# Principles and Practices of Light Microscopy
# Lecture 7: Light sources and Cameras

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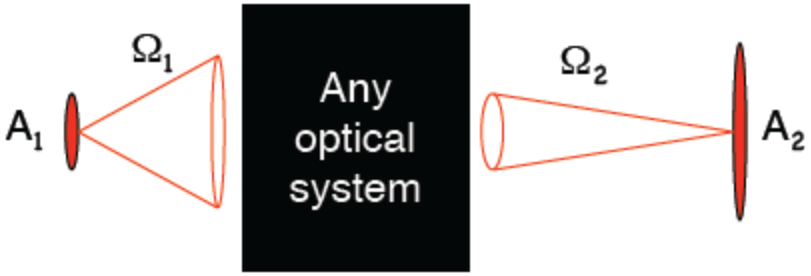
*Image: Susanne Rafelski*

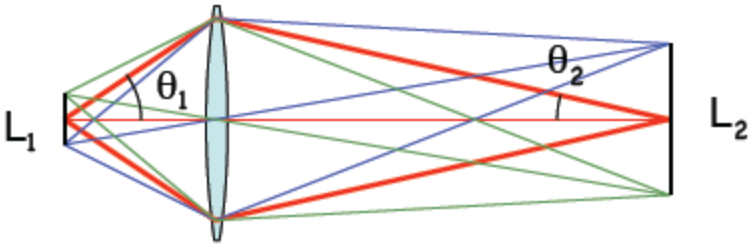
# Light Sources

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

# Light Sources

- Constraint:  area × solid angle is invariant




$L_2 = M\, L_1$

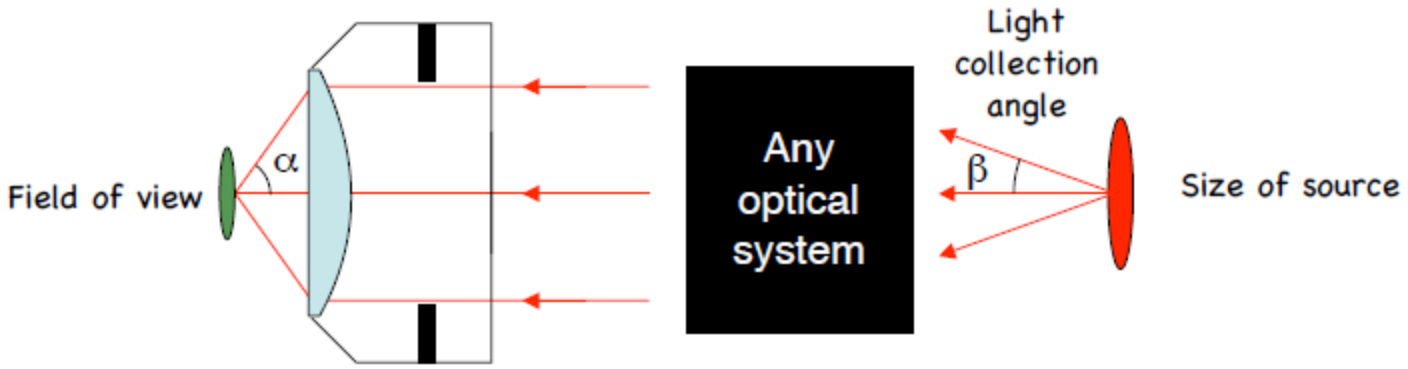
$\theta_2 = \theta_1 / M$

$\Rightarrow$

$L_2\, \theta_2 = L_1\, \theta_1$

# Brightness

- (area × solid angle) at sample ≤ (area × solid angle) at source
- No optical system can increase it

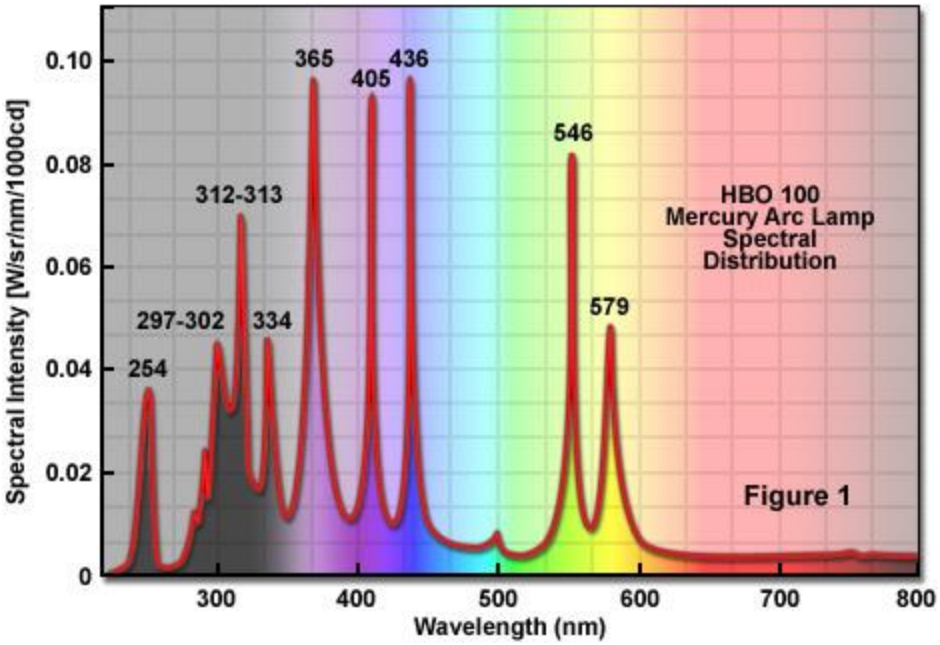


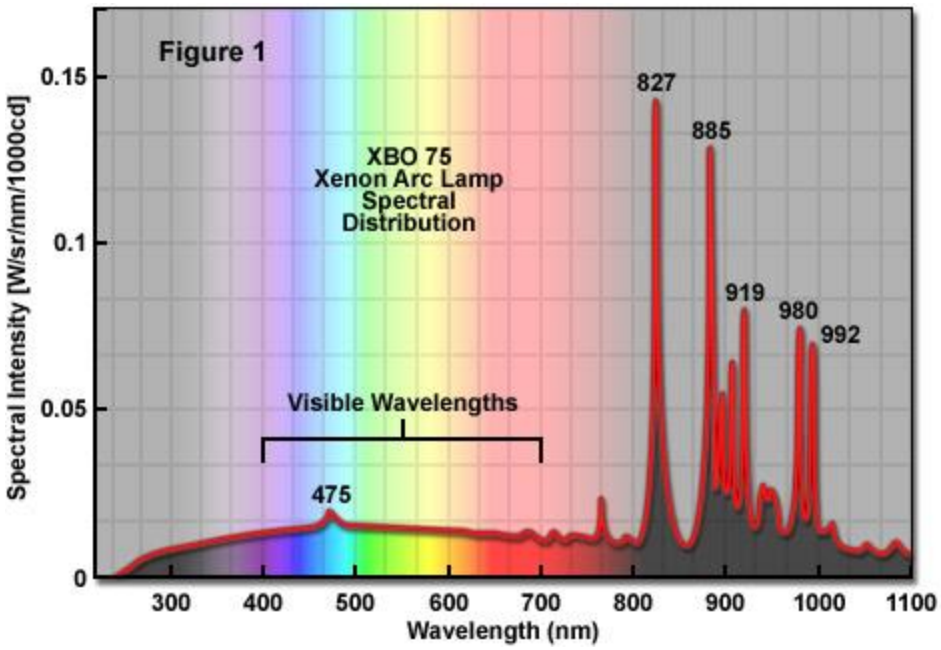

Usable size of source ≈ (Field of view) X NA / sin(β)

# Source and sample size should be similar

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

# Arc Lamp Spectra

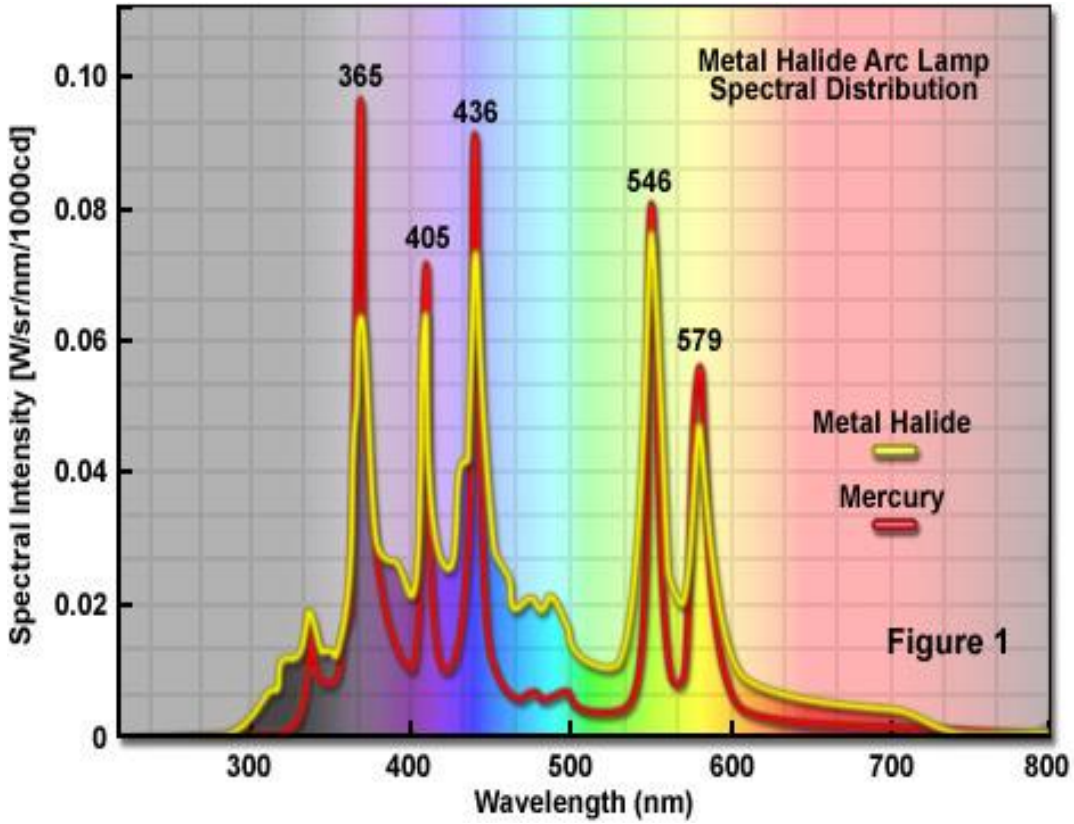



- Hg: Brighter if your excitation spectrum matches one of the lines
- Xe: More stable, longer lifetime, flat emission in visible is sometimes beneficial

