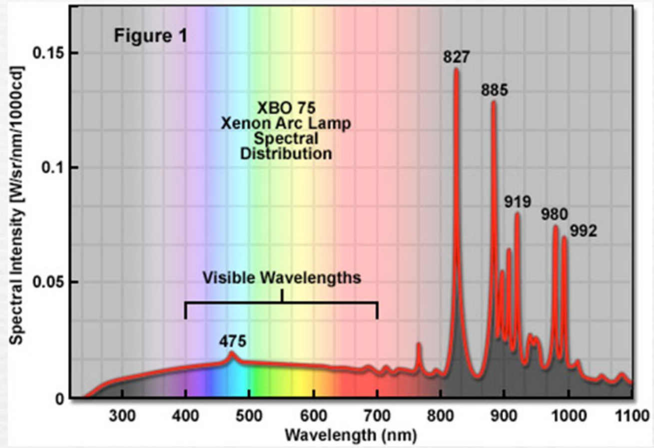


Brightness is determined by size and angle

Arc Lamps



Mercury Arc



Xenon Arc

Cons:

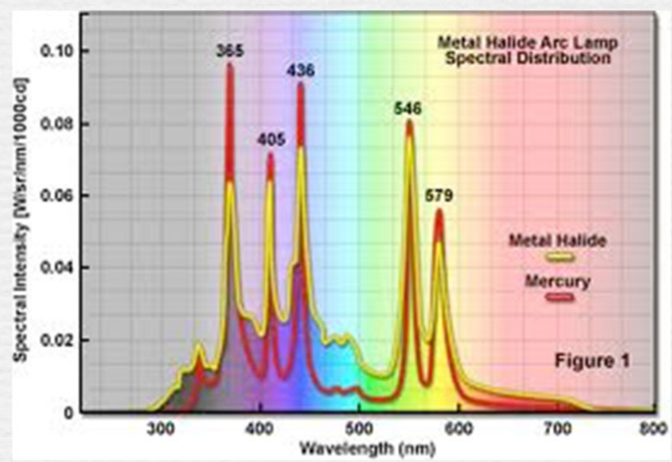
- Short Lifetime
- Dangerous (Hg)
- Hot
- Needs mechanical shutter
- Laborious installation

Metal Halide

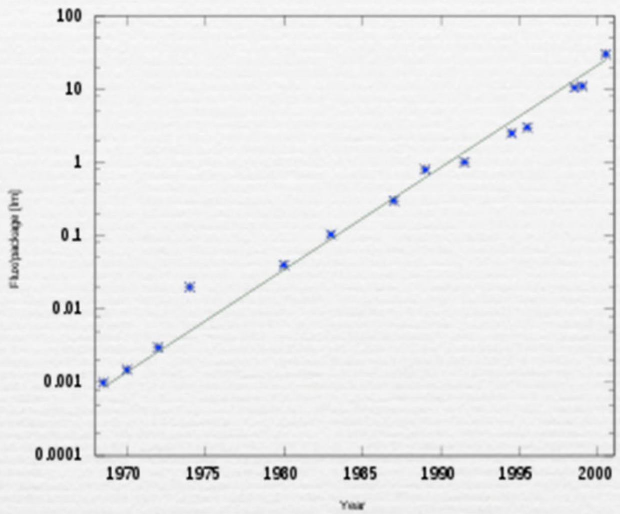
Produces light by an electric arc through a gaseous mixture of vaporized mercury and metal halides (compounds of metals with bromine or iodine).

Step up from Arc lamps, still:

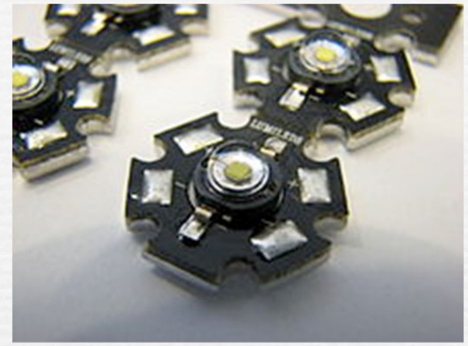
- Hot, loud, lifetime ~1500 hours
- Lamps expensive ($500-800)
- Needs mechanical shutter

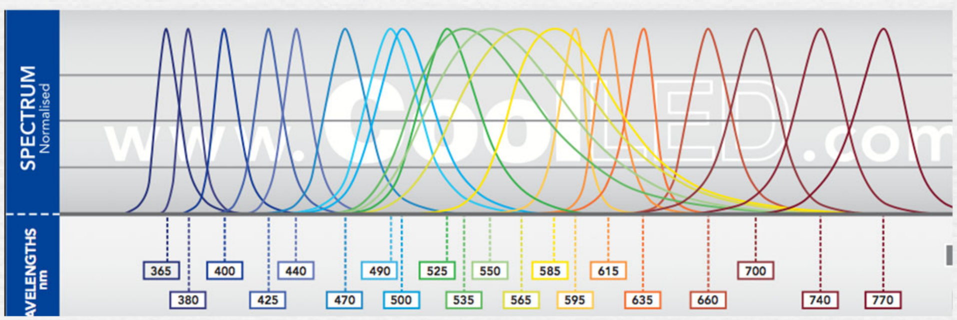


LEDs

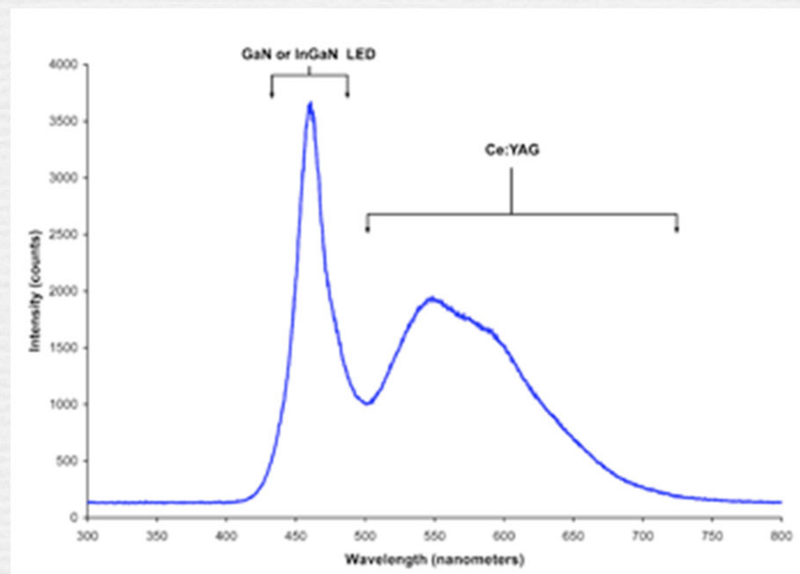


Haitz's law: Every decade the cost per lumen falls a factor of 10, amount of light increases a factor of 20





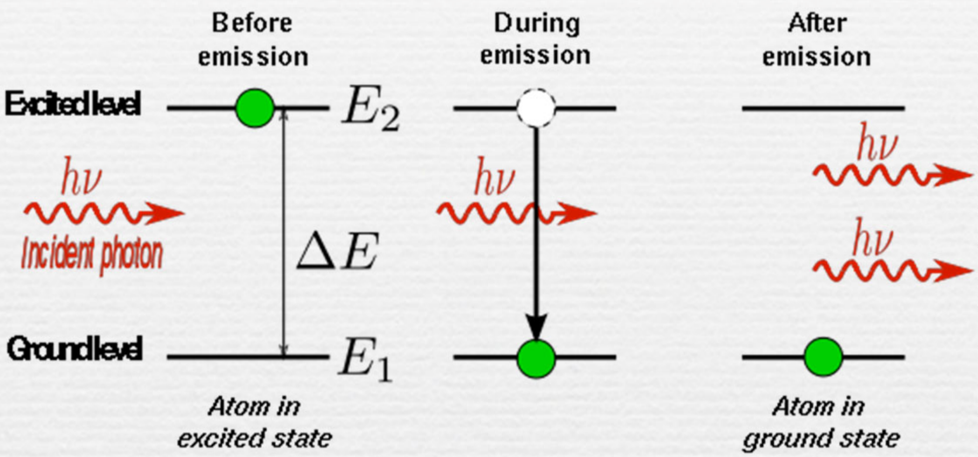
Source: www.coolled.com



White LED with phosphors

Lasers

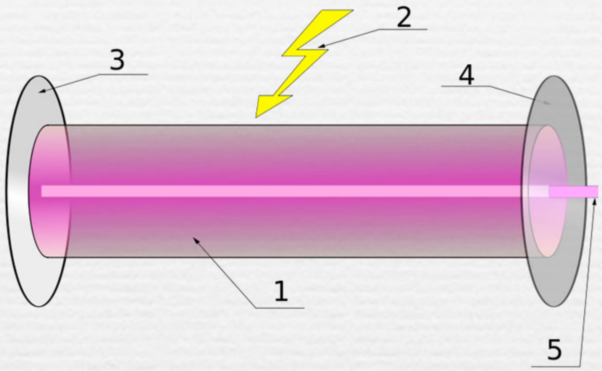
Stimulated Emission



$$E_2 - E_1 = \Delta E = h\nu$$

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

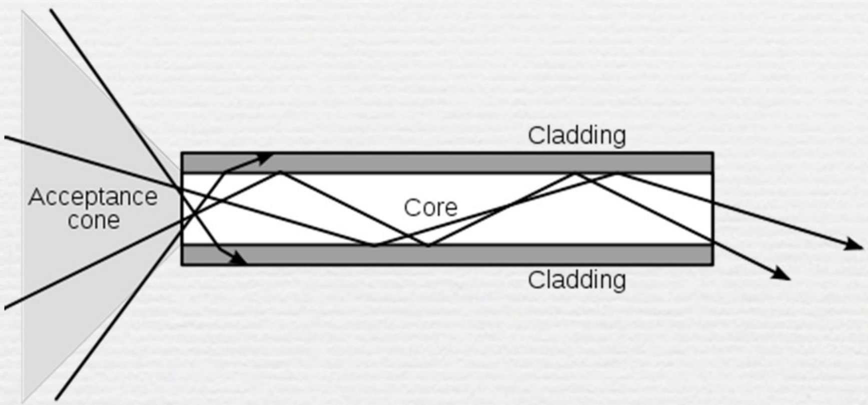
Laser Diode
(electrically pumped)

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

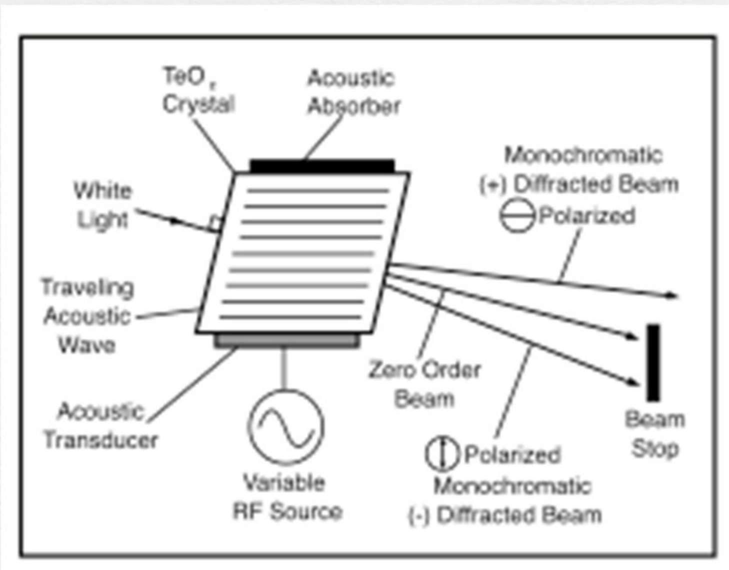


Multi Mode - core > 10 microns

Single Mode - core 3-6 micron (visible)

Lasers, modulation

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



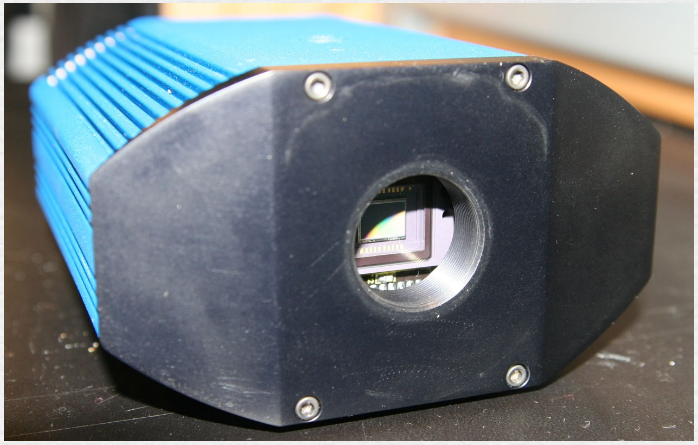
Acousto Optical Tunable Filter

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

Also: AOM or Bragg cell

Imaging Detectors

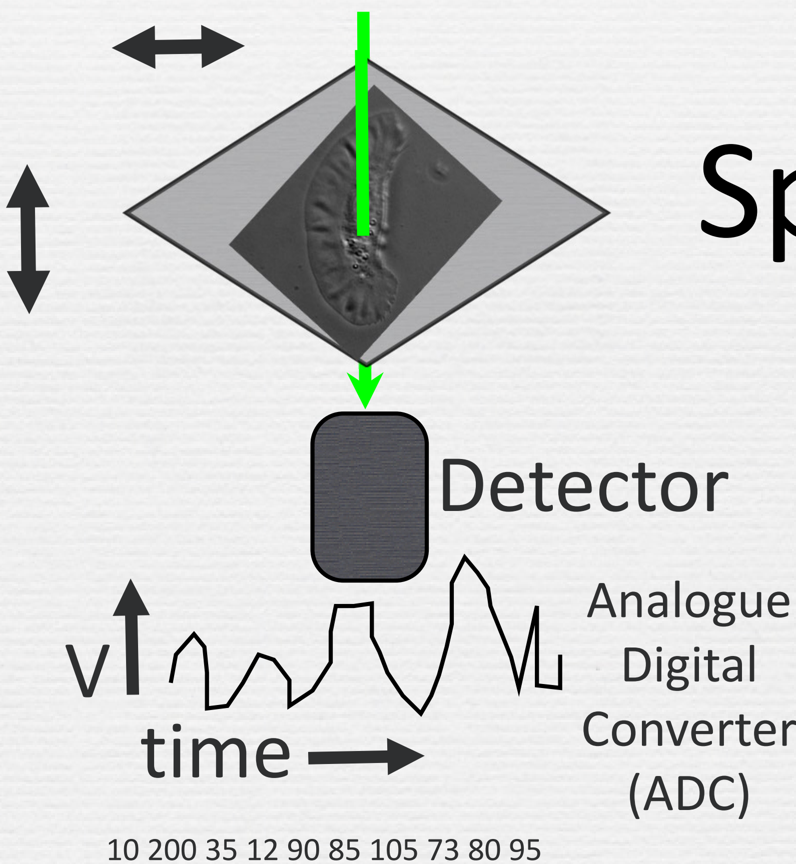
- Detectors:
  - 'Single point' detectors
  - 'Multiple point' detector (cameras)



Imaging Detectors

Photon -> Electrons -> Voltage ->Digital Number

Single point detector



Speed!

