Digital Cameras in Microscopy

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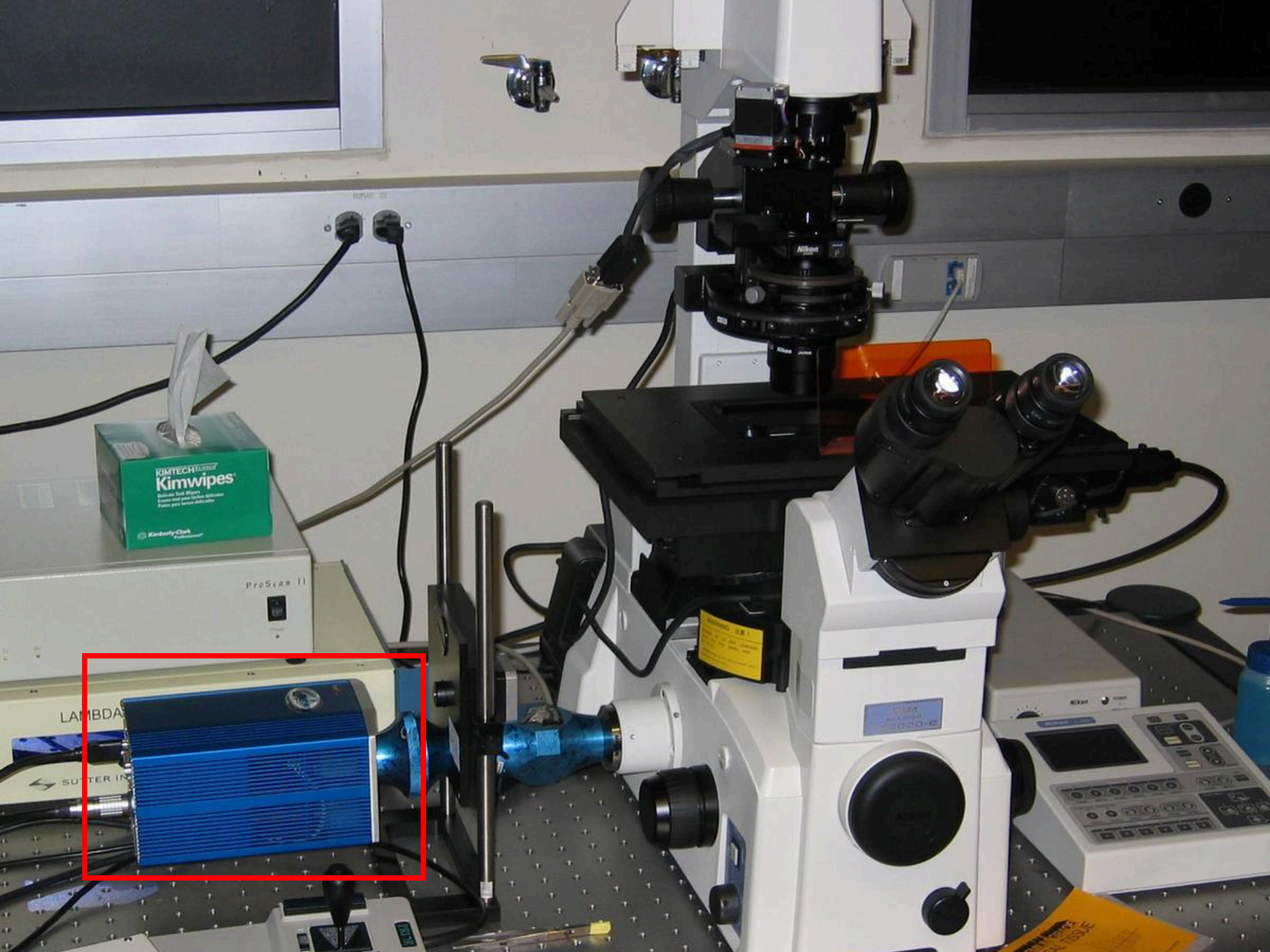
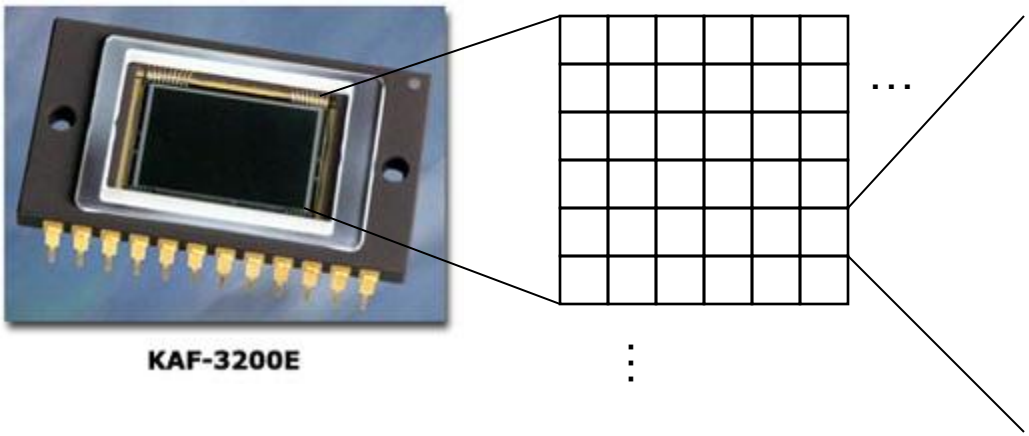

KIMTECH
Kimwipes
Kimberly-Clark
ProScan II
LAMBDA
SUTTER IN

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



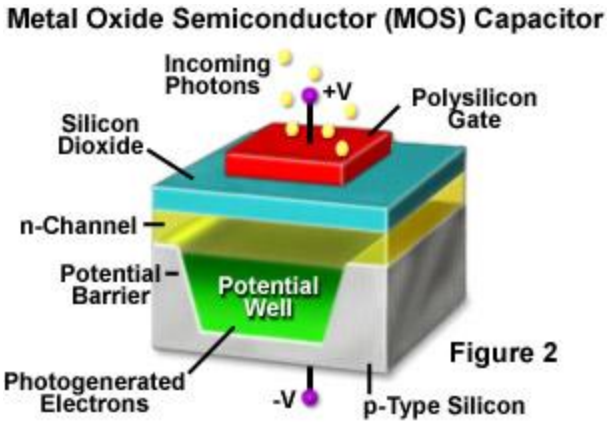


Figure 2

CCD readout “bucket-brigade” analogy

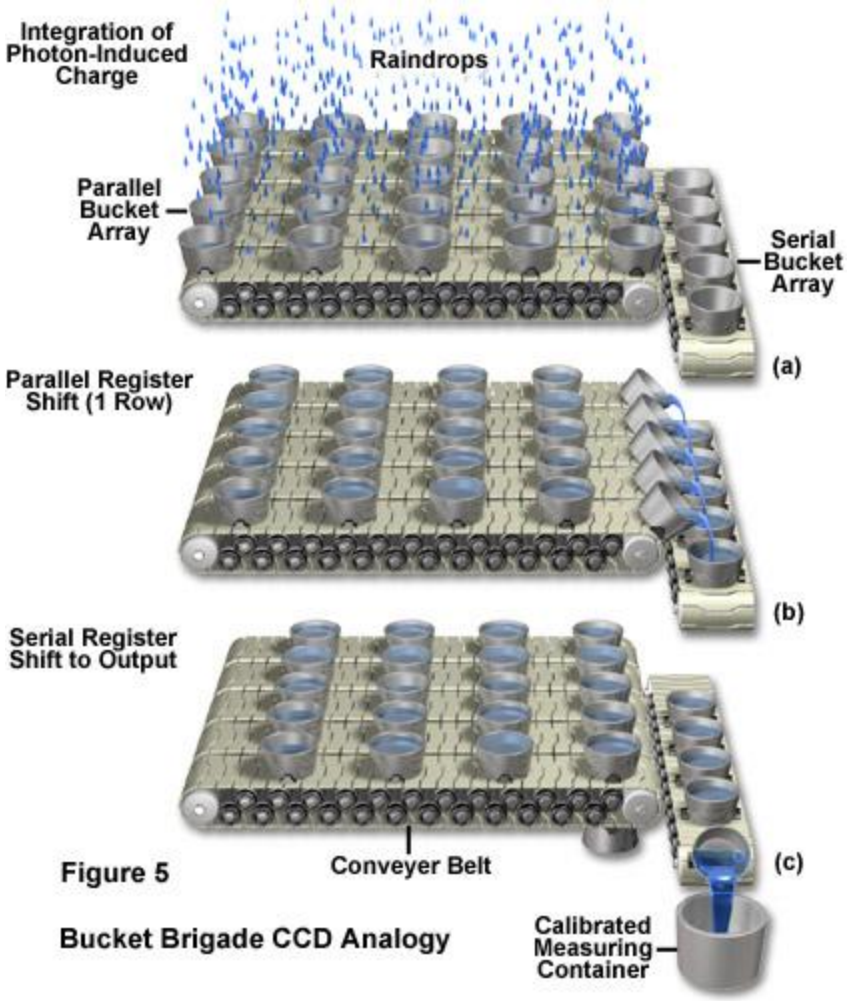


Figure 5

Bucket Brigade CCD Analogy

A little more realistic….