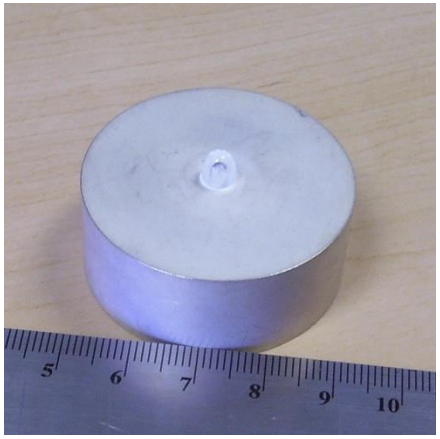
# Metal Halide Arc Lamp

•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

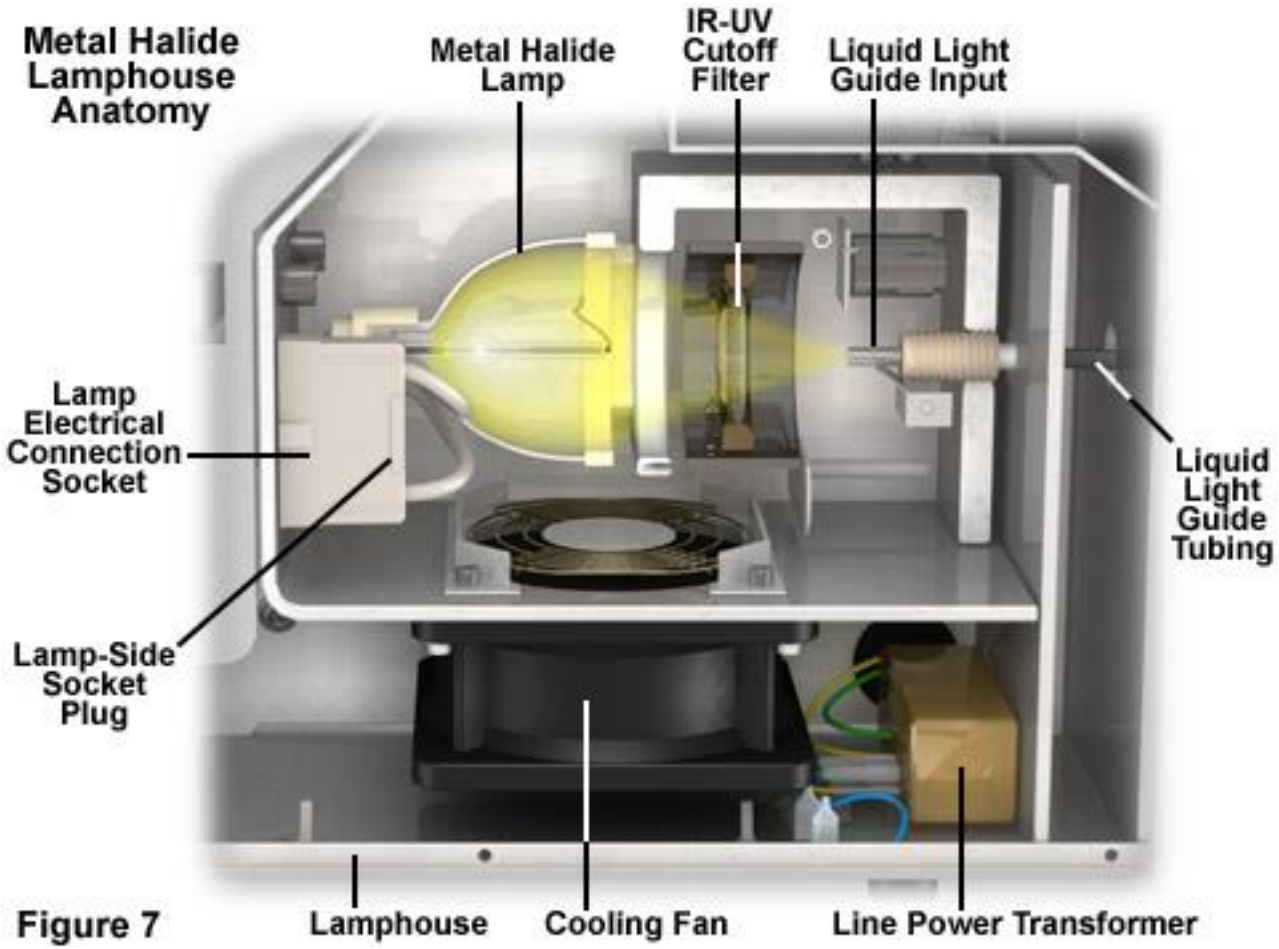
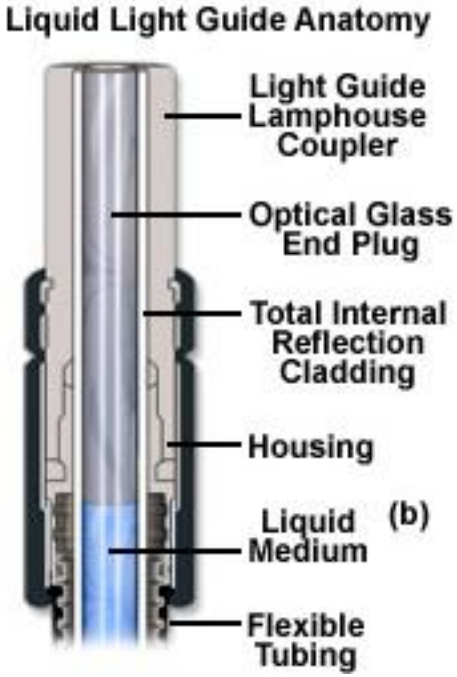
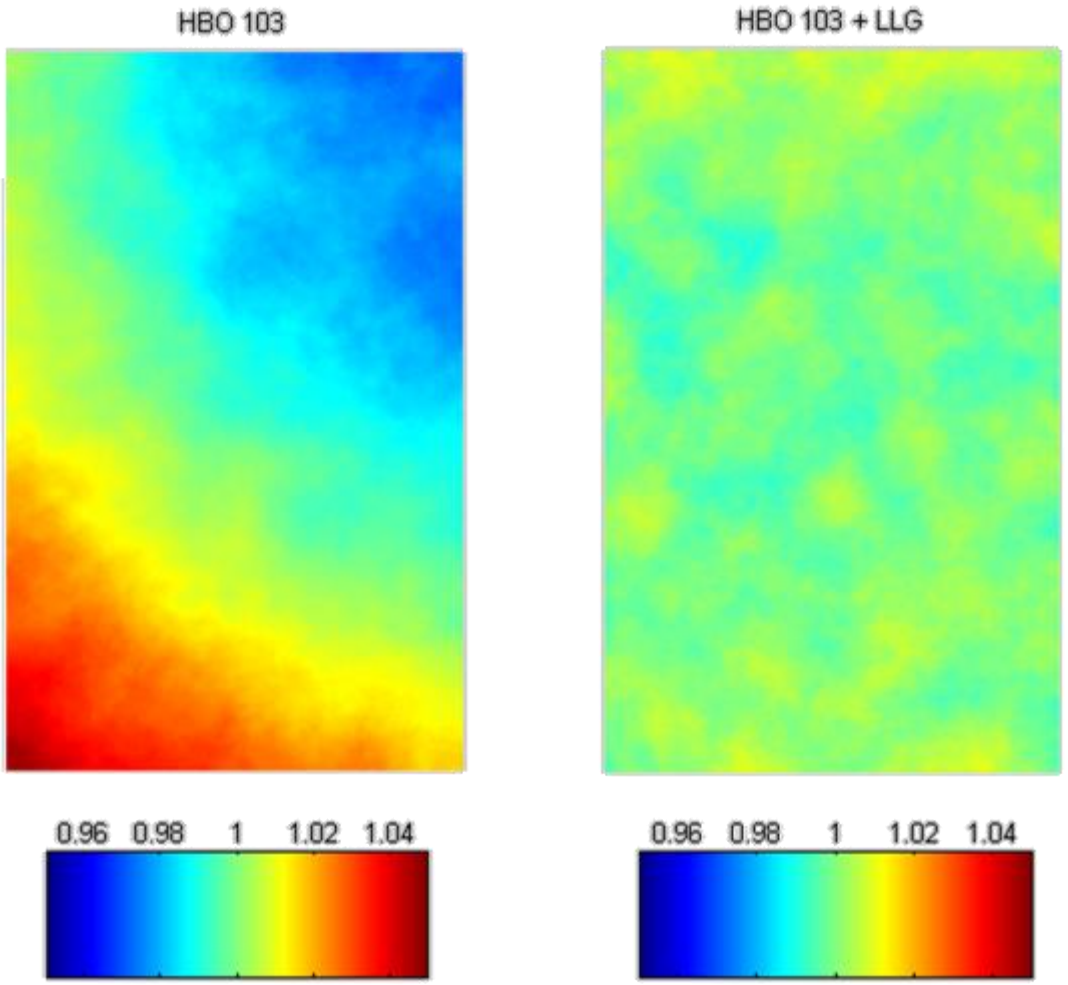



Figure 7

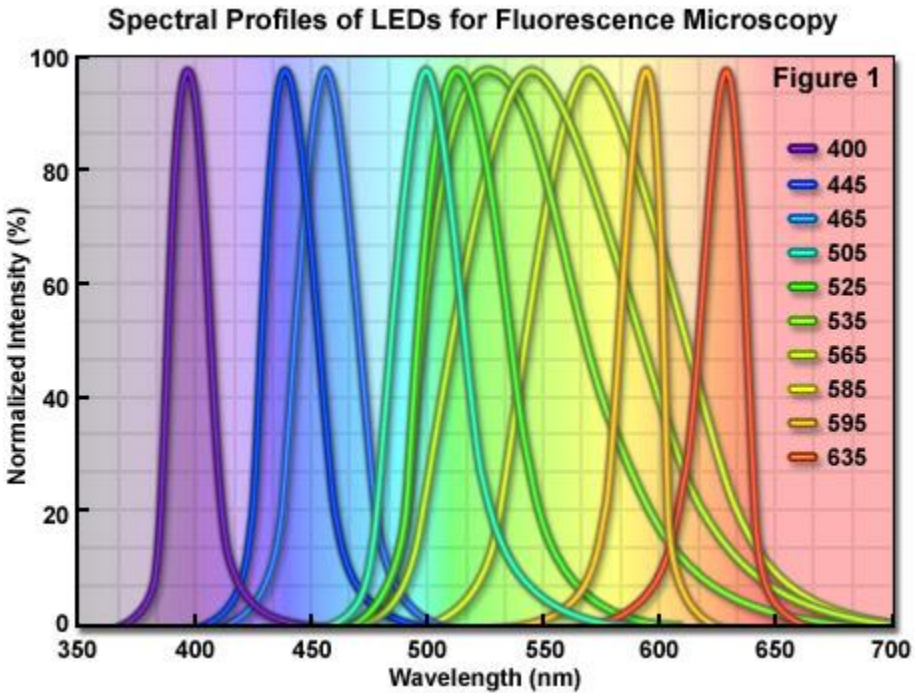


# Benefit of Liquid Light Guide

## Improved illumination uniformity



# LEDs




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

# Relative lamp power

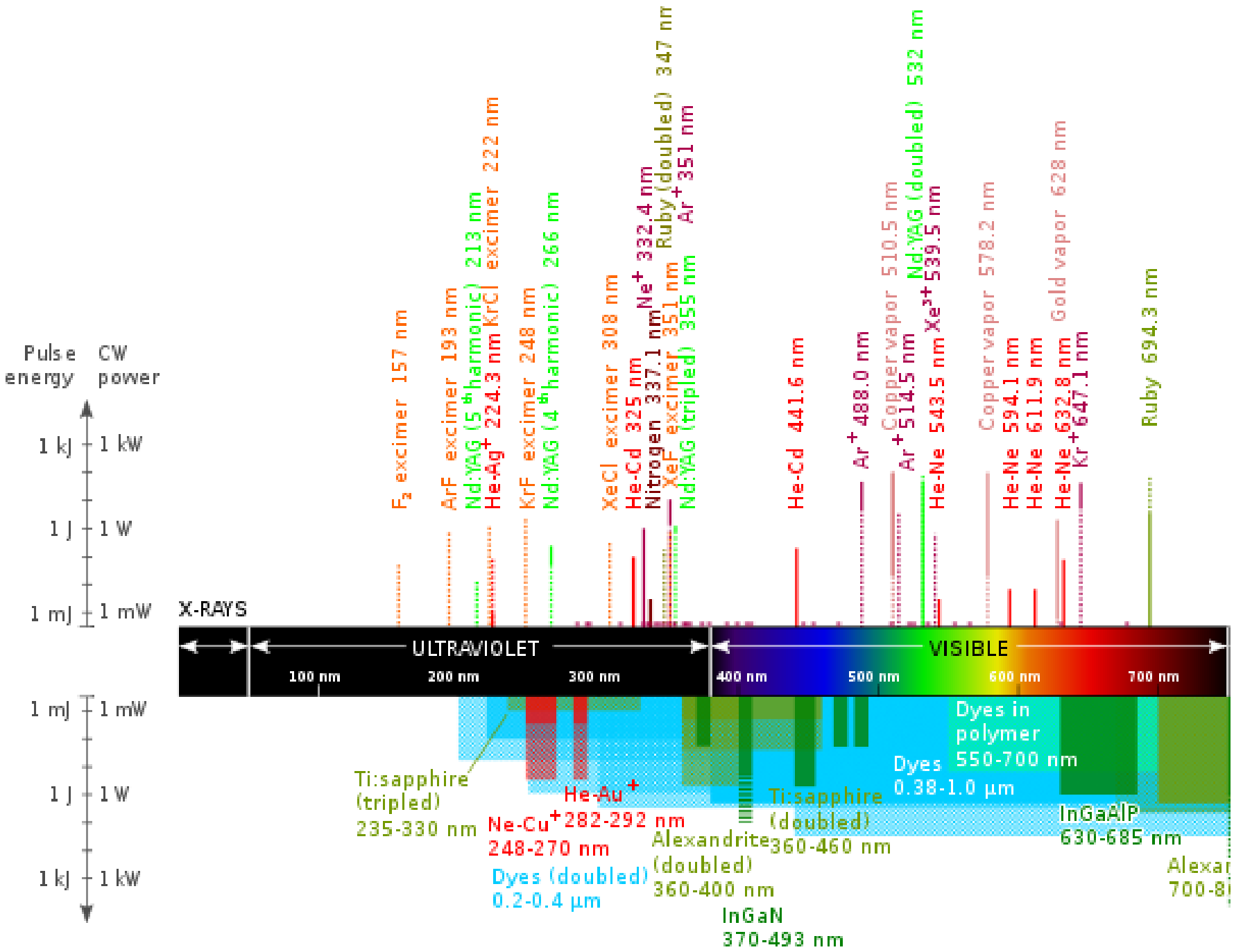
| Filter Set | Excitation Filter (nm) | Dichromatic Mirror (nm) | Mercury HBO Power mW/Cm² | Xenon XBO Power mW/Cm² | Metal Halide Power mW/Cm² | LED Power mW/Cm² | Tungsten HAL Power mW/Cm² |
|---|---|---|---|---|---|---|---|
| DAPI (49)[1] | 365/10 | 395 LP | 23.0 | 5.6 | 14.5 | 0.70 (365)[3] | 0.06[4] |
| 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 |

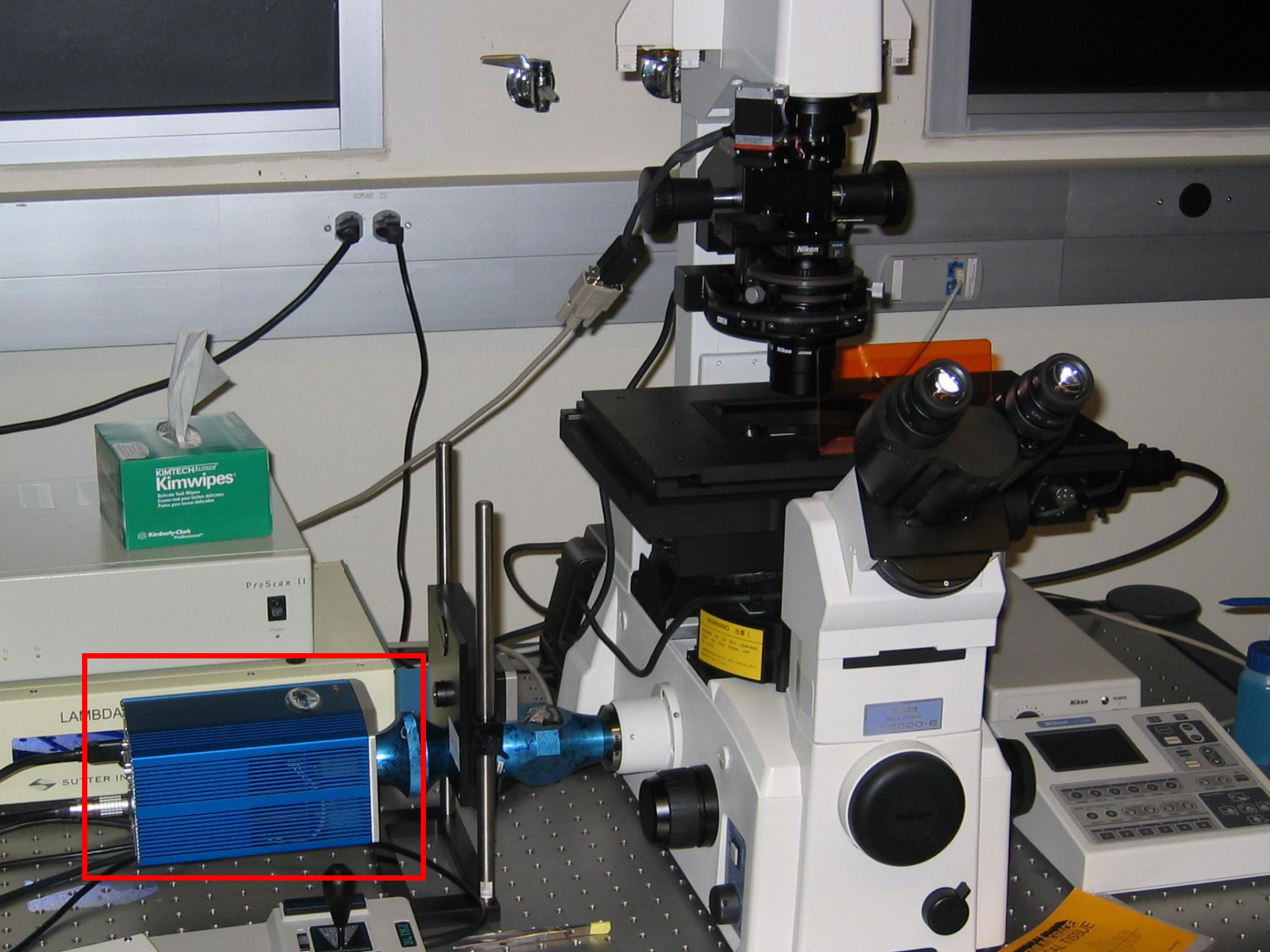
[1]Zeiss Filters [2]Semrock Filters [3]LED Peak Wavelength [4]Tungsten-Halogen Lamp Voltage = 12.2 V

