A microscopic image showing numerous small, circular cells with intricate internal structures, possibly nuclei, stained in a golden-yellow color against a dark background.

Principles and Practices of Light Microscopy

Lecture 7: Light sources and Cameras



Kurt Thorn, NIC

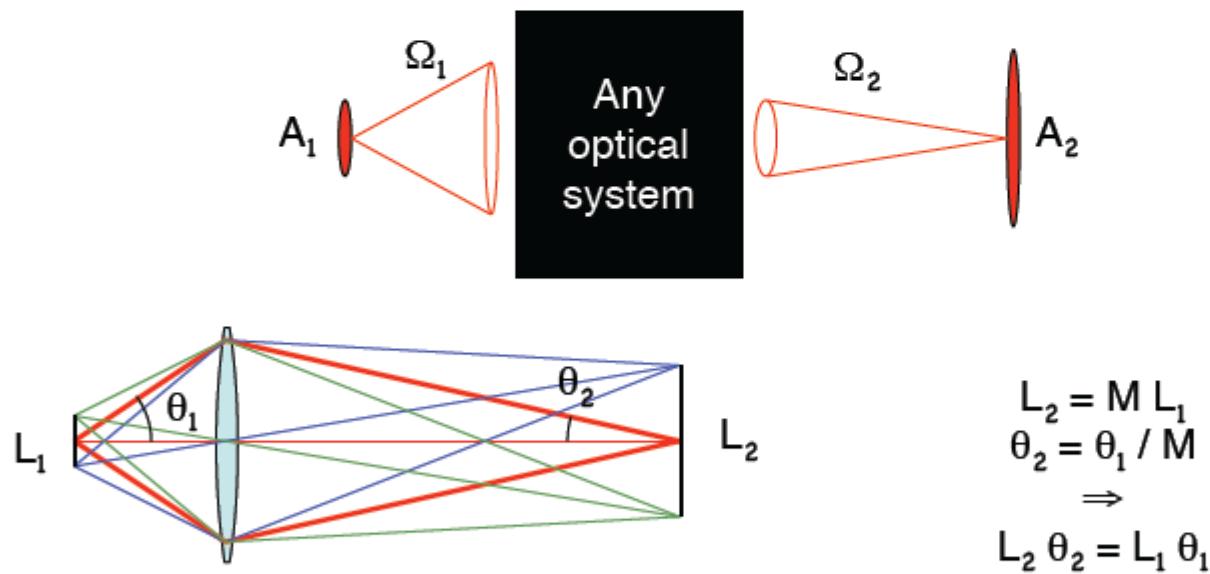
Image: Susanne Rafelski

Light Sources

- Arc Lamps
 - Hg and Xe
 - Metal Halide
- LEDs
- Plasma
- Lasers
 - Generally only for collimated illumination (Confocal, TIRF)

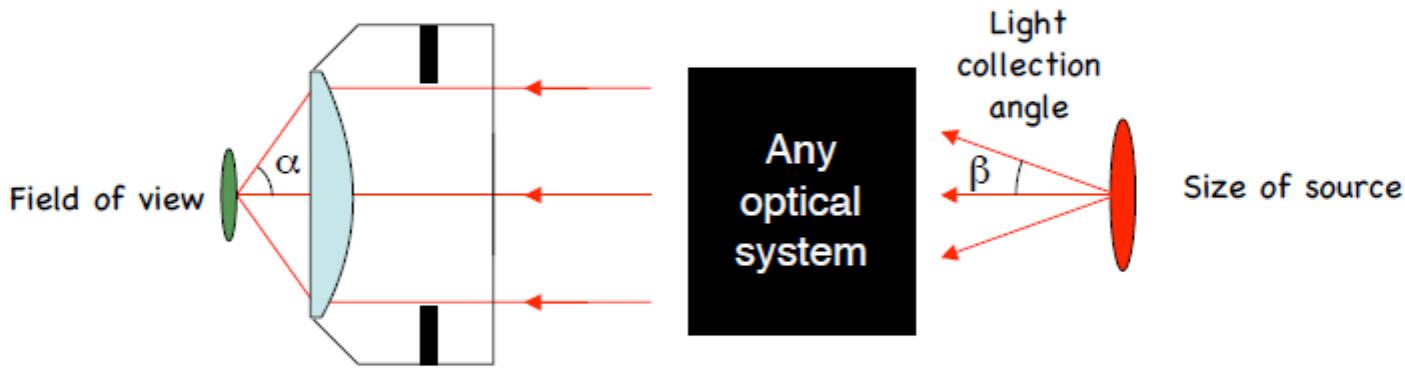
Light Sources

- Constraint: area \times solid angle is invariant



Brightness

- $(\text{area} \times \text{solid angle}) \text{ at sample} \leq (\text{area} \times \text{solid angle}) \text{ at source}$
- No optical system can increase it

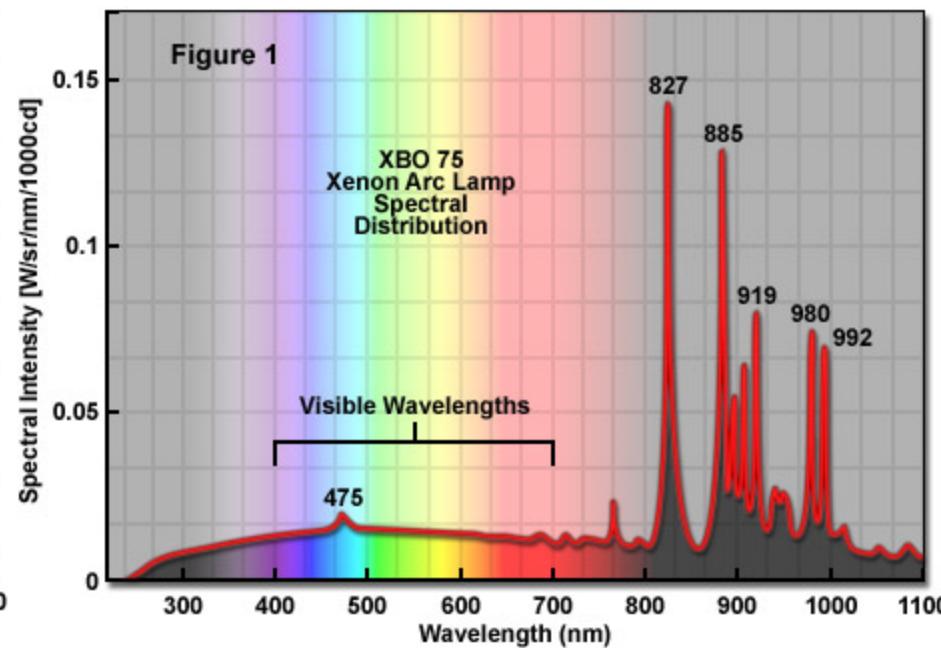
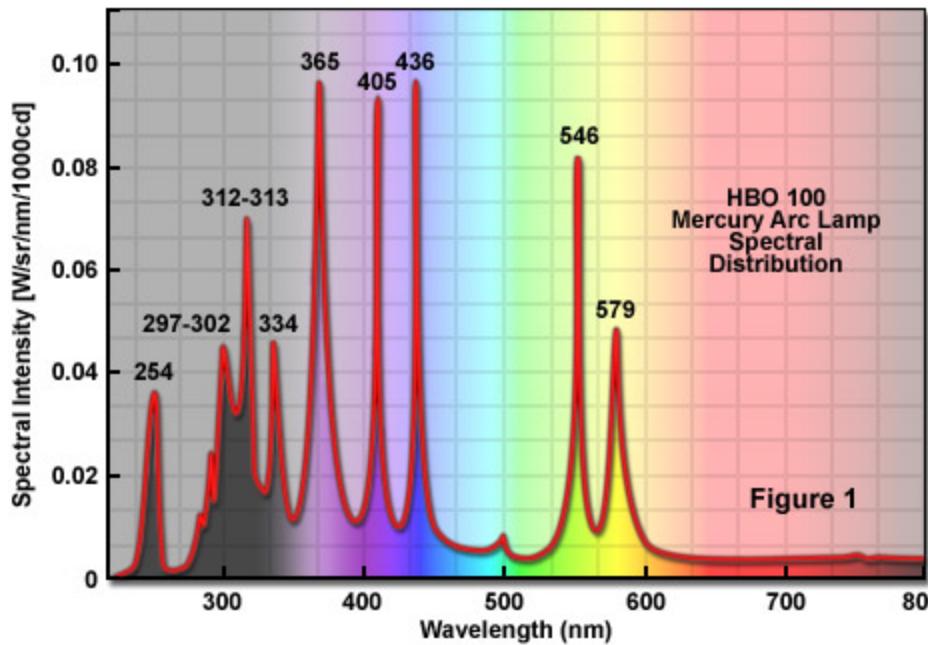


$$\text{Usable size of source} \approx (\text{Field of view}) \times \text{NA} / \sin(\beta)$$

Source and sample size should be similar

- Widefield imaging
 - Extended sources: LEDs, arc lamps, etc.
- Scanning systems
 - Laser sources

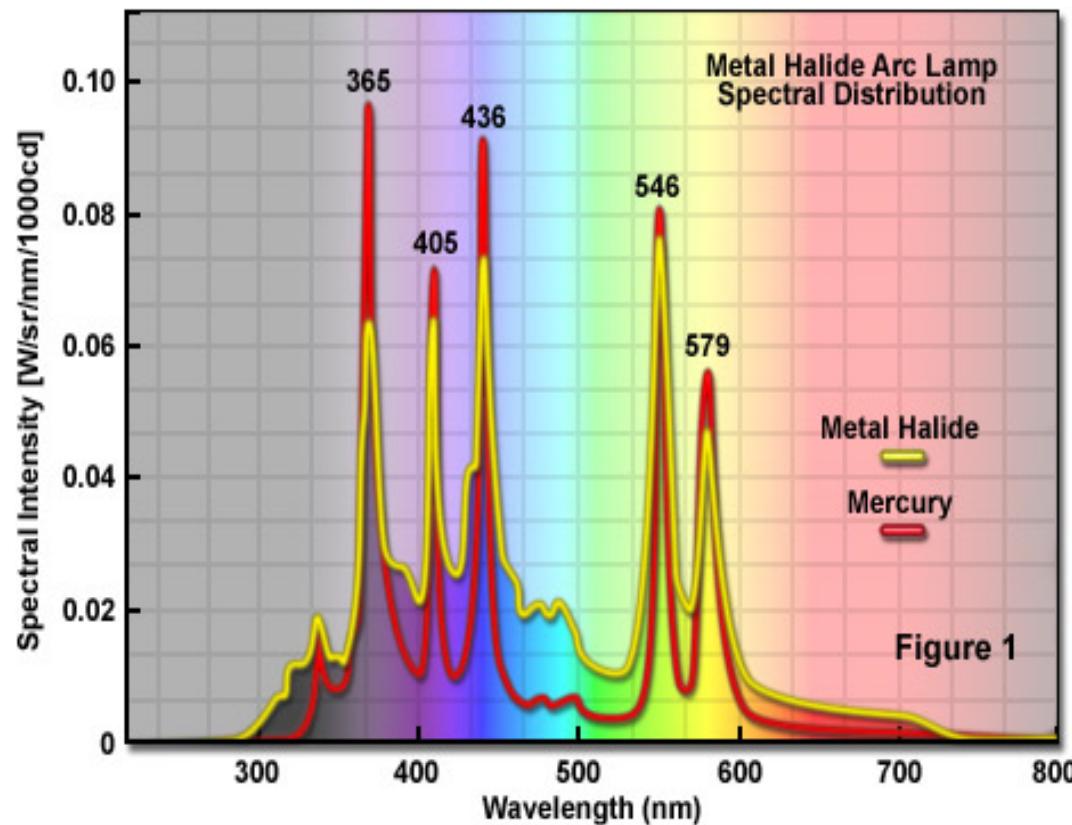
Arc Lamp Spectra



- Hg: Brighter if your excitation spectrum matches one of the lines
- Xe: More stable, longer lifetime, flat emission in visible is sometimes beneficial

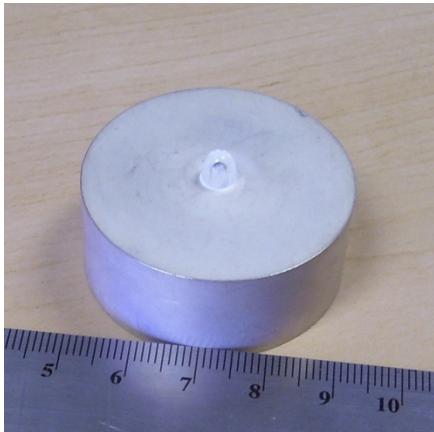
Metal Halide Arc Lamp

- Exfo, Intensilight,
etc.



