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WHAT'S BEHIND THE PICTURE?

{ Choosing and using scientific cameras }

KEITH BENNETT, PH.D. | HAMAMATSU PHOTONICS K.K. | FOCUS ON MICROSCOPY | 04.13.2014

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Choosing and Using Scientific Cameras

1 The image problem

2 Think in photons

3 Real cameras are not perfect

4 Know thyself

5 The Living Image: Case Studies

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The Image Problem…

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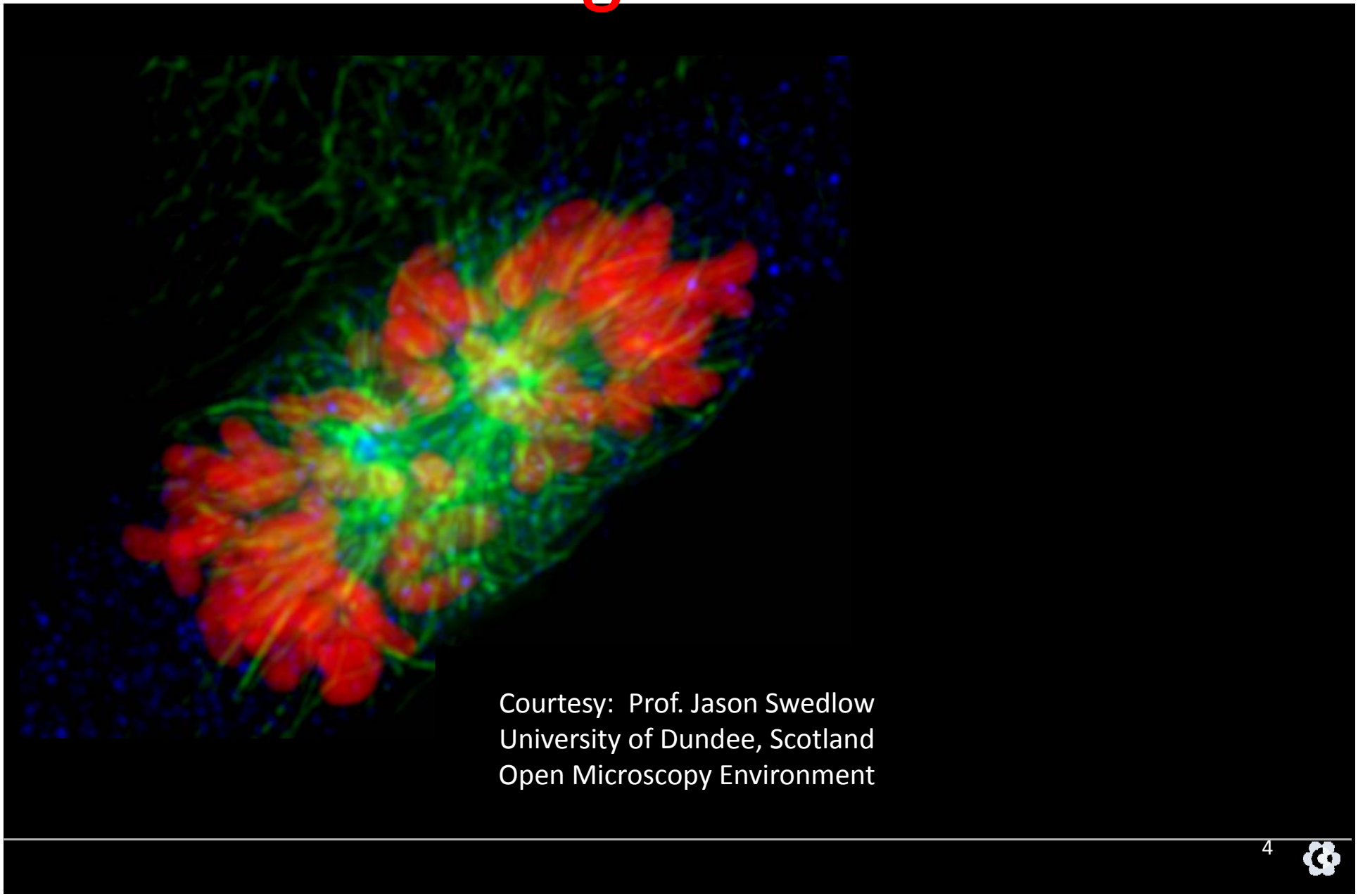
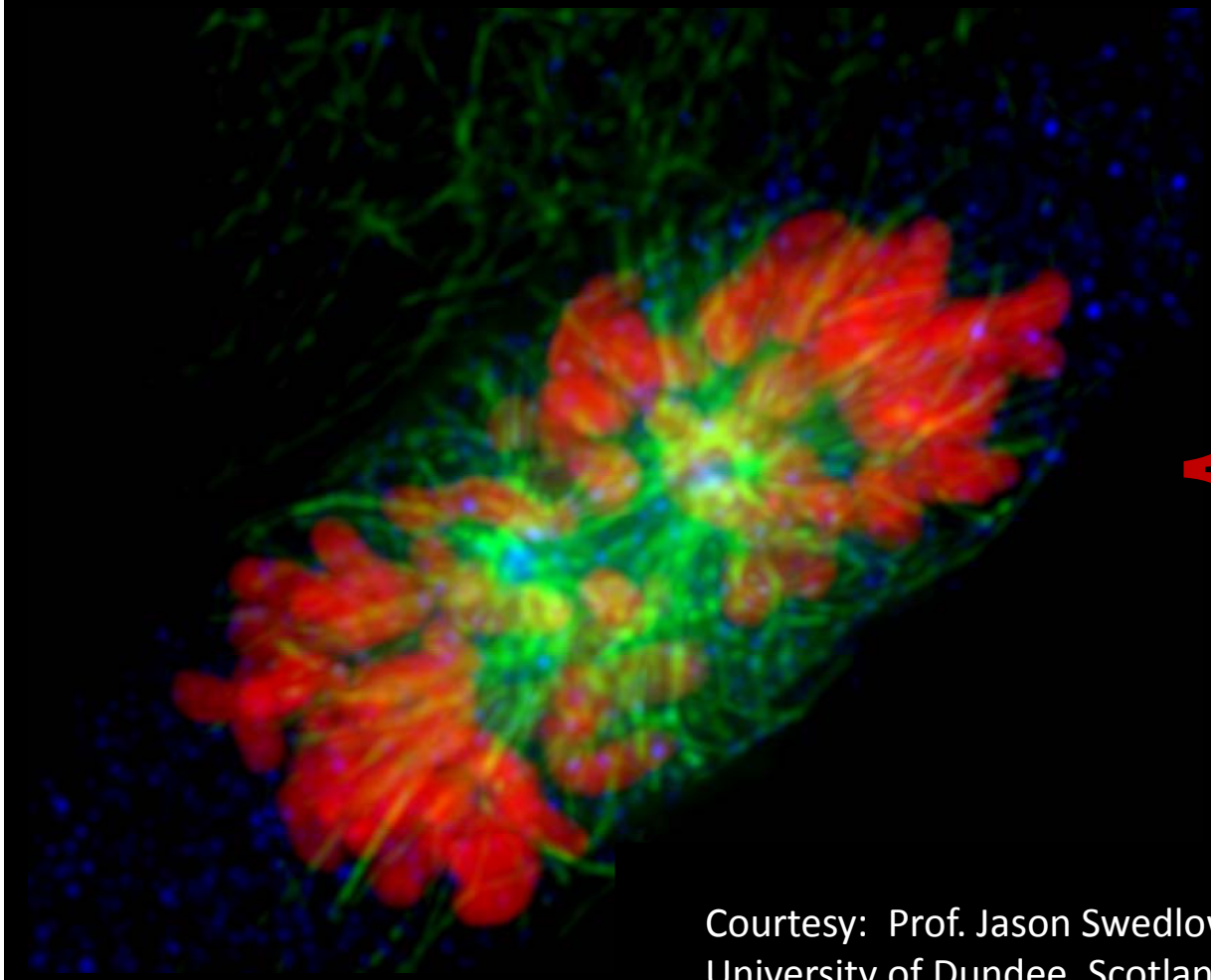


Courtesy: Prof. Jason Swedlow
University of Dundee, Scotland
Open Microscopy Environment

The Image Problem…



A pretty picture?