- 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 $\rightarrow$ 532 nm


Pulse energy
CW power
1 kJ
1 kW
1 J
1 W
1 mJ
1 mW
1 mJ
1 mW
1 J
1 W
1 kJ
1 kW
X-RAYS
ULTRAVIOLET
VISIBLE
100 nm
200 nm
300 nm
400 nm
500 nm
600 nm
700 nm
F2 excimer 157 nm
ArF excimer 193 nm
Nd:YAG (5th harmonic) 213 nm
He-Ag+ 224.3 nm
KrCl excimer 222 nm
KrF excimer 248 nm
Nd:YAG (4th harmonic) 266 nm
XeCl excimer 308 nm
He-Cd 325 nm
Nitrogen 337.1 nm
Ne+ 332.4 nm
Ruby (doubled) 347 nm
XeF excimer 351 nm
Nd:YAG (tripled) 355 nm
Ar+ 351 nm
He-Cd 441.6 nm
Ar+ 488.0 nm
Copper vapor 510.5 nm
Ar+ 514.5 nm
Nd:YAG (doubled) 532 nm
He-Ne 543.5 nm
Xe3+ 539.5 nm
Copper vapor 578.2 nm
He-Ne 594.1 nm
He-Ne 611.9 nm
Gold vapor 628 nm
He-Ne 632.8 nm
Kr+ 647.1 nm
Ruby 694.3 nm
Ti:sapphire (tripled) 235-330 nm
He-Au+
Ne-Cu+ 282-292 nm
248-270 nm
Dyes (doubled) 0.2-0.4 µm
Alexandrite (doubled) 360-400 nm
InGaN 370-493 nm
Ti:sapphire (doubled) 360-460 nm
Dyes 0.38-1.0 µm
Dyes in polymer 550-700 nm
InGaAlP 630-685 nm
Alexa
700-8


KIMTECH
Kimwipes
Kimberly-Clark
ProScan II
Nikon

# Detectors

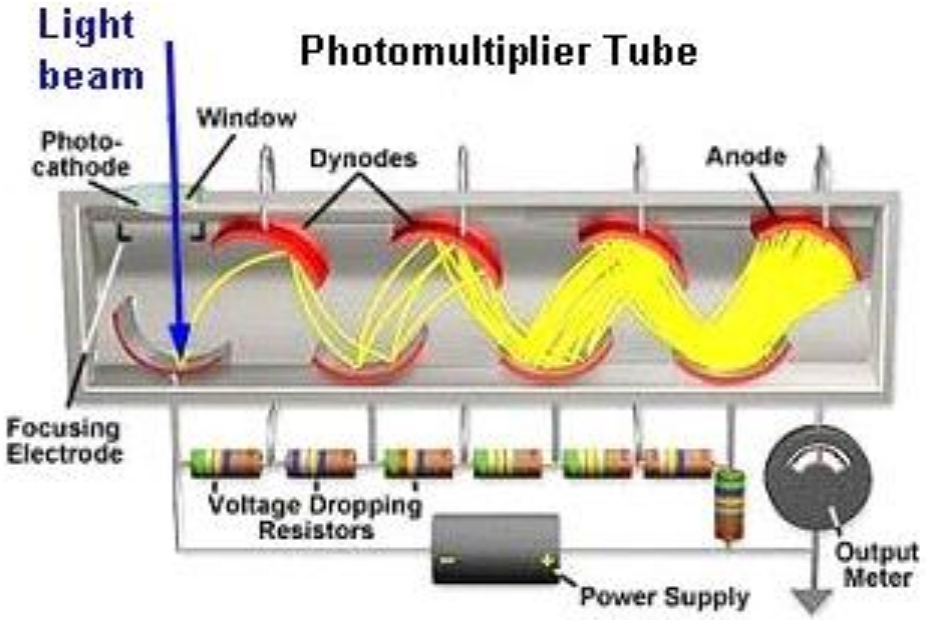
- Must
  - Convert light into an electrical signal
  - Ideally linear, low noise, accurate

- 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

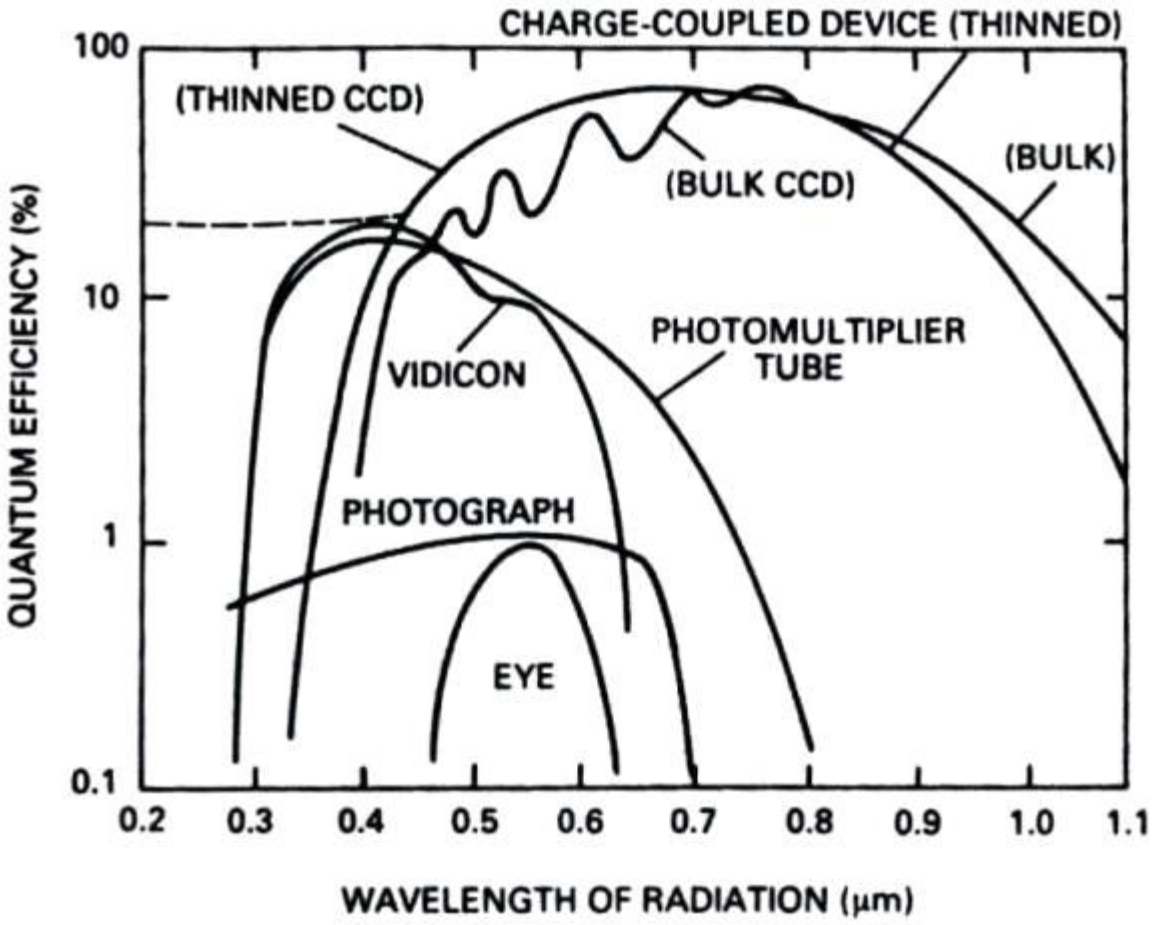
# Detectors - PMTs

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

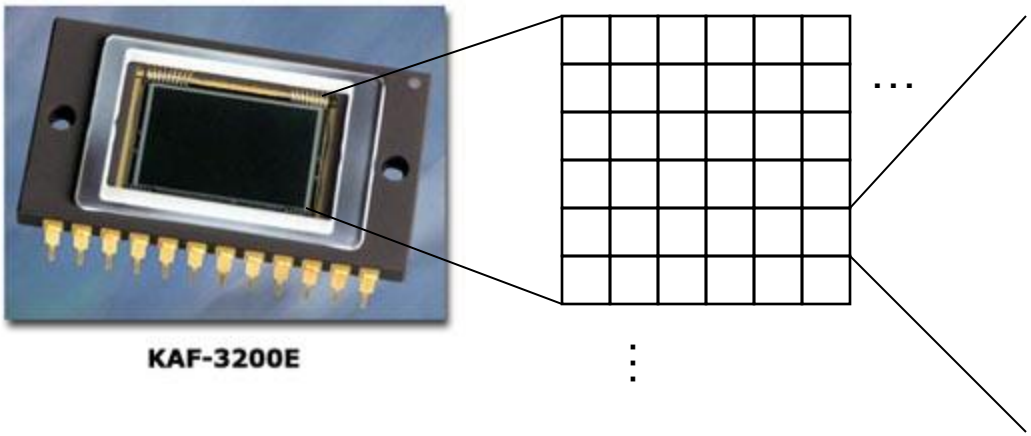


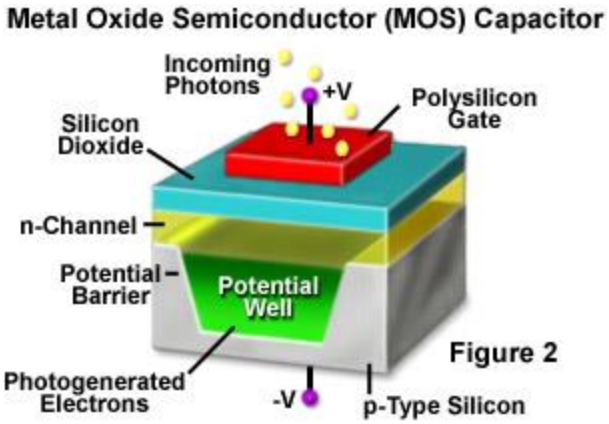

- Pulse width for single photon ~ 10-100ns
- Very linear
- Very high gain
- ~ 0 read noise

