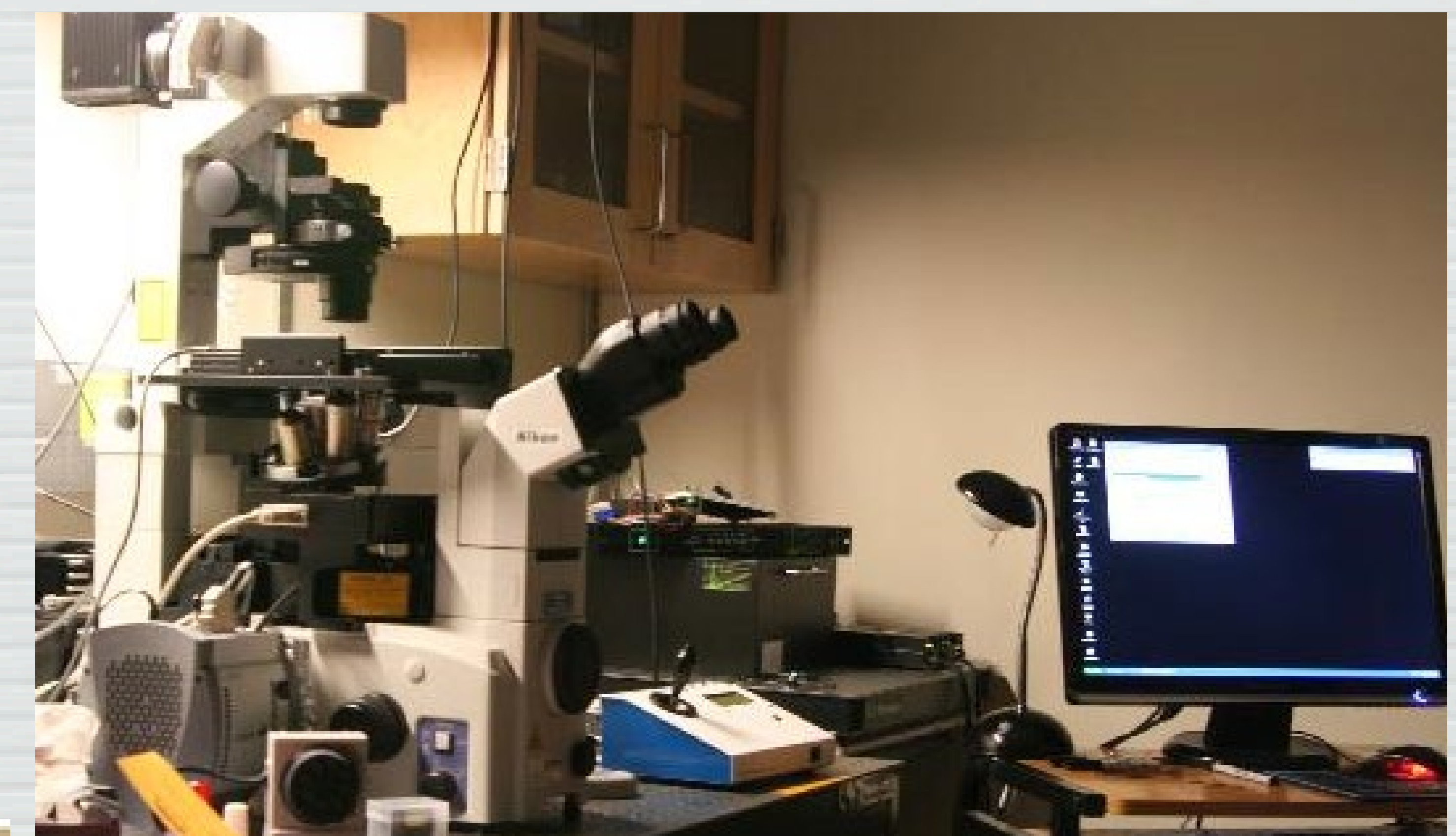
Digital microscopy: Image detectors and software control

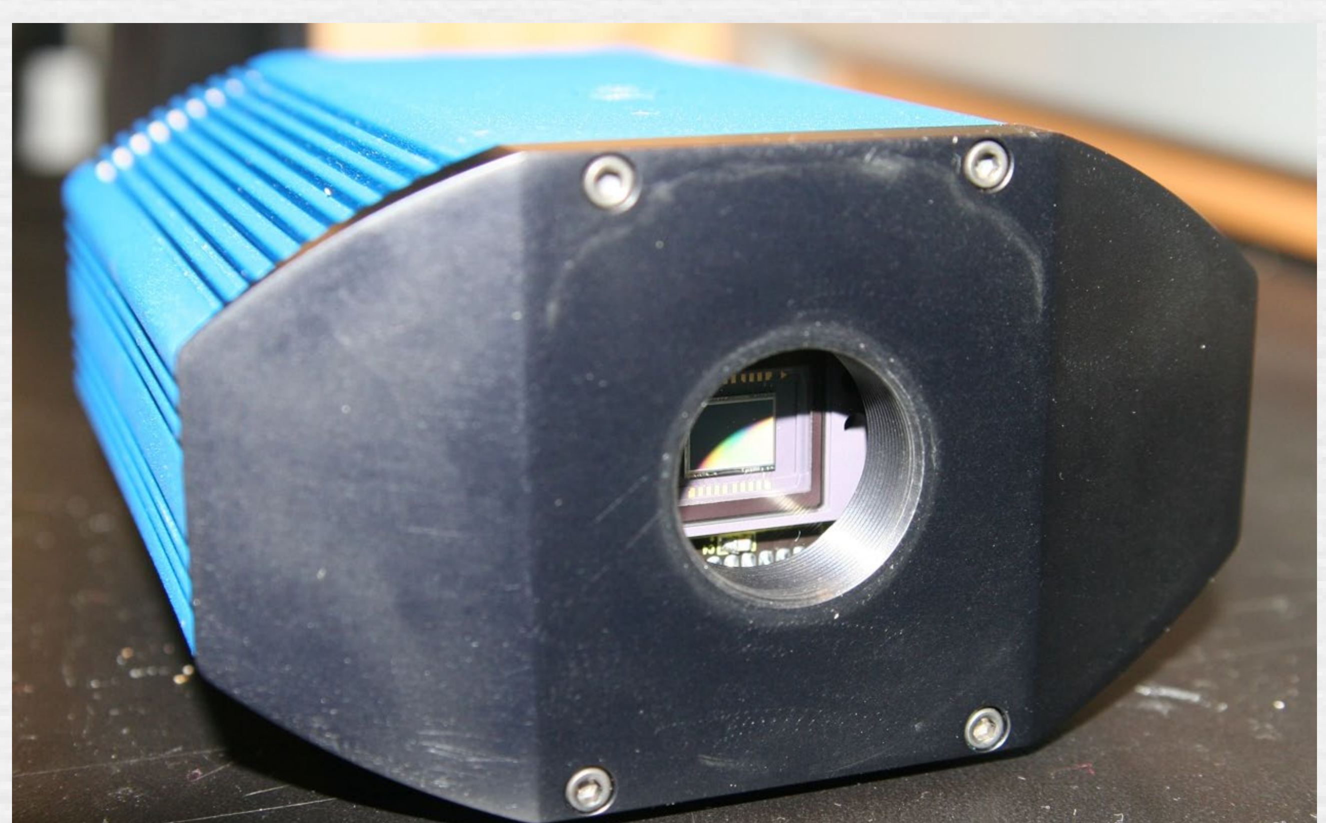


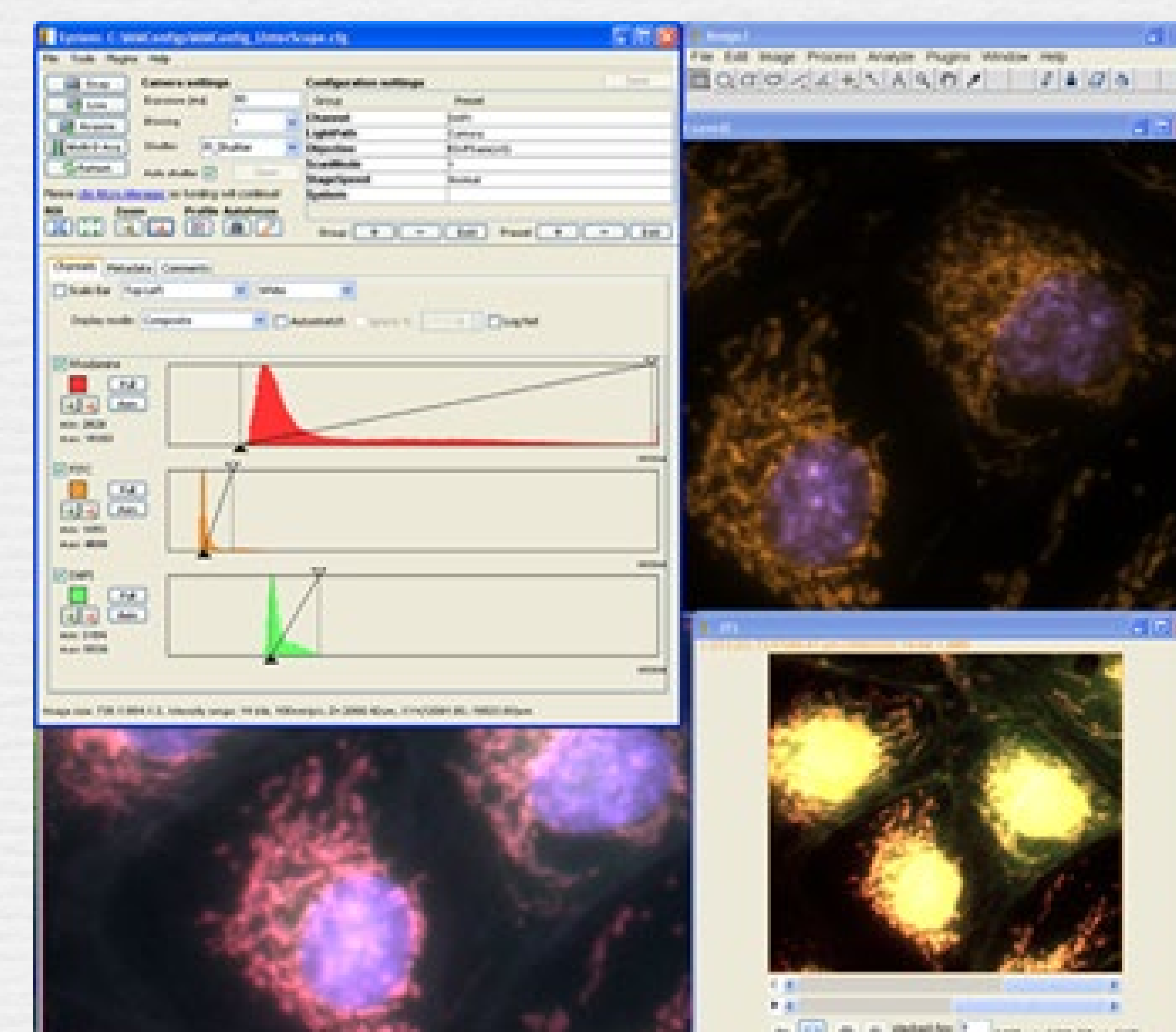
Nico Stuurman, UCSF/HHMI

UCSF, Mar 27, 2012

Outline

- Detectors:
  - ‘Single point’ detectors
  - ‘Multiple point’ detector (cameras)
- Software control of image acquisition

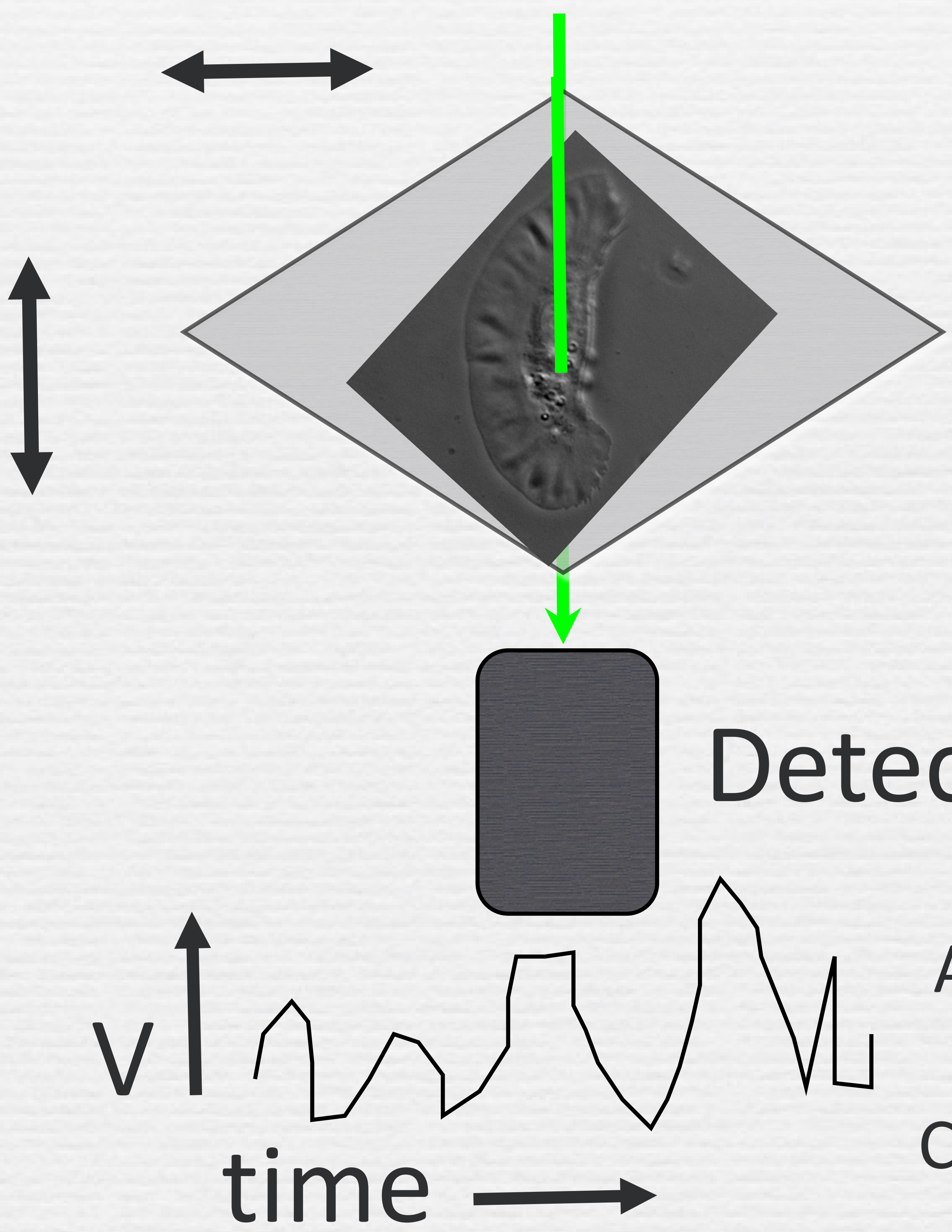




Imaging Detectors

Photon -> Electrons -> Voltage ->Digital Number

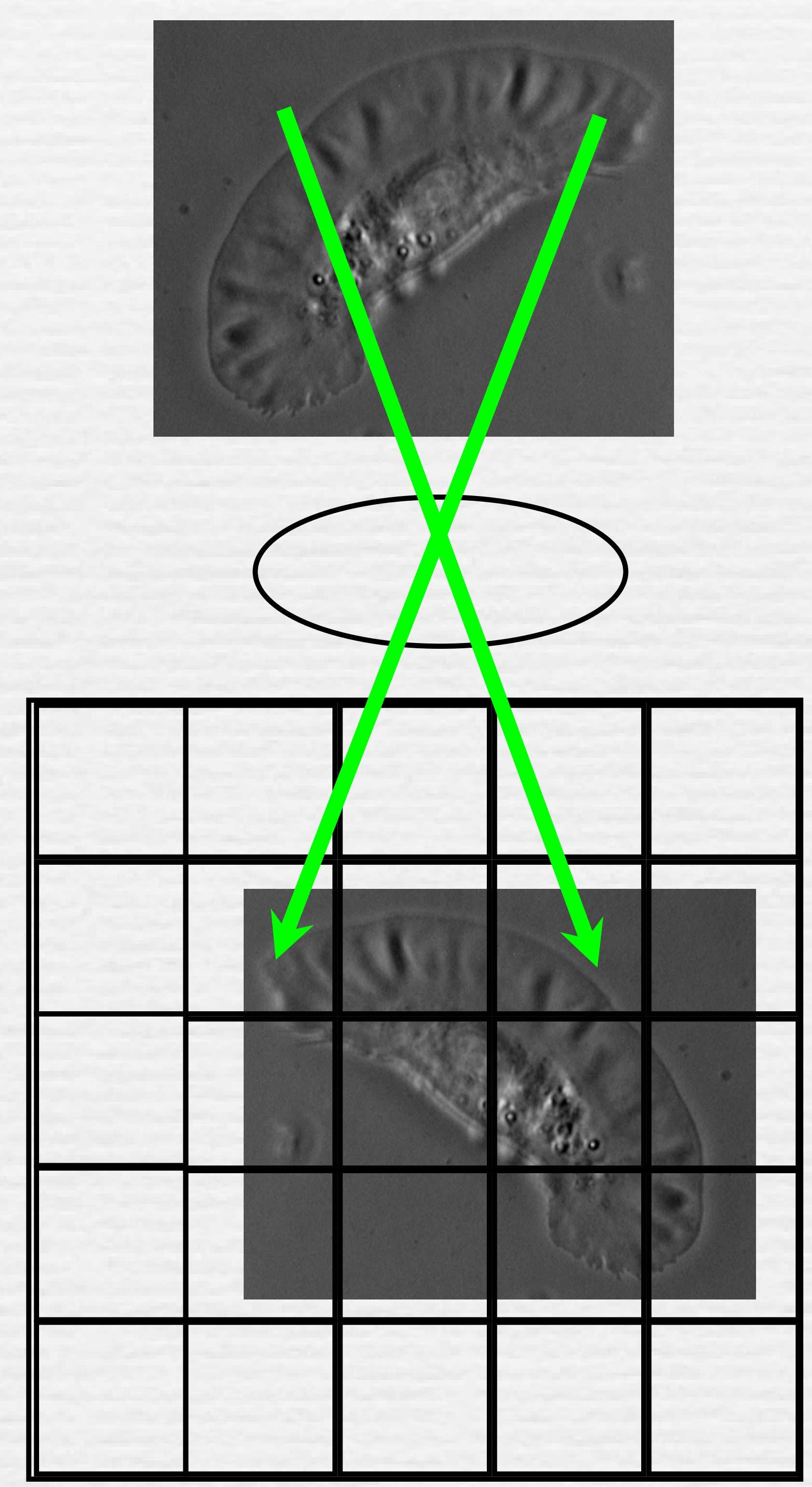
Single point detector



10 200 35 12 90 85 105 73 80 95

Speed!

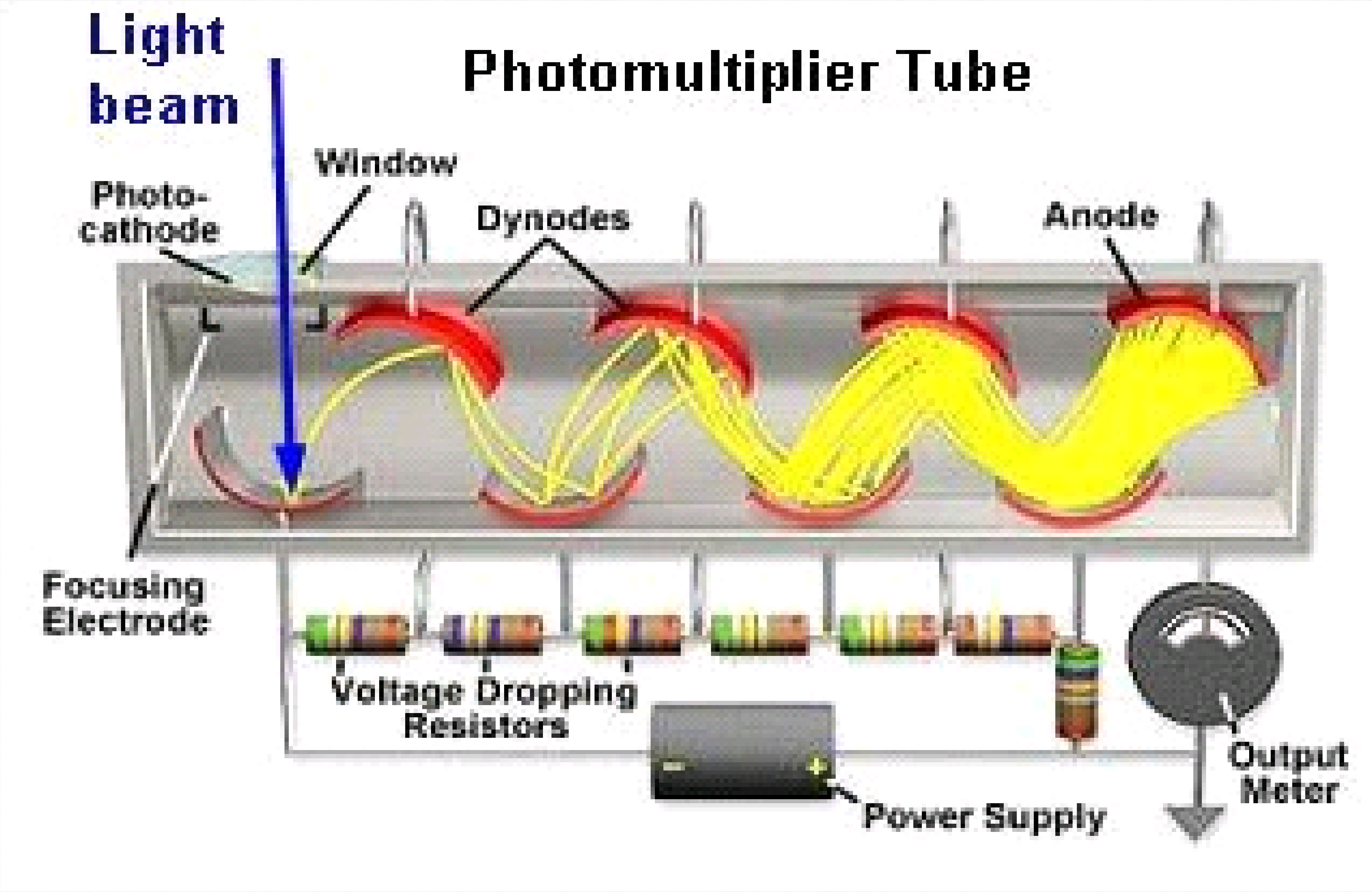
Multi point detector (camera)