High efficiency plasma lamps

- Essentially an arc lamp without electrodes – uses microwave waveguide to create a plasma in a quartz bulb
- Broadband visible emission
- Very long (>10,000 hr) lifetime
- Commercialized by Sutter as XL lamp



Liquid light guide coupling

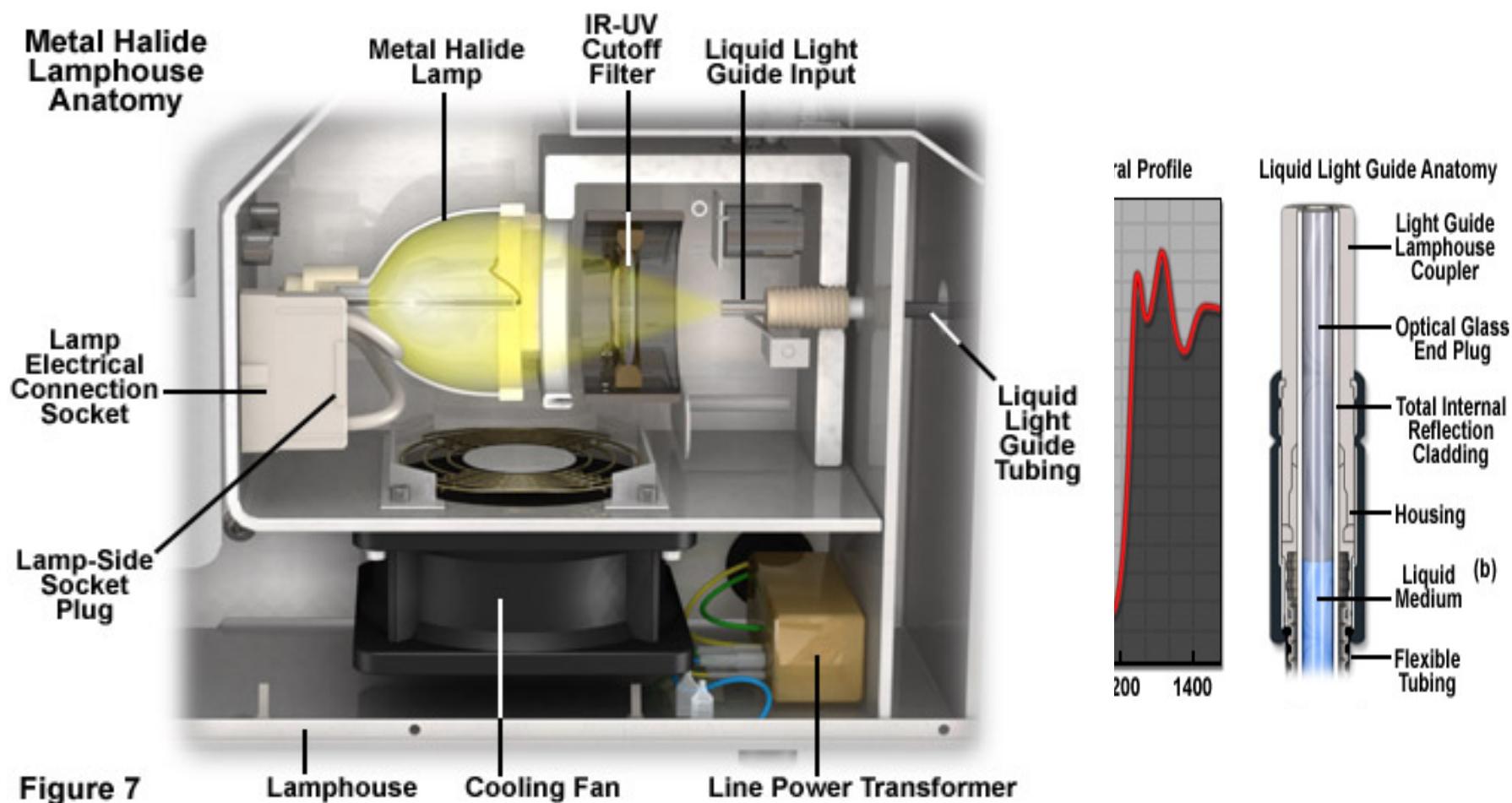
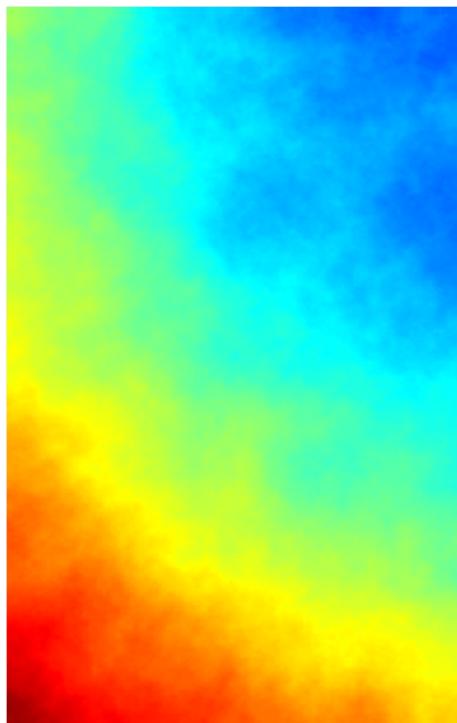


Figure 7

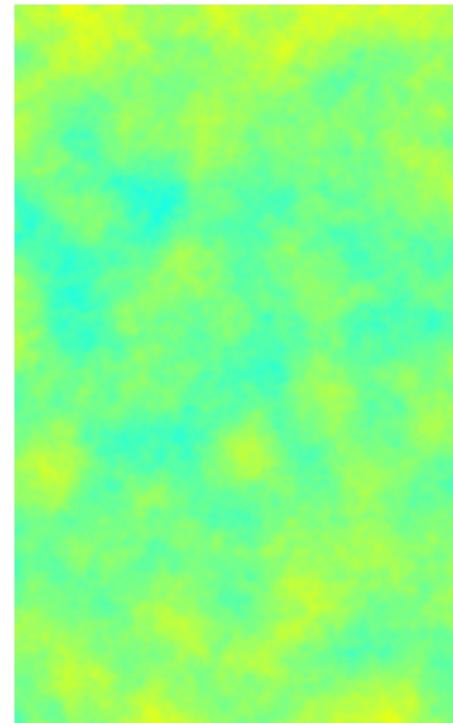
Benefit of Liquid Light Guide

Improved illumination uniformity

HBO 103



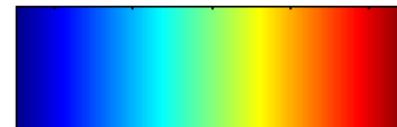
HBO 103 + LLG



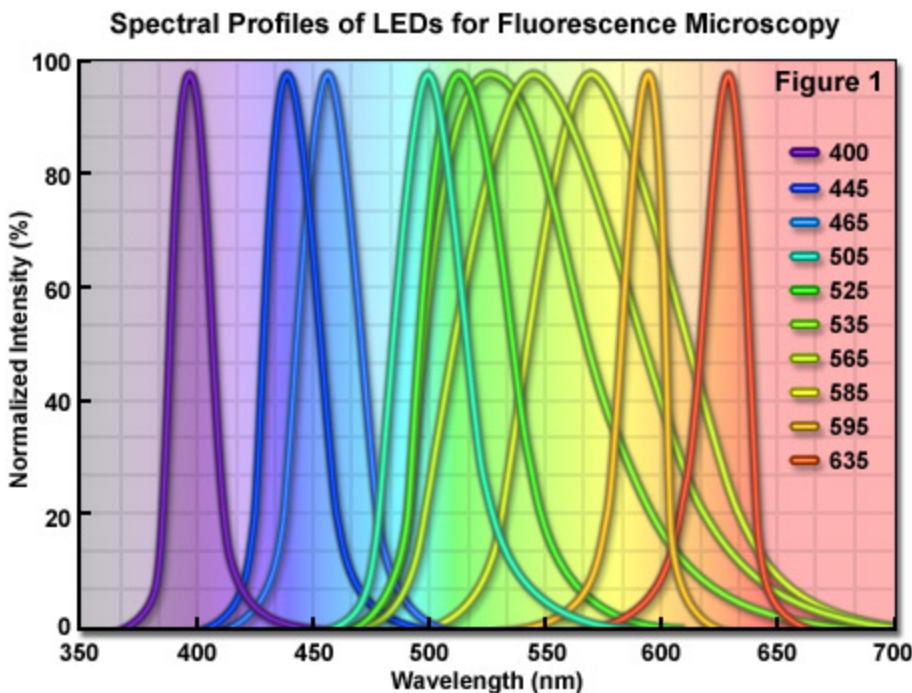
0.96 0.98 1 1.02 1.04



0.96 0.98 1 1.02 1.04



LEDs



- Good in the blue / red
- Not so good in the green / yellow
- Long lifetime
- Fast switching

Relative lamp power

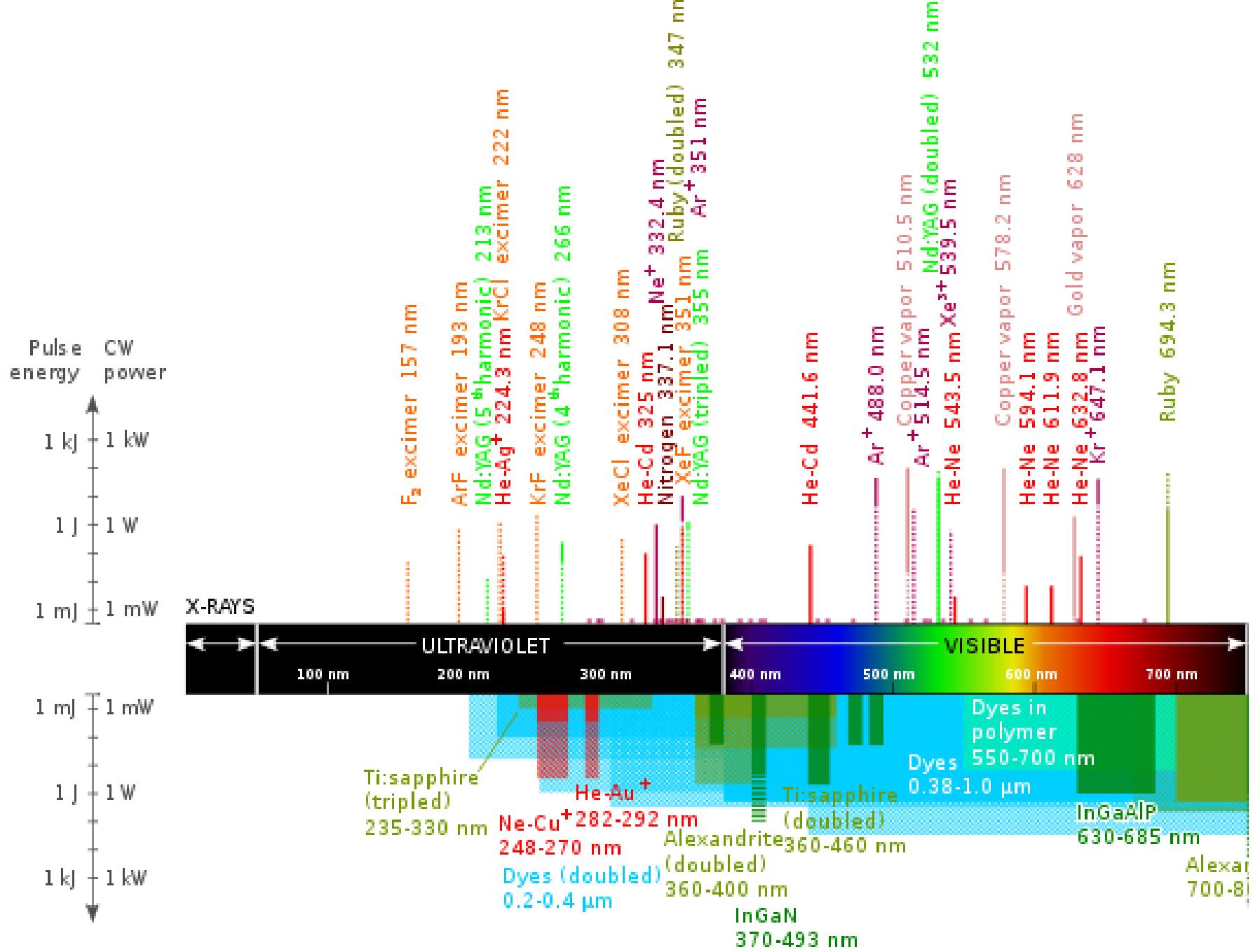
Filter Set	Excitation Filter (nm)	Dichromatic Mirror (nm)	Mercury HBO Power mW/Cm ²	Xenon XBO Power mW/Cm ²	Metal Halide Power mW/Cm ²	LED Power mW/Cm ²	Tungsten HAL Power mW/Cm ²
DAPI (49) ¹	365/10	395 LP	23.0	5.6	14.5	0.70 (365) ³	0.06 ⁴
CFP (47) ¹	436/25	455 LP	79.8	25.0	76.0	26.5 (445) ³	1.0
GFP/FITC (38) ¹	470/40	495 LP	32.8	52.8	57.5	39.2 (465) ³	2.8
YFP (S-2427A) ²	500/24	520 LP	20.0	35.4	26.5	10.9 (505) ³	2.7
TRITC (20) ¹	546/12	560 LP	43.1	12.2	33.5	2.7 (535) ³	1.4
TRITC (S-A-OMF) ²	543/22	562 LP	76.0	31.9	67.5	6.6 (535) ³	3.6
Texas Red (4040B) ²	562/40	595 LP	153.7	54.4	119.5	7.9 (585) ³	6.9
mCherry (64HE) ¹	587/25	605 LP	80.9	29.7	54.5	7.2 (585) ³	4.3
Cy5 (50) ¹	640/30	660 LP	9.1	22.1	13.5	14.9 (635) ³	4.5

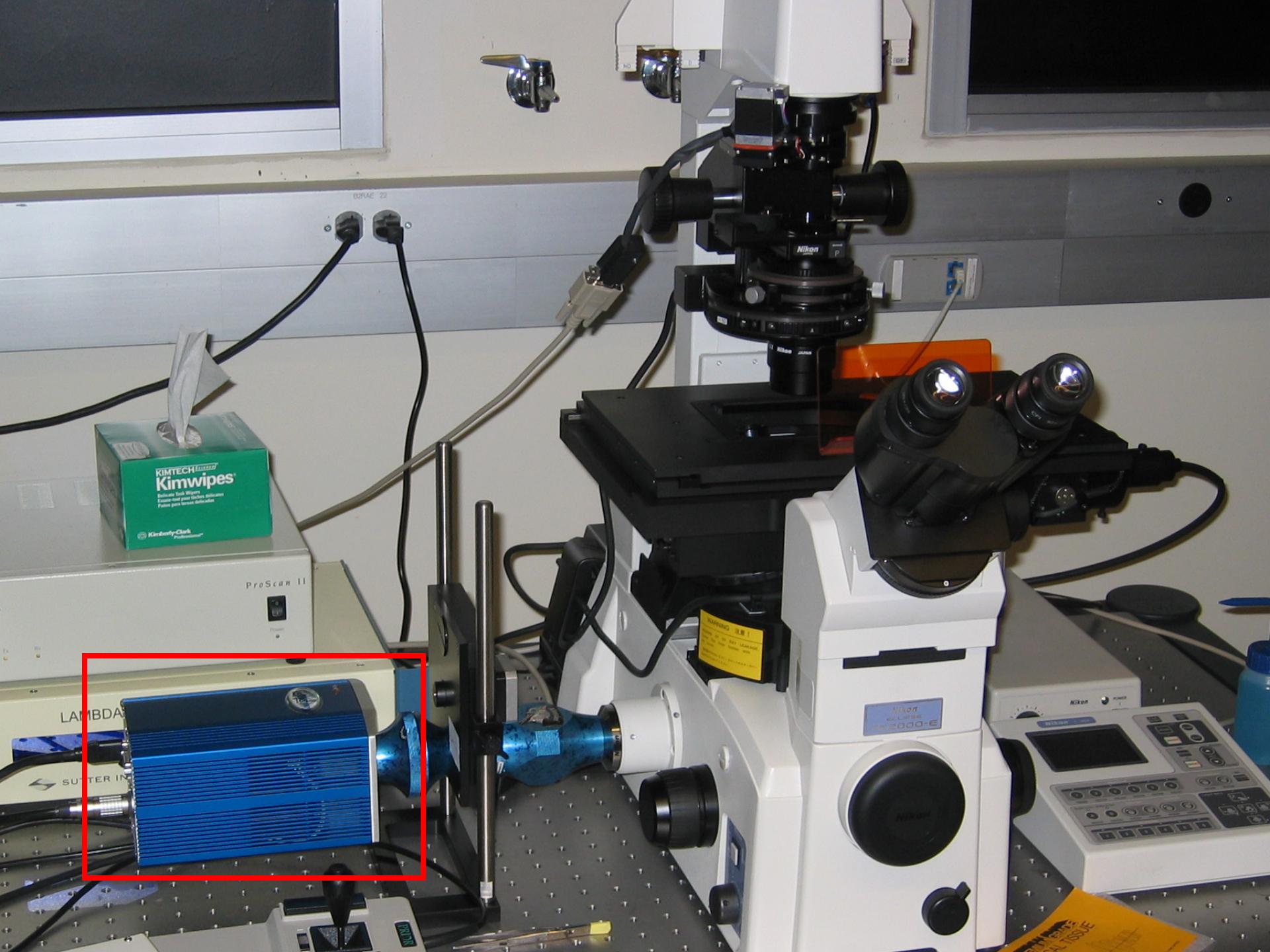
¹Zeiss Filters ²Semrock Filters ³LED Peak Wavelength ⁴Tungsten-Halogen Lamp Voltage = 12.2 V

Summary: Metal Halide best; LEDs still not quite bright enough.