Multi point detector (camera)

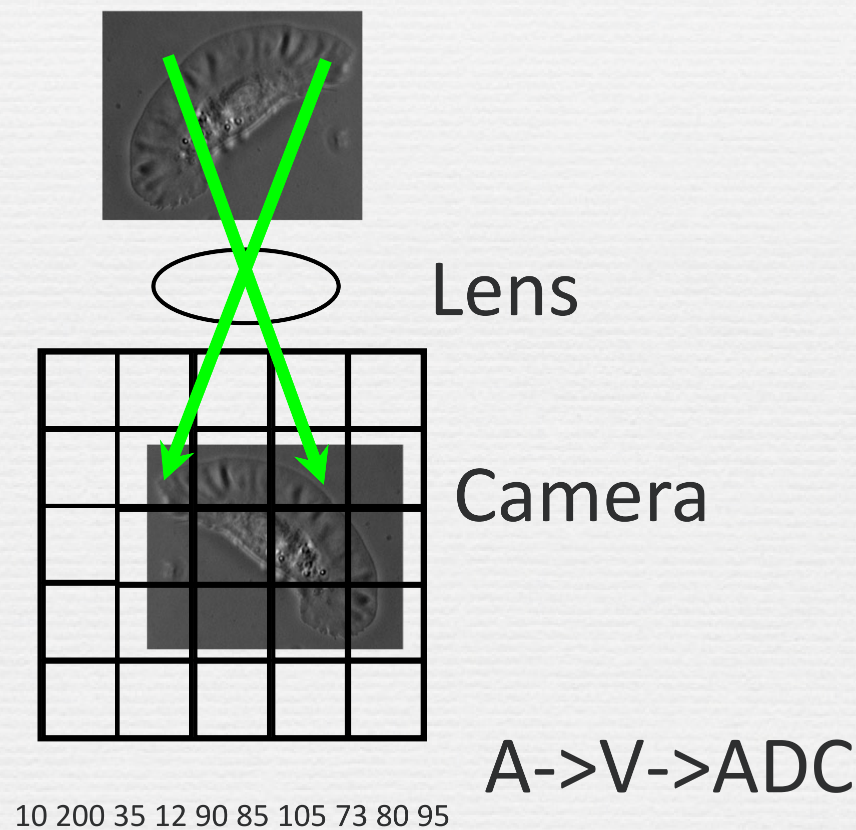
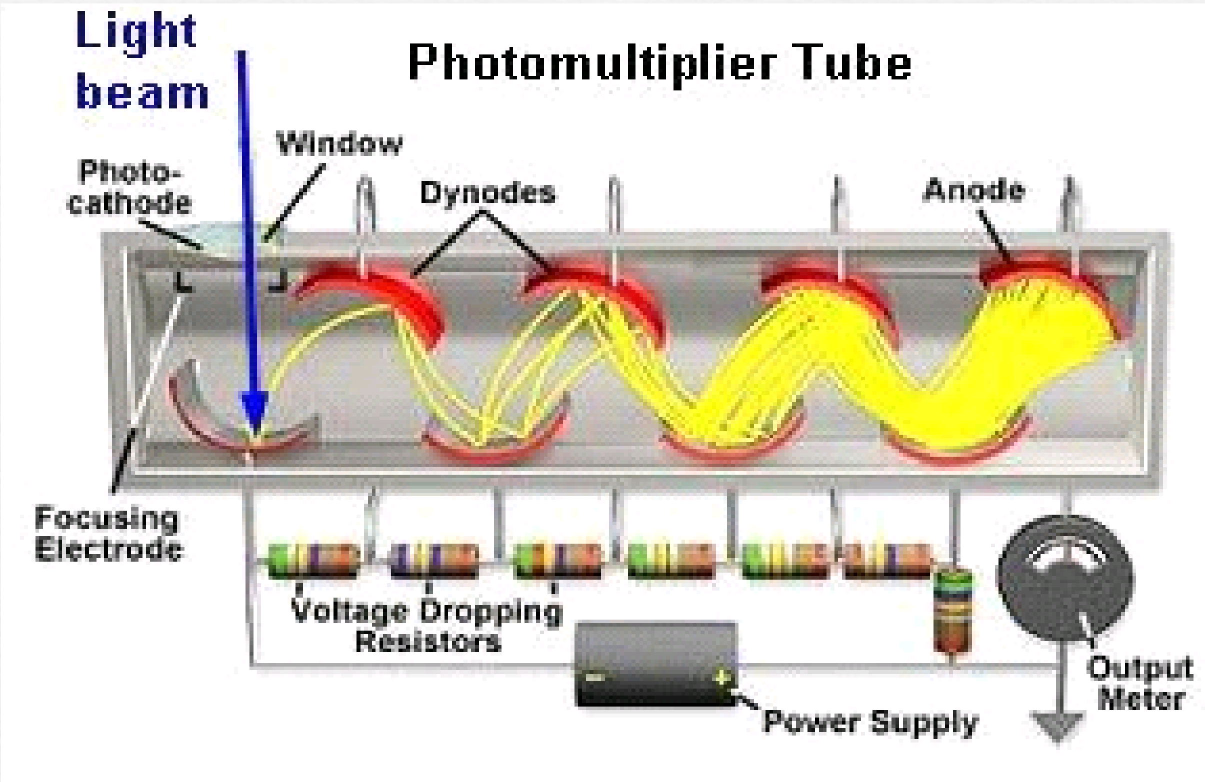
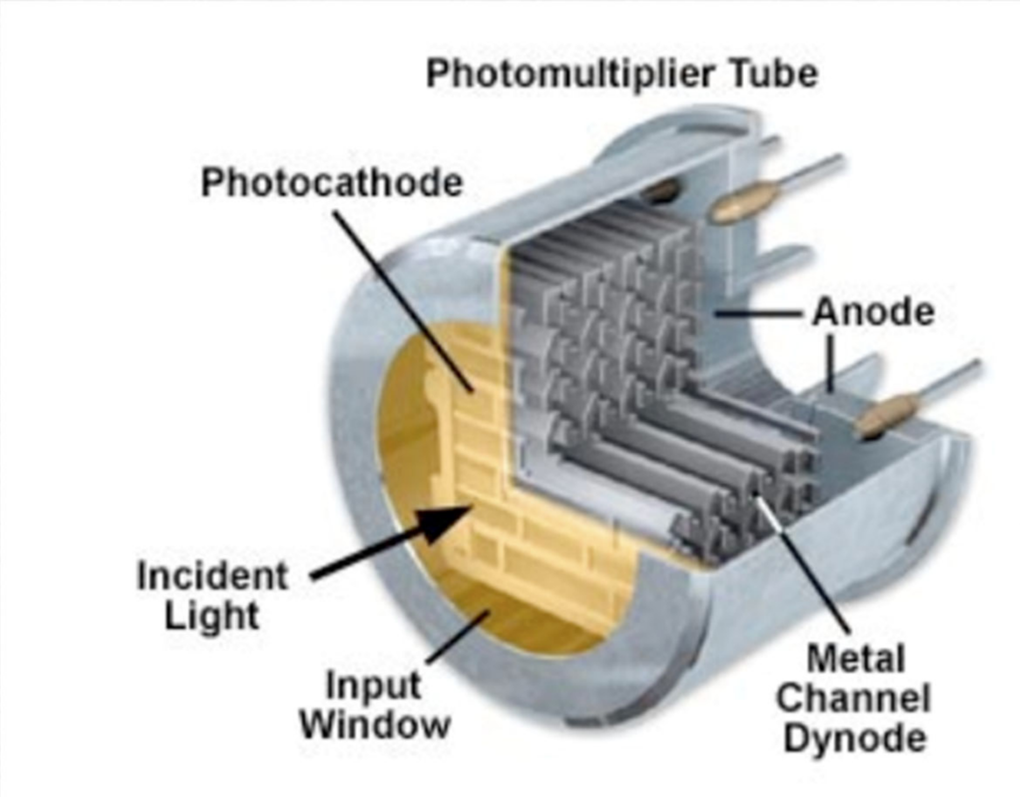


Photo-Multiplier Tube (PMT)



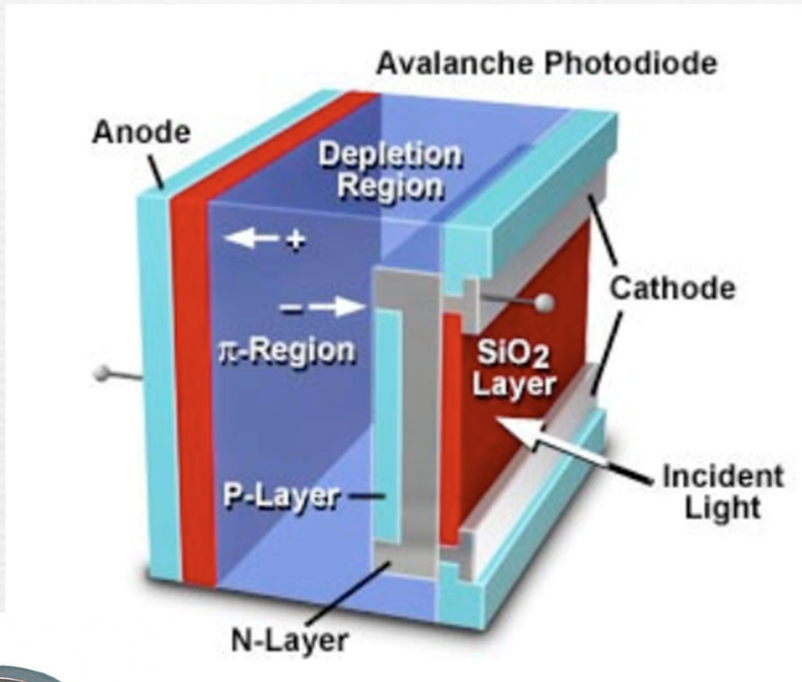
- Very linear
- Very High Gain
- Fast response
- Poor Quantum efficiency (~25%)

PMT modes



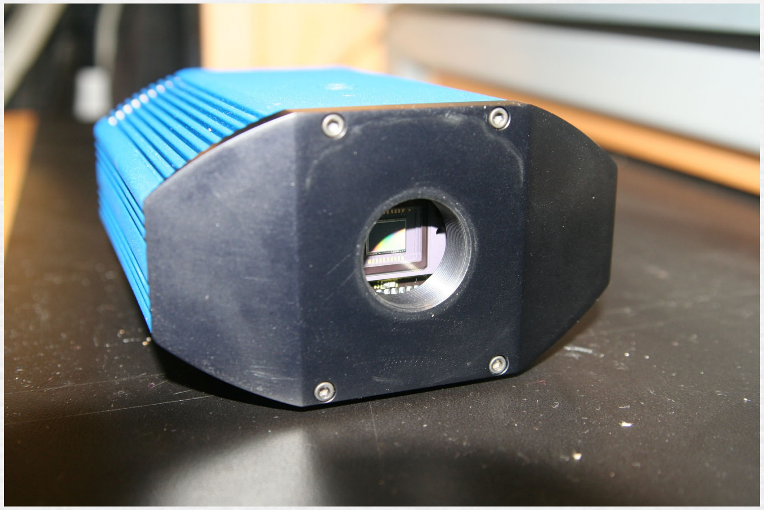
- Photon counting mode:
  - Count pulses
  - Zero background
  - Slow
- Linear mode
- Measure current
- Fast but noisy