A->V->ADC

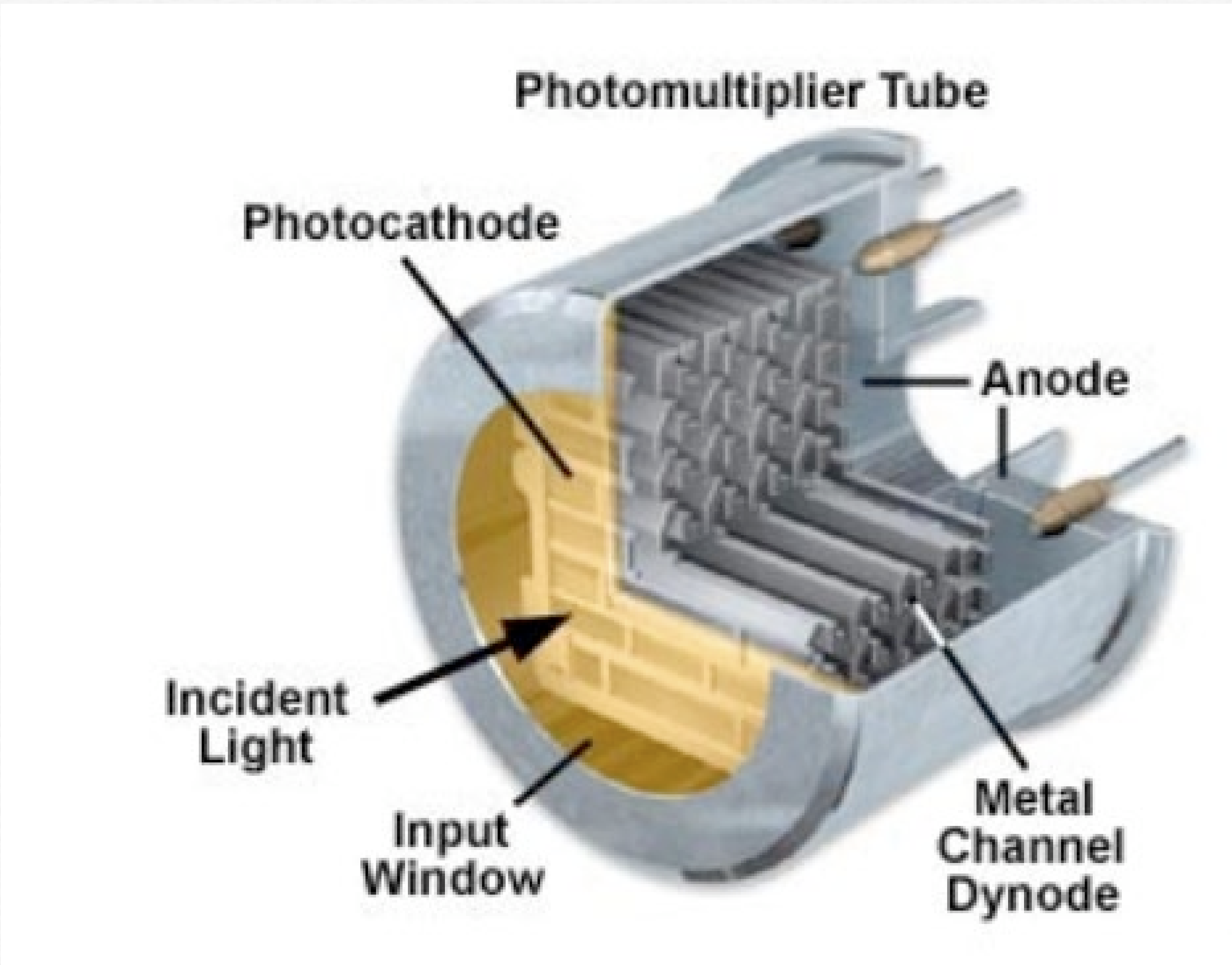
10 200 35 12 90 85 105 73 80 95

Photo-Multiplier Tube (PMT)



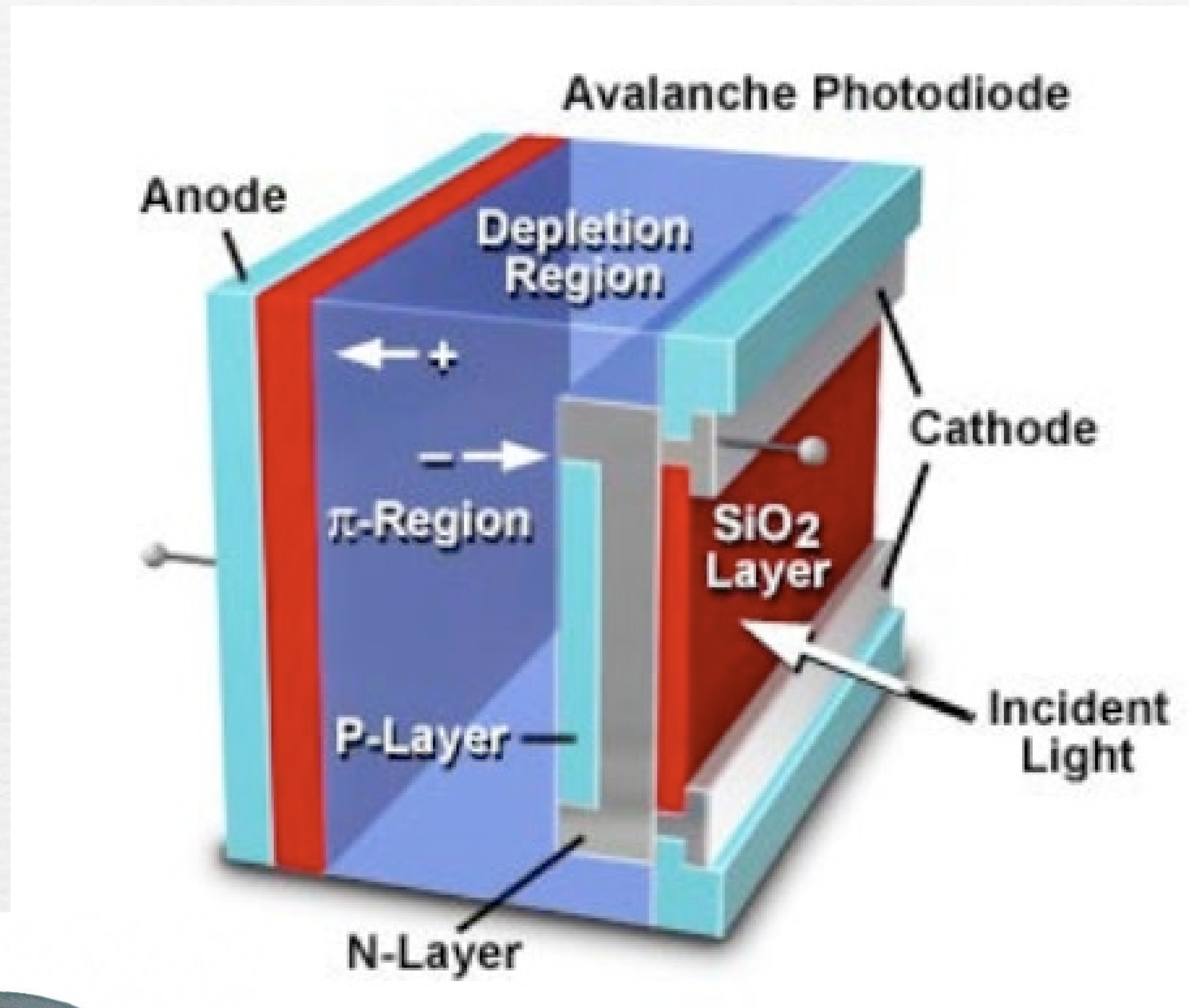
- Very linear
- Very High Gain
- Fast response
- Poor Quantum efficiency (~25%)

PMT modes



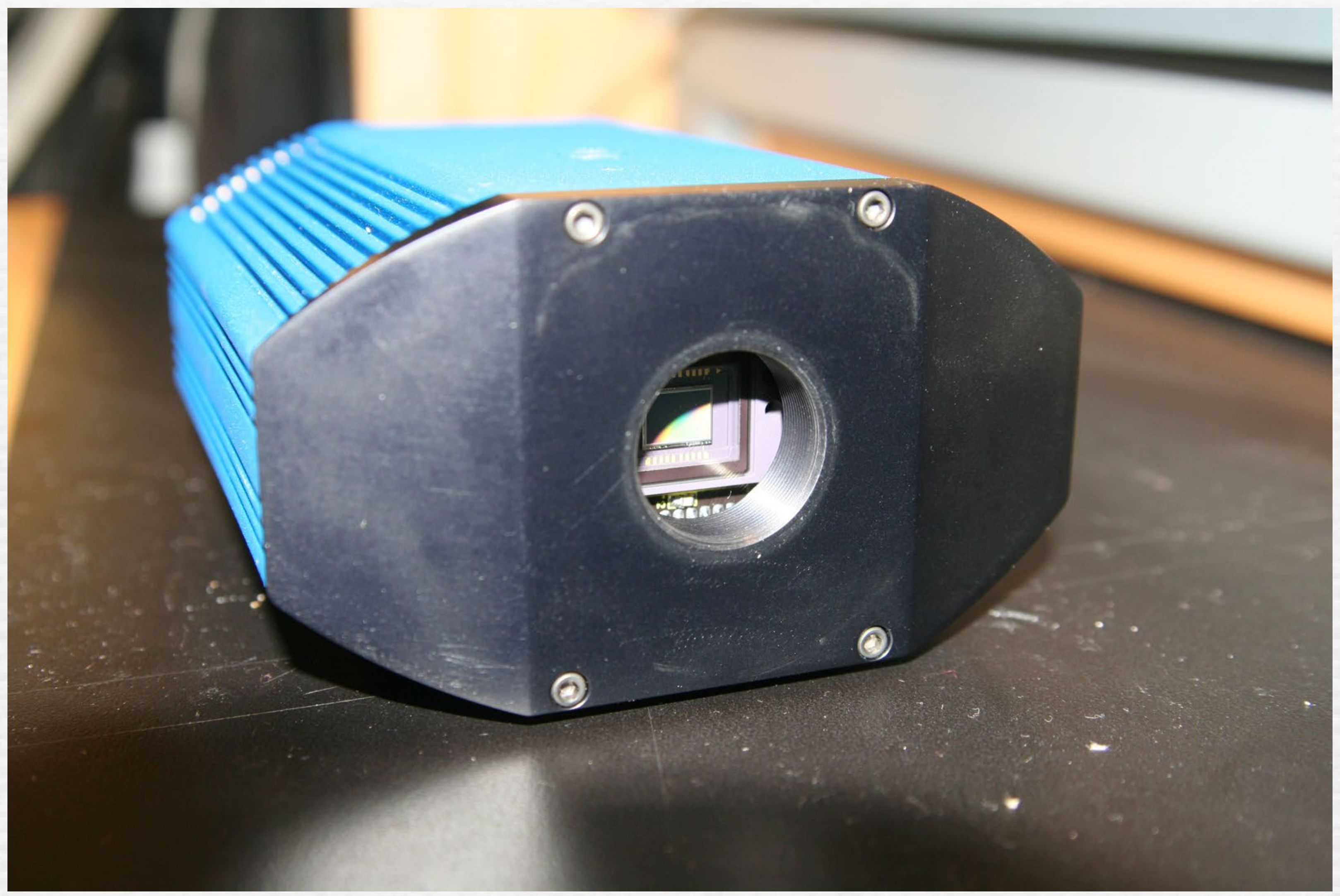
- Photon counting mode:
  - Count pulses
  - Zero background
  - Slow
- Linear mode
- Measure current
- Fast but noisy

Avalanche Photo Diode

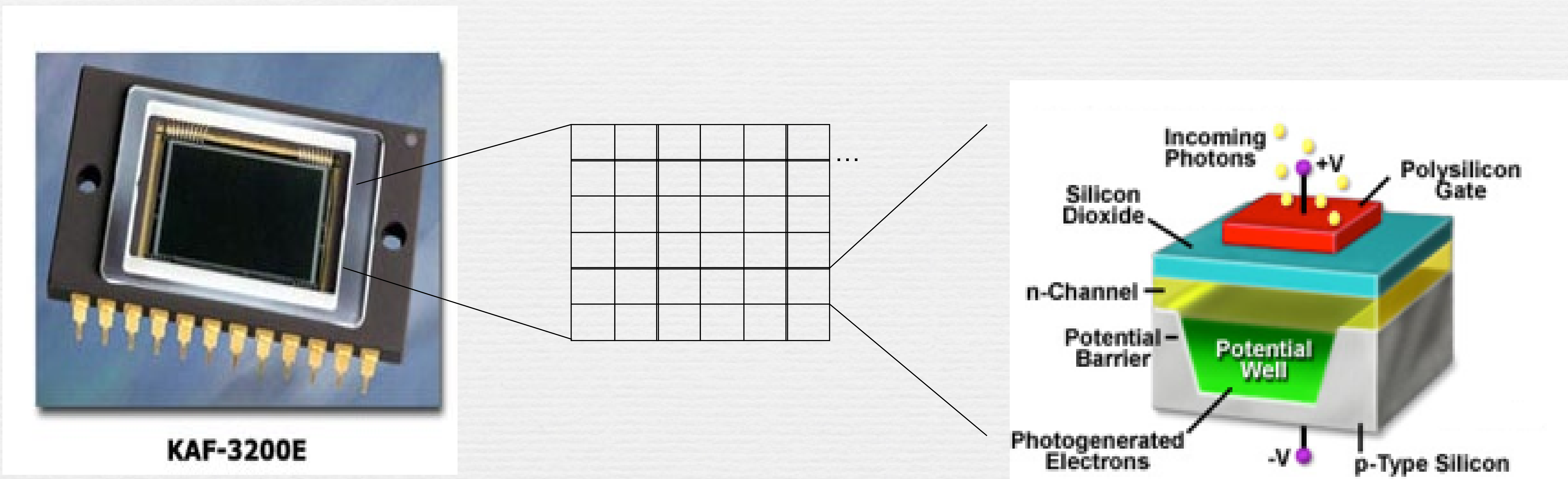


- Absorbed photons->electron
- Electrons amplified by high voltage and ‘impact ionization’
- High QE (~90%)
- Photon-counting ability (different design)
- Overheats if run too fast

Cameras in Microscopy

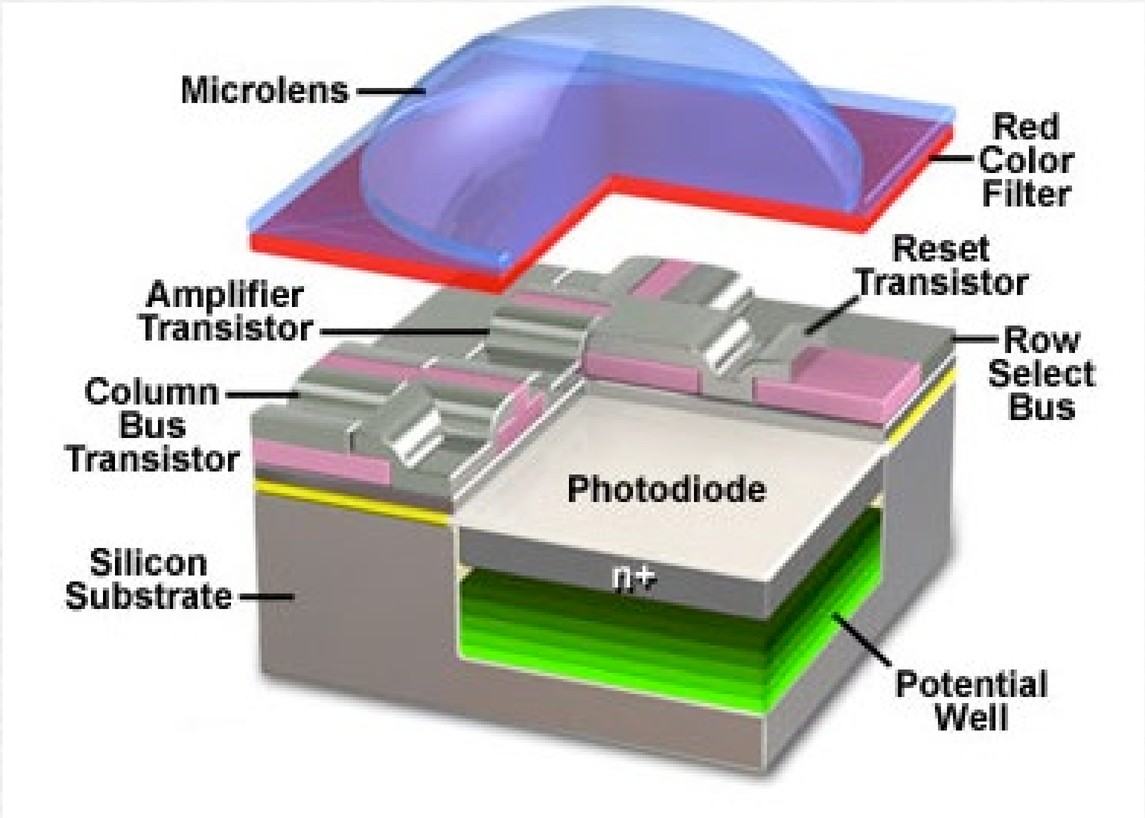


Arrays of photo-sensitive elements



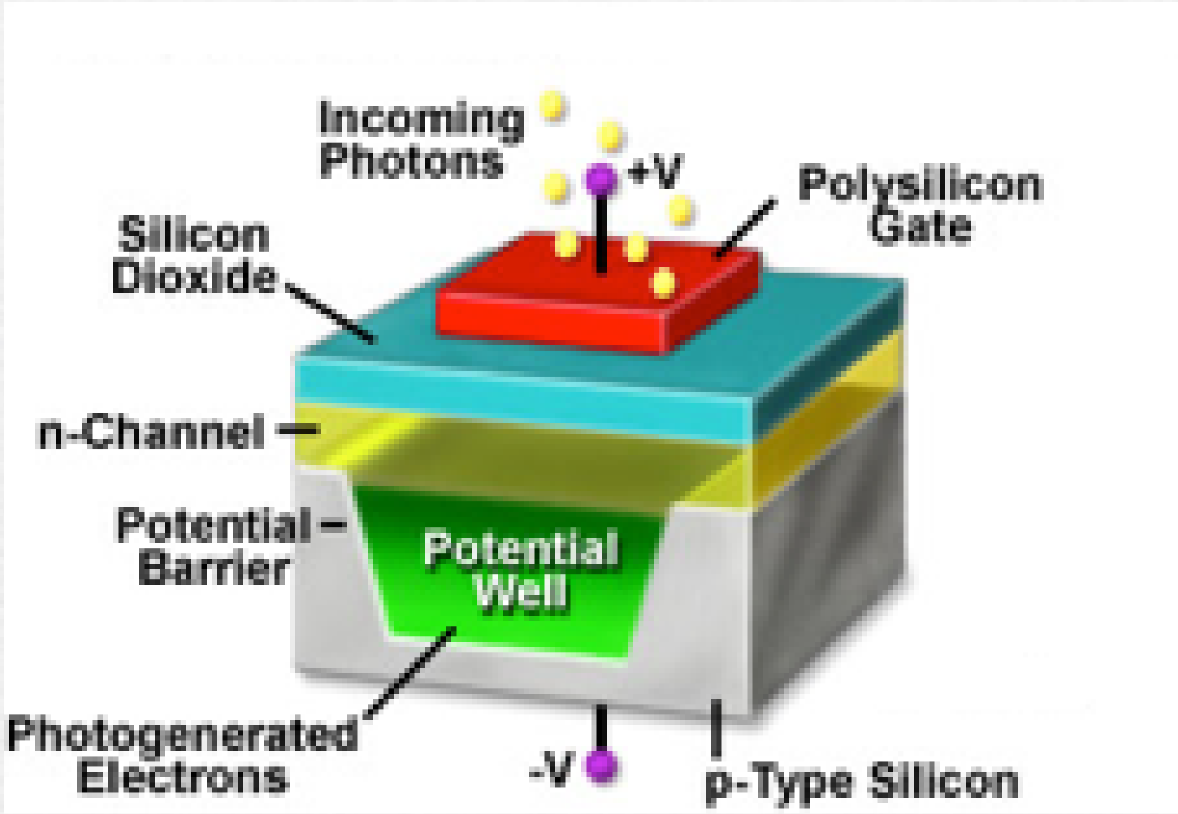
Two Architectures:

Complementary Metal Oxide Semiconductor (CMOS)



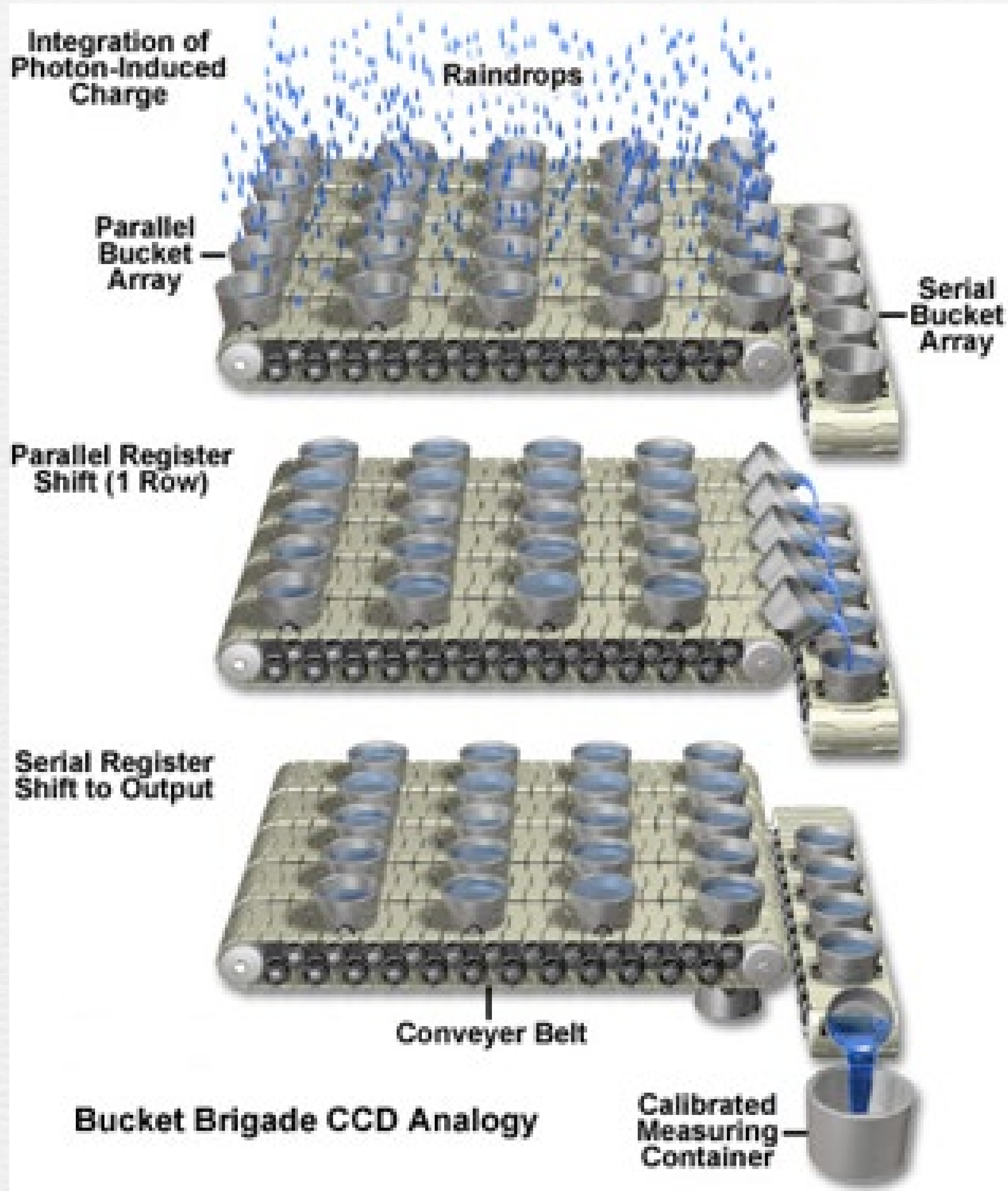
Each pixel has an amplifier
Transfers voltage
Fast
Noisy

Charged Coupled Devices (CCD)