Lasers

- Highly collimated, small source size
- Many wavelengths and technologies
 - Gas lasers (chiefly Ar-ion)
 - Solid state (Nd:YAG, Ti:Sapphire, etc.)
 - Diode
- Can frequency double, triple, quadruple
 - 1064 nm Nd:YAG → 532 nm



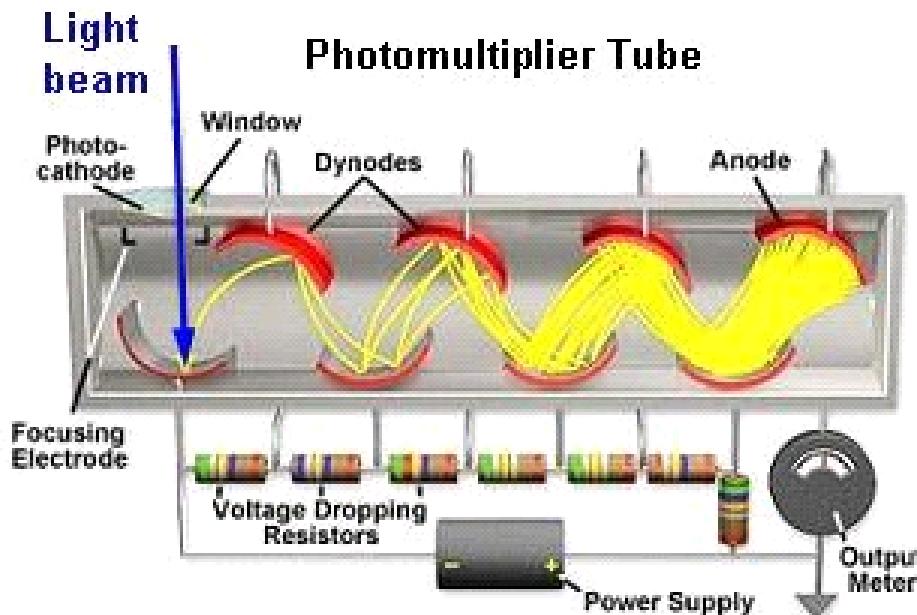


Detectors

- Must
 - Convert light into an electrical signal
 - Ideally linear, low noise, accurate
- Imaging detectors (cameras)
 - CCD : high QE, low noise, slow
 - CMOS : higher noise, fast
- Non-imaging (single point, for scanning confocals)
 - Photomultiplier tubes: fast, moderate QE, low noise

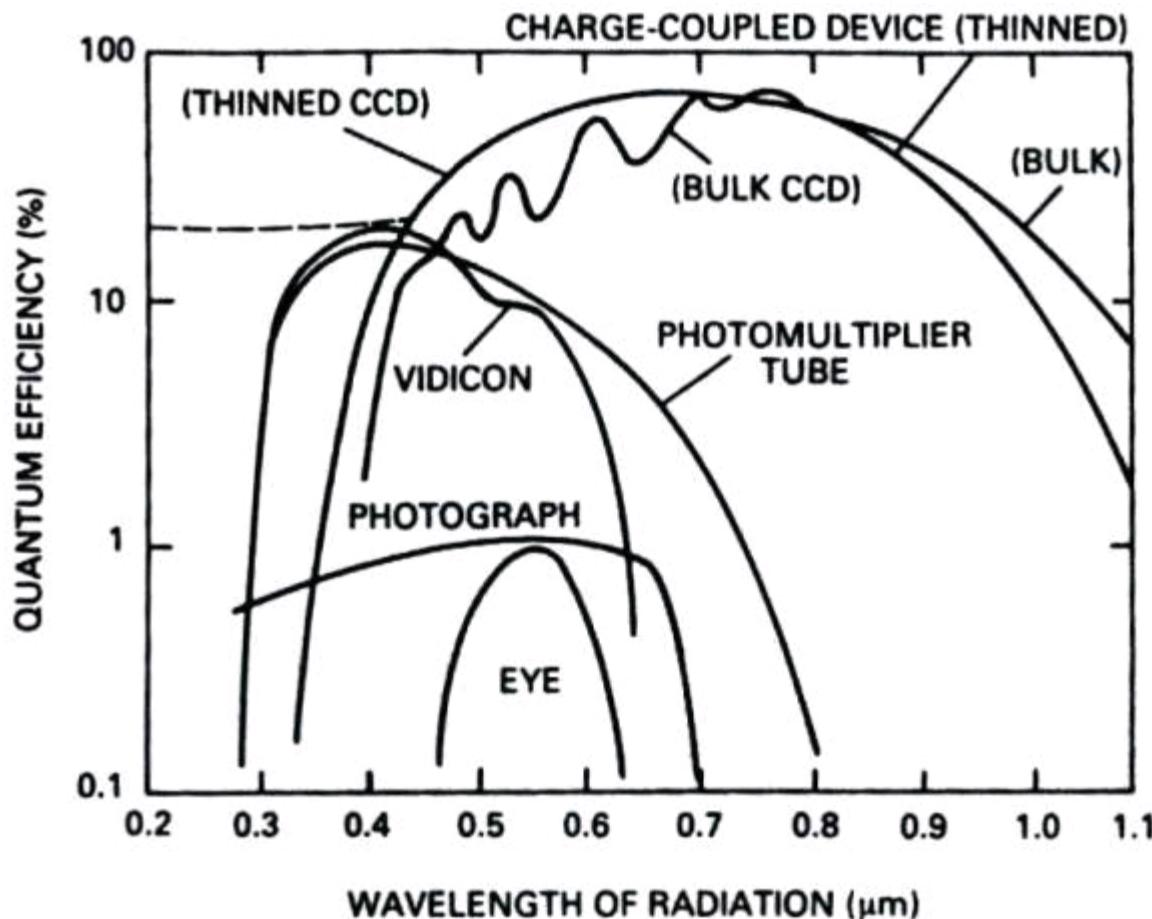
Detectors - PMTs

- Must be fast – confocal beam spends only a few μs on each pixel
 - Photomultiplier tubes

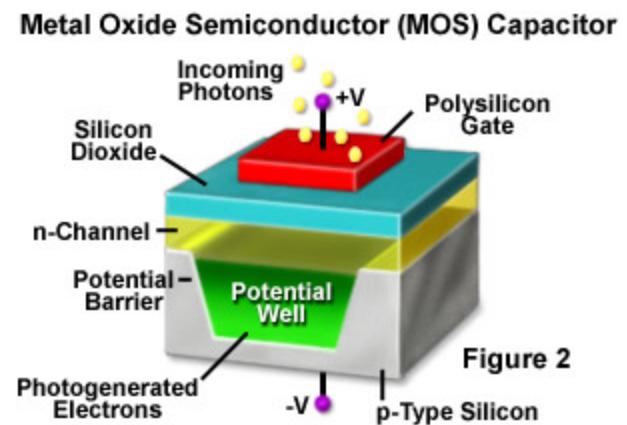
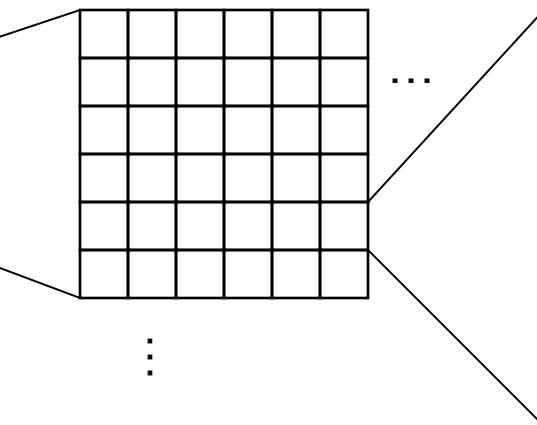
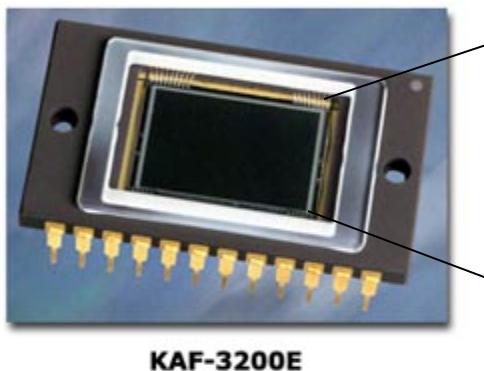


- Pulse width for single photon ~ 10-100ns
- Very linear
- Very high gain
- ~ 0 read noise

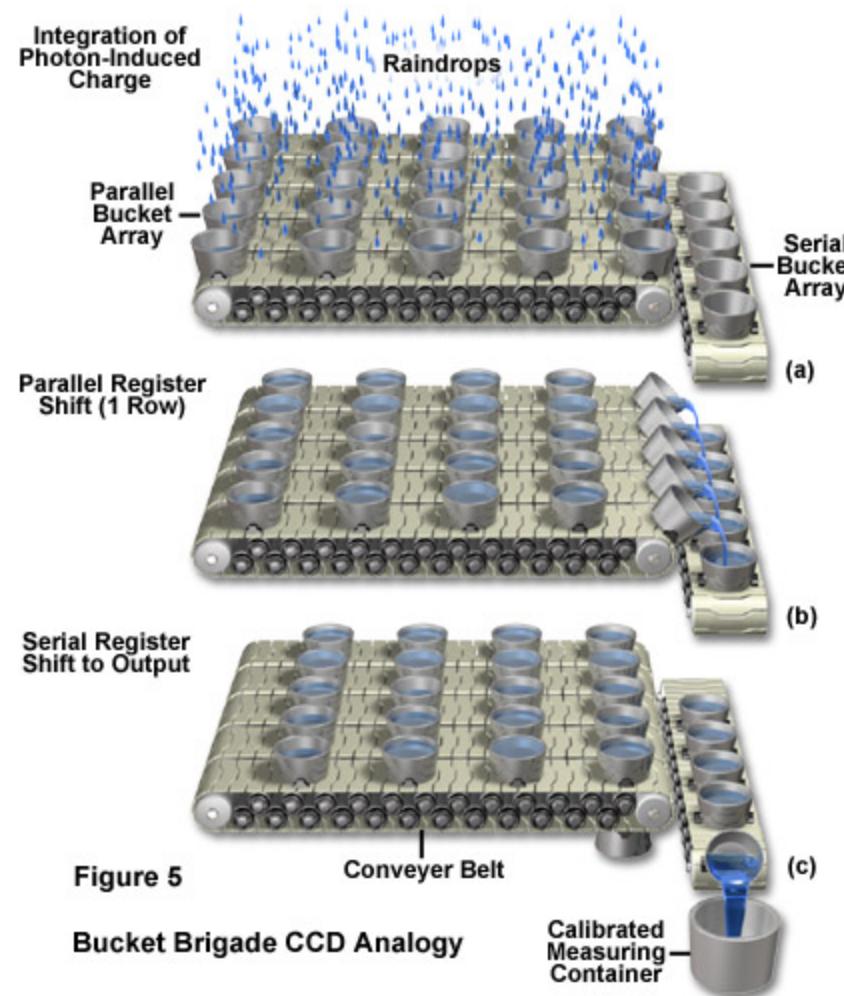
Detectors - PMTs



CCD architecture



CCD readout “bucket-brigade” analogy



A little more realistic....

