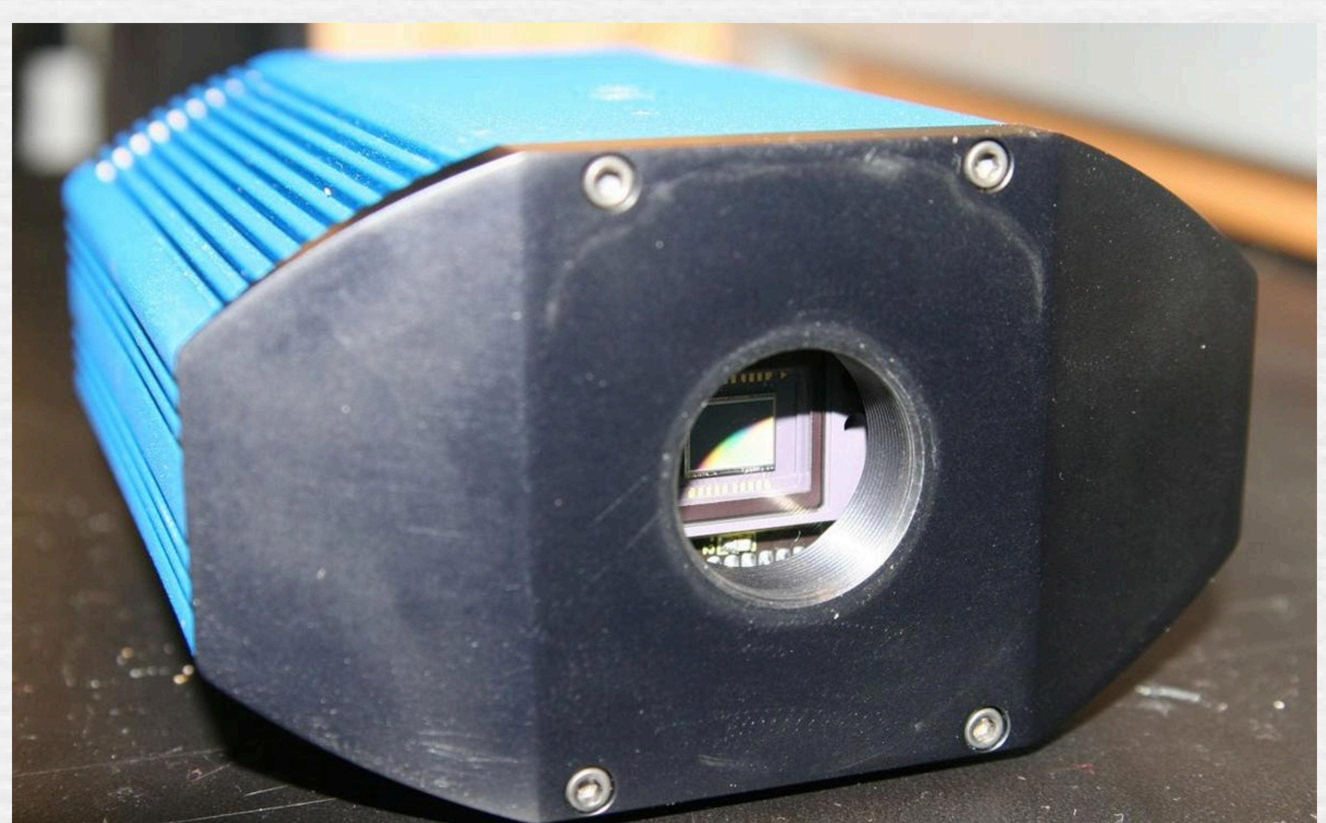
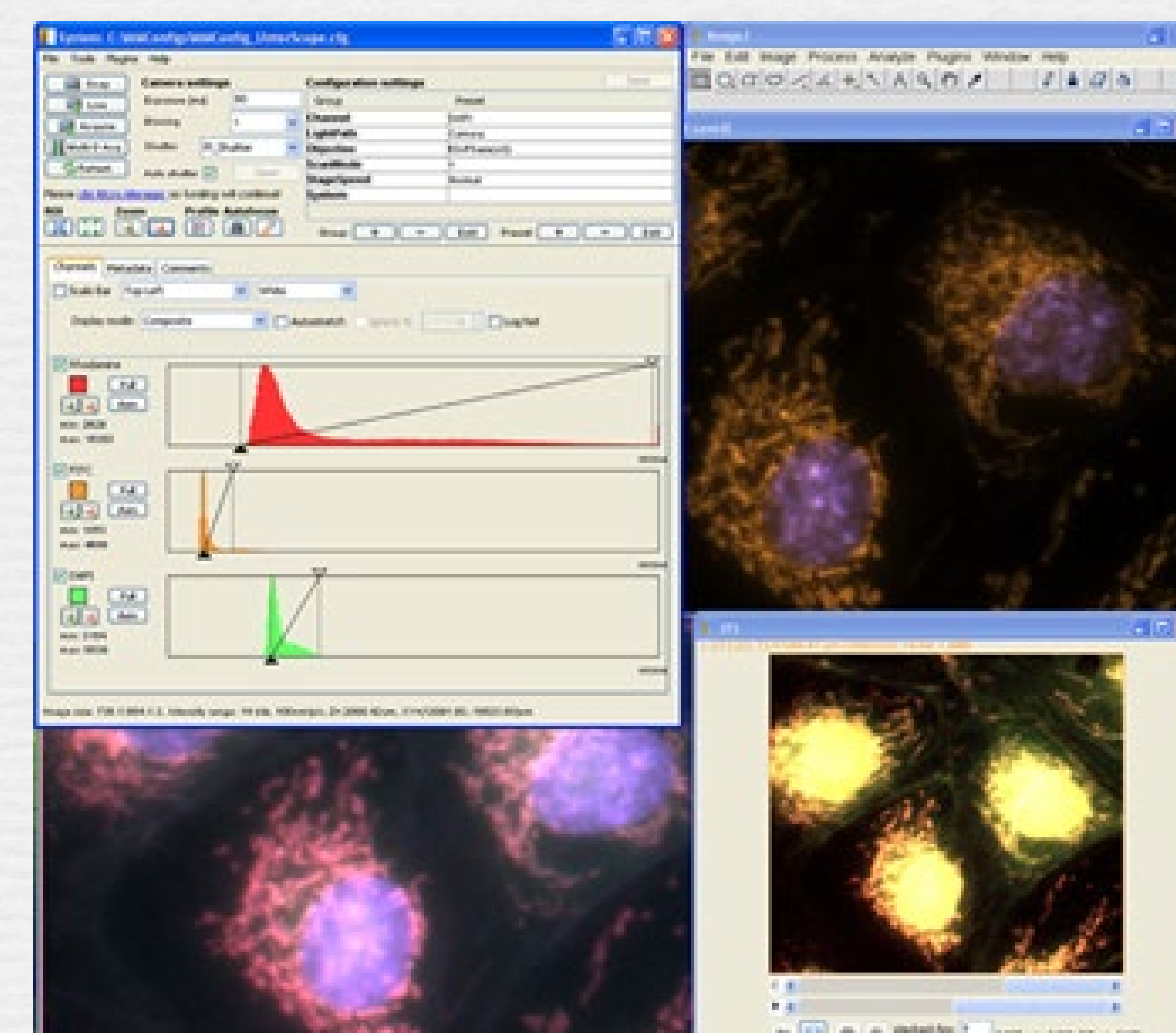
Digital microscopy: Image detectors and software control

Nico Stuurman, UCSF/HHMI
UCSF, Mar 27, 2012

Outline

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

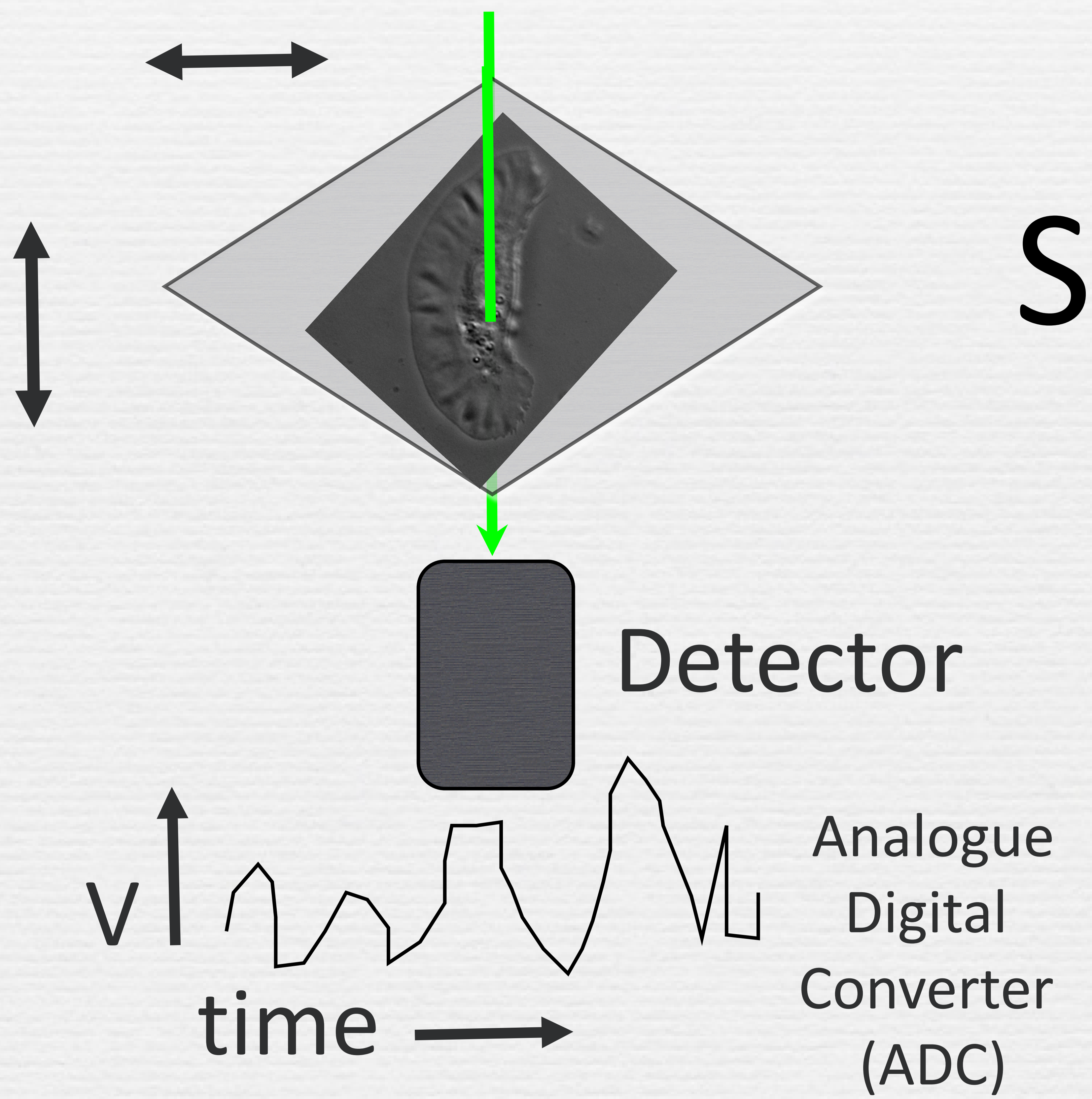




Imaging Detectors

Photon -> Electrons -> Voltage ->Digital Number

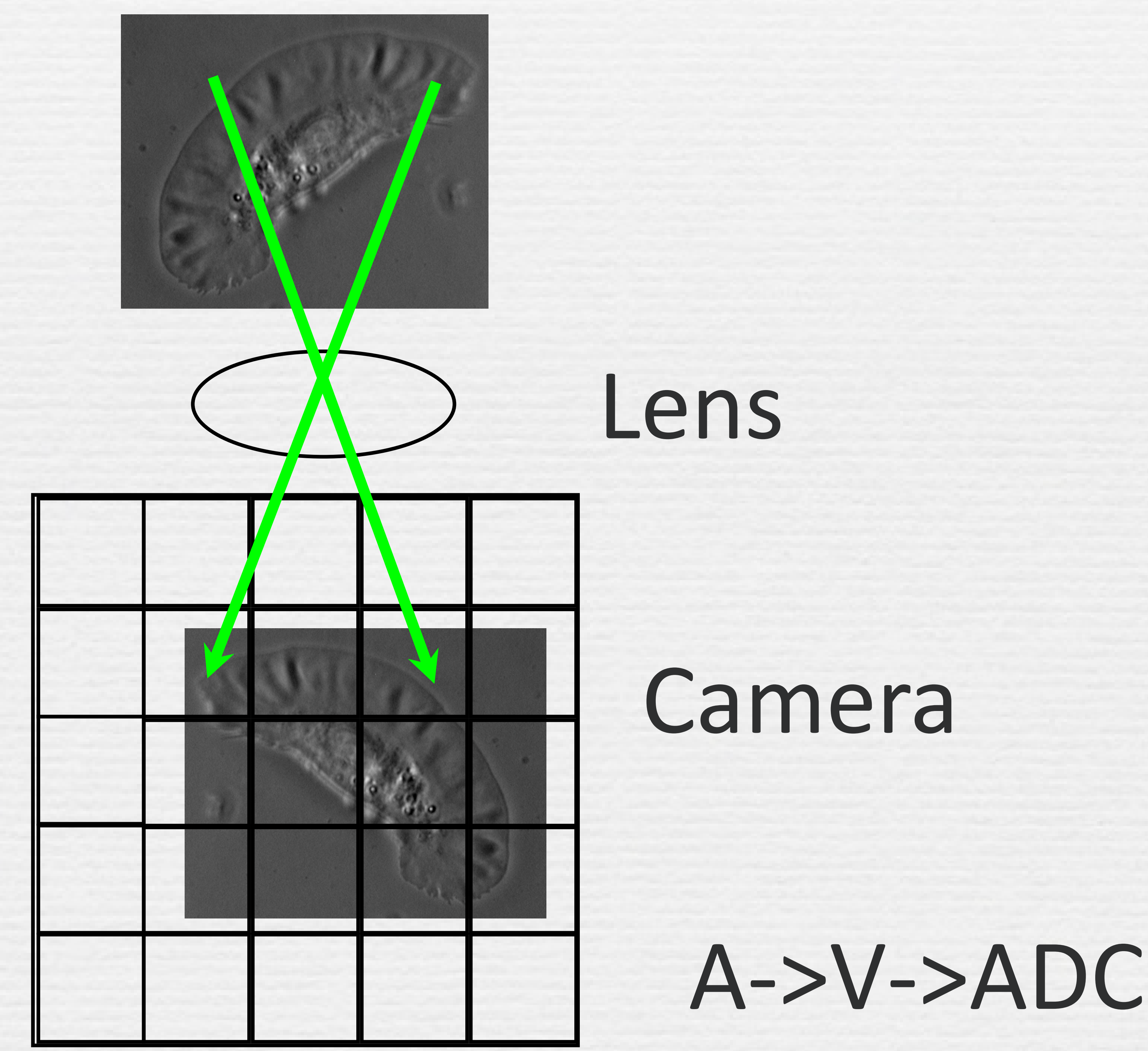
Single point detector



10 200 35 12 90 85 105 73 80 95

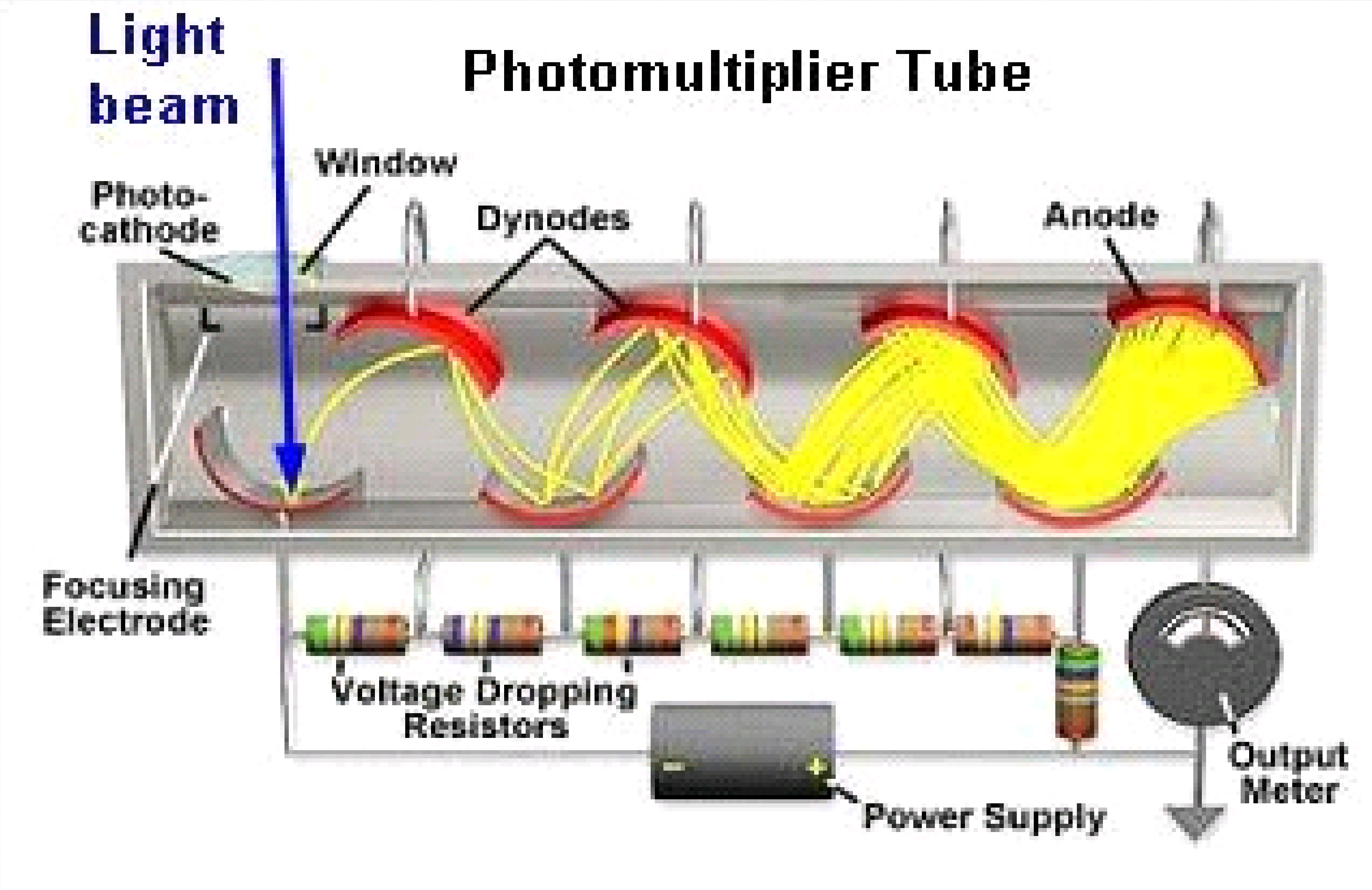
Speed!

Multi point detector (camera)



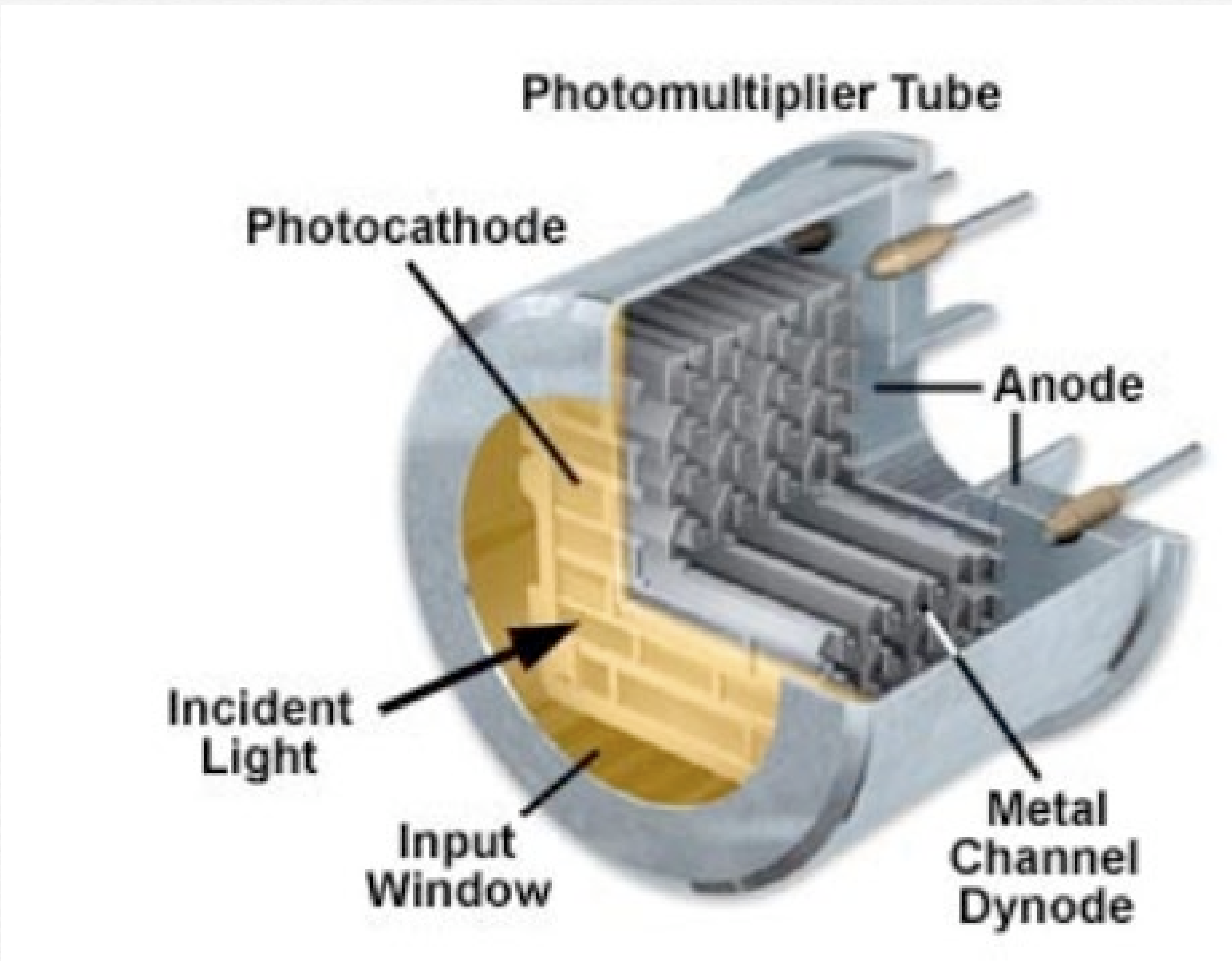
10 200 35 12 90 85 105 73 80 95

Photo-Multiplier Tube (PMT)