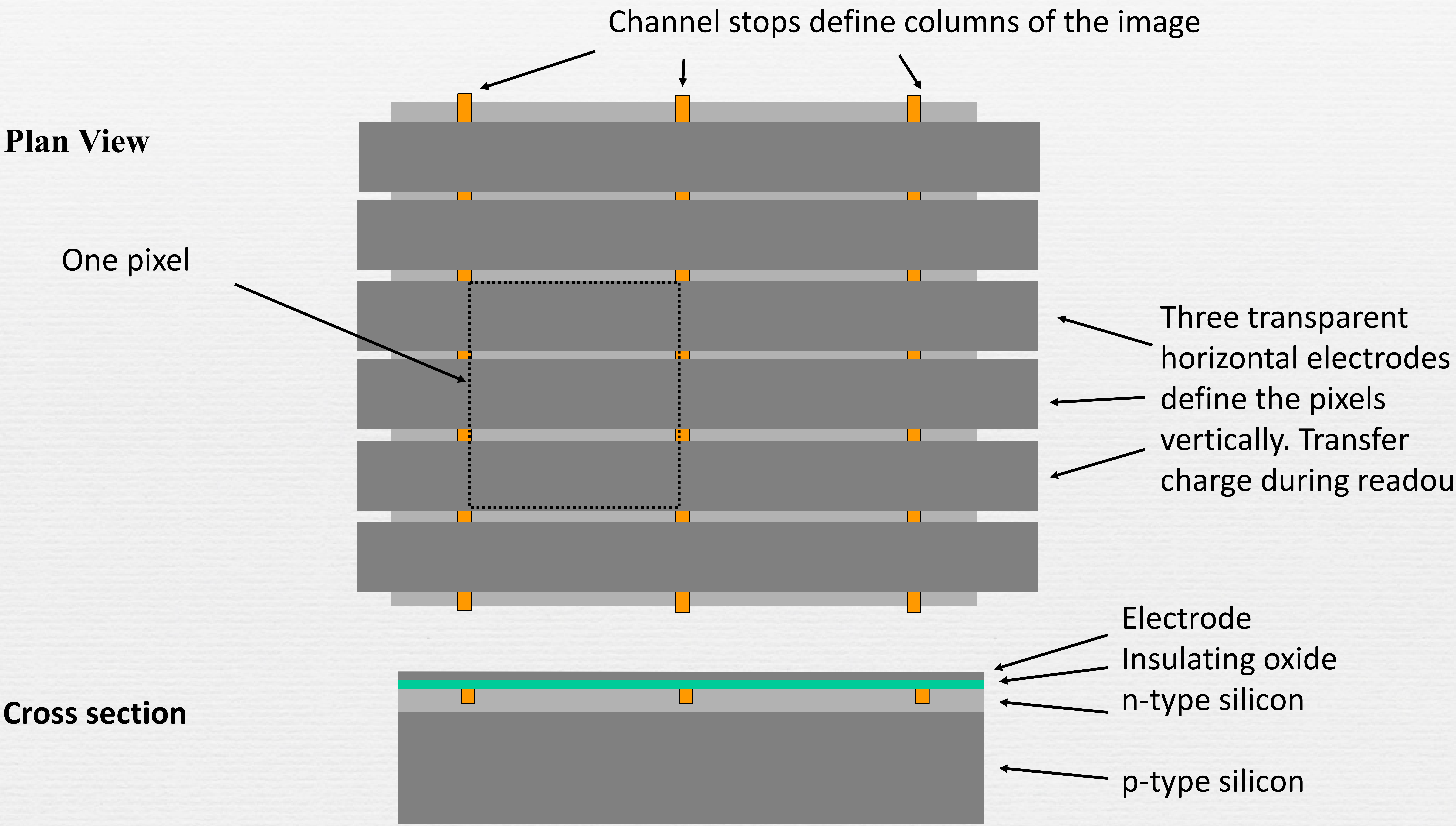


Single read-out amplifier
Transfers charge
Slow
Precise

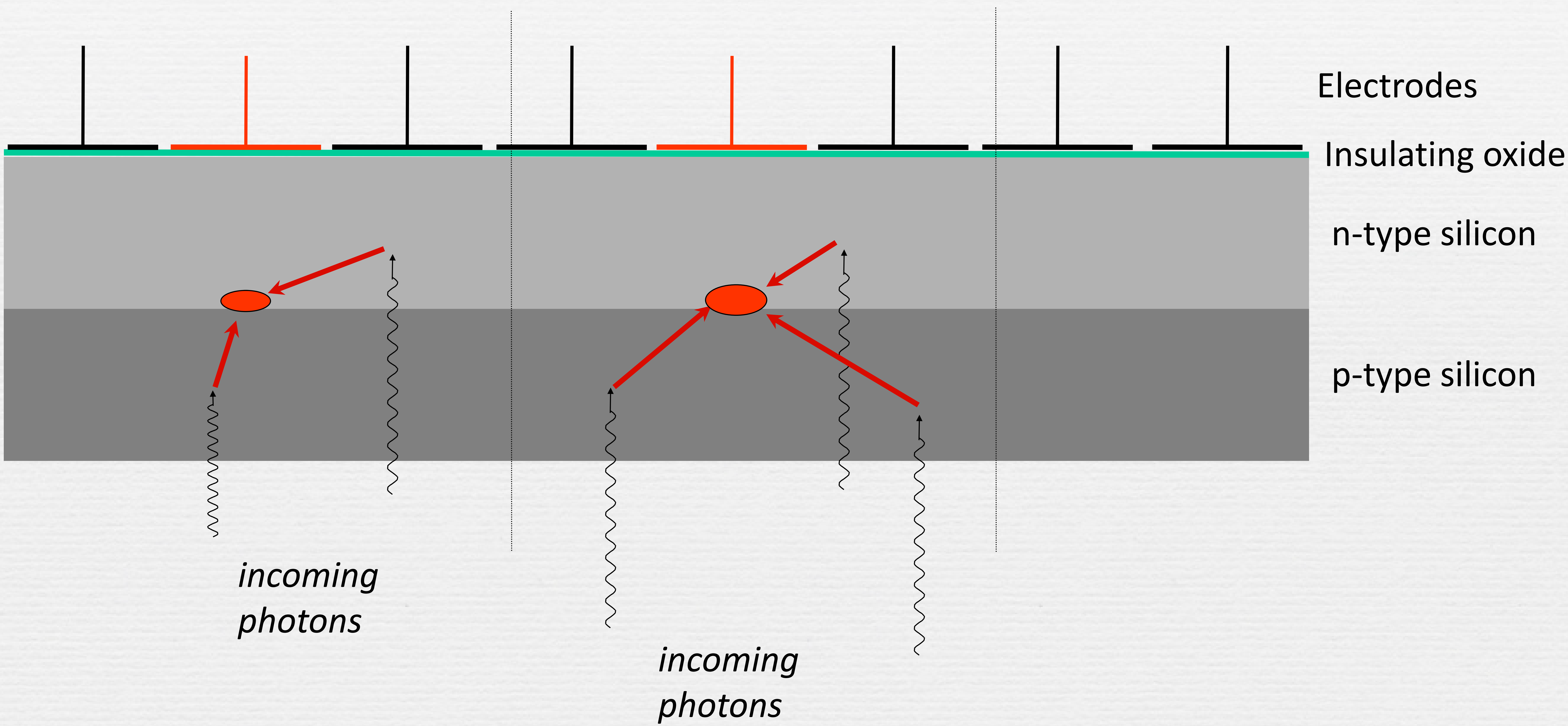
CCD readout “bucket-brigade” analogy



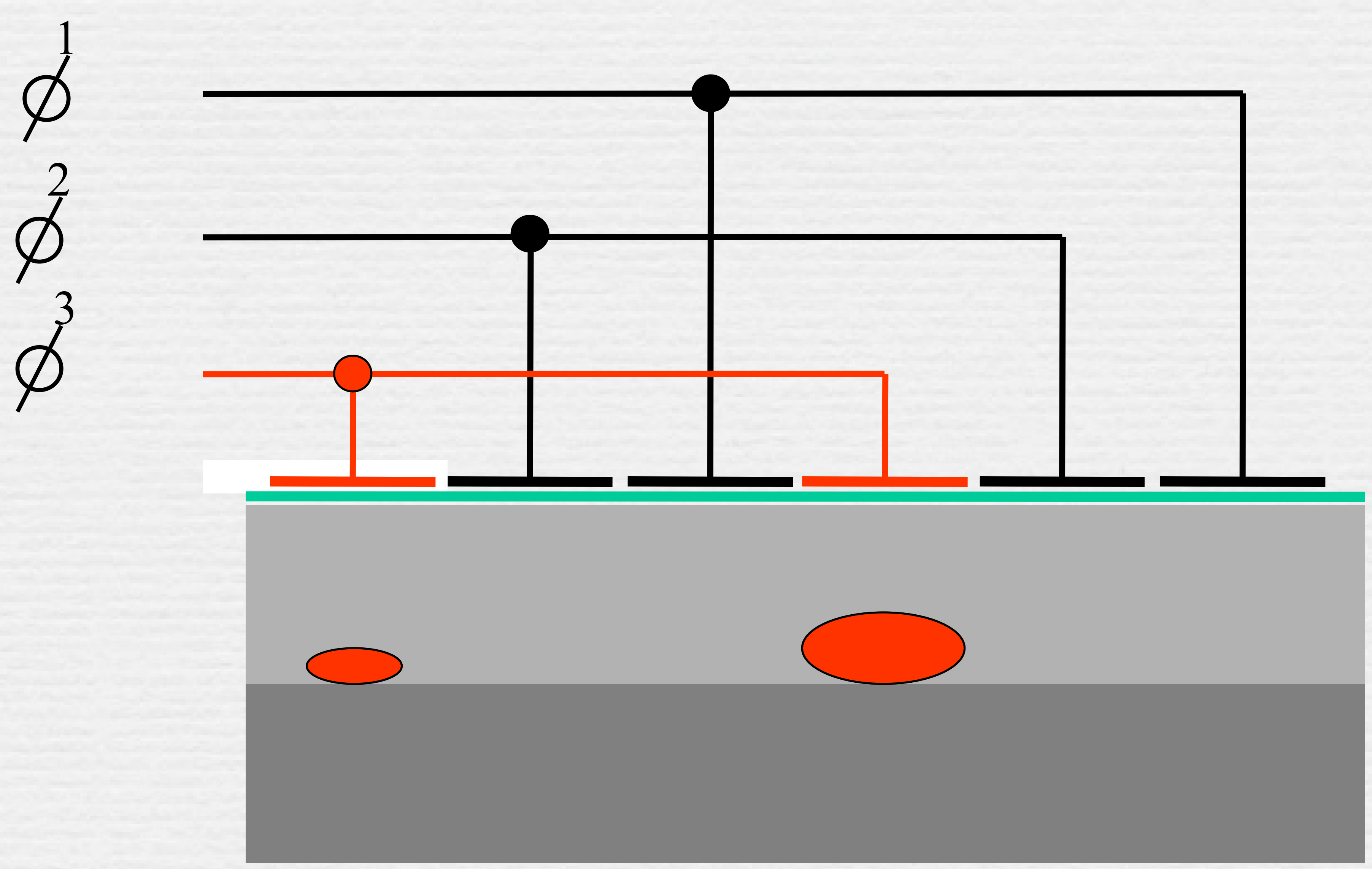
CCD Architecture

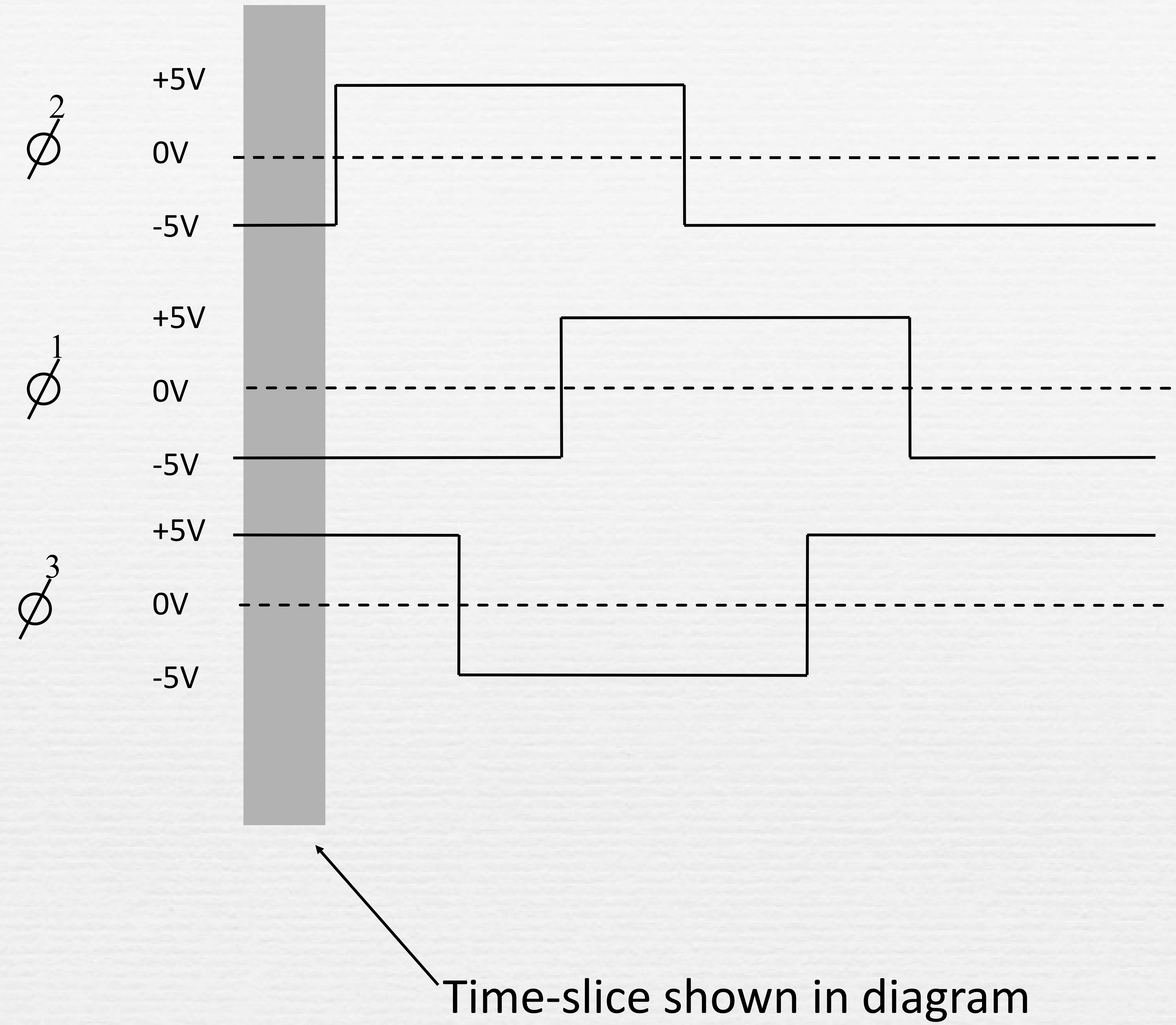


CCD Architecture

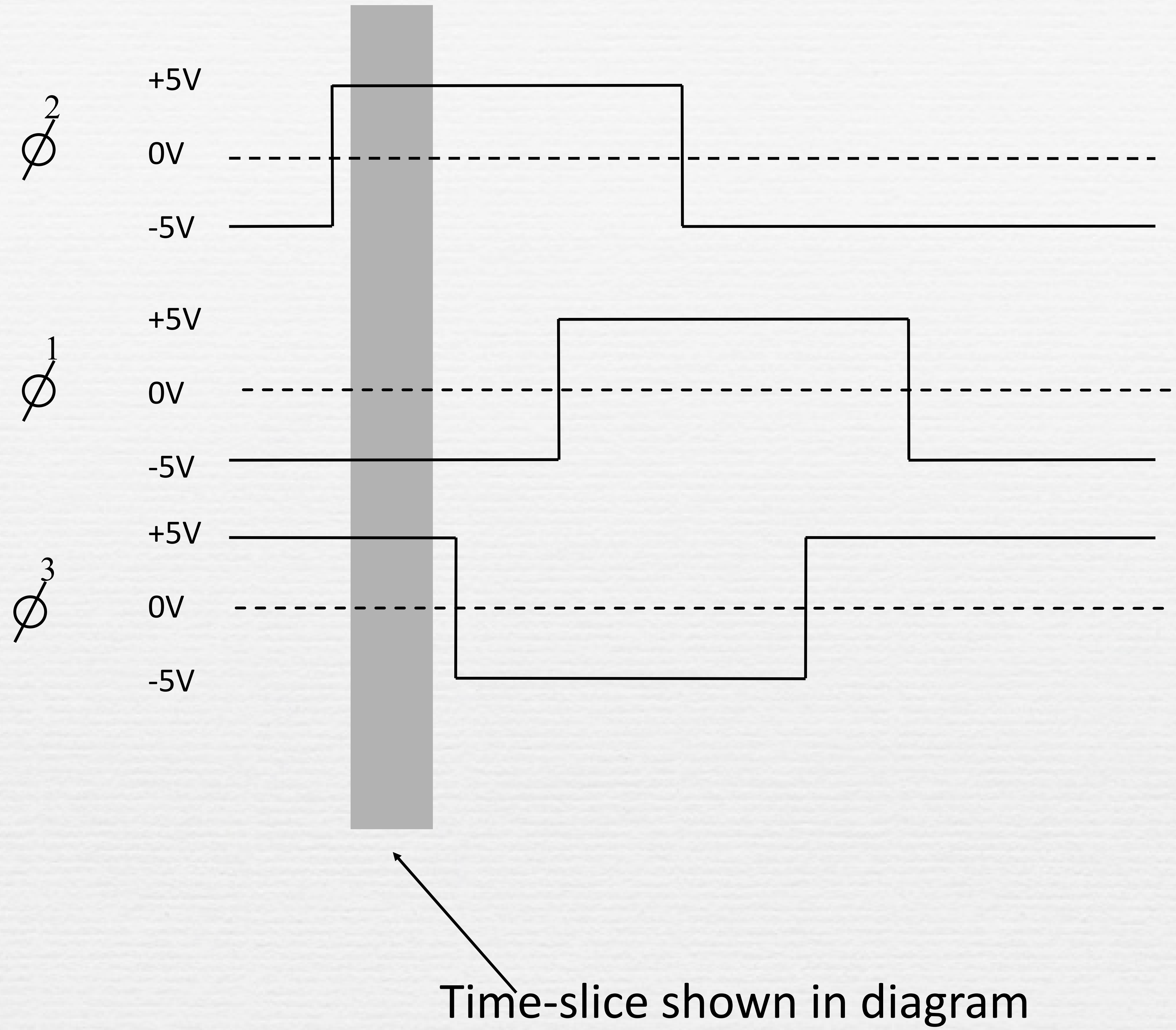


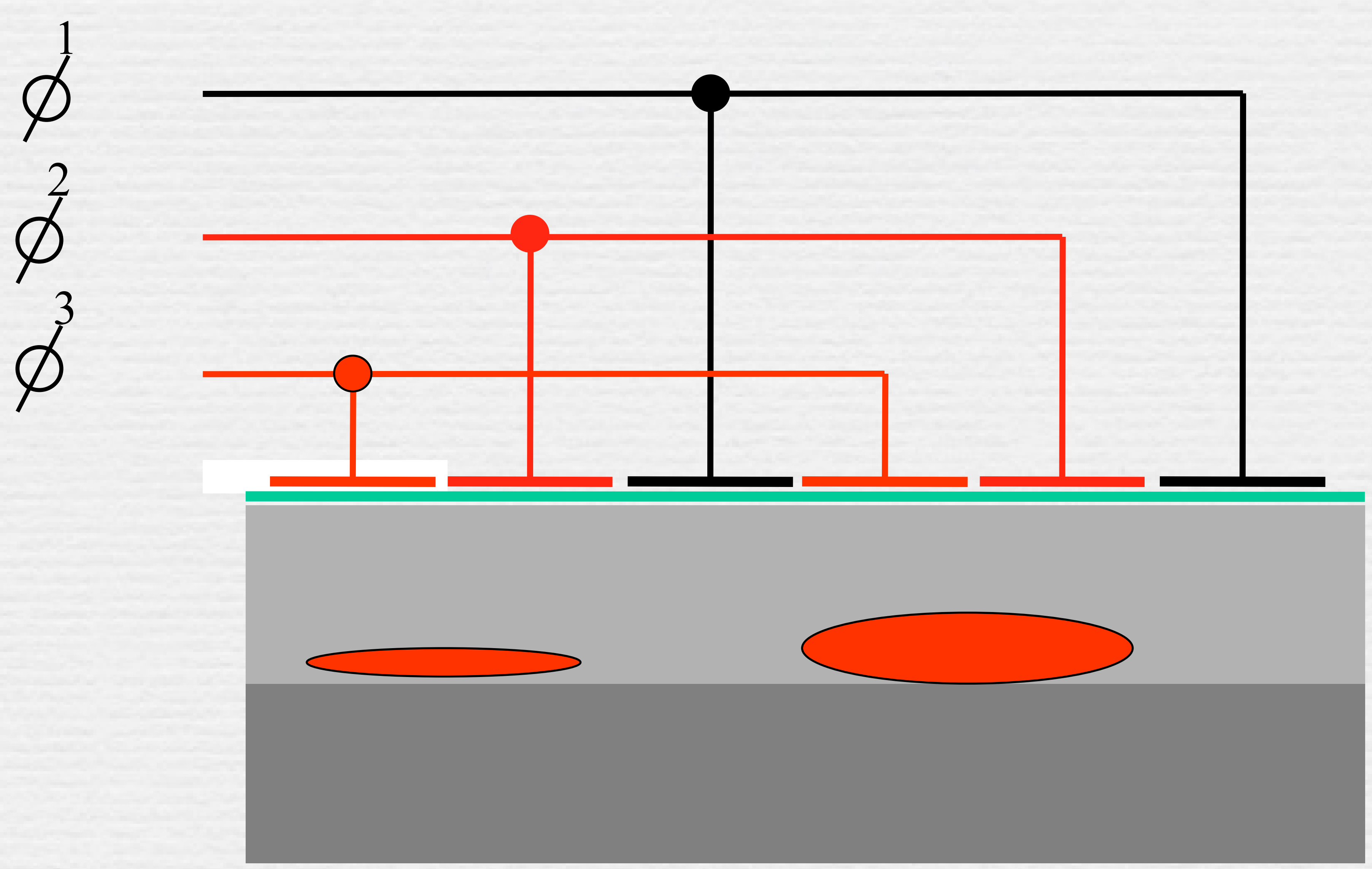
Charge Transfer in a CCD



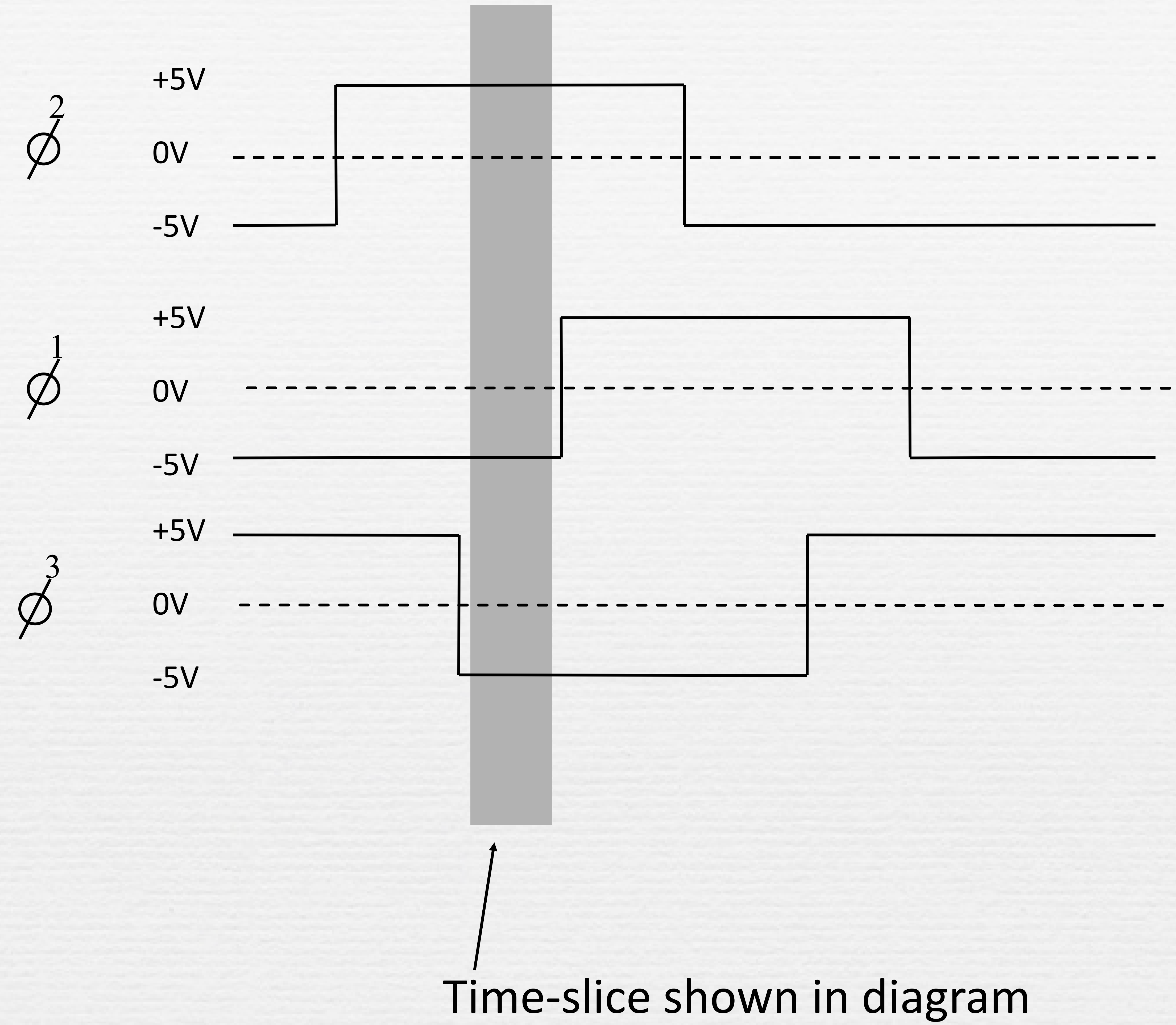


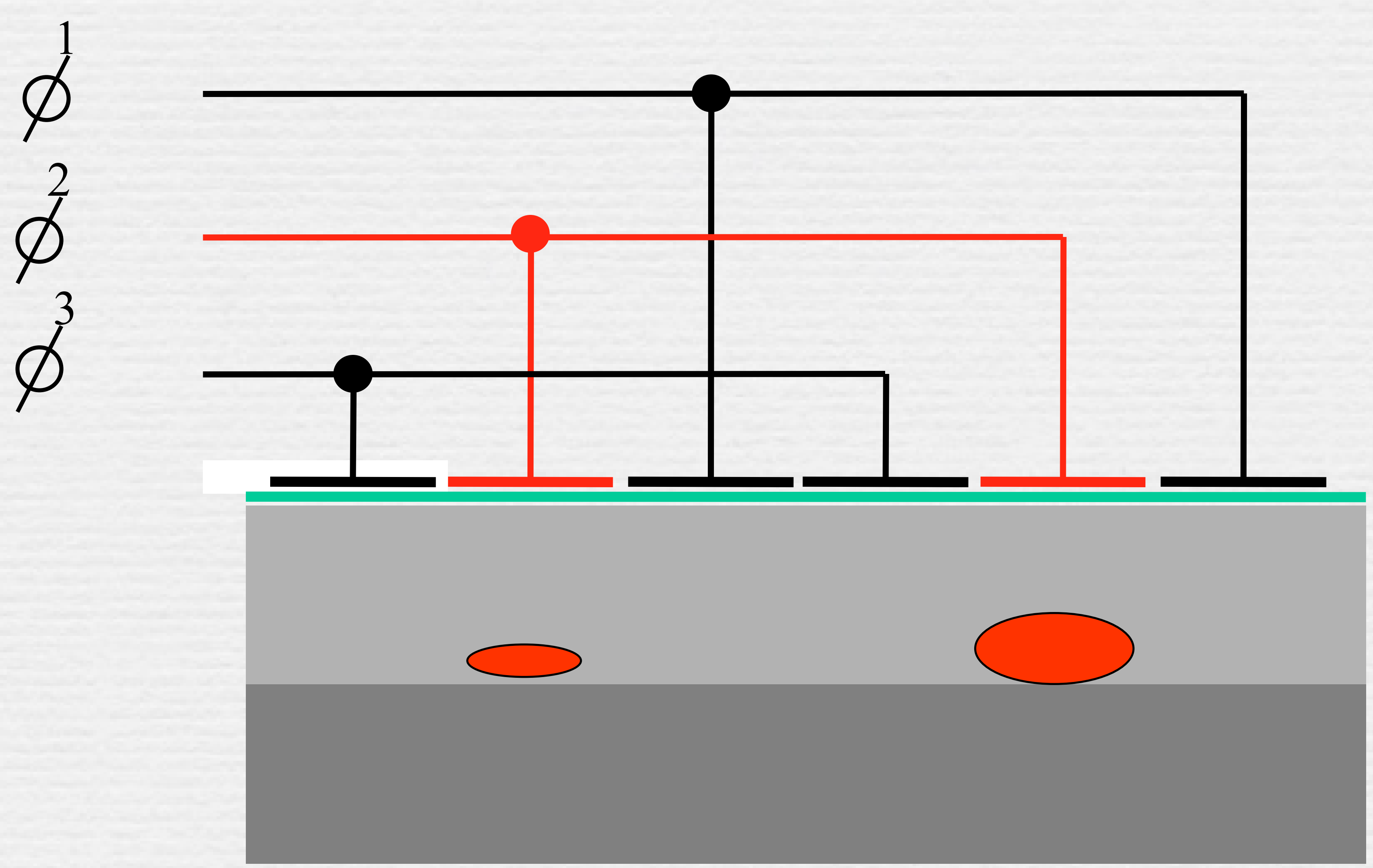
Charge Transfer in a CCD



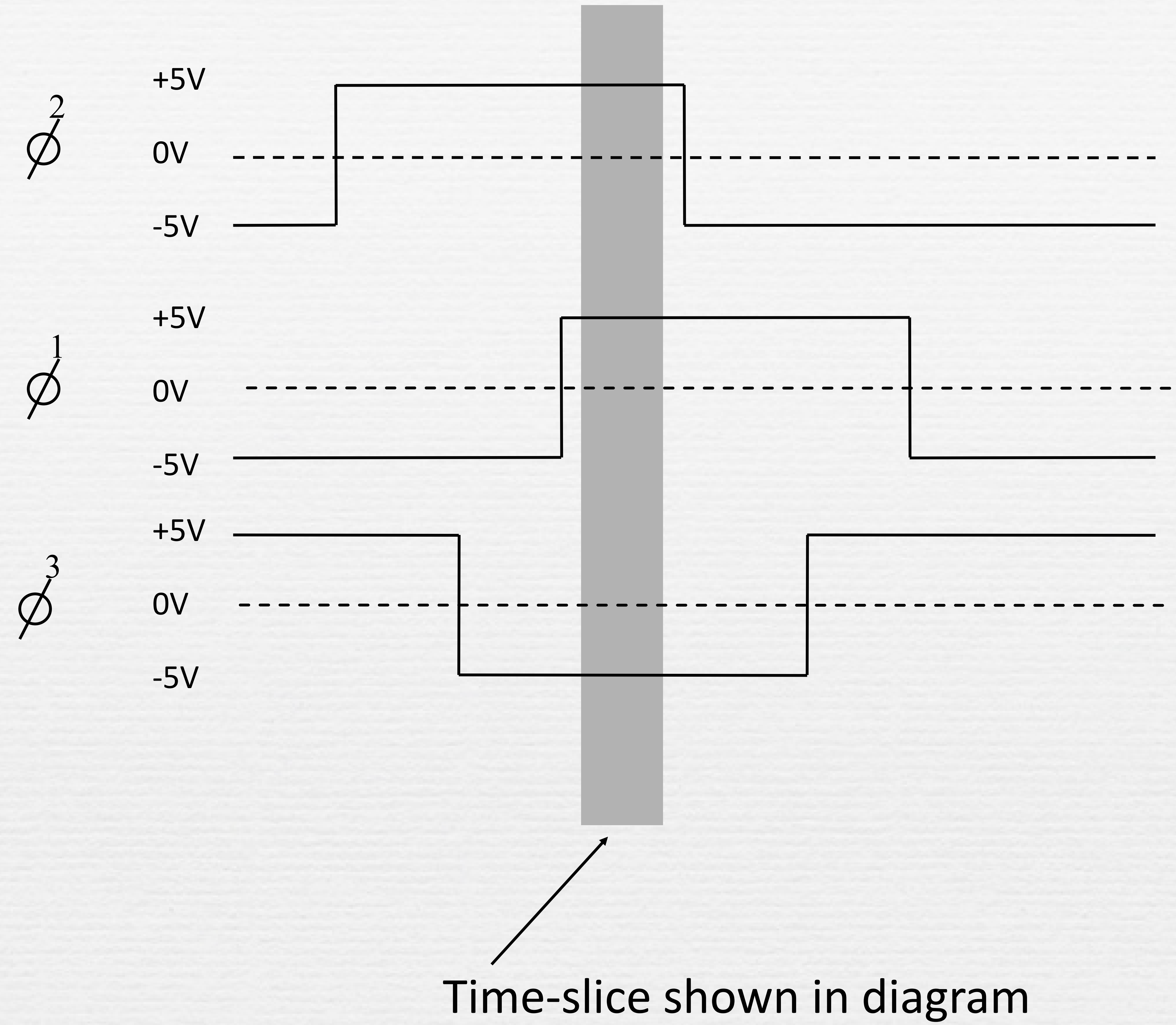


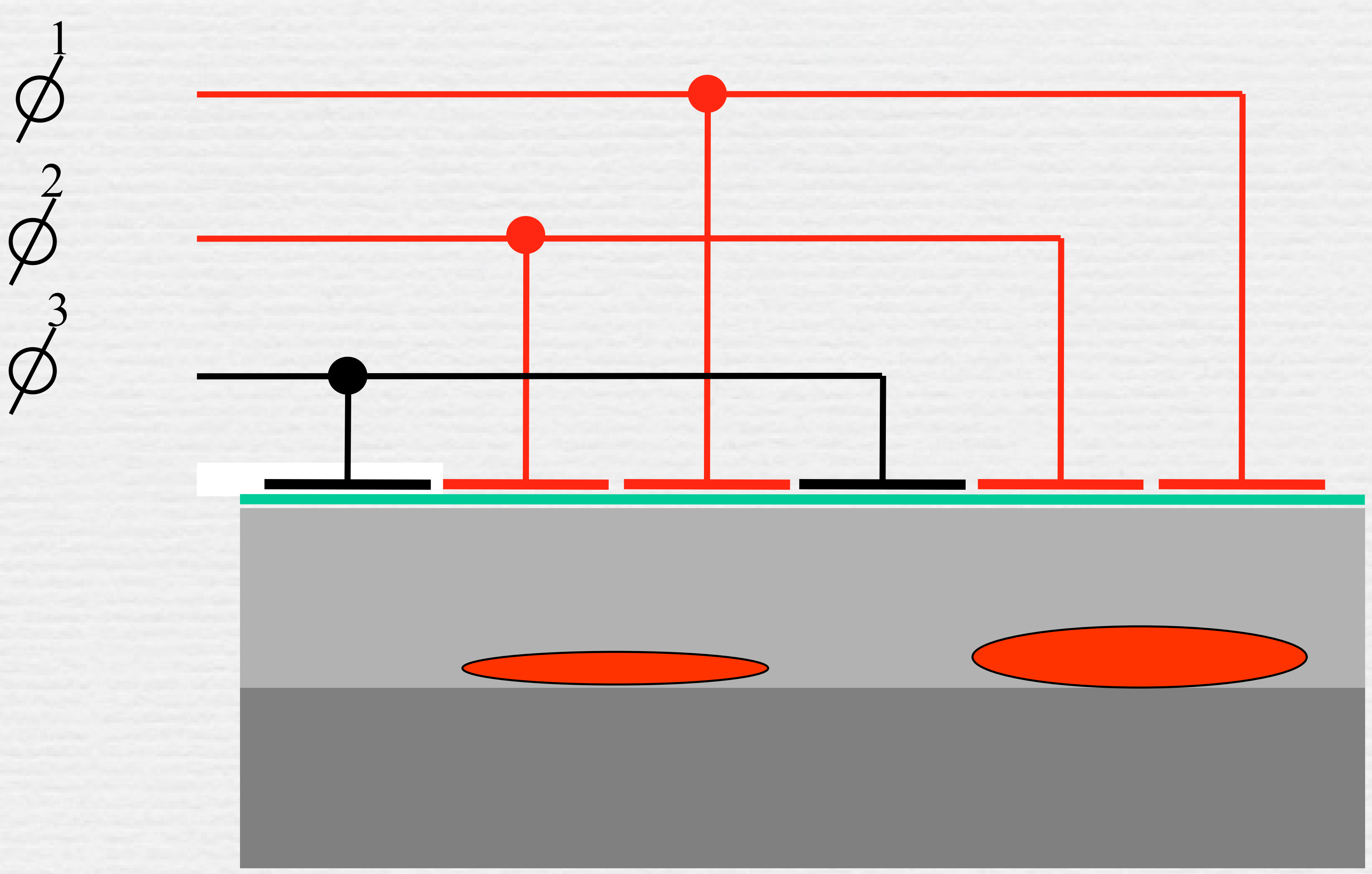
Charge Transfer in a CCD



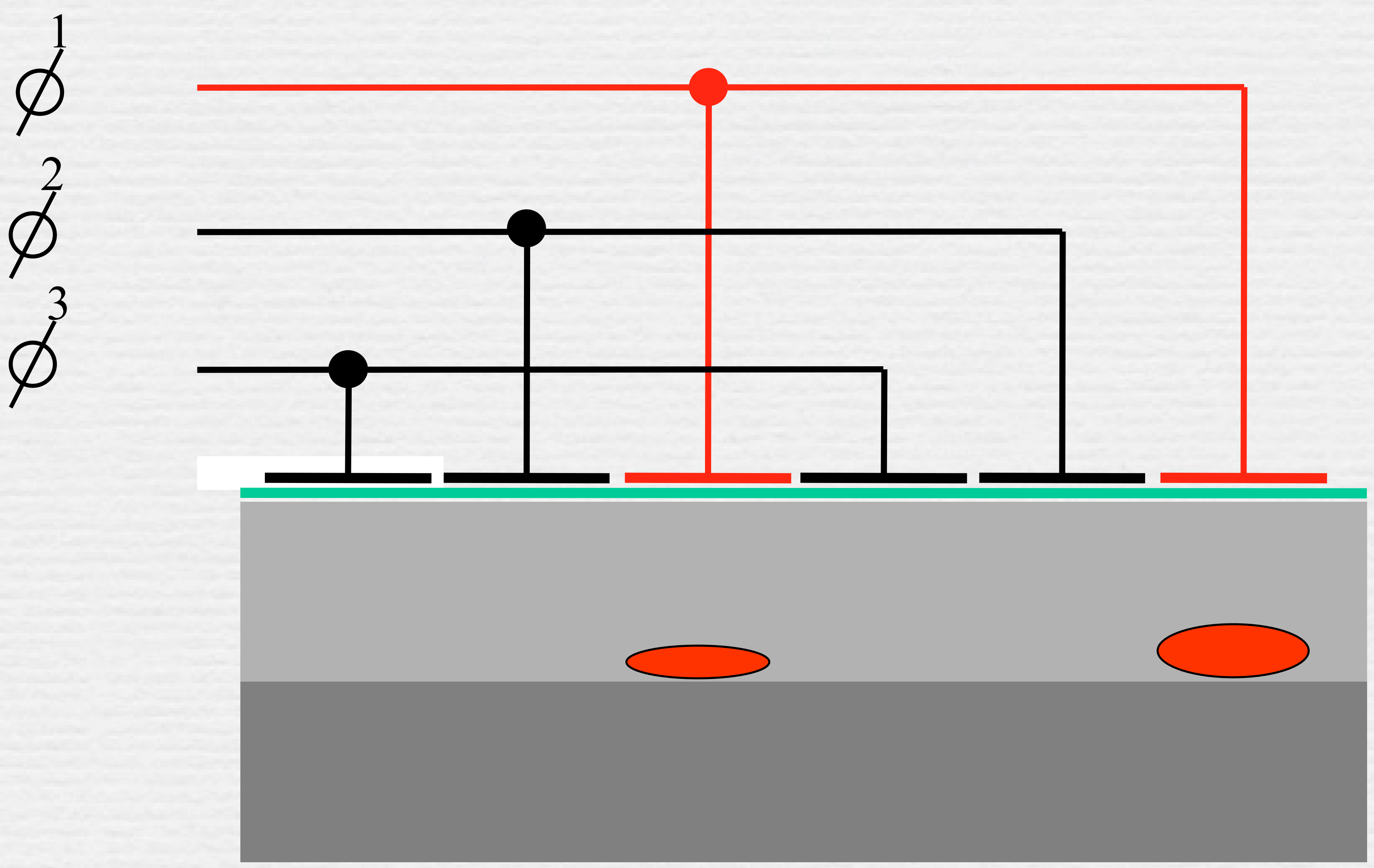