Each pixel is subdivided into three phases

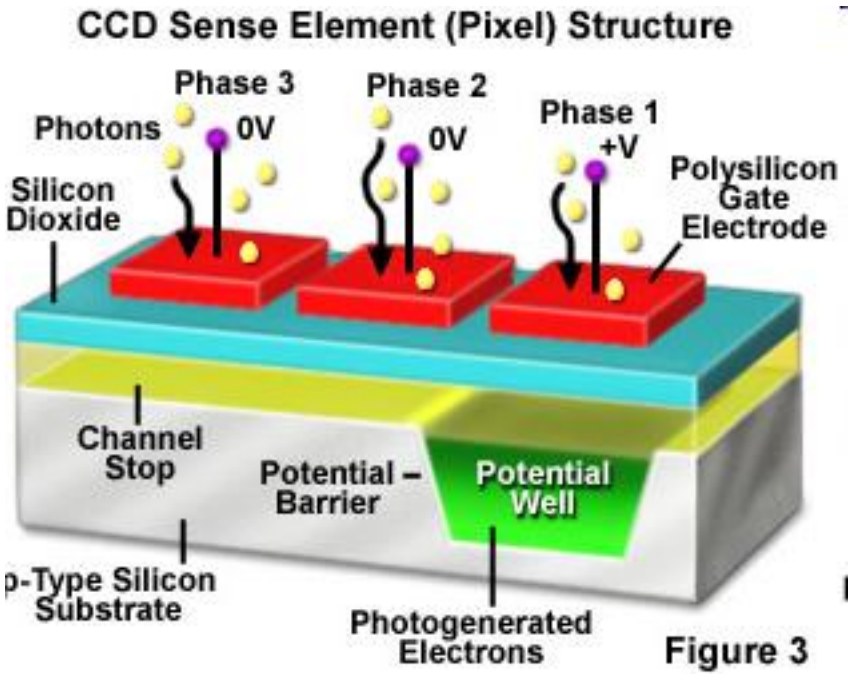
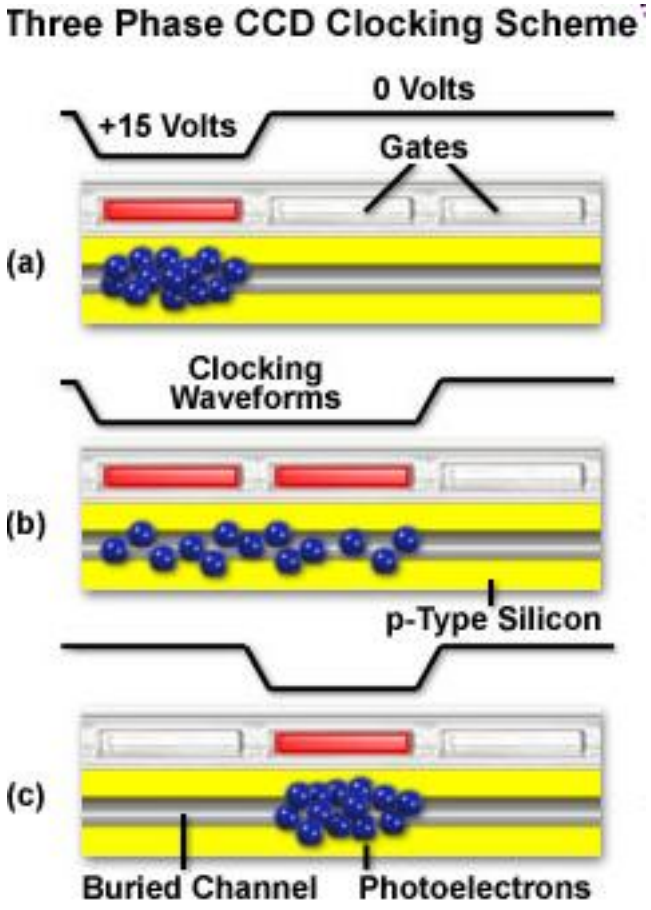
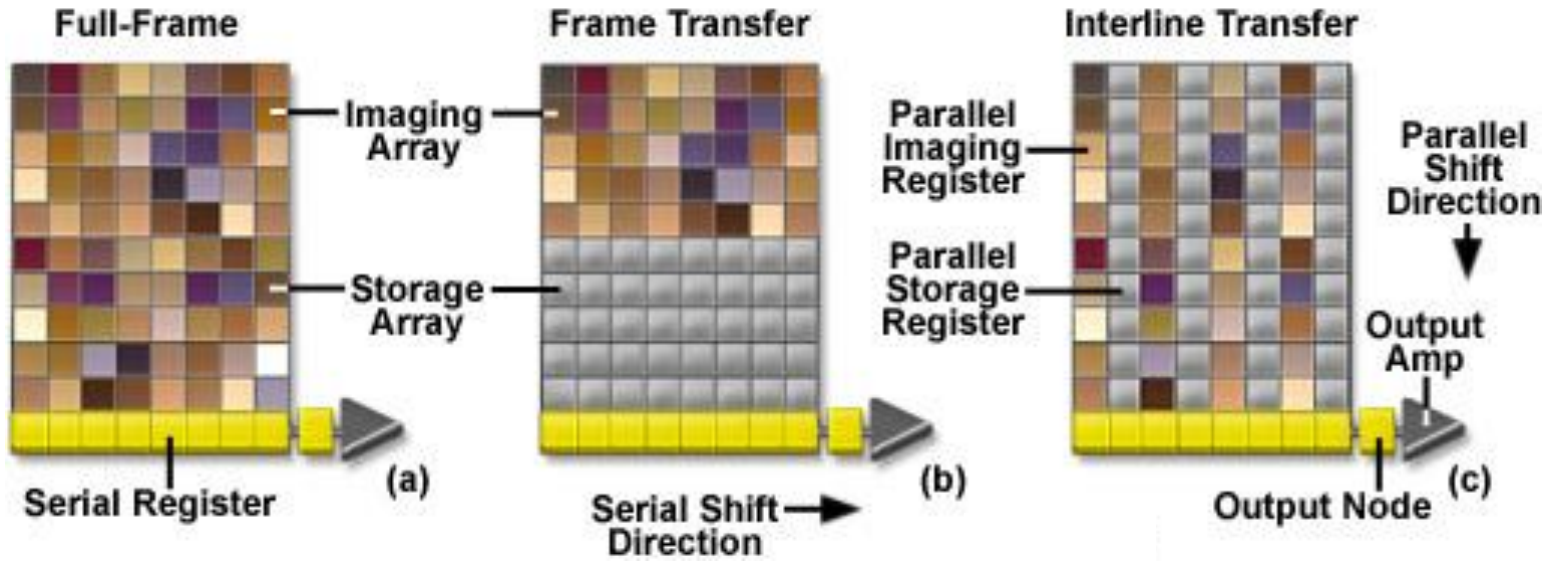