Each pixel is subdivided into three phases

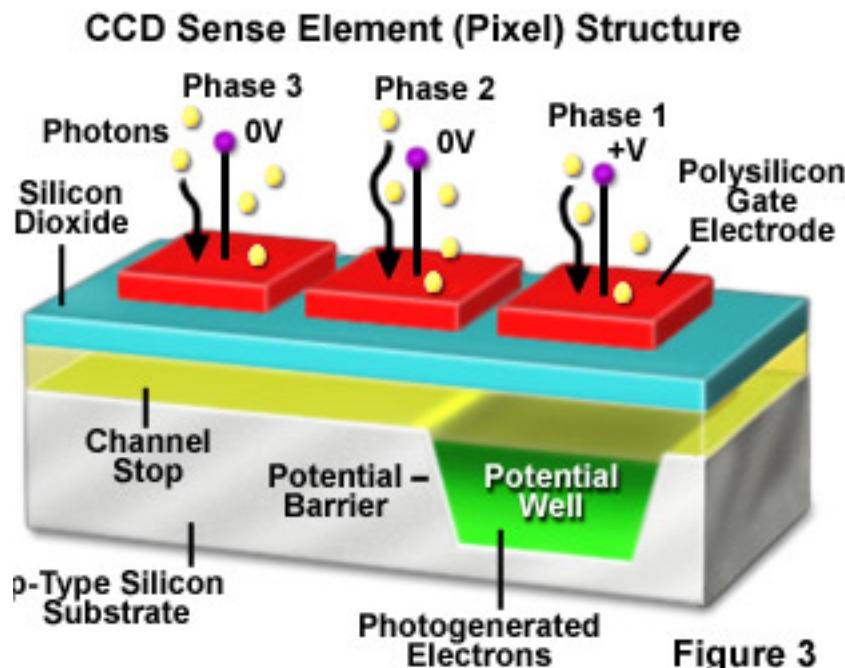
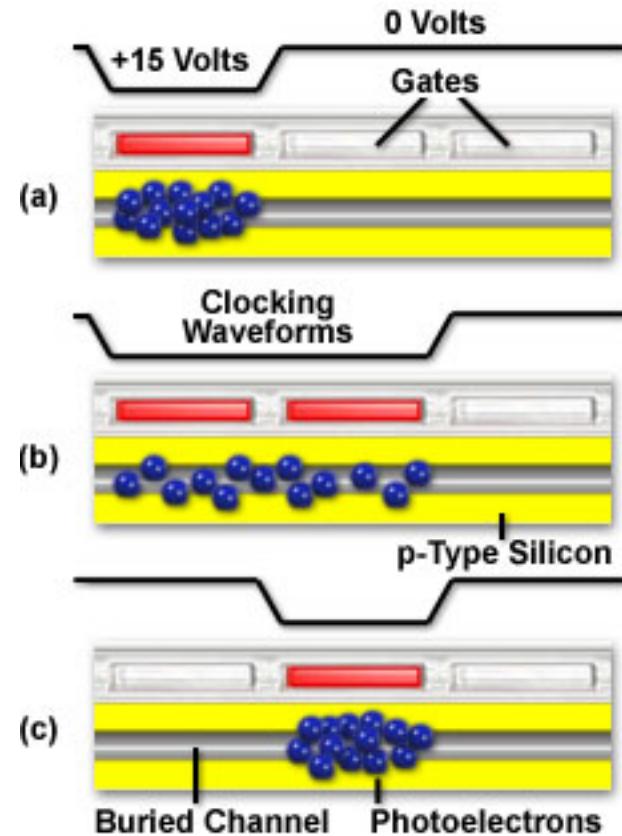
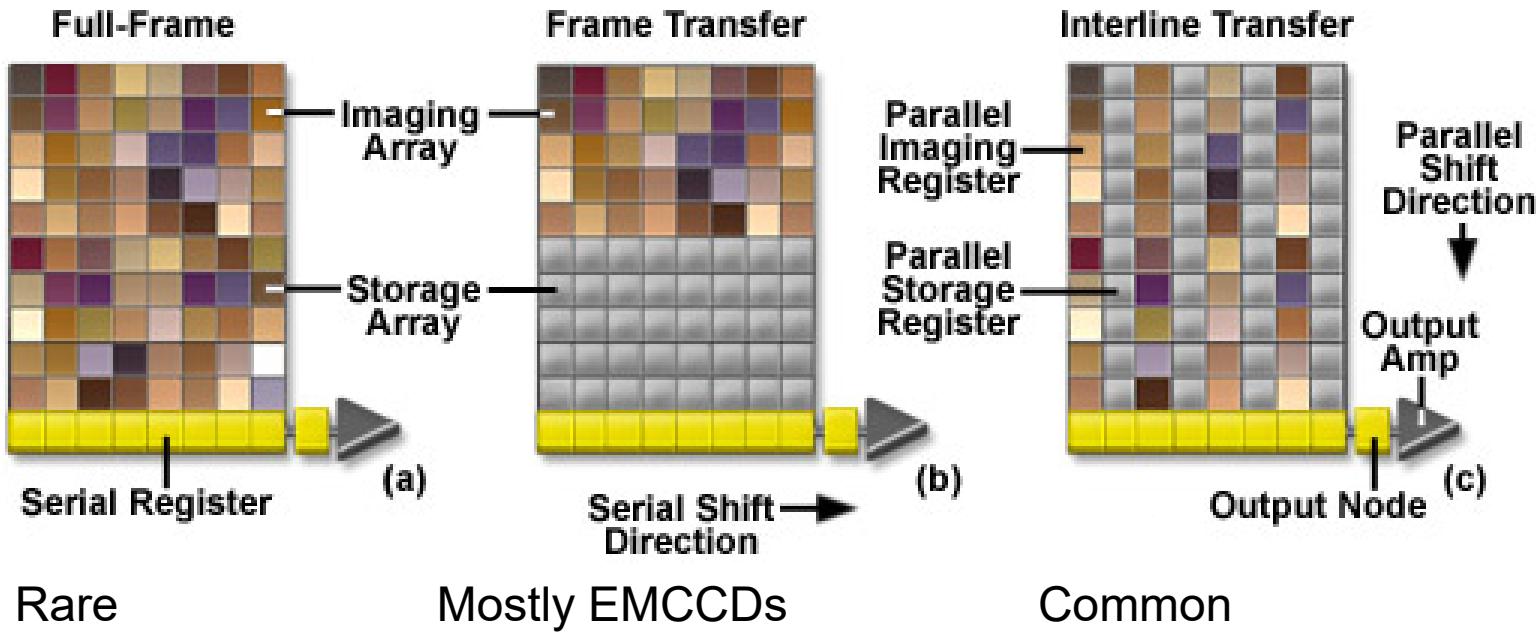


Figure 3

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

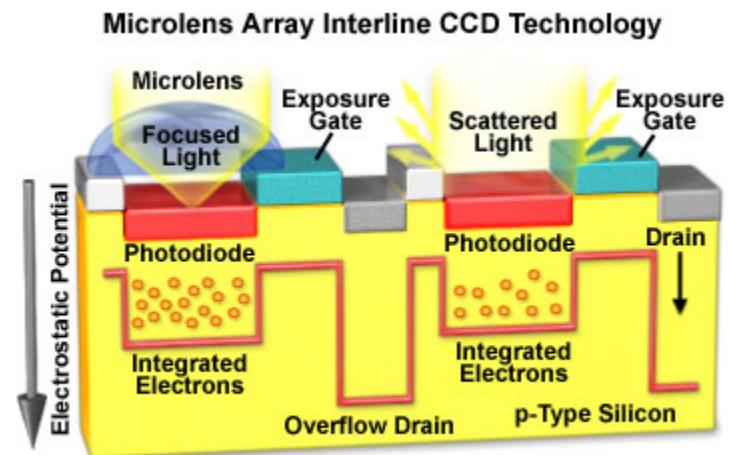
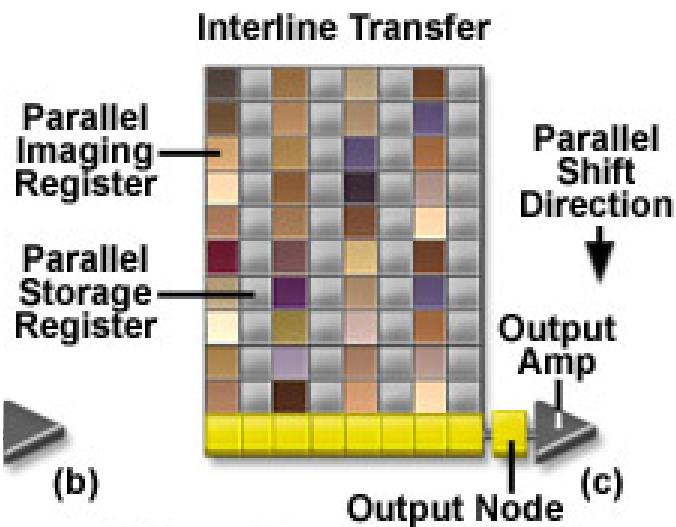


Figure 8

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

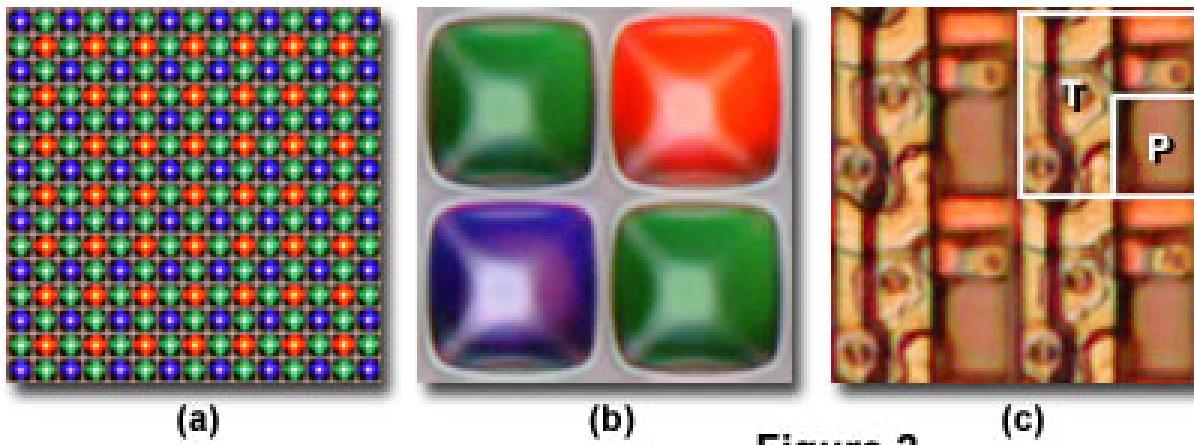


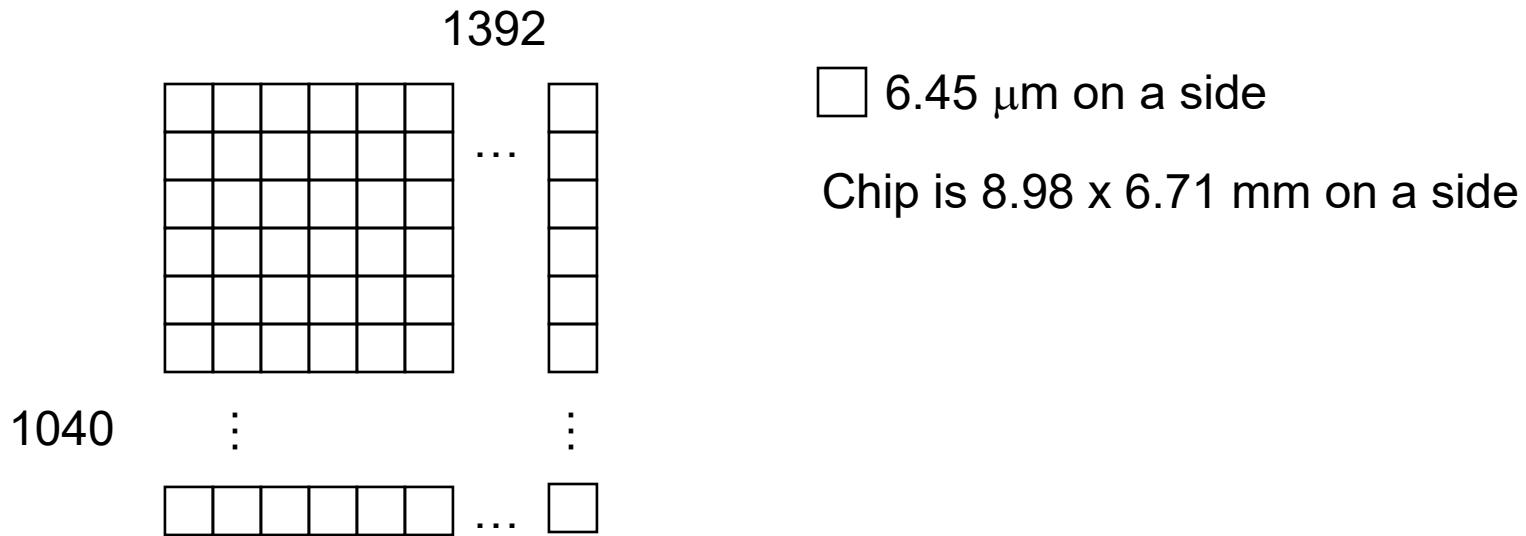
Figure 2

- 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

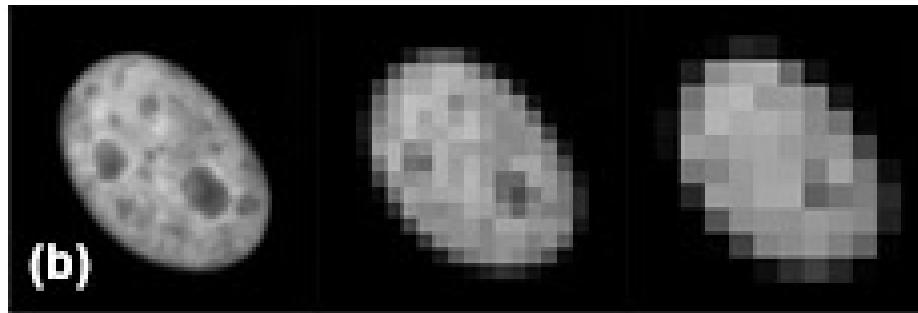
- Pixel size and number
- Quantum efficiency: the fraction of photons hitting the CCD that are converted to electrons
- Full well depth: total number of electrons that can be recorded per pixel
- Read noise
- Dark current (negligible for most biological applications)
- Readout time

Magnification and CCDs



Typical magnification from sample to camera is roughly objective magnification

Resolution and magnification



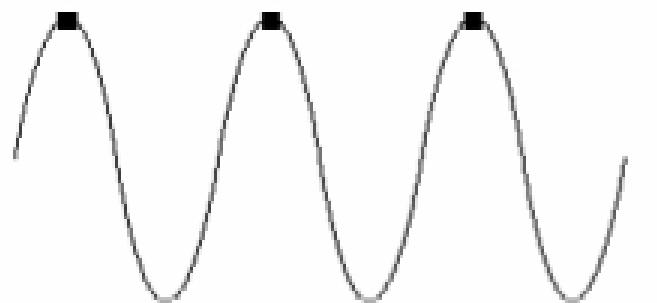
More pixels / resolution element

Where is optimum?