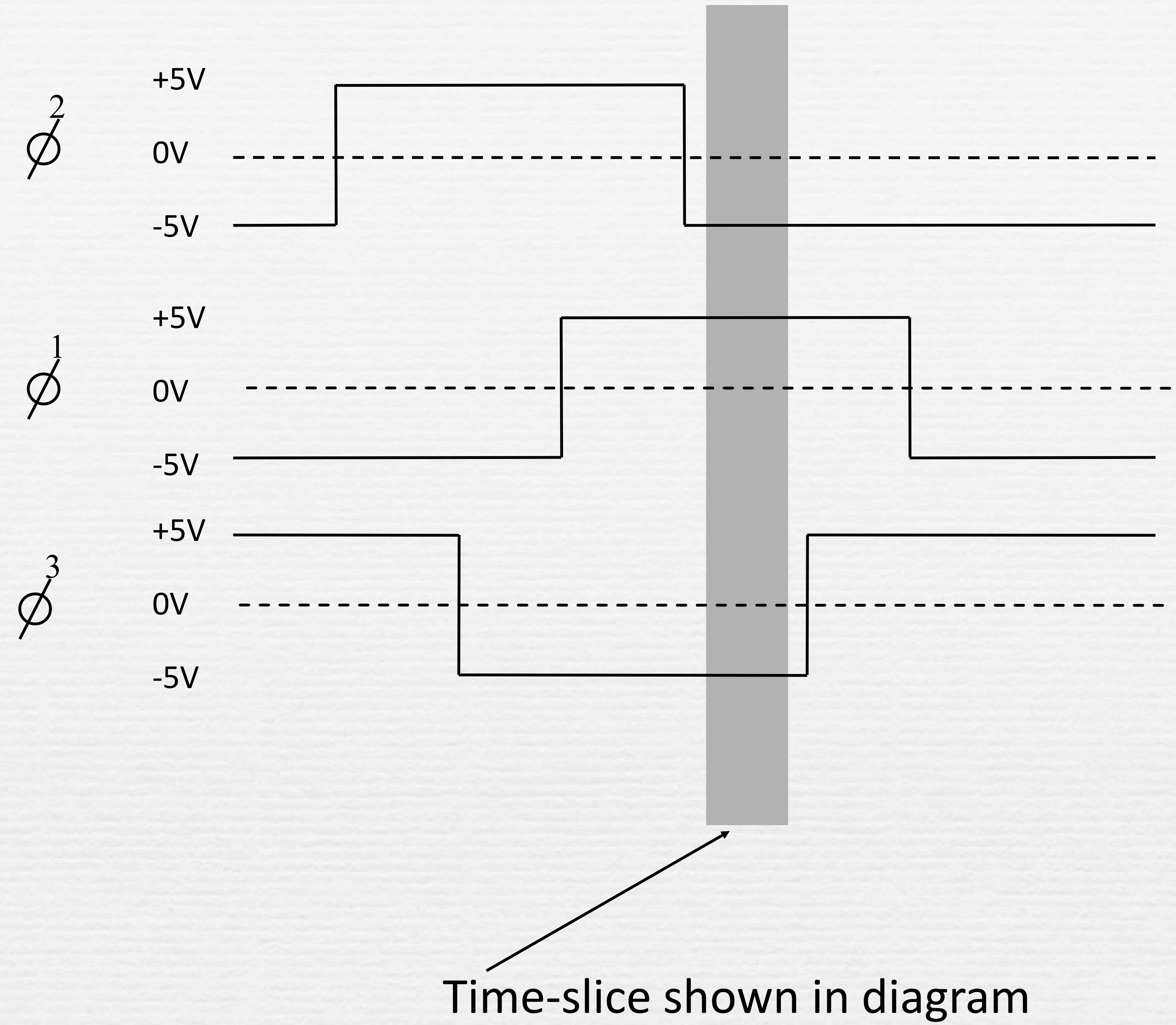
Charge Transfer in a CCD



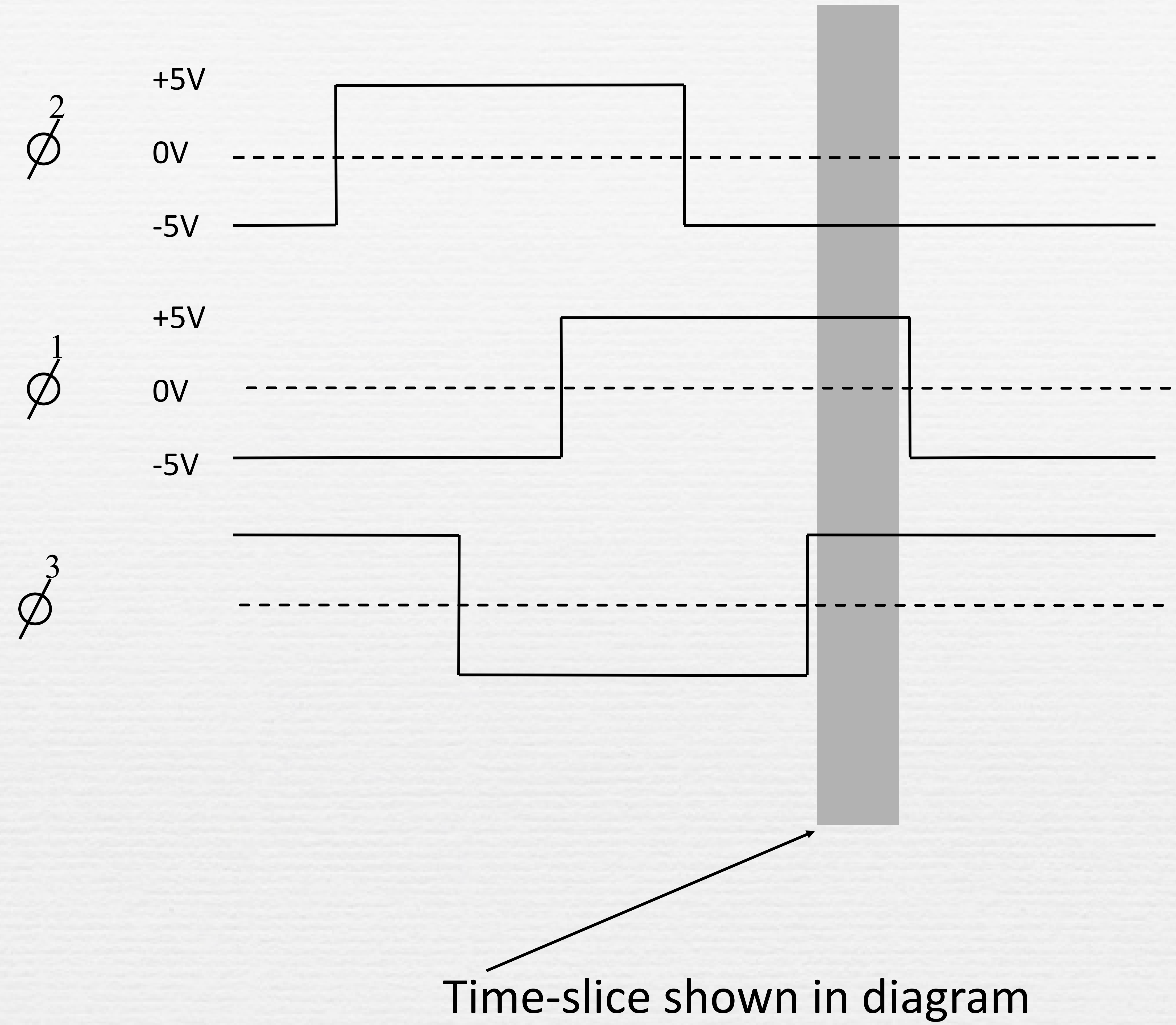


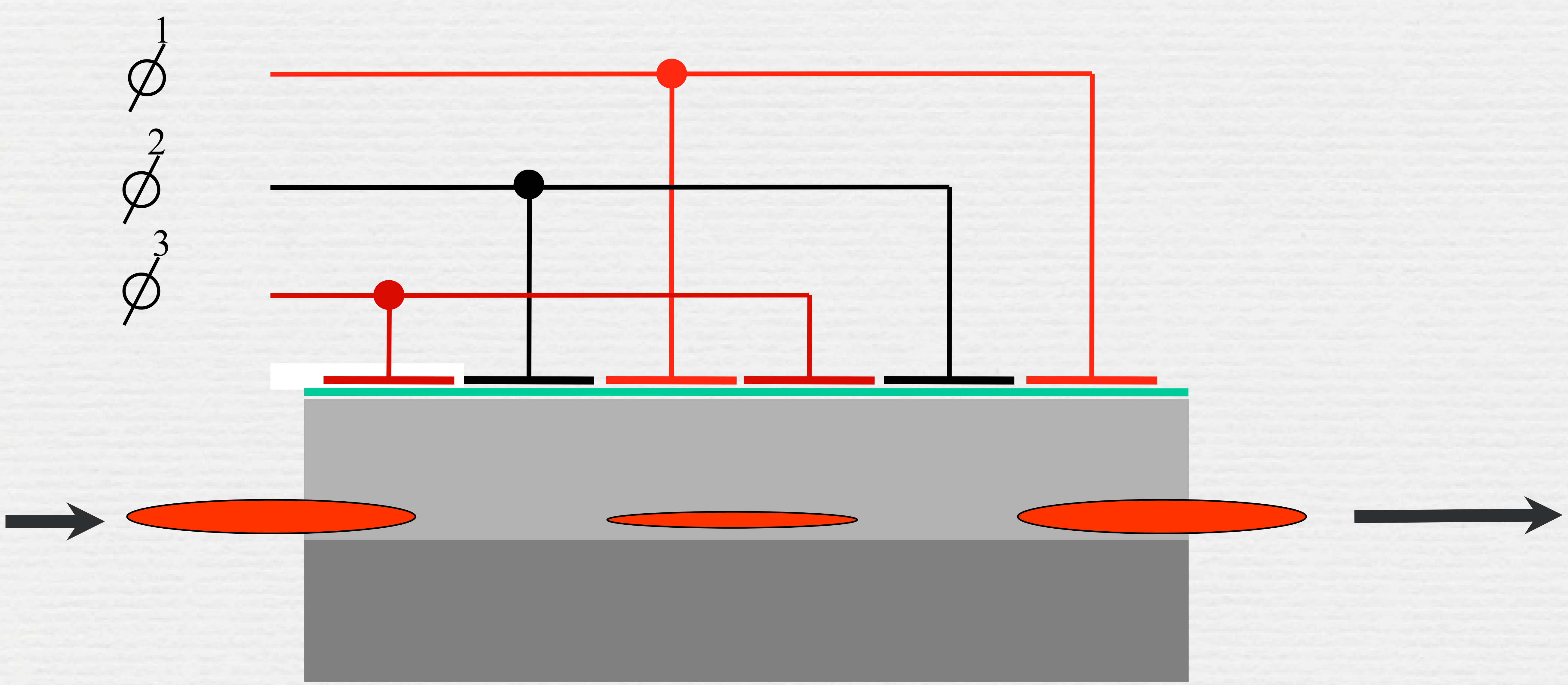
Charge Transfer in a CCD



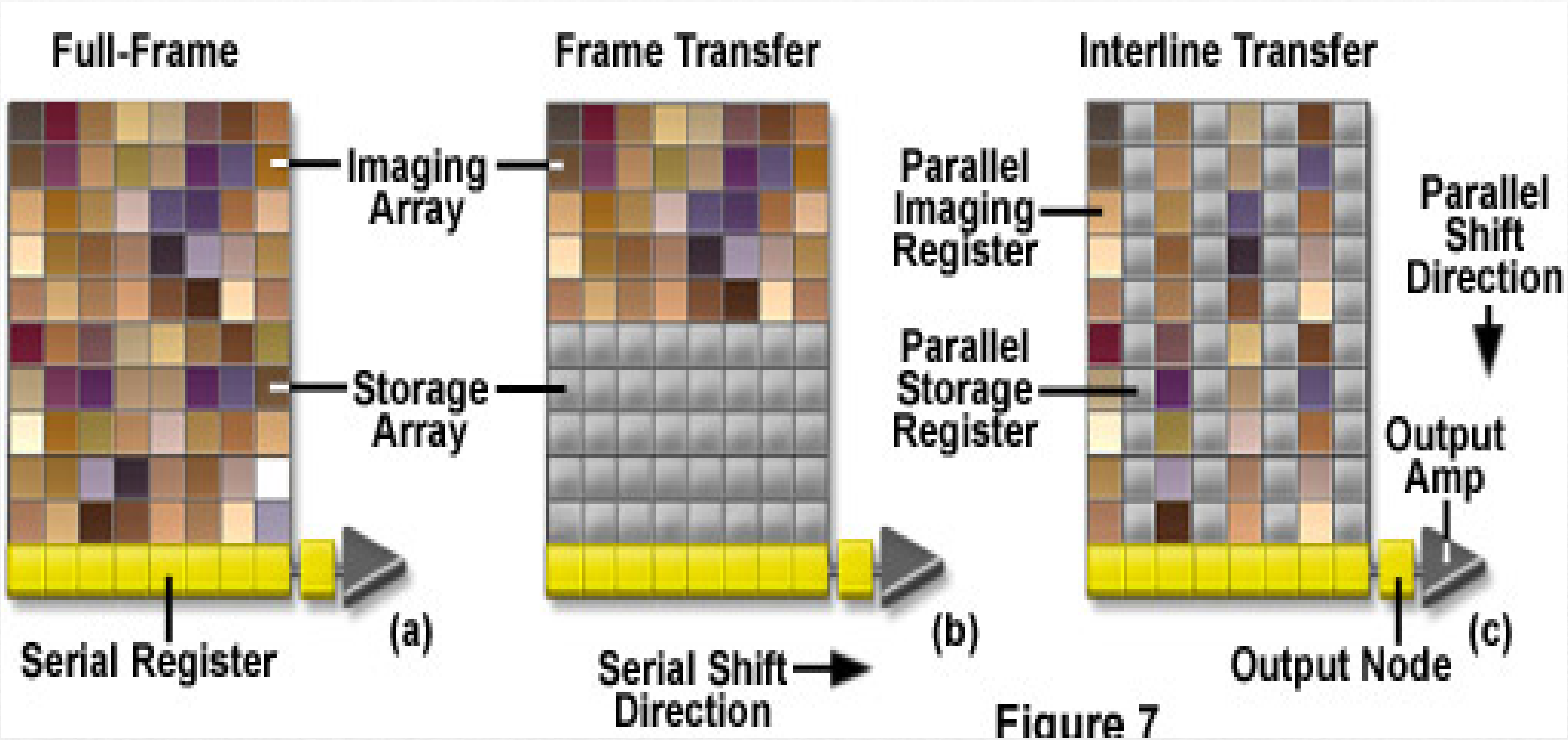


Charge Transfer in a CCD
Transfer charge to the next pixel





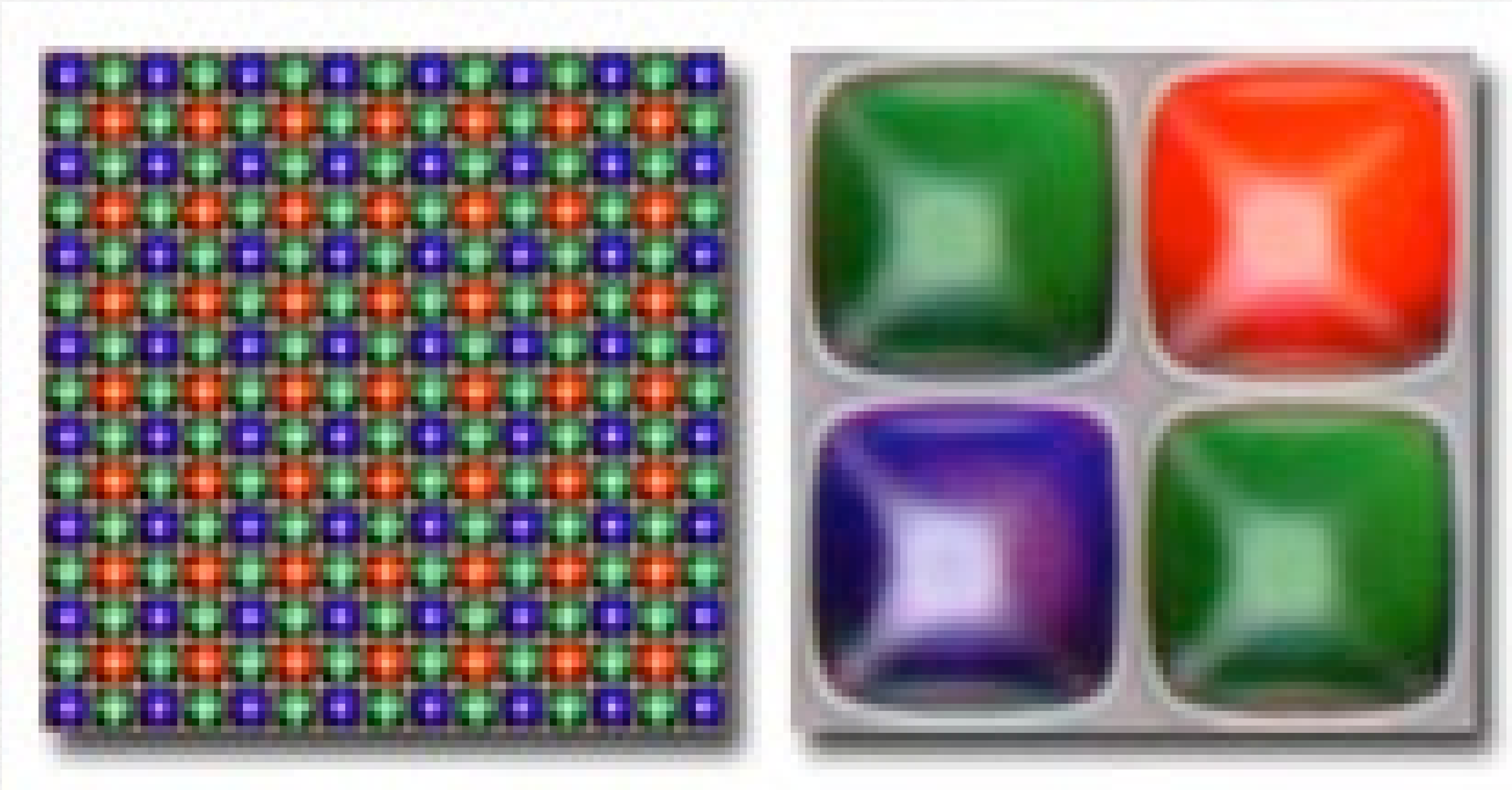
CCD Architectures



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 hitti
  that are converted to photo-electrons
- Full well depth: total number of photo-electr
  be recorded per pixel
- Read noise
- Dark current (negligible for most biological ap
- Readout time (calculate from clock rate and a
- Electron conversion factor (relate digital numbers to electrons)