Figure 3



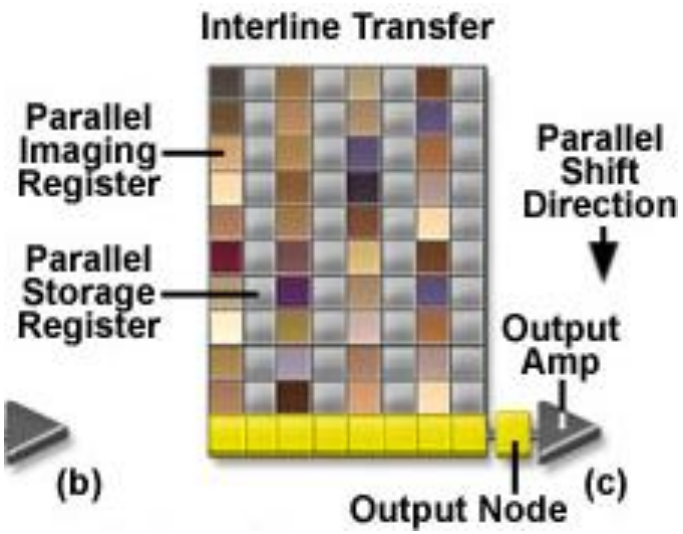
CCD Architectures

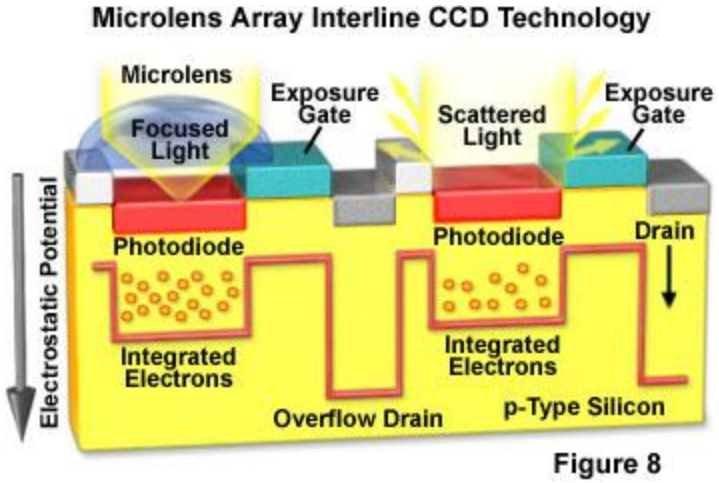


Rare Mostly EMCCDs Common

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

Interline CCDs and microlenses

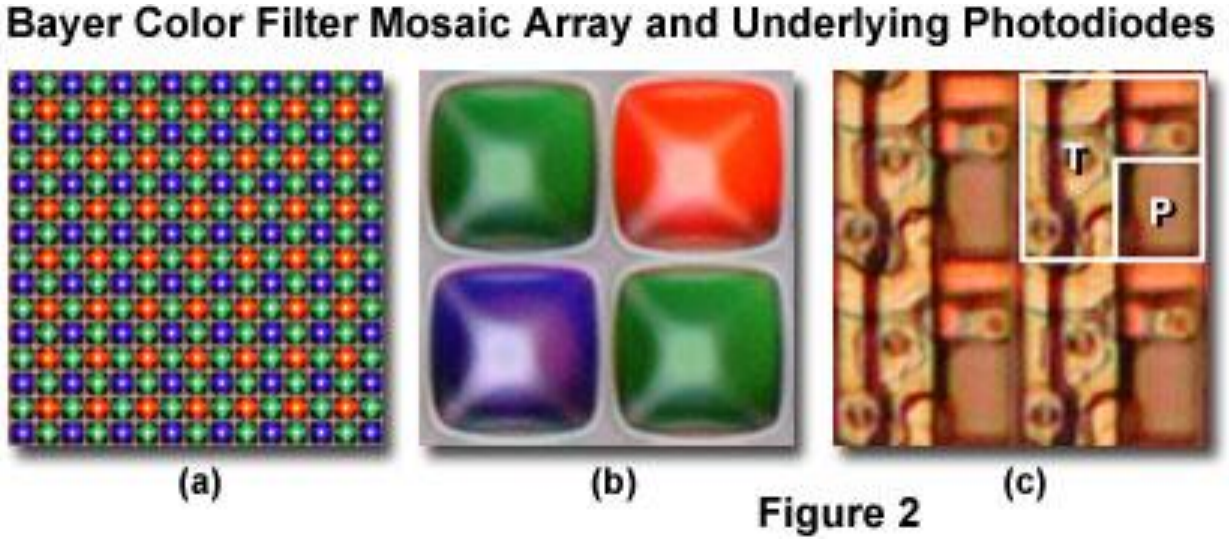




Interline storage registers take up half the light gathering area on the CCD

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

Why don’t we use color CCDs?

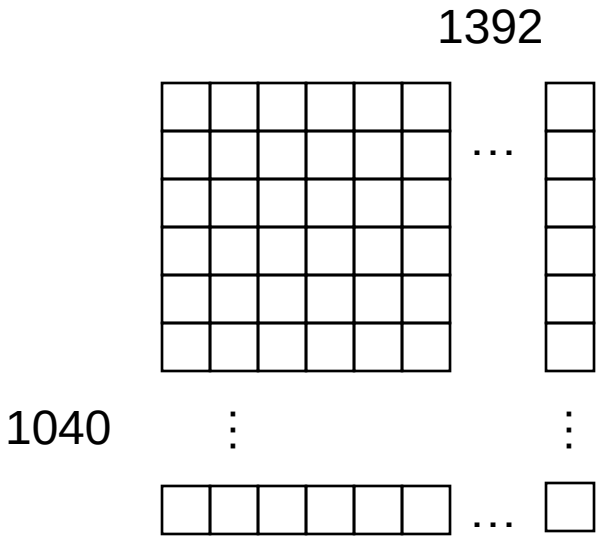


- Four monochrome pixels are required to measure one color pixel
- Your 5MP digital camera really acquires a 1.25 MP red and blue image and a 2.5 MP green image and uses image processing to reconstruct the true color image at 5 MP

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

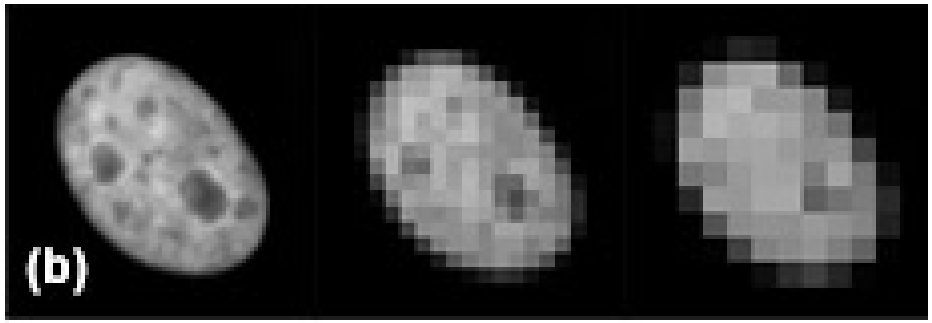


6.45 μm on a side

Chip is 8.98 x 6.71 mm on a side

Typical magnification from sample to camera is roughly objective magnification

Resolution and magnification



More pixels / resolution element

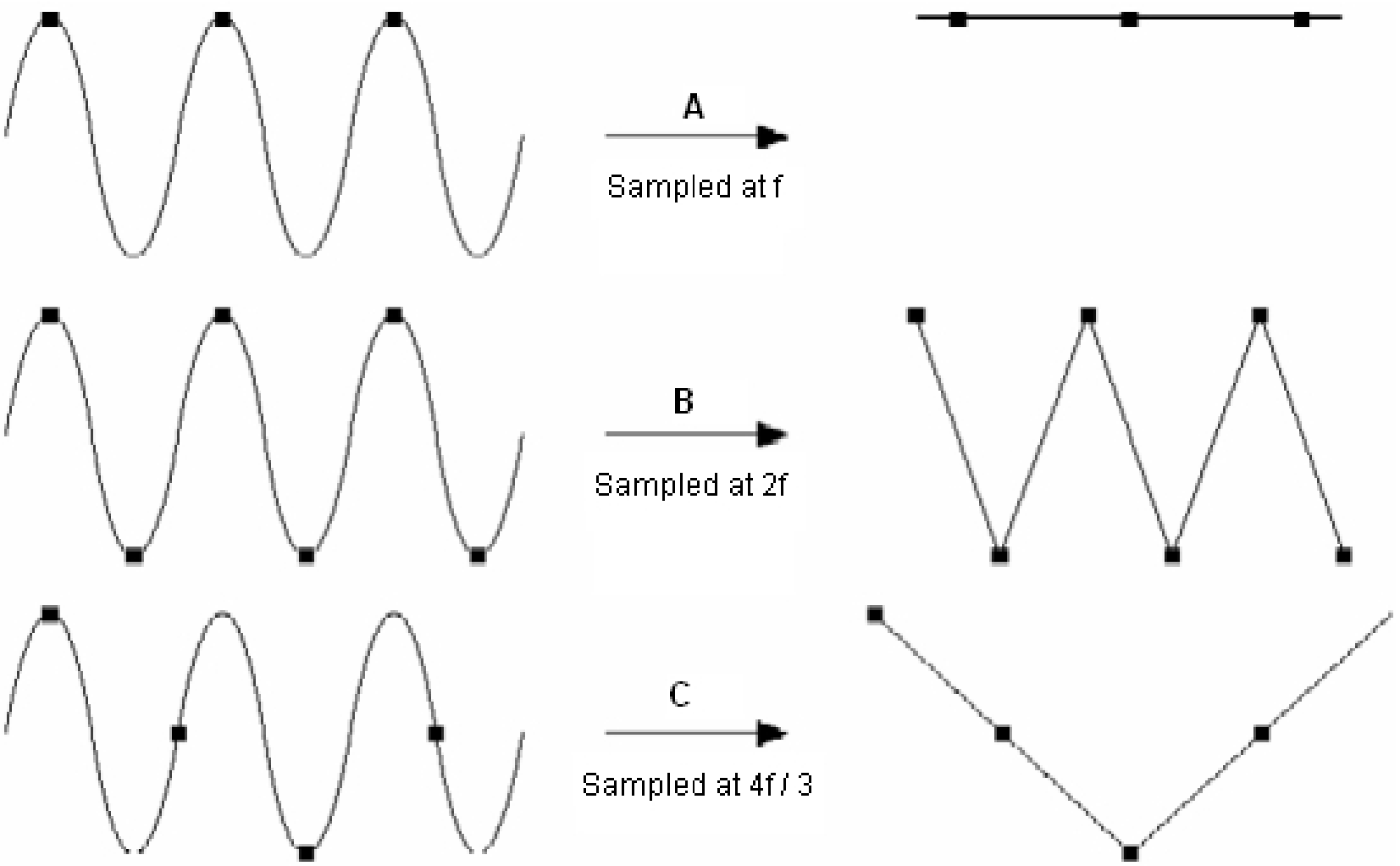
Where is optimum?