A measurement?

A resource?

A reference?

Courtesy: Prof. Jason Swedlow
University of Dundee, Scotland
Open Microscopy Environment

{1}

THE IMAGE PROBLEM

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LOOK CAREFULLY

- Eyes can be fooled
  - Not good at quantifying greys
  - Not objective
  - Emphasizes patterns and colors
  - Viewing environment
- Screens are not capable of displaying full bit depth
- Image display can (and should be!) manipulated for on screen viewing

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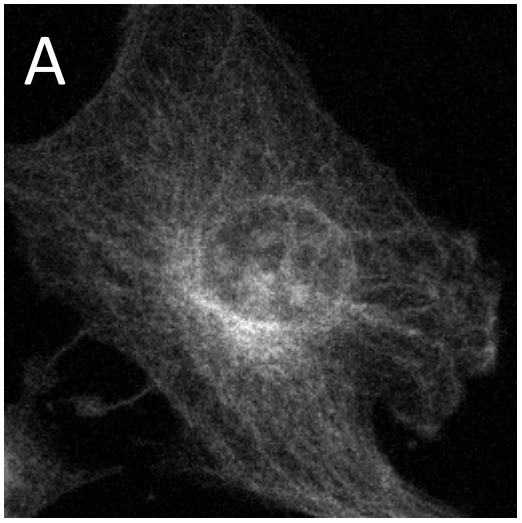
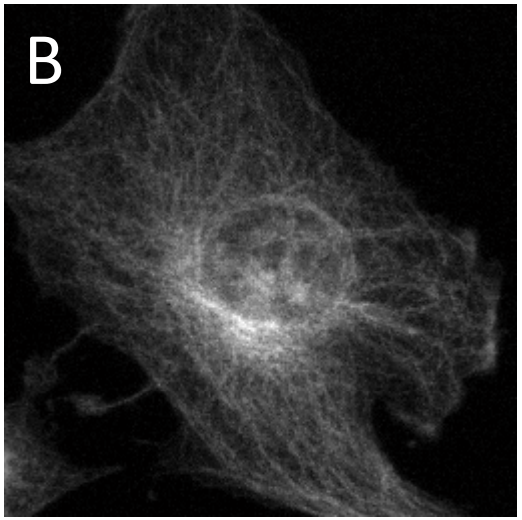
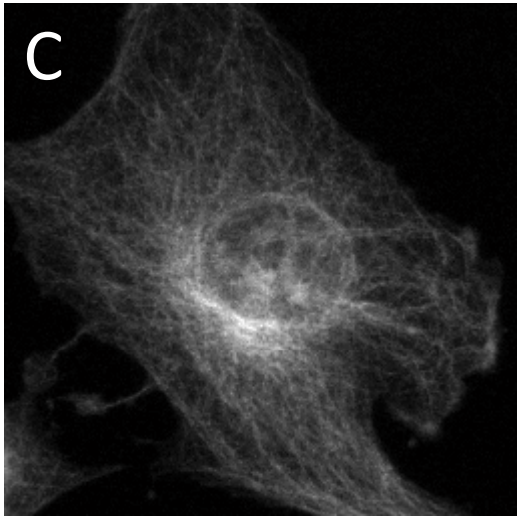
{1} THREE IDENTICAL IMAGES?