# Detectors - PMTs



# CCD architecture



# CCD readout “bucket-brigade” analogy

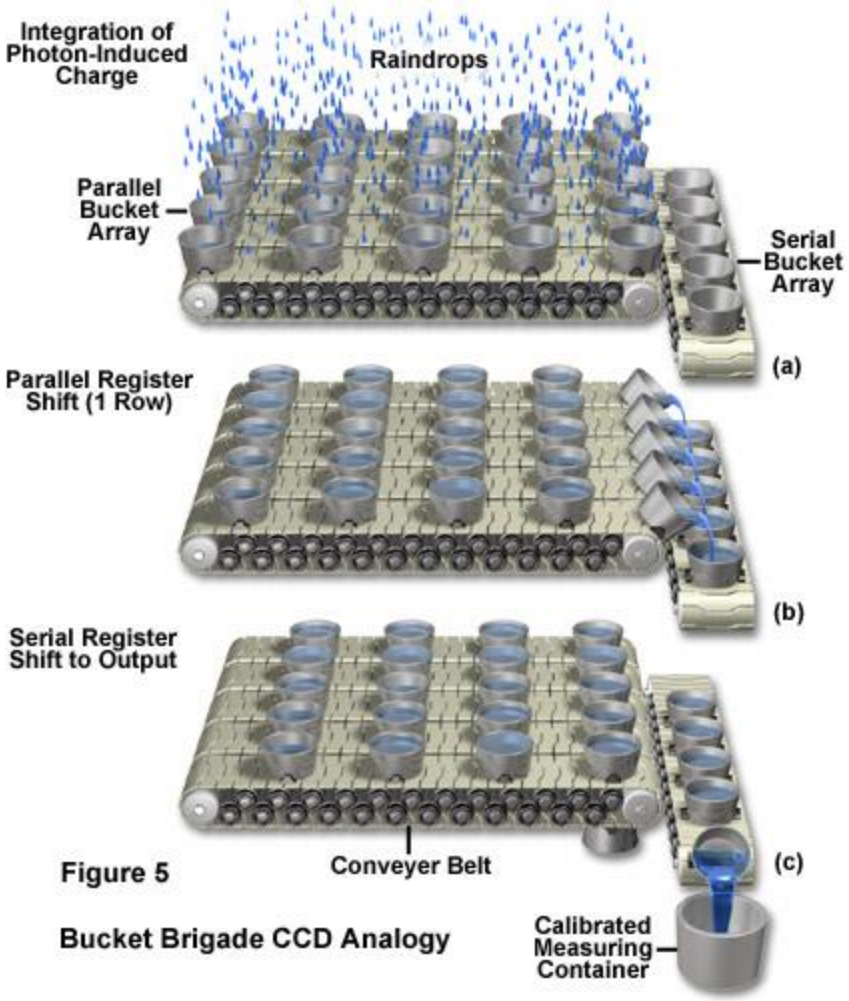



Figure 5

Bucket Brigade CCD Analogy

# A little more realistic….

Each pixel is subdivided into three phases

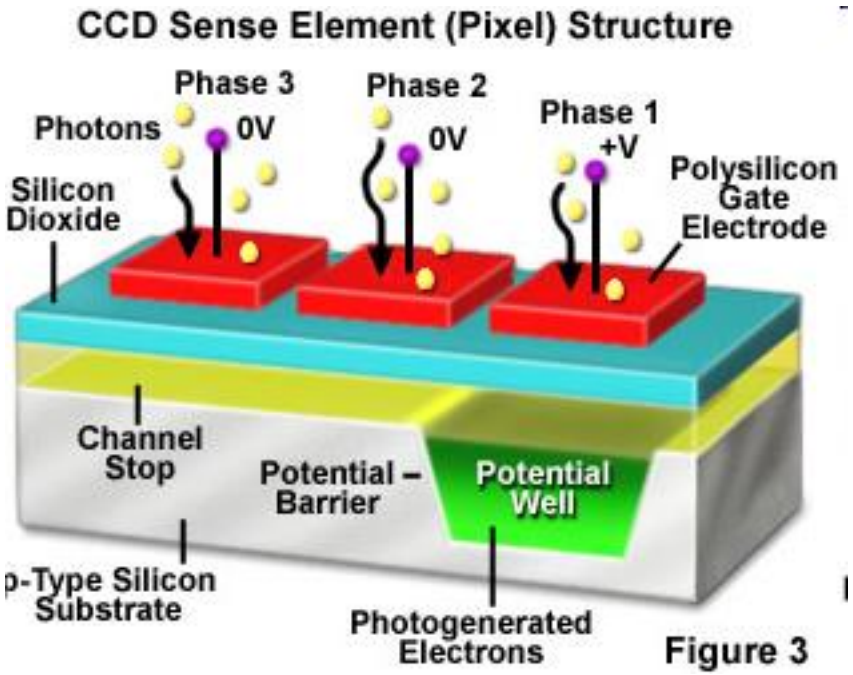
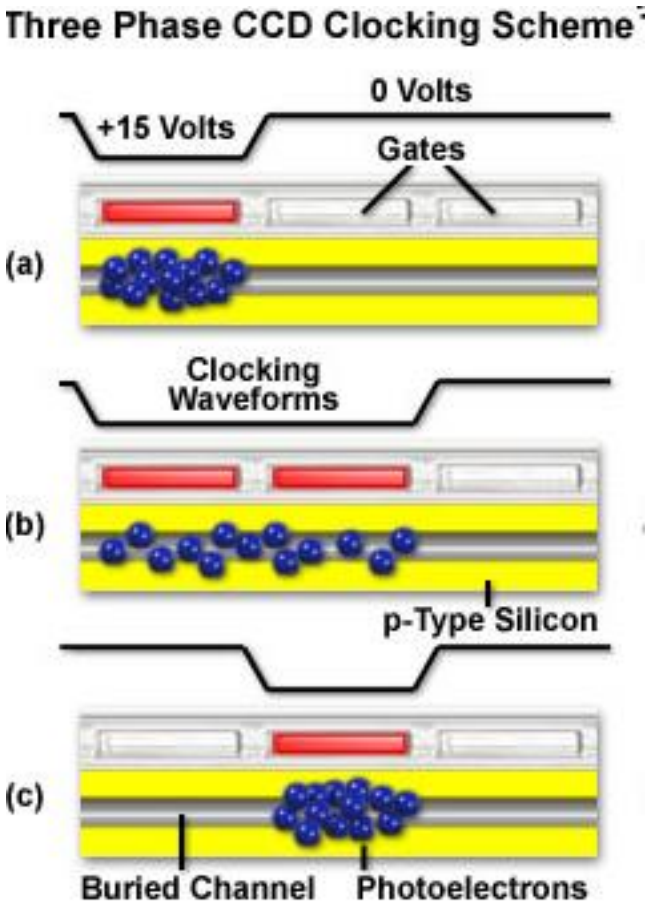
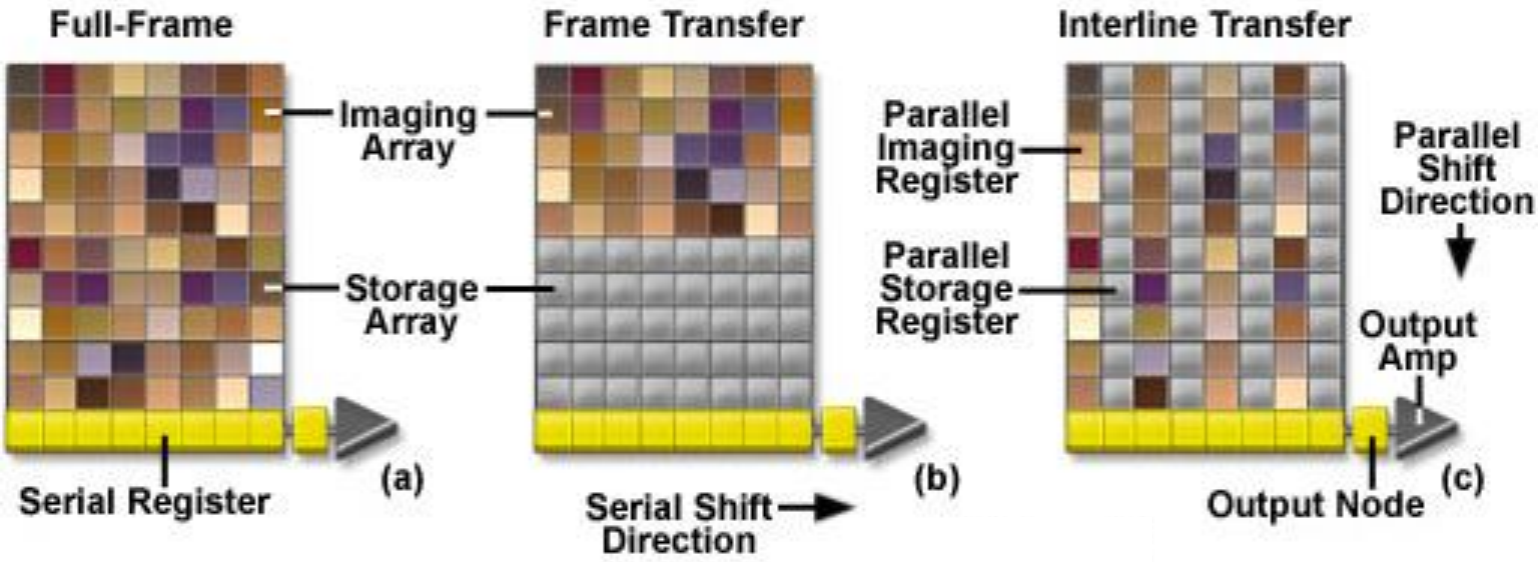



Figure 3



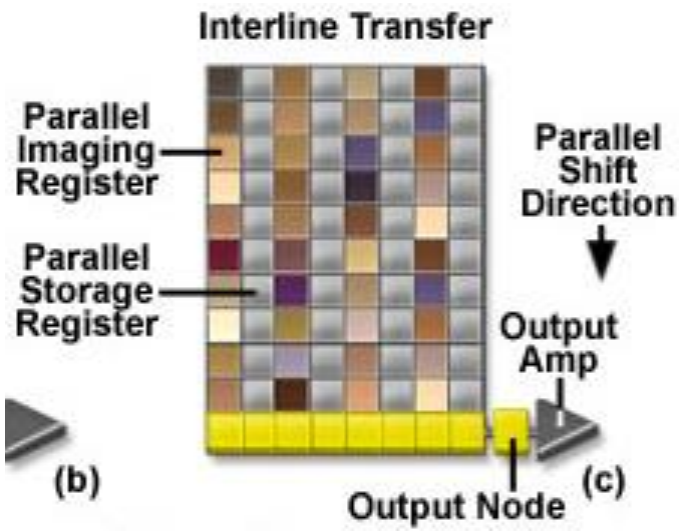
# CCD Architectures

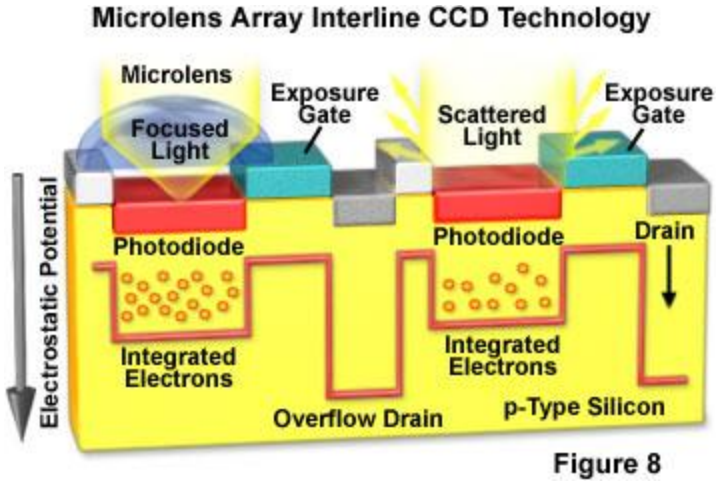



Rare Mostly EMCCDs Common

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?

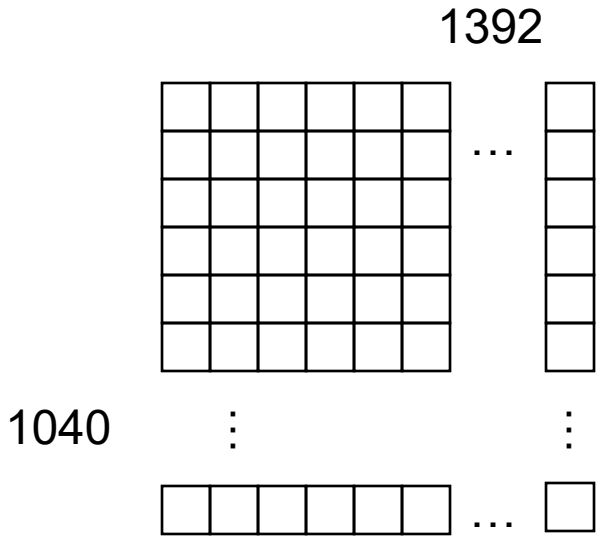
Figure 2

- 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

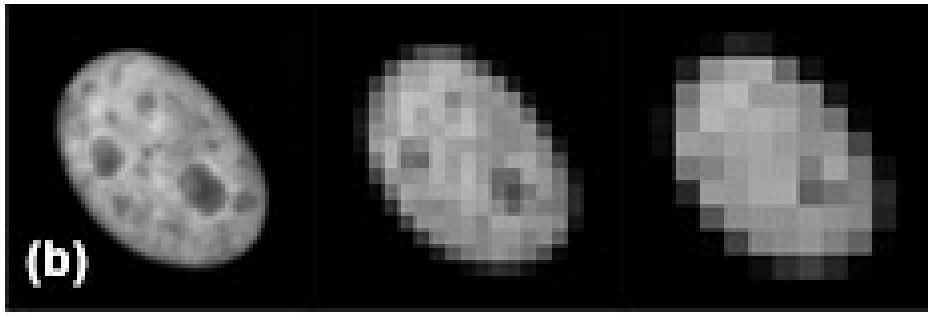



6.45 μm on a side

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

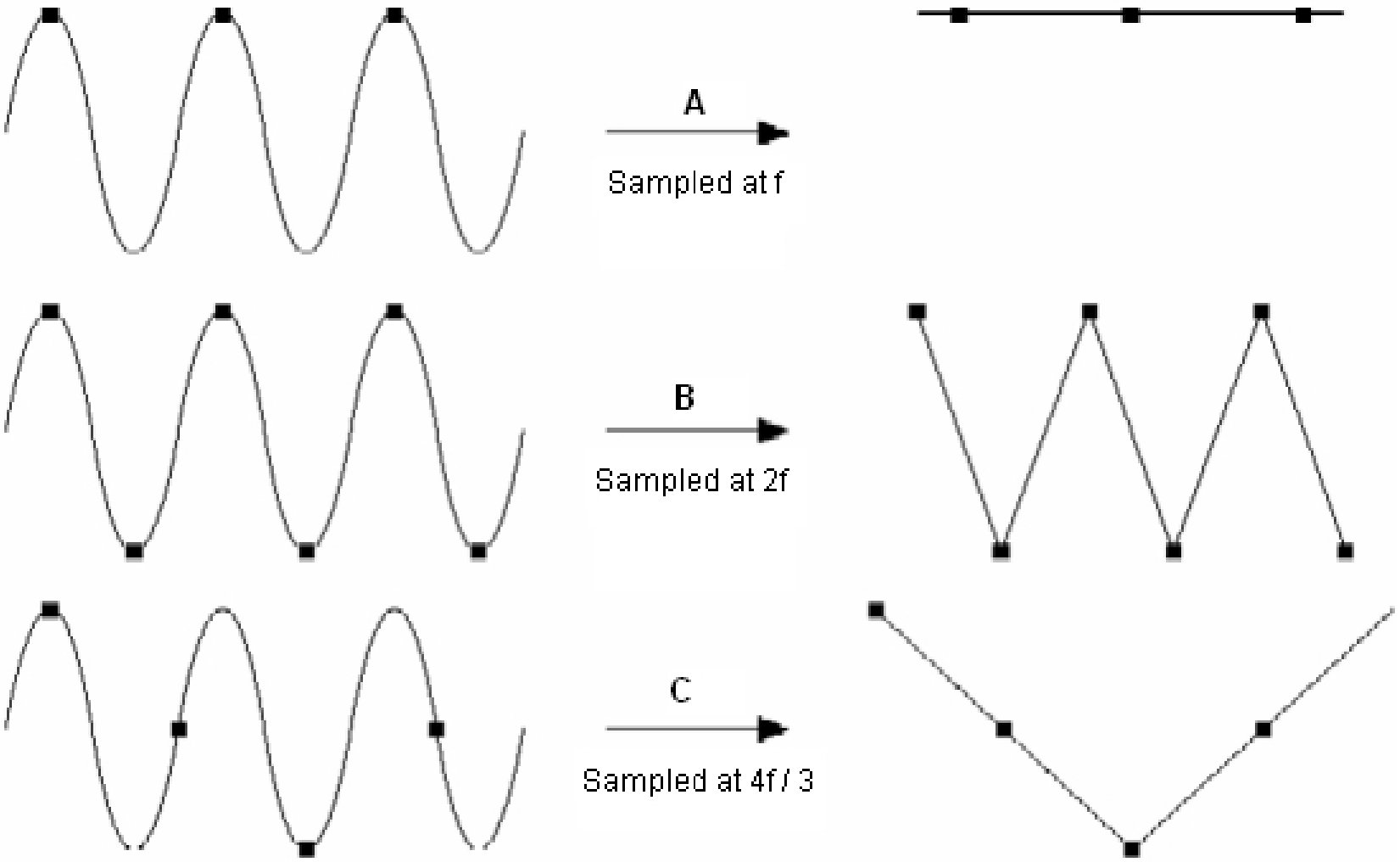
Where is optimum?

