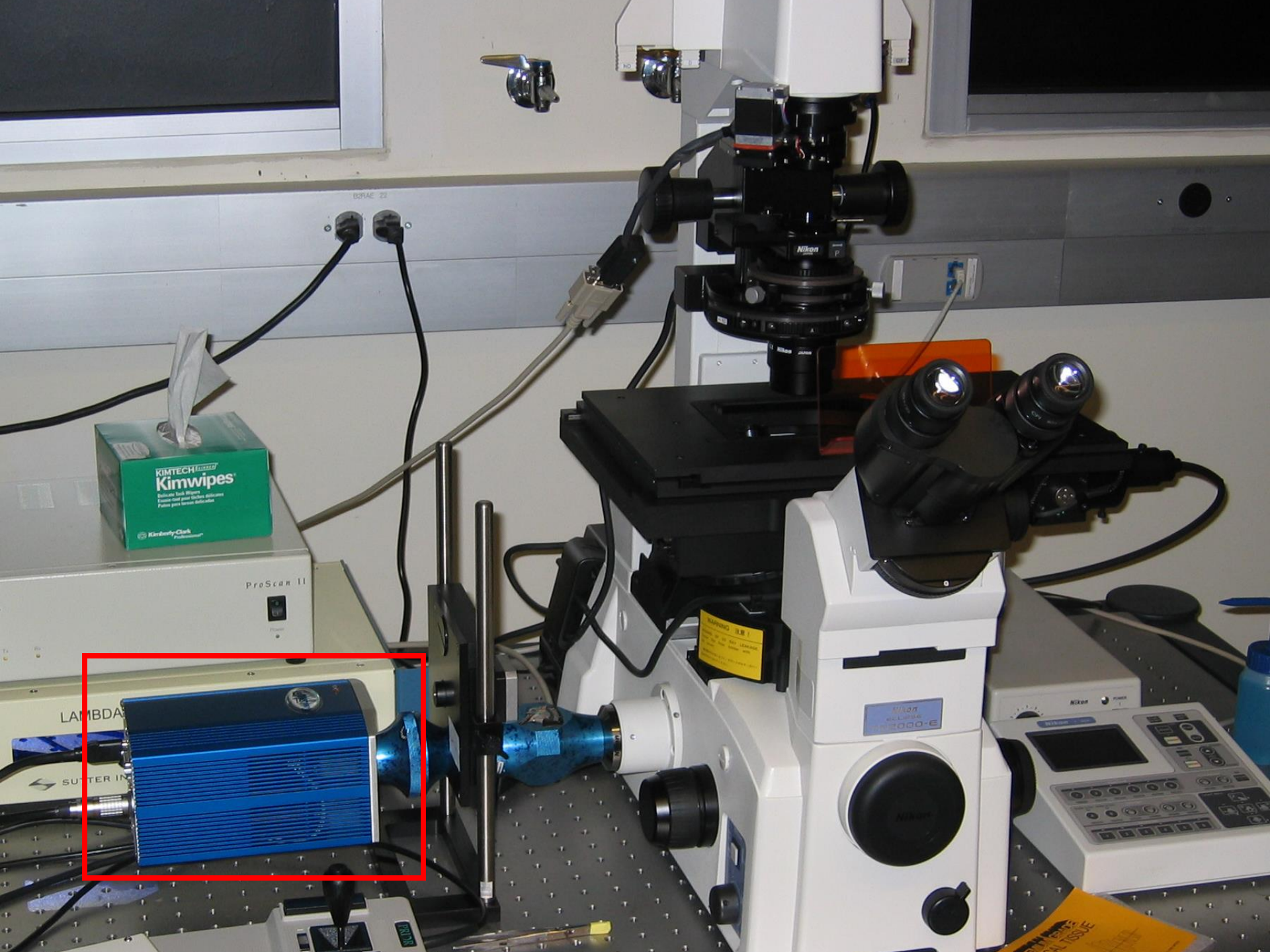


Digital Cameras in Microscopy

Kurt Thorn

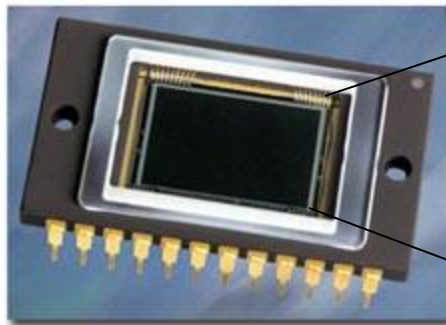
Nikon Imaging Center @ QB3/UCSF



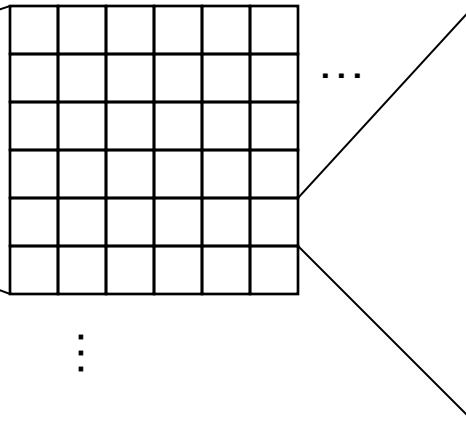
What does a camera need to do?

- Convert light into an electrical signal
- Accurately measure this signal
- Do this in a spatially resolved way