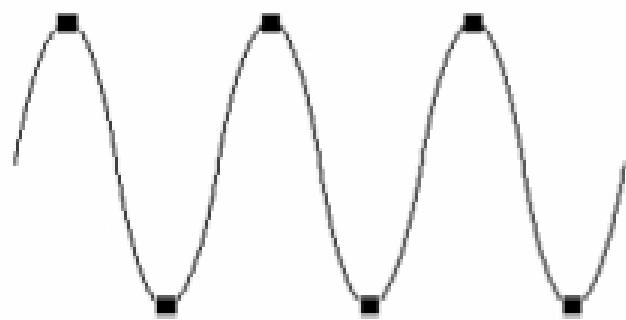
Nyquist-Shannon Sampling

- 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

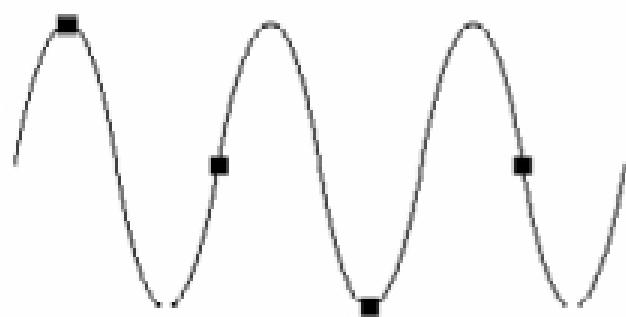
Nyquist-Shannon Sampling



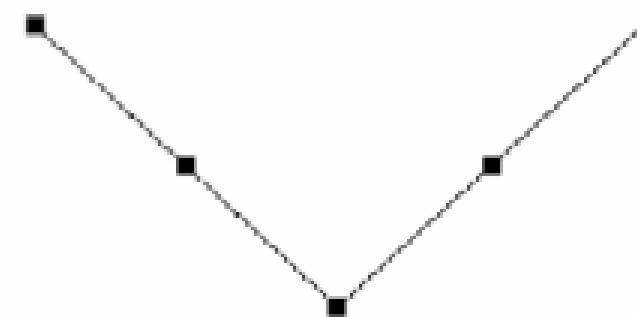
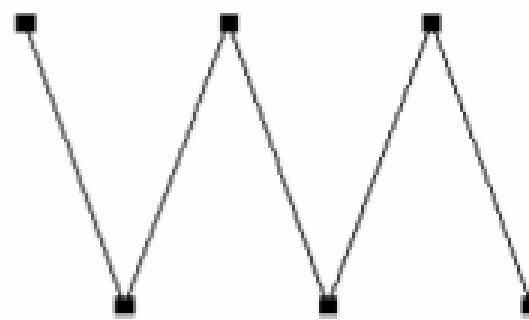
A
Sampled at f



B
Sampled at $2f$



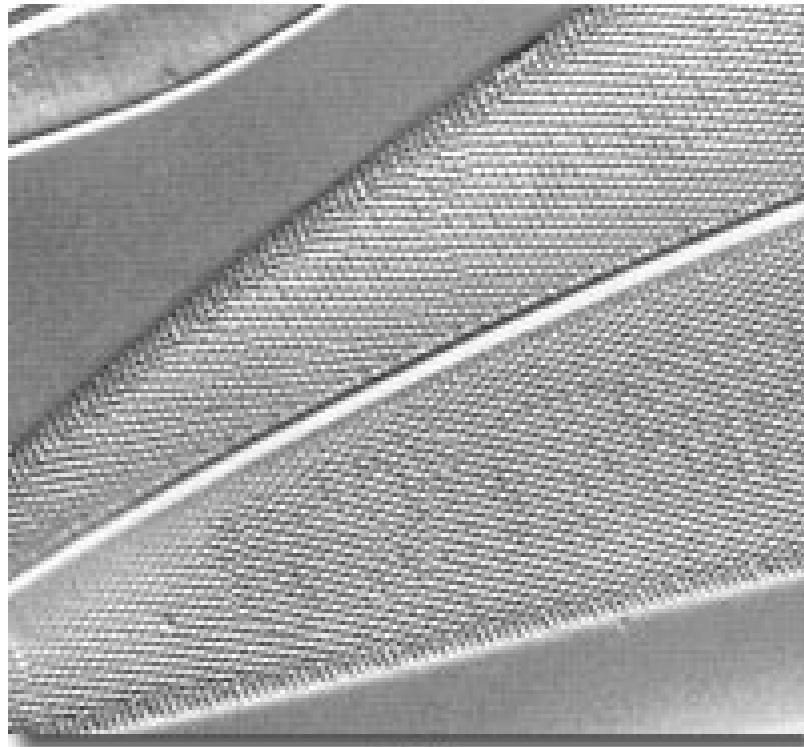
C
Sampled at $4f/3$



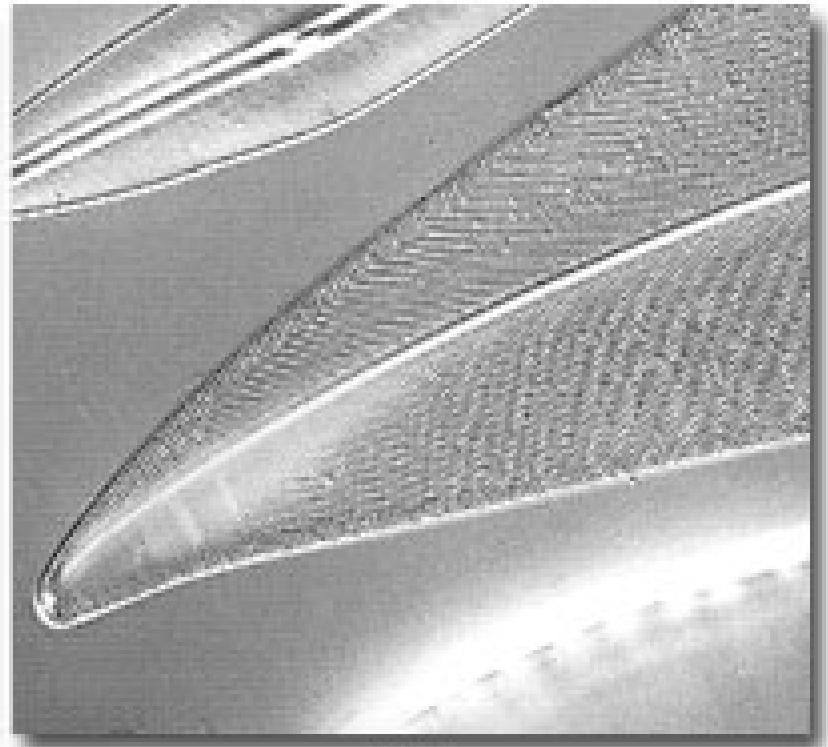
Resolution and CCDs

- 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

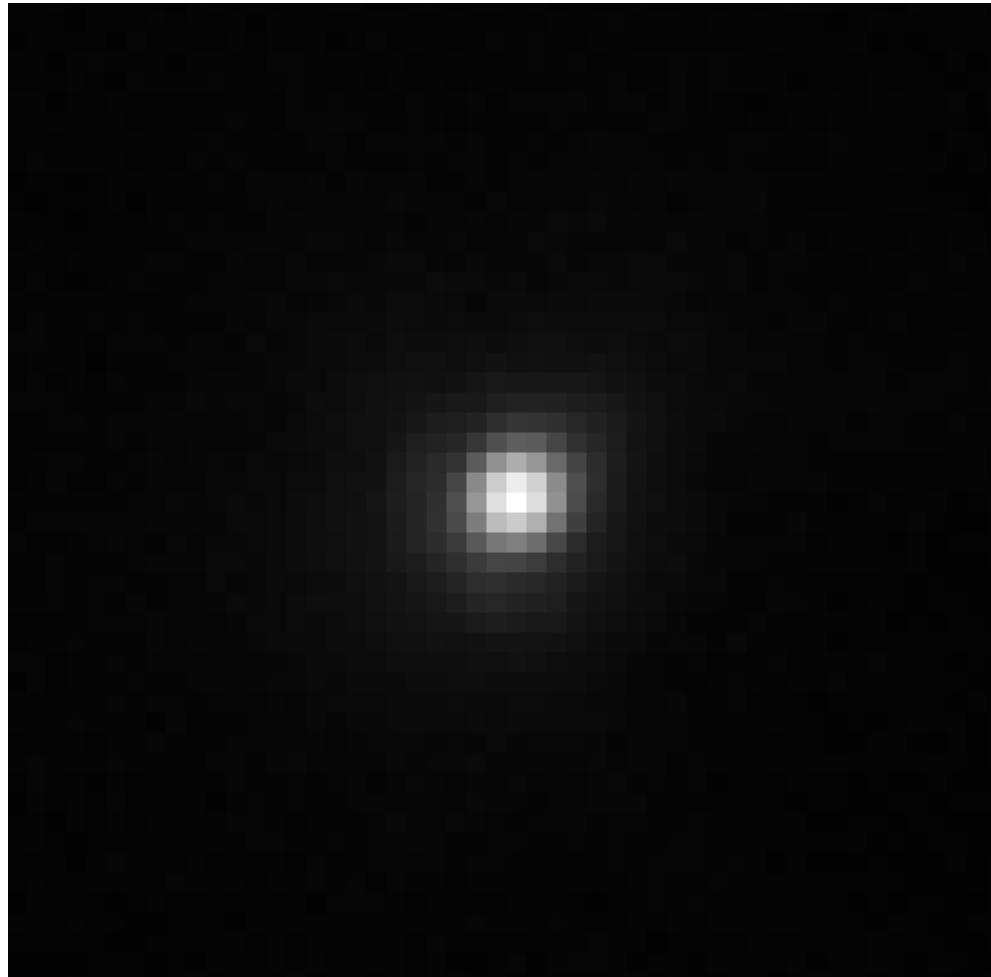


Undersampled

A resolution-centric view of imaging

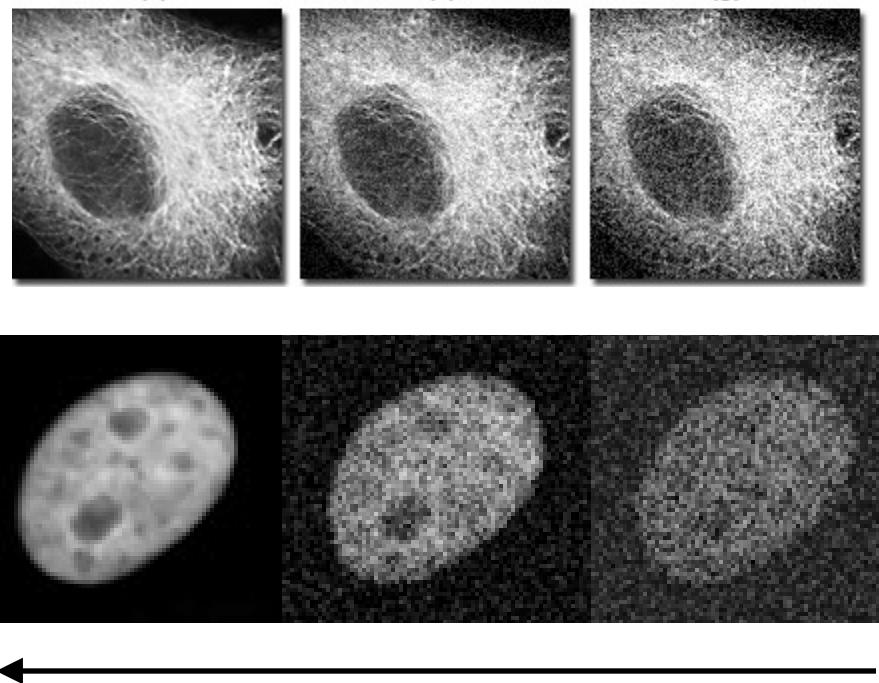
- The objective NA sets the highest resolution you can measure ($1.4 \text{ NA} \sim 220 \text{ nm}$)
- To achieve this resolution, 220 nm in your image must cover 2 pixels
- Choose your magnification to achieve this
- For $6.45 \mu\text{m}$ pixels, we need a total magnification of $6450/110 = 58.6$
- 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?

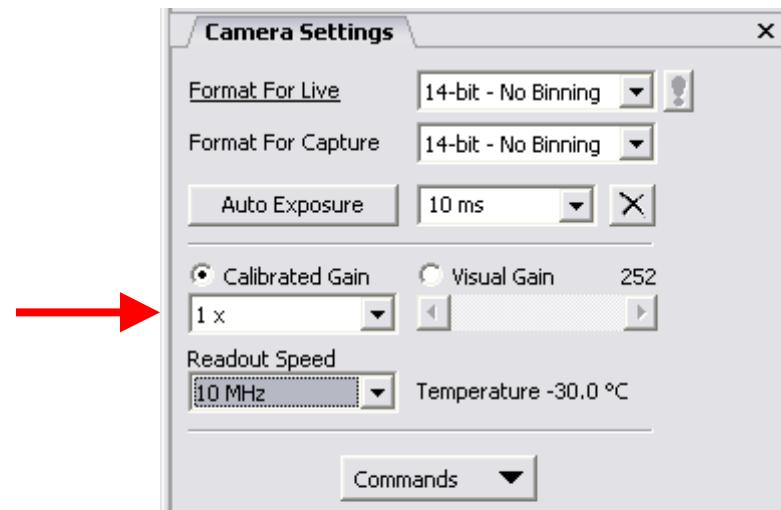


Noise

- 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 (90 ms readout)
 - 5.5 e- / pixel @ 20MHz (180ms readout)
- Dark current – thermal accumulation of electrons
 - Cooling helps, so negligible for most applications
 - CoolSNAP HQ2: 0.001 e- / pixel / s (@ -30°C)