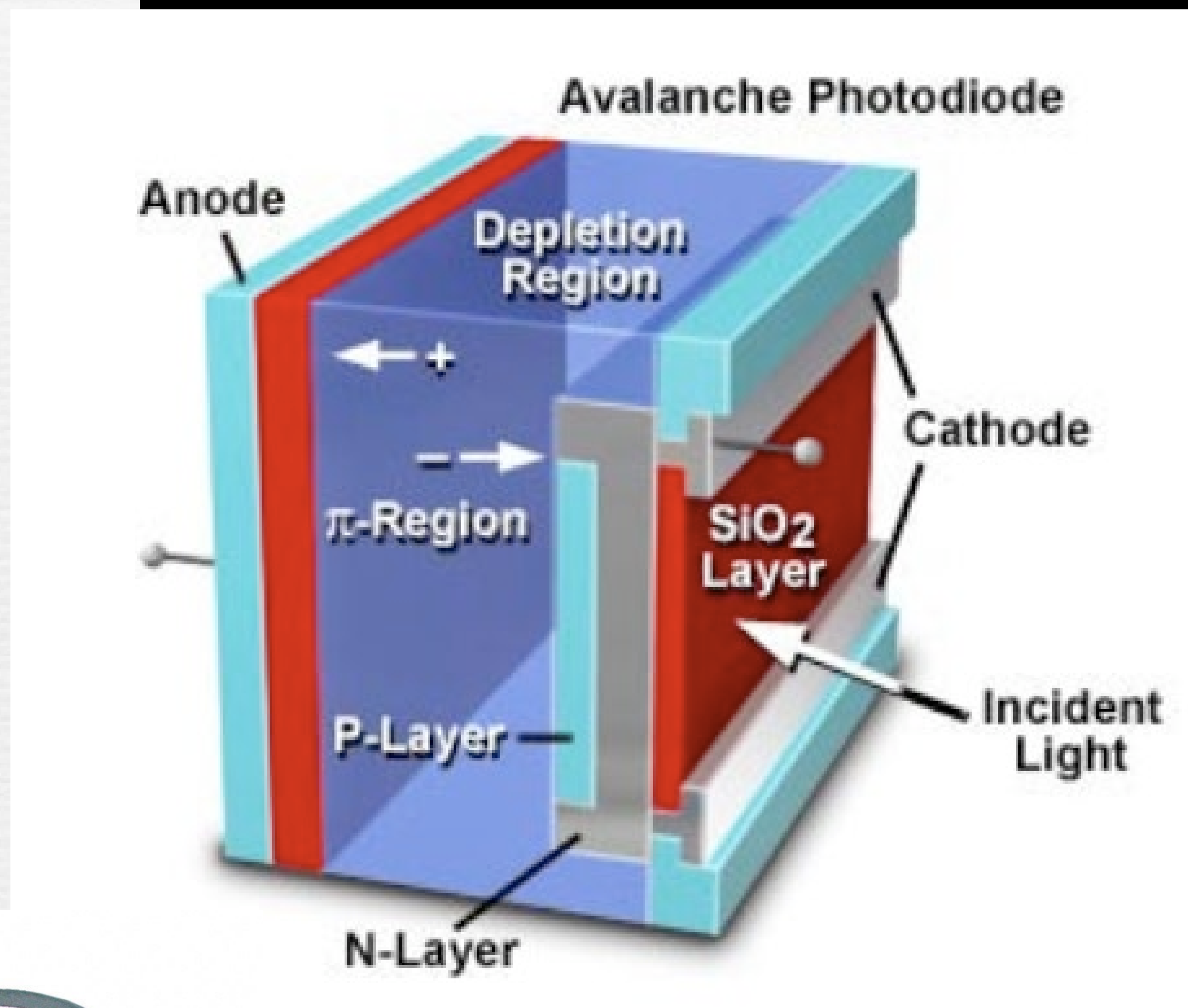
- Very linear
- Very High Gain
- Fast response
- Poor Quantum efficiency (~25%)

PMT modes



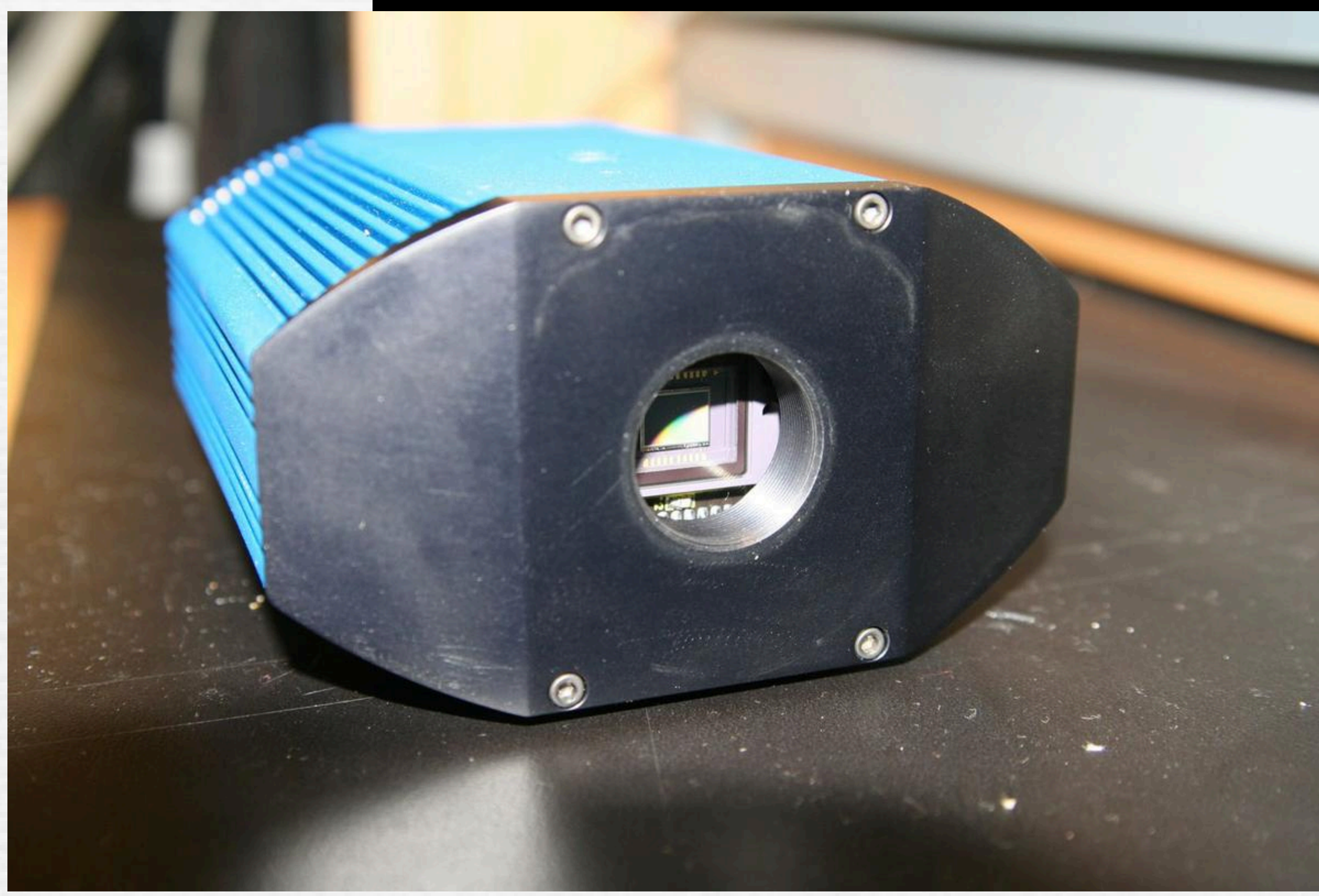
- Photon counting mode:
  - Count pulses
  - Zero background
  - Slow
- Linear mode
- Measure current
- Fast but noisy

Avalanche Photo Diode

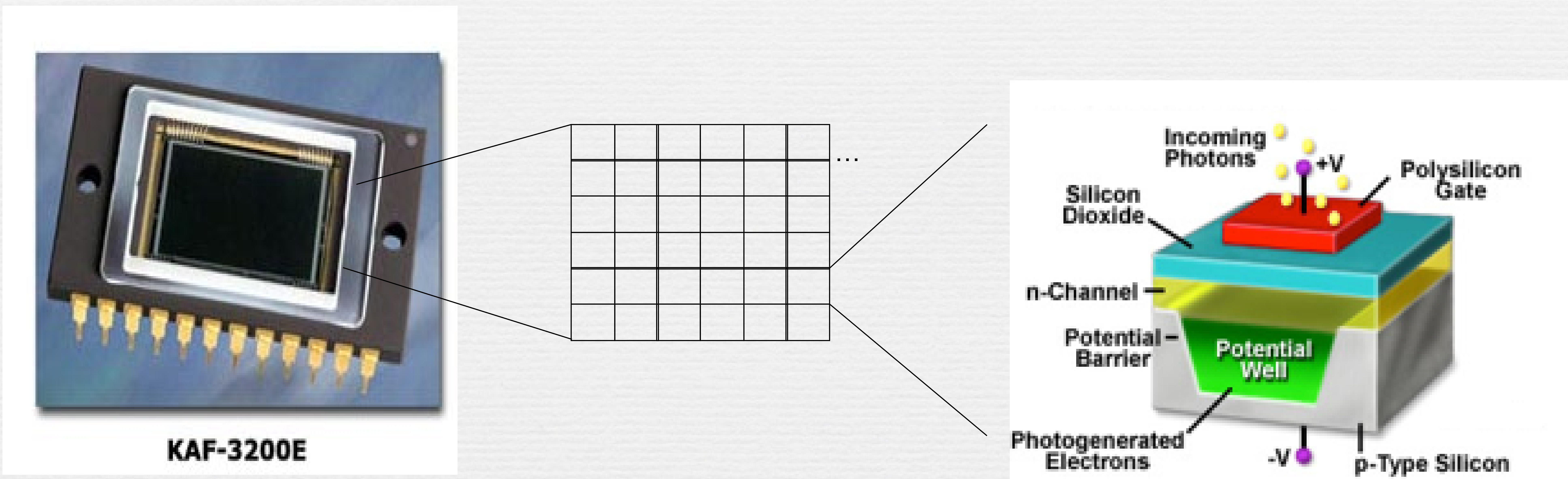


- Absorbed photons->electron
- Electrons amplified by high voltage and 'impact ionization'
- High QE (~90%)
- Photon-counting ability (different design)
- Overheats if run too fast

Cameras in Microscopy

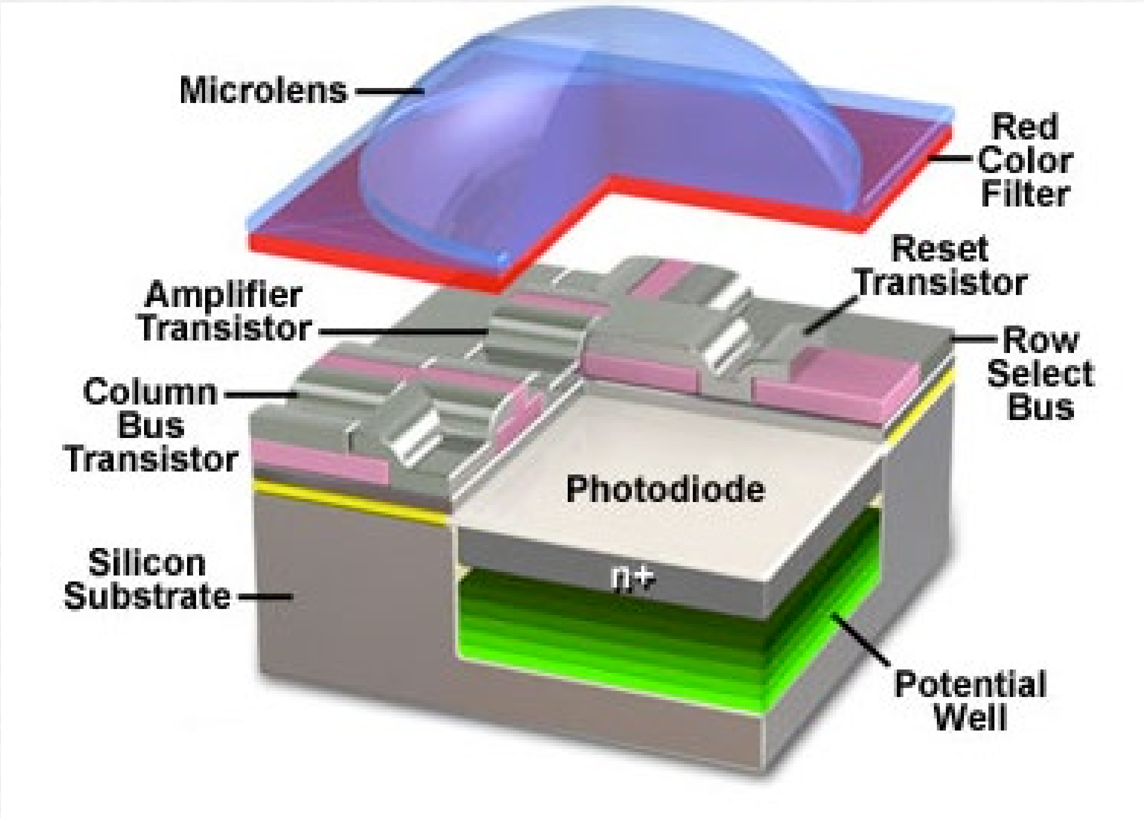


Arrays of photo-sensitive elements



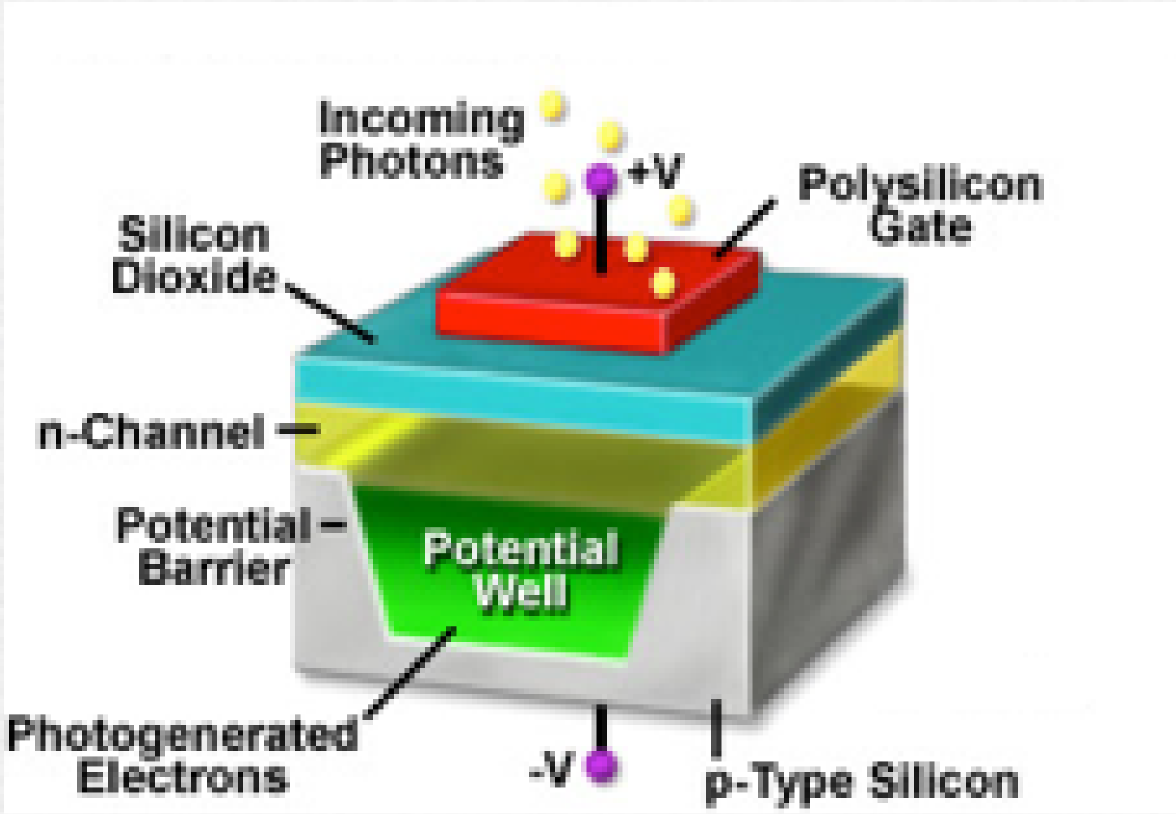
Two Architectures:

Complementary Metal Oxide Semiconductor (CMOS)



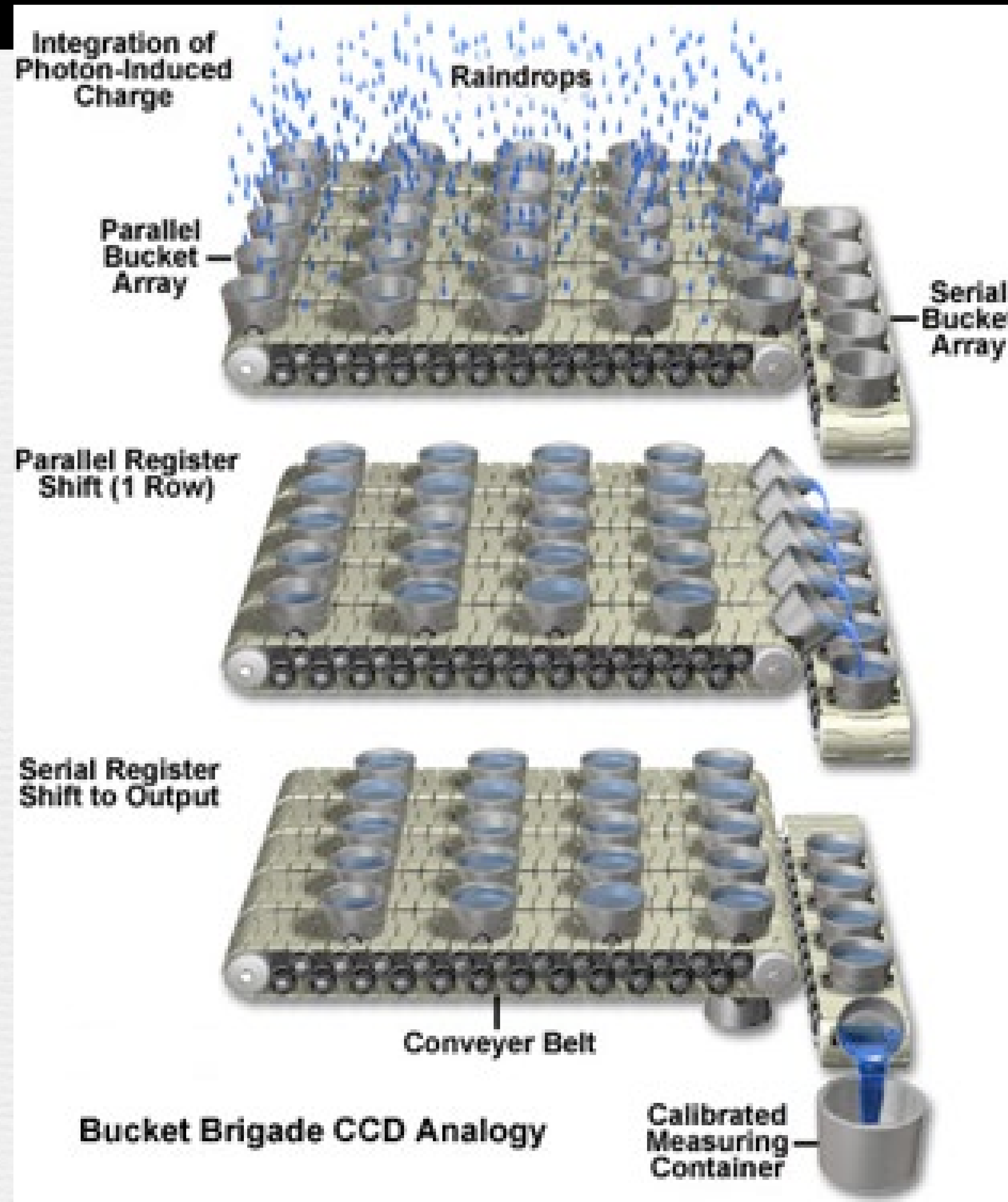
Each pixel has an amplifier
Transfers voltage
Fast
Noisy

Charged Coupled Devices (CCD)