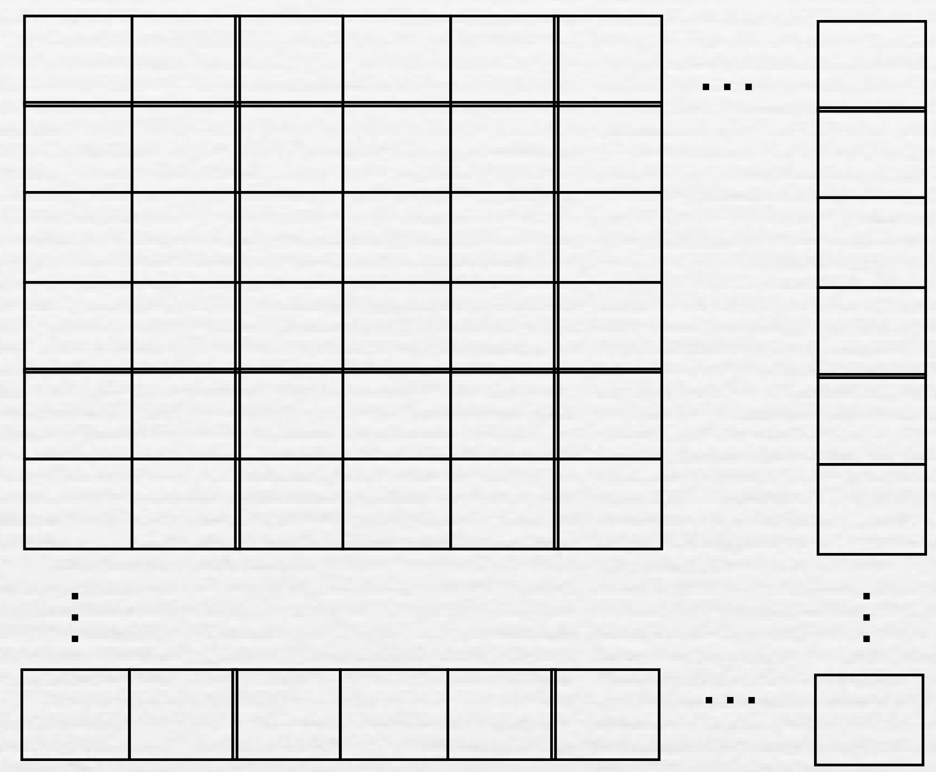
SPECIFICATIONS

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



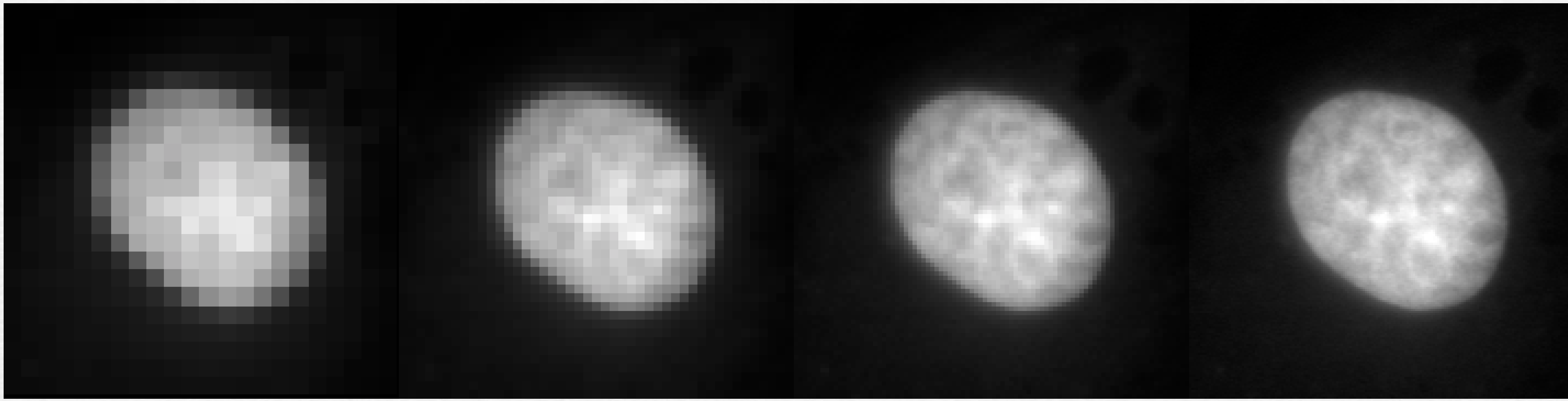
Sony Interline Chip ICX285

6.45 µm on a side

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



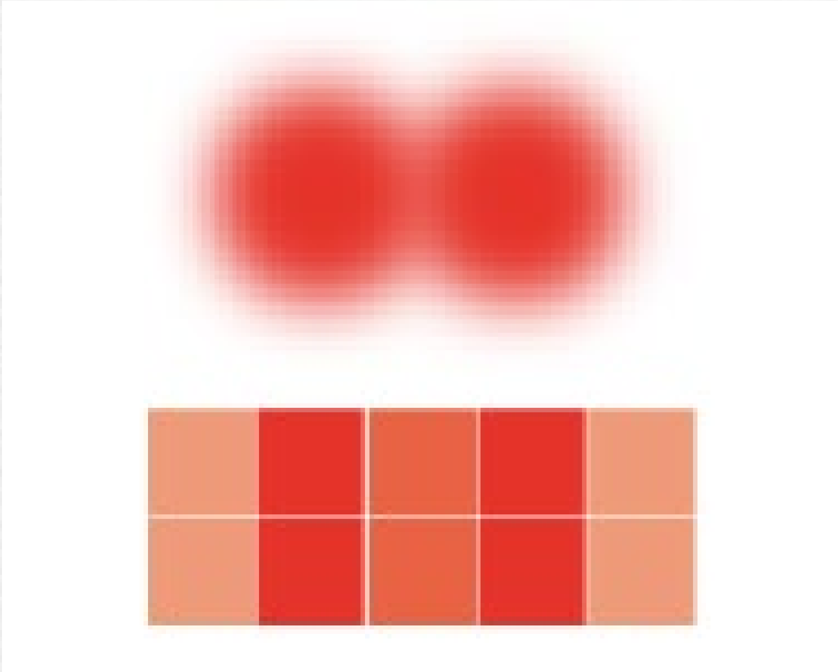
More pixels / resolution element

Where is optimum?

Digital Sampling

- How many CCD pixels are needed to accurately reproduce the smallest object that can be resolved by the scope?

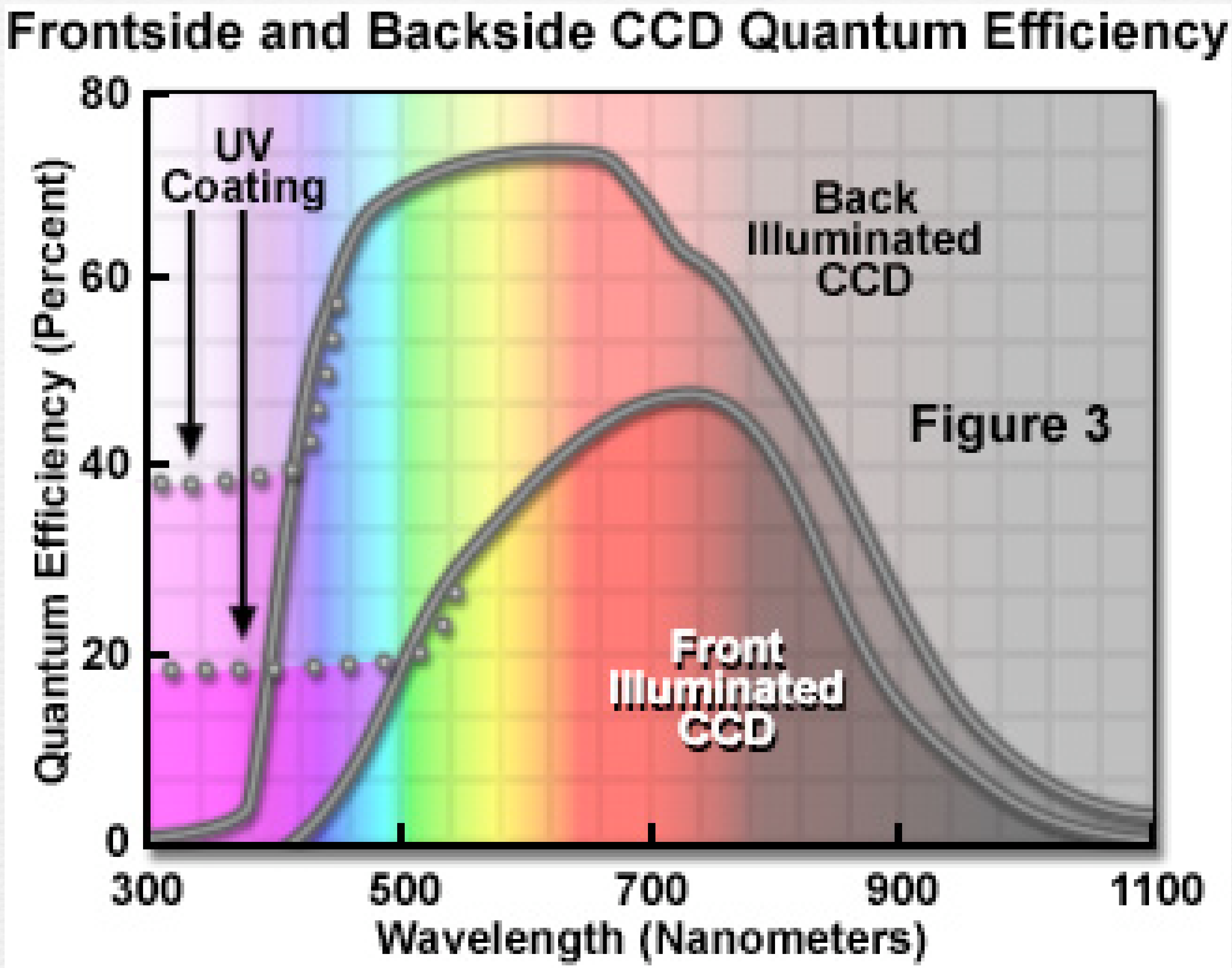
- Nyquist-Shannon Sampling theorem: Must have at least two pixels per resolvable element
- 2.5 – 3 is preferable