Noise

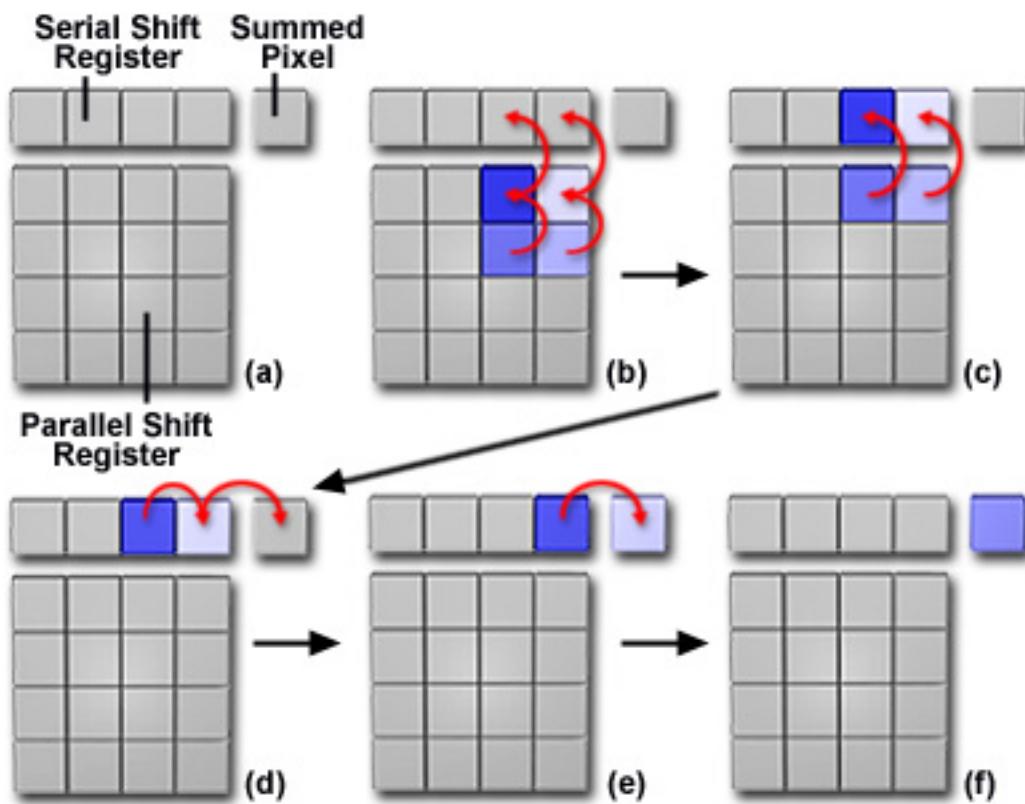
- Photon Shot Noise: Due to the fact that photons are particles and collected in integer numbers
 - Square root of the number of photons
- 1 photon \neq 1 count in your image – depends on the camera (A/D) gain
- Zero photons collected doesn't result in zero being measured on the camera – it has an offset



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,
$$\begin{aligned}\text{SNR} &= (\text{\# of photons}) / \sqrt{(\text{\# of photons})} \\ &= \sqrt{(\text{\# of photons})}\end{aligned}$$
- So, to double your SNR, you need to acquire four times as long (or 2x2 bin)

Binning



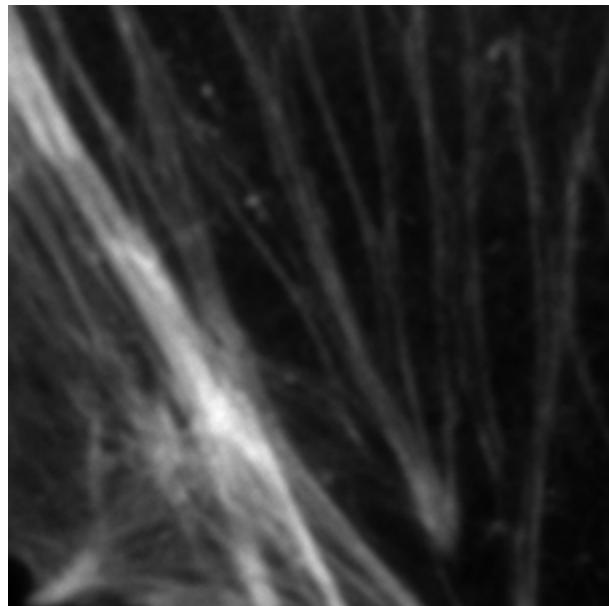
- Read out 4 pixels as one
- Increases SNR by 2x
- Decreases read time by 2 or 4x
- Decreases resolution by 2x

Signal/Noise Ratio (SNR)

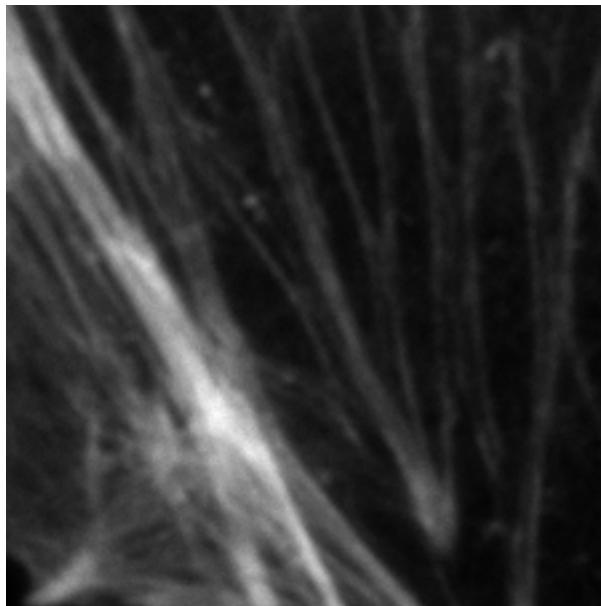
- Read noise dominates whenever
 $\text{read noise}^2 = \# \text{ of photons}$
- 8 e- read noise → 64 photons
- 16 e- read noise → 256 photons
- 50 e- read noise → 2500 photons
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

What does this look like?

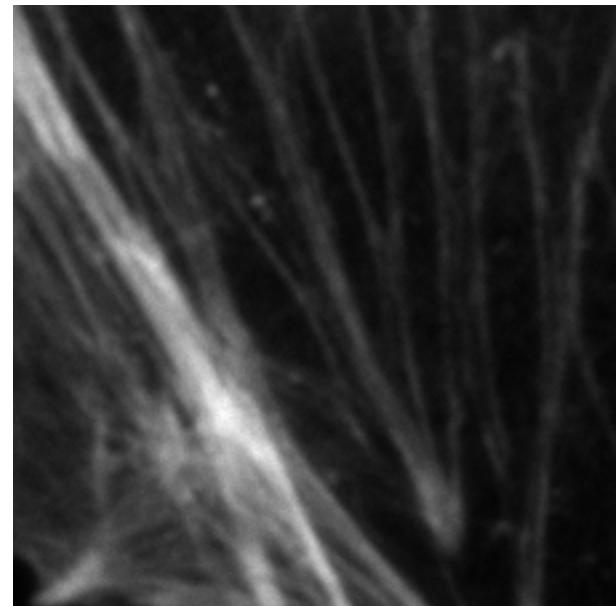
1000 photons / pixel on average; ~5000 in brightest areas



Test image



no read noise

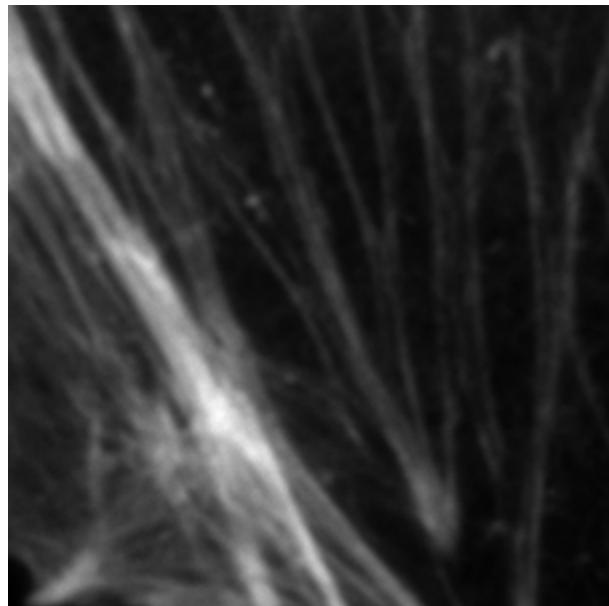


5 e- read noise

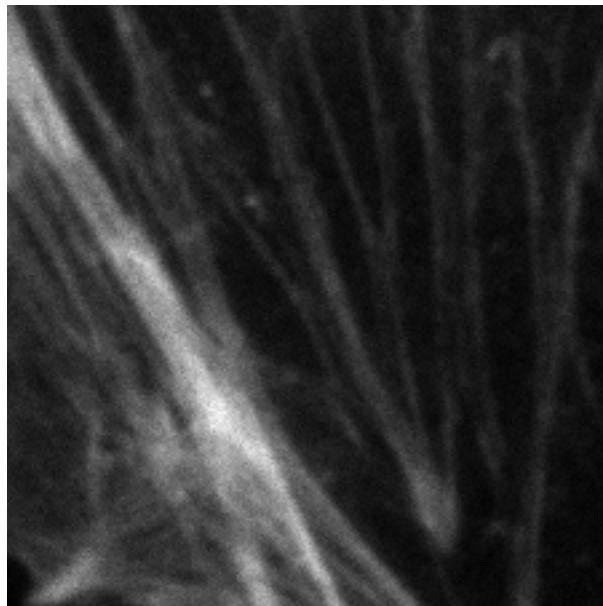
Photon shot noise \sim 6x read noise

What does this look like?

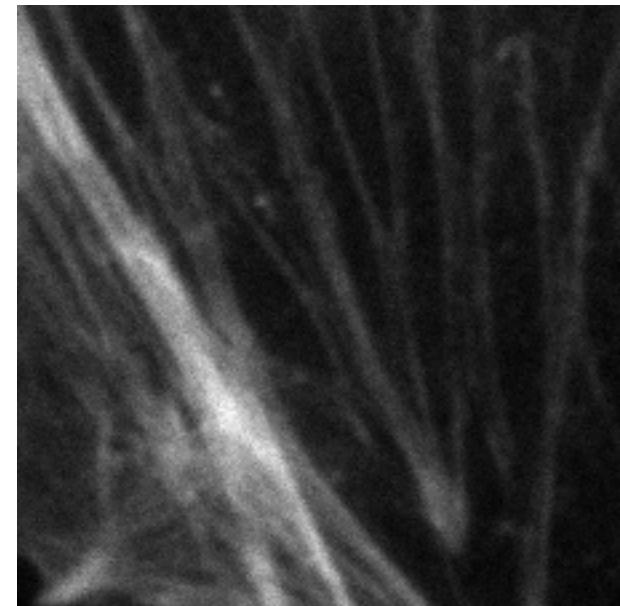
100 photons / pixel on average; ~500 in brightest areas



Test image



no read noise

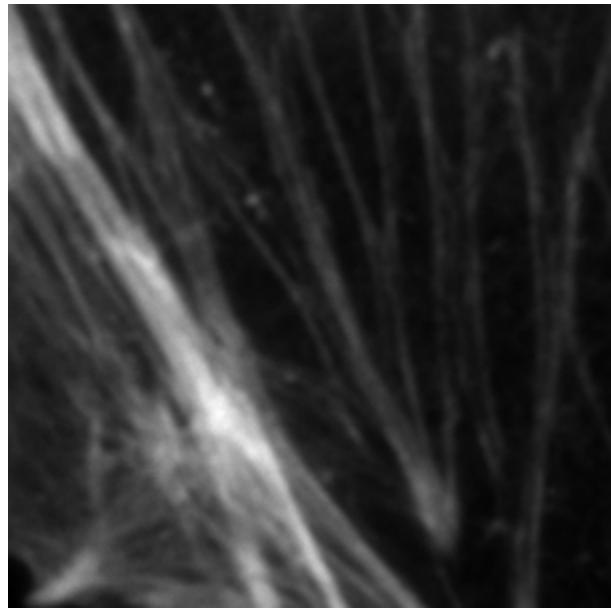


5 e- read noise

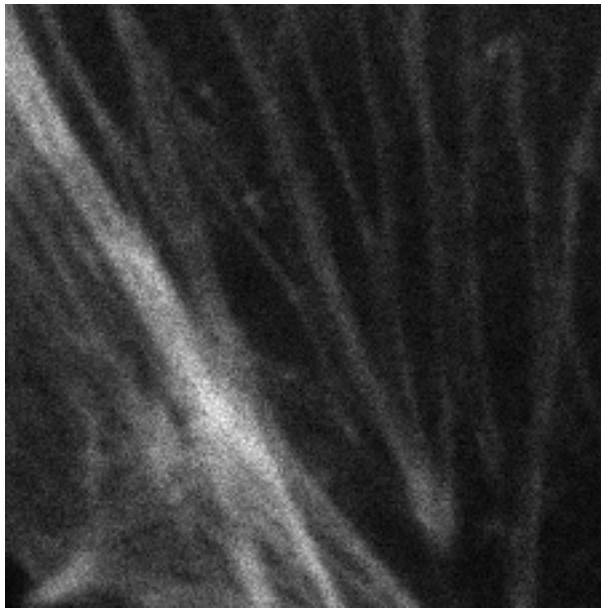
Photon shot noise = 2x read noise

What does this look like?

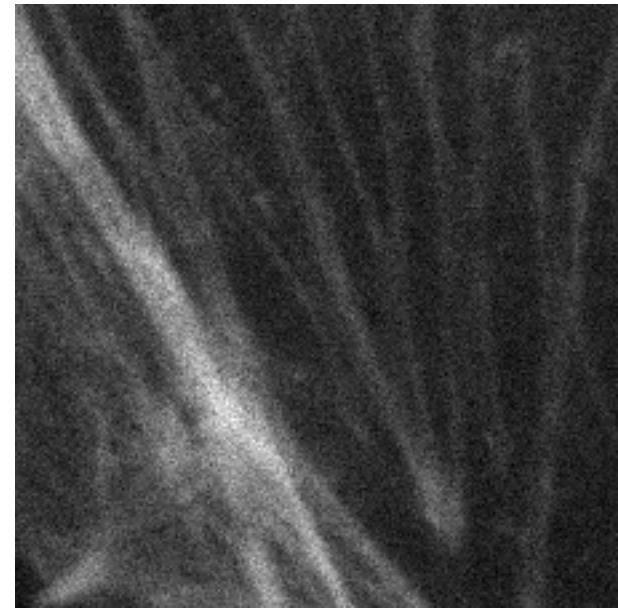
25 photons / pixel on average; ~125 in brightest areas



Test image



no read noise

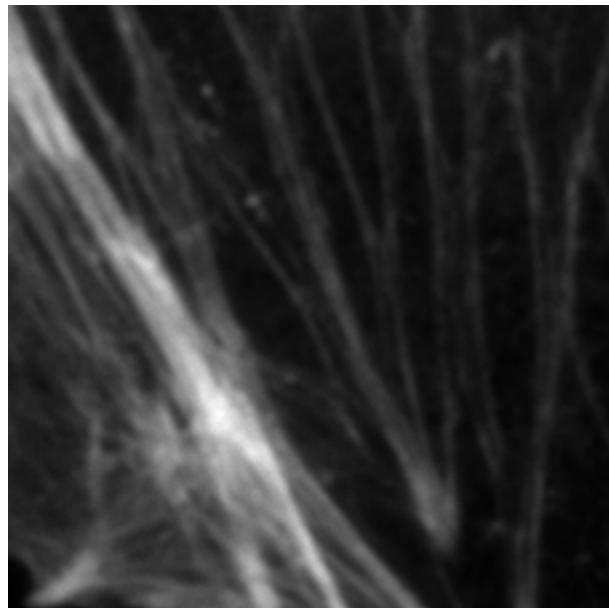


5 e- read noise

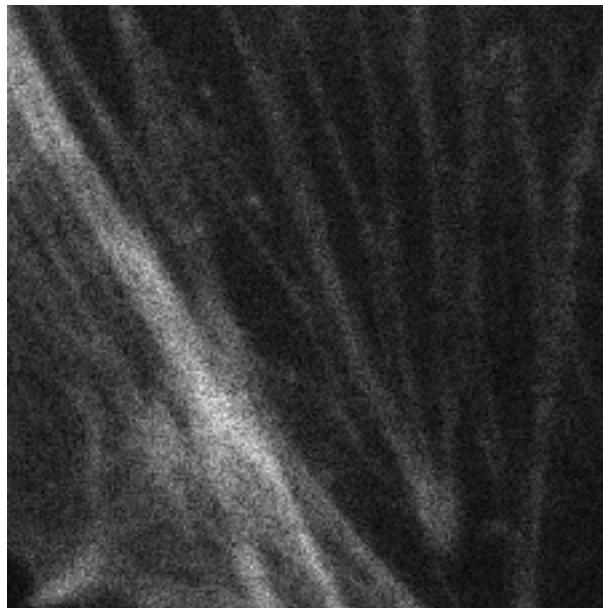
Photon shot noise = read noise

What does this look like?