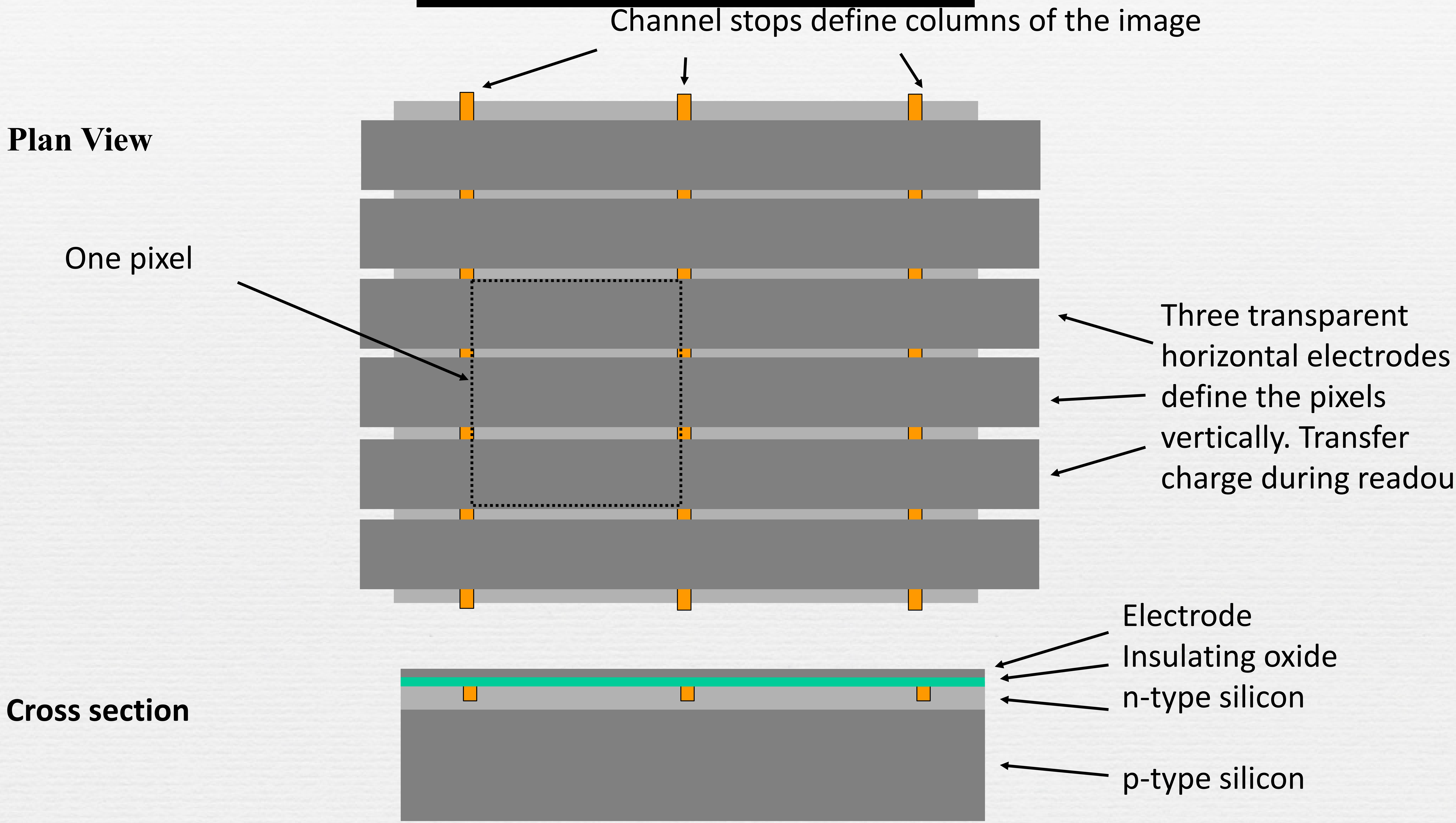


Single read-out amplifier
Transfers charge
Slow
Precise

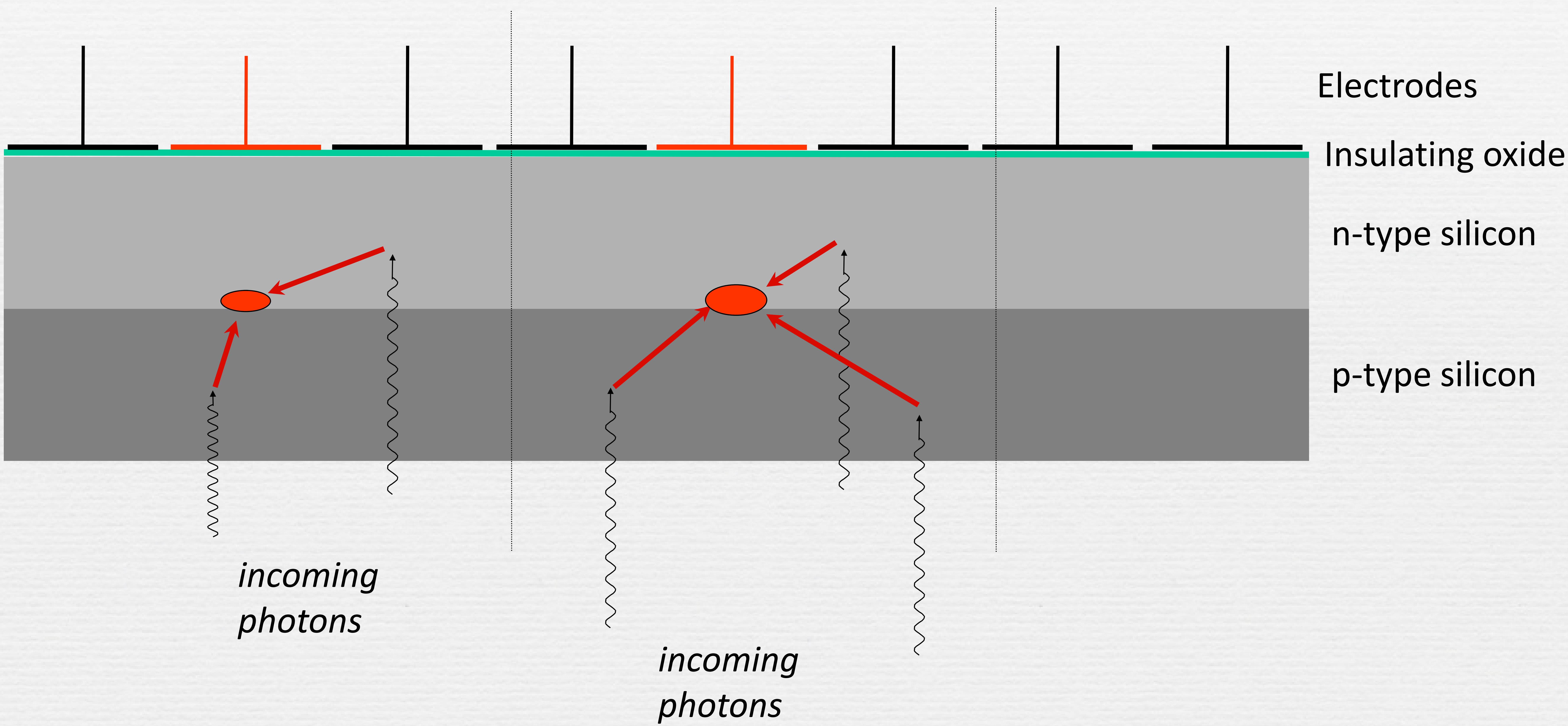
CCD readout "bucket-brigade" analogy



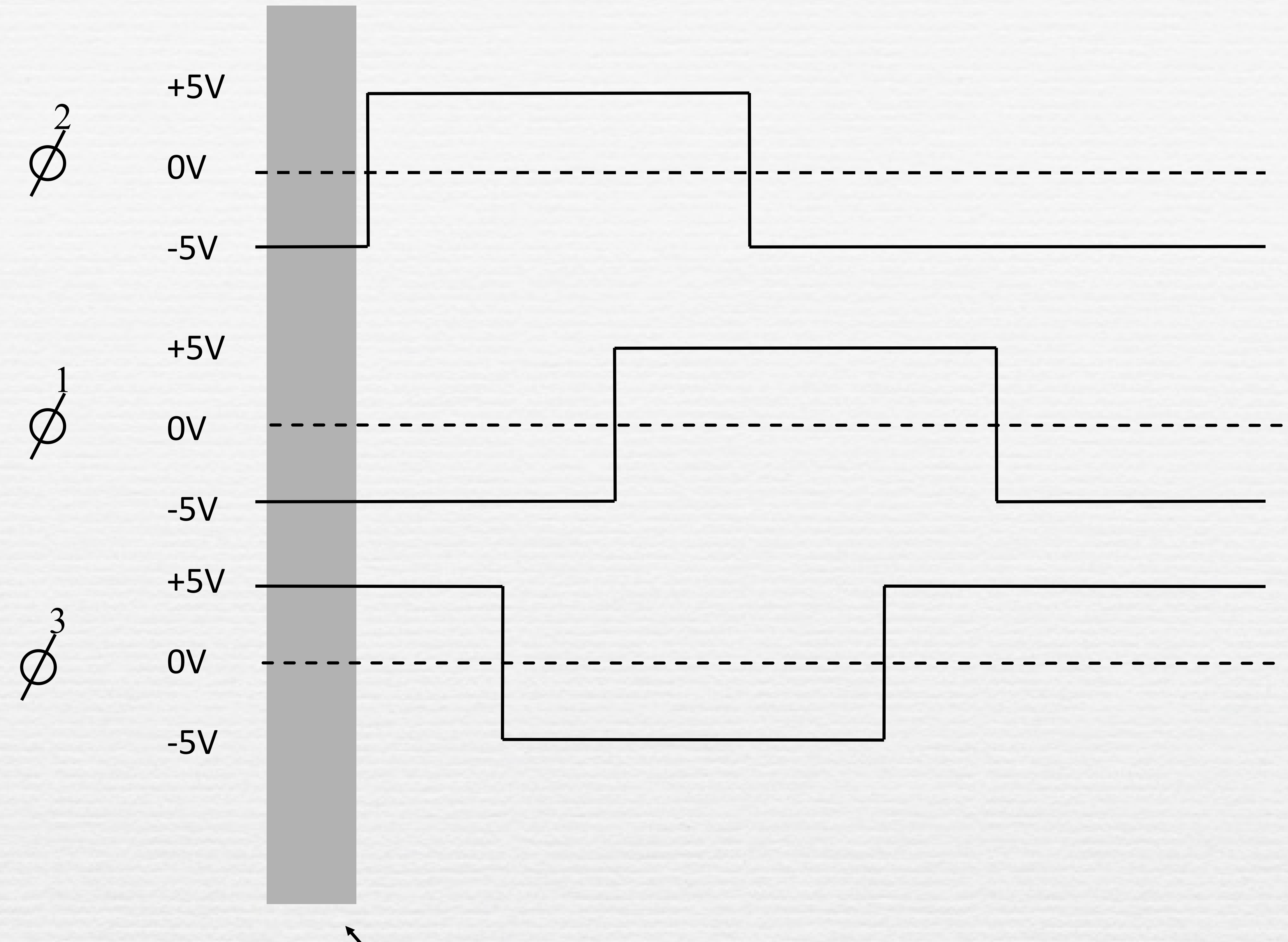
CCD Architecture



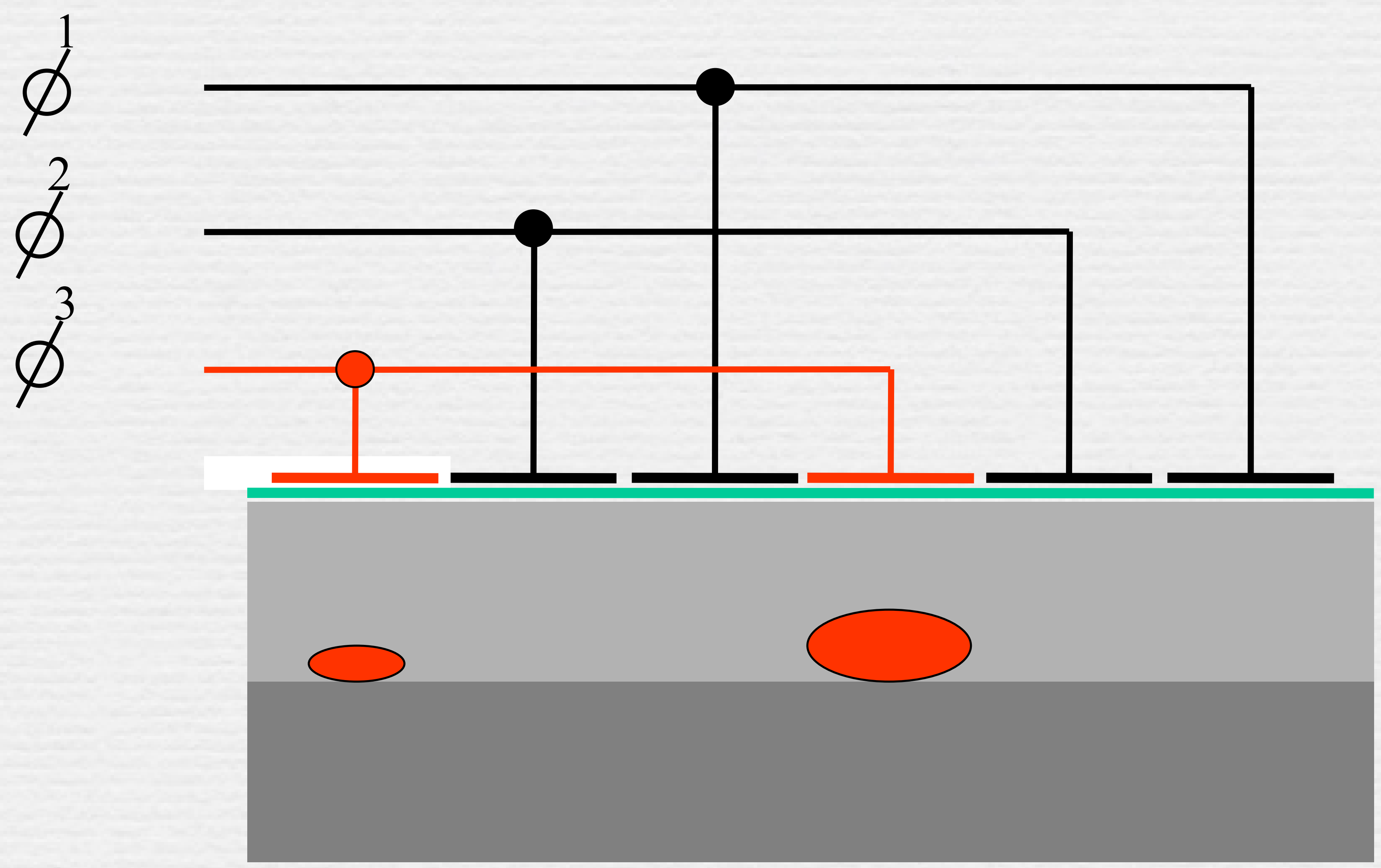
CCD Architecture



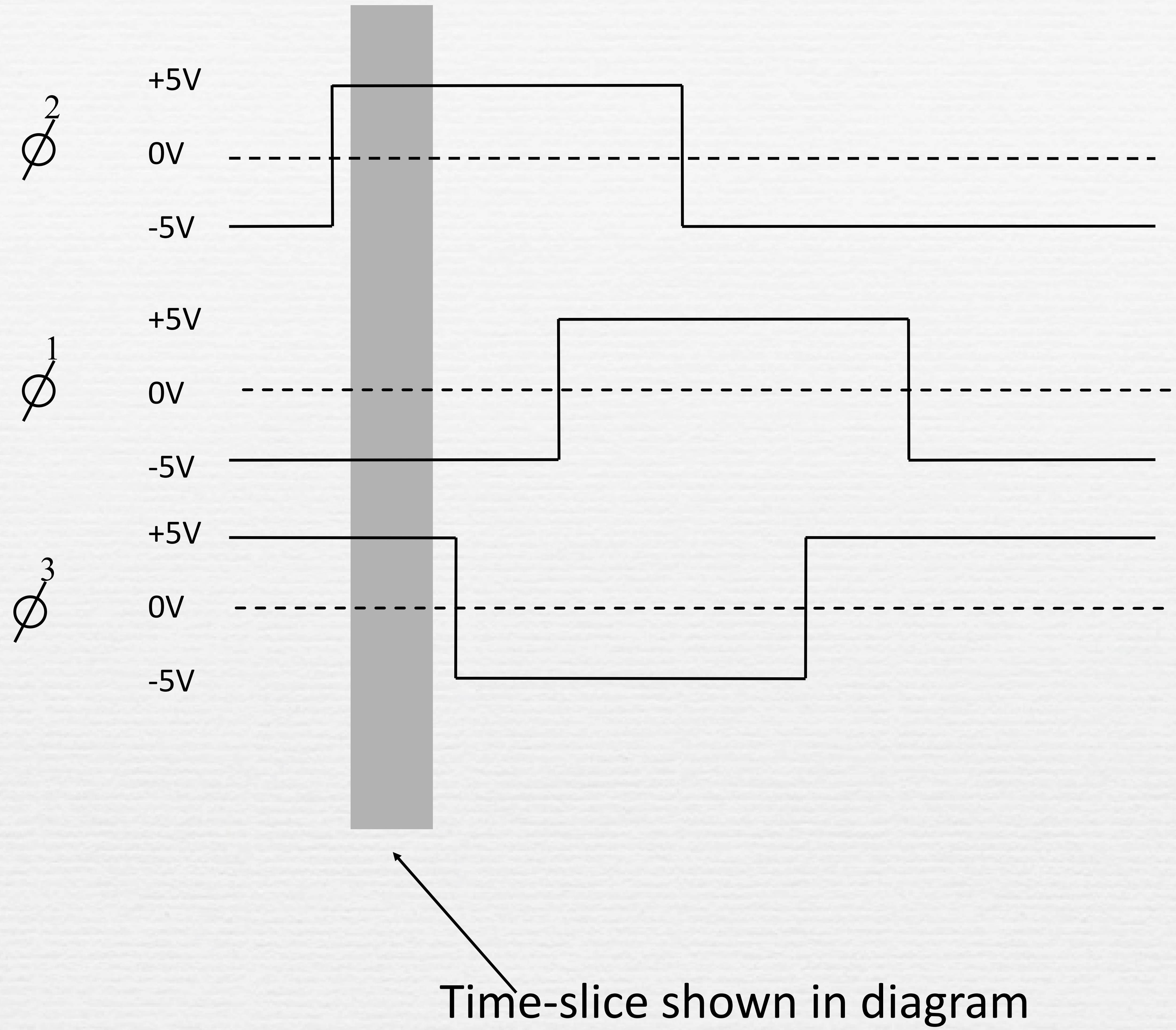
Charge Transfer in a CCD

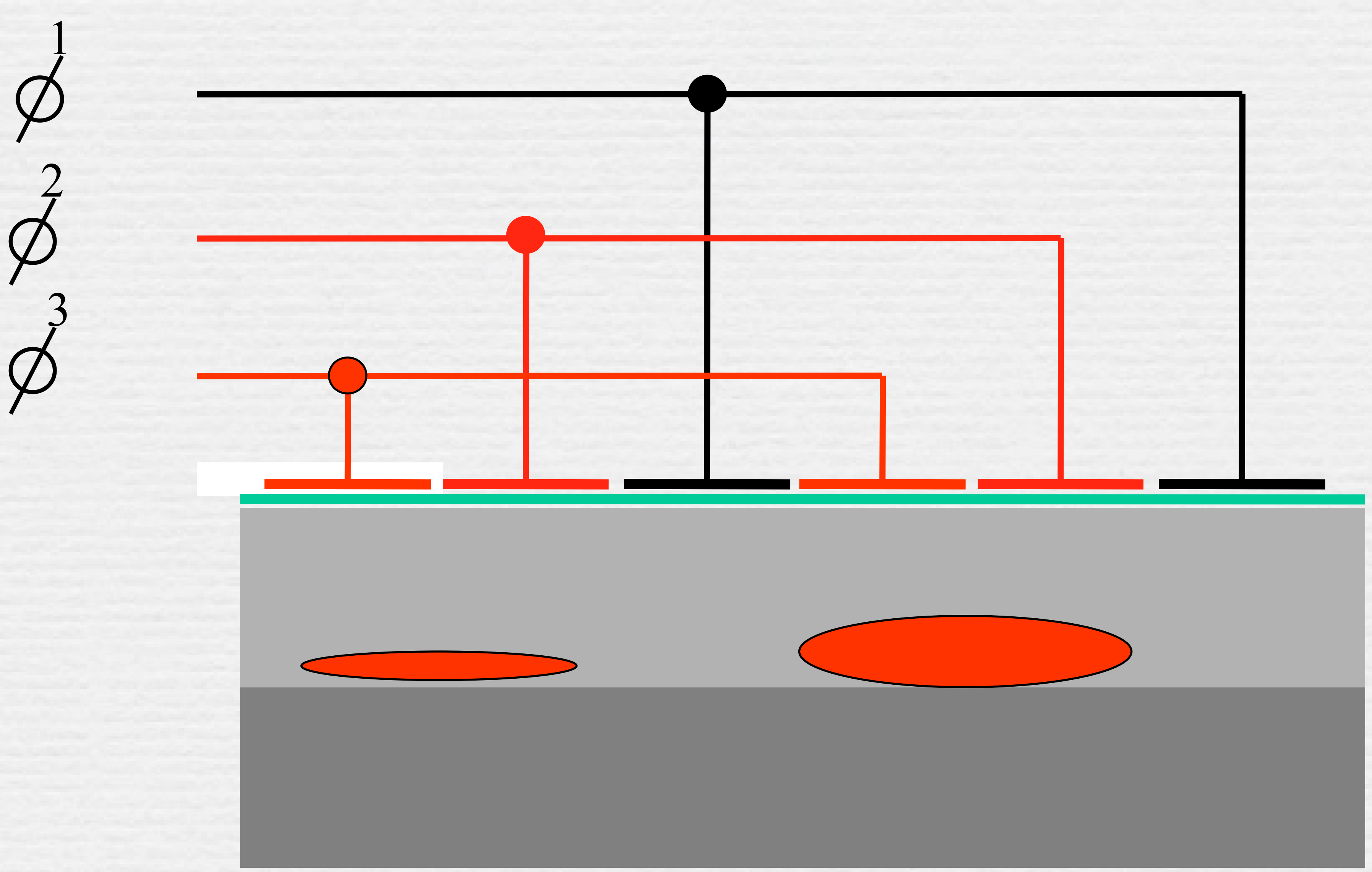


Time-slice shown in diagram

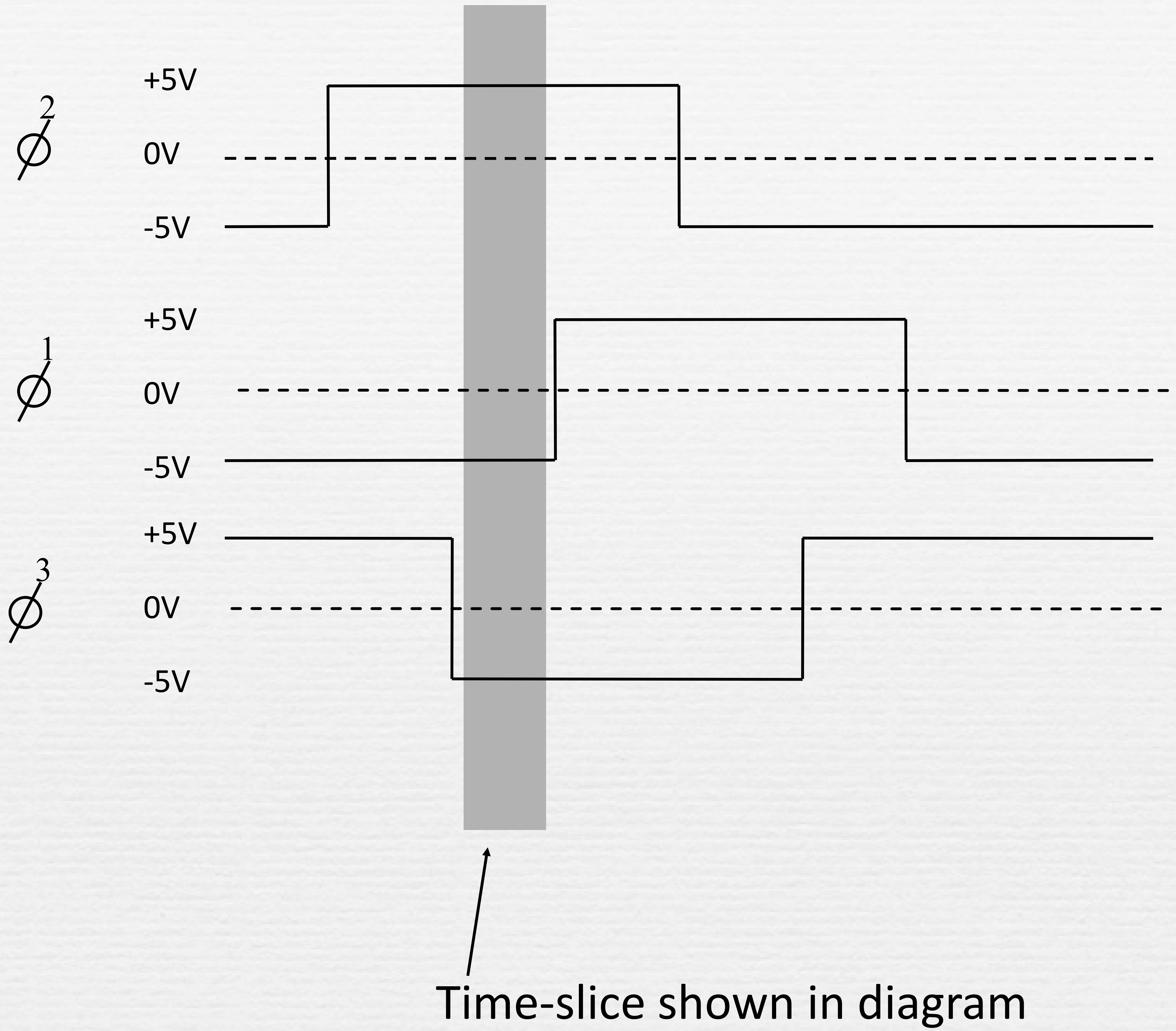


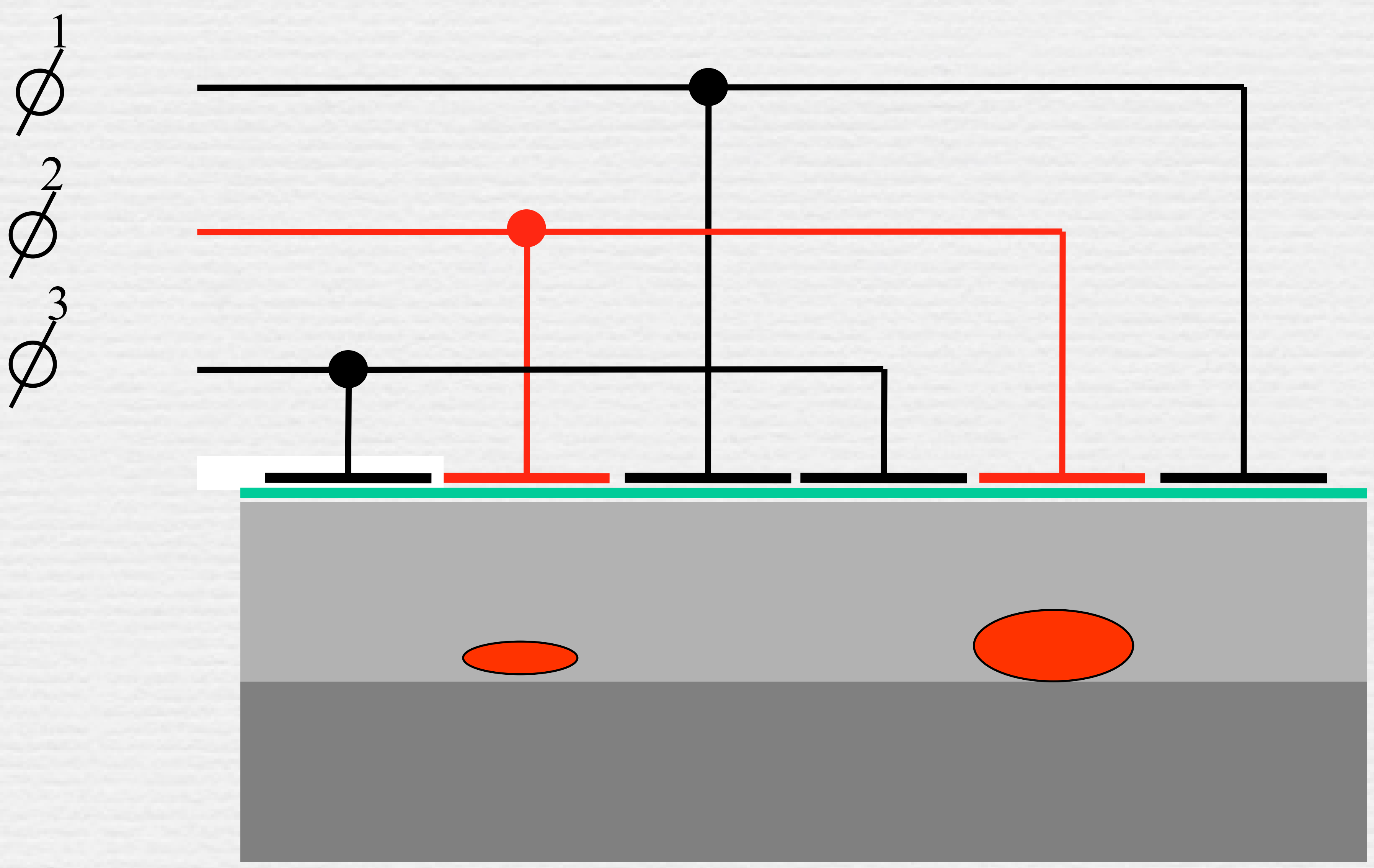
Charge Transfer in a CCD



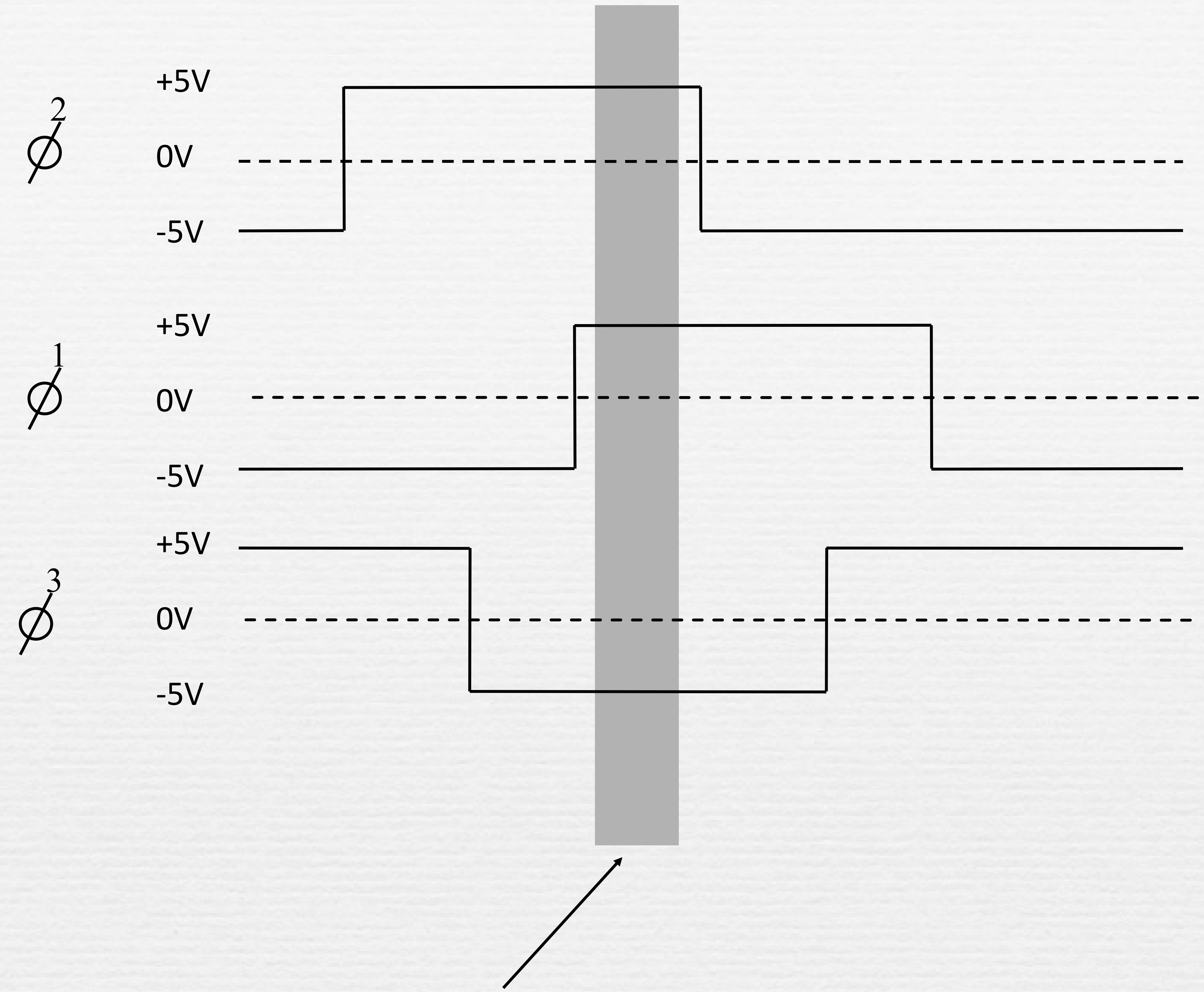