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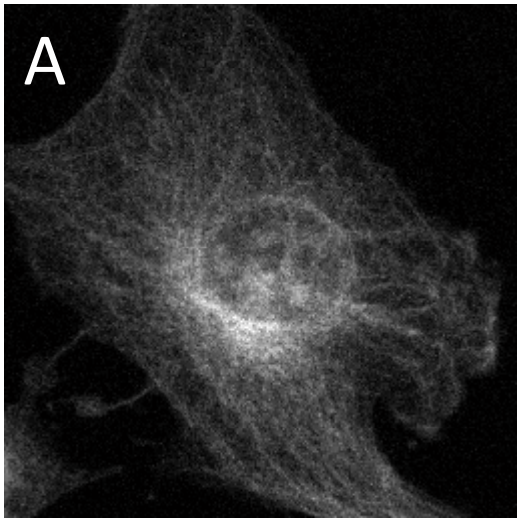
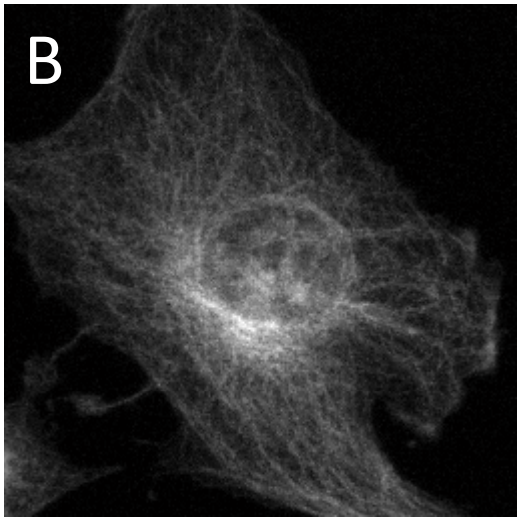
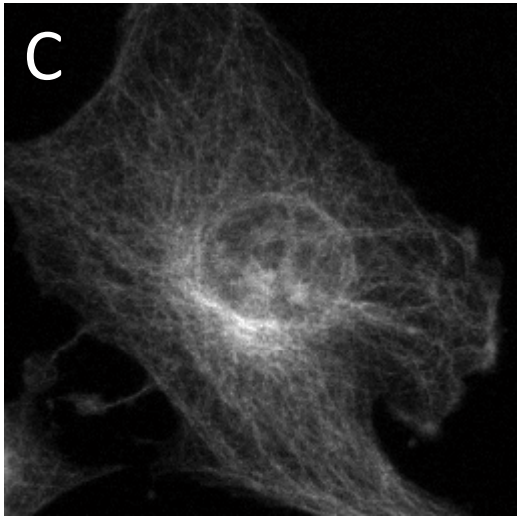
{1} THREE IDENTICALLY DISPLAYED IMAGES!



200 photons



500 photons



1000 photons

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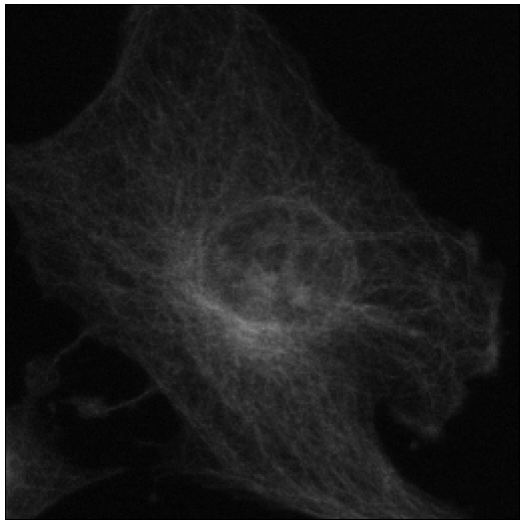
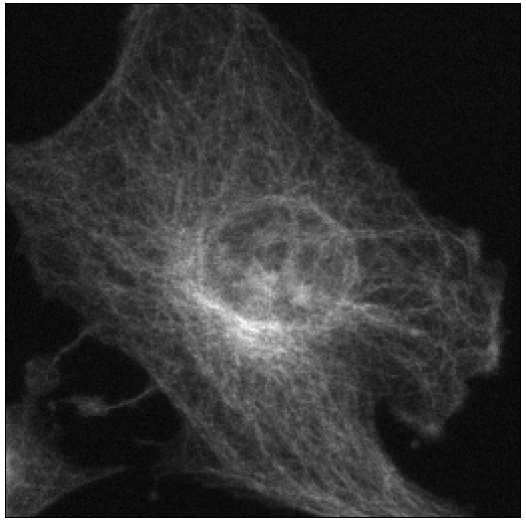
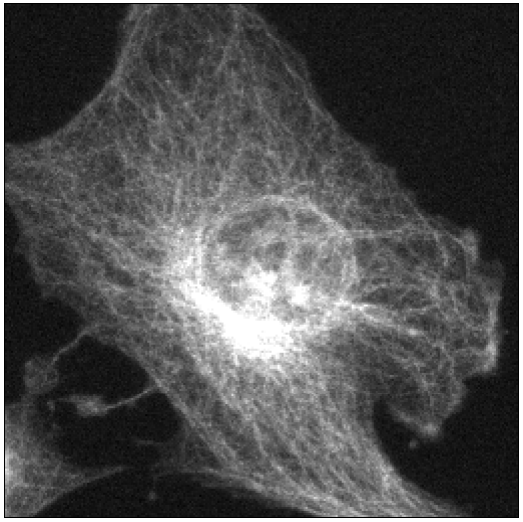
{1} Three Different Intensities?