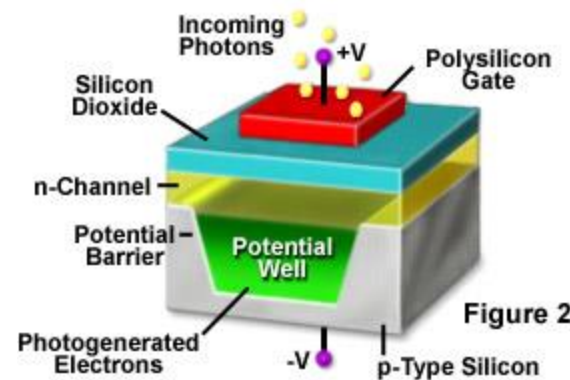
CCD architecture



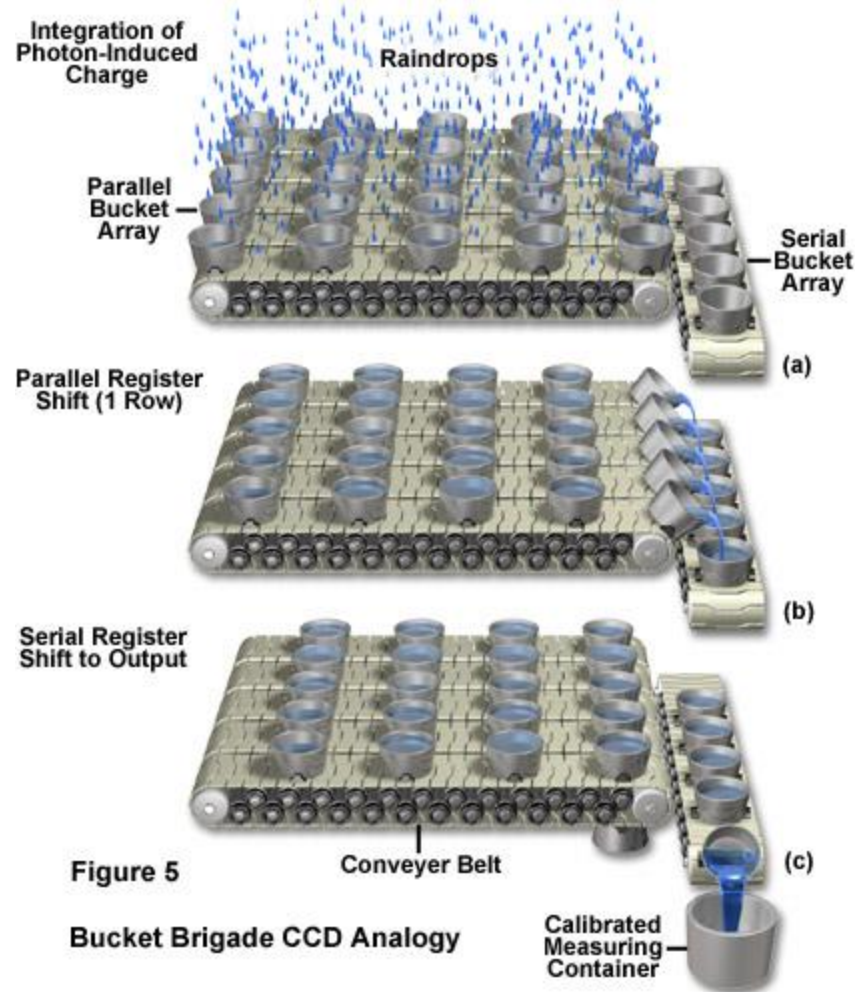
KAF-3200E



Metal Oxide Semiconductor (MOS) Capacitor

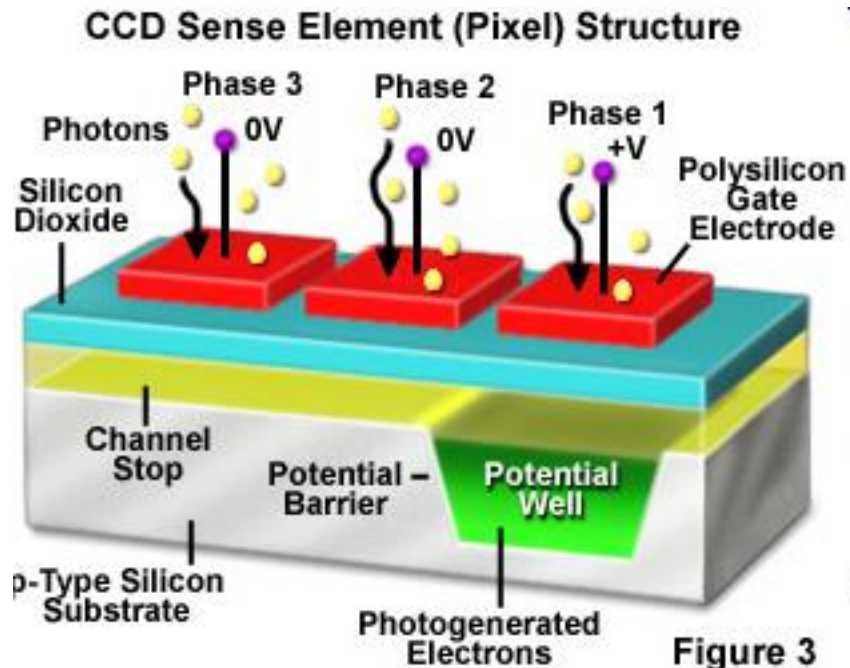


CCD readout “bucket-brigade” analogy

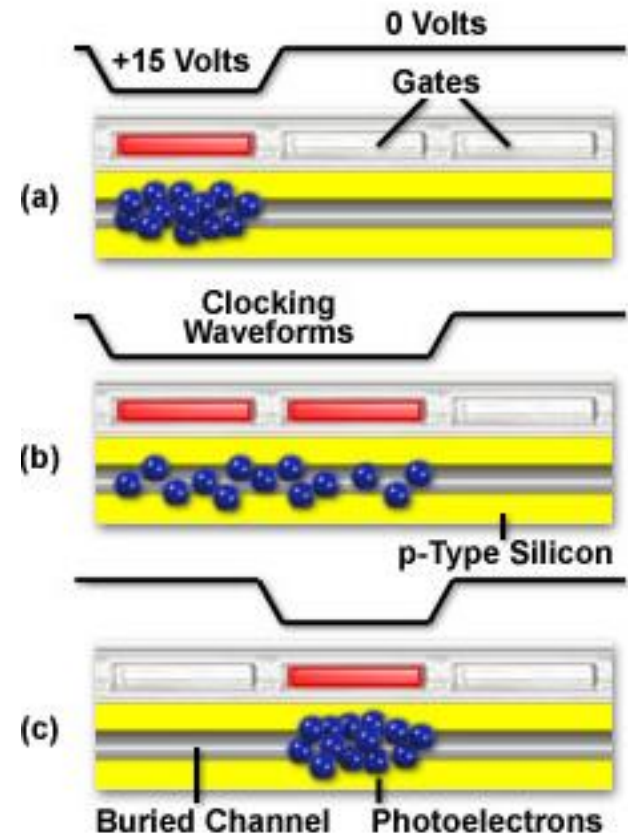


A little more realistic....

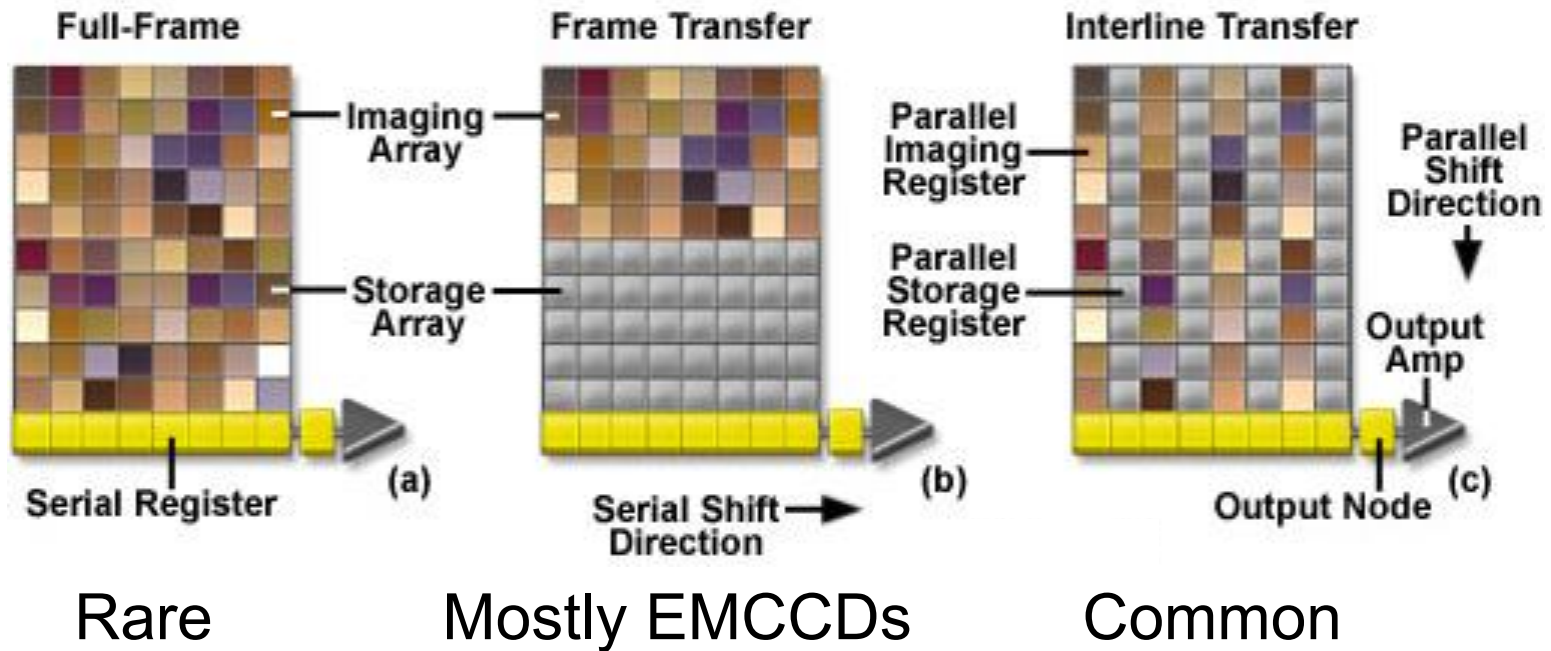
Each pixel is subdivided into three phases



Three Phase CCD Clocking Scheme

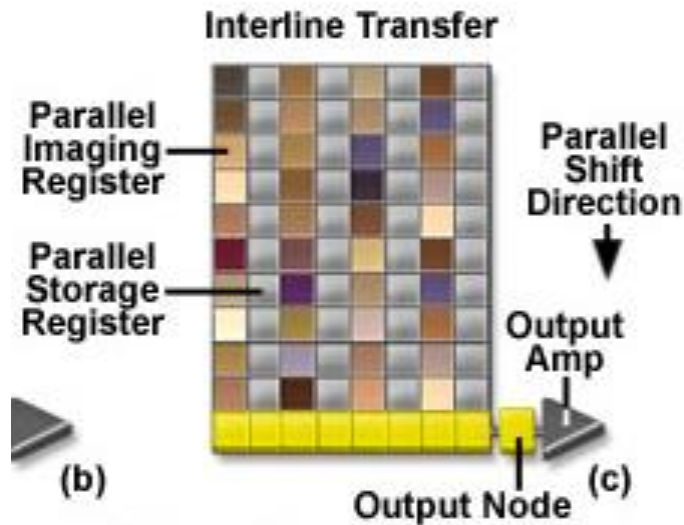


CCD Architectures

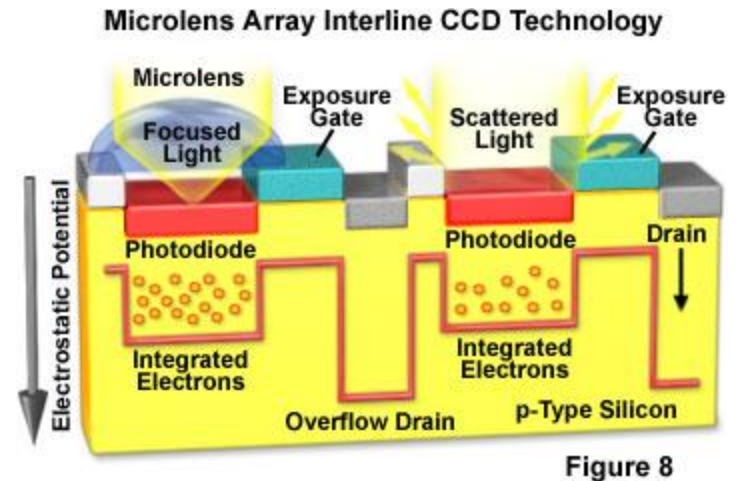


Full frame CCDs cannot acquire while being read out;
They also require a mechanical shutter to prevent smearing
during readout.

Interline CCDs and microlenses



Interline storage registers take up half the light gathering area on the CCD



Solution: use microlenses to focus light onto the light-gathering areas

Why don't we use color CCDs?