Charge Transfer in a CCD

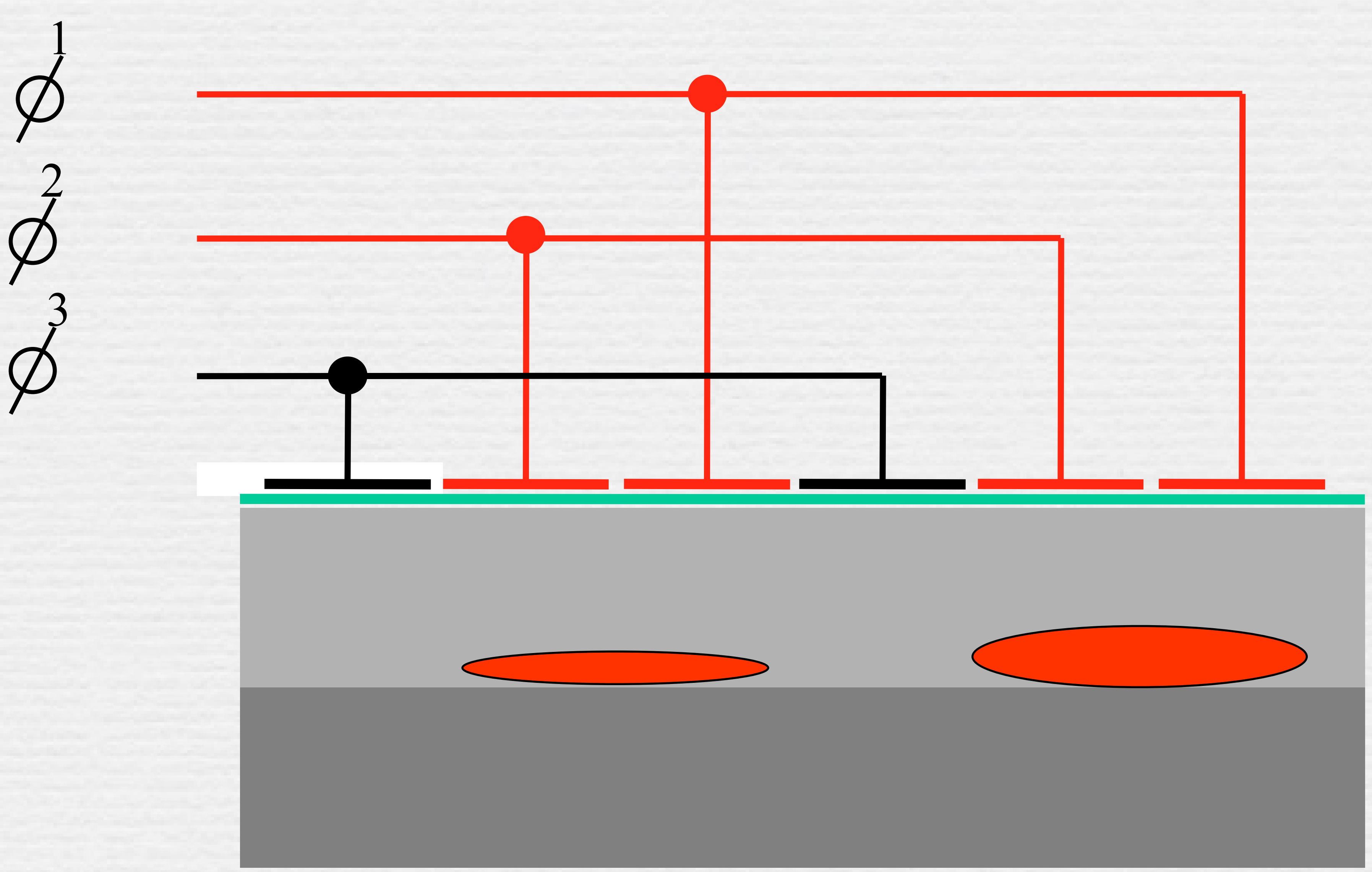




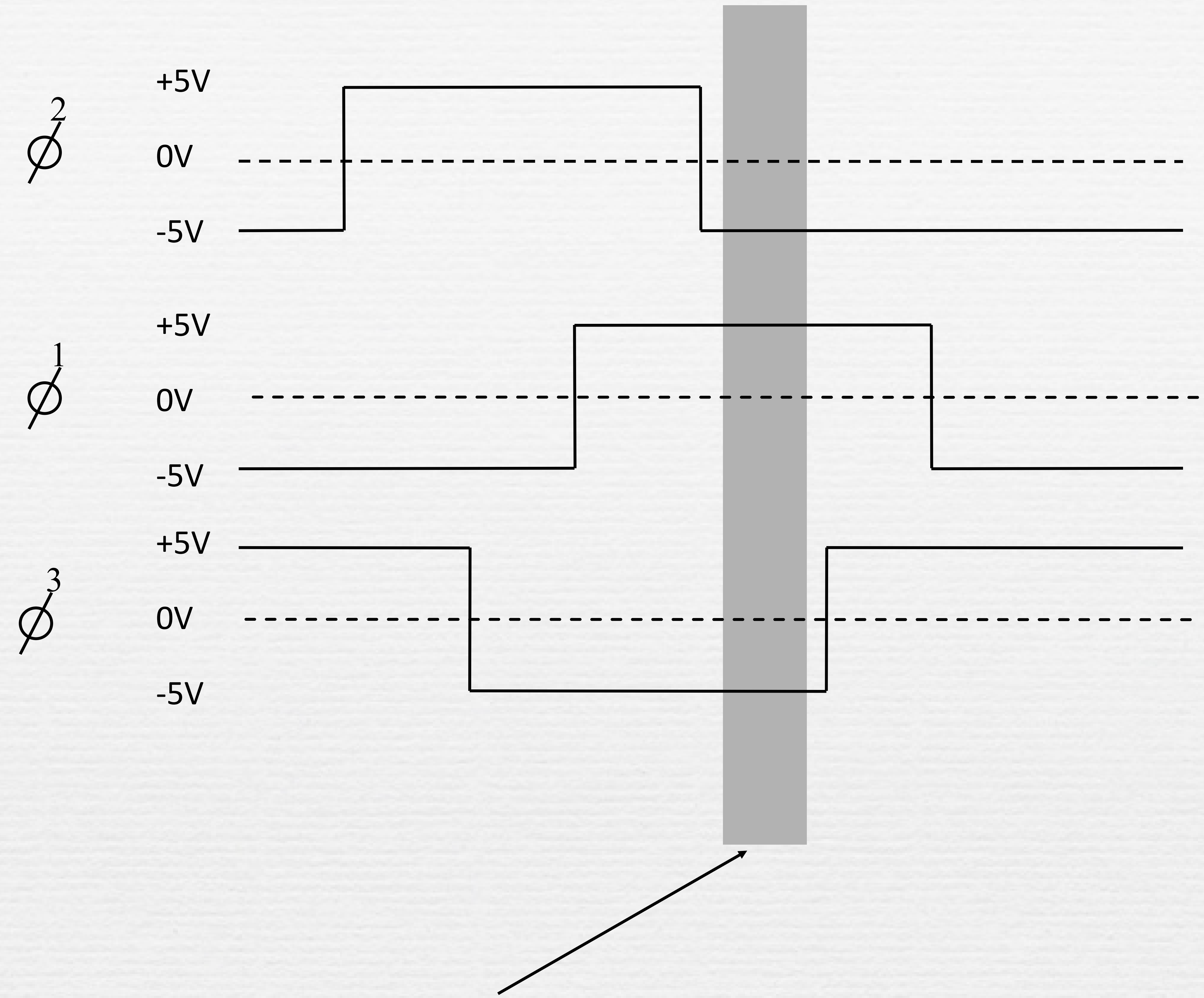
Charge Transfer in a CCD



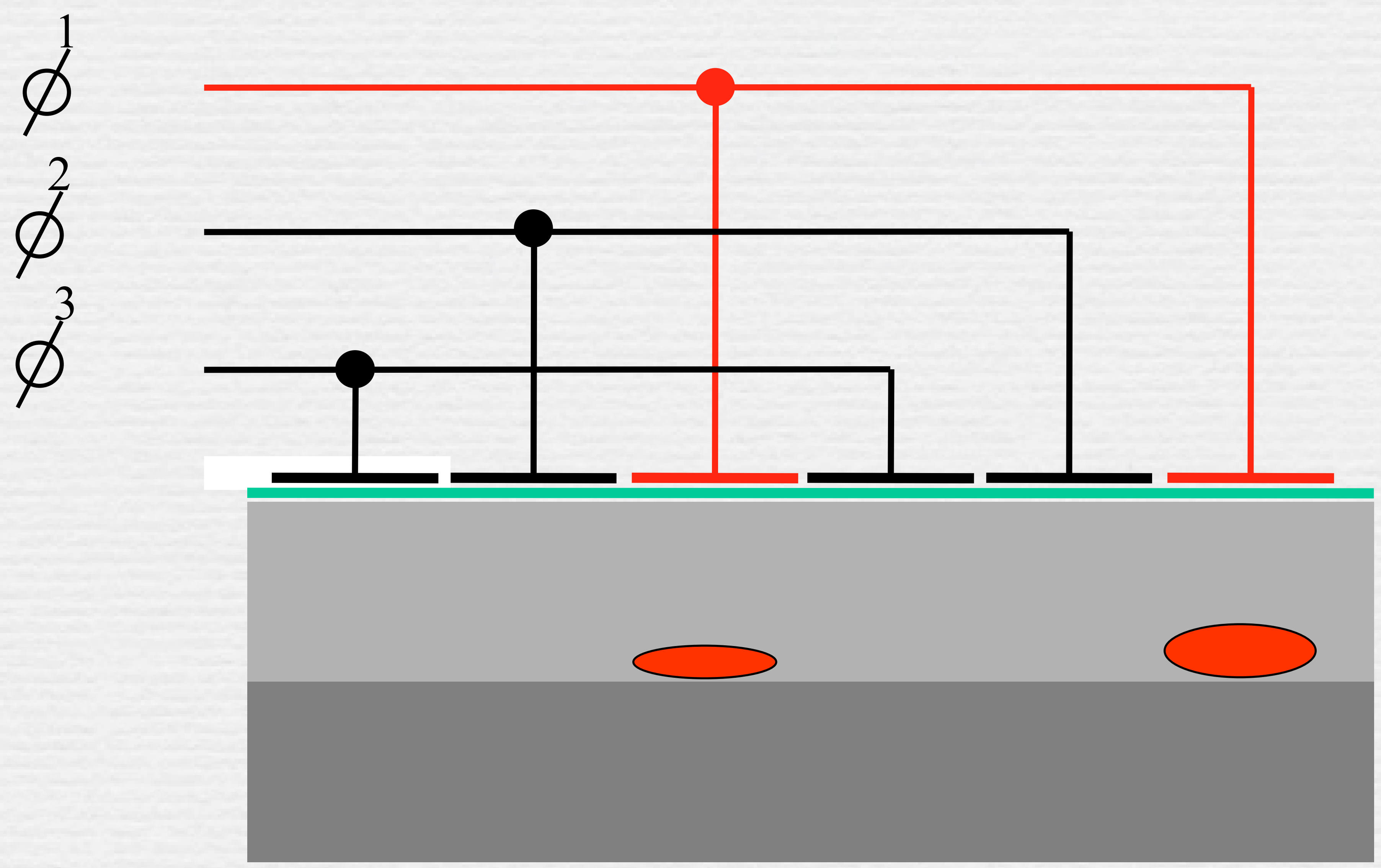
Time-slice shown in diagram



Charge Transfer in a CCD

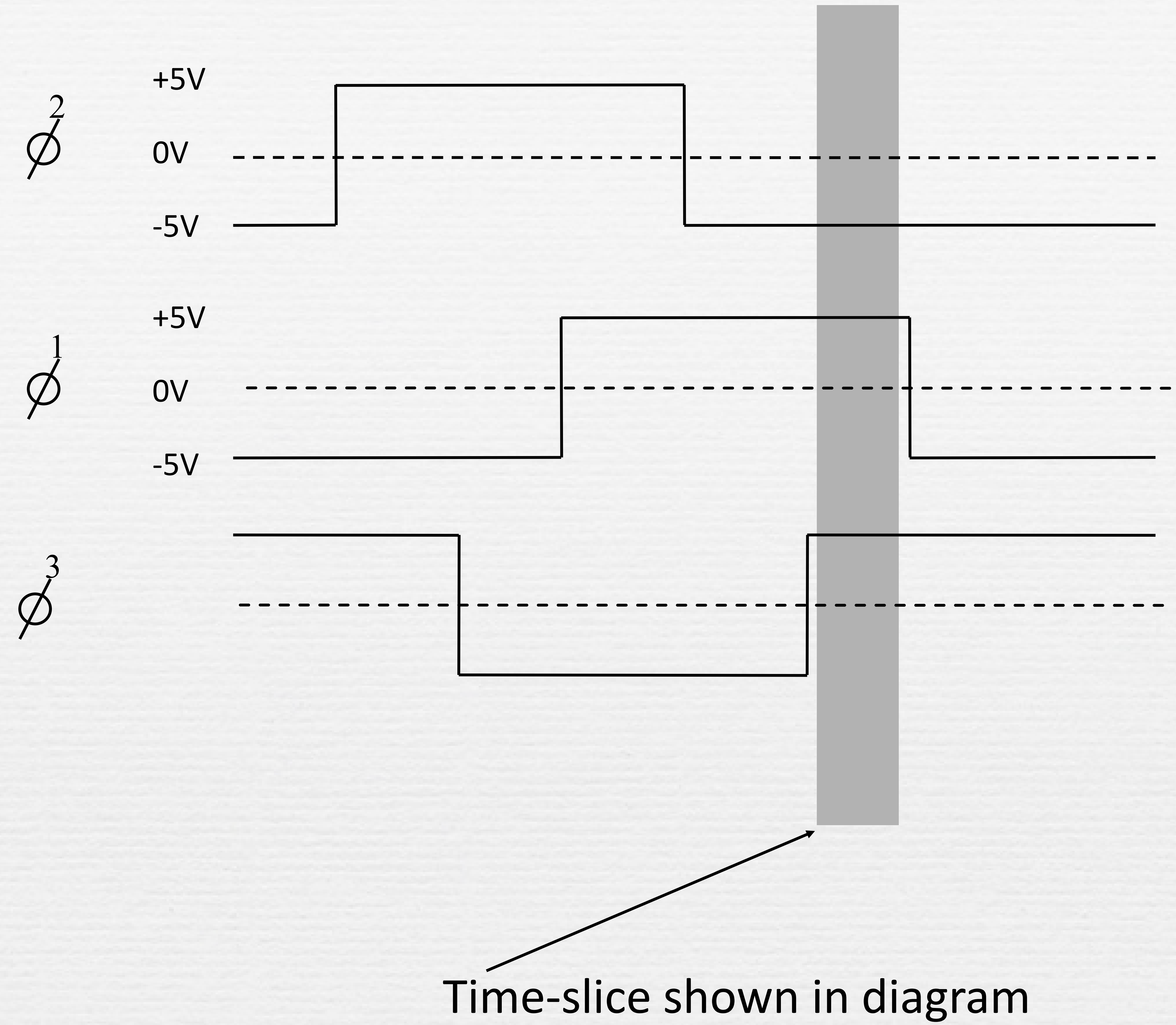


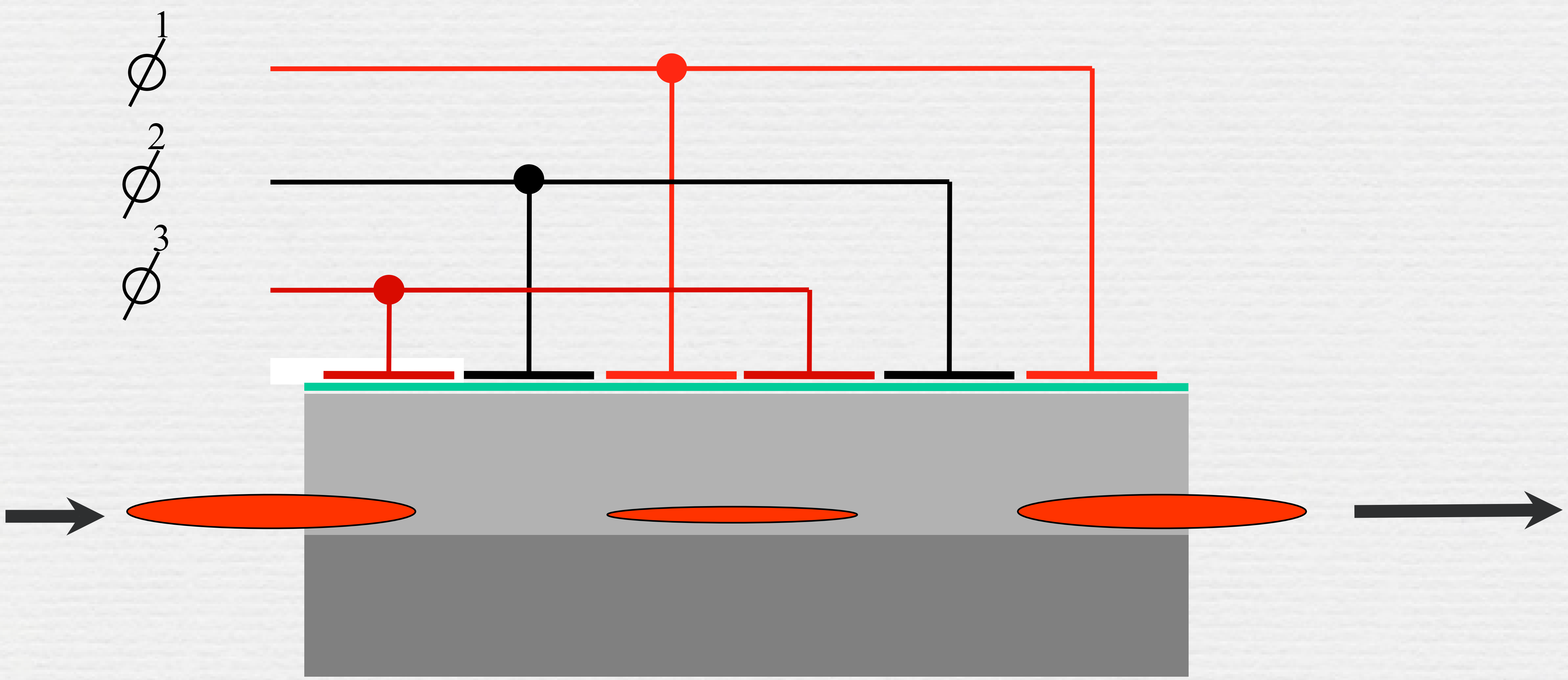
Time-slice shown in diagram



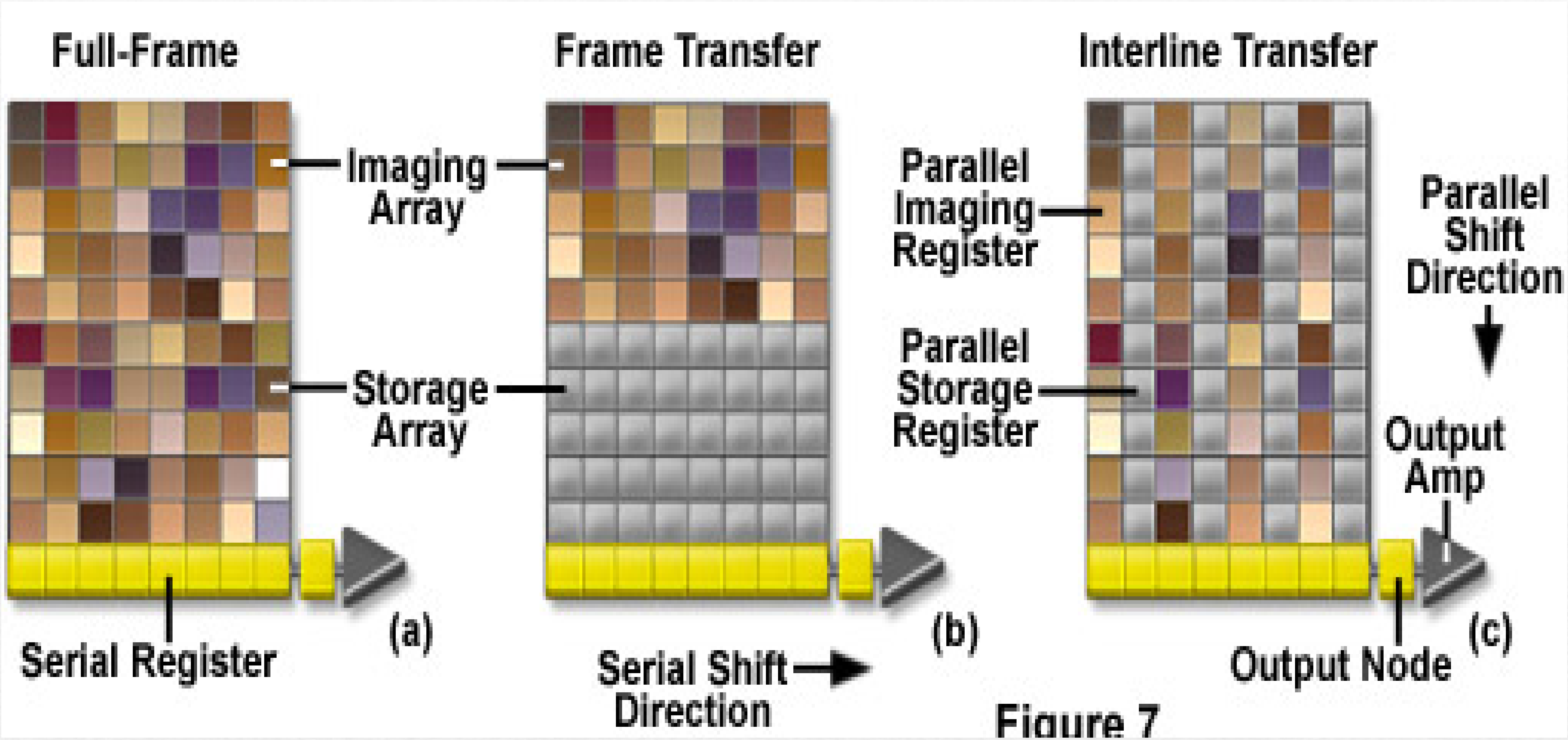
Charge Transfer in a CCD

Transfer charge to the next pixel





CCD Architectures



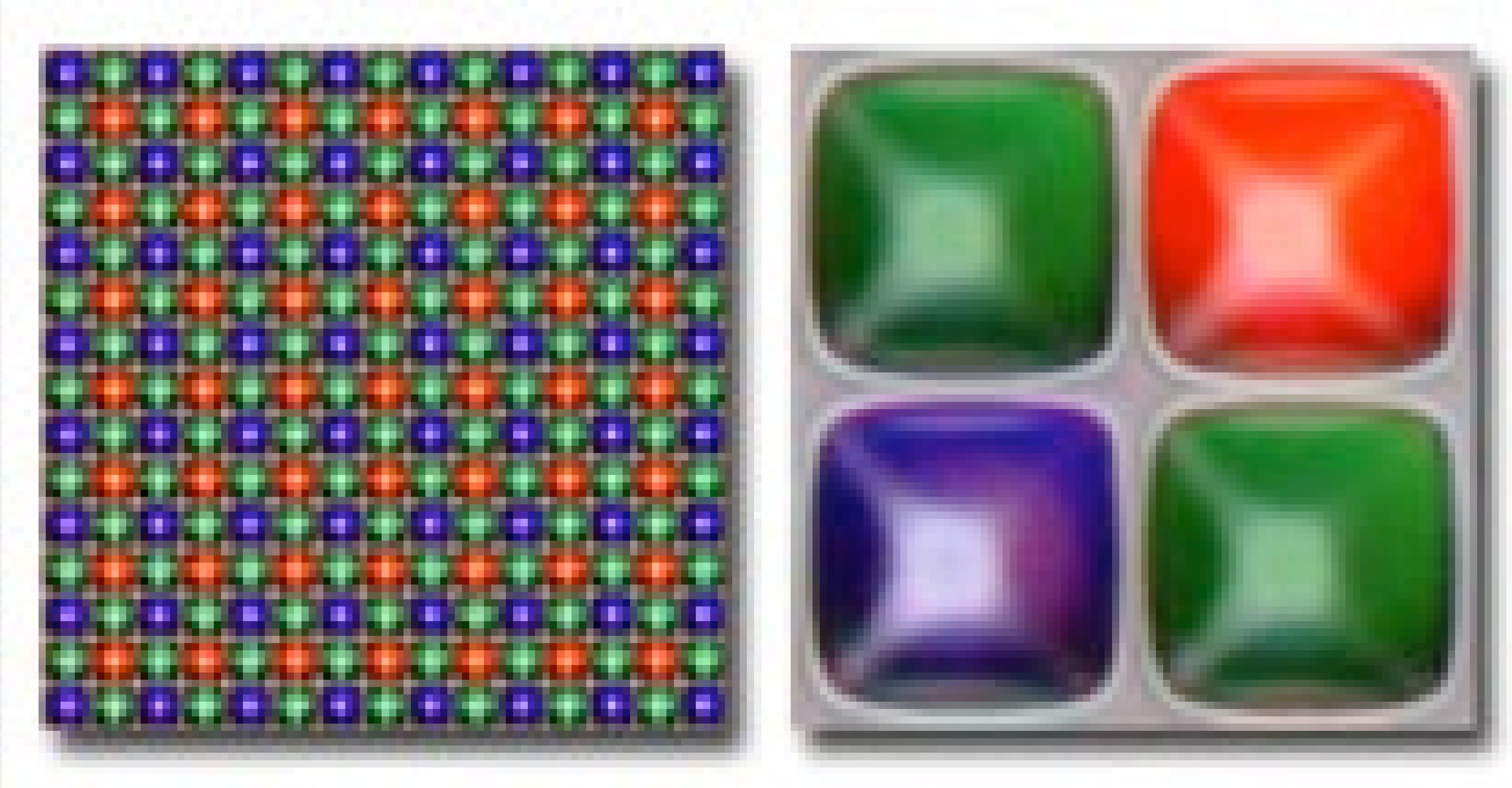
Rare

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