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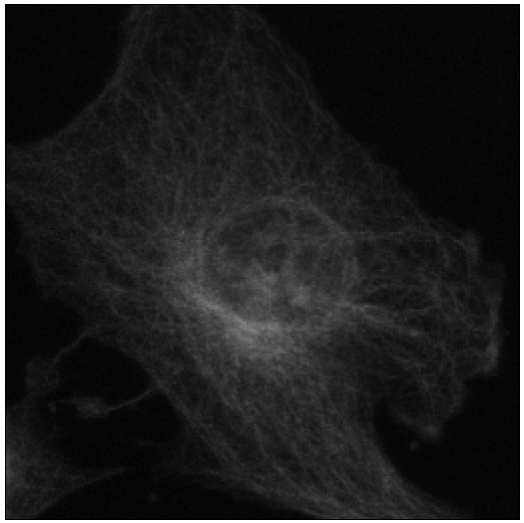
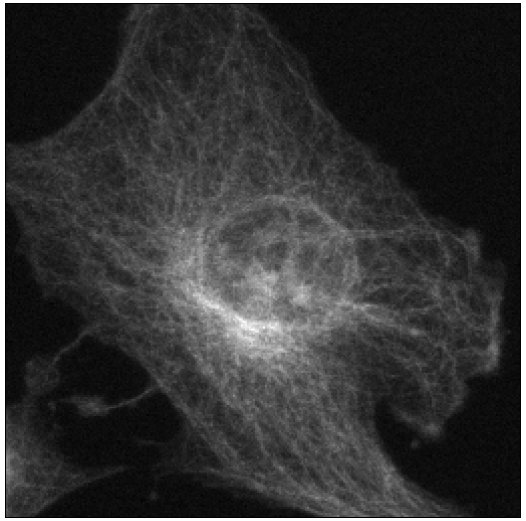
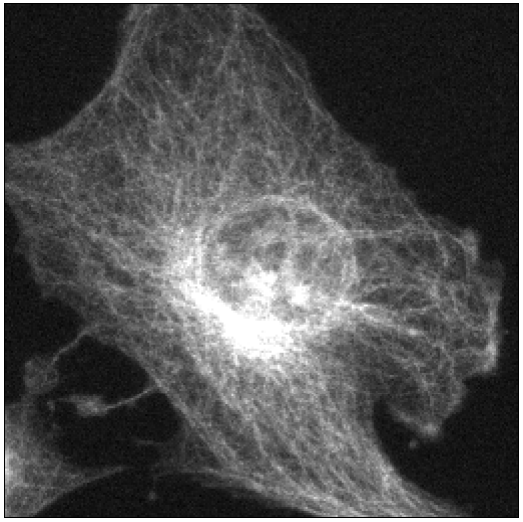
{1} THREE DIFFERENT DISPLAYS OF THE SAME INTENSITY!