Bayer Color Filter Mosaic Array and Underlying Photodiodes

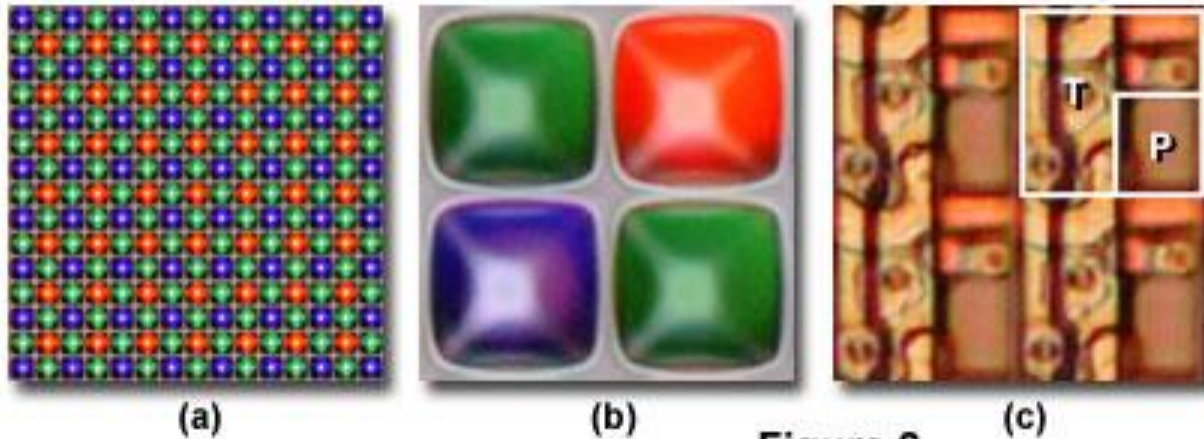


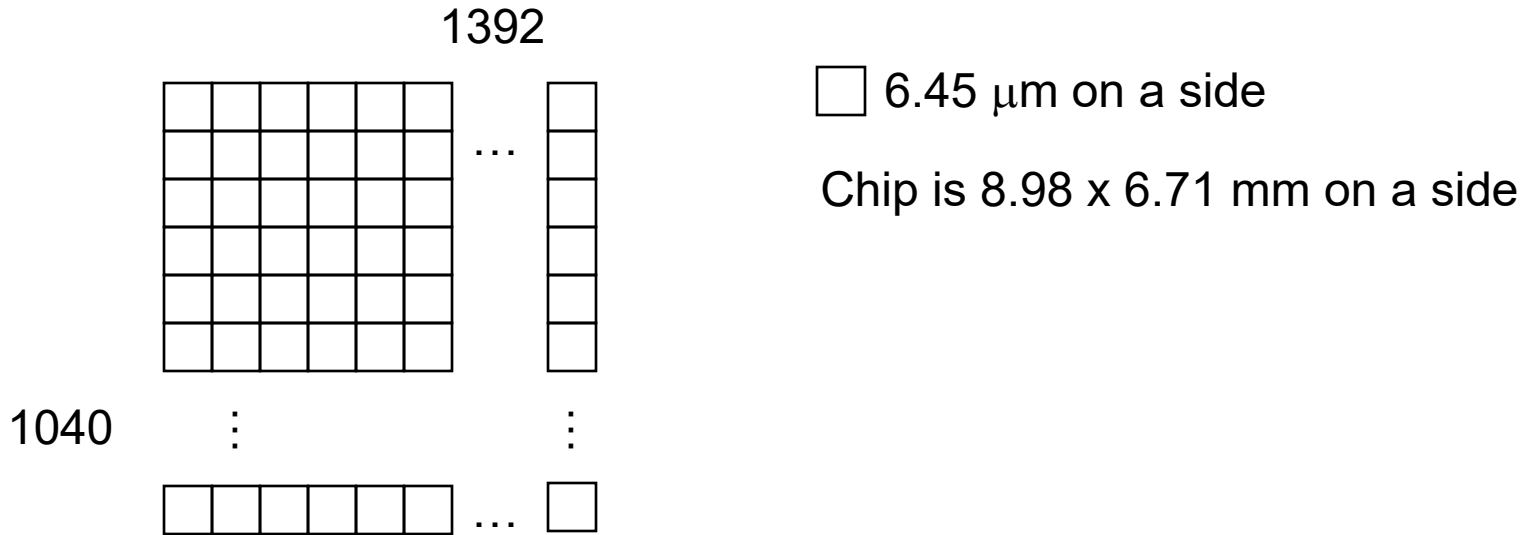
Figure 2

- 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

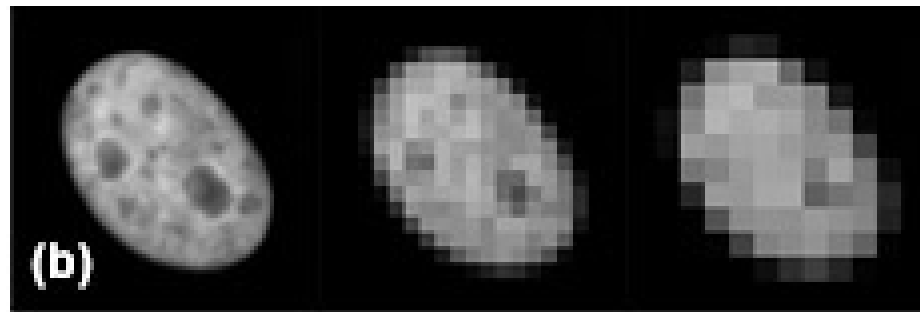
- Pixel size and number
- Quantum efficiency: the fraction of photons hitting the CCD that are converted to electrons
- Full well depth: total number of electrons that can be recorded per pixel
- Read noise
- Dark current (negligible for most biological applications)
- Readout time

Magnification and CCDs



Typical magnification from sample to camera is roughly objective magnification

Resolution and magnification



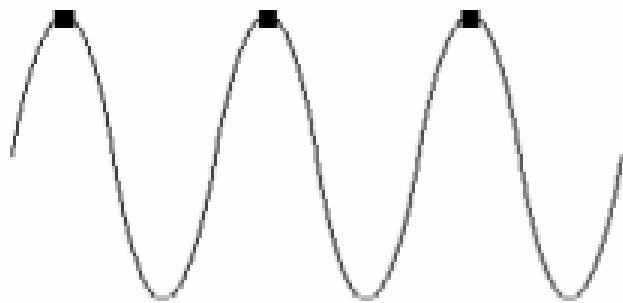
←
More pixels / resolution element

Where is optimum?

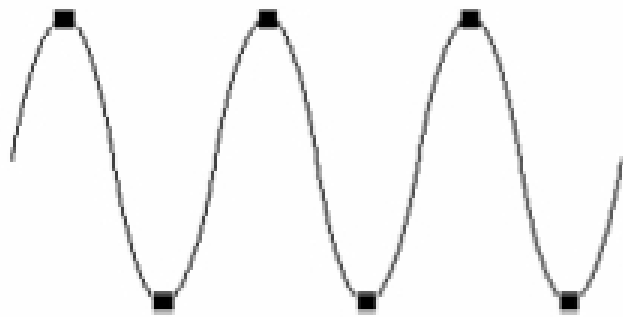
Nyquist-Shannon Sampling

- 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

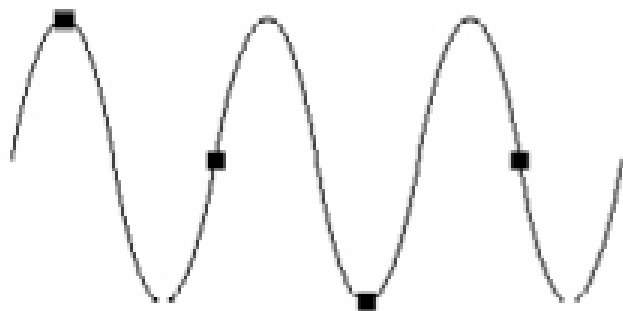
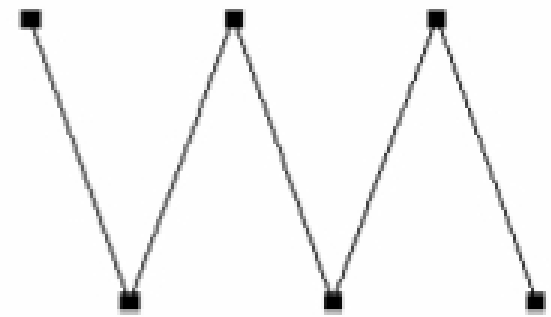
Nyquist-Shannon Sampling