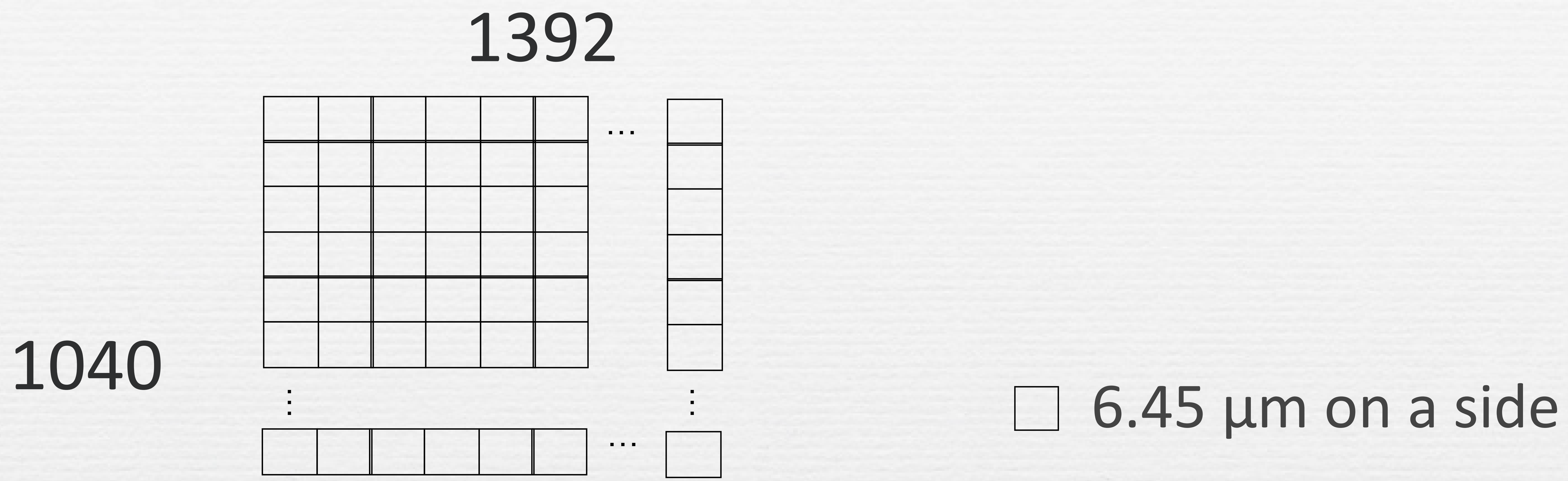
Vital Statistics for CCDs

- 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

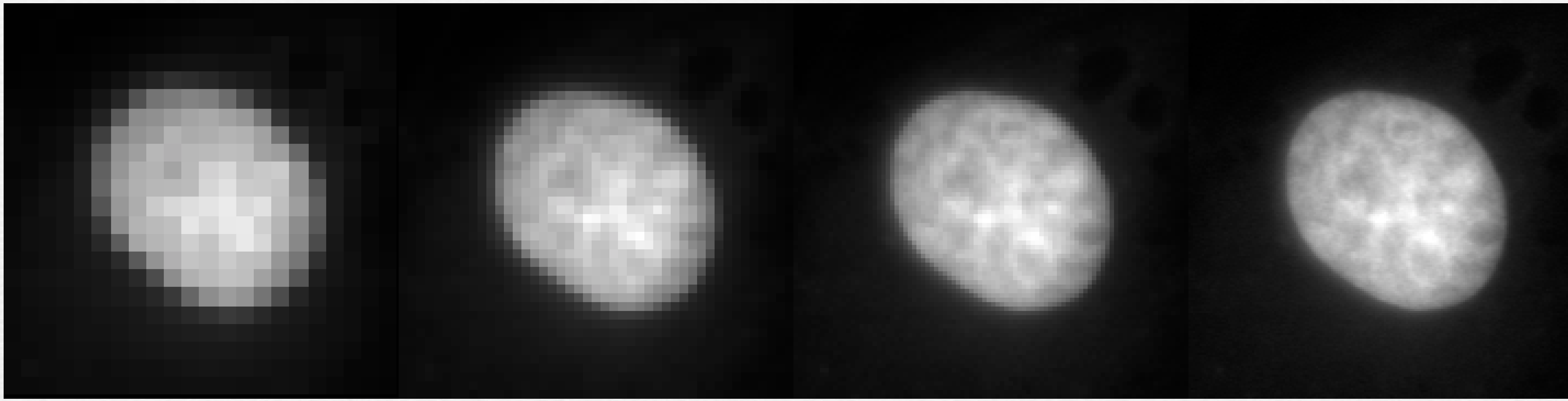


Sony Interline Chip ICX285

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



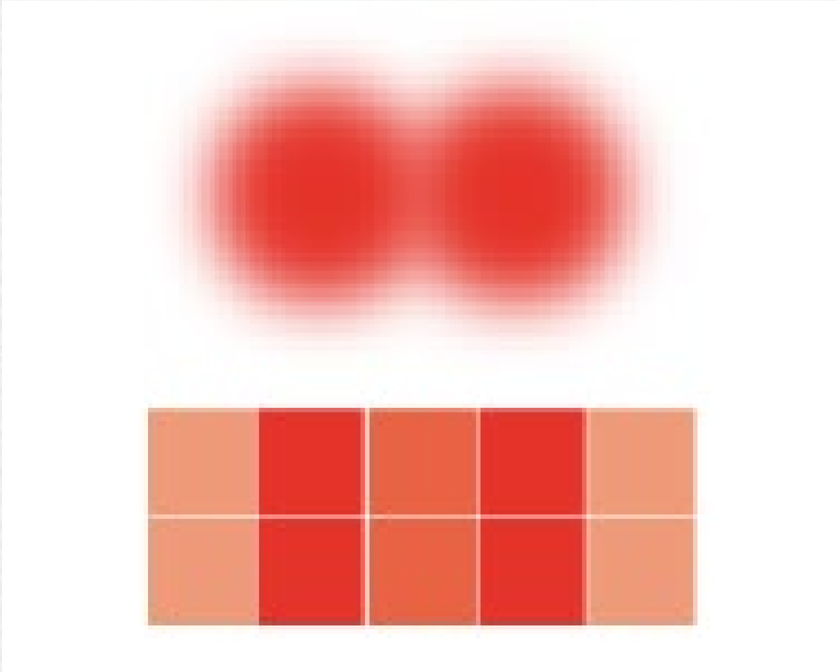
More pixels / resolution element

Where is optimum?

Digital Sampling

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

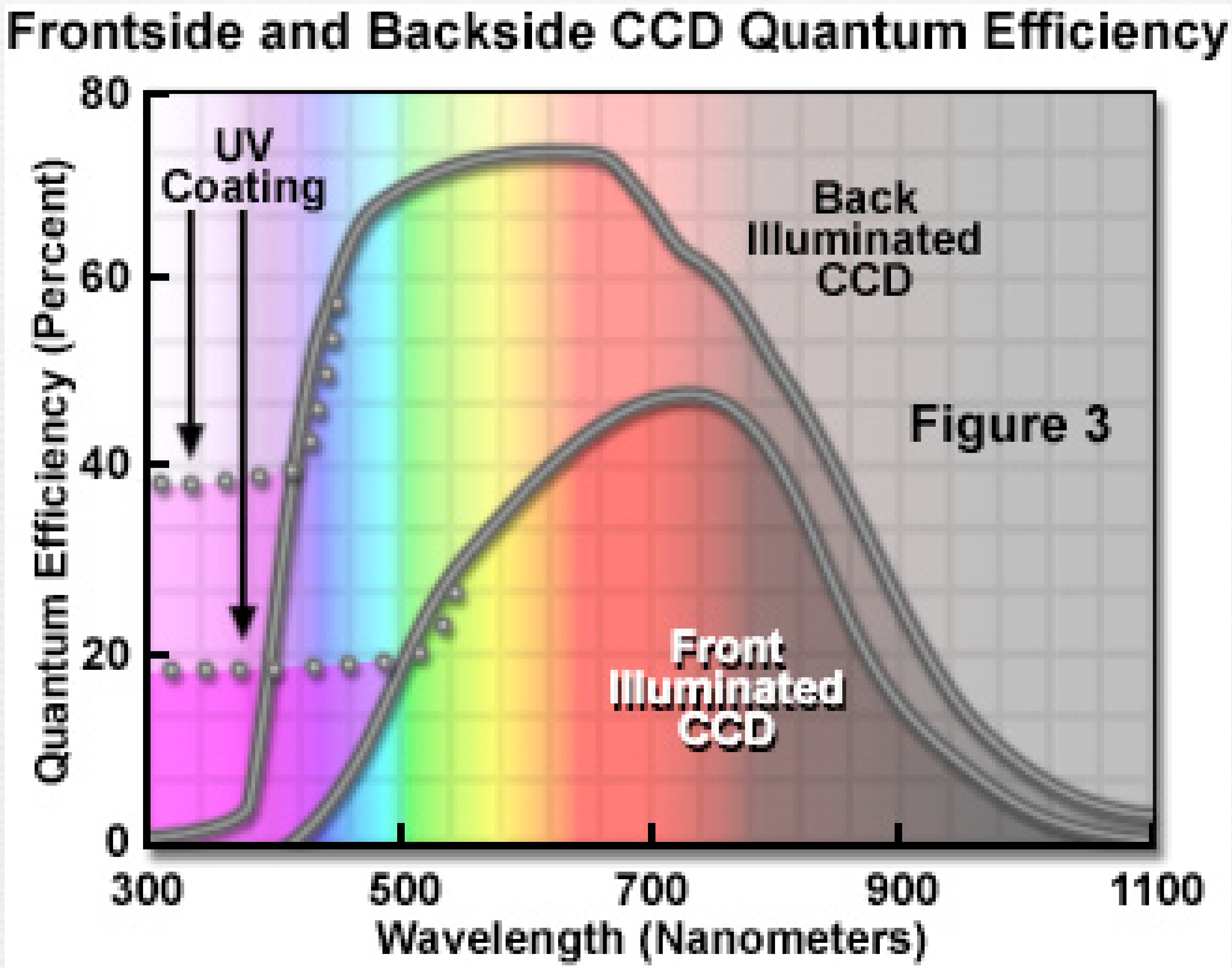
- Nyquist-Shannon Sampling theorem: Must have at least two pixels per resolvable element
- 2.5 – 3 is preferable