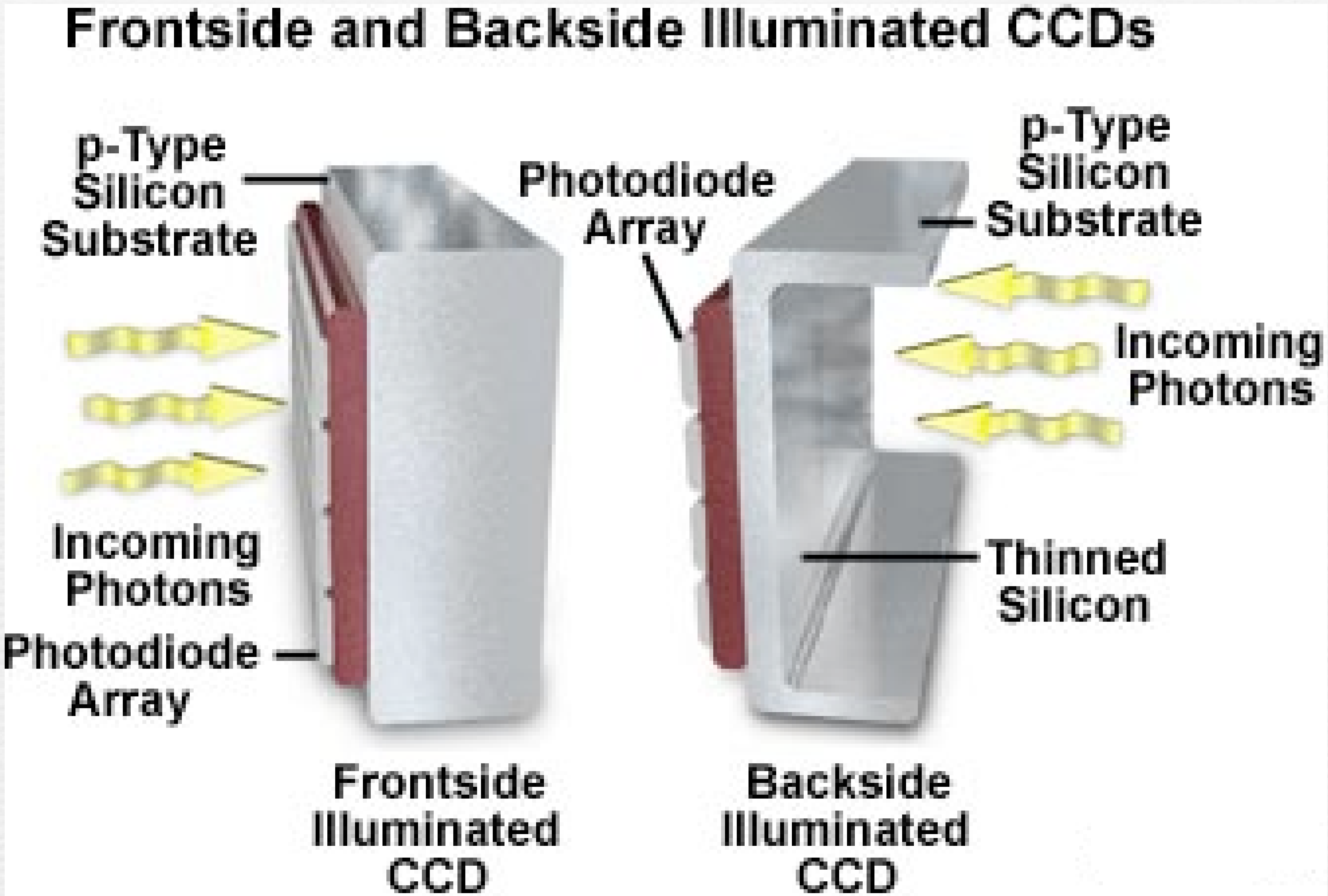
A resolution-centric view of imaging

- 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 undersampled, a 60x would be just at the Nyquist limit, and a 100x lens would oversample

Quantum Efficiency

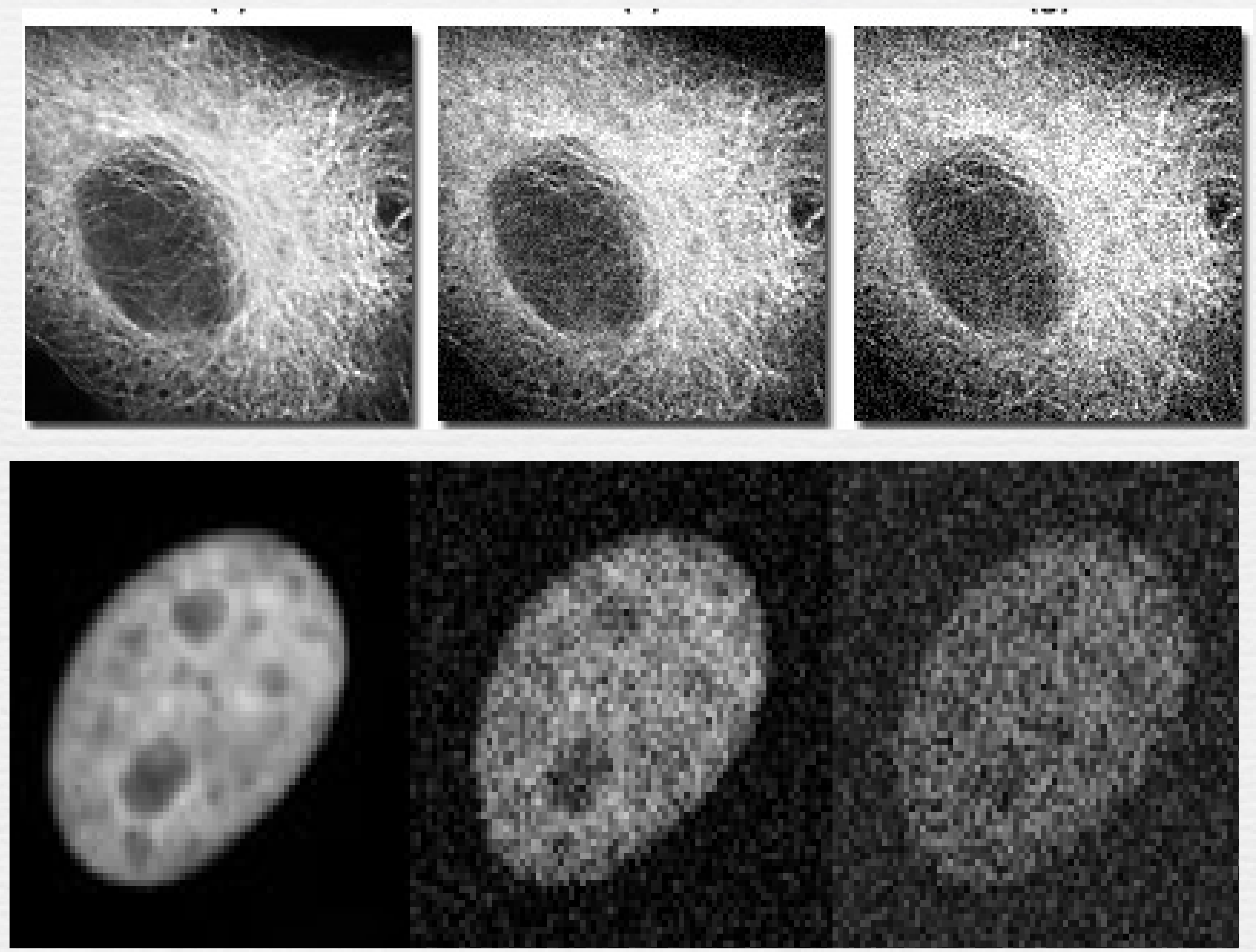


Back-thinning increases QE



Noise

- Longer exposure times are better – why?



Decreasing exposure time

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

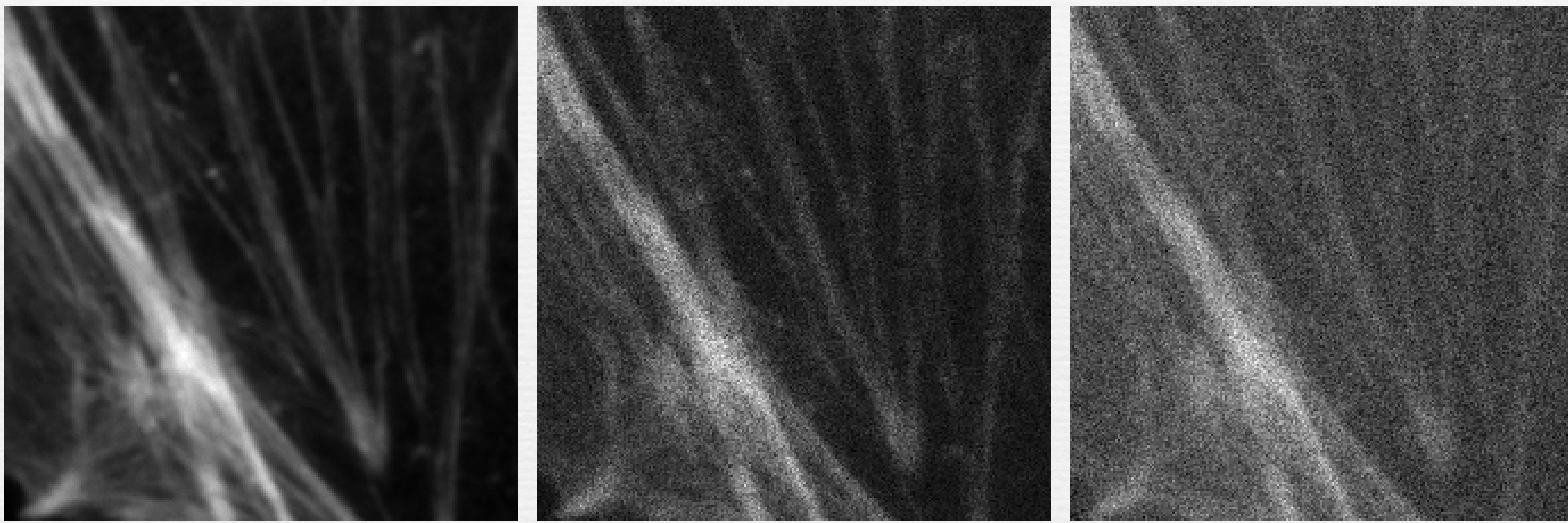
- Dark current – thermal accumulation of electrons
  - Cooling helps, so negligible for most applications

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

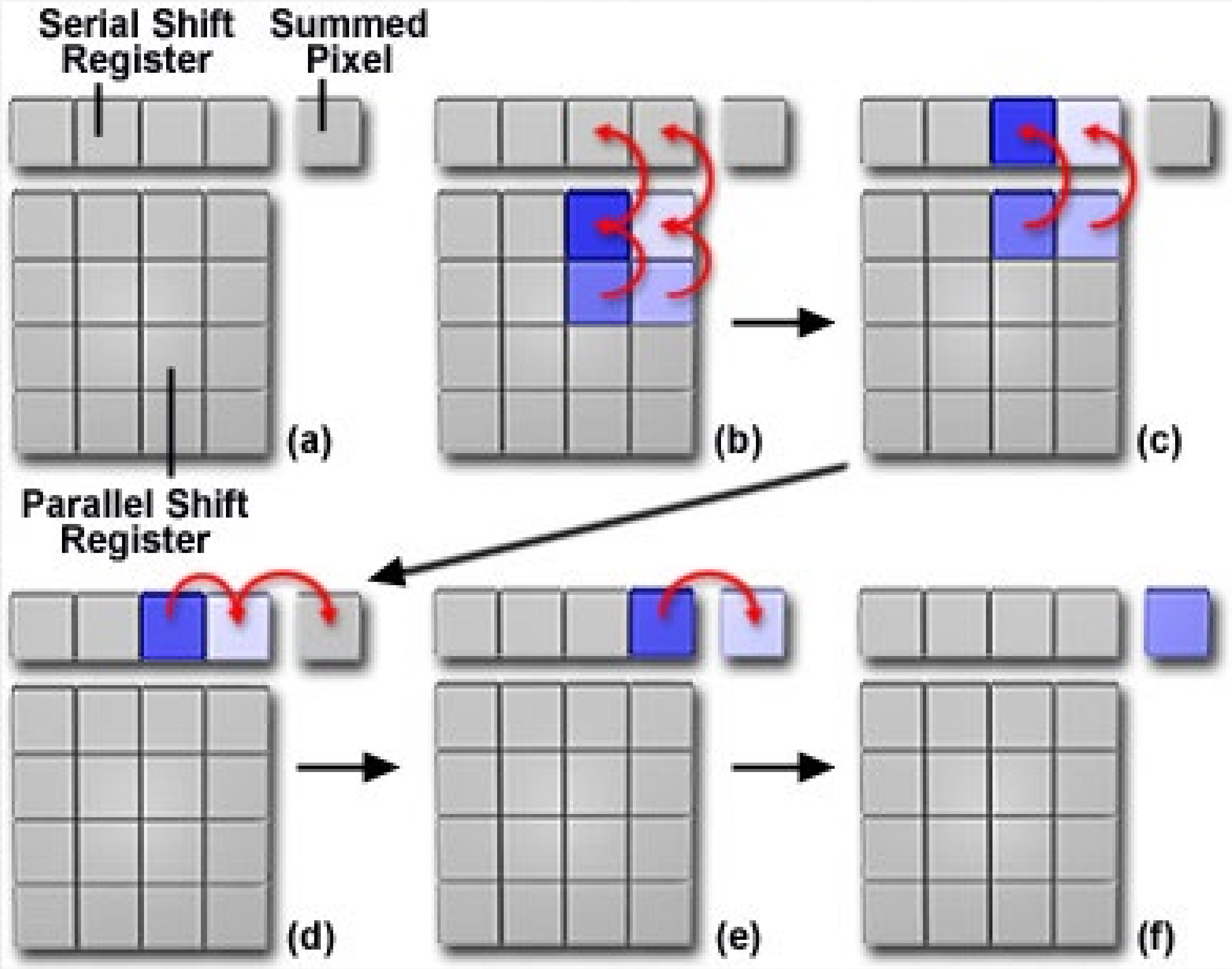
10 photons / pixel on average; ~50 in brightest areas