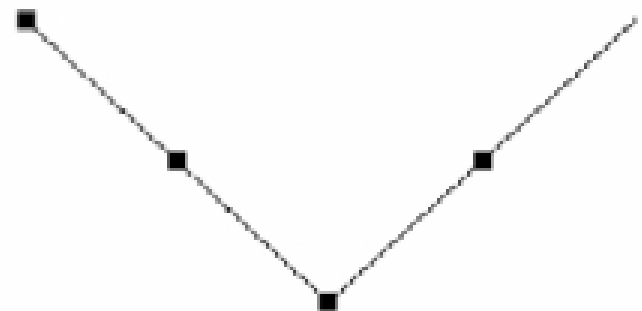
A
→
Sampled at f



B
→
Sampled at $2f$



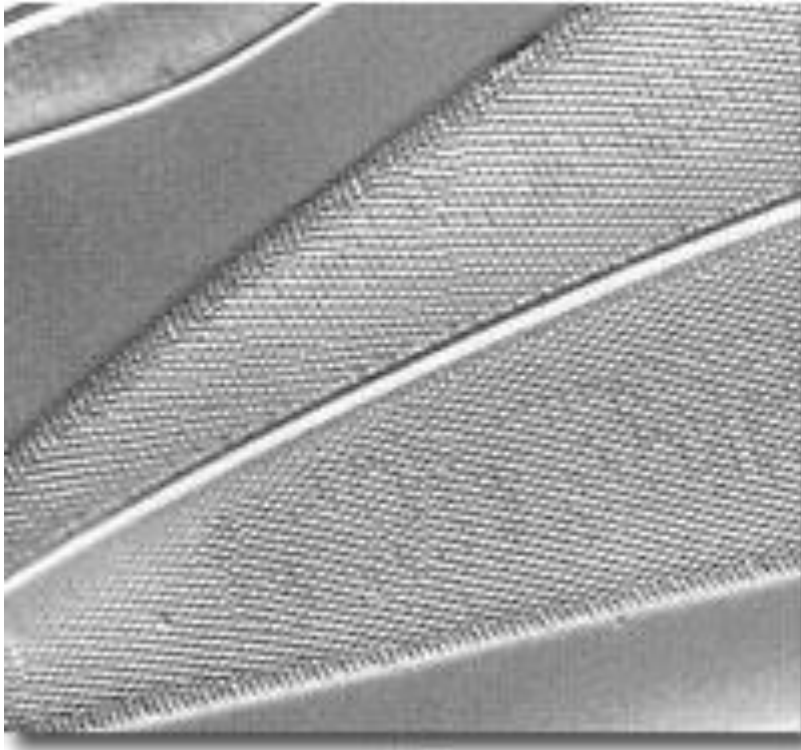
C
→
Sampled at $4f/3$



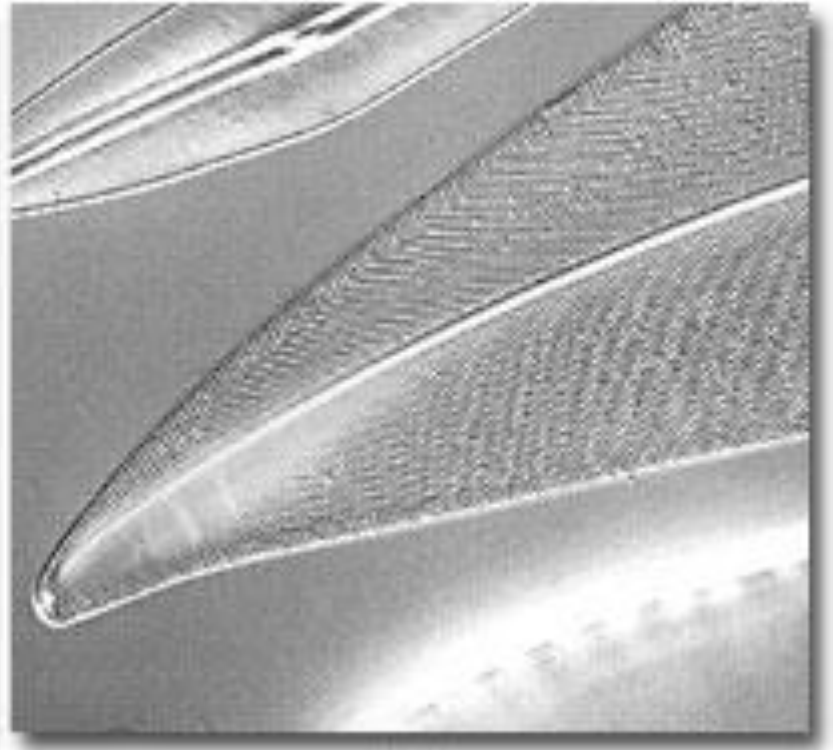
Resolution and CCDs

- Nyquist-Shannon Sampling theorem:
Must have at least two pixels per resolvable element
- E.g: if your resolution is 300 nm, your image should be magnified to so that 150 nm in the sample corresponds to at least one pixel on the camera
- If you fail to do this, you will miss features smaller than twice your sampling size
- You can also run into aliasing problems

Aliasing



Nyquist sampled

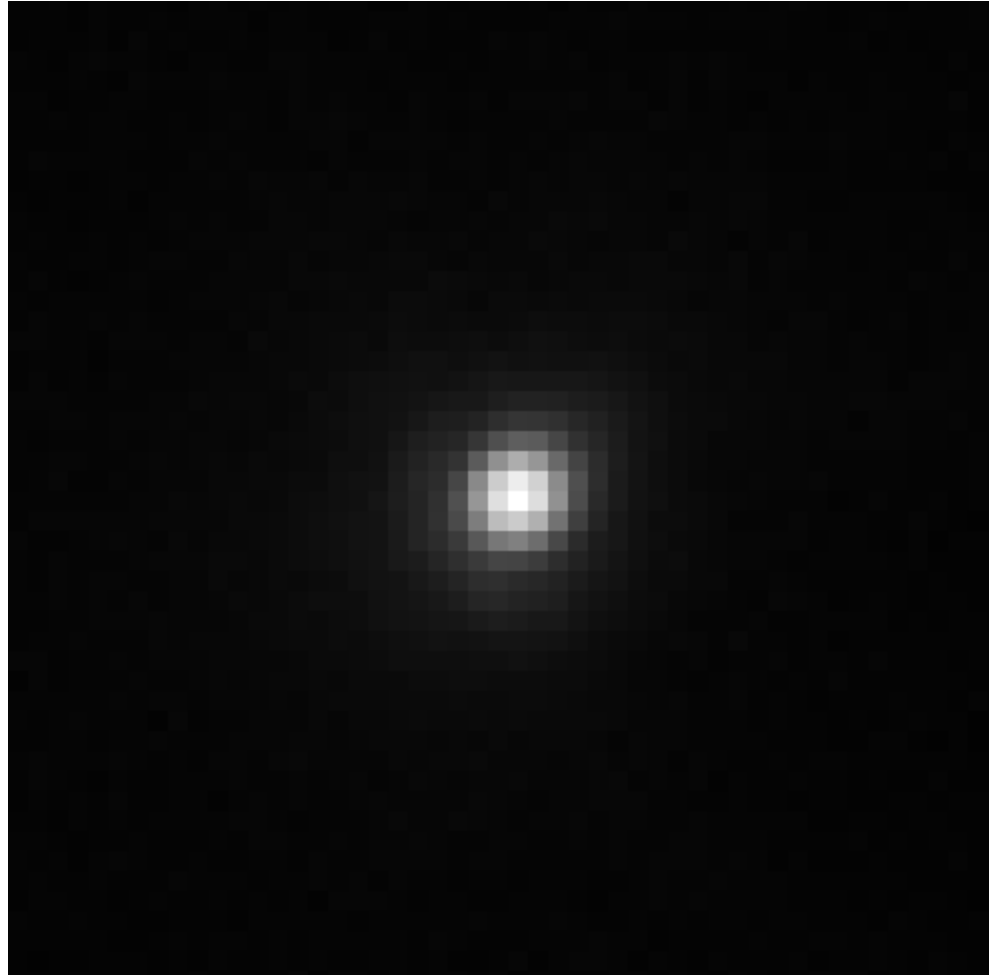


Undersampled

A resolution-centric view of imaging

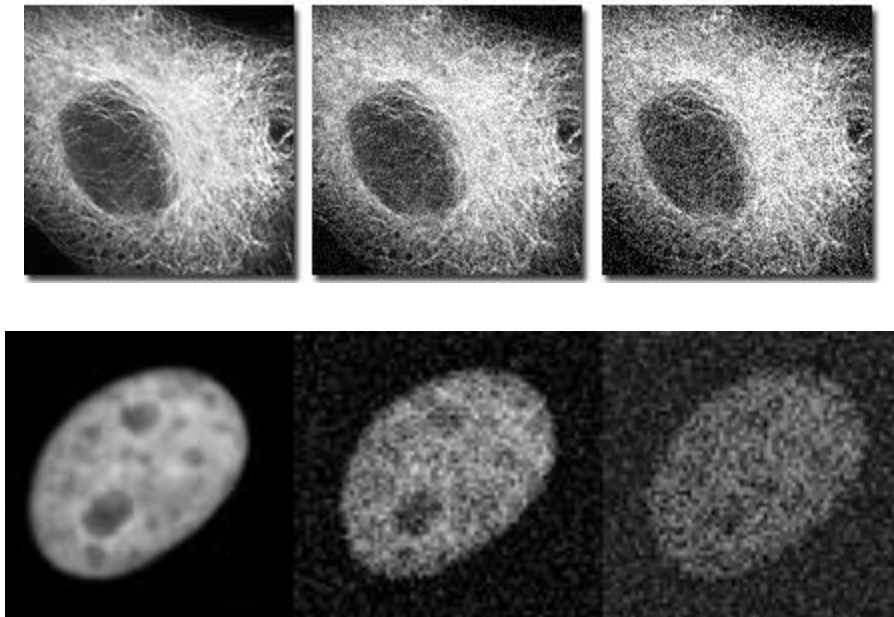
- The objective NA sets the highest resolution you can measure (1.4 NA ~ 220 nm)
- To achieve this resolution, 220 nm in your image must cover 2 pixels
- Choose your magnification to achieve this
- For 6.45 μm pixels, we need a total magnification of $6450/110 = 58.6$
- So for 1.4 NA, a 40x lens would be undersampled, a 60x would be just at the Nyquist limit, and a 100x lens would oversample

Actual PSF



Noise

- Longer exposure times are better – why?



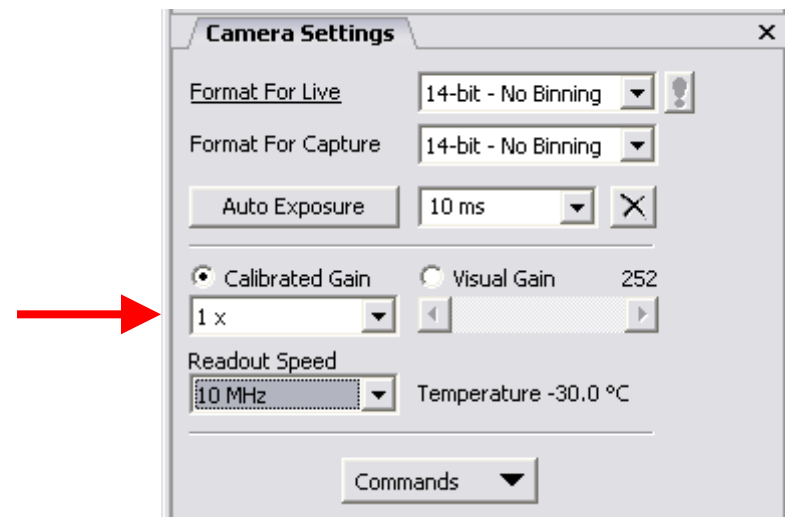
← Increasing exposure time

Noise

- Read noise – inherent in reading out CCD
 - Scales as the square root of readout speed (faster = noisier)
 - For CoolSNAP HQ2: $4.5 \text{ e}^- / \text{pixel}$ @ 10MHz (180 ms readout)
 - $5.5 \text{ e}^- / \text{pixel}$ @ 20MHz (90ms readout)
- Dark current – thermal accumulation of electrons
 - Cooling helps, so negligible for most applications
 - CoolSNAP HQ2: $0.001 \text{ e}^- / \text{pixel} / \text{s}$ (@ -30°C)