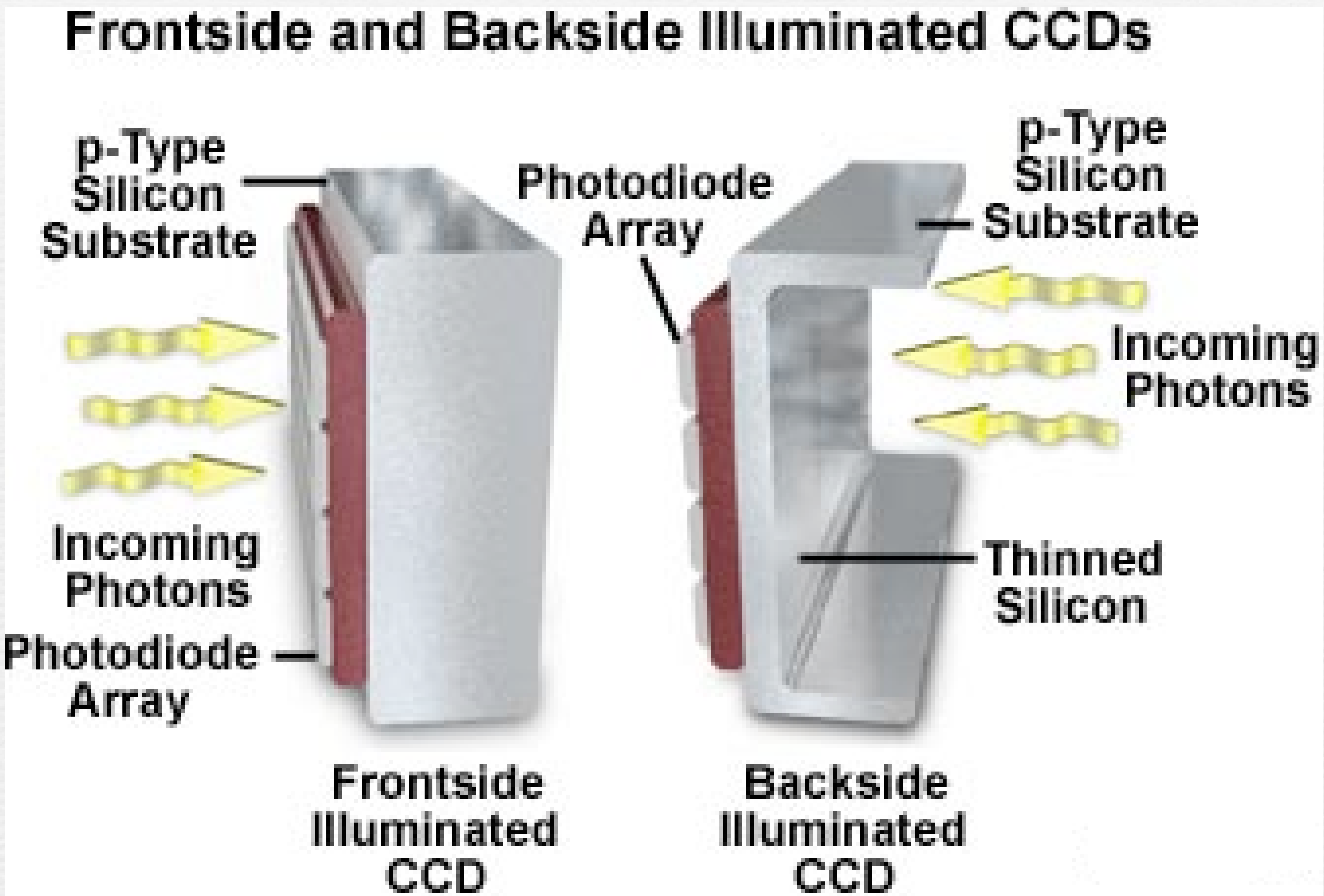
A resolution-centric view of imaging

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

Quantum Efficiency

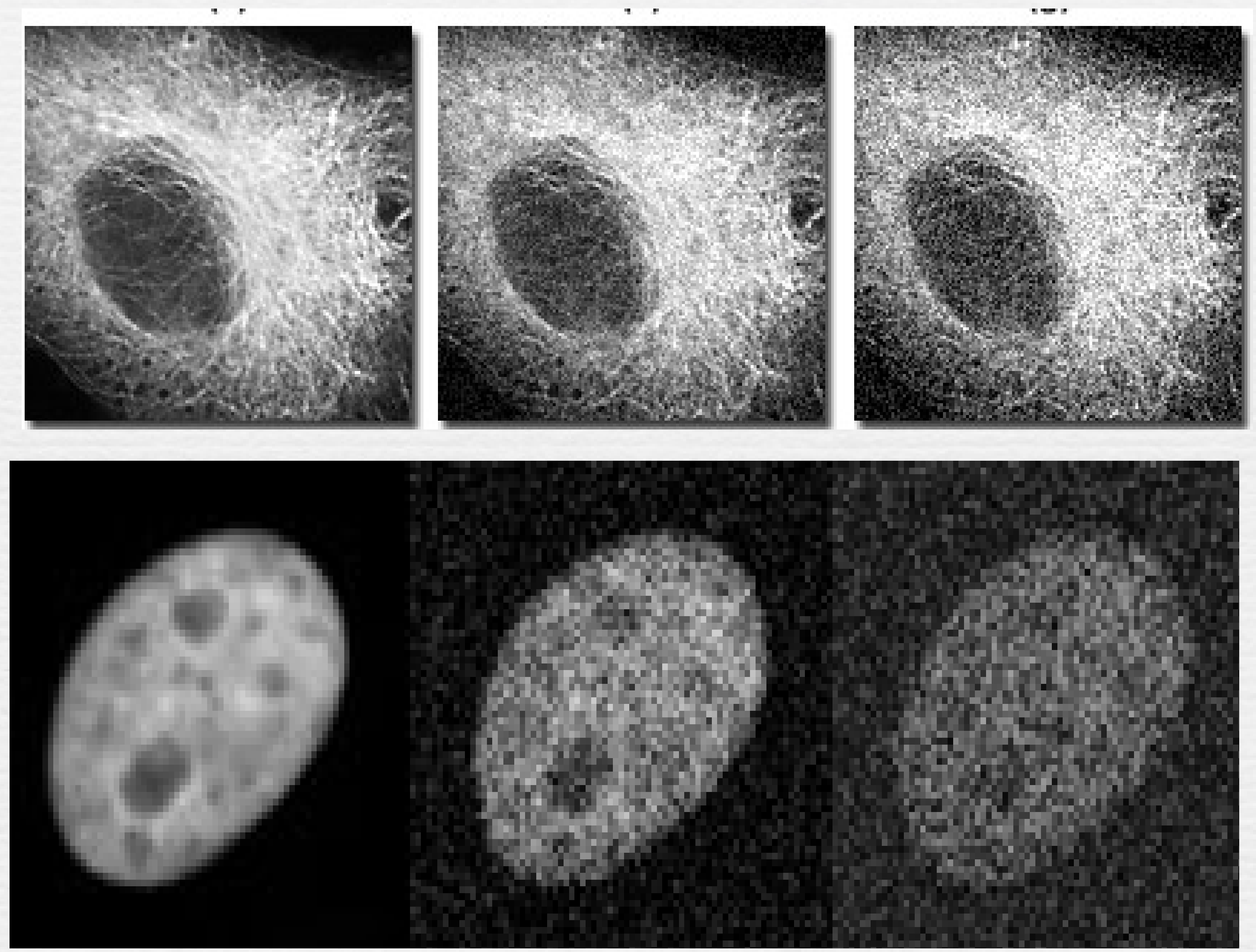


Back-thinning increases QE



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 = $\sqrt{(\text{read noise}^2 + N)}$

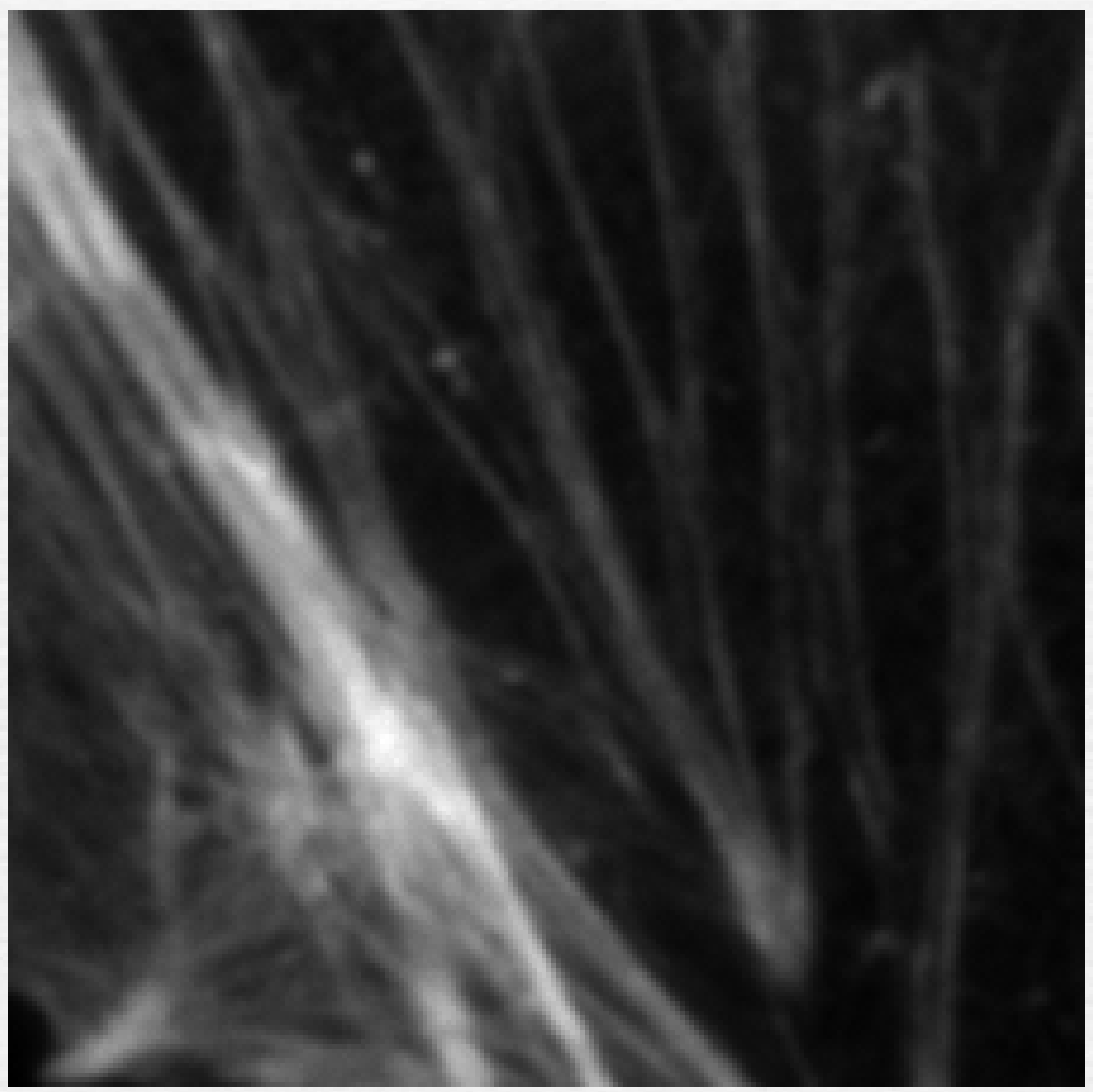
- When # of photons << read noise^2 -> Read noise dominates

- When # of photons >> read noise^2 -> Shot noise dominates

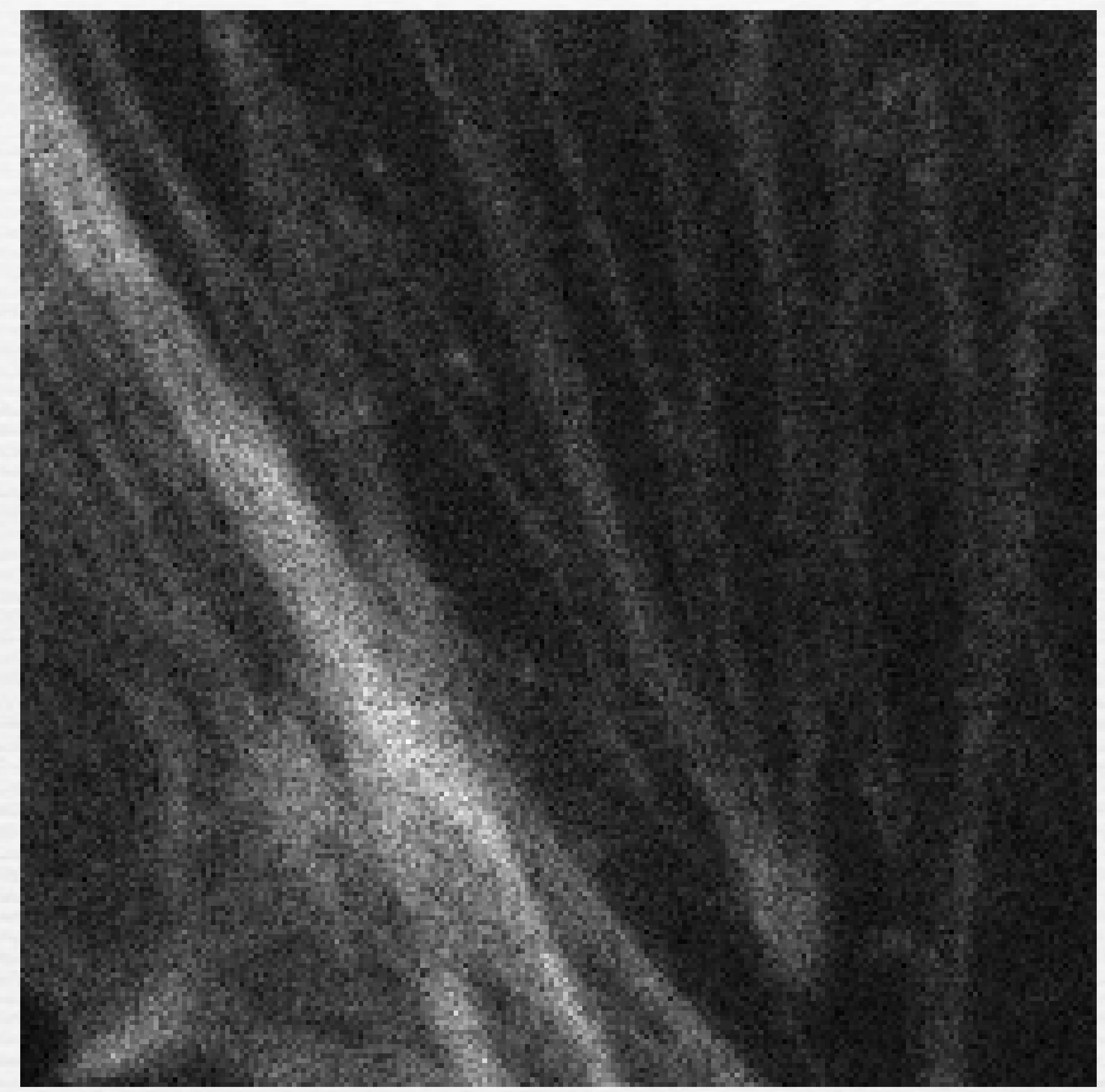
- When shot noise dominates (Signal/Noise = $N/\sqrt{N}$), to double your SNR, you need to acquire four times as long (or 2x2 bin)

Often, read-noise dominates

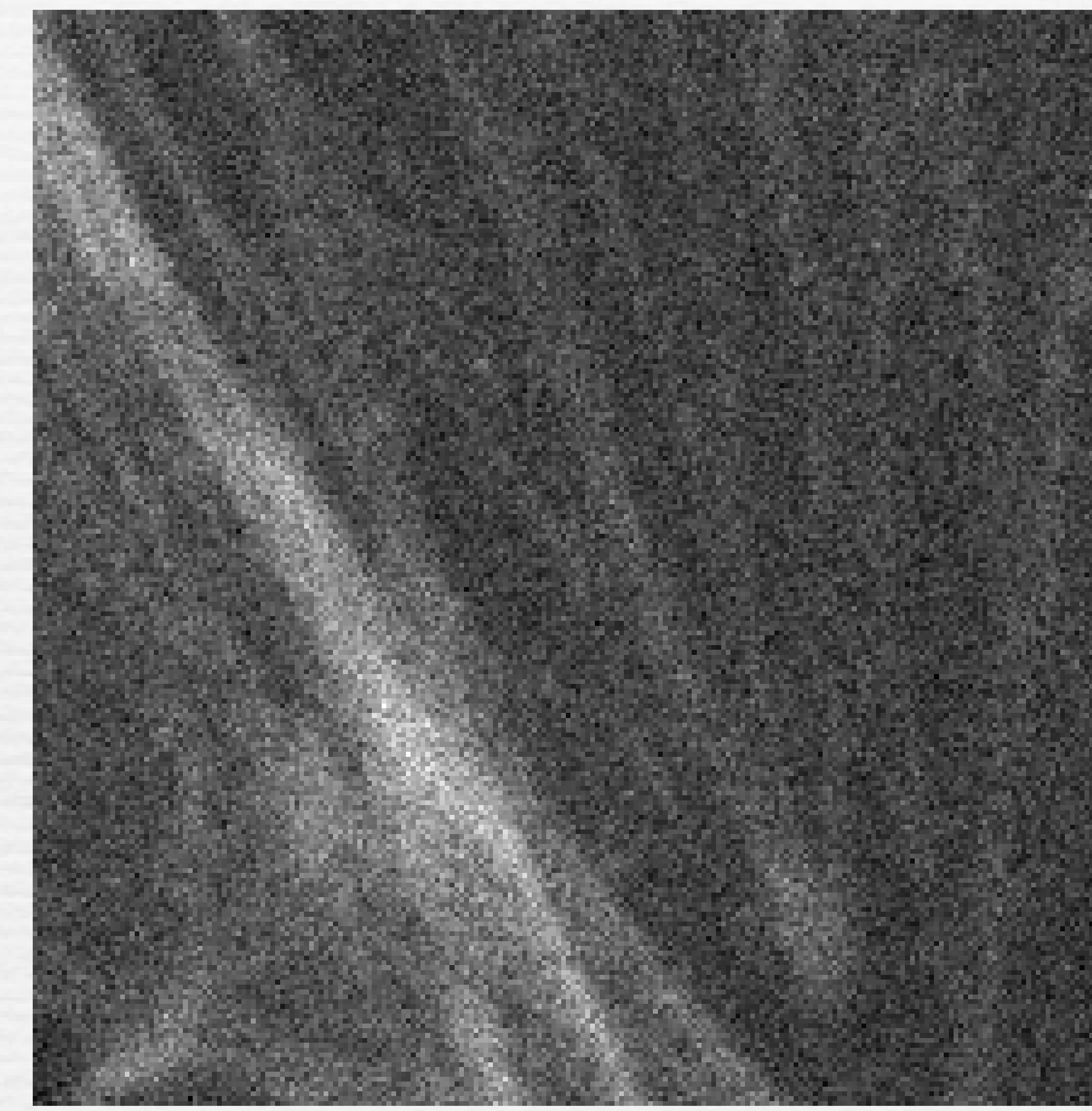
10 photons / pixel on average; ~50 in brightest areas