Avalanche Photo Diode

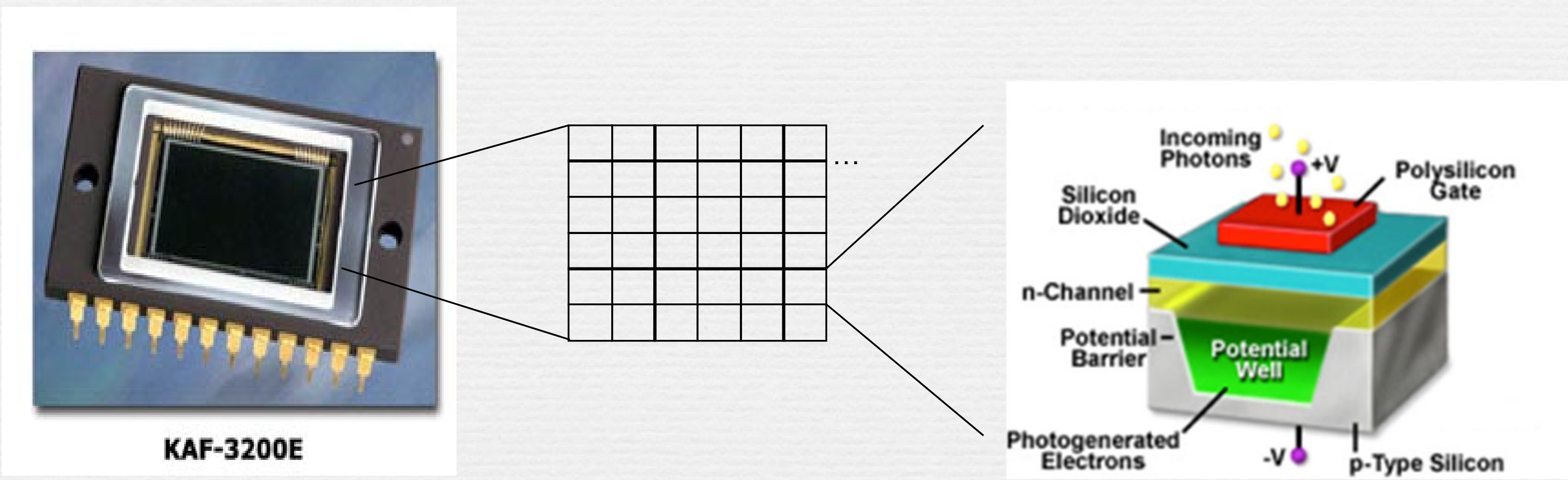


- Absorbed photons->electron
- Electrons amplified by high voltage and 'impact ionization'
- High QE (~90%)
- Photon-counting ability (different design)
- Overheats if run too fast

Cameras in Microscopy

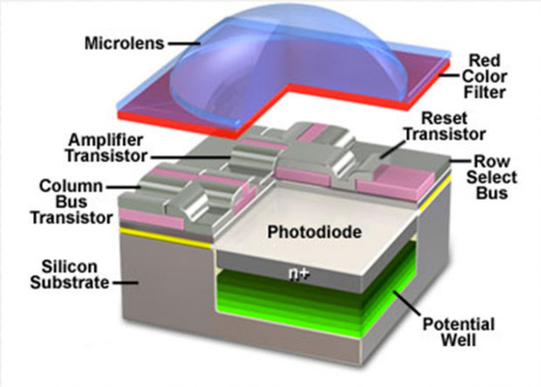


Arrays of photo-sensitive elements



Two Architectures:

Complementary Metal Oxide Semiconductor (CMOS)



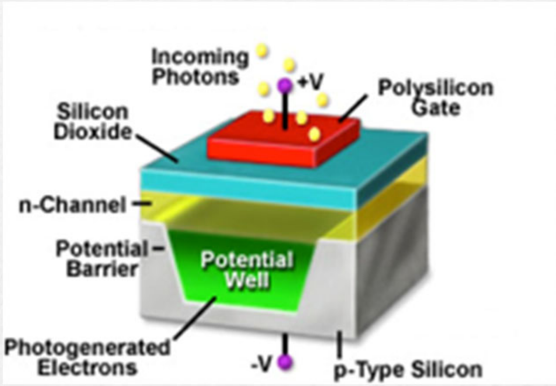
Each pixel has an amplifier

Transfers voltage

Fast

Noisy

Charged Coupled Devices (CCD)