Noise

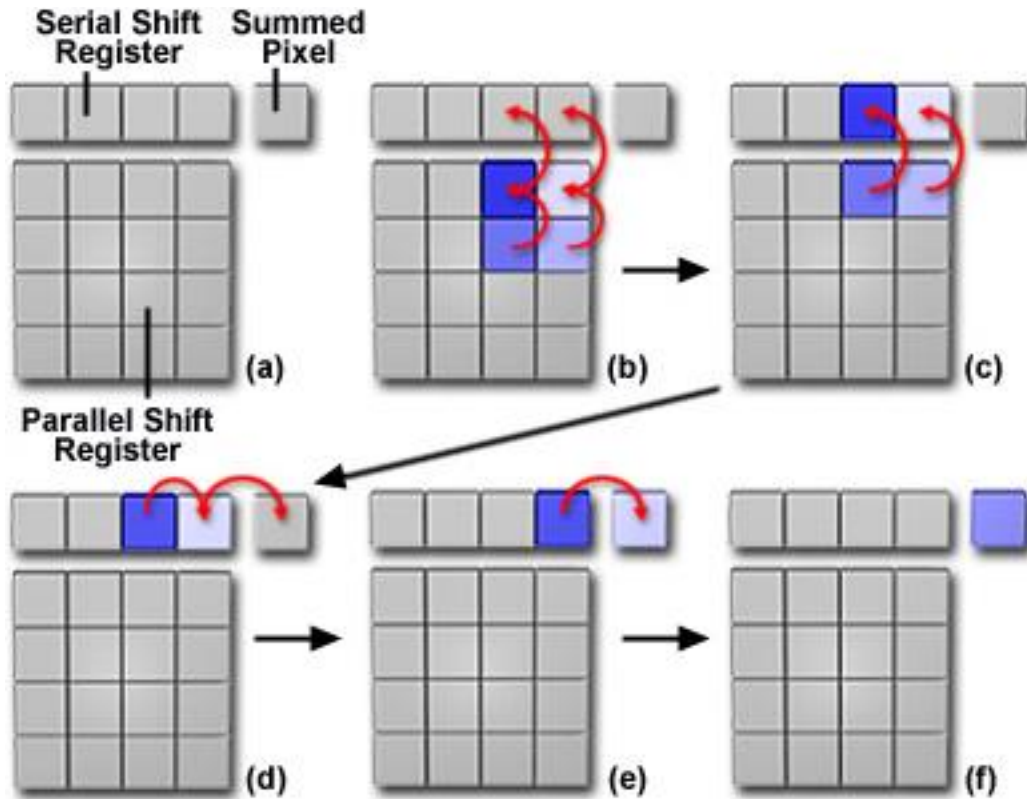
- Photon Shot Noise: Due to the fact that photons are particles and collected in integer numbers
 - Square root of the number of photons
- 1 photon \neq 1 count in your image – depends on the camera (A/D) gain
- Zero photons collected doesn't result in zero being measured on the camera – it has an offset



Signal/Noise Ratio (SNR)

- Signal = # of photons
- Noise = $\sqrt{(\text{read noise}^2 + (\# \text{ of photons}))}$
- At low photon numbers, read noise dominates
- At high photon numbers,
SNR = $(\# \text{ of photons}) / \sqrt{(\# \text{ of photons})}$
= $\sqrt{(\# \text{ of photons})}$
- So, to double your SNR, you need to acquire four times as long (or 2x2 bin)

Binning



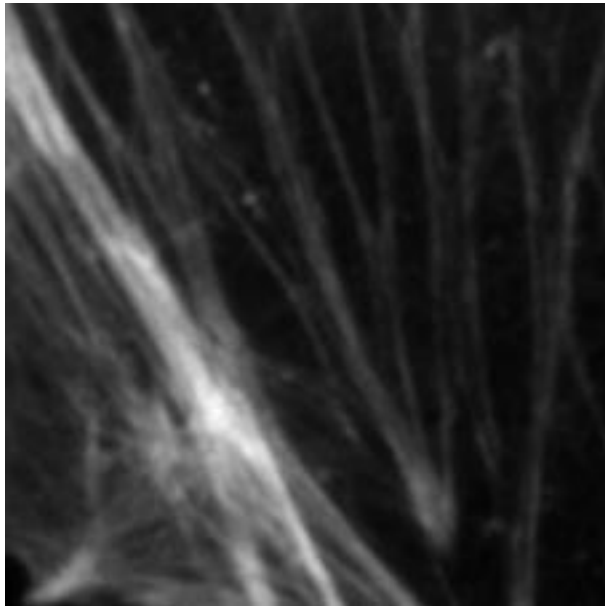
- Read out 4 pixels as one
- Increases SNR by 2x
- Decreases read time by 2 or 4x
- Decreases resolution by 2x

Signal/Noise Ratio (SNR)

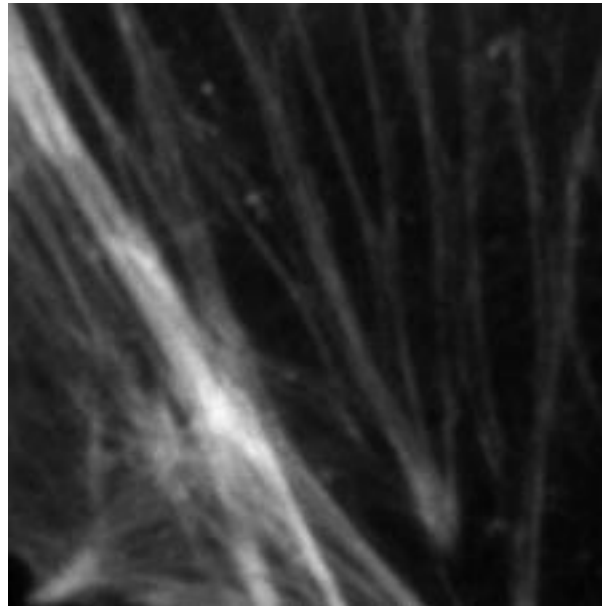
- Read noise dominates whenever $\text{read noise}^2 = \# \text{ of photons}$
- 8 e- read noise \rightarrow 64 photons
- 16 e- read noise \rightarrow 256 photons
- 50 e- read noise \rightarrow 2500 photons
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

What does this look like?

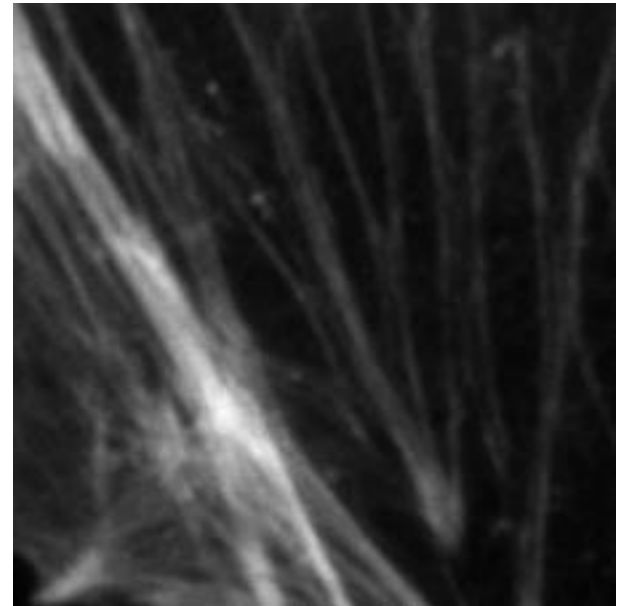
1000 photons / pixel on average; ~ 5000 in brightest areas



Test image



no read noise

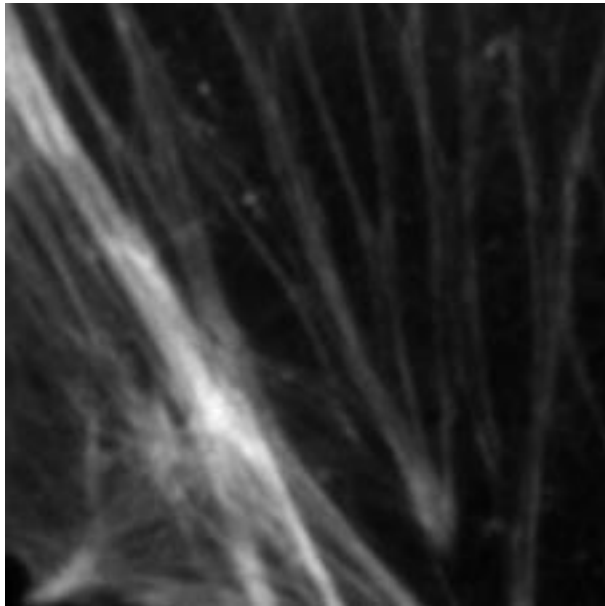


5 e⁻ read noise

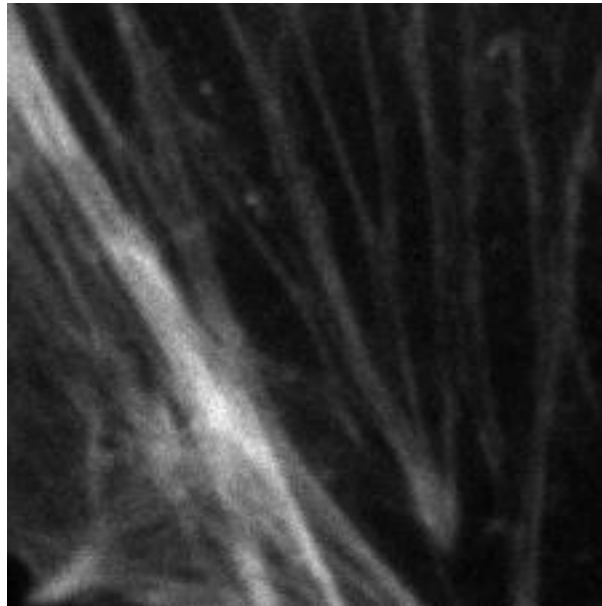
Photon shot noise $\sim 6\times$ read noise

What does this look like?