# 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

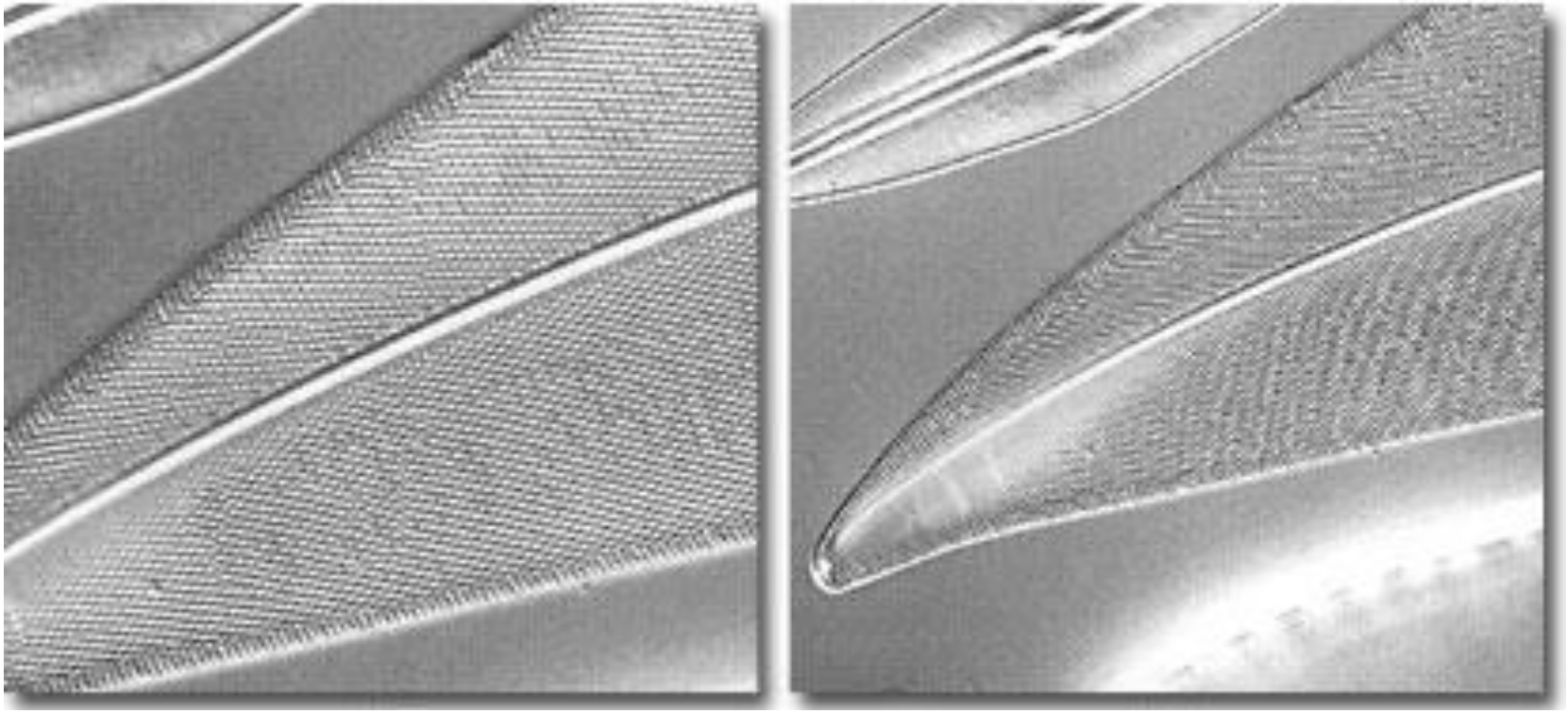


# Resolution and CCDs

- 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



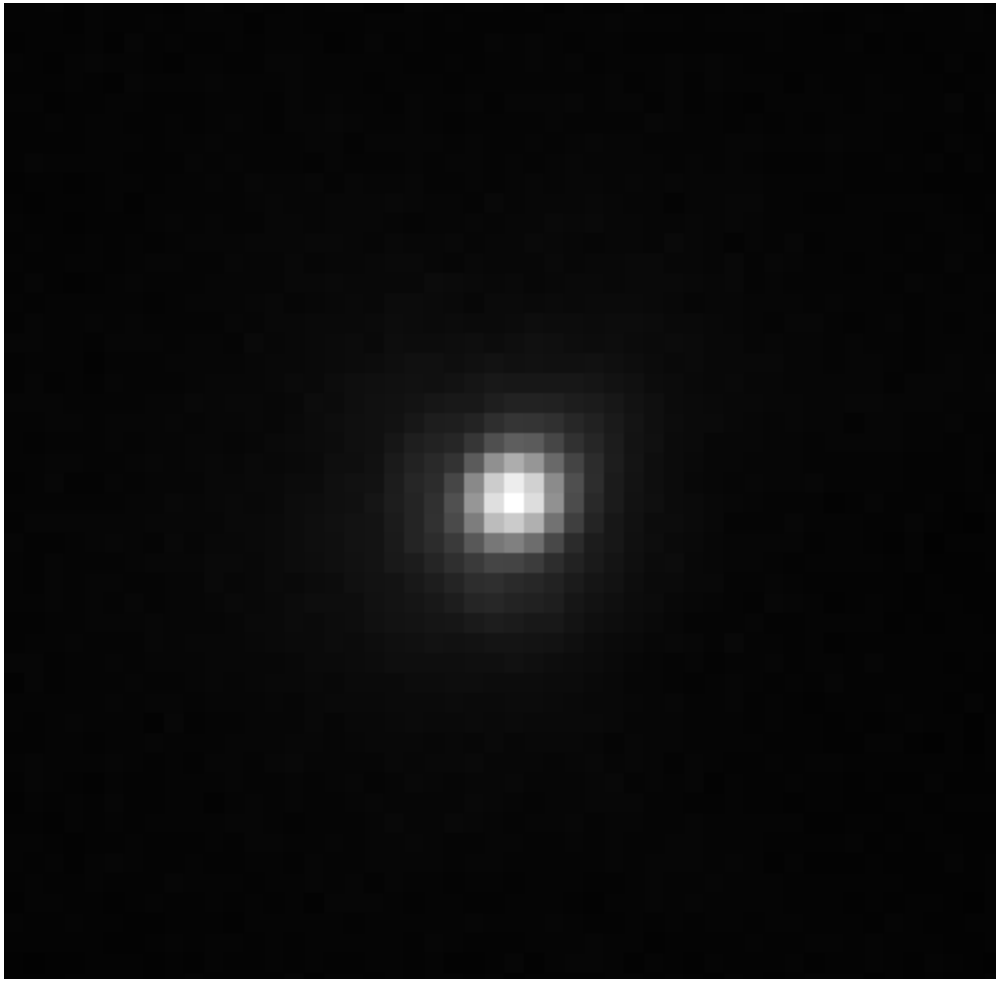
Nyquist sampled

Undersampled

# A resolution-centric view of imaging

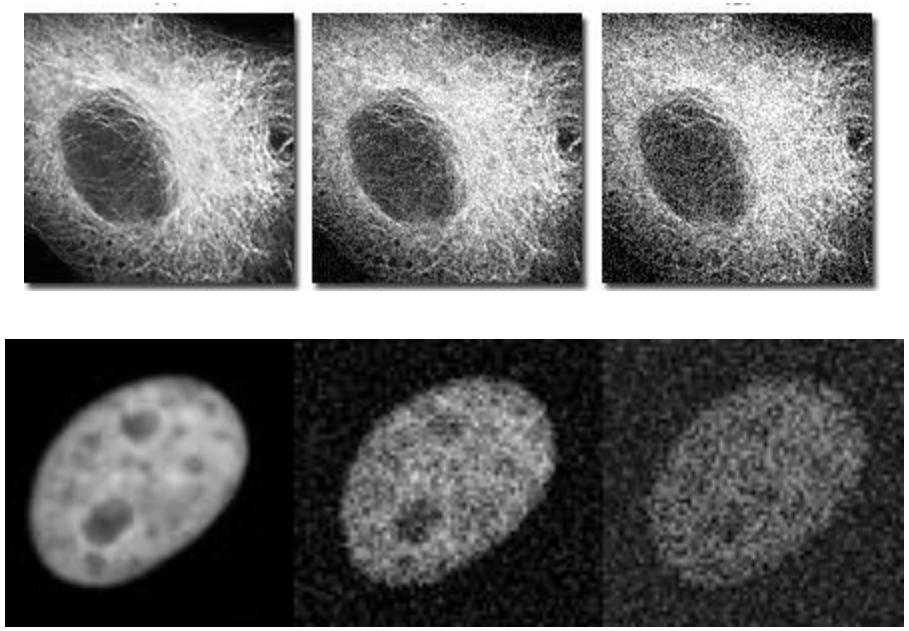
- The objective NA sets the highest resolution you can measure (1.4 NA ~ 220 nm)
- To achieve this resolution, 220 nm in your image must cover 2 pixels
- Choose your magnification to achieve this
- For 6.45 μm pixels, we need a total magnification of 6450/110 = 58.6
- So for 1.4 NA, a 40x lens would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



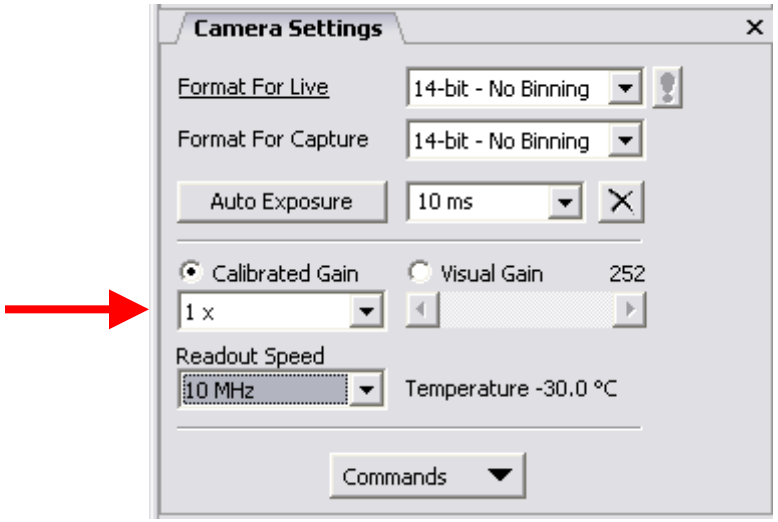
# Noise

- 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 (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 ≠ 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)

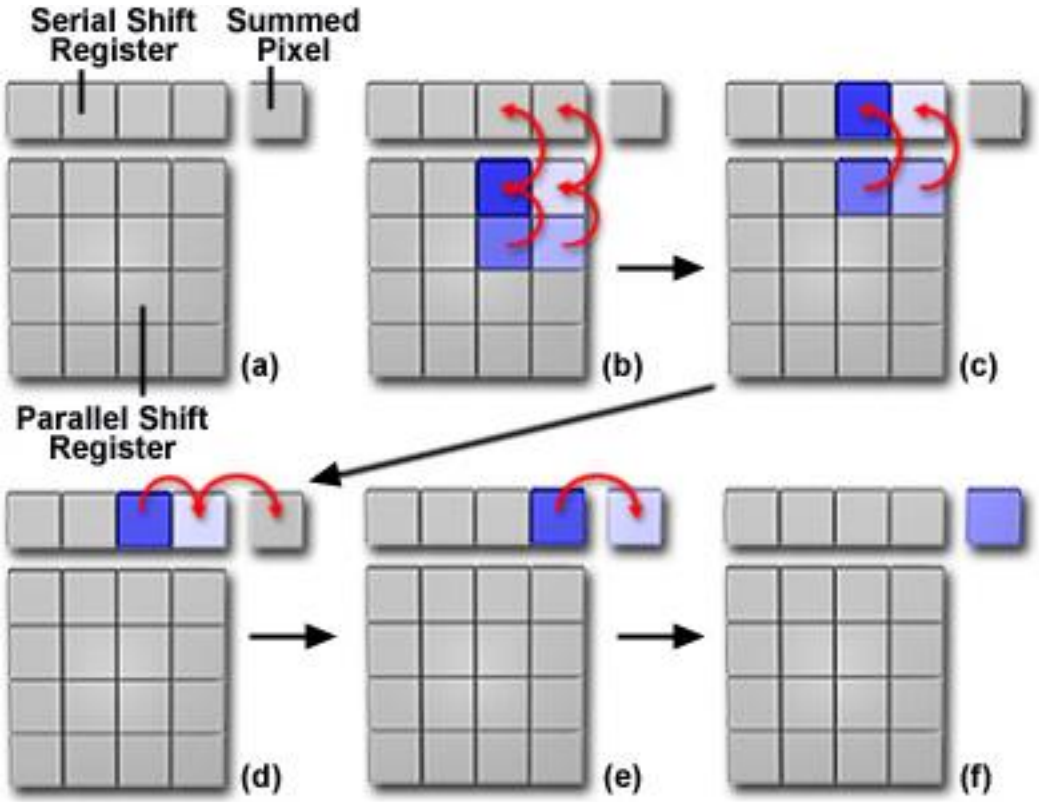
- Signal = # of photons
- Noise = $\sqrt{}$ (read noise$^2$ +(# of photons))