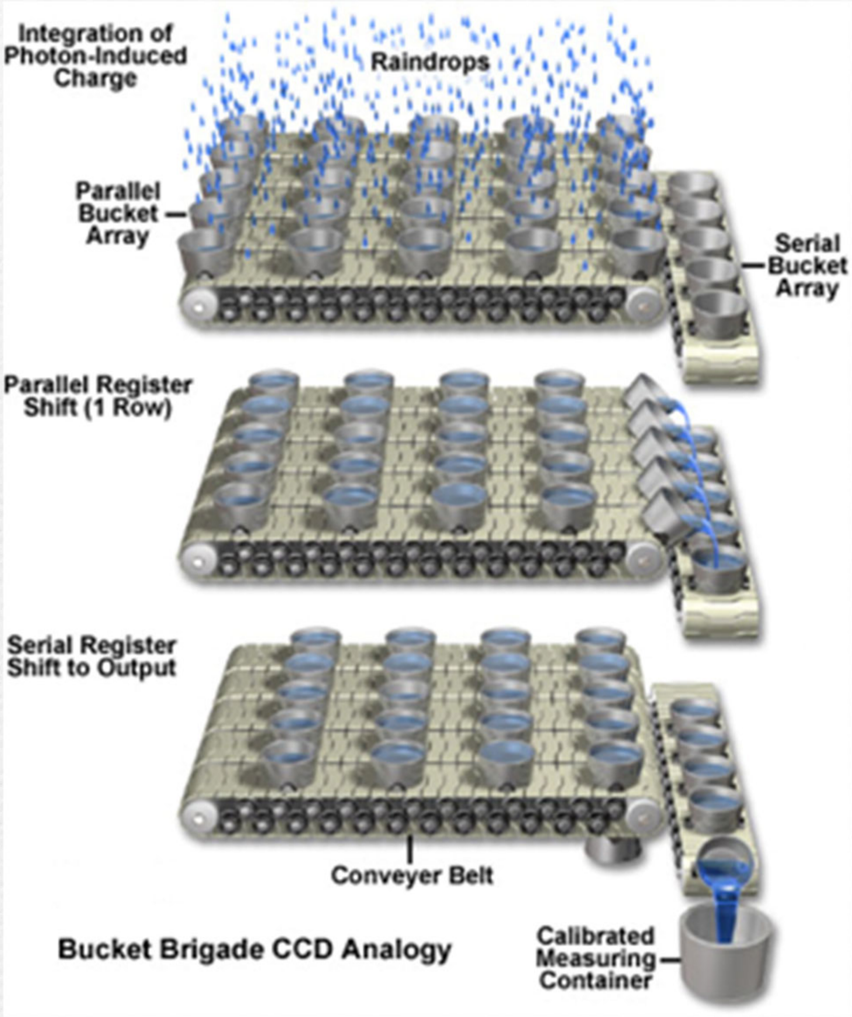
Single read-out amplifier

Transfers charge

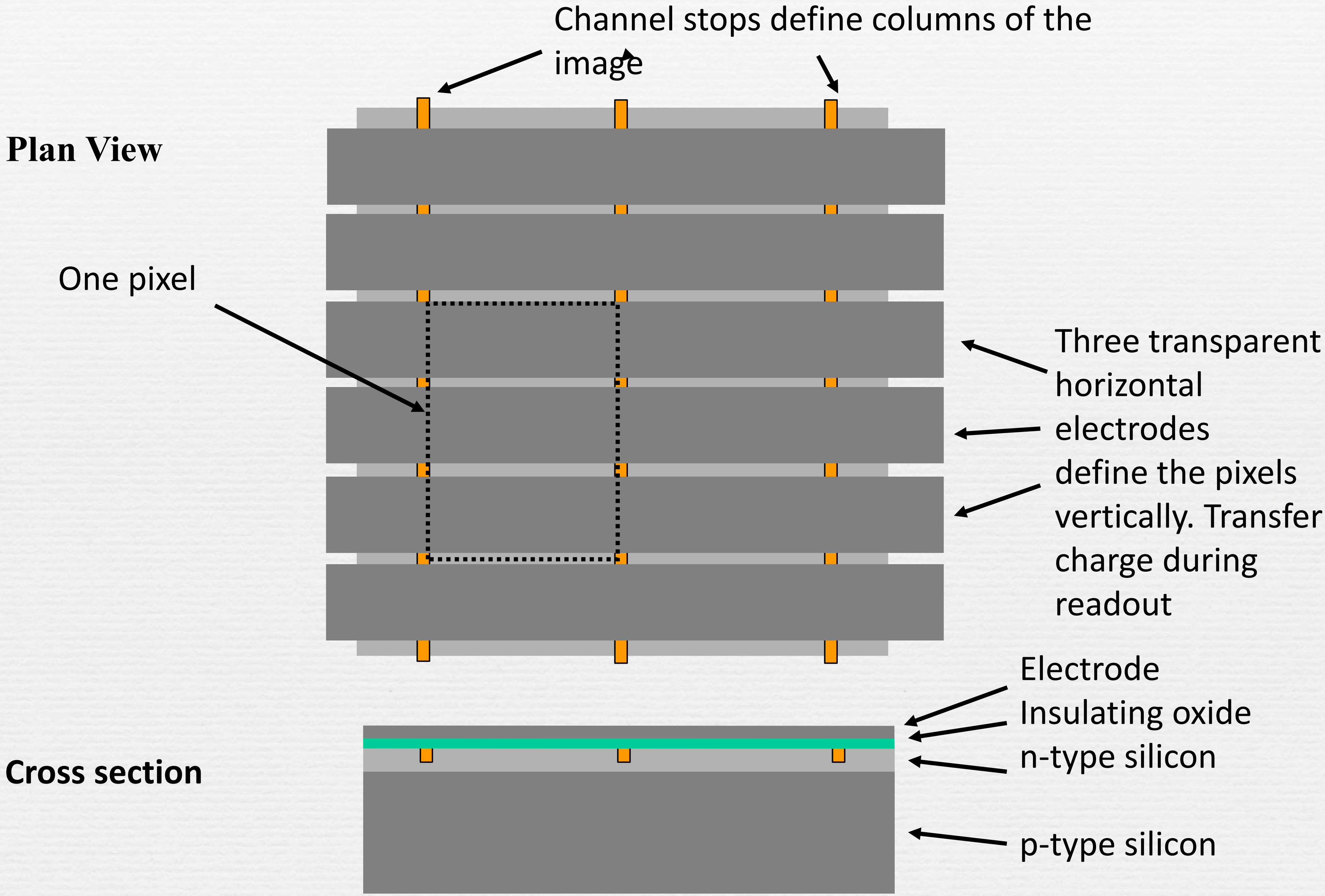
Slow

Precise

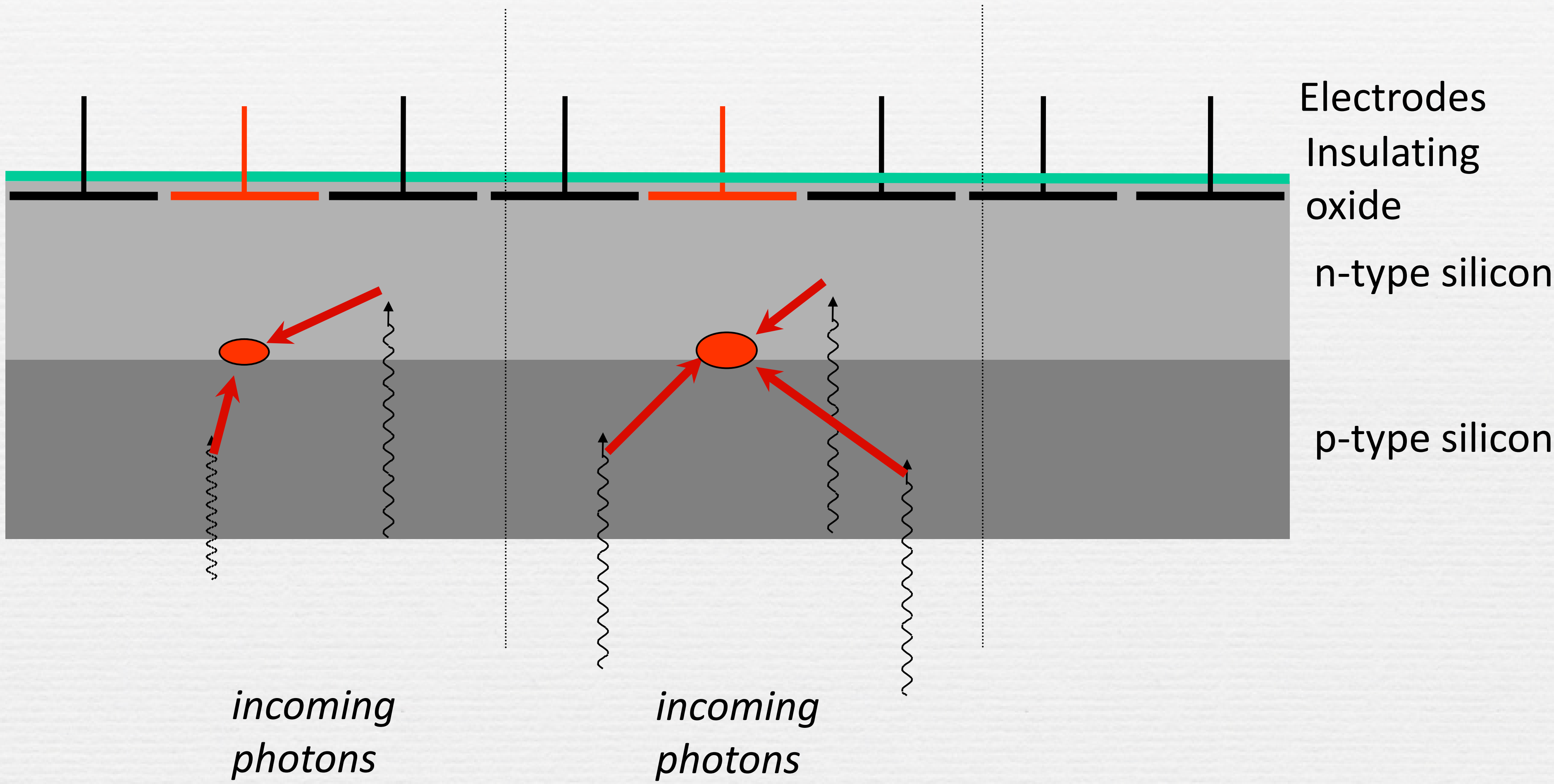
CCD readout “bucket-brigade” analogy



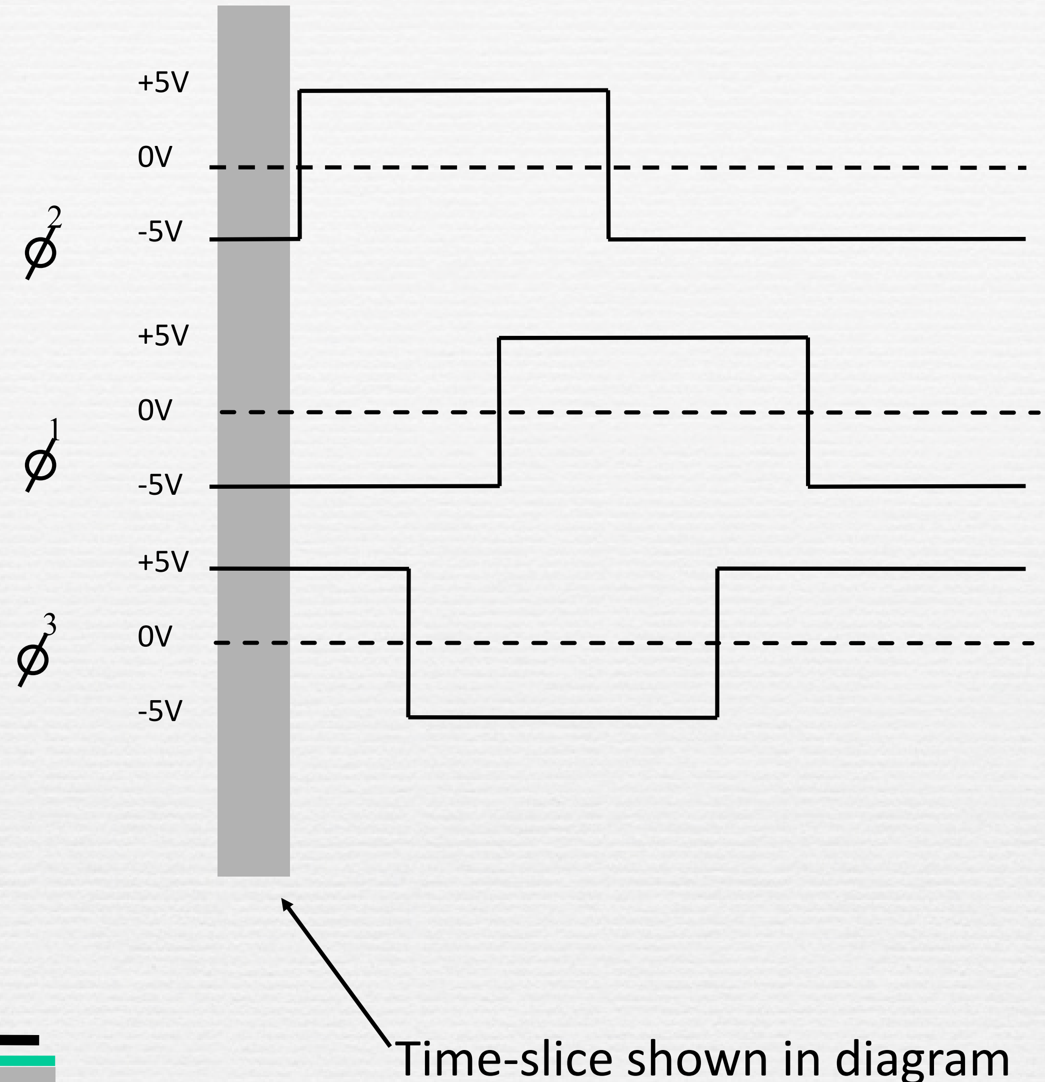
CCD Architecture

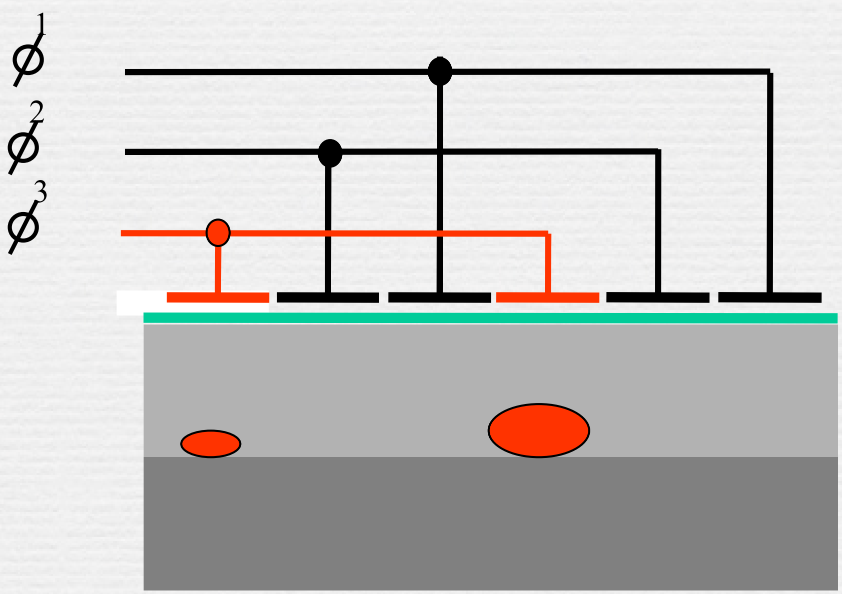


CCD Architecture

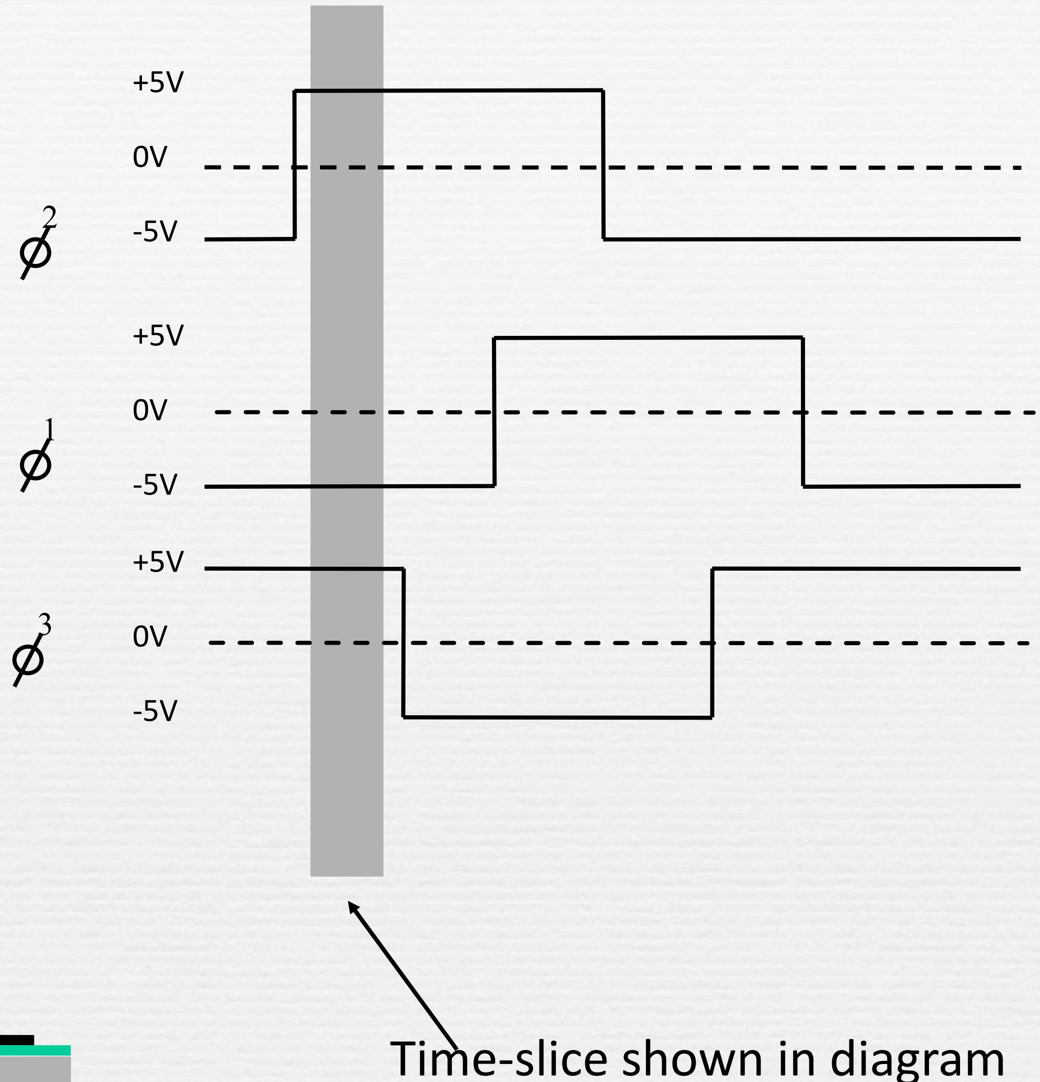


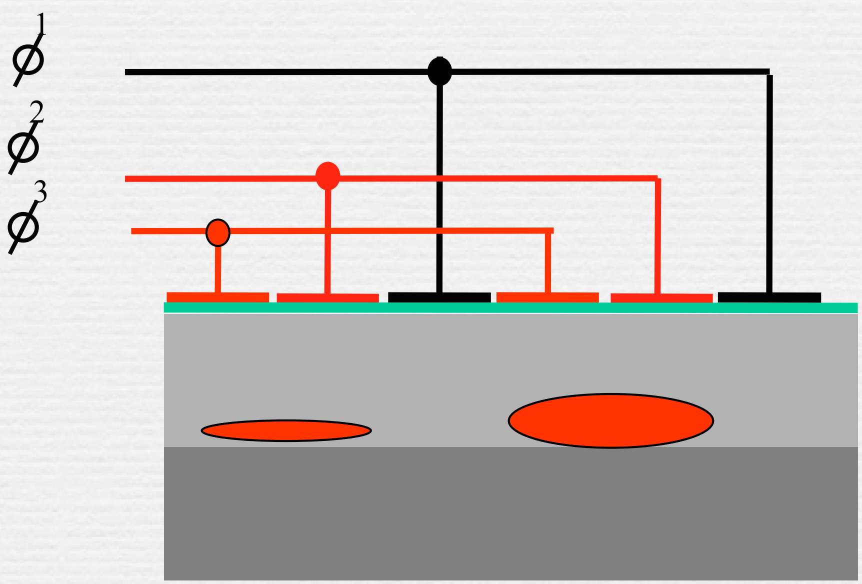
Charge Transfer in a CCD



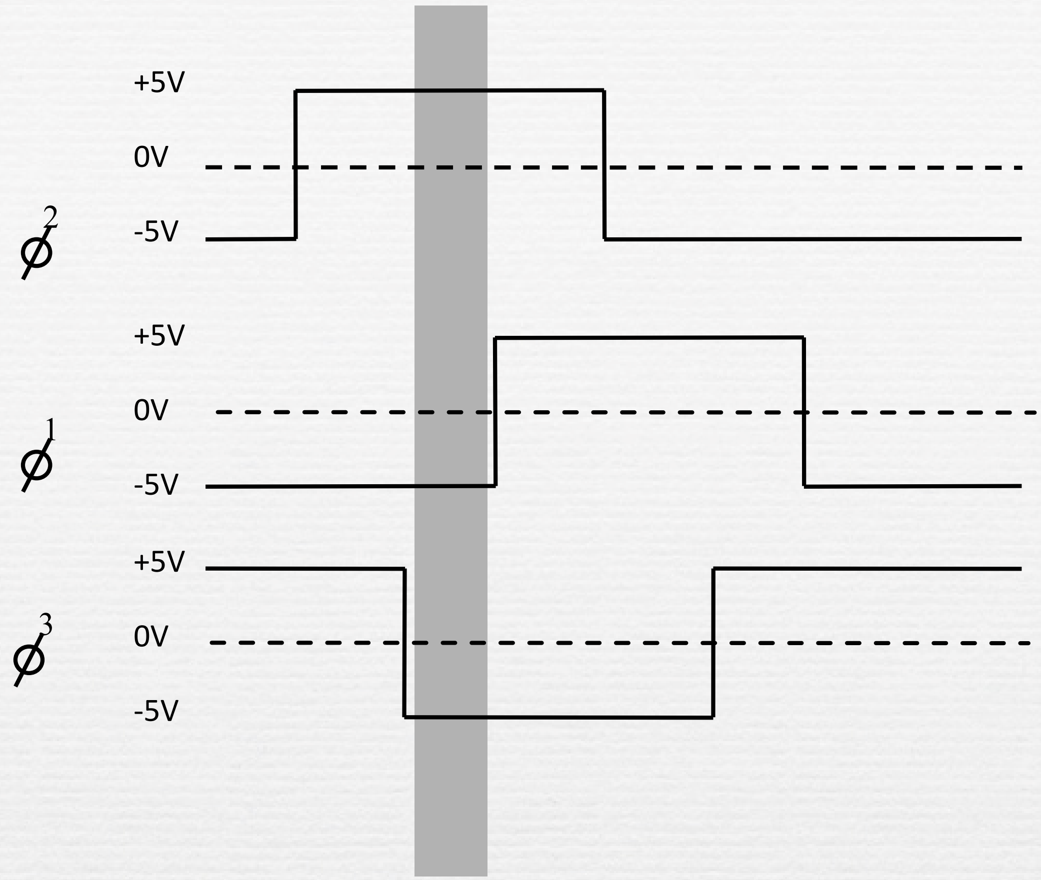