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

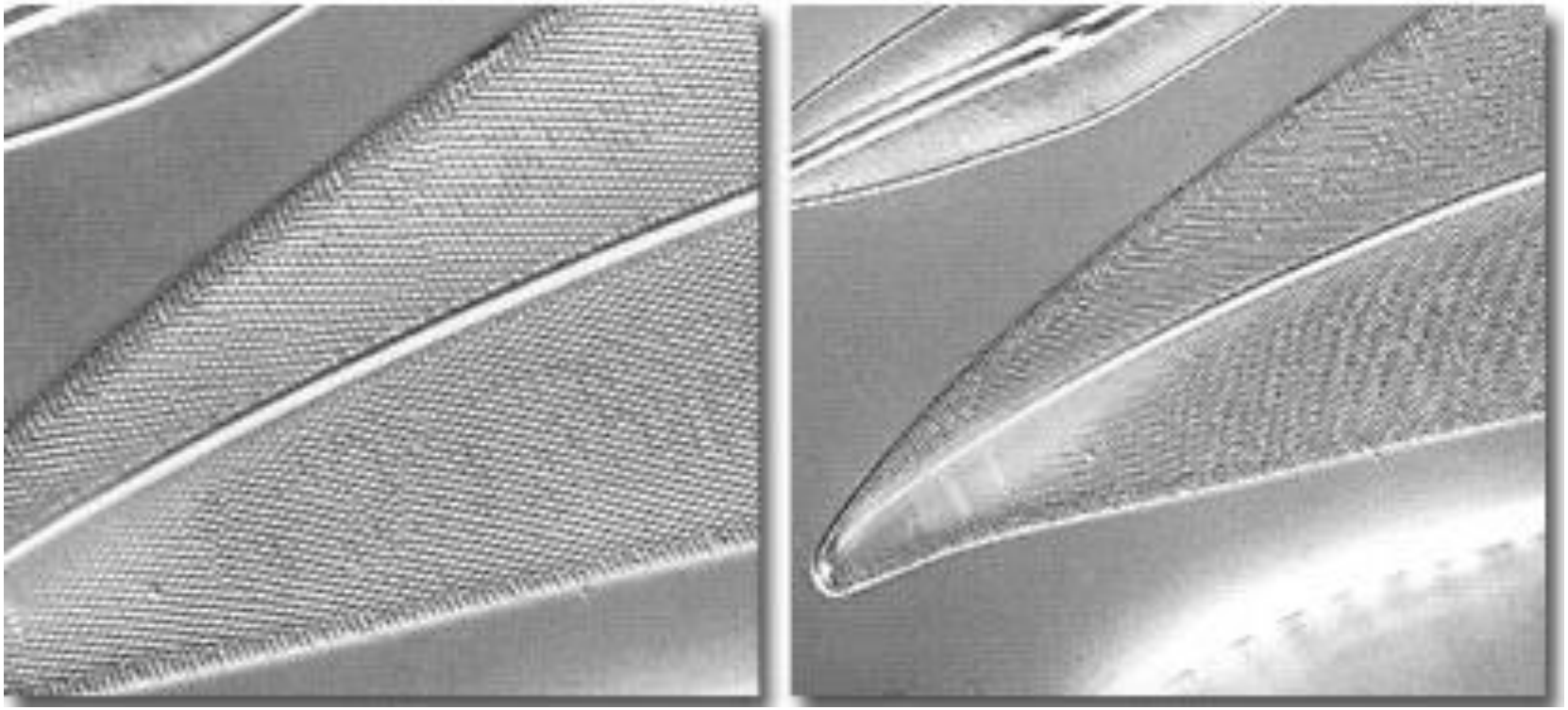


Resolution and CCDs

- 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



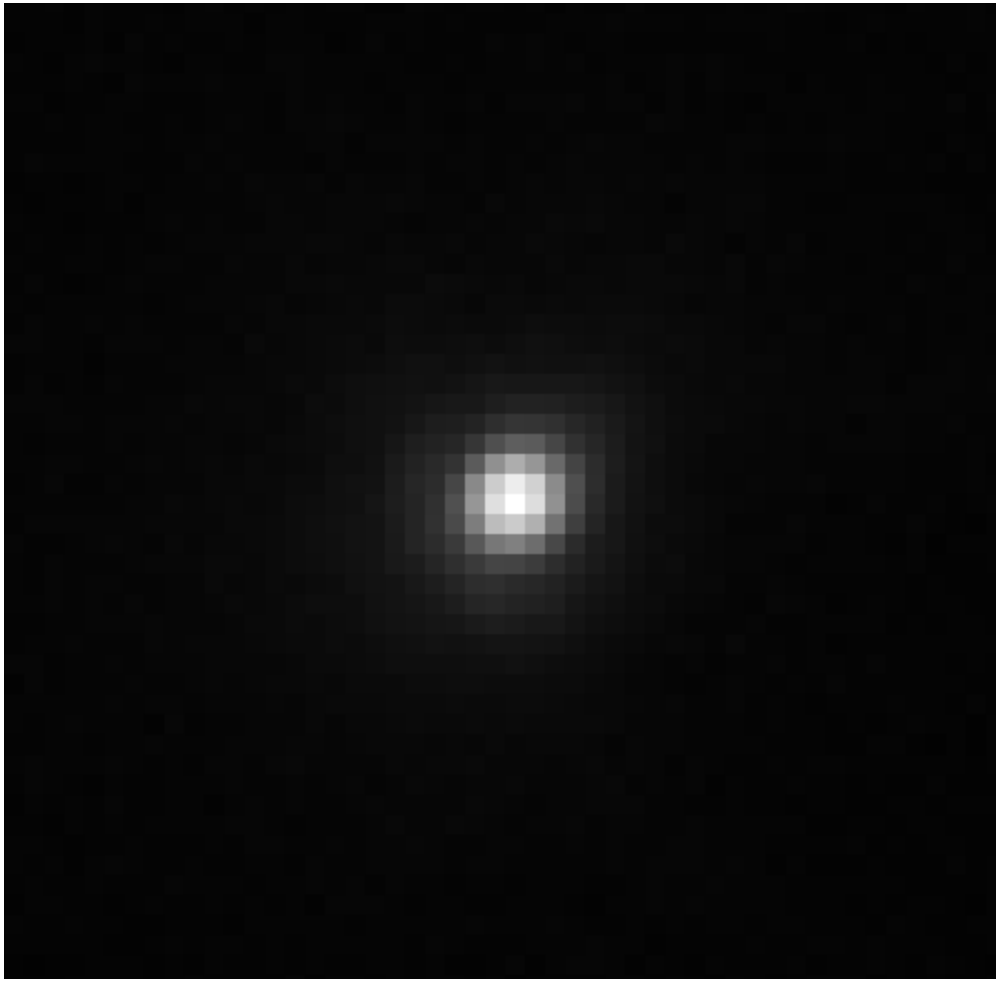
Nyquist sampled

Undersampled

A resolution-centric view of imaging

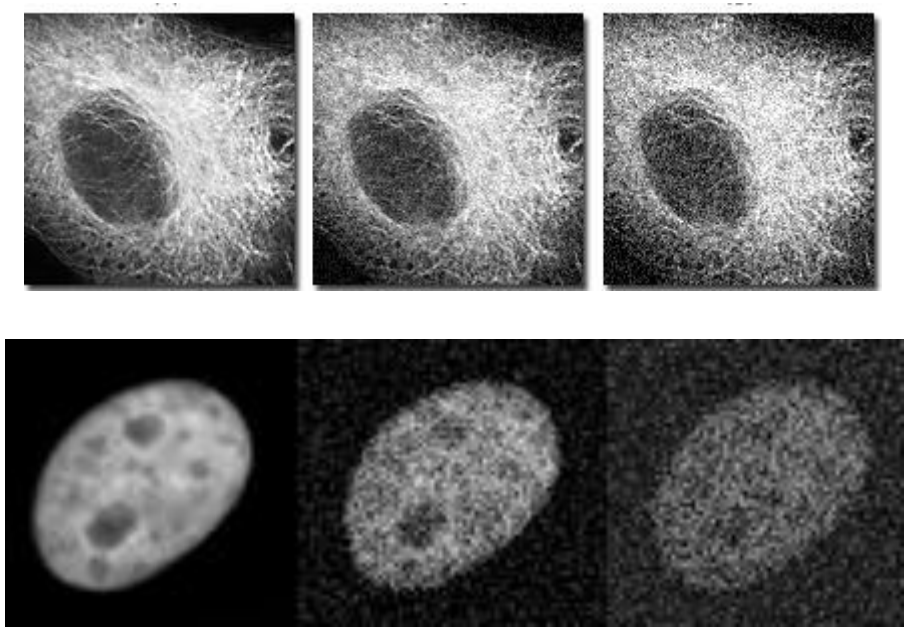
- The objective NA sets the highest resolution you can measure (1.4 NA ~ 220 nm)
- To achieve this resolution, 220 nm in your image must cover 2 pixels
- Choose your magnification to achieve this
- For 6.45 μm pixels, we need a total magnification of 6450/110 = 58.6
- So for 1.4 NA, a 40x lens would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



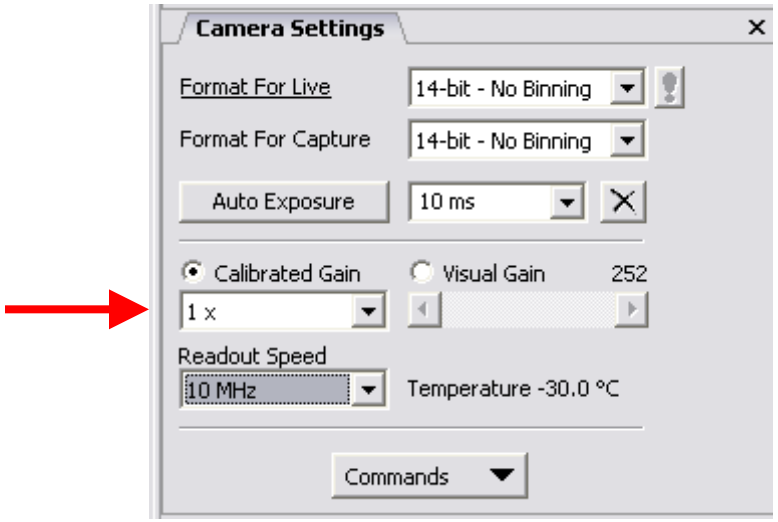
Noise

- Read noise – inherent in reading out CCD
  - Scales as the square root of readout speed (faster = noisier)
  - For CoolSNAP HQ2: 4.5 e^- / pixel @ 10MHz (180 ms readout)
  - 5.5 e^- / pixel @ 20MHz (90ms readout)