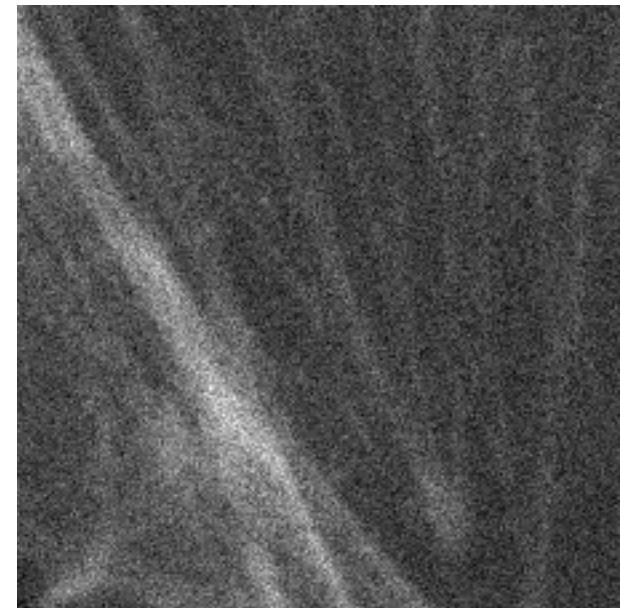
10 photons / pixel on average; ~50 in brightest areas



Test image



no read noise

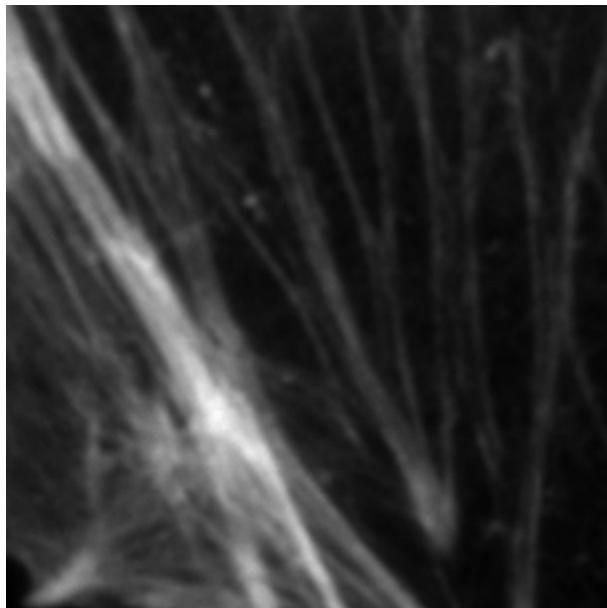


5 e- read noise

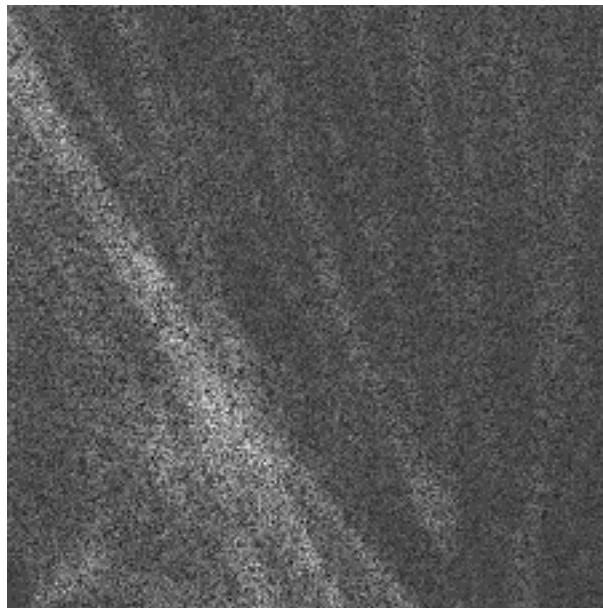
Photon shot noise \sim 2/3 read noise

What does this look like?

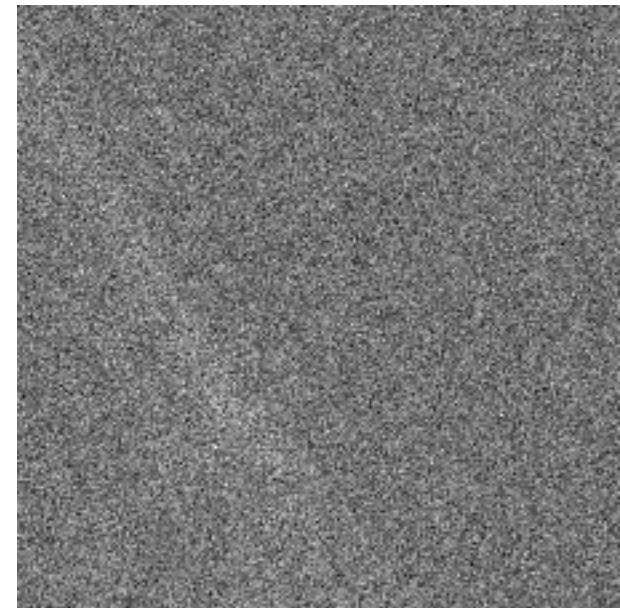
1 photon / pixel on average; ~5 in brightest areas



Test image



no read noise



5 e- read noise

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

Beating the read-out noise EMCCD

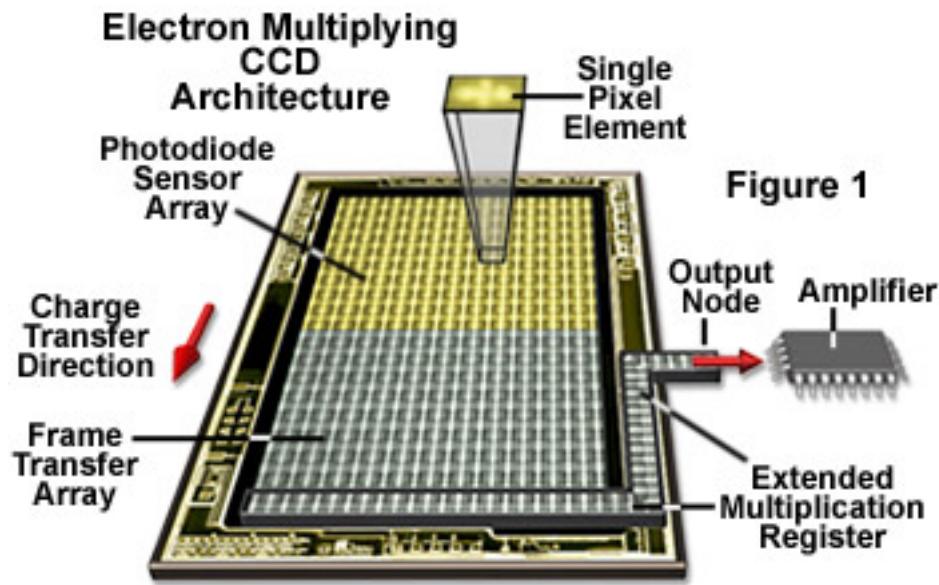


Figure 1

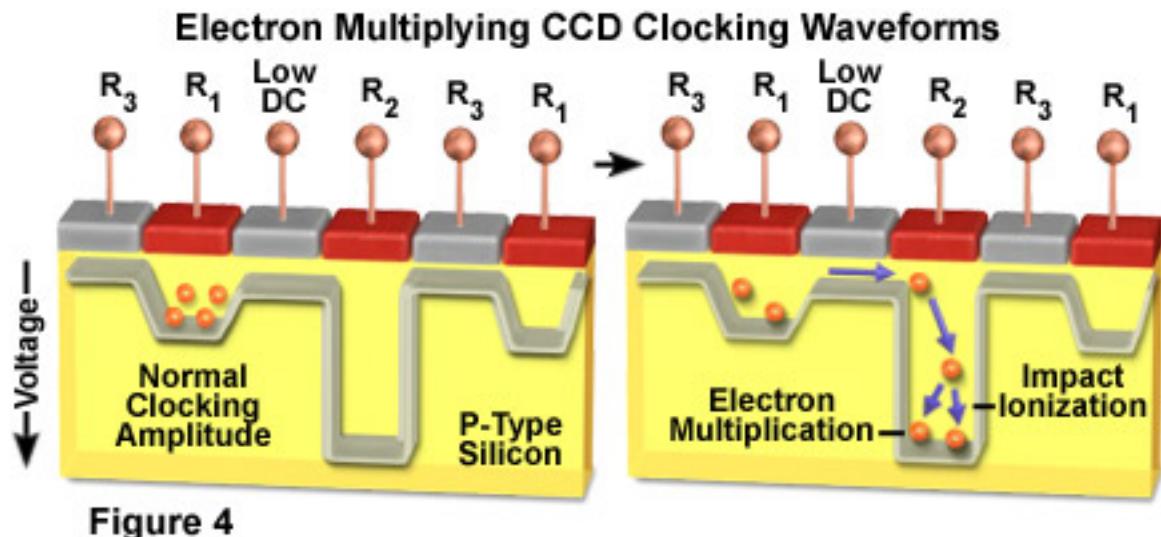


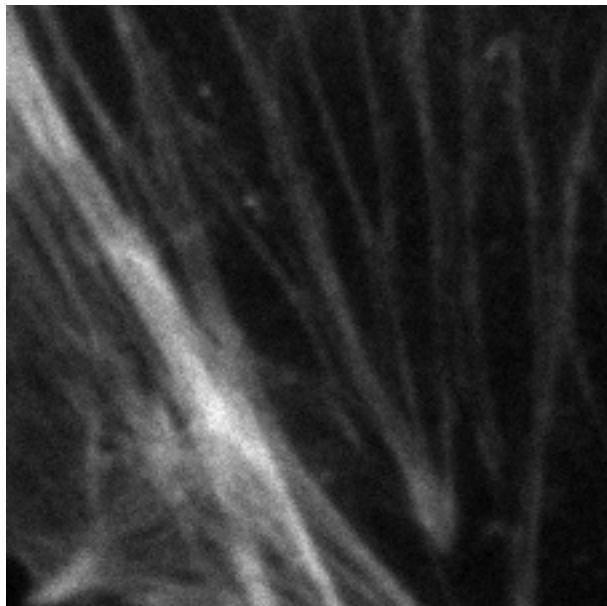
Figure 4

EMCCD result

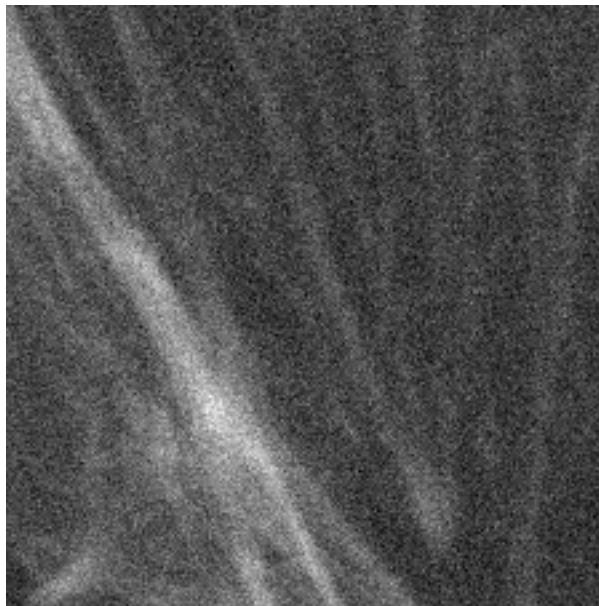
- Fast noisy CCD – runs at 30 fps, but 50 e- read noise
- Multiply signal by 100-fold – now read noise looks like 0.5 e-
- Downside – multiplication process adds additional Poisson noise, so your QE looks like it's halved
- Upside – you get to image fast without worrying about read noise

Hypothetical CCD/EMCCD comparison

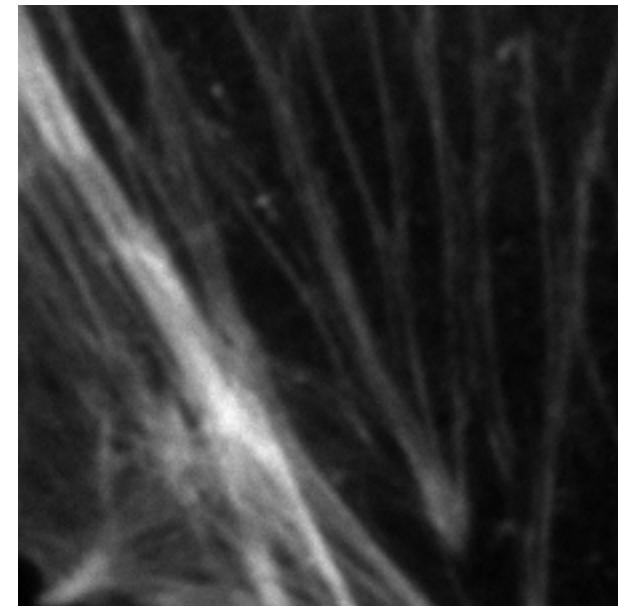
100 photon / pixel on average; ~500 in brightest areas