Charge Transfer in a CCD

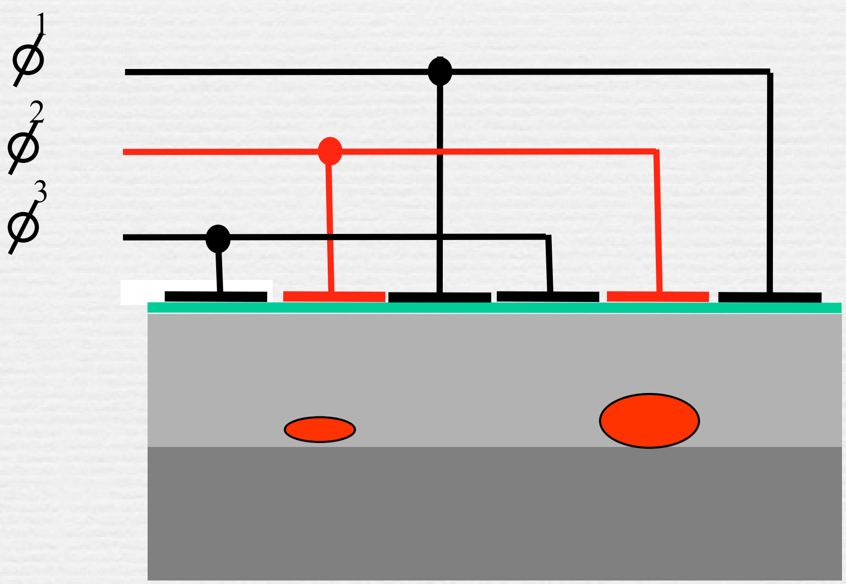




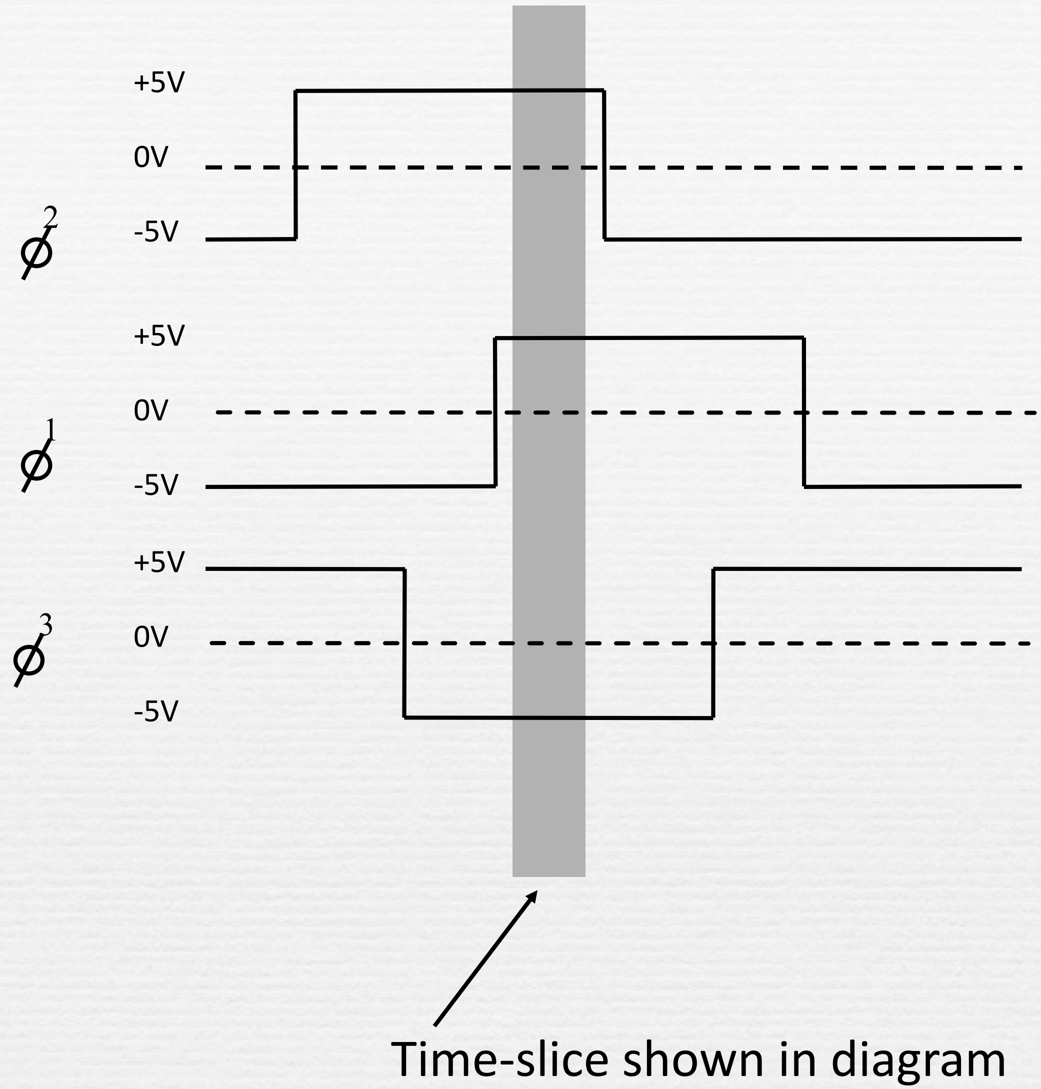
Charge Transfer in a CCD

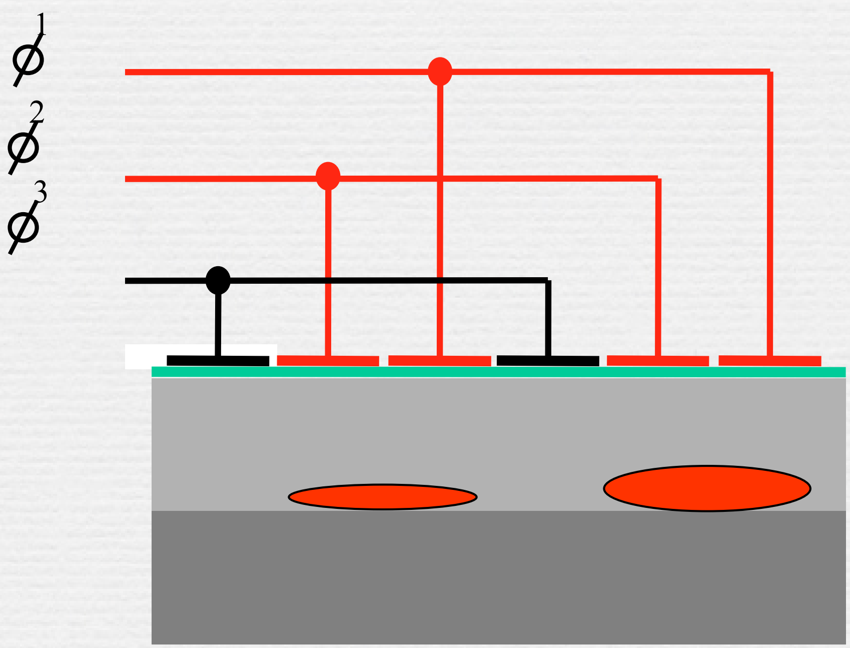


Time-slice shown in diagram

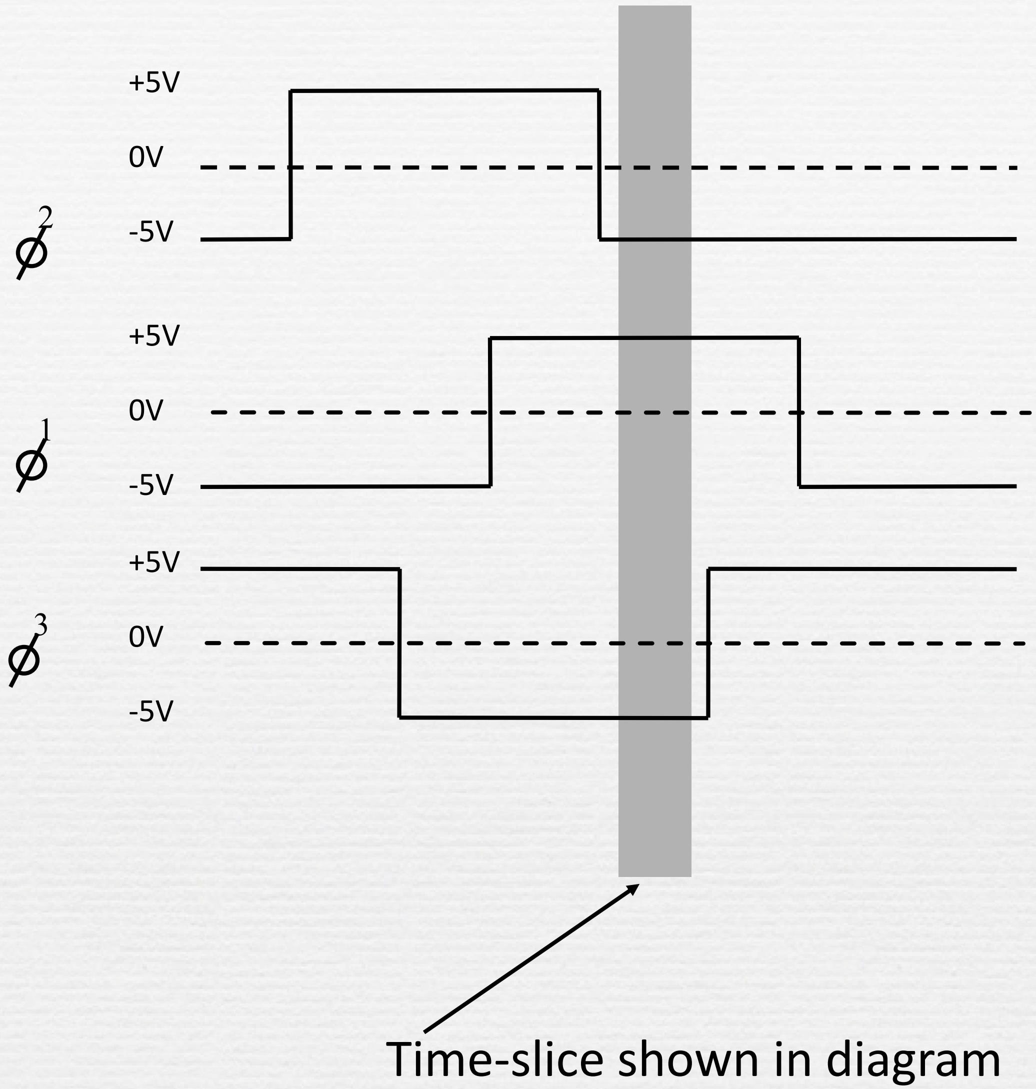


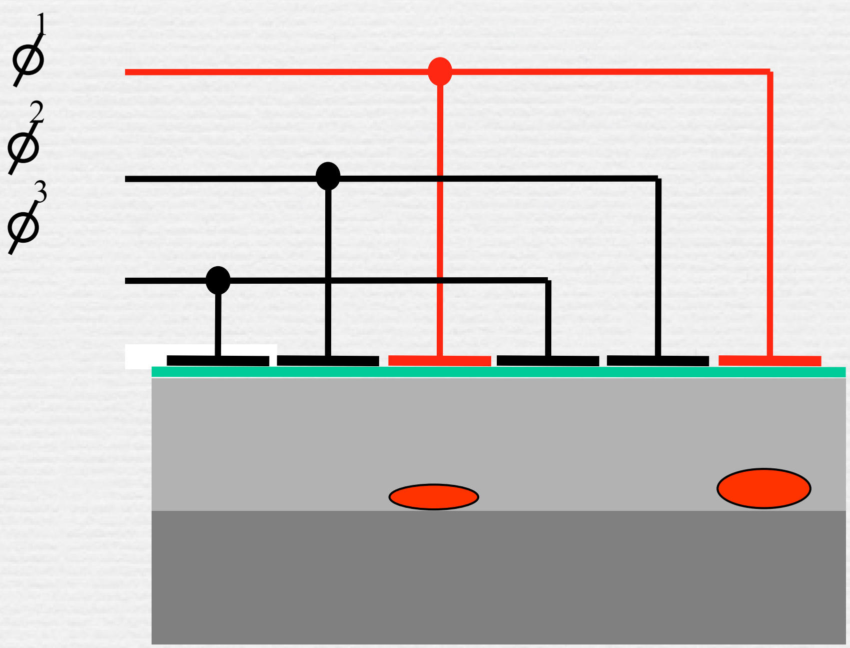
Charge Transfer in a CCD



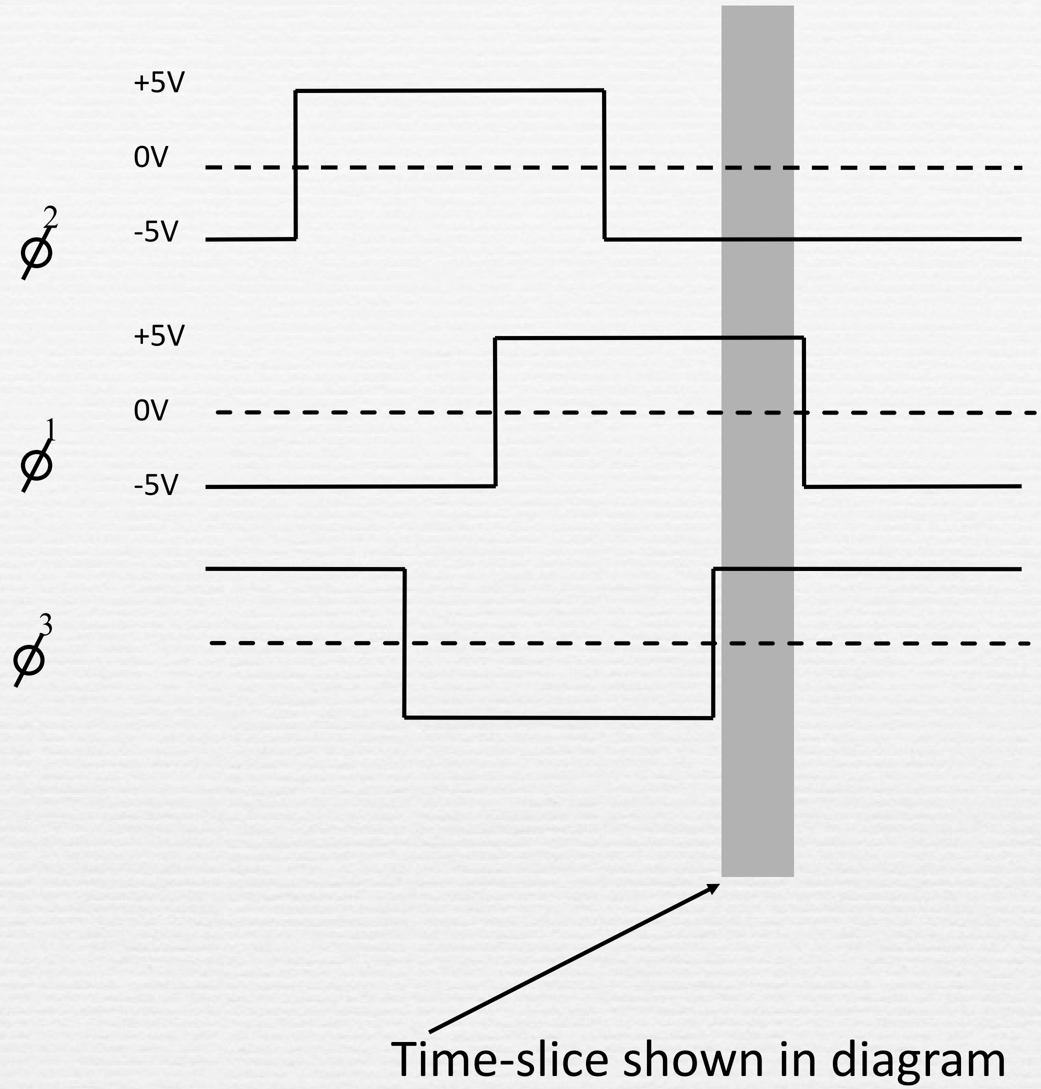


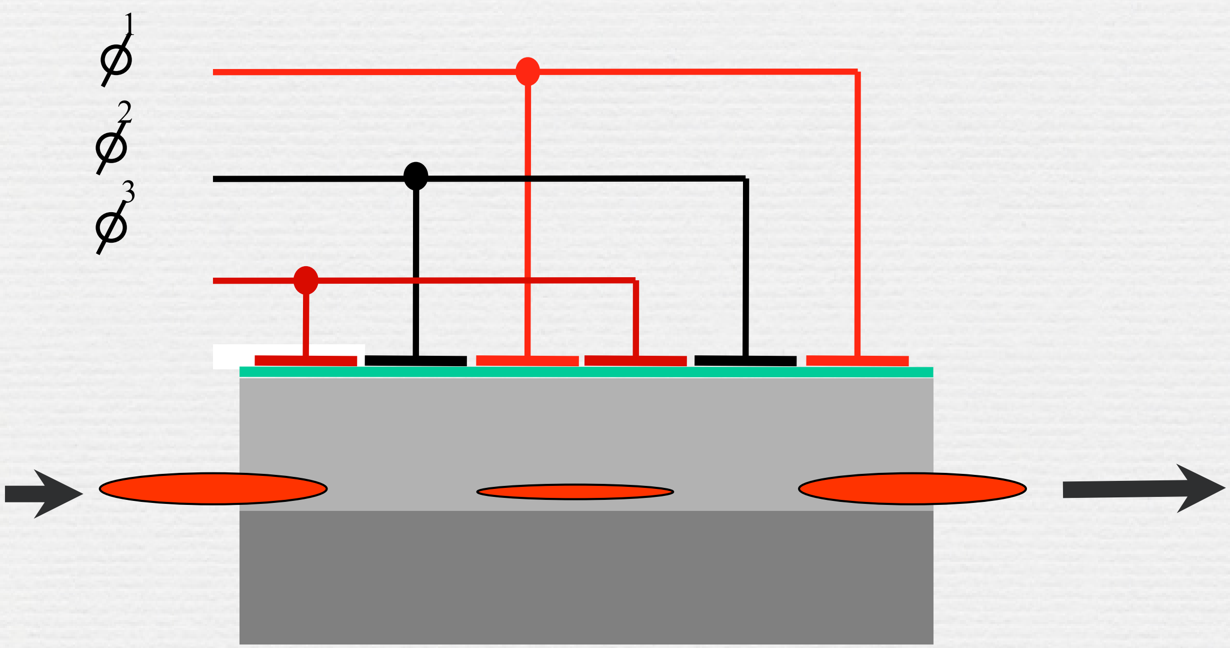
Charge Transfer in a CCD



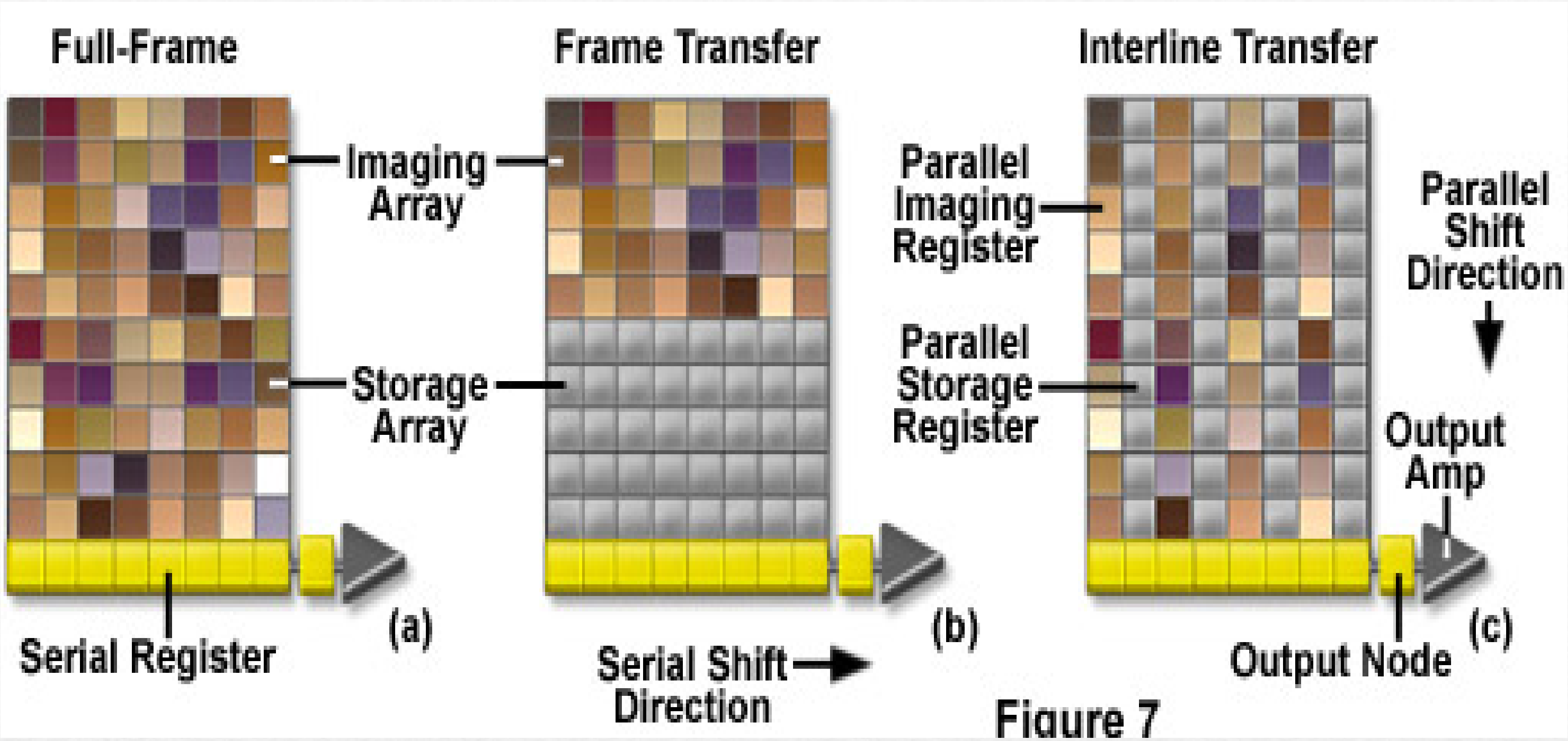


Charge Transfer in a CCD
Transfer charge to the next pixel