- 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)

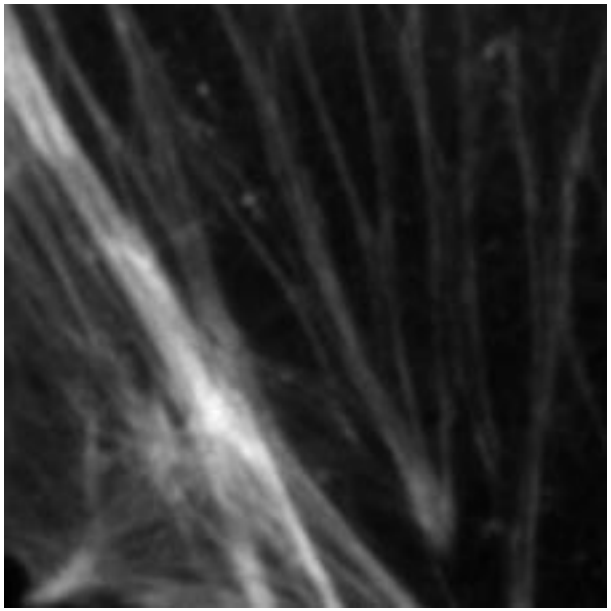
- Read noise dominates whenever
  read noise$^2$ = # of photons

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

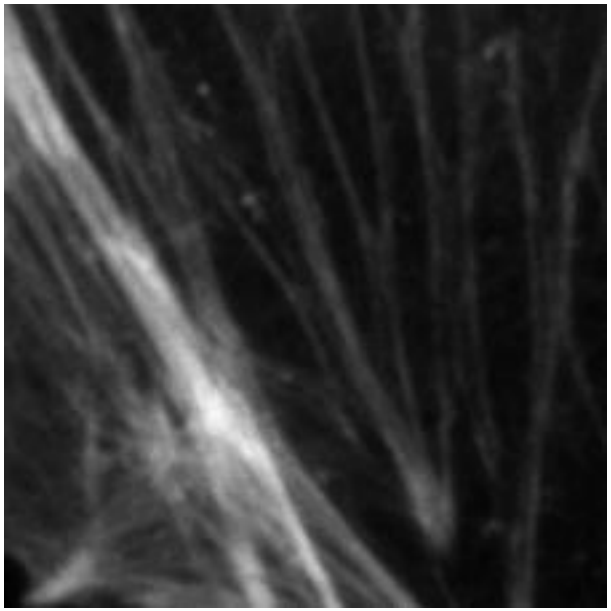
- Full range on Coolsnap HQ2 with 4x gain: 4095 photons

# What does this look like?

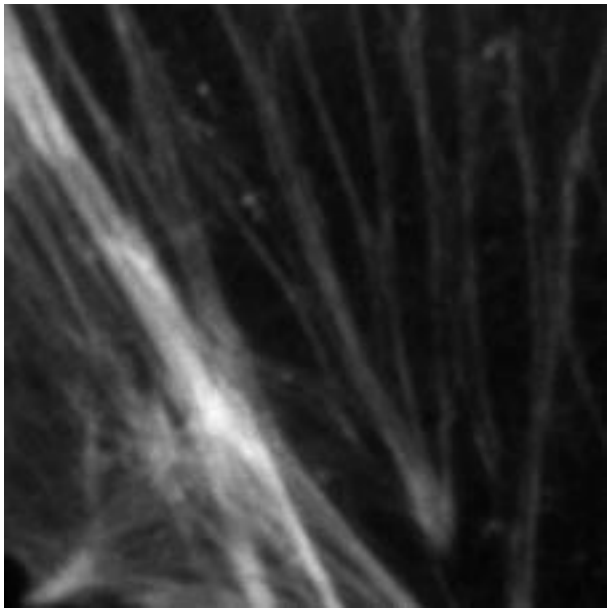
1000 photons / pixel on average; ~5000 in brightest areas



Test image



no read noise

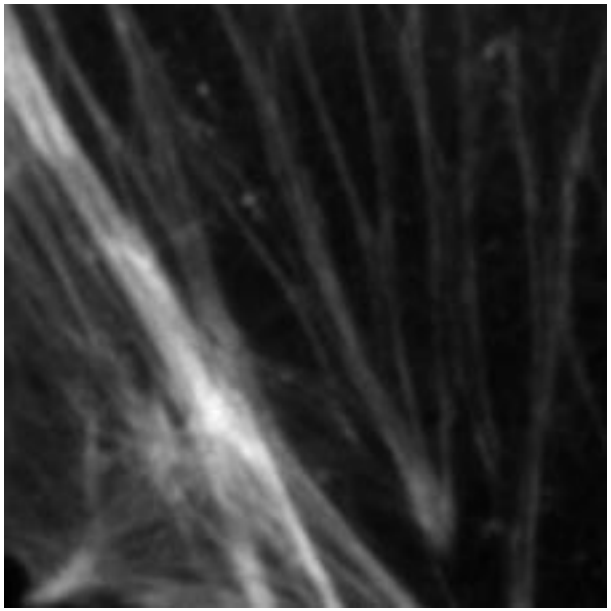


5 $e^-$ read noise

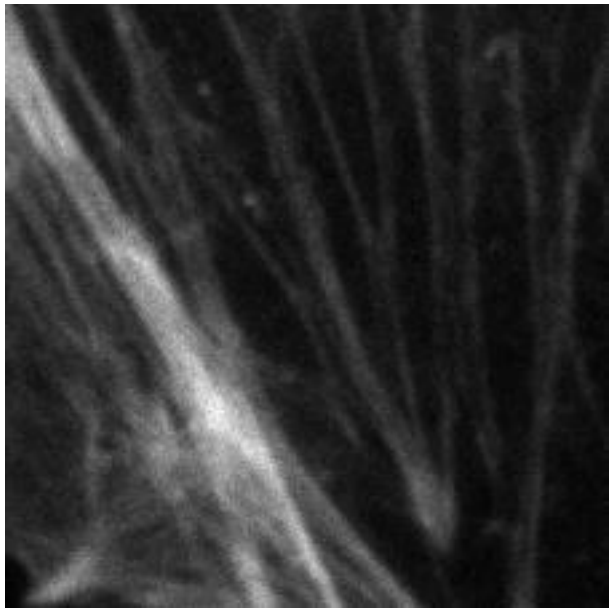
Photon shot noise ~ 6x read noise

# What does this look like?

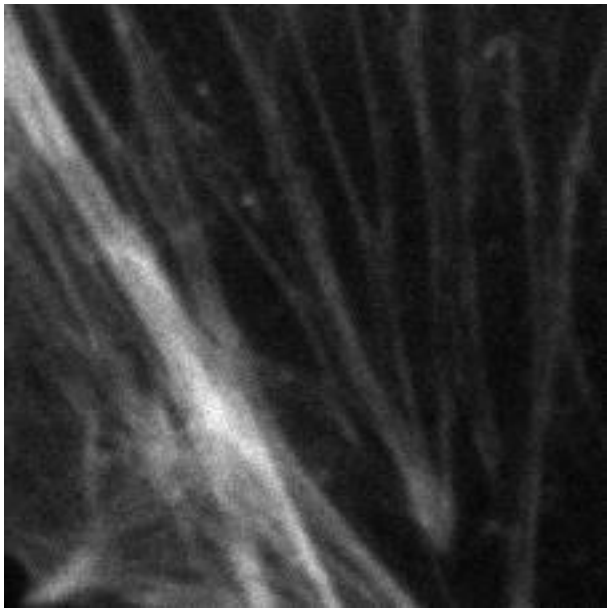
100 photons / pixel on average; ~500 in brightest areas



Test image



no read noise

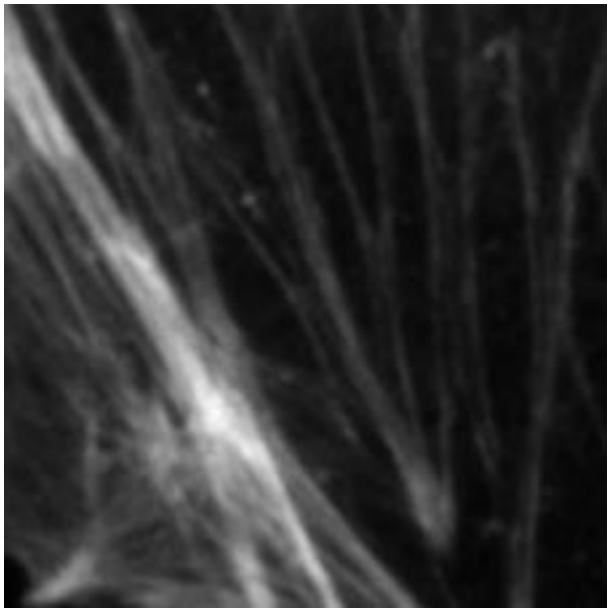


5 $e^-$ read noise

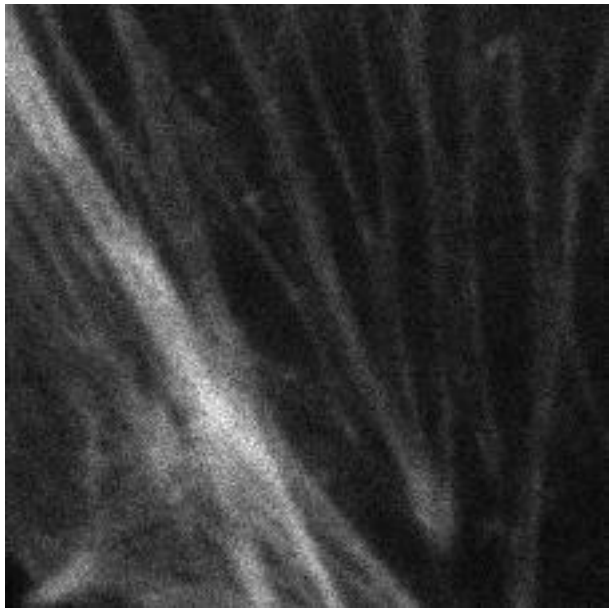
Photon shot noise = 2x read noise

# What does this look like?

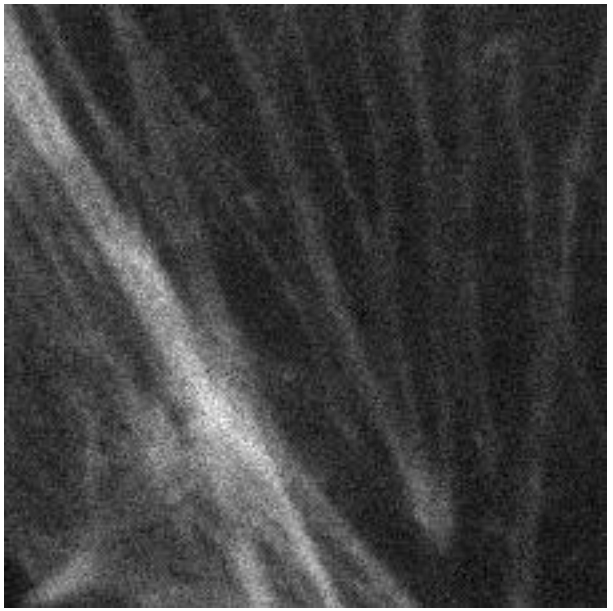
25 photons / pixel on average; ~125 in brightest areas



Test image



no read noise

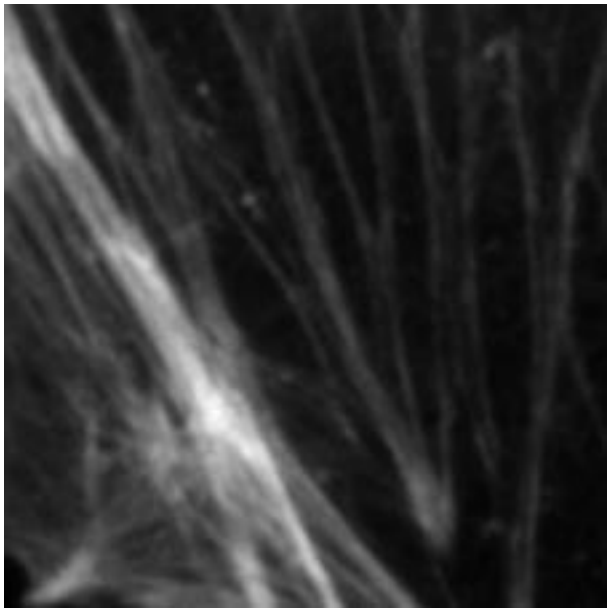


5 $e^-$ read noise

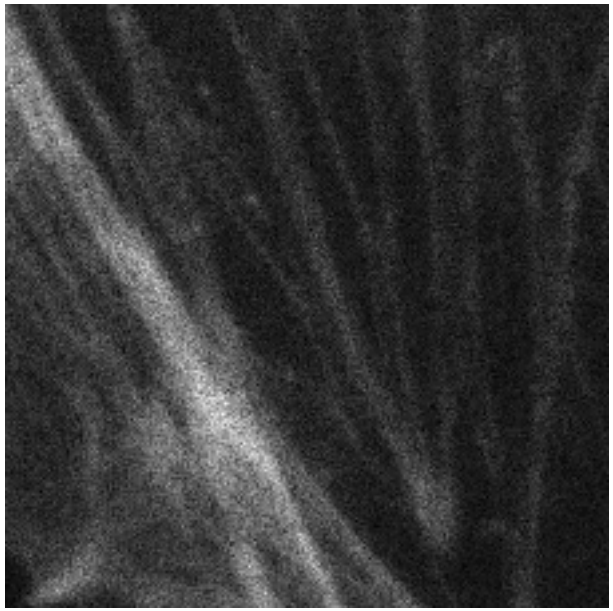
Photon shot noise = read noise

# What does this look like?

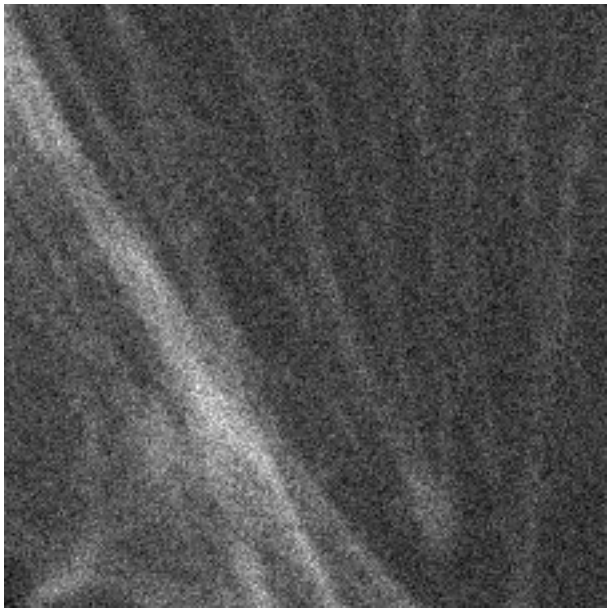
10 photons / pixel on average; ~50 in brightest areas