Test image　　　no read noise　　　5 e^- read noise

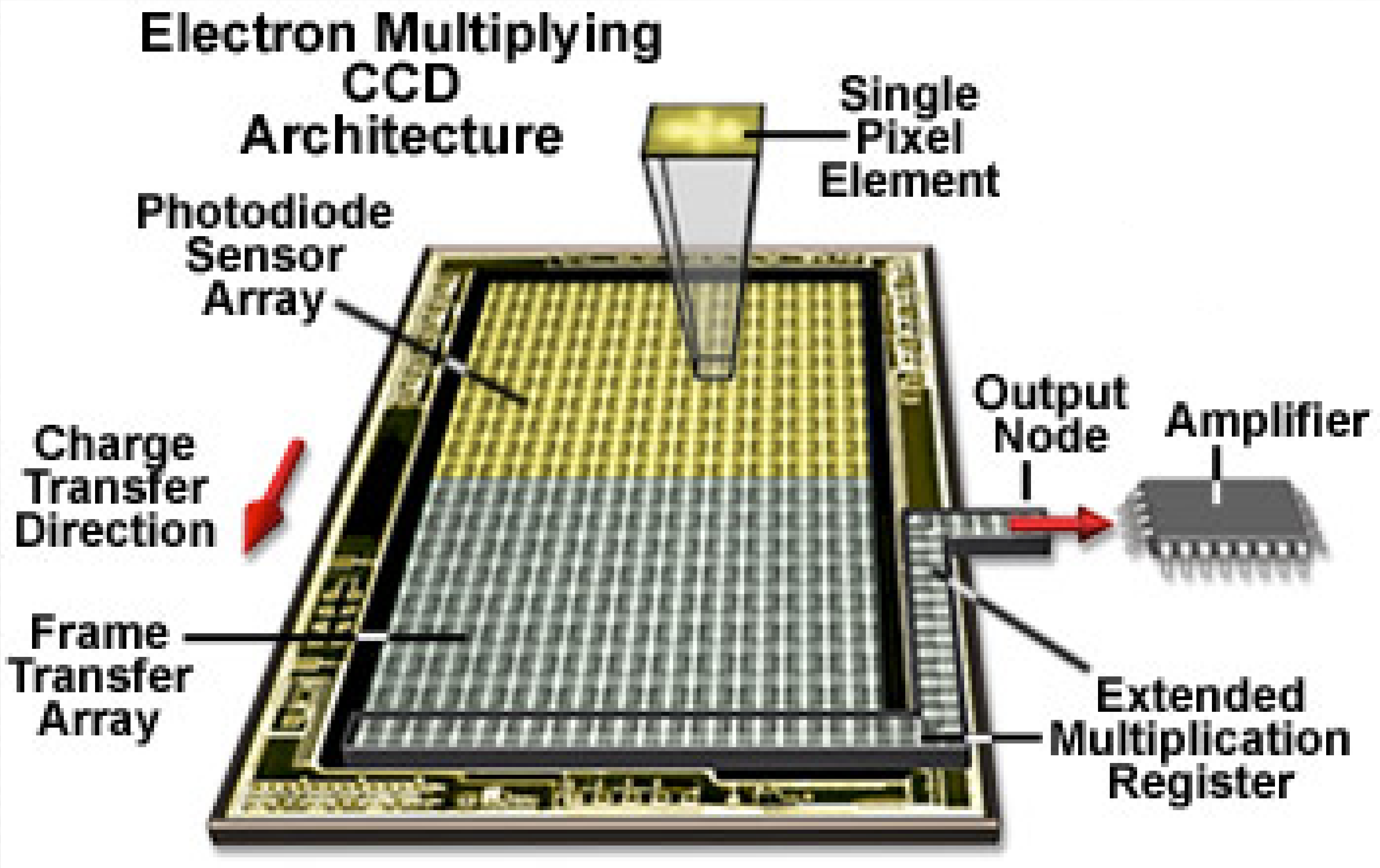
Photon shot noise ~ 3/5 read noise

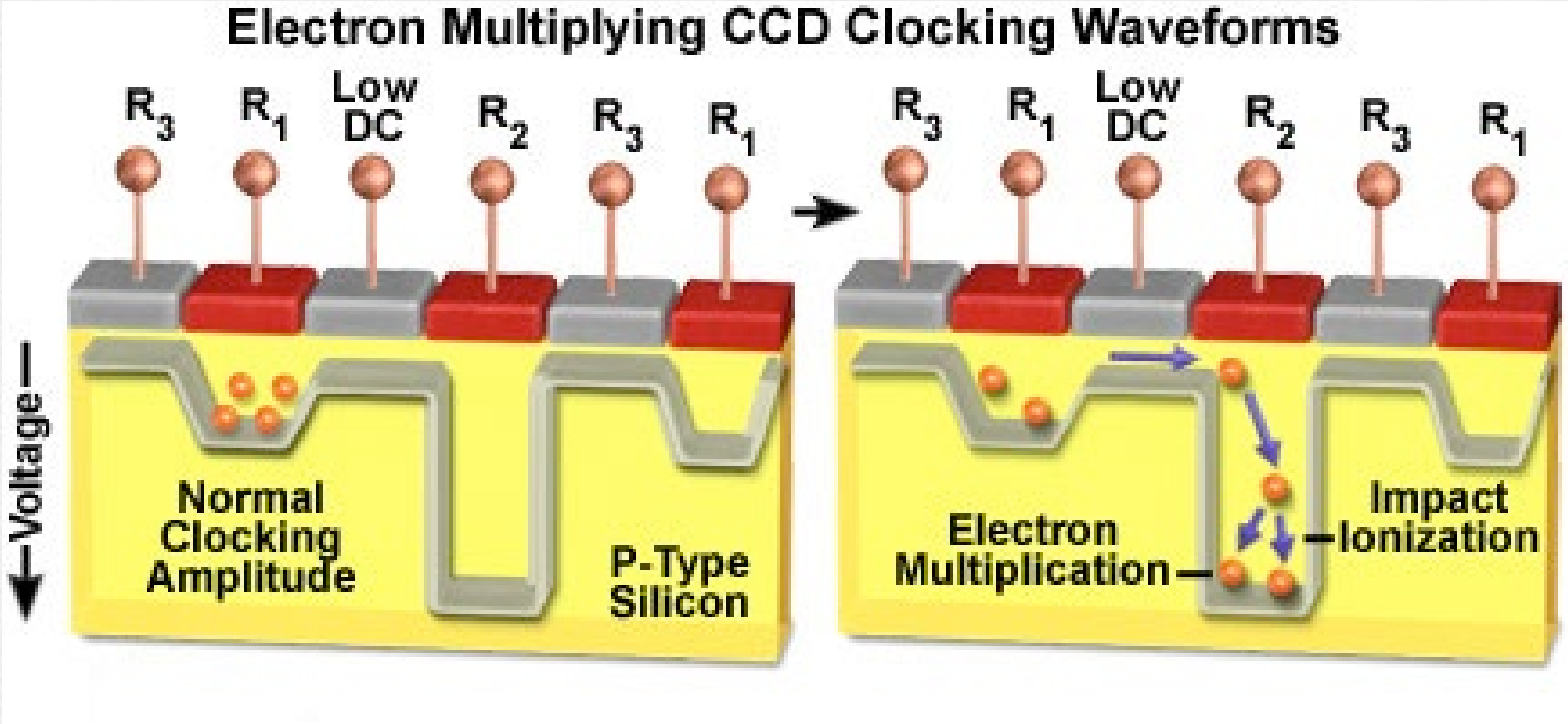
Binning



- 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





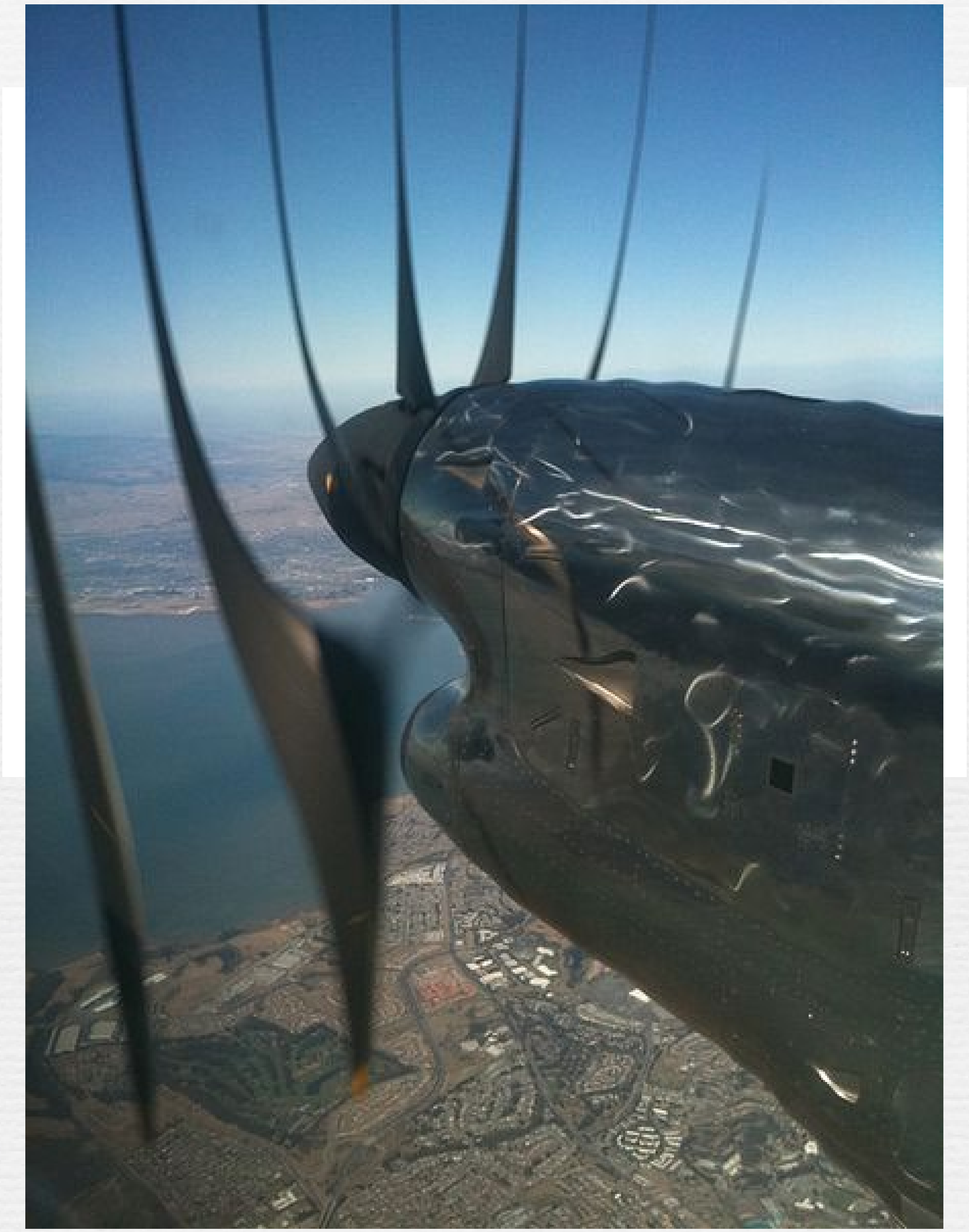
EMCCD result

- 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



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

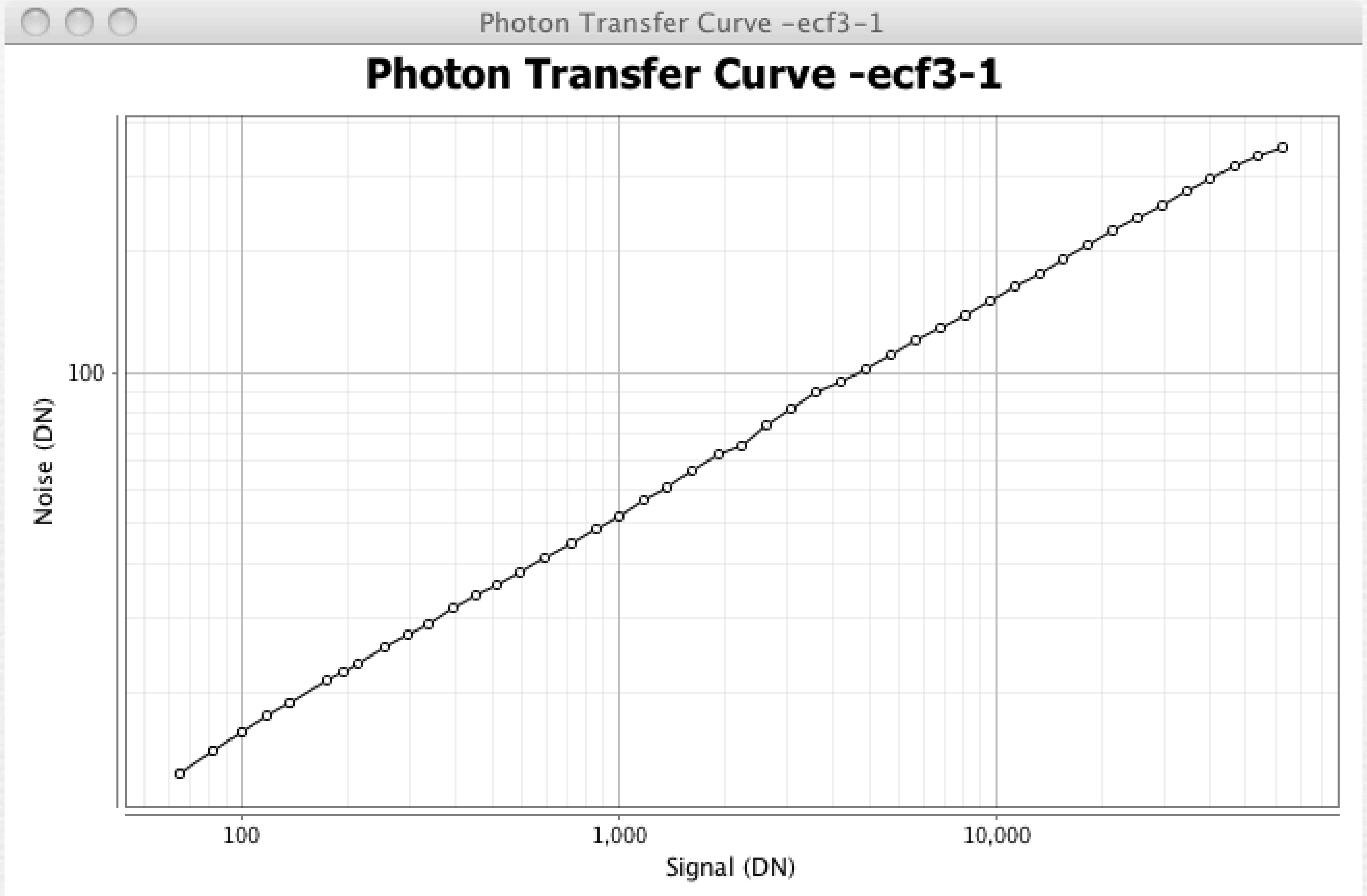
- Zero photons collected doesn't result in number zero. Offset can often be changed
- 1 photon does not necessarily equal 1 count in your image – electron conversion factor - depends on camera gain

Measure the electron conversion factor:

When the dominant noise source is Photon Shot noise:

$\sigma(N) = \sqrt{(N)}$	Photon Shot noise
$N = c \cdot DN$	c = electron conversion factor
$\sigma(N) = c \cdot \sigma(DN)$	
$c = DN/\sigma^2(DN)$	

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

Software control of microscopes: µManager as an example

- Nico Stuurman, Arthur Edelstein, Ziah Dean, Henry Pinkard, Ron Vale. Dept. of Cellular and Molecular Pharmacology, UCSF/HHMI - San Francisco

Why micro-manager.org?

Started summer 2005 at Vale Lab, UCSF

- Single Interface for all microscopes
- Standard and open plug-in interface
- Choice in hardware
- (Real) extensibility
- Quality
- Cost

μManager features

- Simple user interface to important imaging strategies: Snap Image, Time-lapse, z-series, multi-channel, multi-positions
- Controls many microscope hardware components
- Hardware support can be added by anyone
- Integrated with ImageJ
- Cross-platform (Windows, Mac, Linux)
- Open Source
- Modular architecture: Extensible by third parties: at hardware support level and User Interface
- Powerful scripting interface
- Programmatic interfaces to 3rd-party analysis environments such as Matlab enabling analysis driven acquisition
- Free!

µManager
THE OPEN SOURCE MICROSCOPY SOFTWARE

Supported Hardware

- **Microscopes:** Nikon: TE2000, TI, AZ100
  - Zeiss: AxioPlan, AxioVert, AxioObserver, AxioImager
  - Leica: most motorized scopes
  - Olympus IX81, BX81
- **Cameras:**
  - Andor
  - ABSCamera
  - Hamamatsu
  - Roper/Photometrics
  - QImaging
  - dc1394 (firewire)

Mad City Labs
Maerzhauser
Physik Instrumente

Communication ports, IO:
Serial, parallel, USB port
DTOpenlayer
Velleman K8055 and K8061
National Instruments

Other devices:
Neos AOTF controller
Spectral LMM5
Yokogawa CSU22 and CSUX
Pecon environmental control

See: http://micro-manager.org

µManager
THE OPEN SOURCE
MICROSCOPY SOFTWARE

Support and Statistics

Website: http://micro-manager.org
Wiki: http://valelab.ucsf.edu/~nico/MMwiki
Source code:
https://valelab.ucsf.edu/svn/micromanager2/branches/micromanager1.4
Mailing list: https://lists.sourceforge.net/lists/listinfo/micro-manager-general
Support/Help: info@micro-manager.org

10,000 registered users (~250 new users every month)
600 subscribers to the mailing list

Thanks!

- 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

File Formats

- Most portable: TIFF
  - 8 or 16-bit, lossless, supports grayscale and RGB
- OK: JPEG2000, custom formats (nd2, ids, zvi, lsm, etc.)
  - Lossless, supports full bitdepth
  - Custom formats often support multidimensional images
  - Not so portable
- Bad: Jpeg, GIF, BMP, etc.
  - Lossy and / or 8-bit