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



Video rate CCD,
50e- read noise

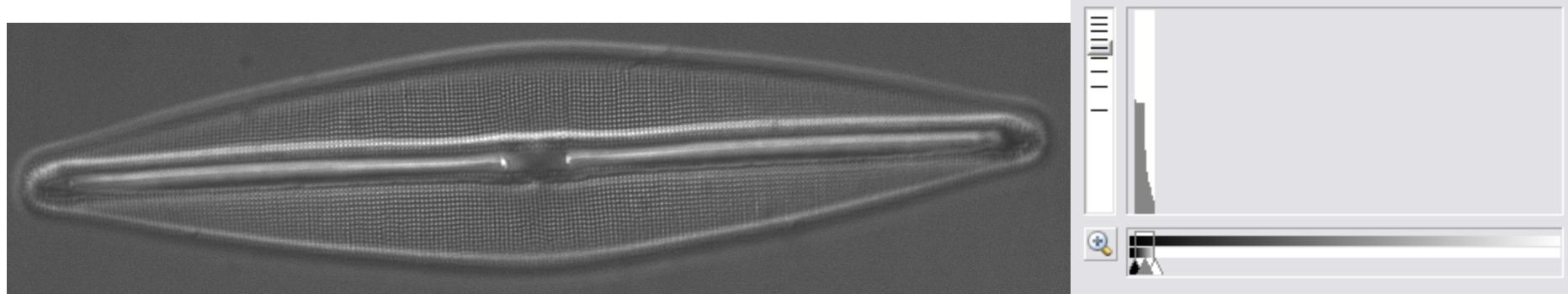
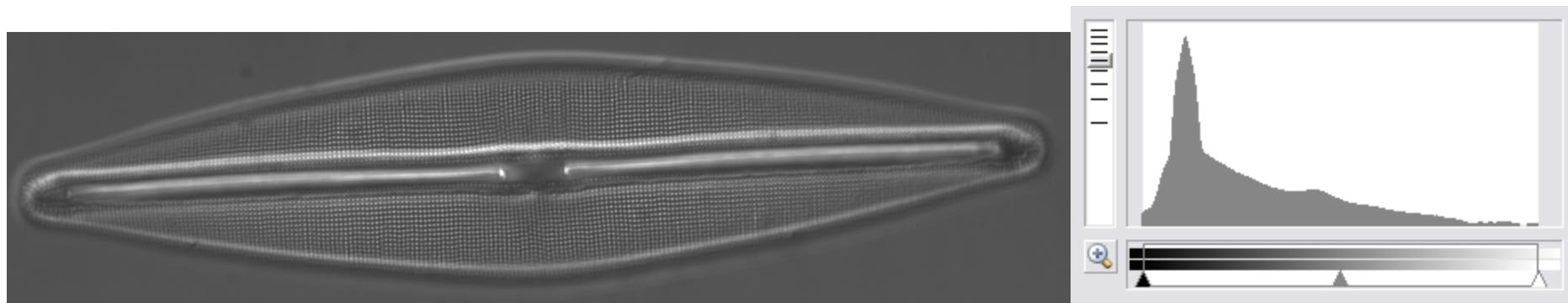


Video rate EMCCD
50e- read noise
200x gain

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)

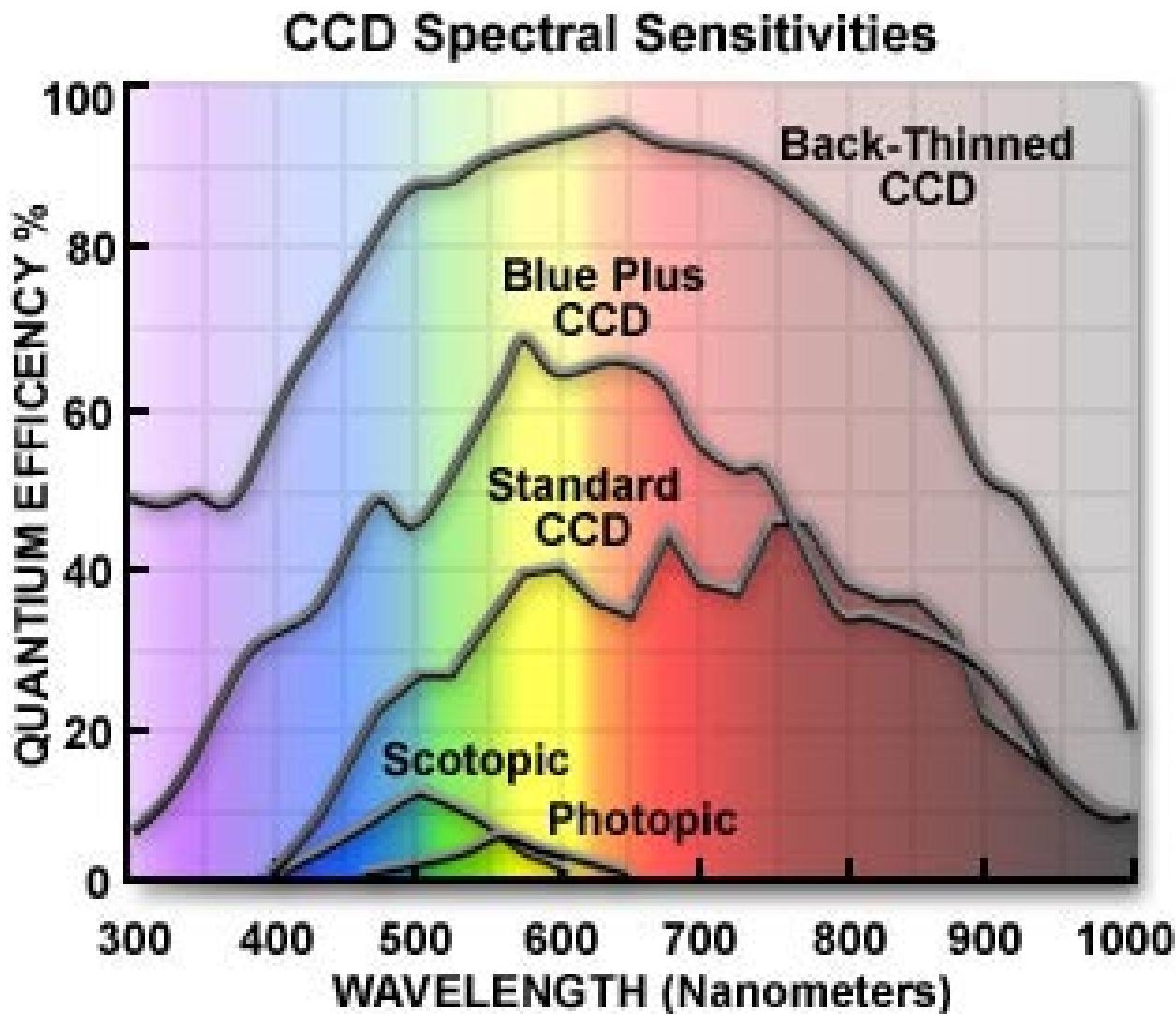
Check your histogram



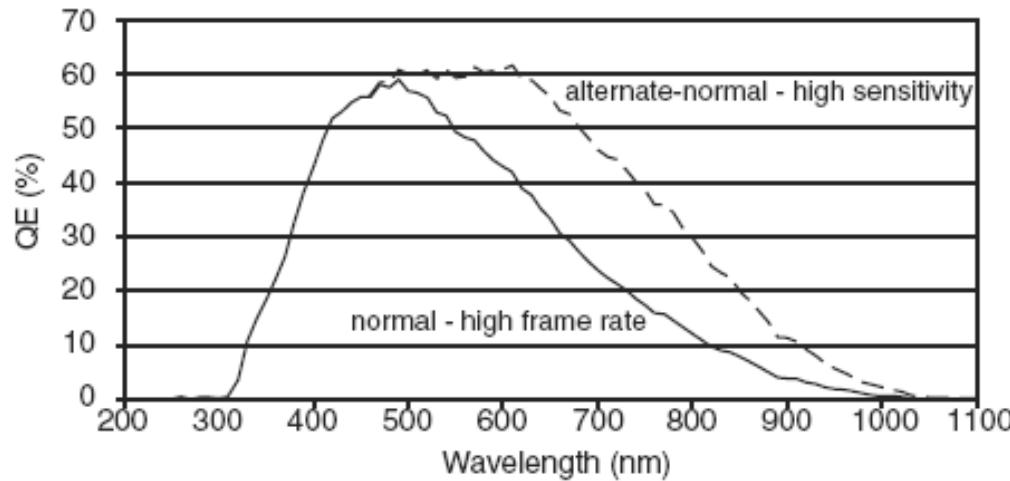
of pixels
↑

Intensity →

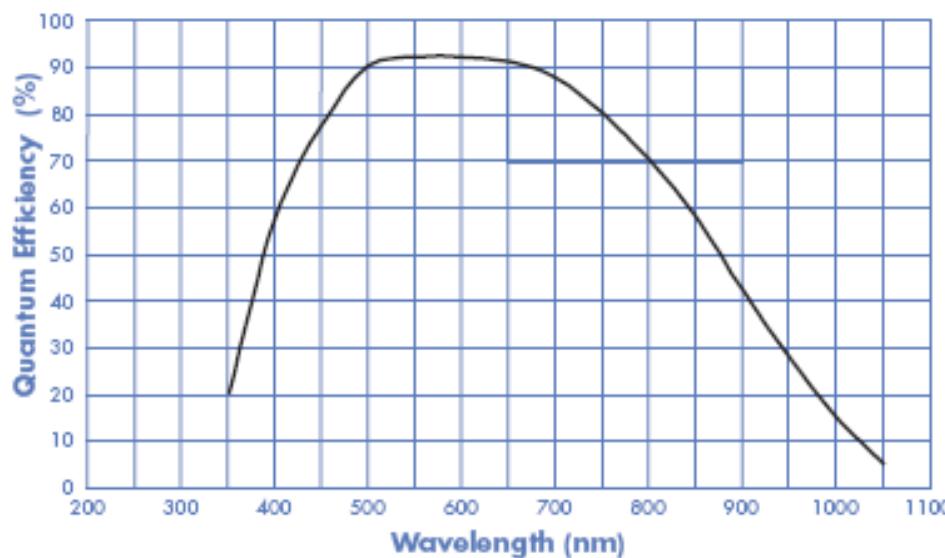
Quantum Efficiencies



Quantum efficiency



HQ2

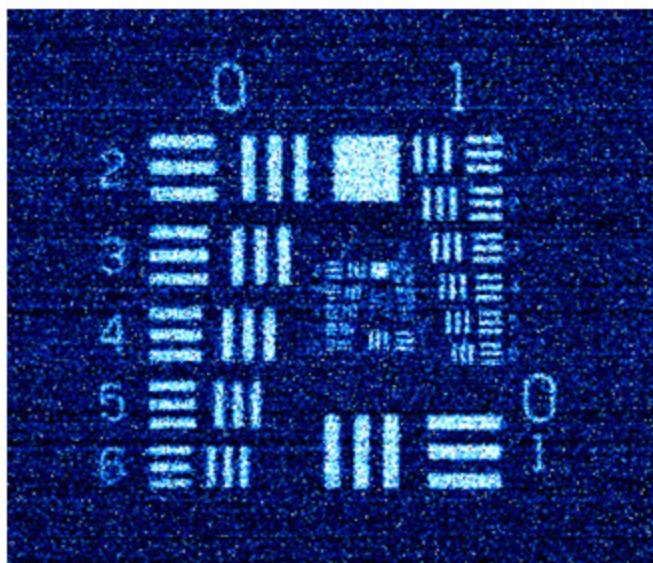


Cascade II

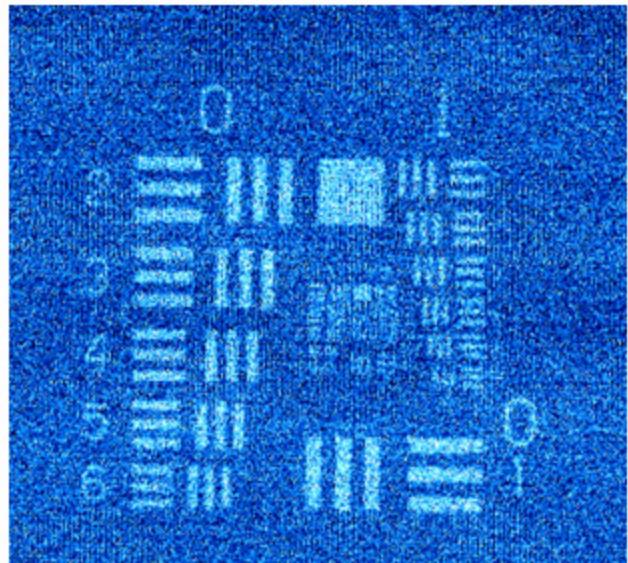
Forthcoming technology: sCMOS

- From Andor / Fairchild / PCO
- Claims:
 - 5.5 Megapixel sensor
 - < 2 e- read noise
 - 60% QE
 - 30 – 100 frame / sec readout

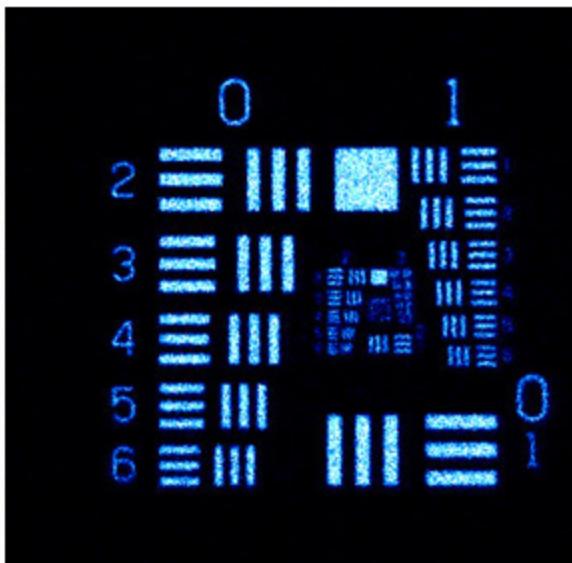
Forthcoming technology: sCMOS



sCMOS (1.5 e^- noise)



Interline CCD (5 e^- noise)



Back-illuminated
EMCCD ($<1 \text{ e}^-$ noise)

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)

More reading

- www.microscopyu.com
- 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)

Acknowledgements

- Nico Stuurman, Mike Davidson