Test image



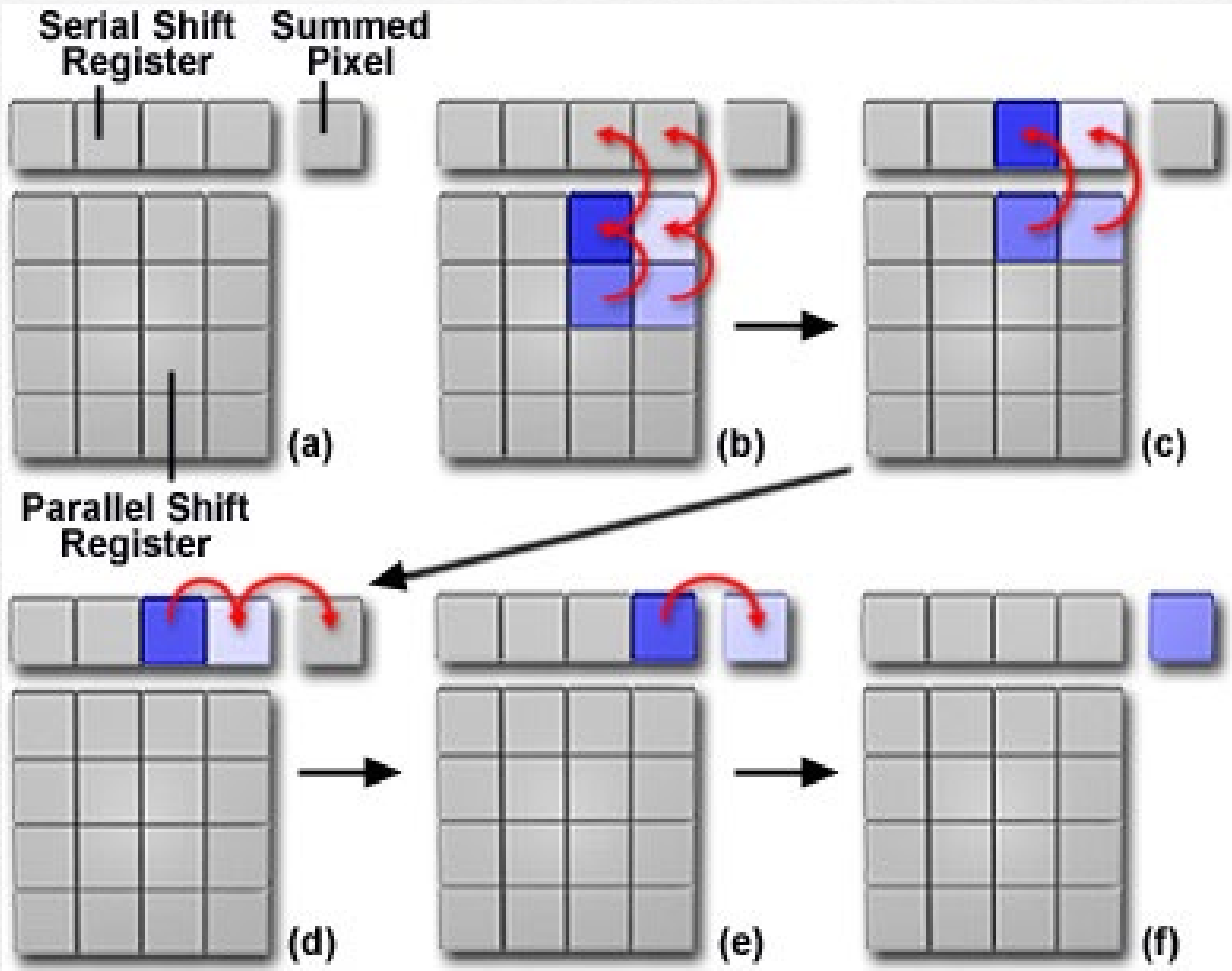
no read noise



5 e^- read noise

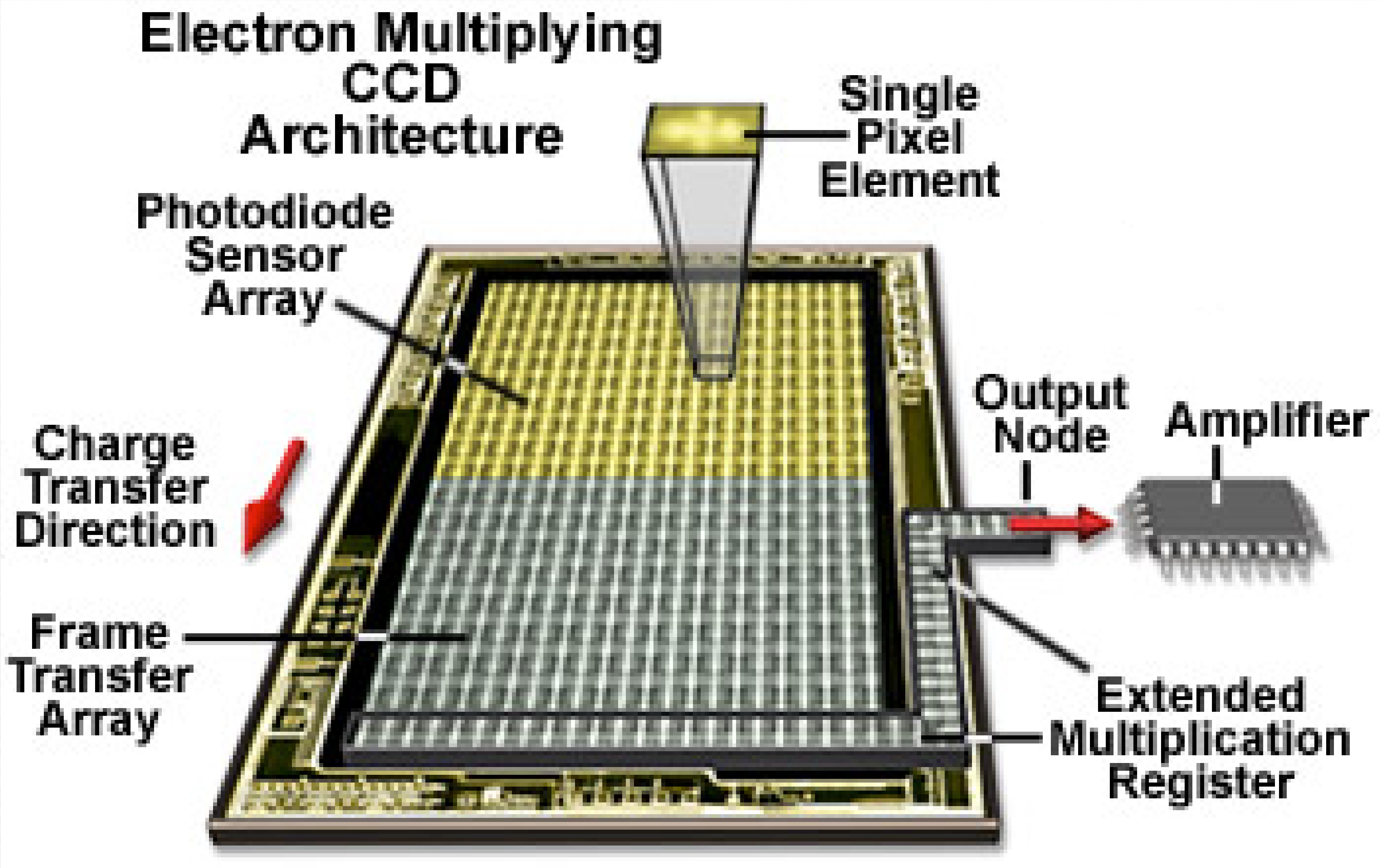
Photon shot noise ~ 3/5 read noise

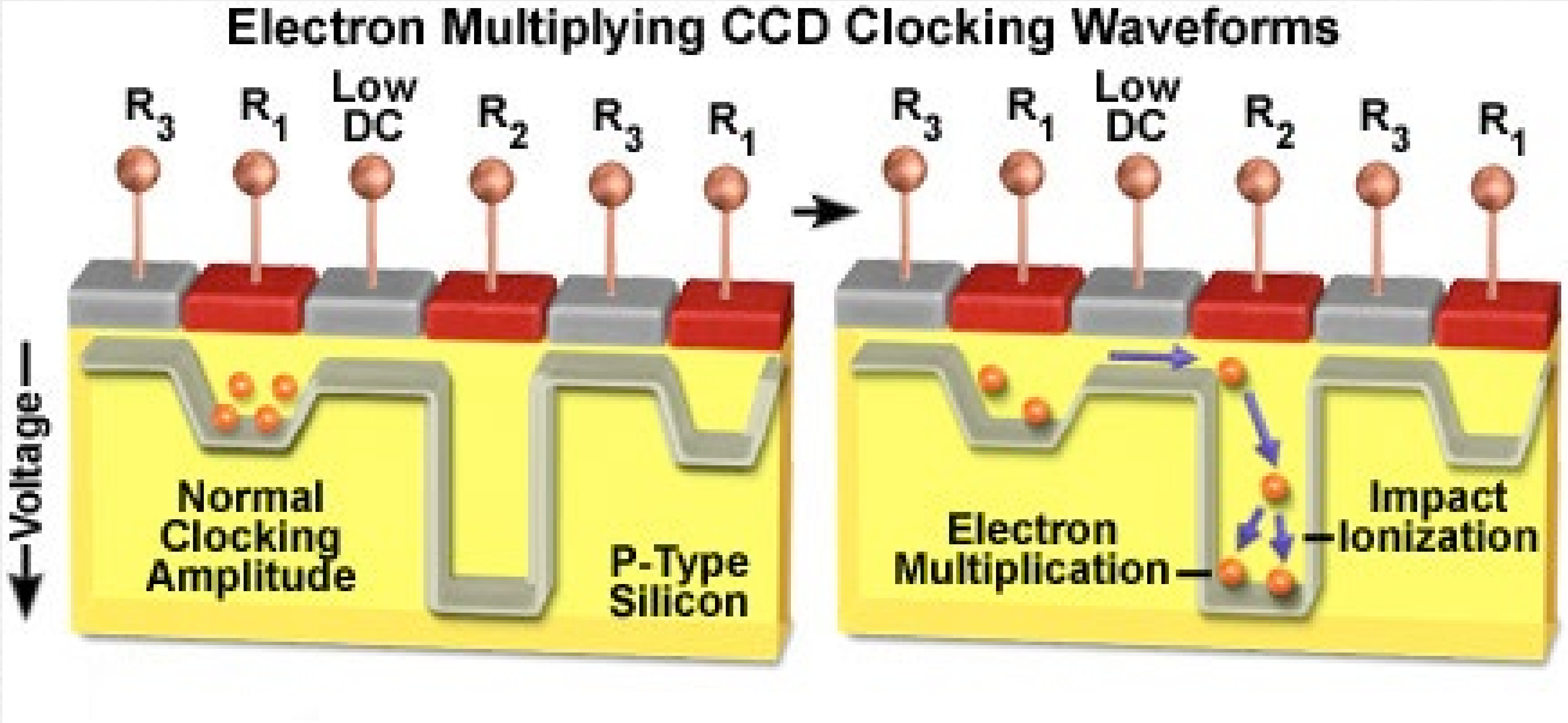
Binning



- 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





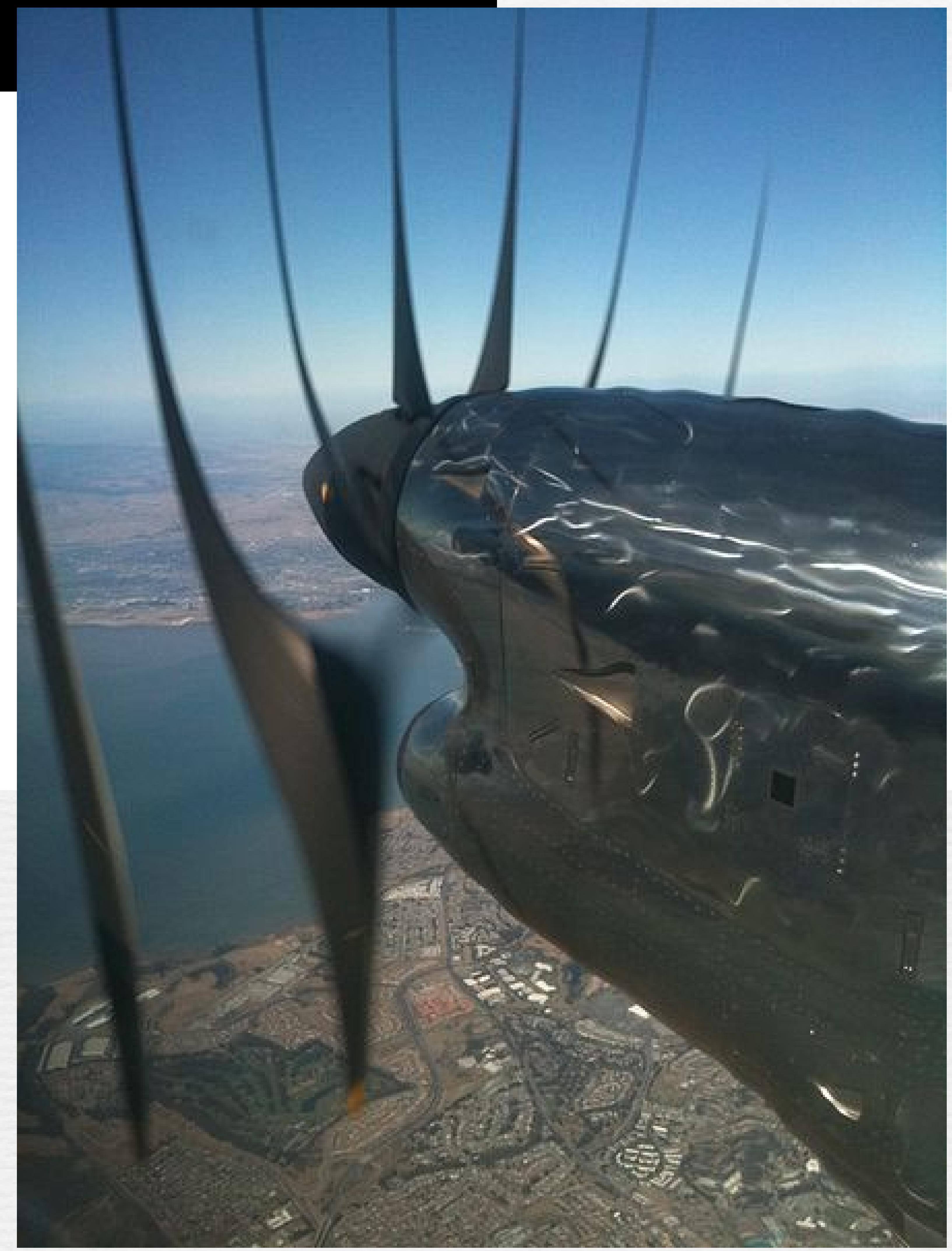
EMCCD result

- 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:

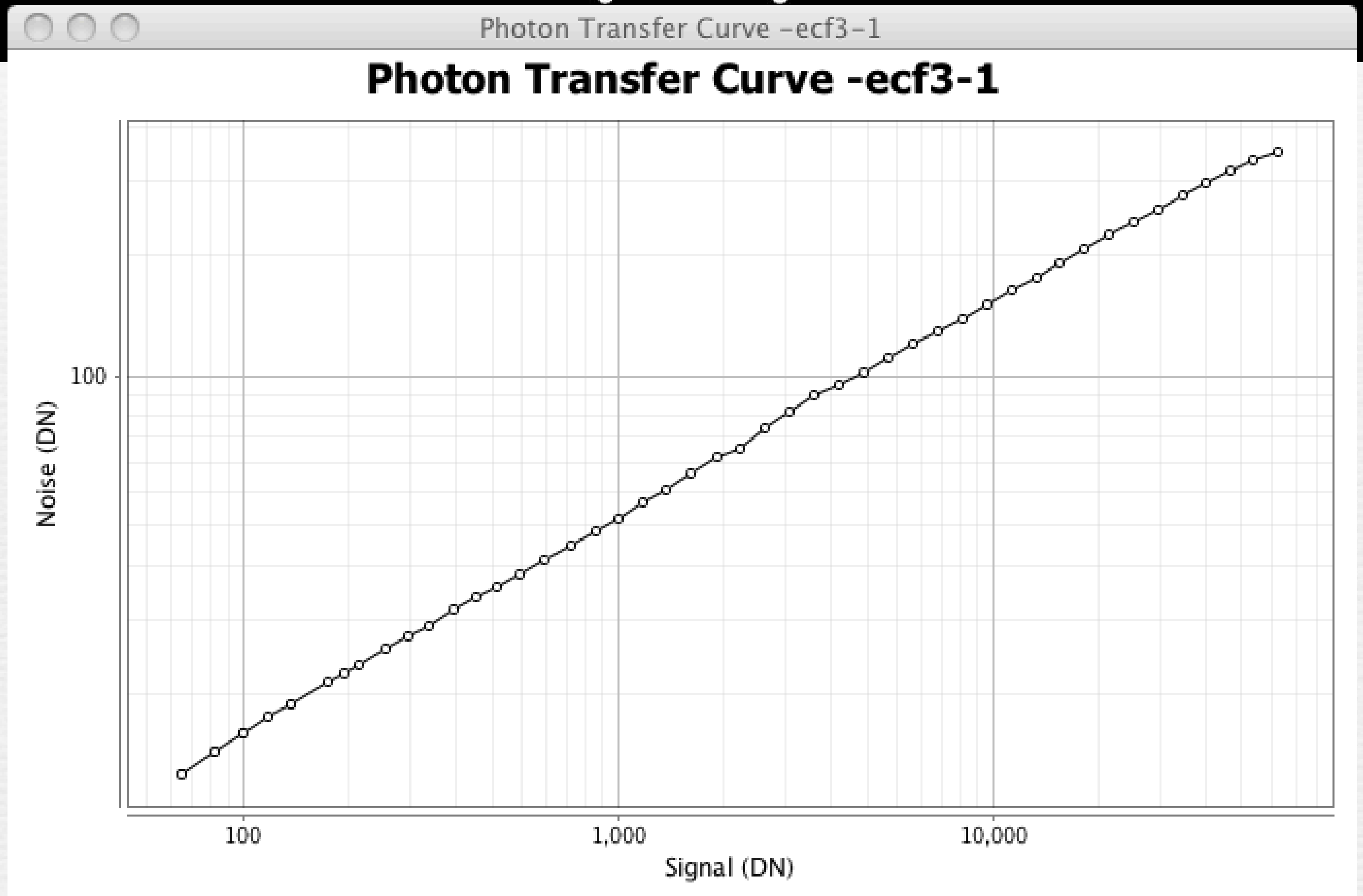
$\sigma(N) = \sqrt{(N)}$ Photon Shot noise