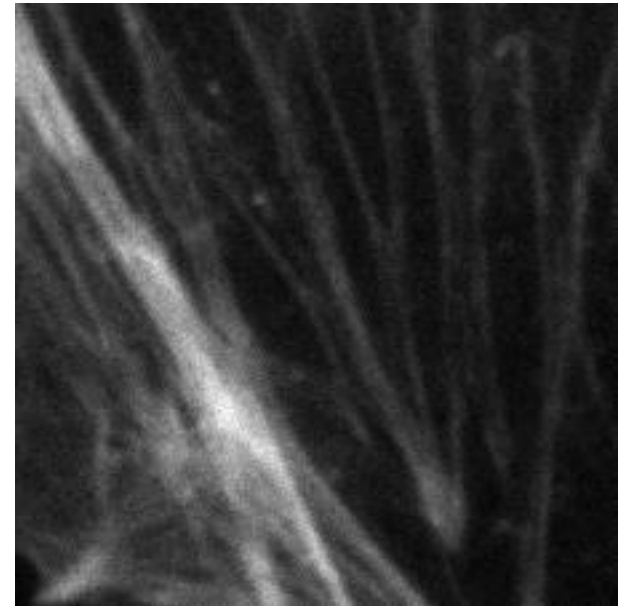
100 photons / pixel on average; ~ 500 in brightest areas



Test image



no read noise

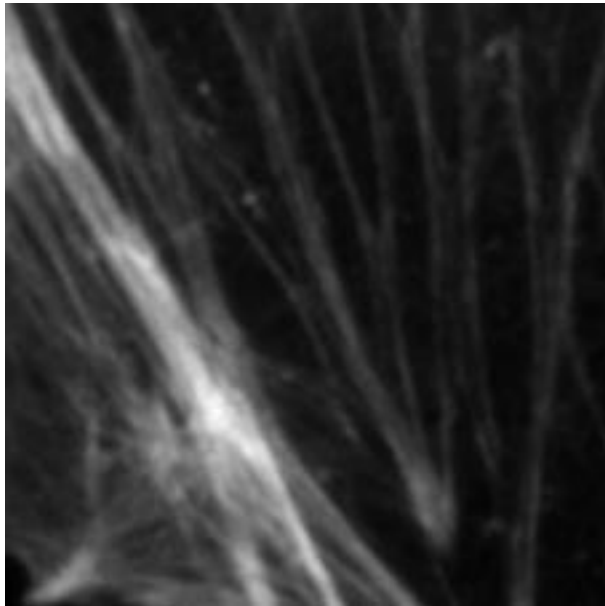


5 e⁻ read noise

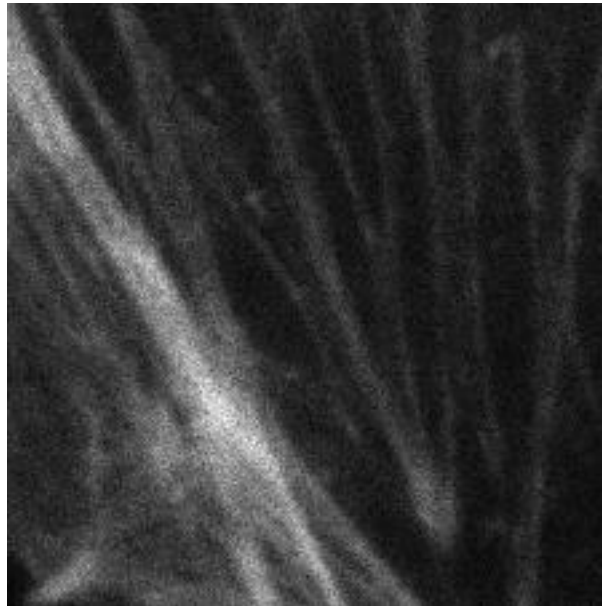
Photon shot noise = 2x read noise

What does this look like?

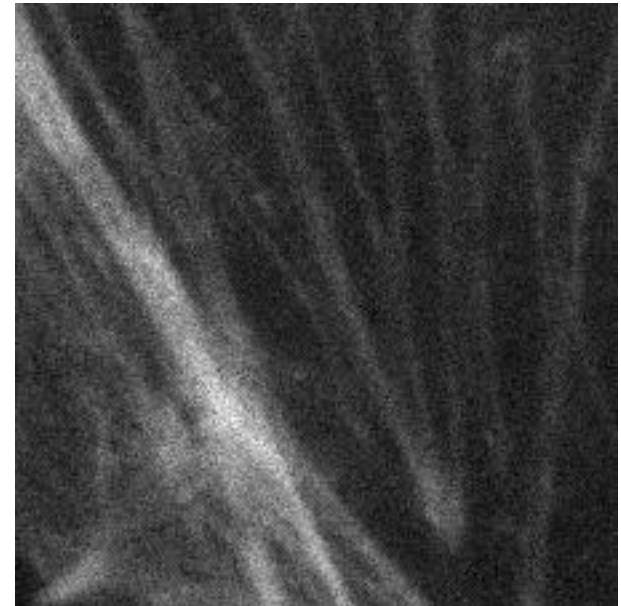
25 photons / pixel on average; ~ 125 in brightest areas



Test image



no read noise

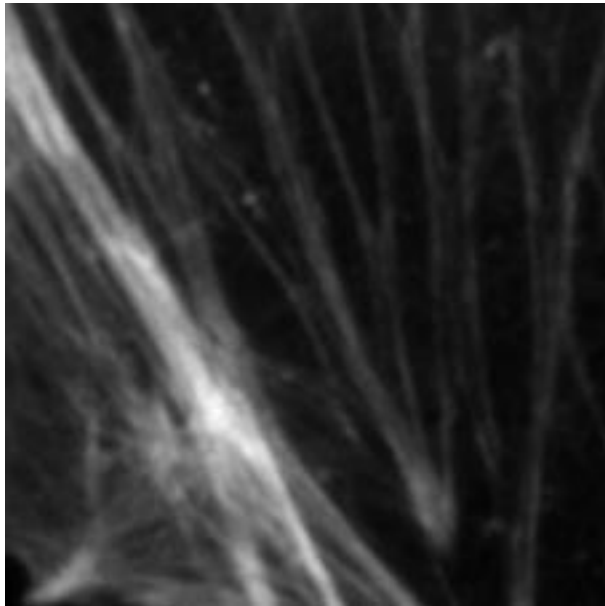


5 e⁻ read noise

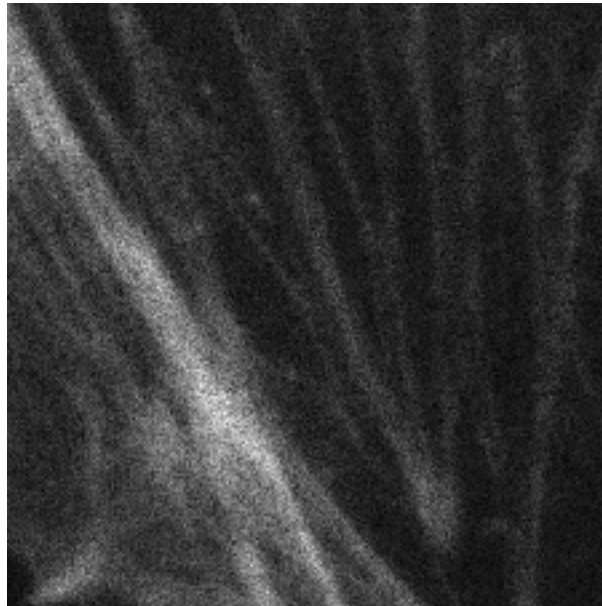
Photon shot noise = read noise

What does this look like?