CCD Architectures



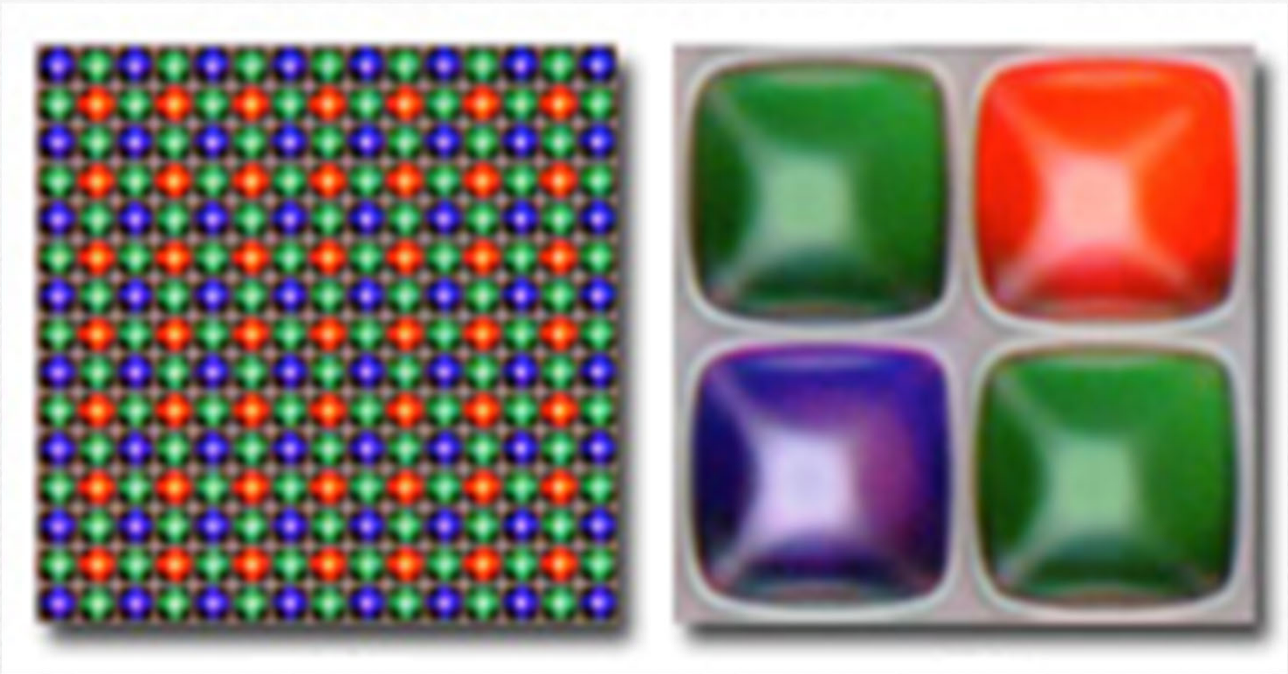
Mostly EMCCDs

Rare

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 pix
- Read noise
- Dark current (negligible for most biological applications)
- Readout time (calculate from clock rate and array size)
- Electron conversion factor (relate digital

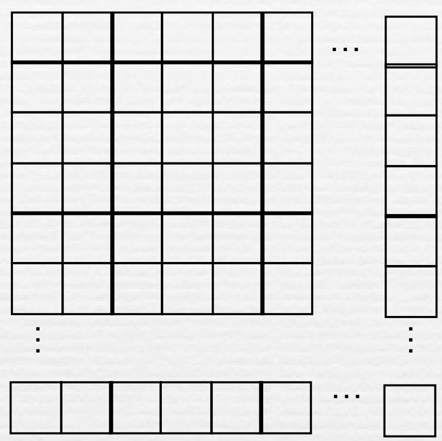
SPECIFICATIONS

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 / 1×1)
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