$N = c \cdot DN$ 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

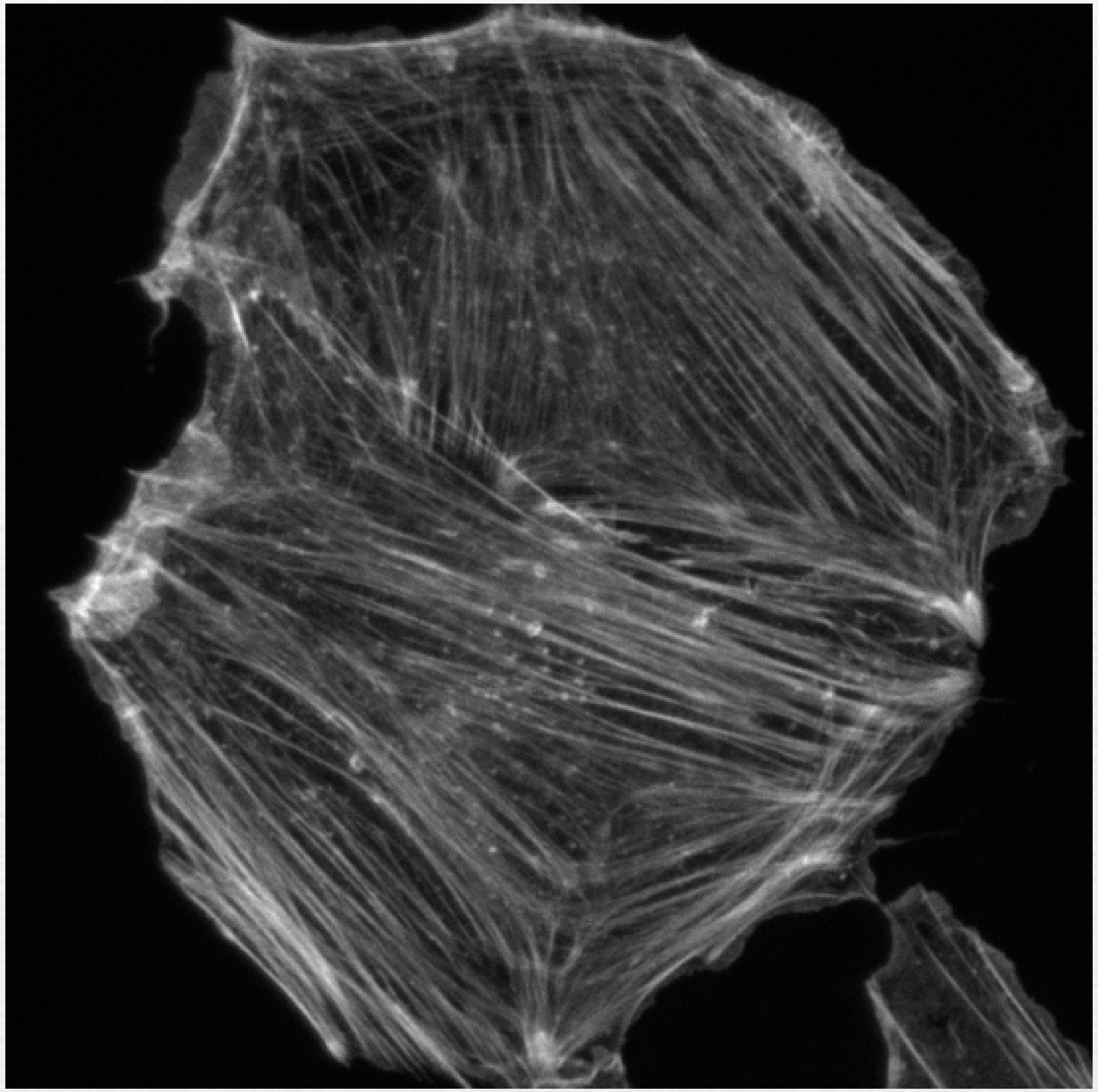
1	1	1
1	1	1
1	1	1

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

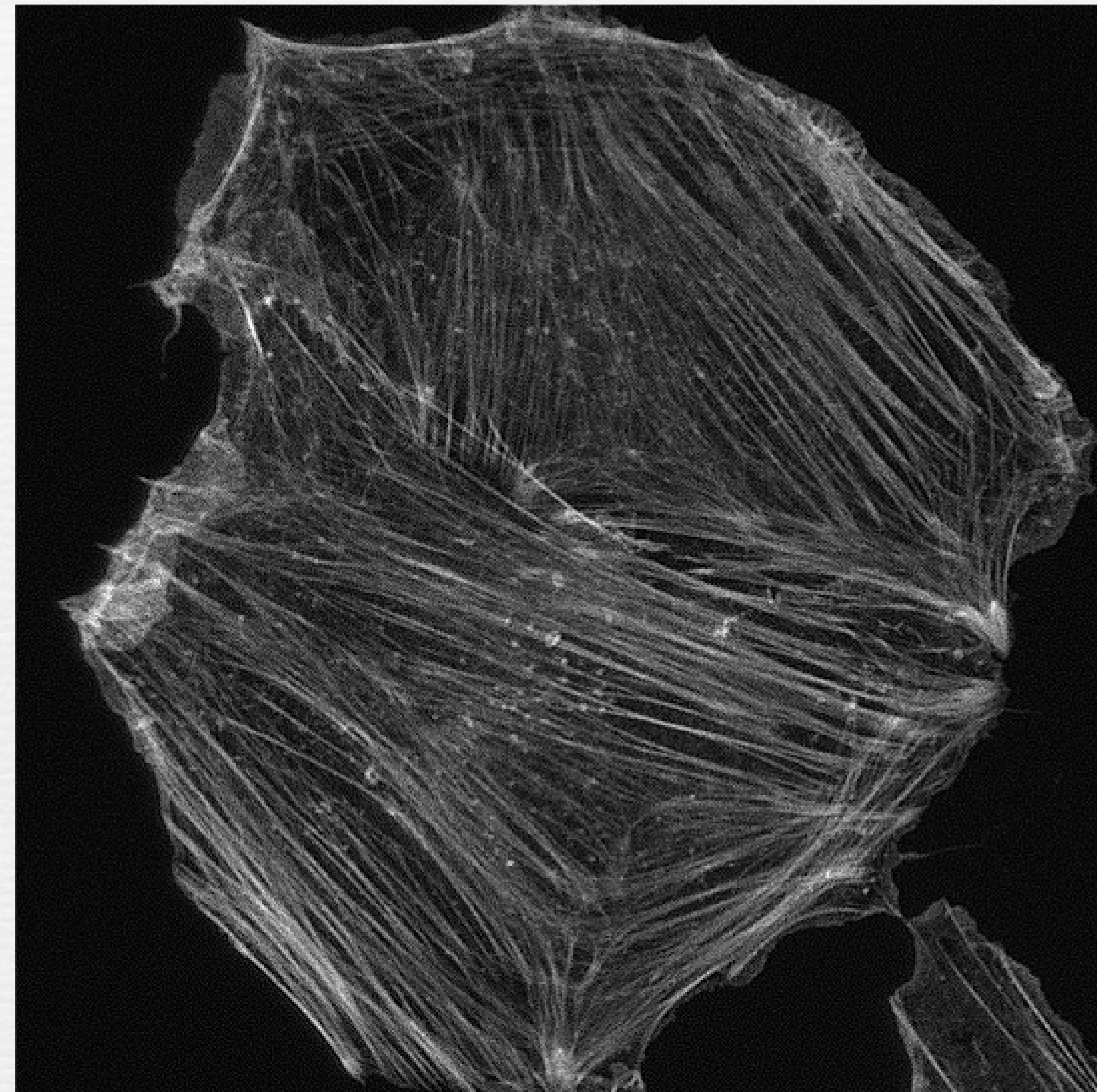
Multiply corresponding pixels and sum

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

Example: Unsharp masking



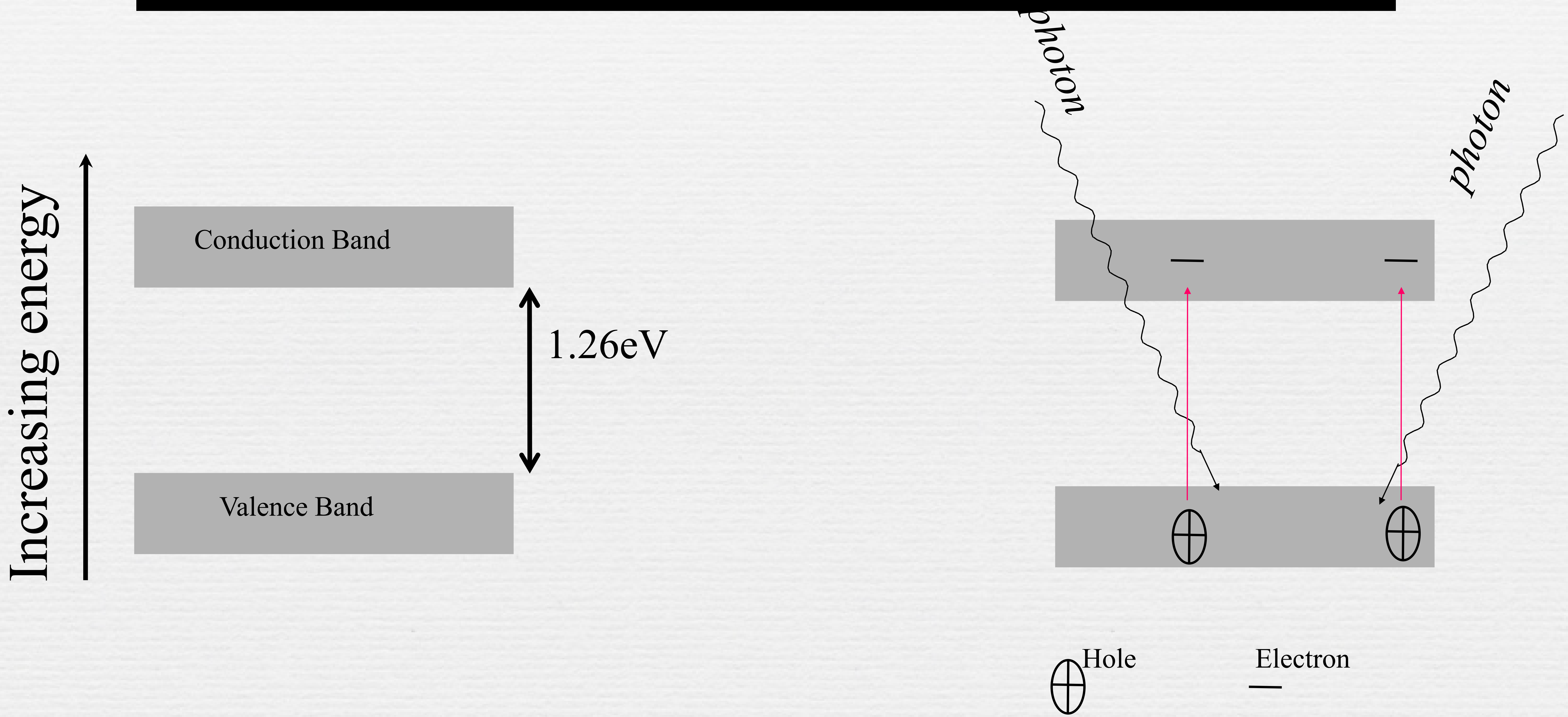
Original



Unsharp masked

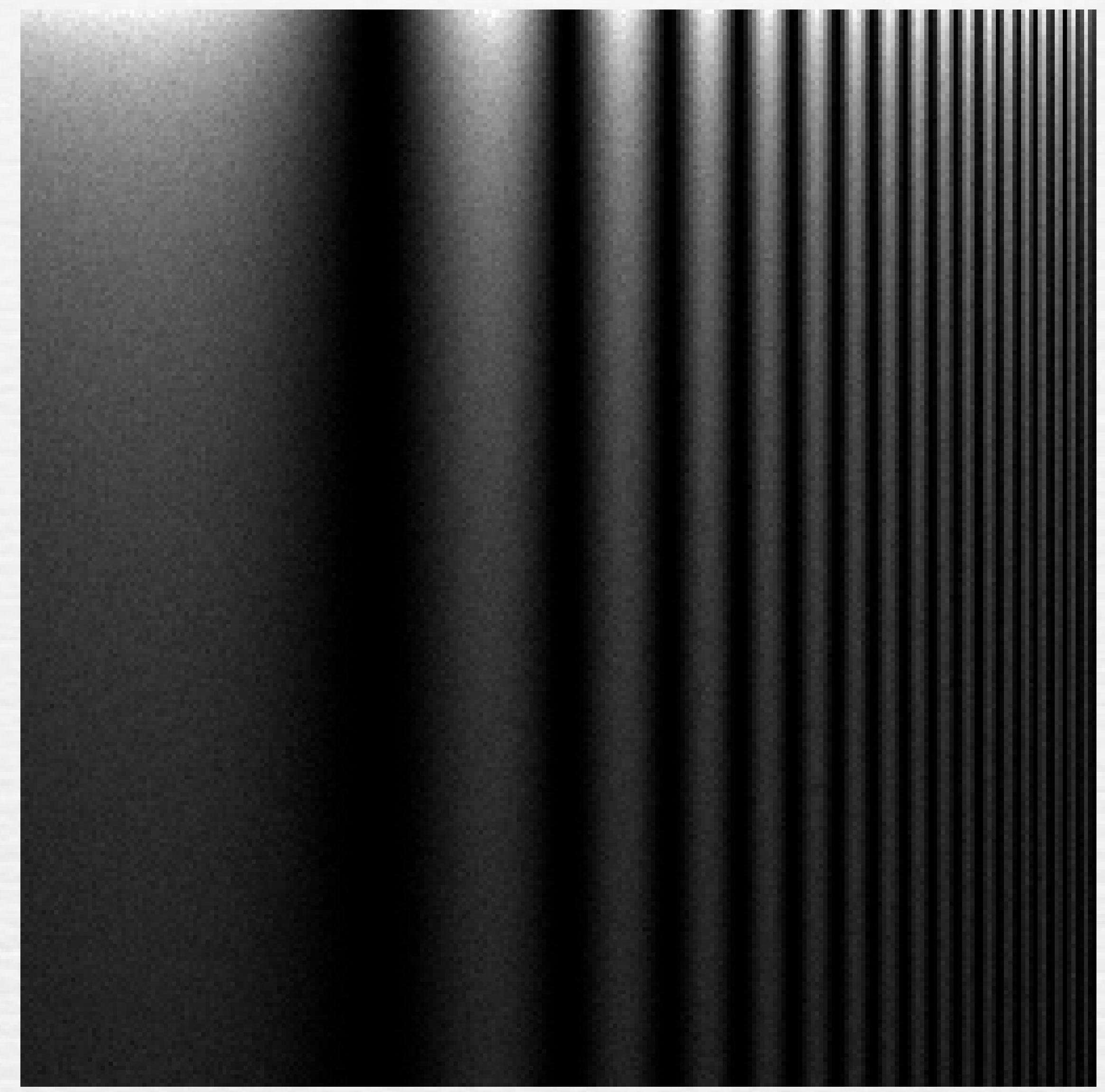
-1	-4	-1
-4	26	-4
-1	-4	-1

Photo-electric Effect

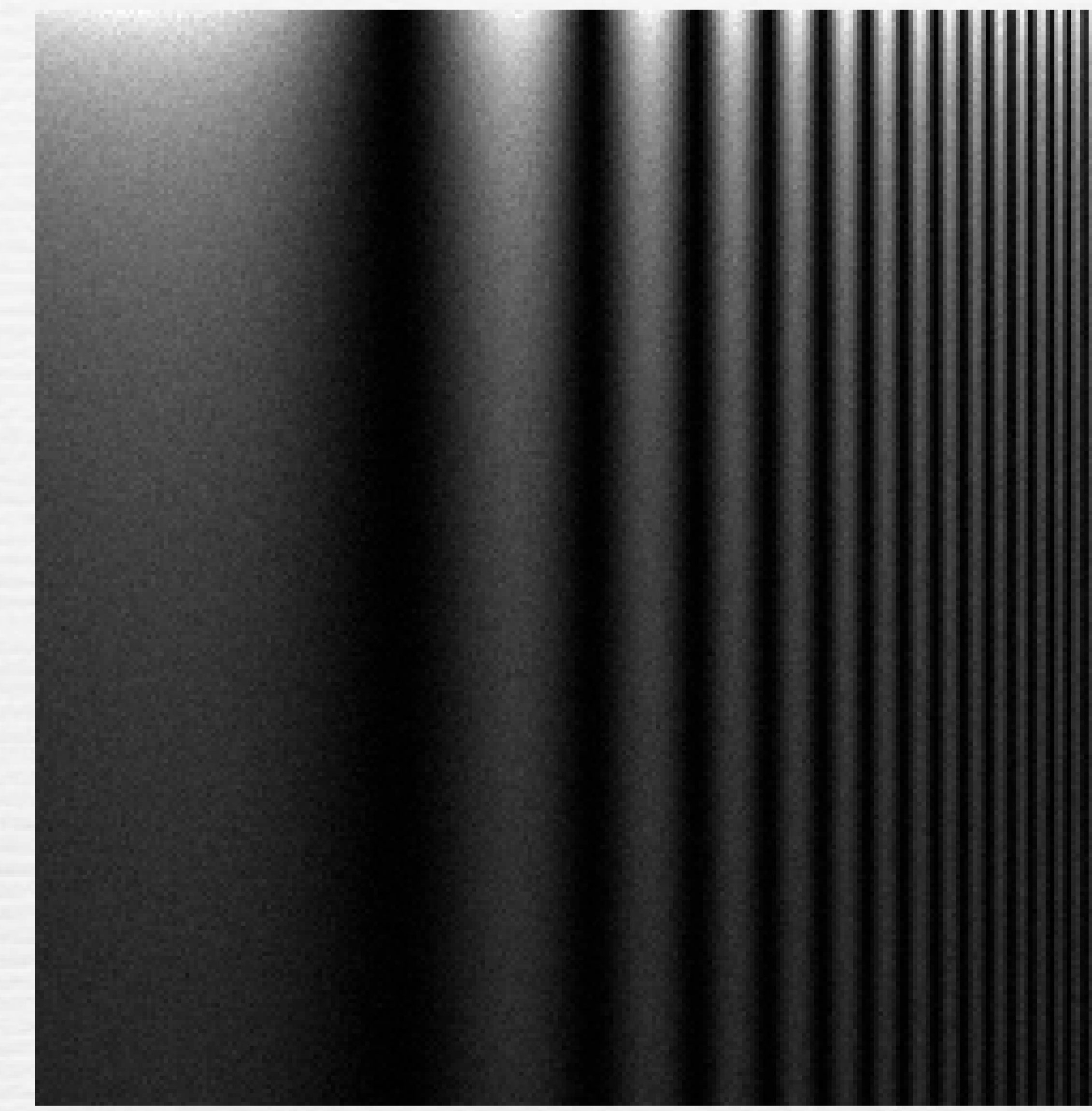


What does this look like?

Test image



1000 ph/pixel,
no read noise

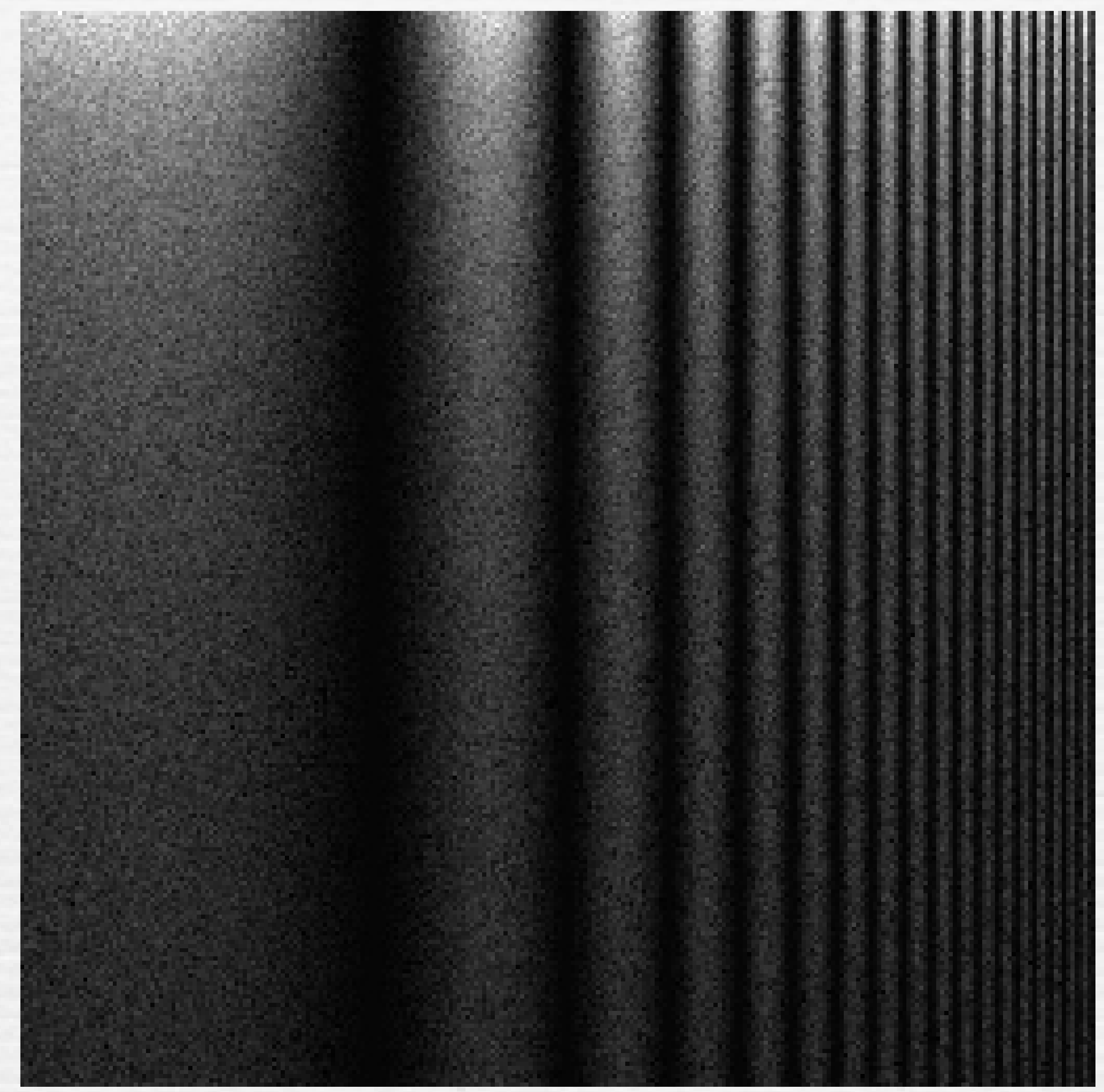


1000 ph/pixel,
5 e^- read noise

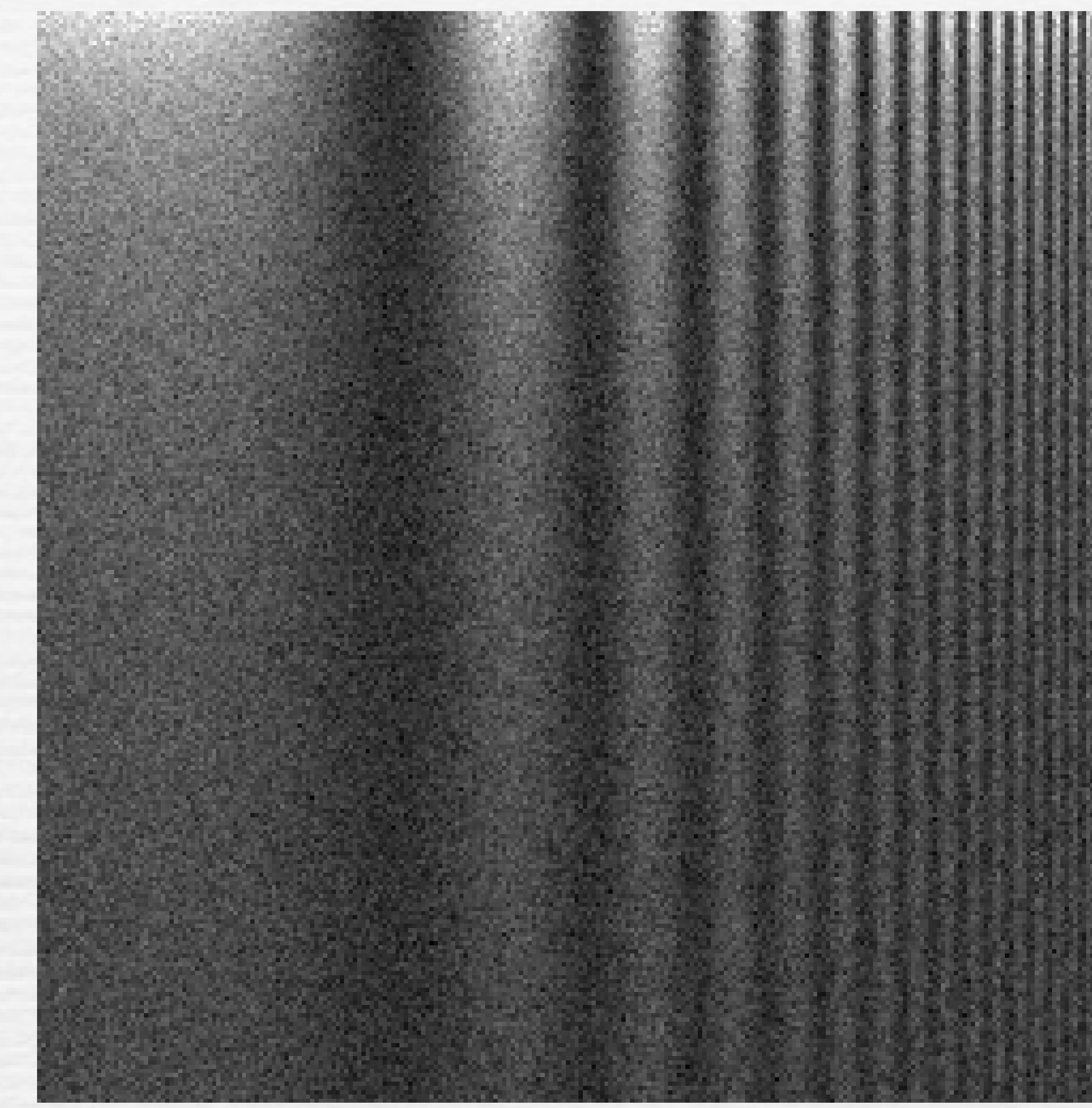
Photon shot noise = 6x read noise

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)

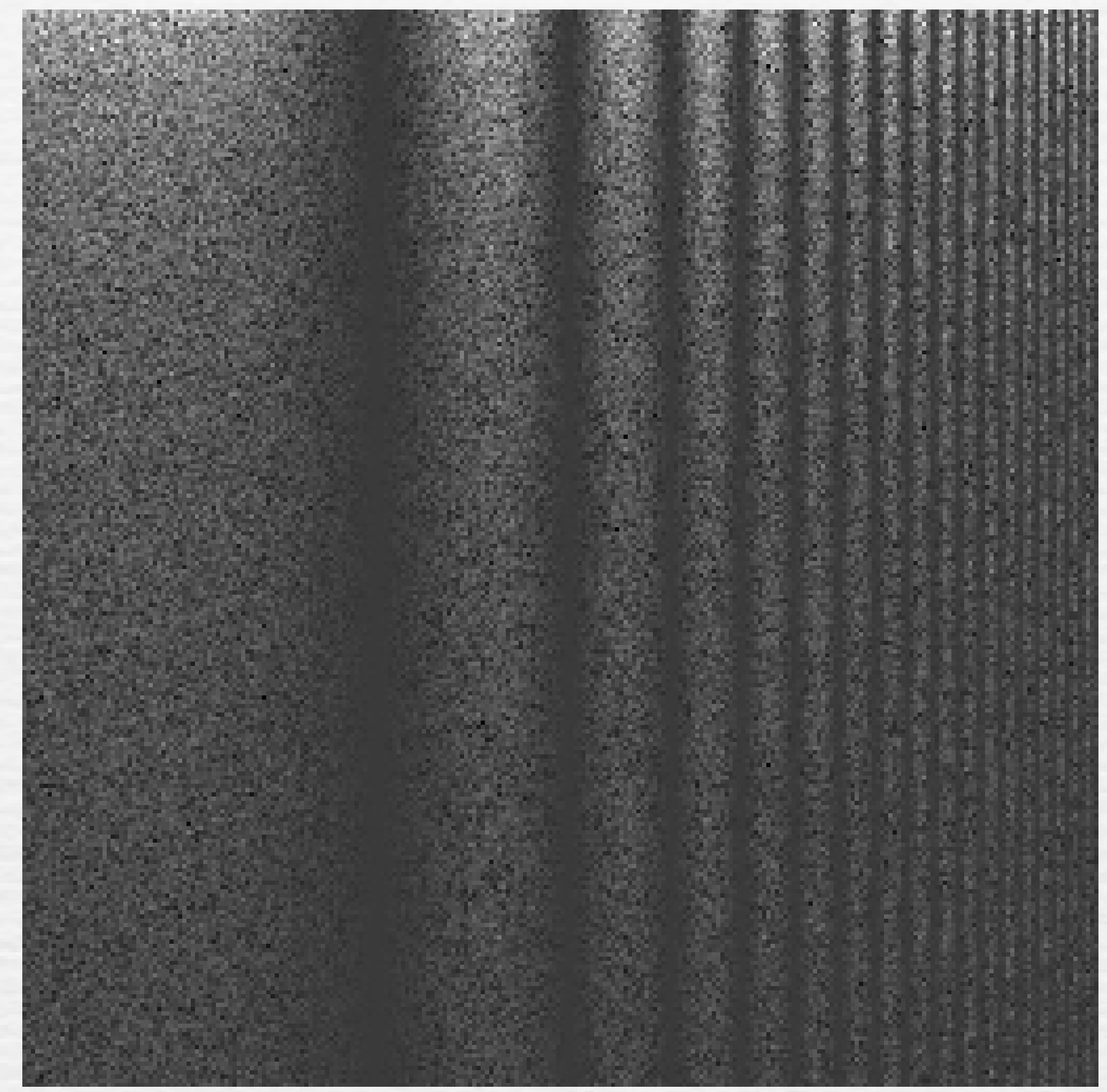
- Read noise dominates whenever read noise^2 >= # of photons

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

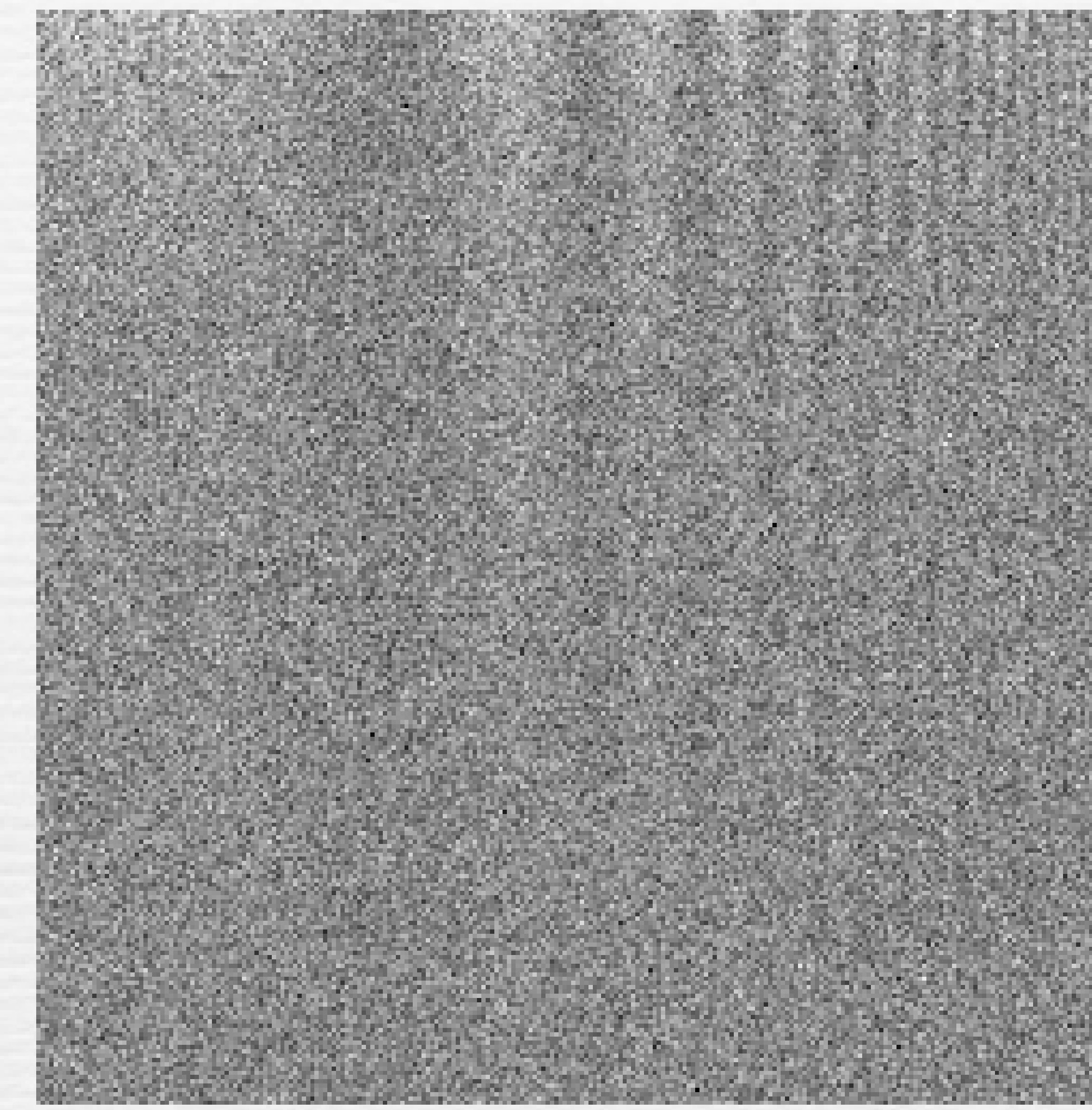
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

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