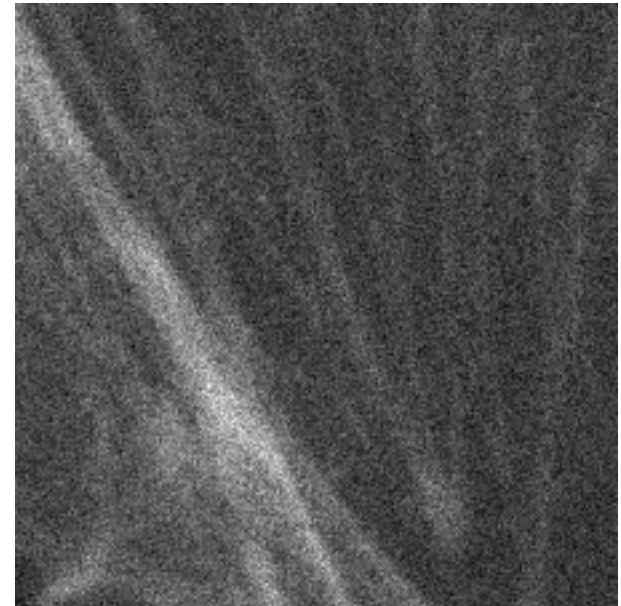
10 photons / pixel on average; ~ 50 in brightest areas



Test image



no read noise

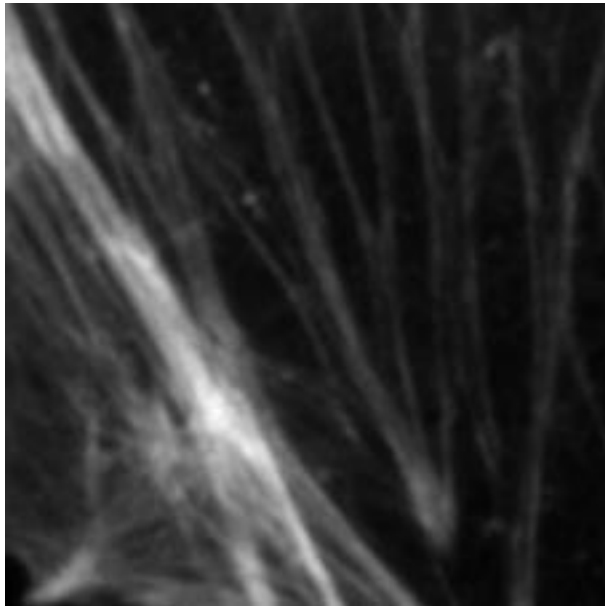


5 e⁻ read noise

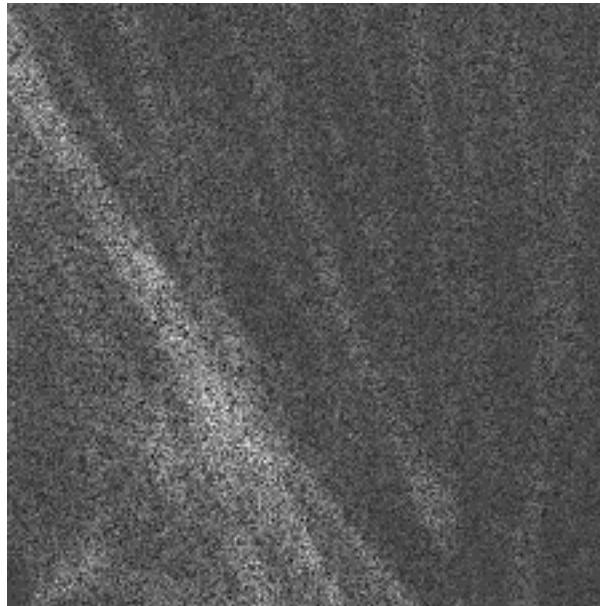
Photon shot noise $\sim 2/3$ read noise

What does this look like?

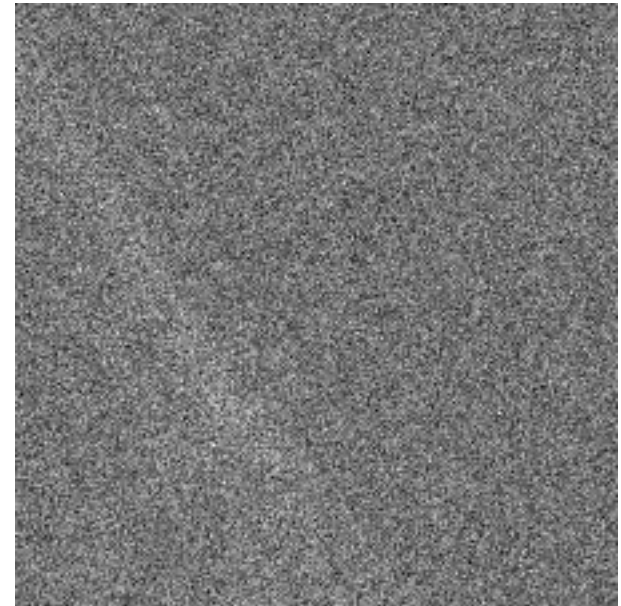
1 photon / pixel on average; ~ 5 in brightest areas



Test image



no read noise



5 e⁻ read noise

Photon shot noise $\sim 1/5$ read noise

Beating the read-out noise EMCCD

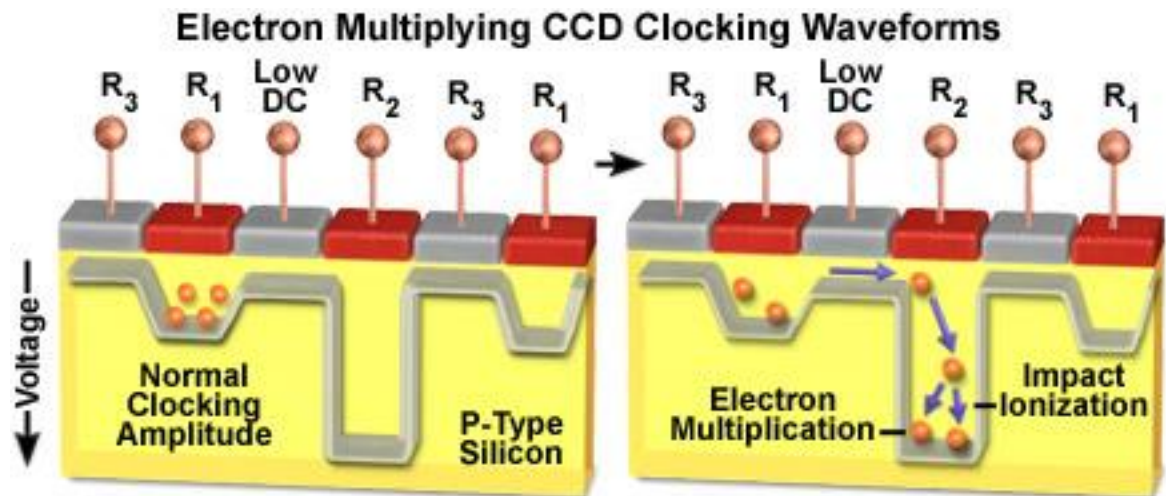
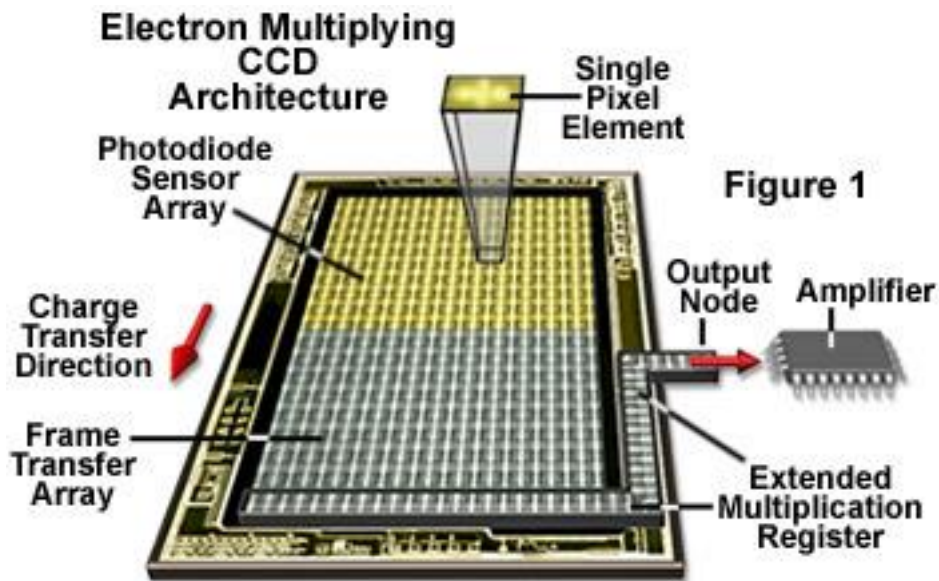


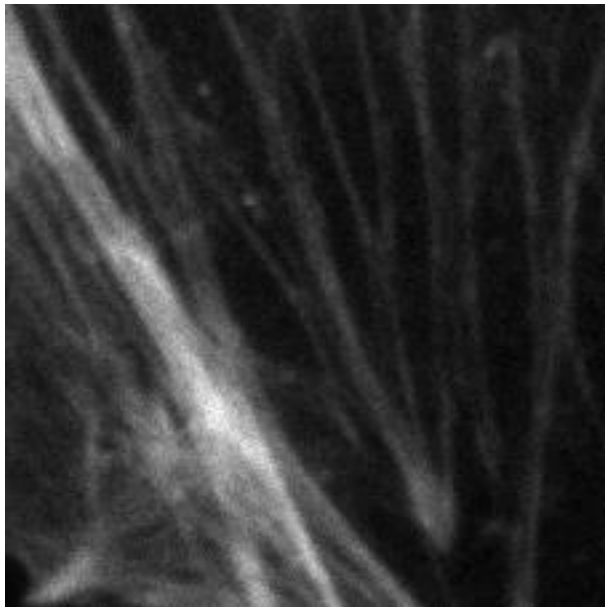
Figure 4

EMCCD result

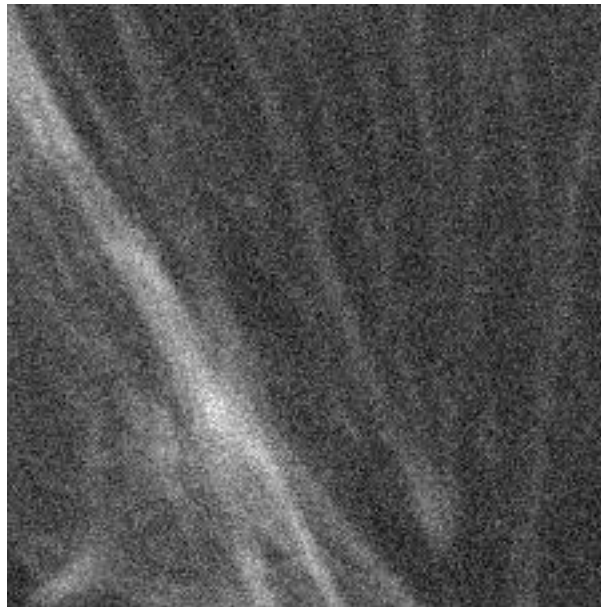
- Fast noisy CCD – runs at 30 fps, but 50 e⁻ read noise
- Multiply signal by 100-fold – now read noise looks like 0.5 e⁻
- Downside – multiplication process adds additional Poisson noise, so your QE looks like it's halved
- Upside – you get to image fast without worrying about read noise