Test image



no read noise

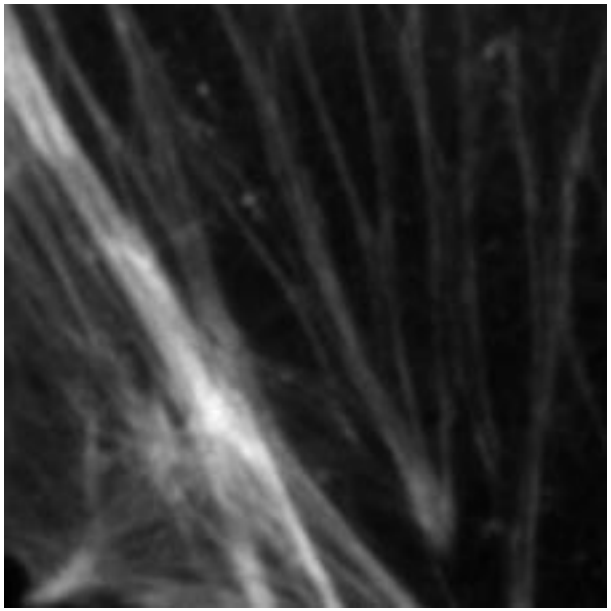


5 $e^-$ read noise

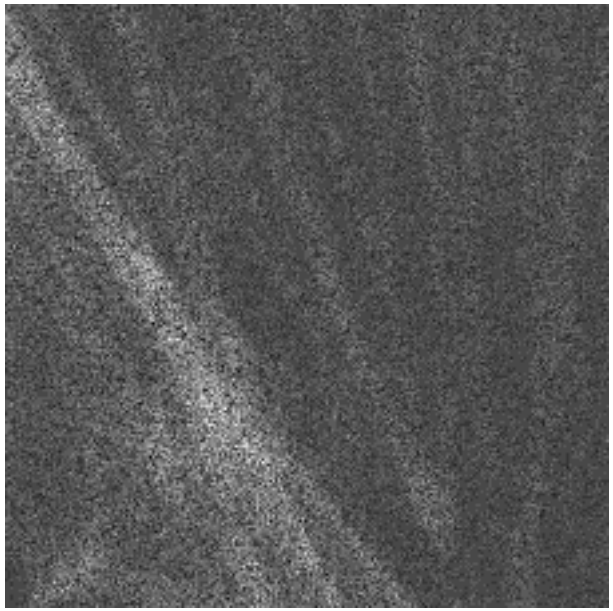
Photon shot noise ~ 2/3 read noise

# What does this look like?

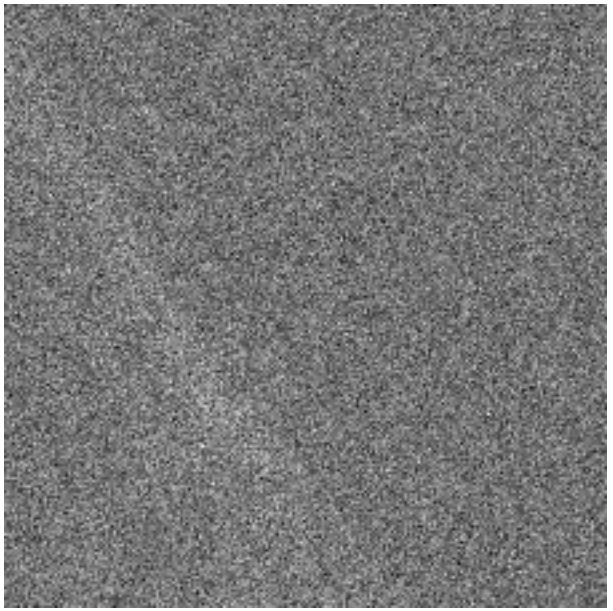
1 photon / pixel on average; ~5 in brightest areas



Test image



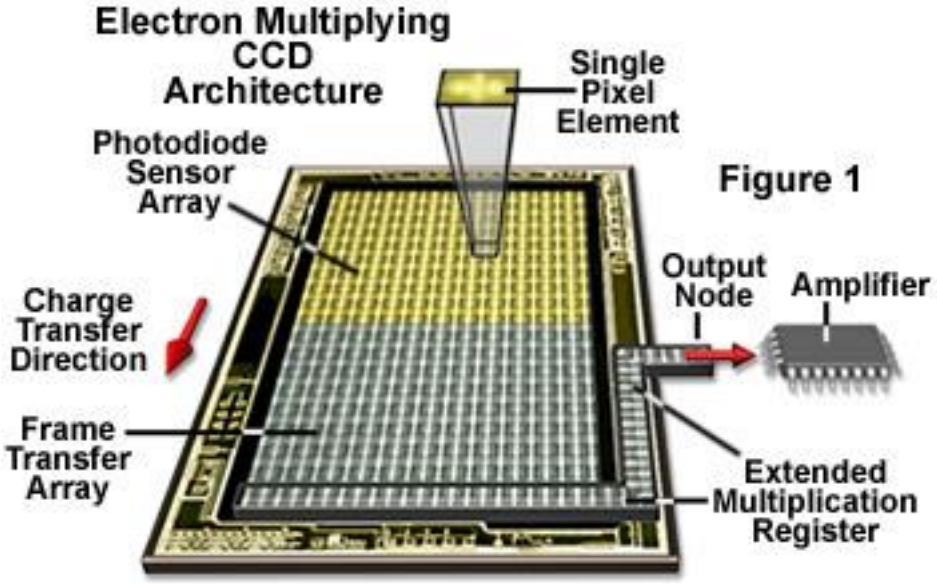
no read noise



5 $e^-$ read noise

Photon shot noise ~ 1/5 read noise

# Beating the read-out noise
# EMCCD



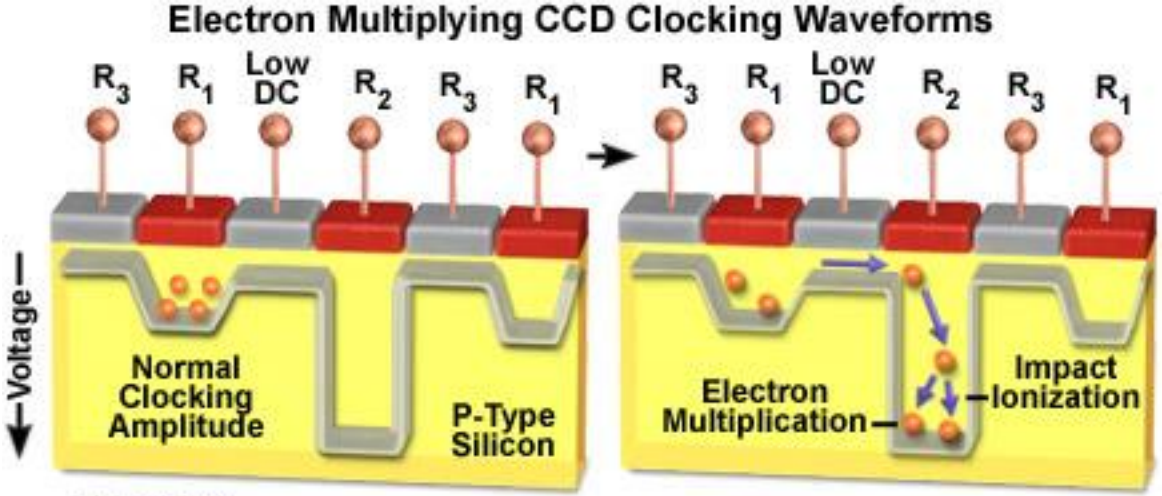



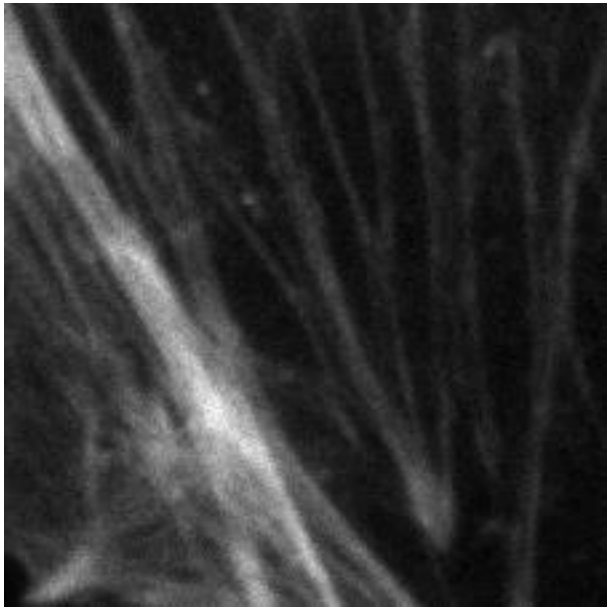

Figure 4

# 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

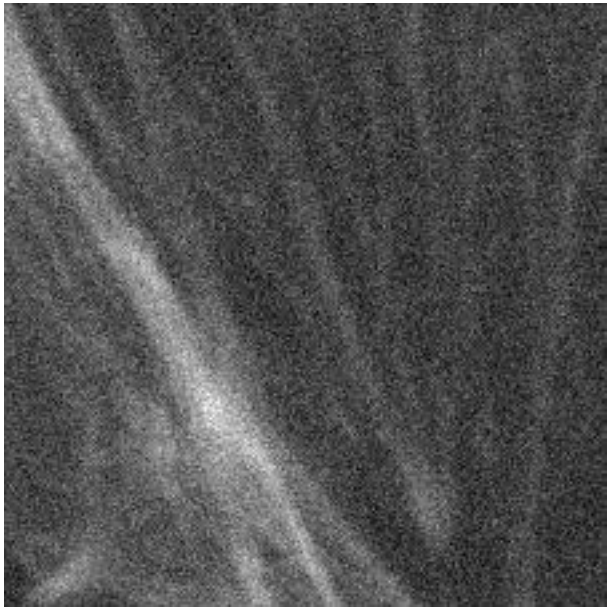
100 photon / pixel on average; ~500 in brightest areas



Slow scan CCD

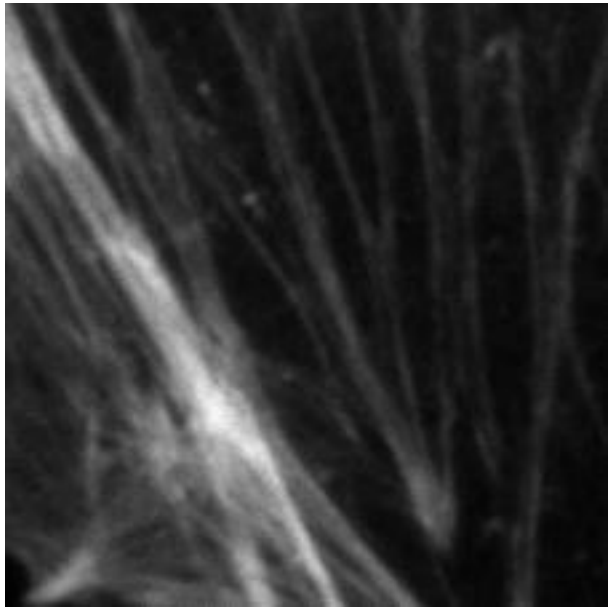
4$e^-$ read noise

(1 sec read time)



Video rate CCD,

50$e^-$ read noise



Video rate EMCCD

50$e^-$ read noise

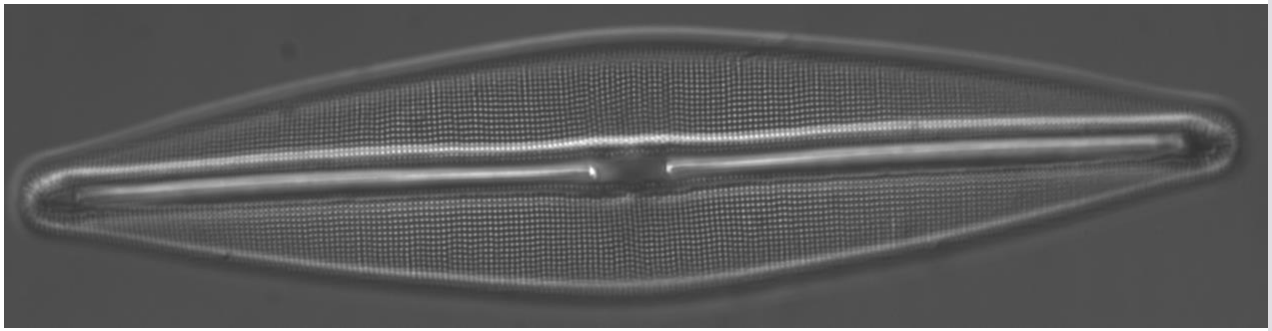
200x gain

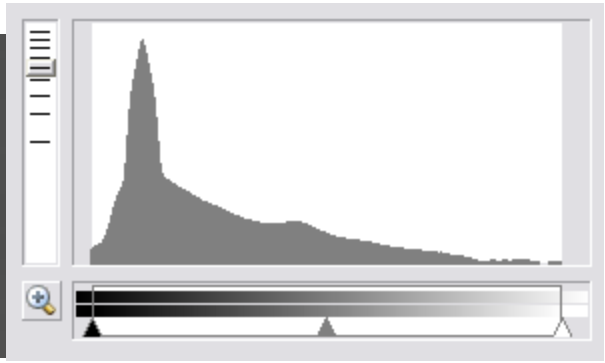
# How many intensity levels can you distinguish?