- Dark current – thermal accumulation of electrons
  - Cooling helps, so negligible for most applications
  - CoolSNAP HQ2: 0.001 e^- / pixel / 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 ≠ 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)

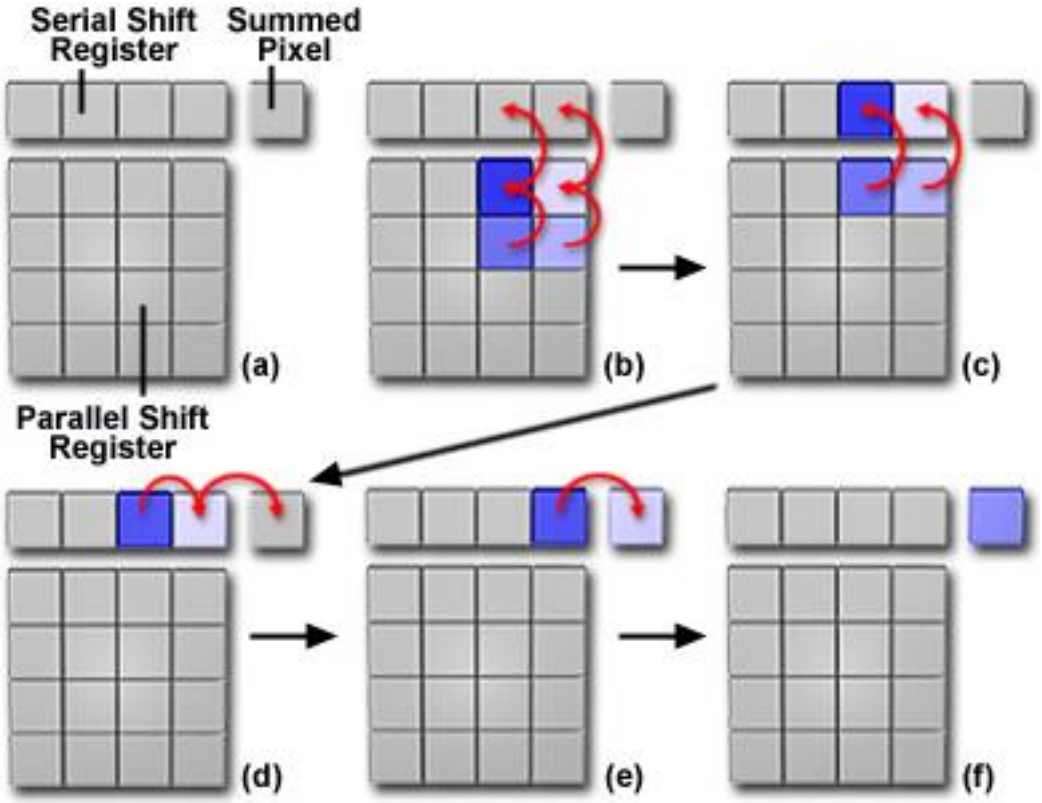
- Signal = # of photons
- Noise = $\sqrt{}$ (read noise^2 +(# of photons))

- At low photon numbers, read noise dominates

- At high photon numbers,
  SNR = (# of photons)/ $\sqrt{}$(# of photons)
  = $\sqrt{}$(# 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)

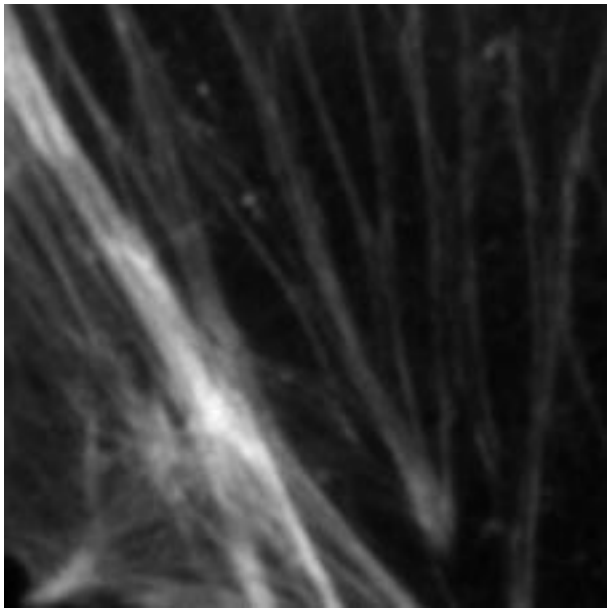
- Read noise dominates whenever
  read noise^2 = # of photons

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

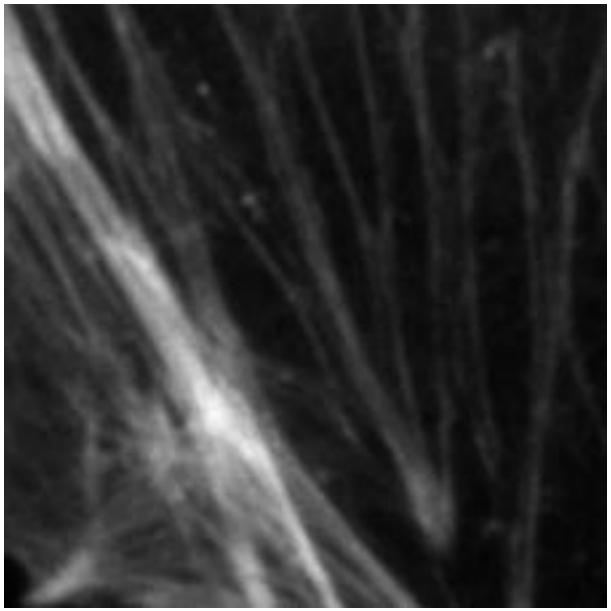
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

What does this look like?

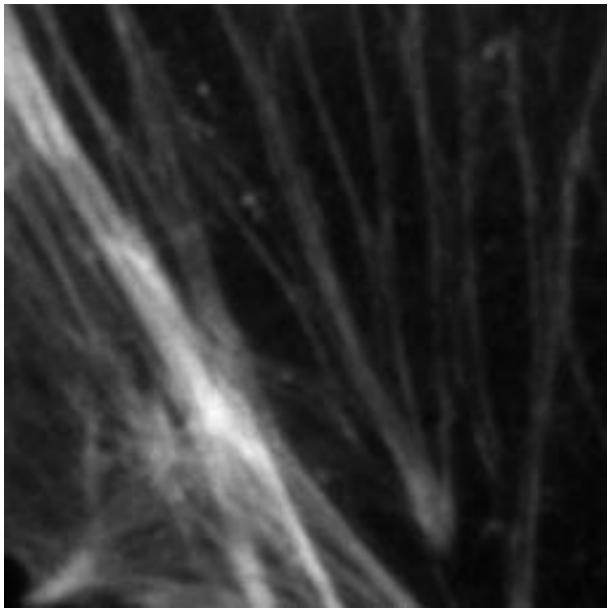
1000 photons / pixel on average; ~5000 in brightest areas



Test image



no read noise

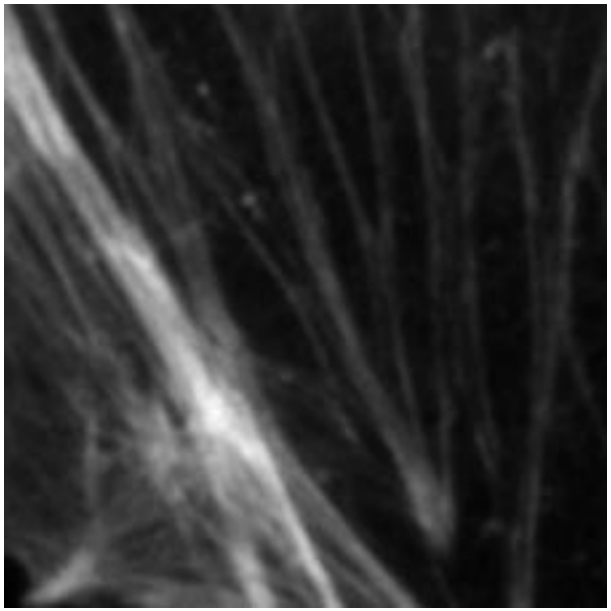


5 e^- read noise

Photon shot noise ~ 6x read noise

What does this look like?