1000 photons



1000 photons



1000 photons

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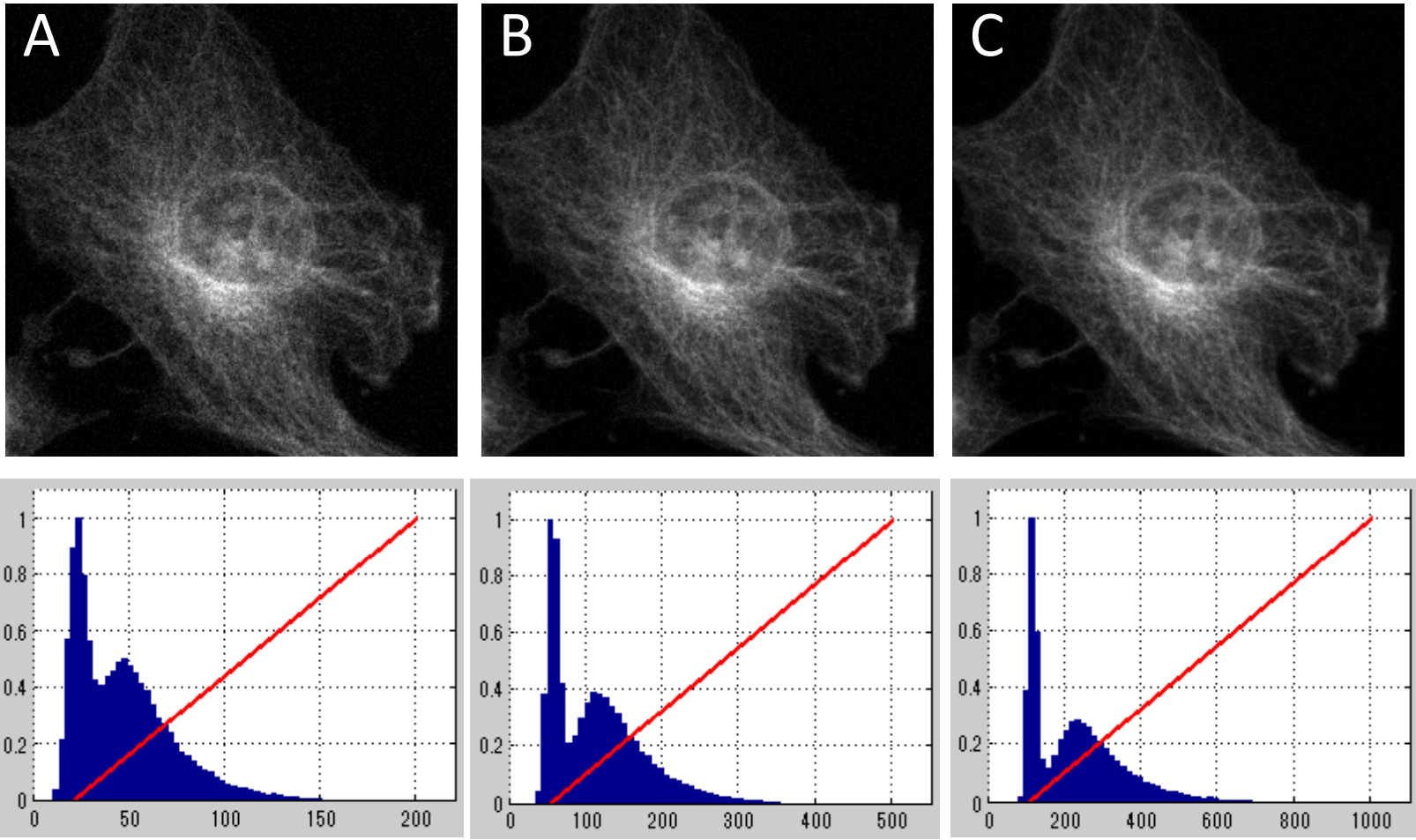
{1} Histogram and Area Statistics



	A	B	C
Peak (photons)	200	500	1000
Mean (photons)	47.0	117.4	234.7

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Choosing and Using Scientific Cameras

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{2}

THINKING IN PHOTONS

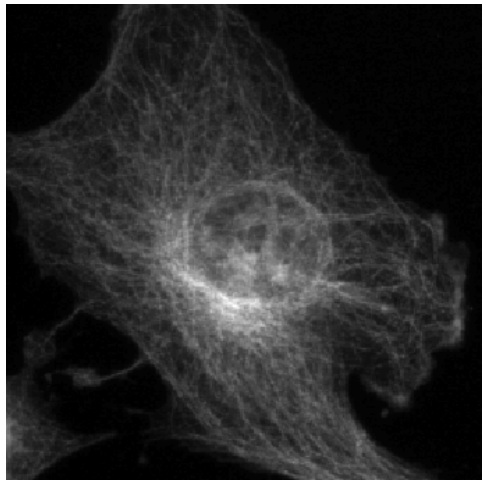
IS THINKING IN PHOTONS REALLY NECESSARY?