Hypothetical CCD/EMCCD comparison

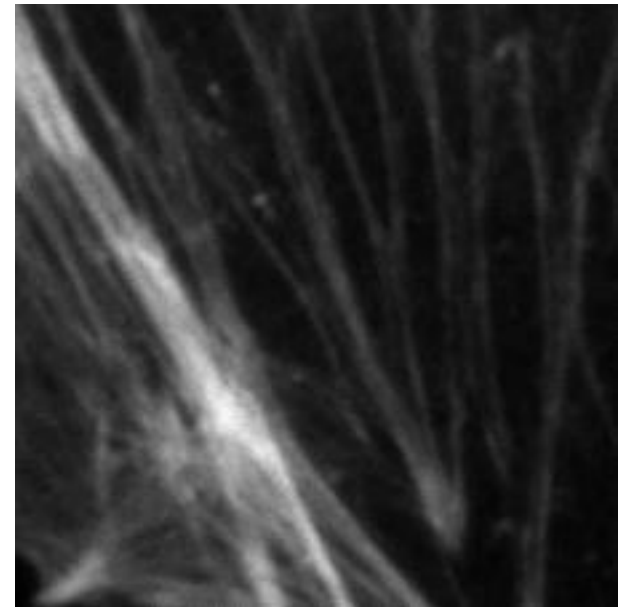
100 photon / pixel on average; ~500 in brightest areas



Slow scan CCD
4e⁻ read noise
(1 sec read time)



Video rate CCD,
50e⁻ read noise



Video rate EMCCD
50e⁻ read noise
200x gain

CCDs vs. CMOS

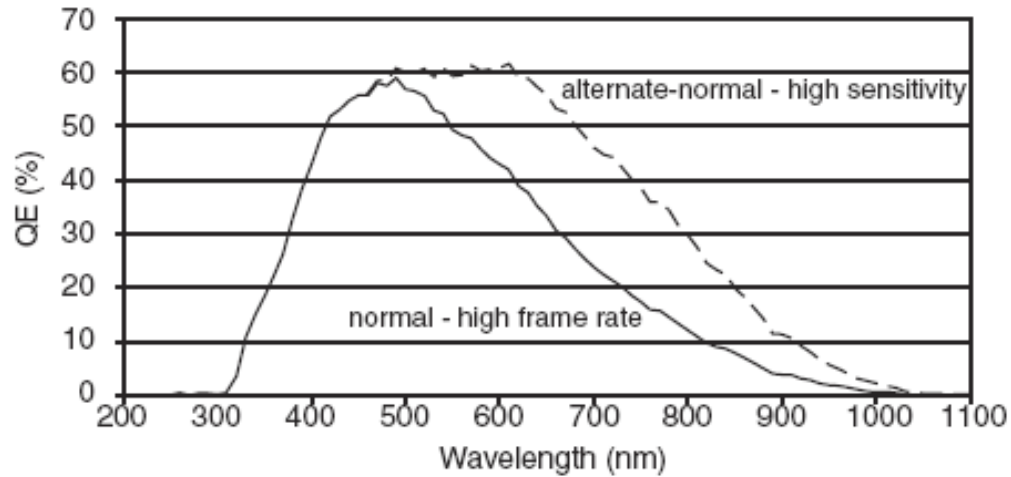
- CCDs:
 - Output electrons
 - Off chip amplifier and A/D converter generate output signal
 - Slow, low noise
- CMOS
 - Outputs digital signal
 - Each pixel has its own amplifier; each row has its own A/D converter
 - Fast, noisy

New: sCMOS

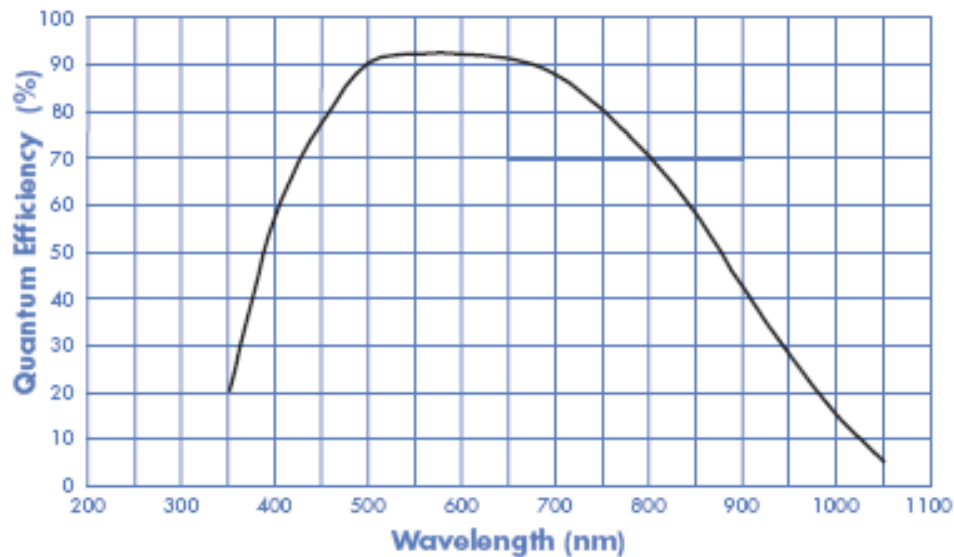
Scientific CMOS

- Like CMOS, but better
 - Differences are proprietary
 - Vendors: Hamamatsu, Andor, PCO
- Specs (Andor Neo):
 - 5.5 megapixels (2560 x 2160)
 - 6.5 μm pixels
 - 100 fps readout
 - 1.5 e^- read noise

Quantum efficiency



HQ2

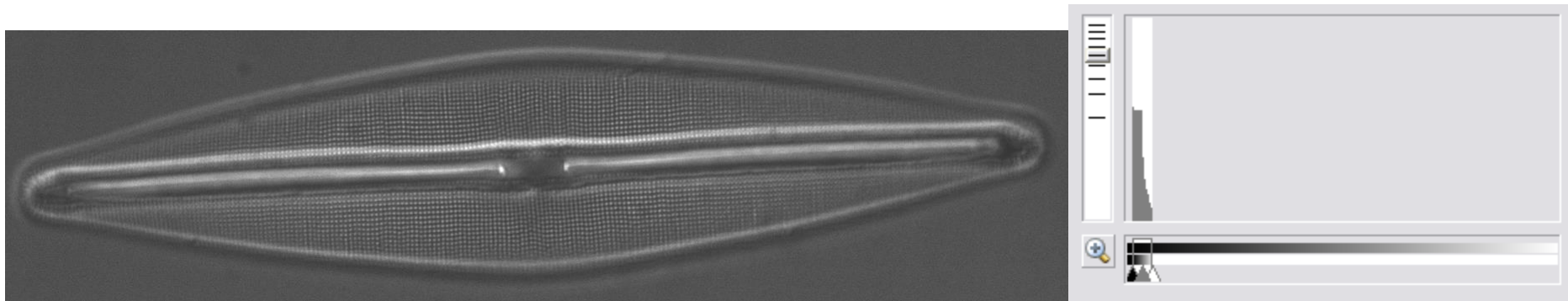
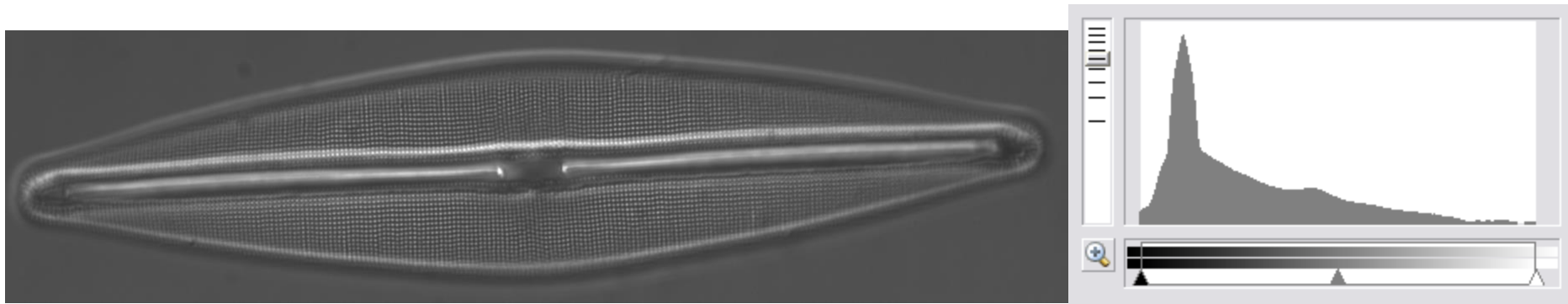


Cascade II

How many intensity levels can you distinguish?

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

Check your histogram



Improve Signal/noise

- Use bright, non-bleaching fluorophores
- Best possible optics (high NA lenses, high QE camera, high transmission filters, reduce spherical aberration, no phase!)
- Minimize optical elements between your sample and the camera (use bottom port!)
- Work in the dark, use clean cover slips, reagents, etc..
- Increase exposure or use frame averaging
- Binning (at the expense of spatial resolution)

Acknowledgements

- www.microscopyu.com
- Nico Stuurman
- James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.), especially appendix 3: “More than you ever really wanted to know about charge-coupled devices”
- James Janesick, “Scientific Charge Coupled Devices” (if you really, really, want to know about CCDs)