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



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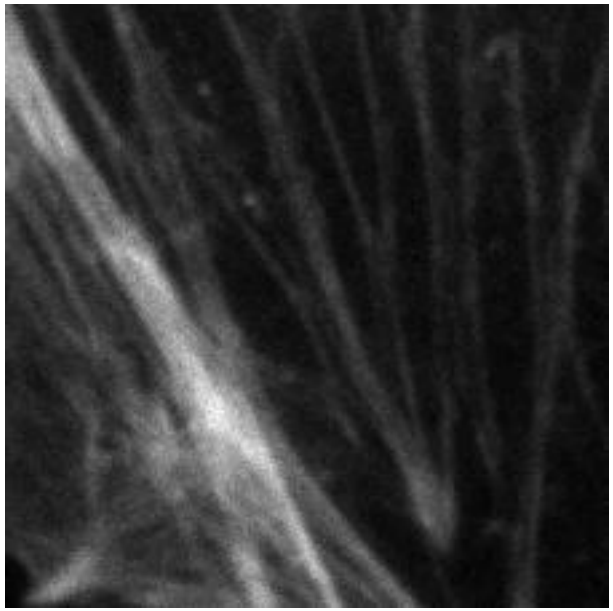
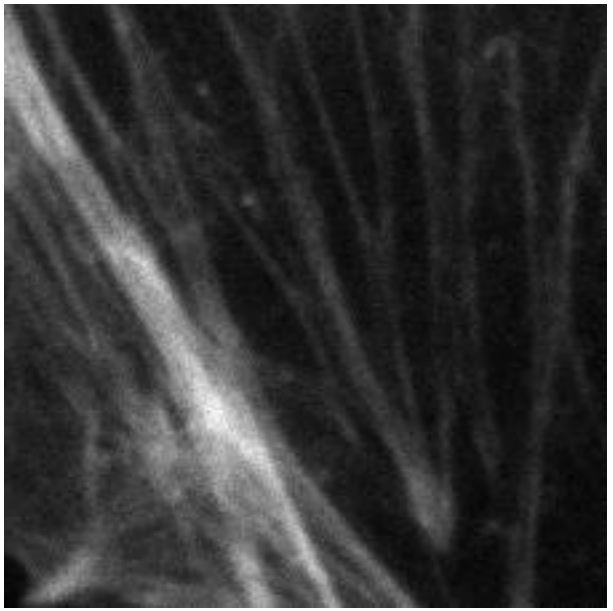


no read noise

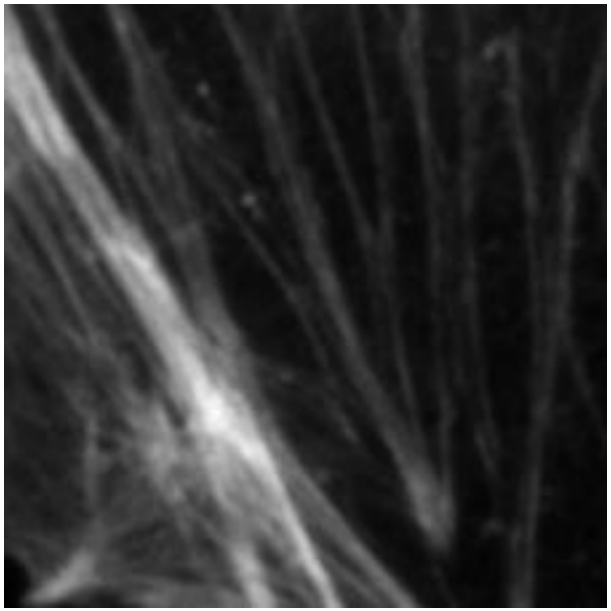


5 e^- read noise

Photon shot noise = 2x read noise

What does this look like?

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



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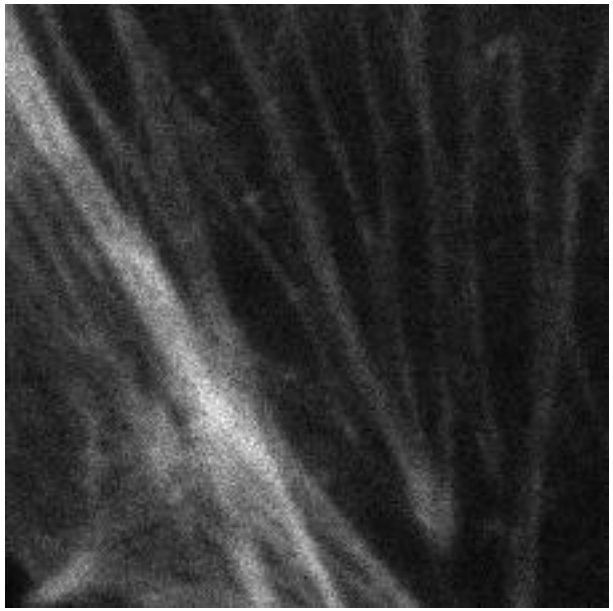
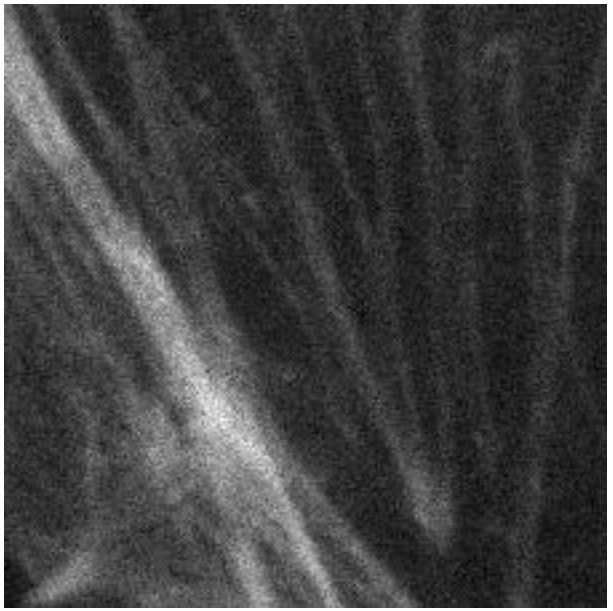


no read noise

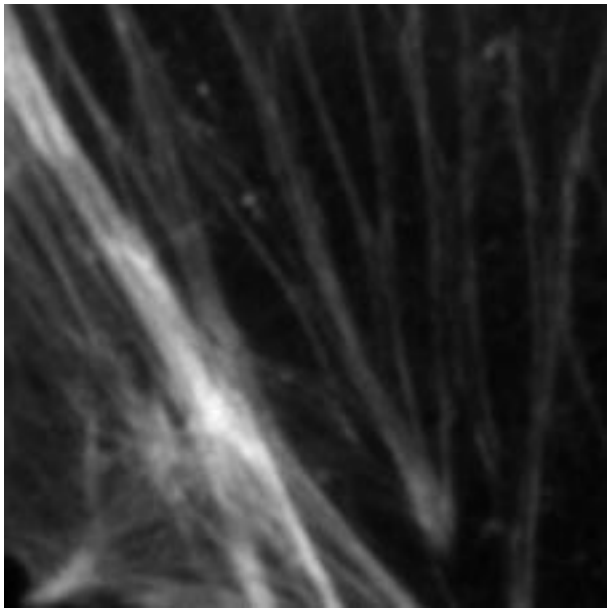


5 e^- read noise

Photon shot noise = read noise

What does this look like?

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



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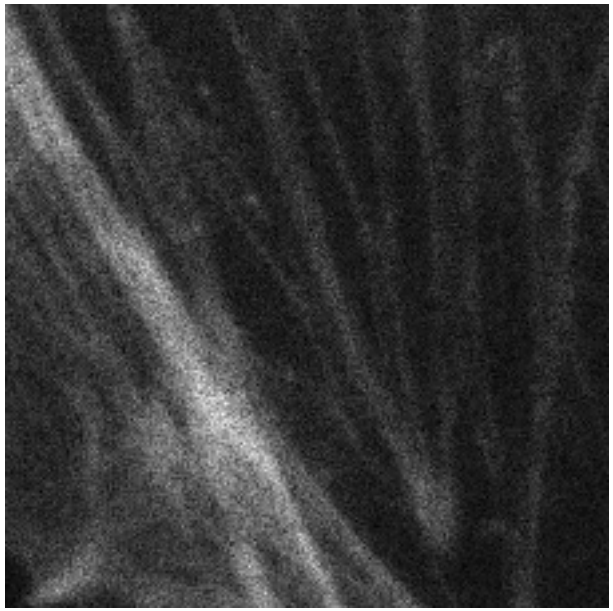
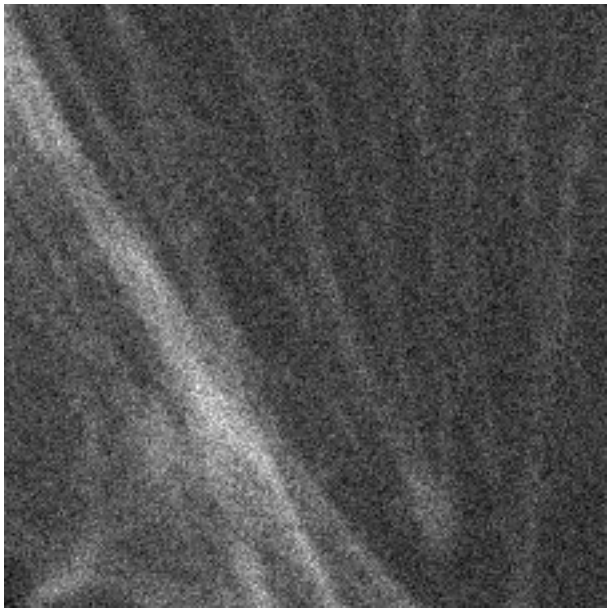


no read noise

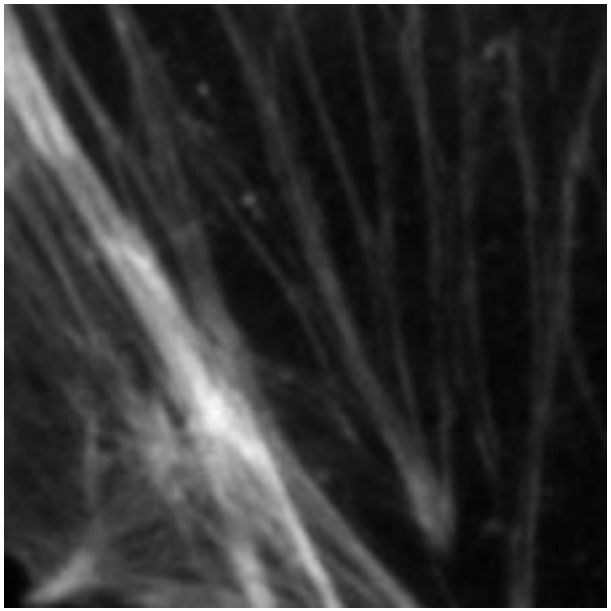


5 e^- read noise

Photon shot noise ~ 2/3 read noise

What does this look like?