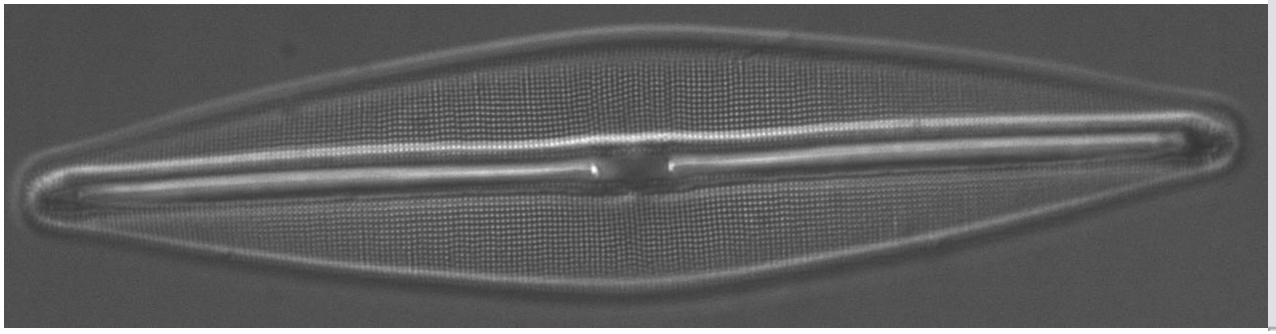
- Full well capacity (16 000 $e^-$)
- Readout noise: 5e-

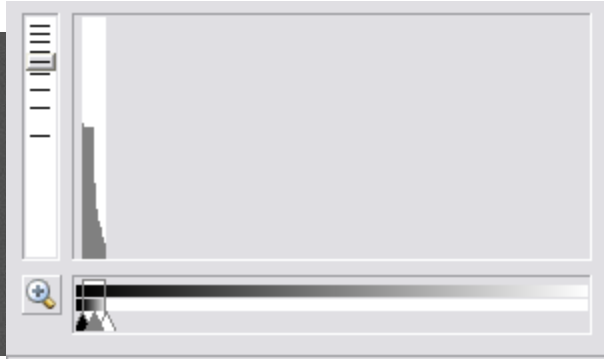
- Dynamic range:
  - FWC/readout noise: 3200
  - 0.9 * FWC / (3 * readout noise) = 960
- (Human eye ~ 100)

# Check your histogram

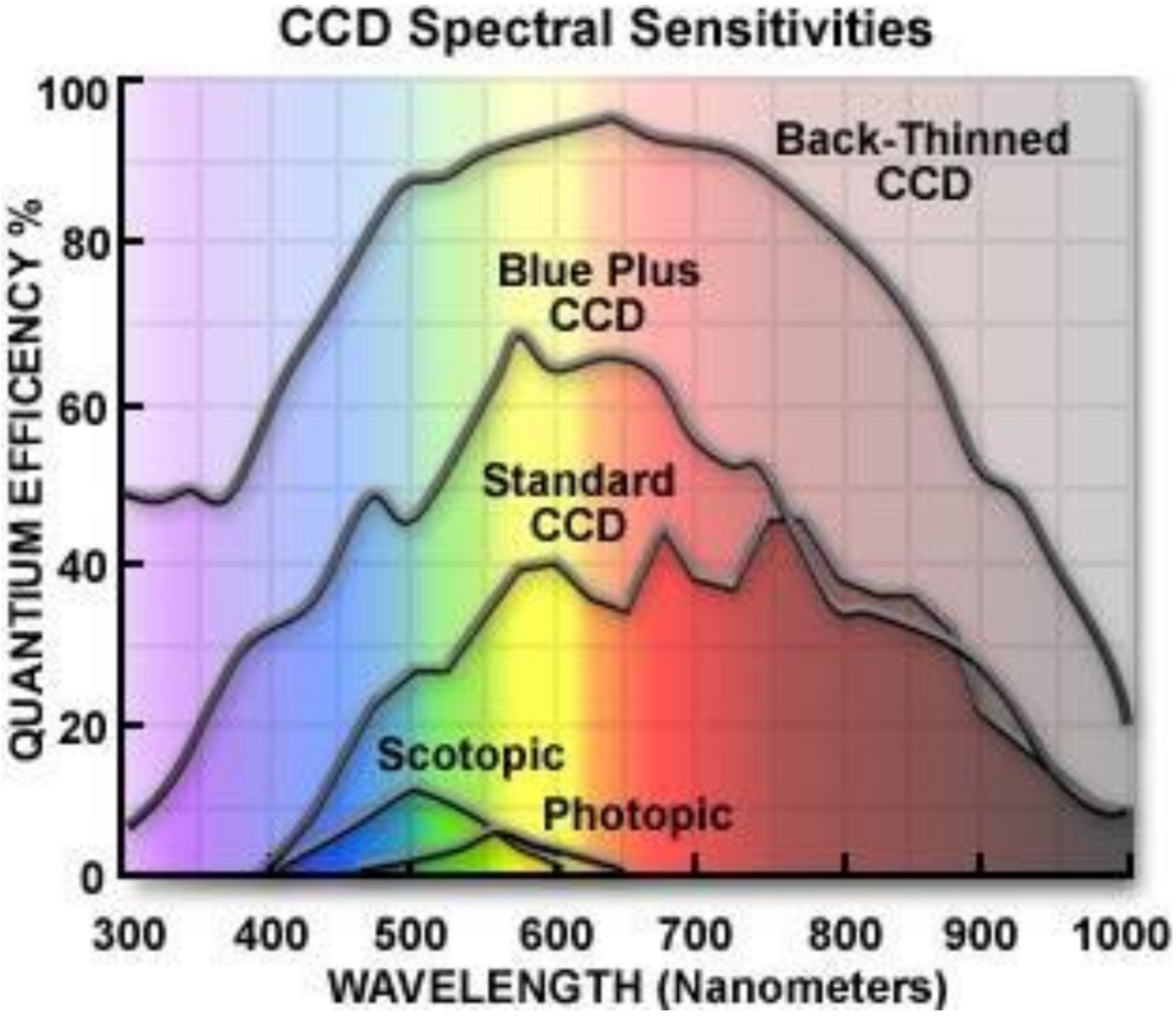




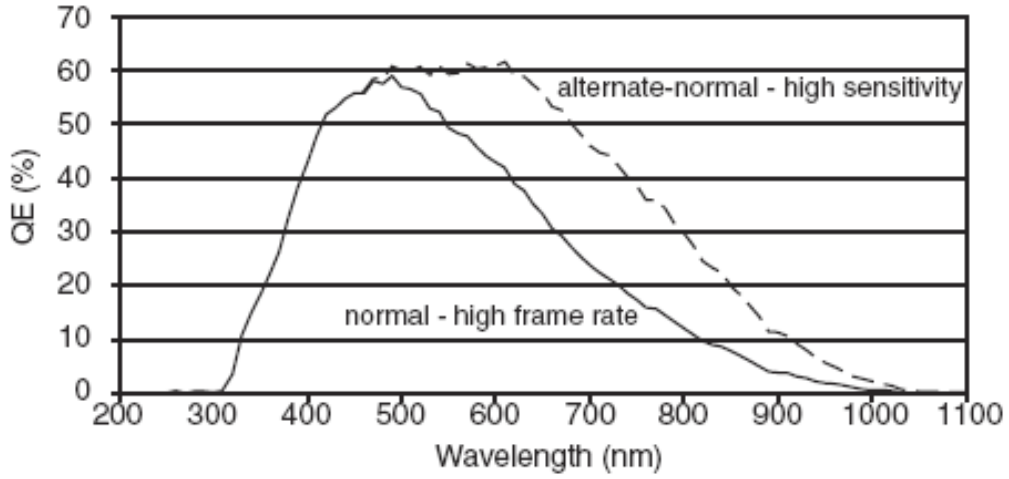




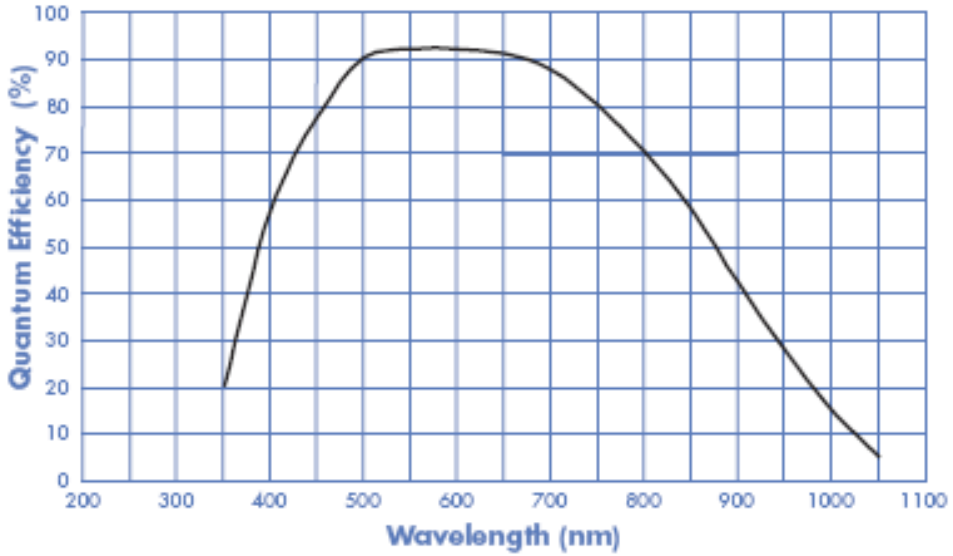
# Quantum Efficiencies



# Quantum efficiency




HQ2

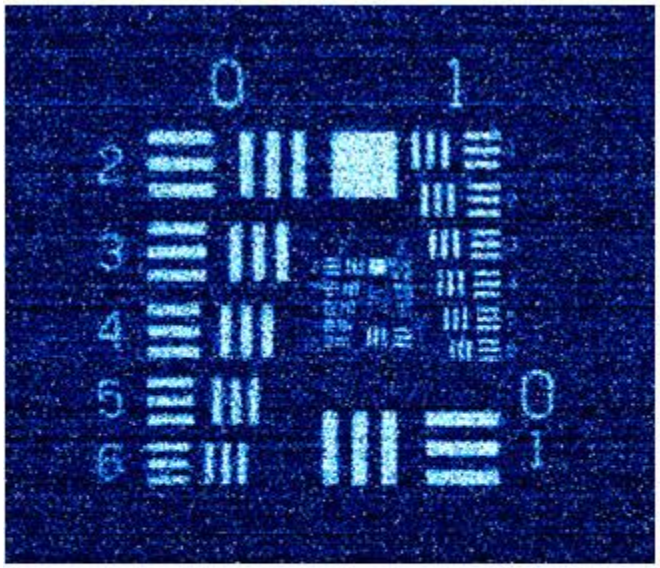



Cascade II

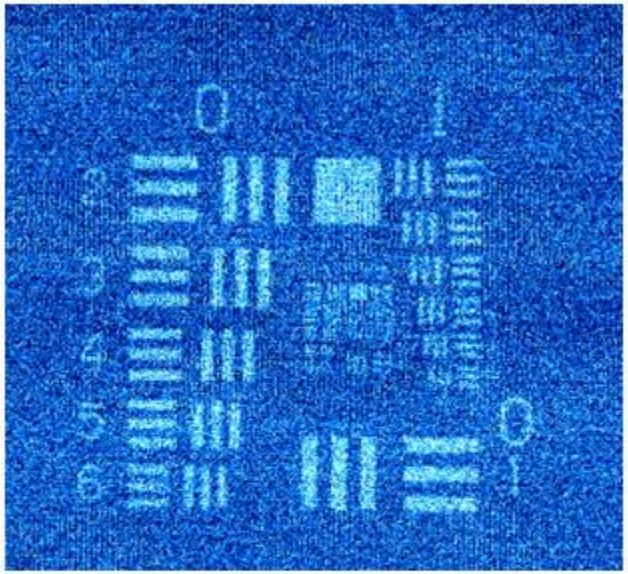
# Forthcoming technology: sCMOS

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

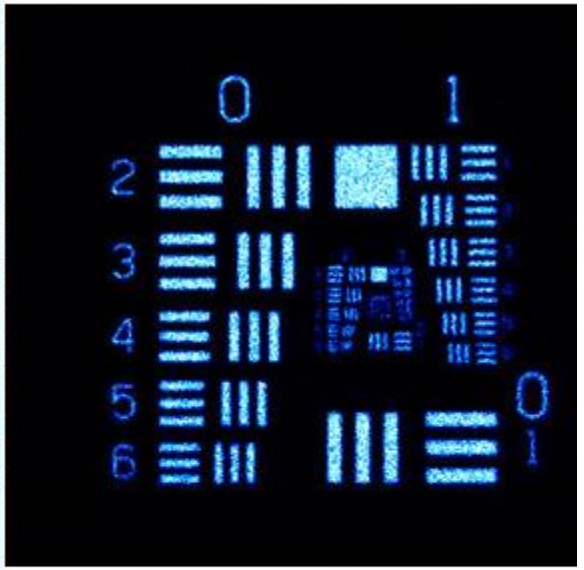
# Forthcoming technology: sCMOS



sCMOS (*1.5 e⁻ noise*)



Interline CCD (*5 e⁻ noise*)



Back-illuminated
EMCCD (*<1 e⁻ noise*)

*www.scmos.com*

# 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