(Linear) digital filters

- Kernels

1	1	1	Averaging / Smoothing
1	1	1	
1	1	1	

0	1	2	1	0	
1	6	10	6	1	
2	10	16	10	2	Gaussian smoothing
1	6	10	6	1	
0	1	2	1	0	

How this works

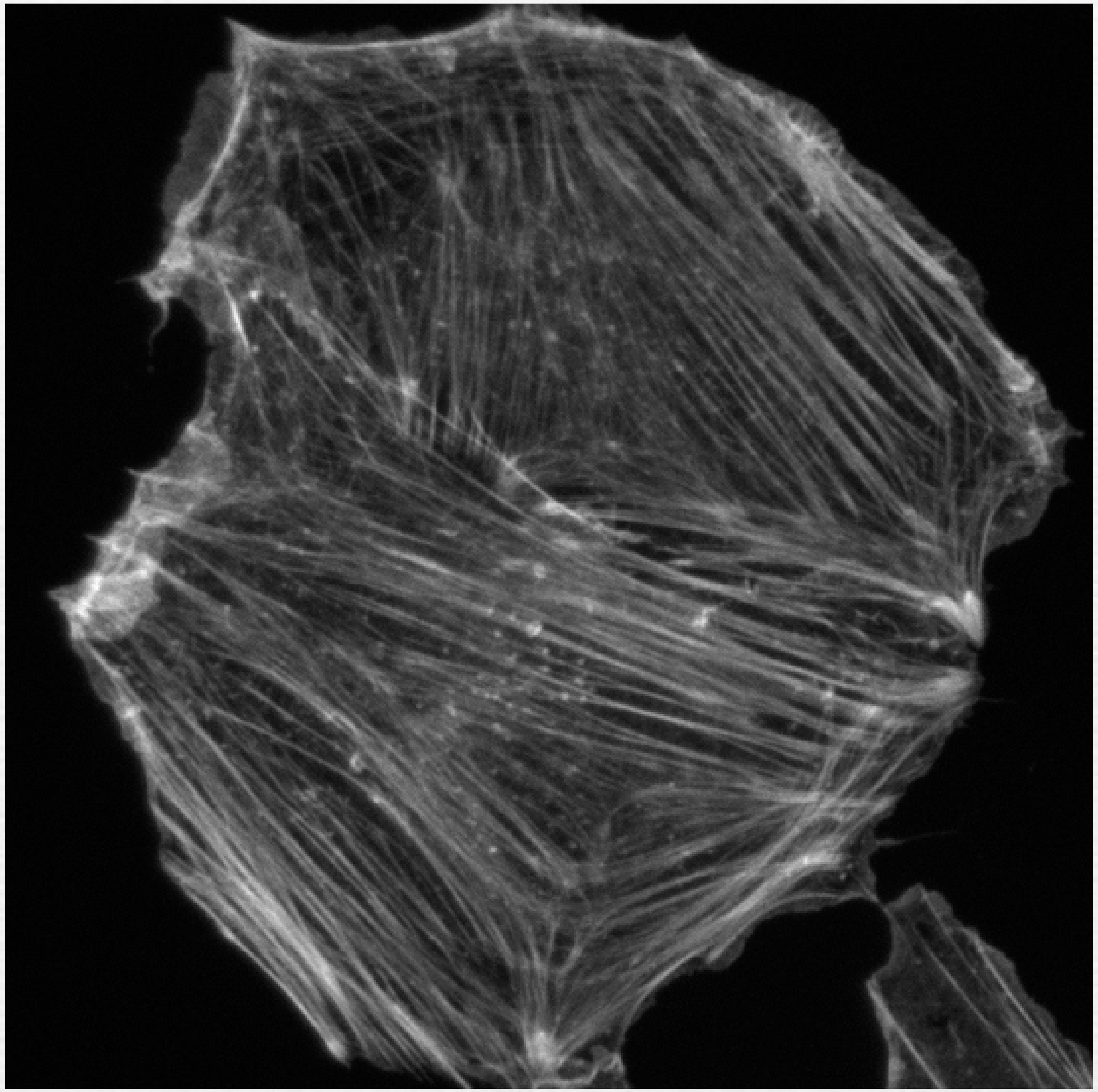
1	1	1
1	1	1
1	1	1

10	11	22	5	7
13	14	10	5	24
20	20	15	23	14
0	3	17	15	8
7	11	6	15	12

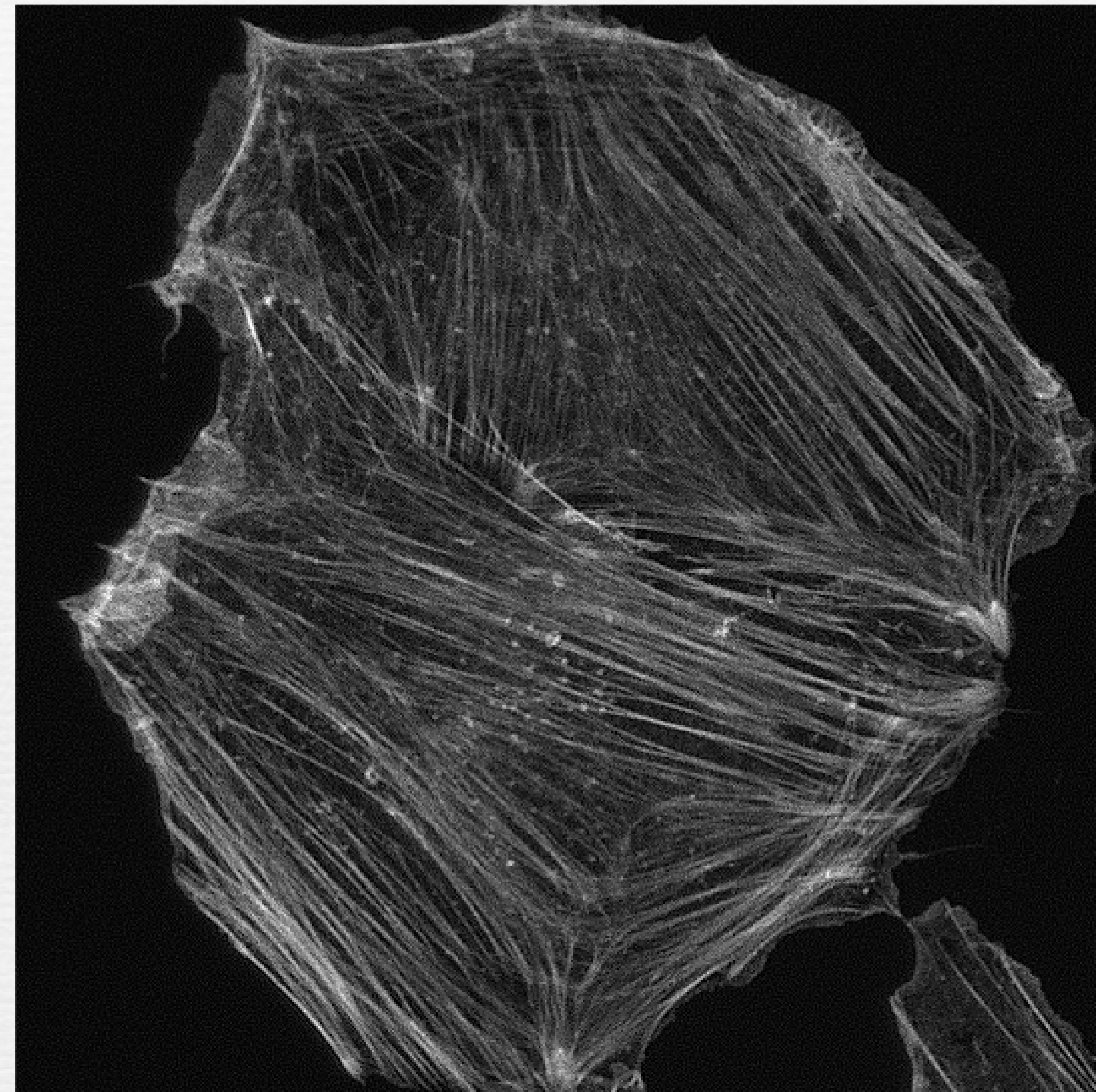
Multiply corresponding pixels and sum

$(10+11+22+13+8+10+20+20+15)/9 = 14$

Example: Unsharp masking



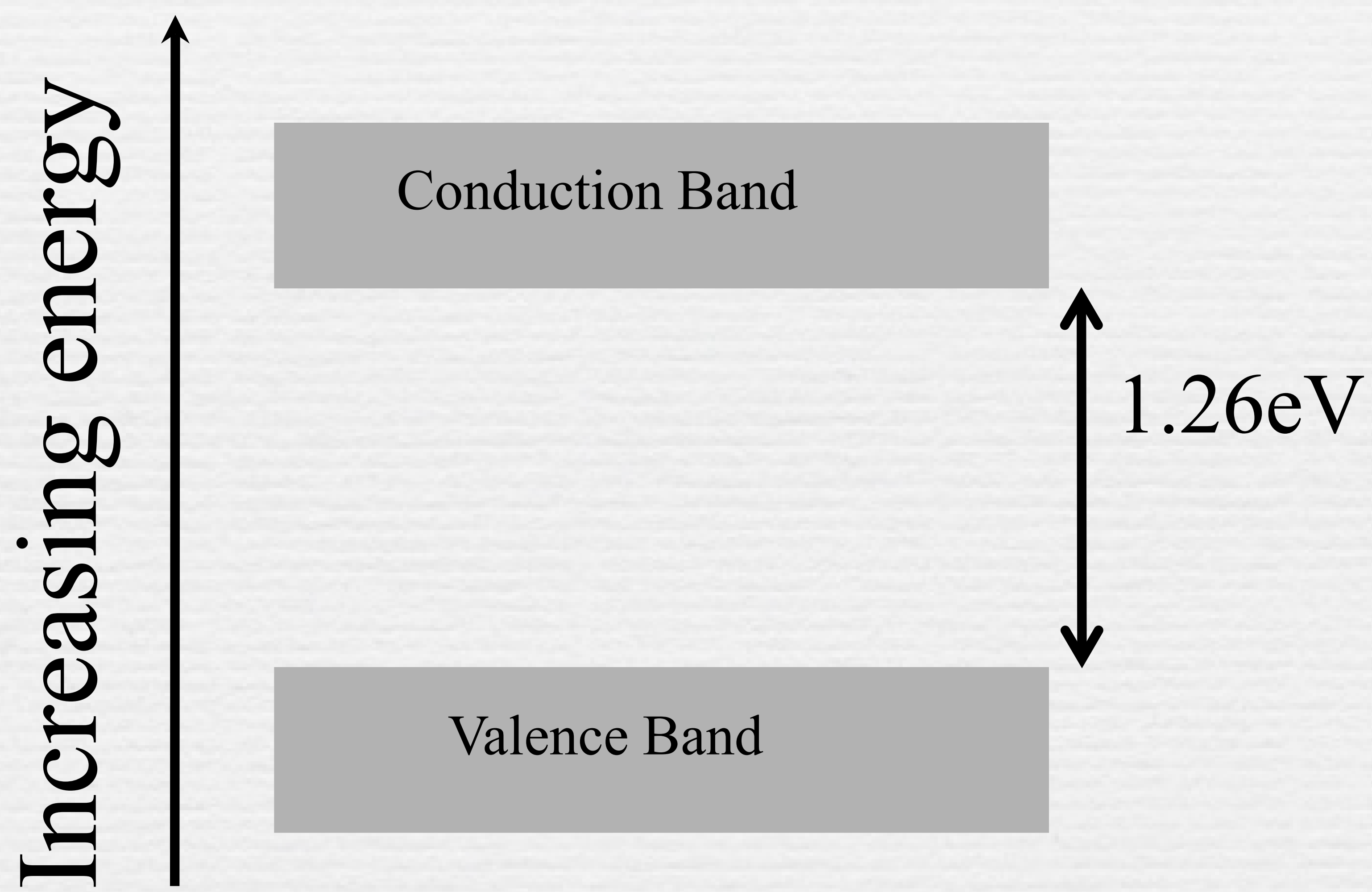
Original

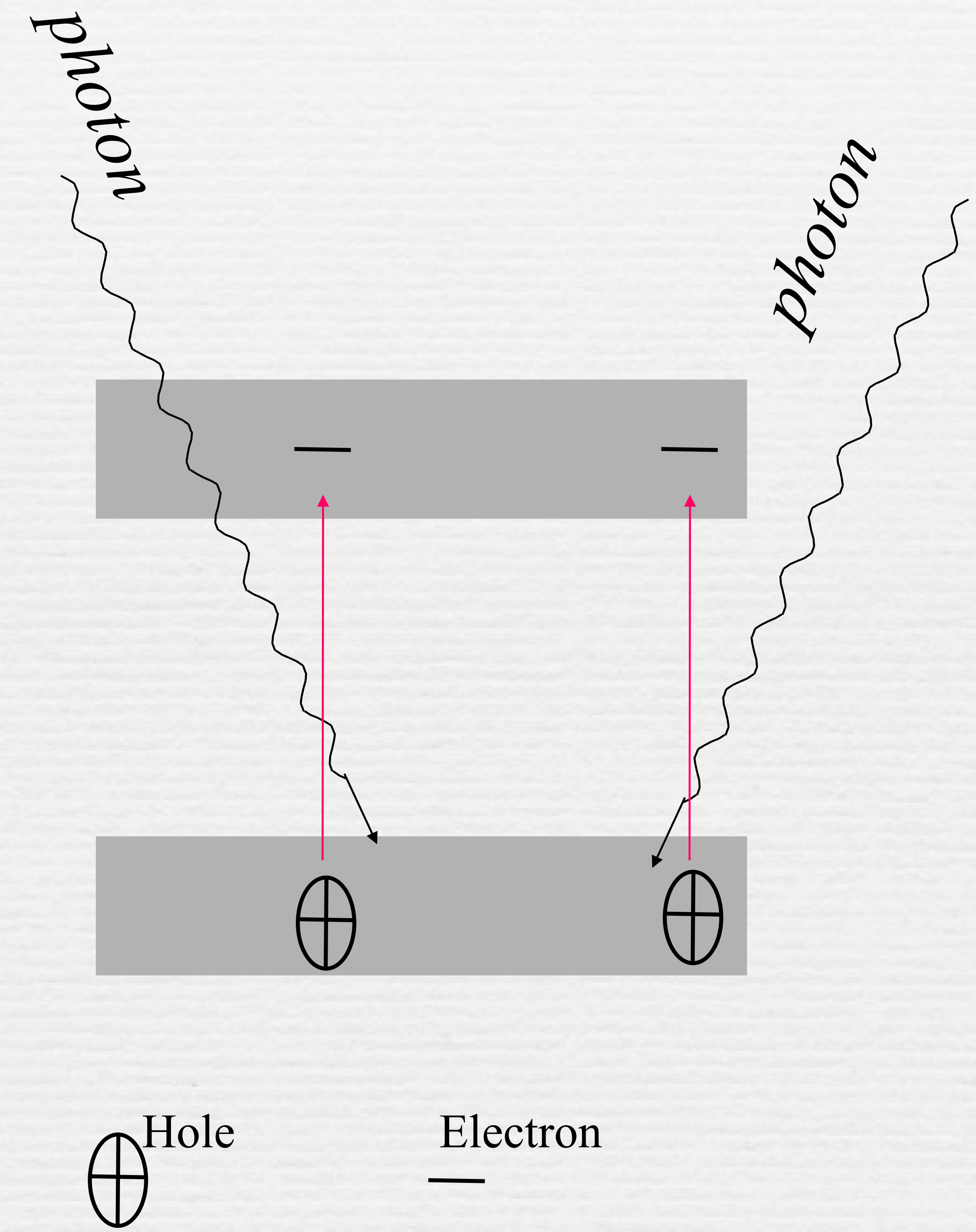


Unsharp masked

$$\begin{matrix} -1 & -4 & -1 \\ -4 & 26 & -4 \\ -1 & -4 & -1 \end{matrix}$$

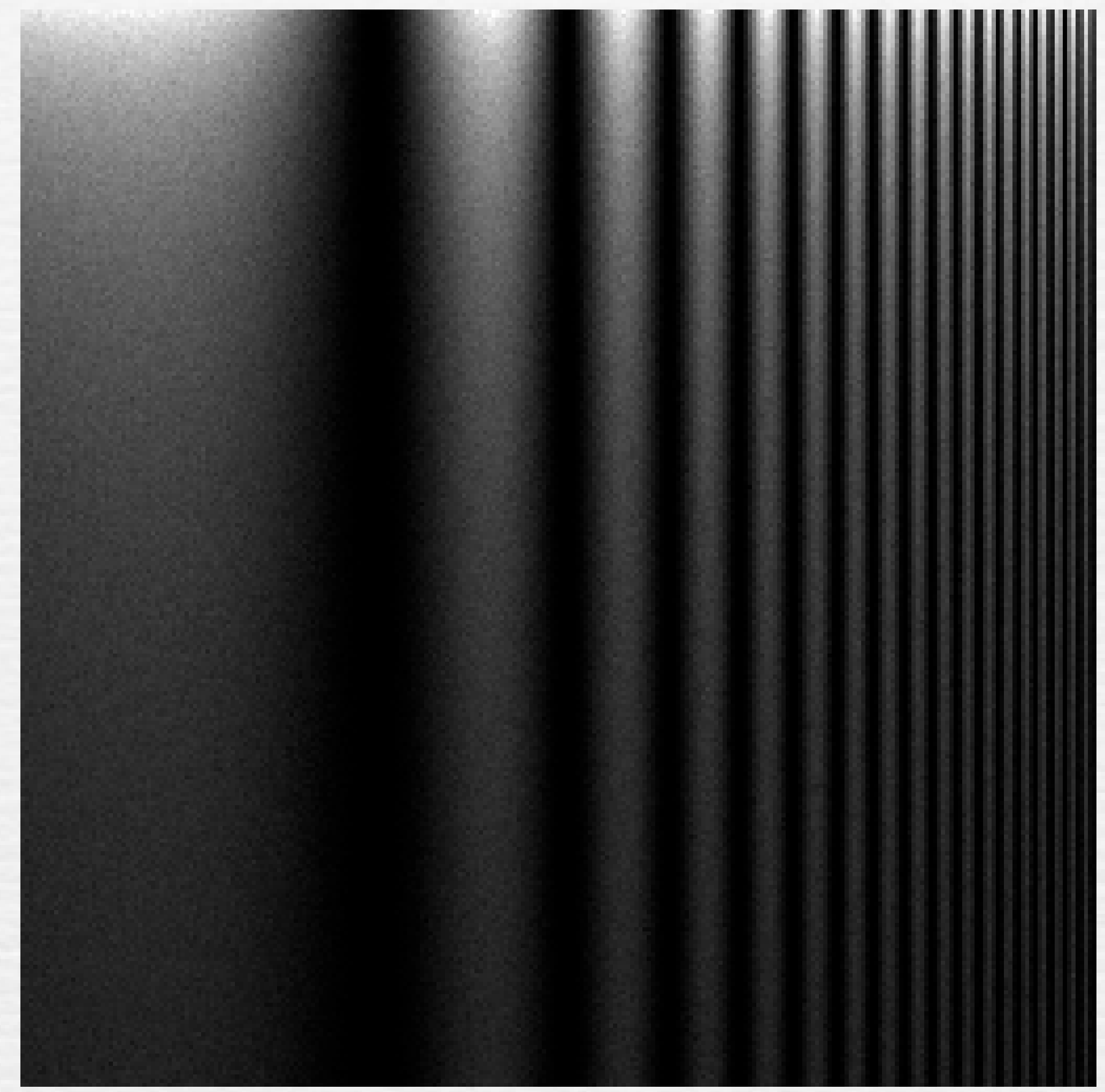
Photo-electric Effect



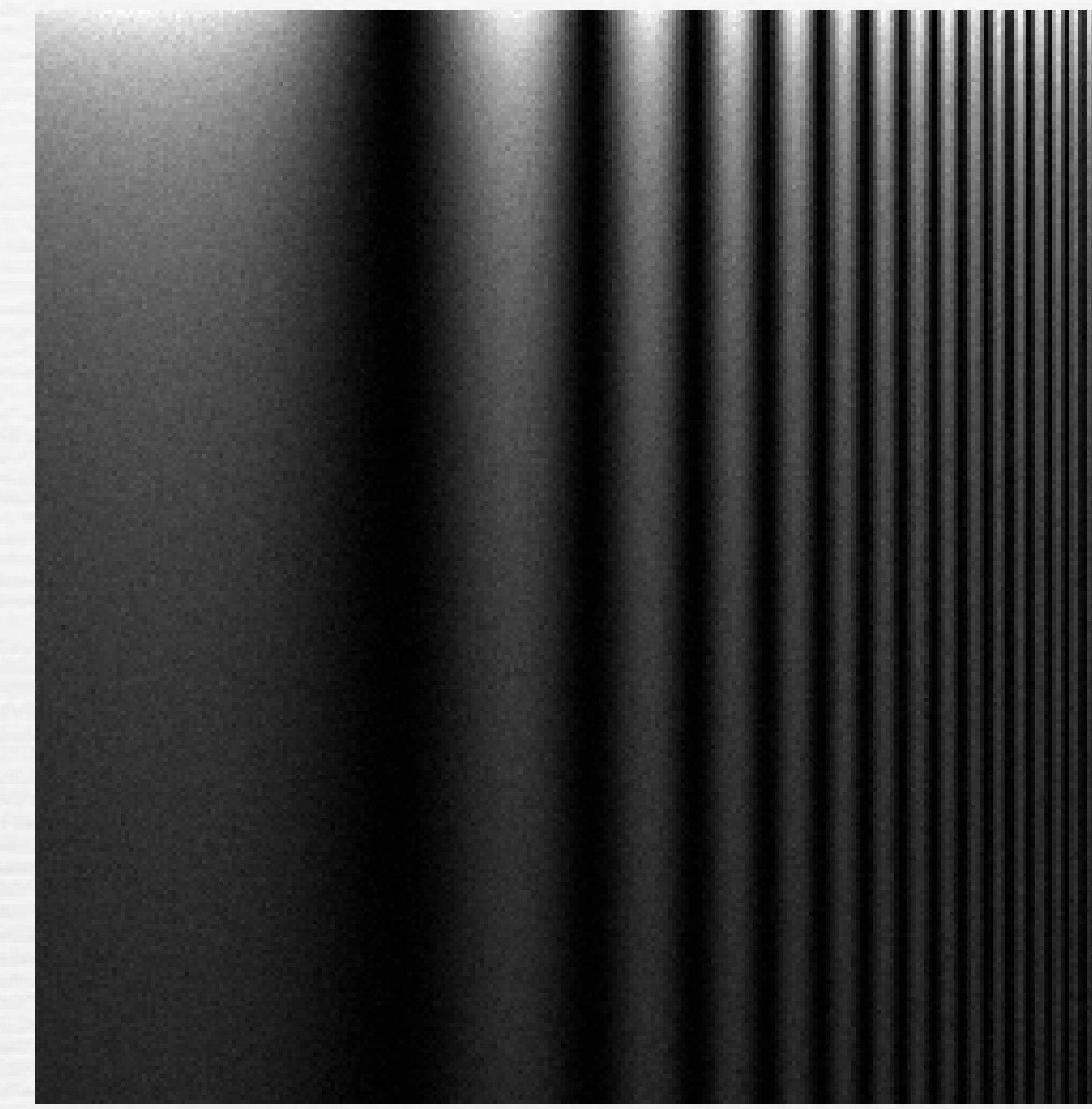


What does this look like?

Test image



1000 ph/pixel,
no read noise

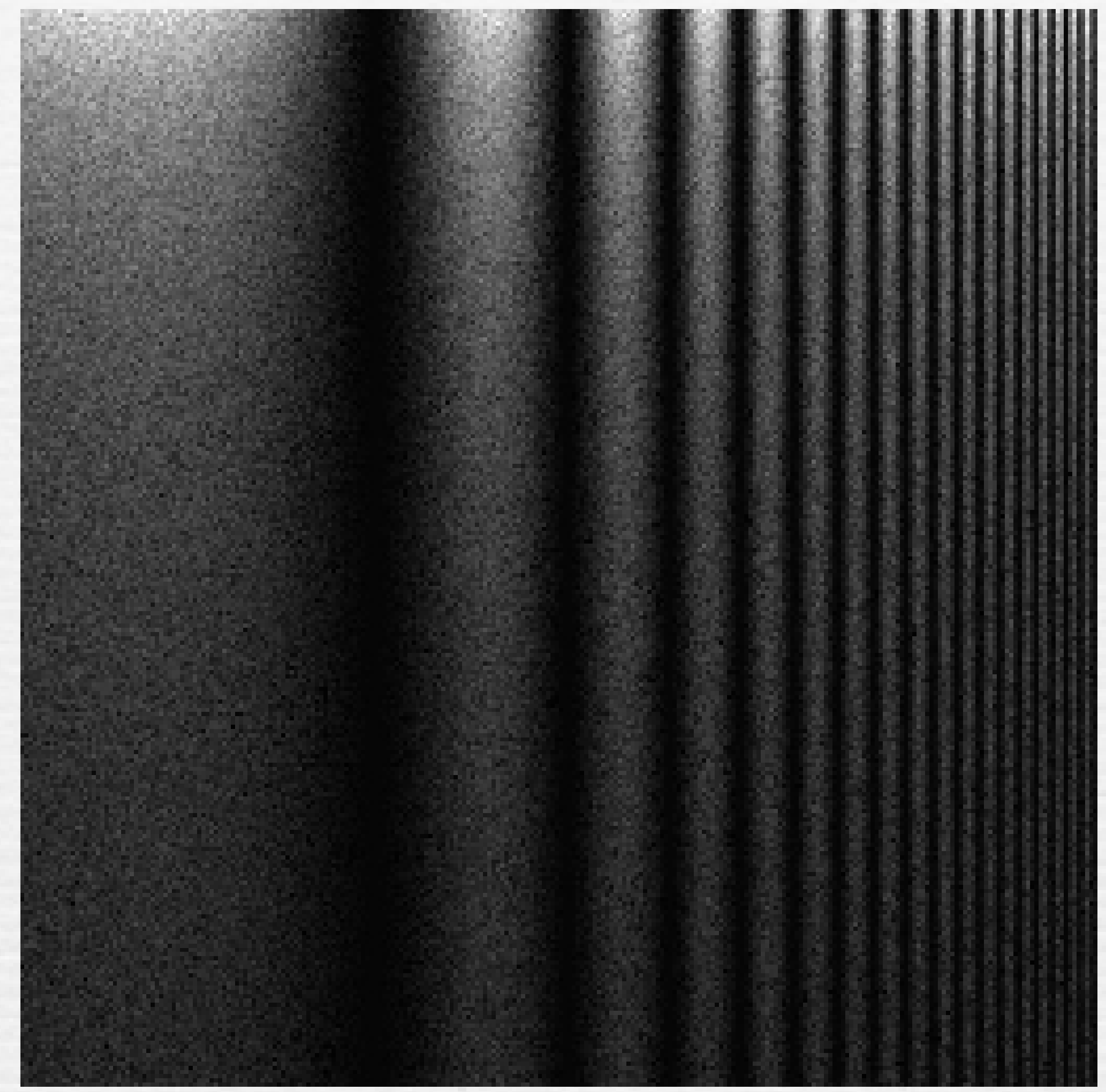


1000 ph/pixel,
5 e^- read noise

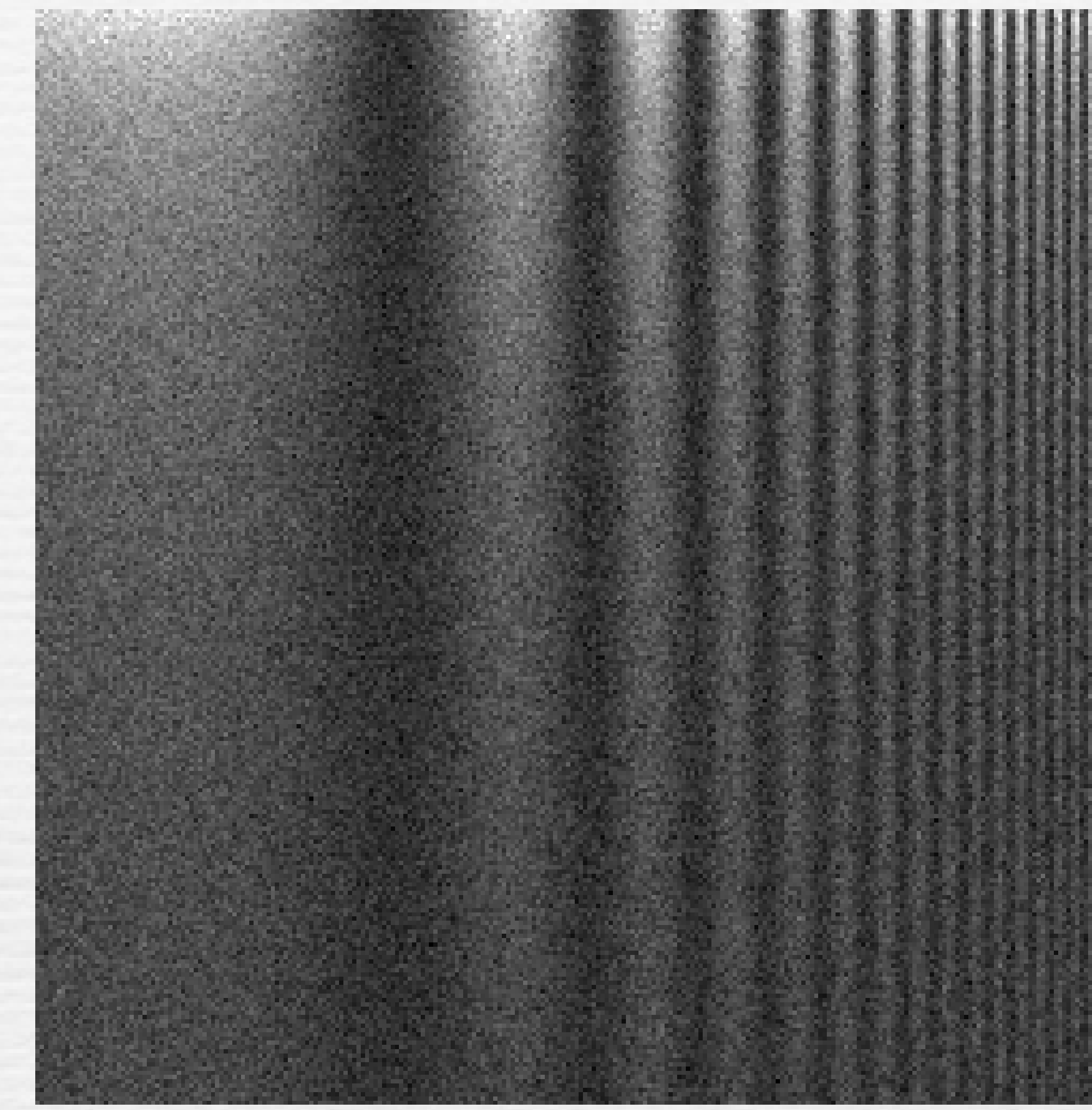
Photon shot noise = 6x read noise

What does this look like?

Test image



100 ph/pixel,
no read noise



100 ph/pixel,
5 e^- read noise

Photon shot noise = 2x read noise

Beating the read-out noise
Intensified CCD (ICCD)

The picture can't be displayed.

Signal/Noise Ratio (SNR)

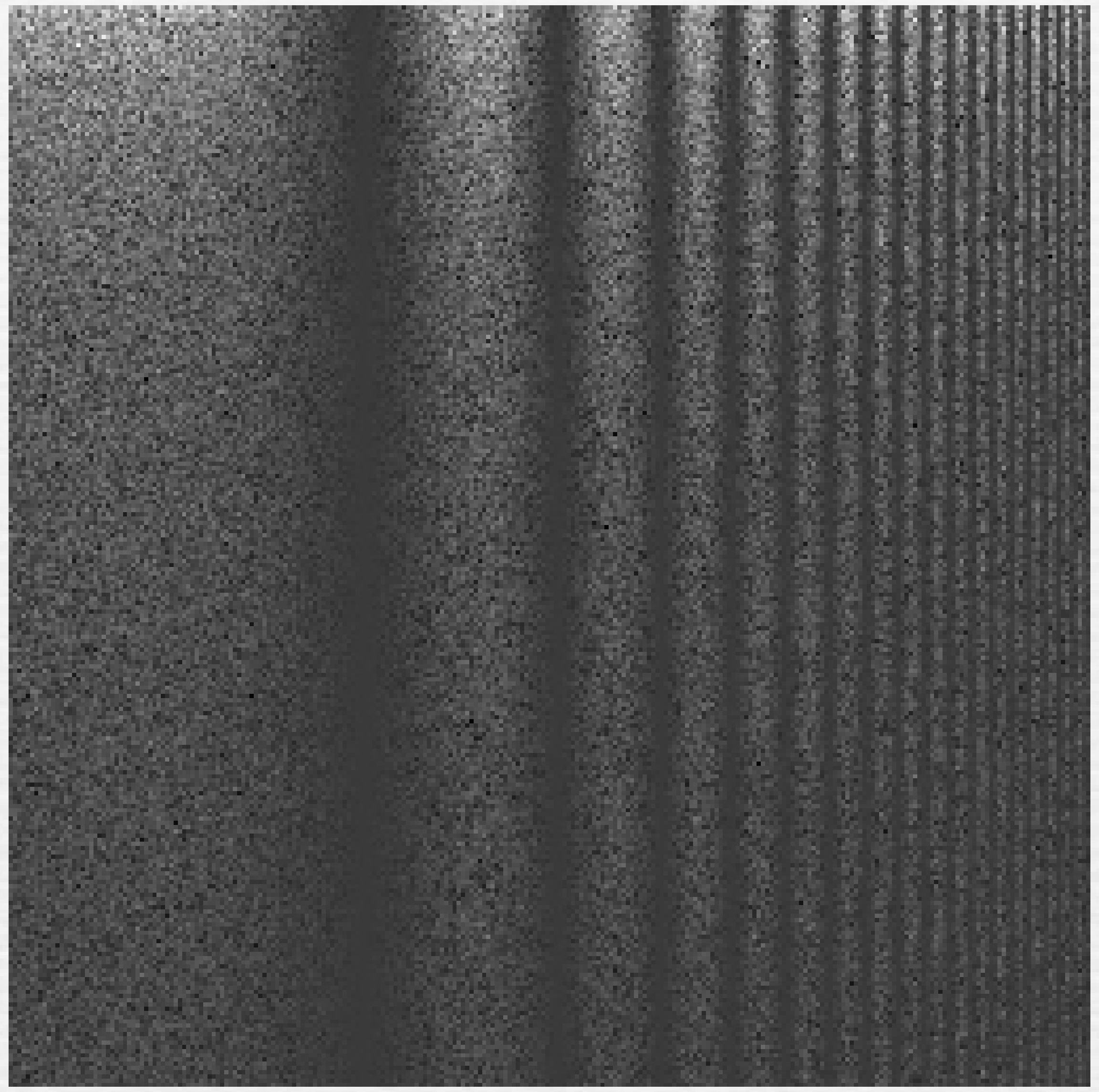
- Read noise dominates whenever read noise^2 >= # of photons

- 8 e- read noise → 64 photons
- 16 e- read noise → 256 photons
- 50 e- read noise → 2500 photons

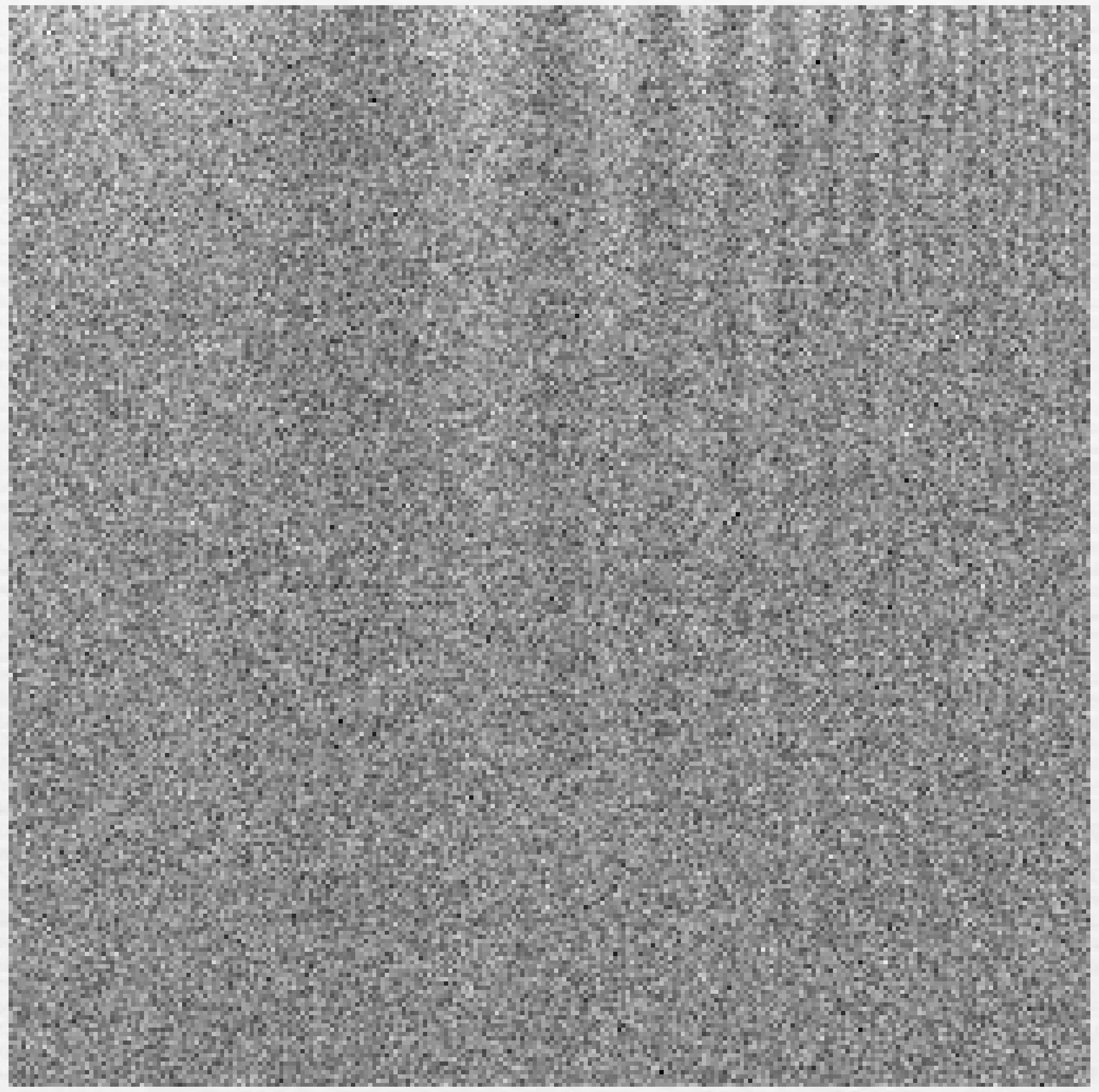
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

Read-noise

Test image



10 ph/pixel,
no read noise



10 ph/pixel,
5 e$^-$ read noise

Photon shot noise = 1/3 x read noise