1392

1040



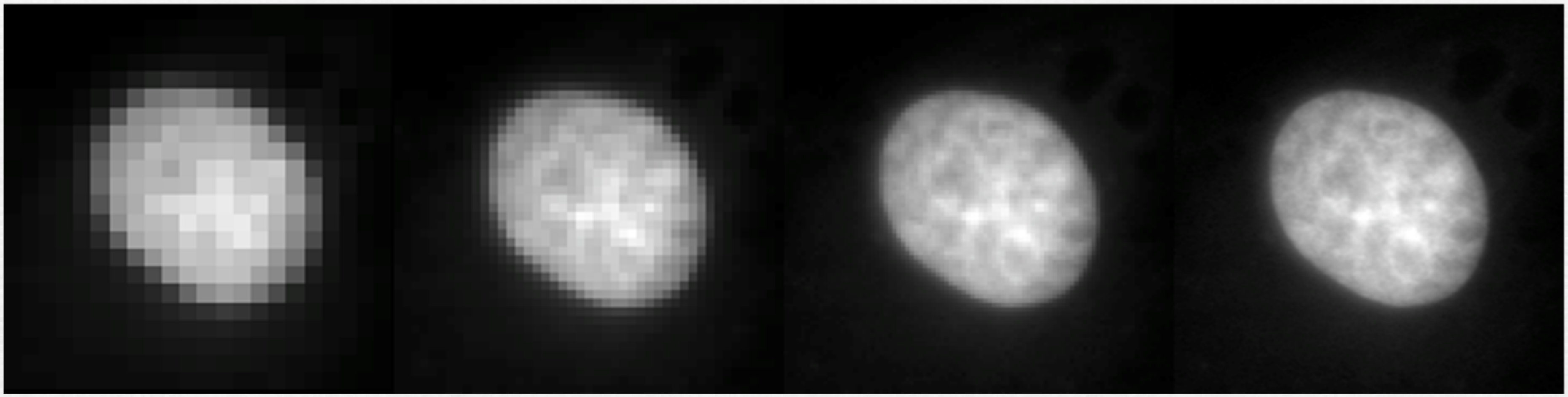
□ 6.45 µm on a side

Sony Interline Chip ICX285

Chip is 8.98 x 6.71 mm

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

Resolution and magnification



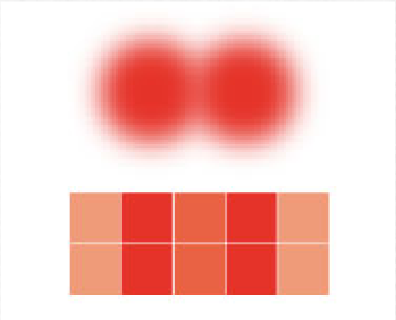
More pixels / resolution element

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

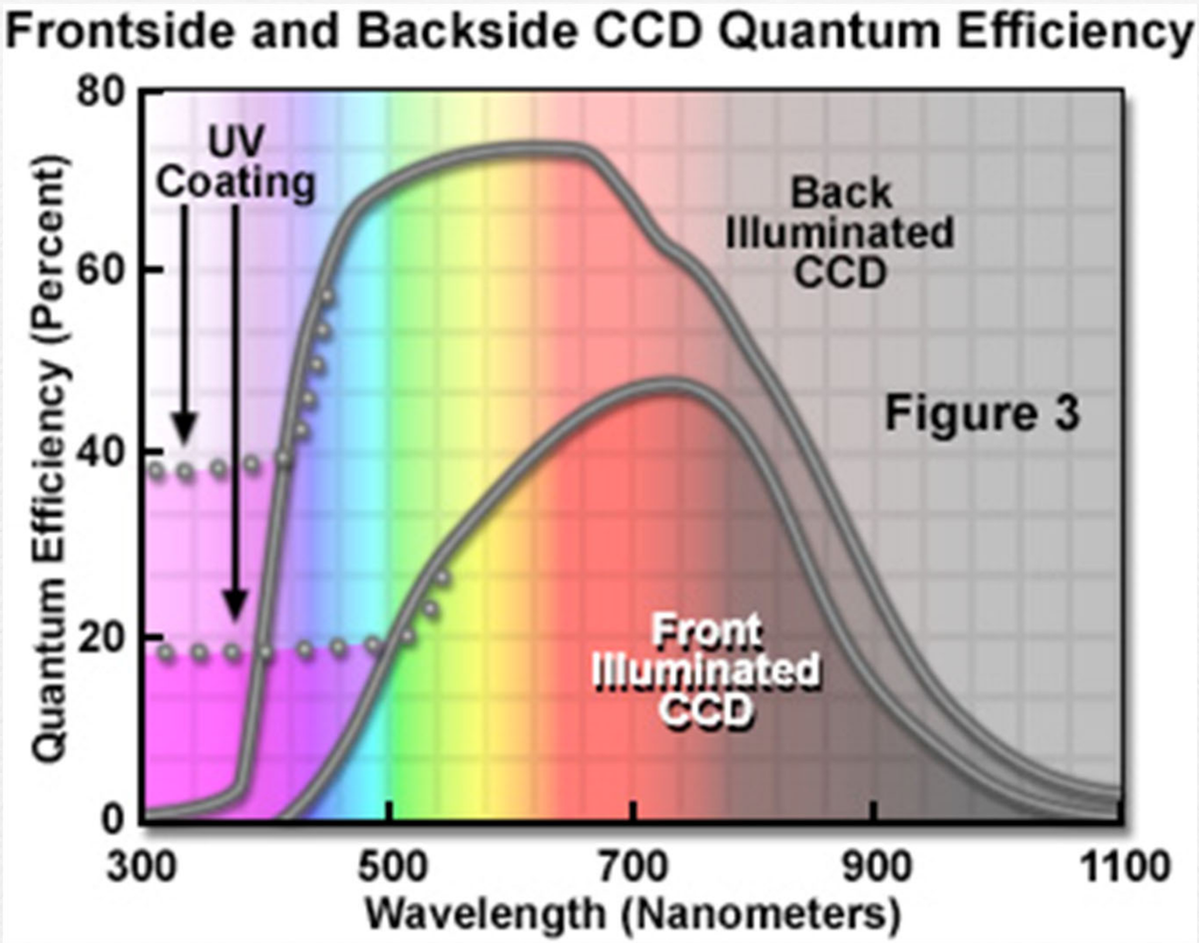


2.5 – 3 is preferable

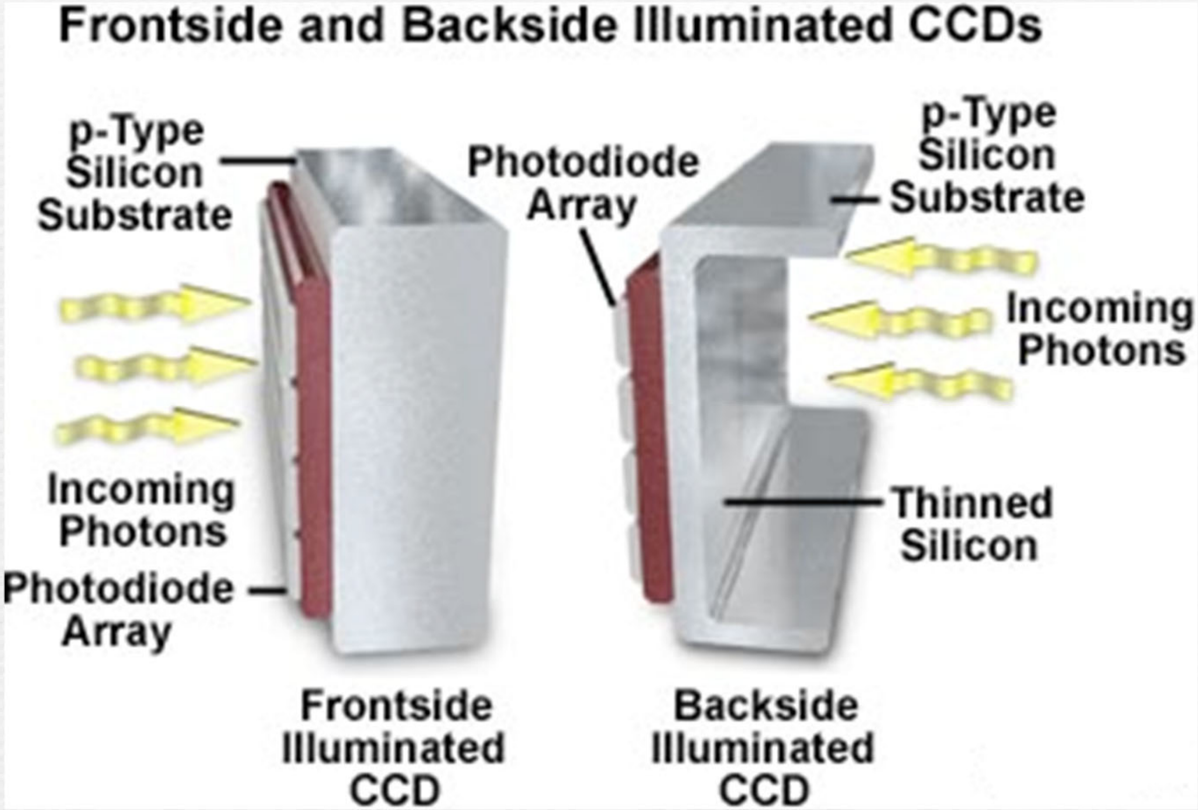
A resolution-centric view of imaging

- Resolution is a function of the objective NA and wavelength (e.g. 1.4 NA with 500 nm light -> ~ 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 µm 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

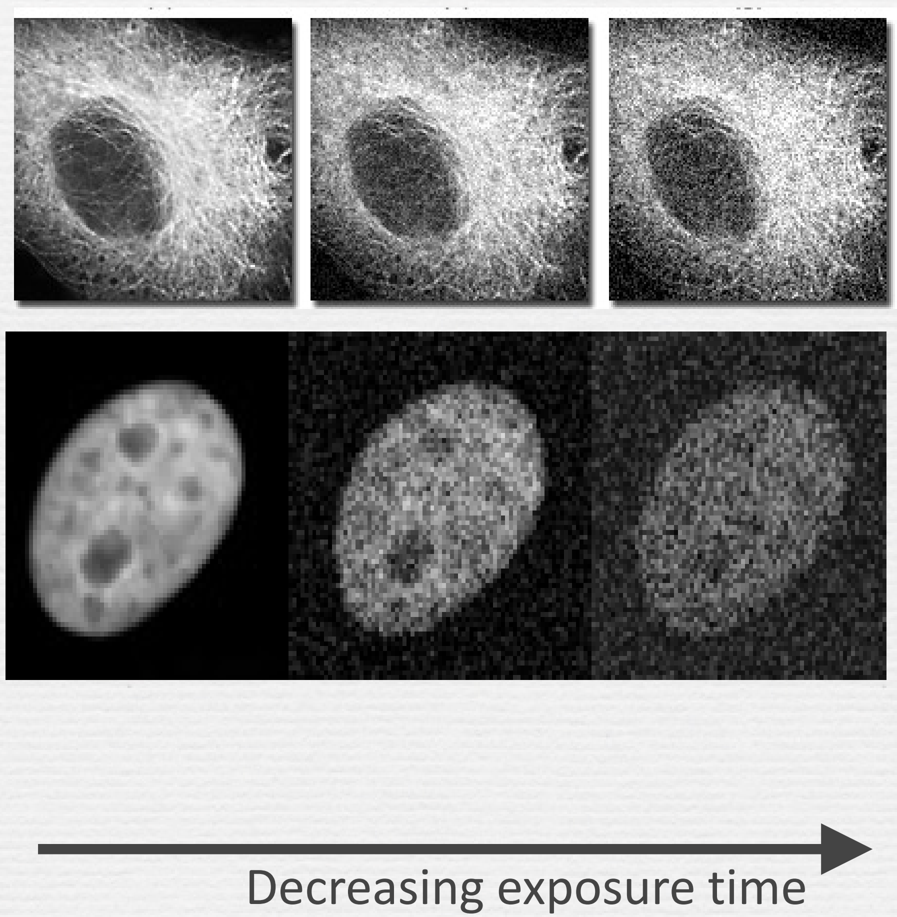


Back-thinning increases QE



Noise

- Longer exposure times are better – why?



Noise

- Photon Shot Noise: Due to the fact that photons are particles and collected in integer numbers. Unavoidable!
  - Scales with √ 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

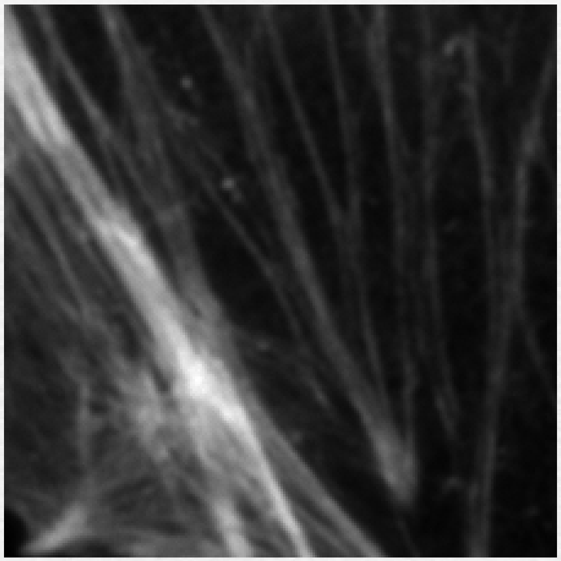
- Dark current – thermal accumulation of electrons
  - Cooling helps, so negligible for most applications

Signal/Noise Ratio (SNR)

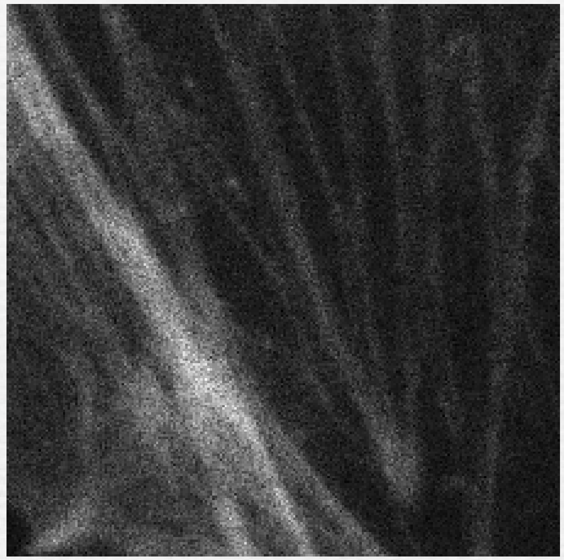
- Signal = # of photons = N
- Noise = $\sqrt{(\text{read noise}^2 + N)}$
- When # of photons << read noise^2 -> Read noise dominates
- When # of photons >> read noise^2 -> Shot noise dominates
- When shot noise dominates (Signal/Noise = $N/\sqrt{N}$), to double your SNR, you need to acquire four times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

Often, read-noise dominates

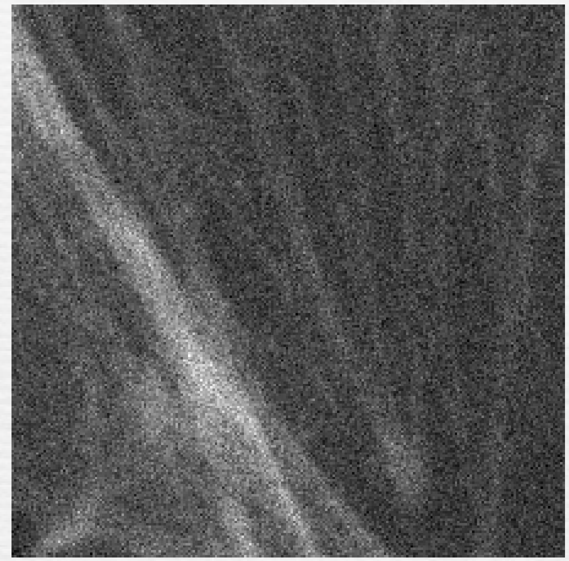
10 photons / pixel on average; ~50 in brightest areas