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



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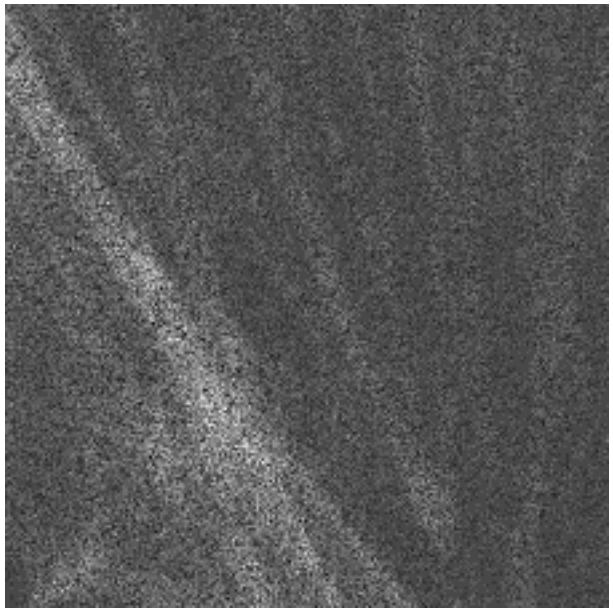
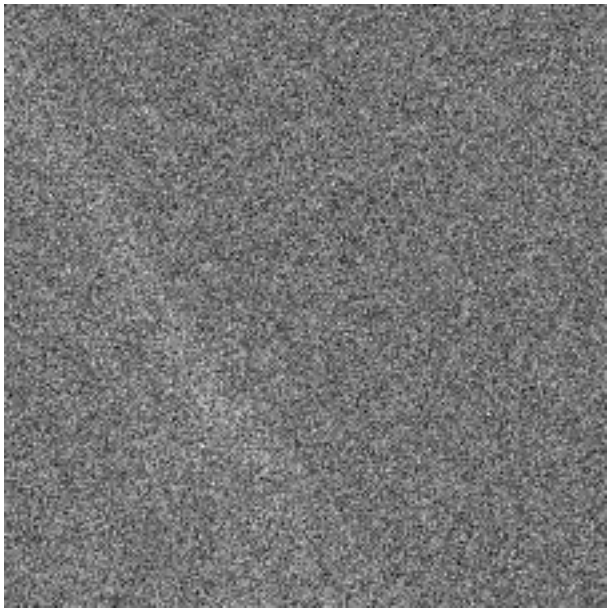


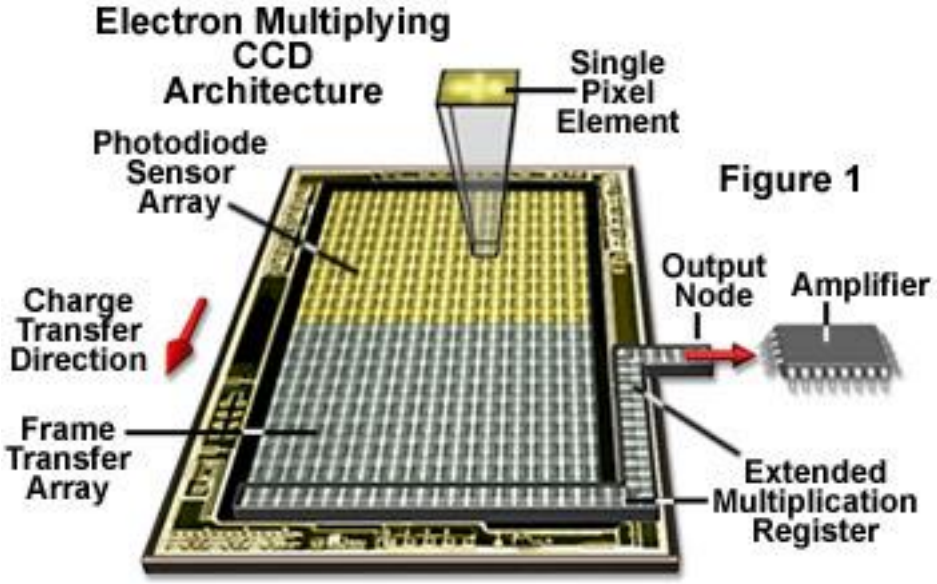
no read noise

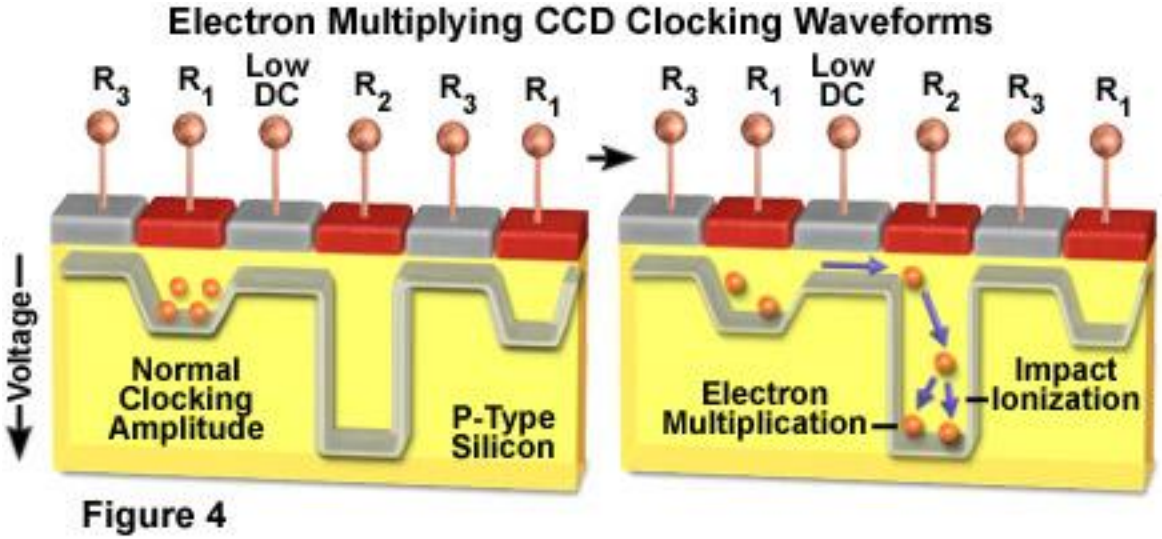


5 e^- read noise

Photon shot noise ~ 1/5 read noise

Beating the read-out noise
EMCCD



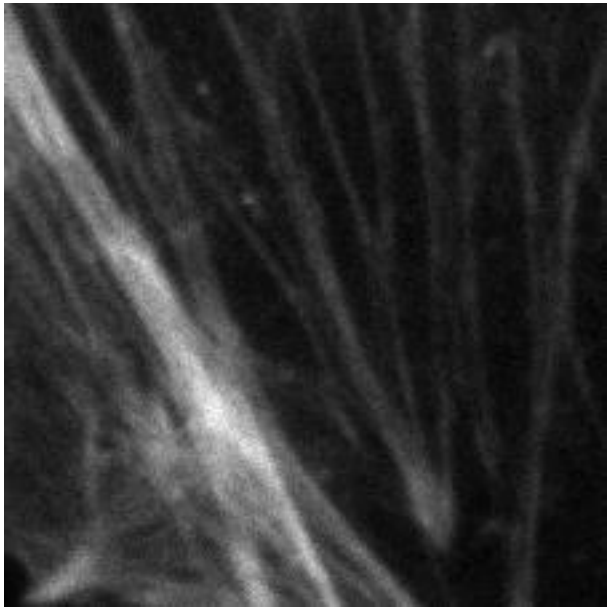


EMCCD result

- 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, so your QE looks like it’s halved
- Upside – you get to image fast without worrying about read noise

Hypothetical CCD/EMCCD comparison

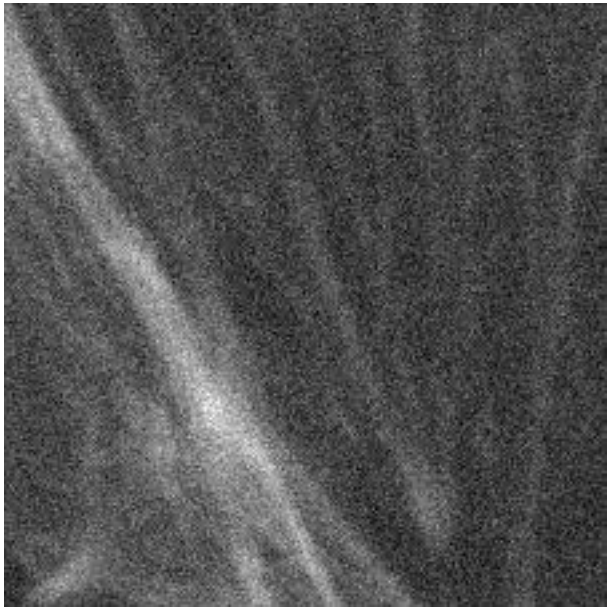
100 photon / pixel on average; ~500 in brightest areas