Test image



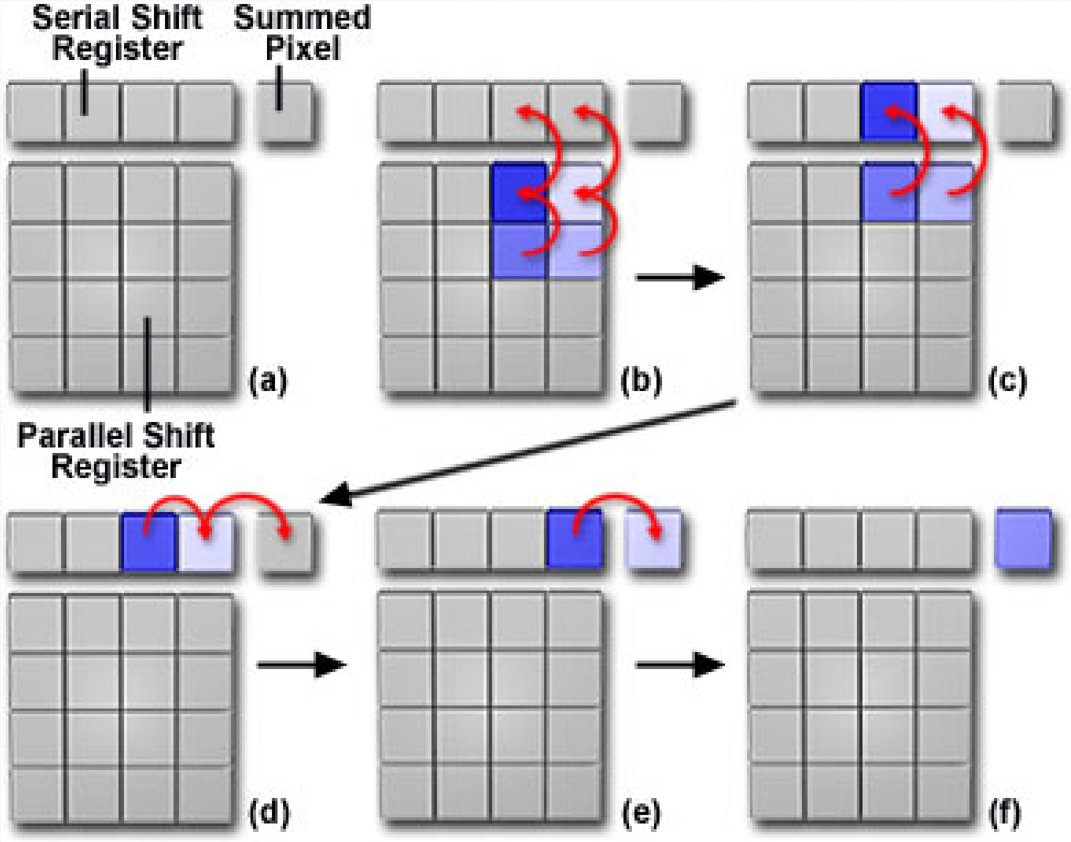
no read noise



5 e^- read noise

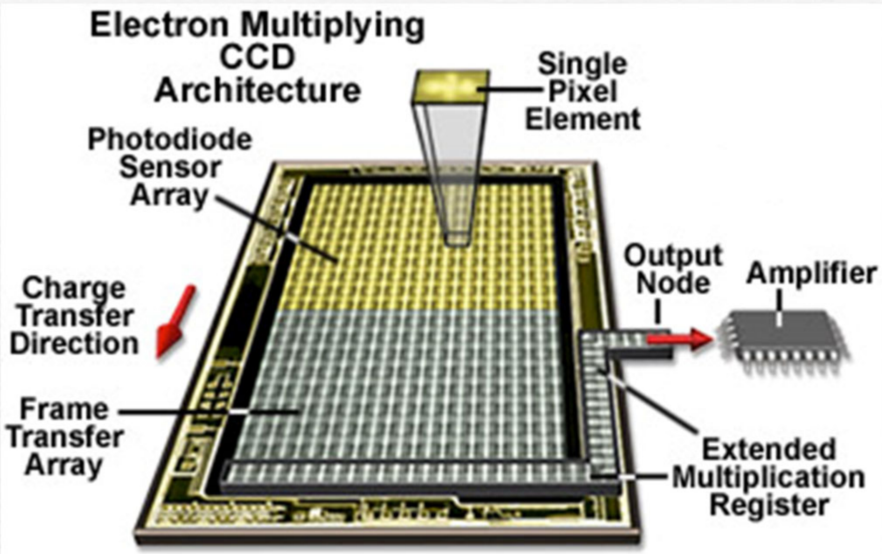
Photon shot noise ~ 3/5 read noise

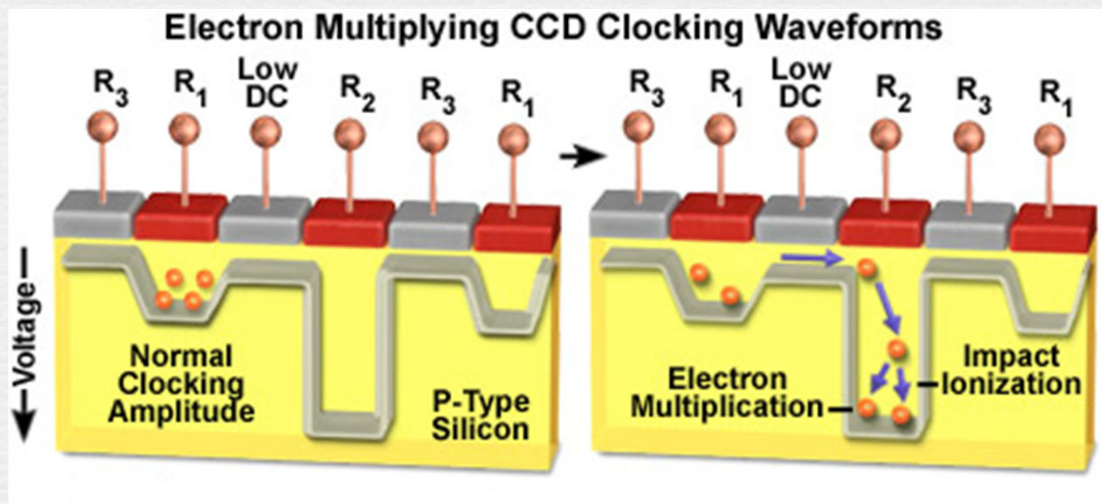
Binning



- 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





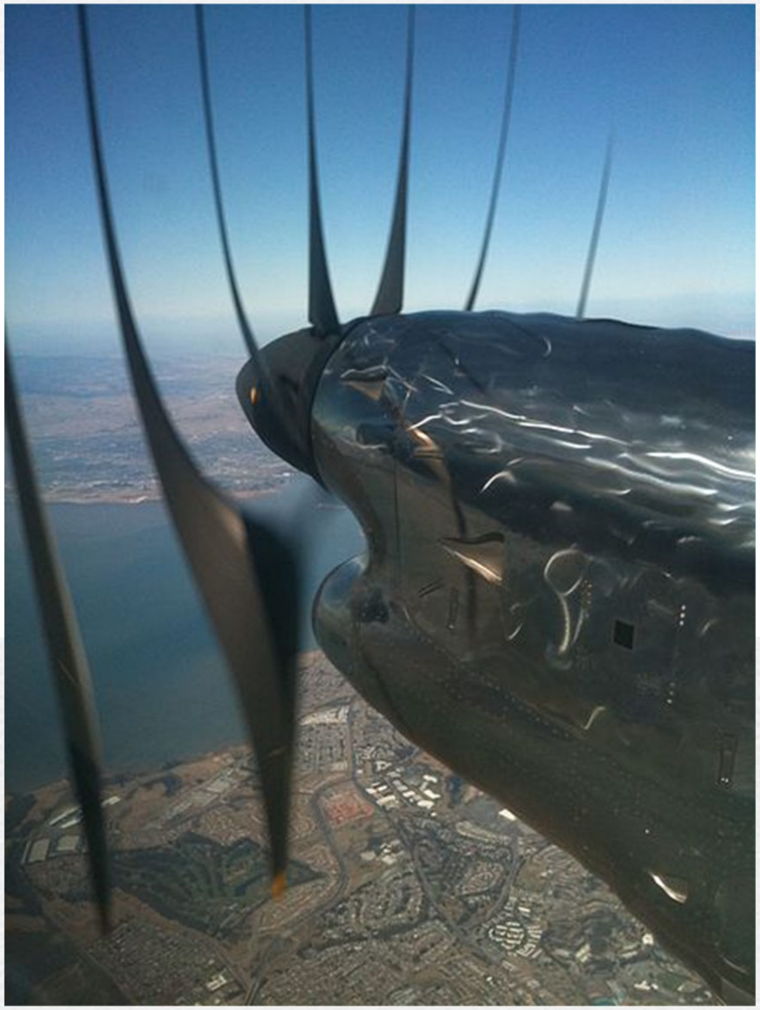
EMCCD result

- Fast noisy CCD – runs at 30 fps, but 50 e^- read noise
- Multiply signal by 100-fold – now read noise looks like 0.5 e^-
- Downside – multiplication process adds additional Poisson noise (looks like QE is halved)
- 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



The picture can't be displayed.

Dynamic Range: How many intensity levels can you distinguish?

- Full well capacity (16 000 e^-)
- Readout noise: 5e-
- Dynamic range:
  - FWC/readout noise: 3200
  - 0.9 * FWC / (3 * readout noise) = 960
- (Human eye ~ 100)

Bitdepth

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

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

$N = c \cdot DN$

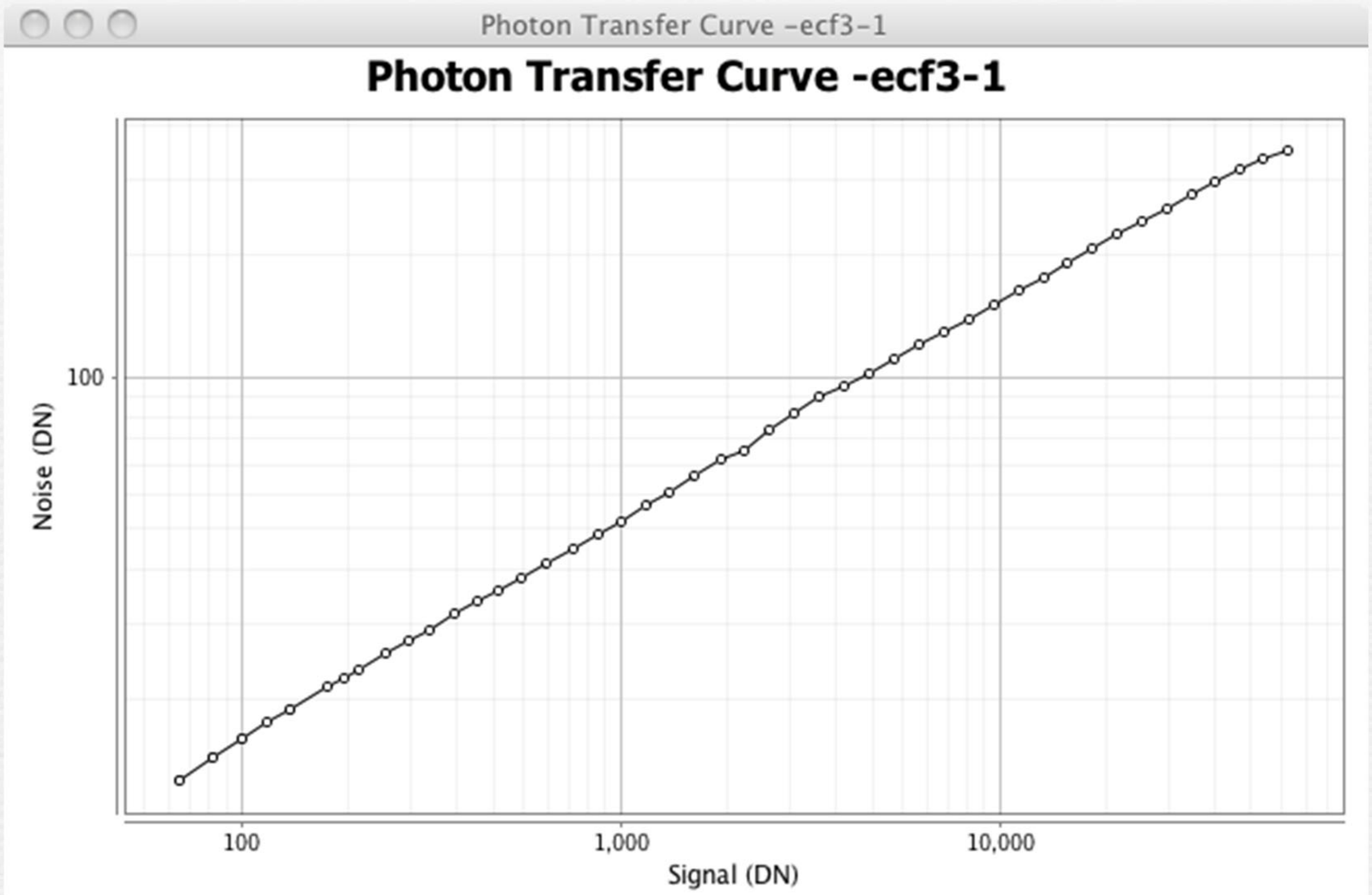
$\sigma(N) = c \cdot \sigma(DN)$

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

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 -> λ. SPIE Press, 2007

http://valelab.ucsf.edu/~MM/MMwiki/index.php/Measuring_camera_specifications

Credits and resources:

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