Slow scan CCD

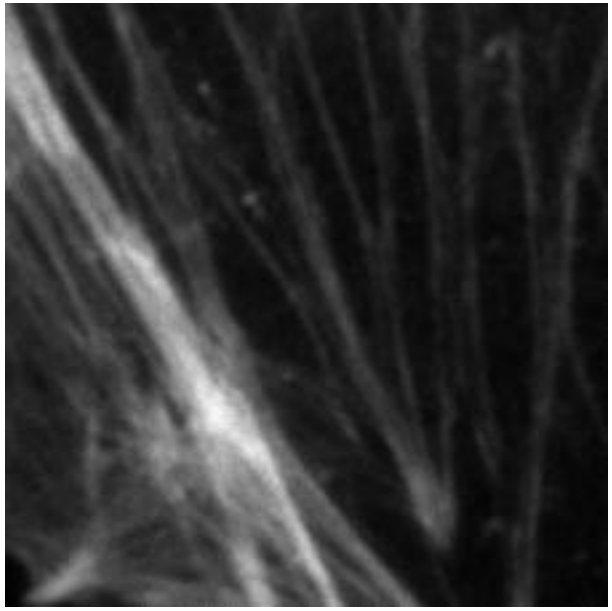
4e^- read noise

(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

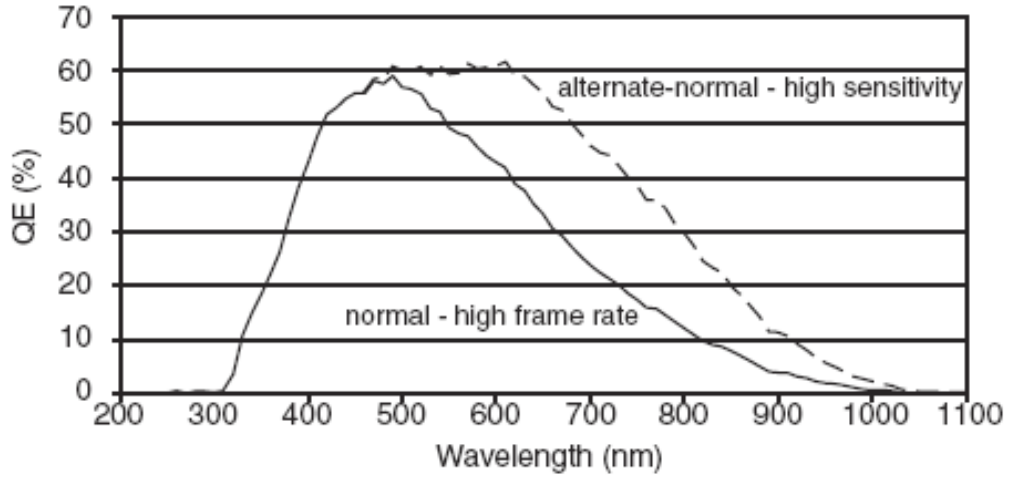
New: sCMOS

Scientific CMOS

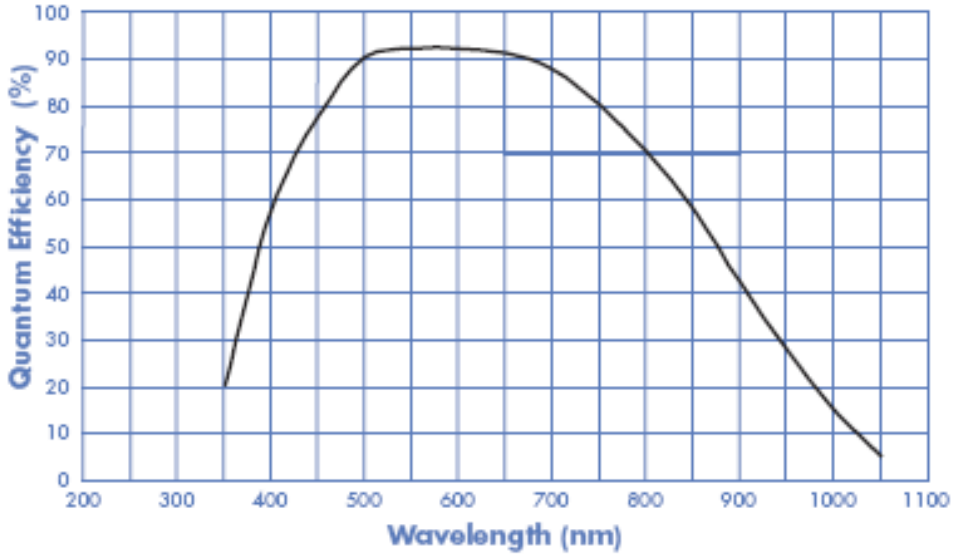
- Like CMOS, but better
  - Differences are proprietary
  - Vendors: Hamamatsu, Andor, PCO

- Specs (Andor Neo):
  - 5.5 megapixels (2560 x 2160)
  - 6.5 μm pixels
  - 100 fps readout
  - 1.5 e^- read noise

Quantum efficiency



HQ2



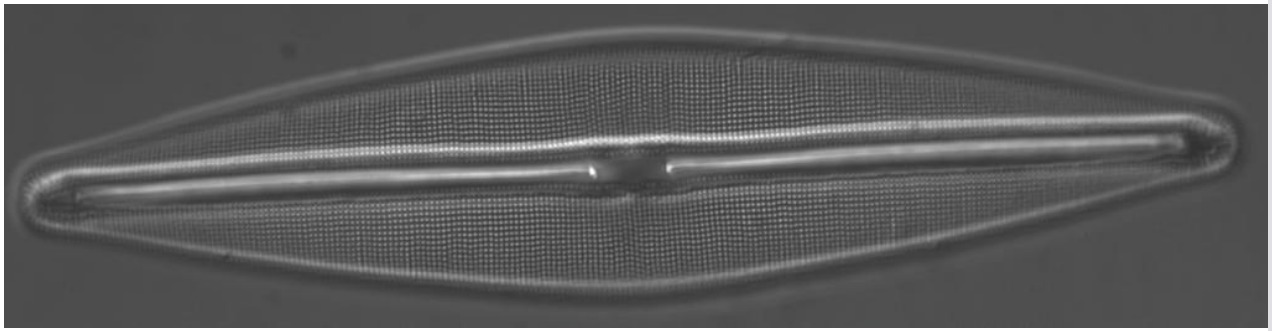
Cascade II

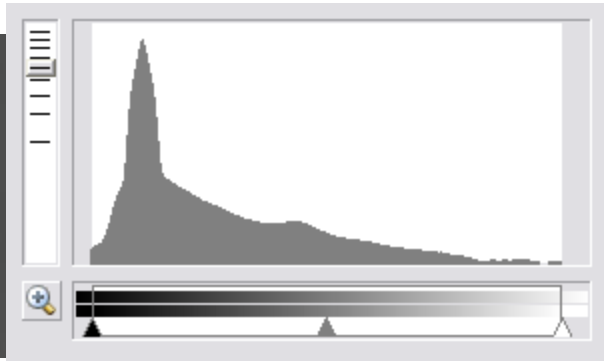
How many intensity levels can you distinguish?

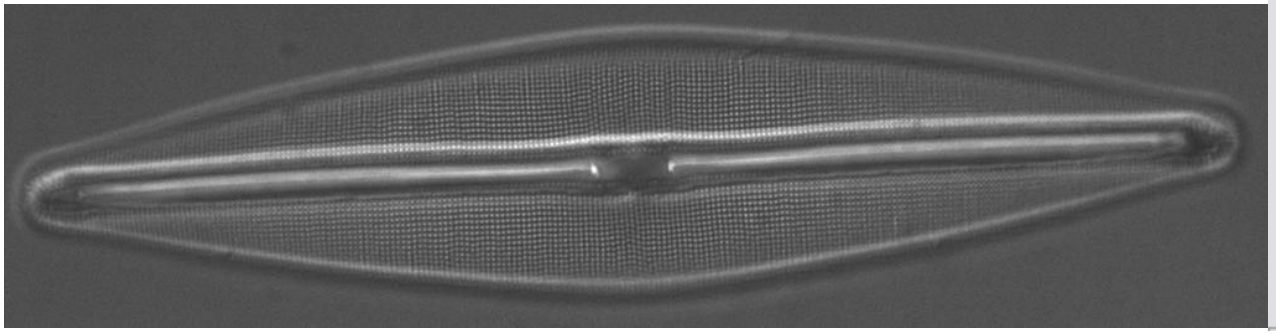
- Full well capacity (16 000 e^-)
- Readout noise: 5e-

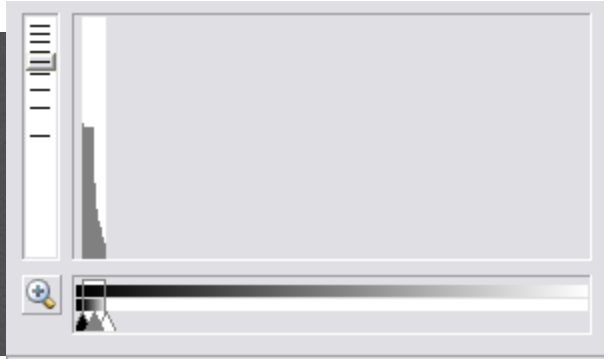
- Dynamic range:
  - FWC/readout noise: 3200
  - 0.9 * FWC / (3 * readout noise) = 960
- (Human eye ~ 100)

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)

Acknowlegdements

- 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)