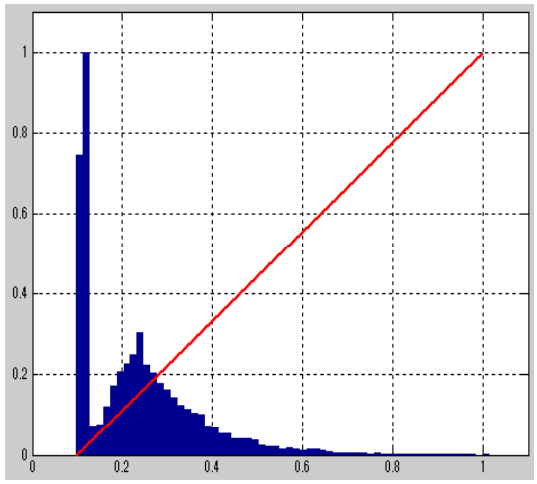
- Aren't ADU's or grey levels good enough?

SCIENTIFIC CAMERAS SHOULD MEASURE **PHOTONS**

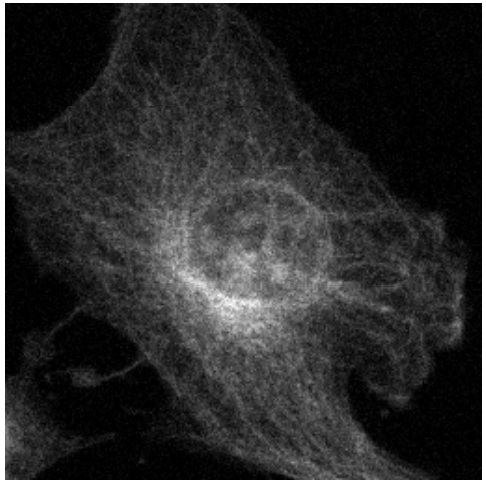
{2}

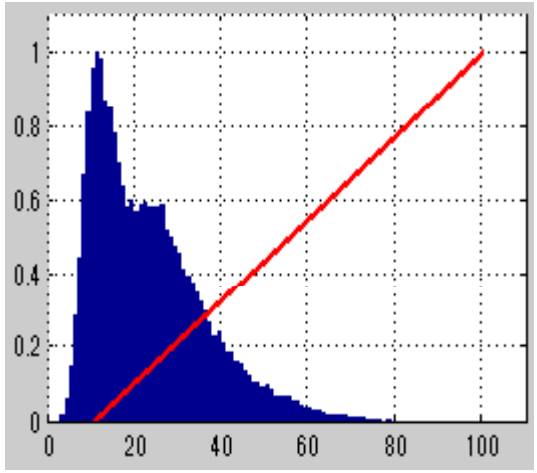
PHOTONS REALLY MATTER





Truth





100 photons peak

Looks similar, but

- The histogram is different
- Information is different
- Quantification different
- Lower image contrast

Perfect camera

Background = Peak/10

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REMEMBER SHOT NOISE

$$N_S = \sqrt{S}$$

{2}

THINKING IN PHOTONS

WHAT'S **LIMITING** MY SCIENCE?

- The information in an image is limited by the number of photons.
- A perfect camera does **not** produce a perfect image, especially if photons are limited.
- The minimum number of photons **needed** depends upon the object imaged, resolution and measurement requirements (i.e. your experiment).

{2}

PHOTONS REALLY MATTER

ARE YOU CONVINCED?

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{3} Real Cameras: Thinking in Photons

How does this make Me a **Better** Microscopist?

- Makes comparisons among cameras meaningful. (ADUs are arbitrary)
- Brings relevance to your data.
- Knowing the number of photons and contrast in sample is key to picking the correct camera.

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{3} Real Cameras

Is Thinking in Photons Really Necessary?

- Can't we figure everything out from a camera specs (QE and electronic specs)?

[Hint: Maybe, but there's a better way]

Scientific Cameras Should Measure Photons

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{3} REAL CAMERAS ARE NOT PERFECT

THE WHAT AND HOW

- The Gap
- Electron multiplying CCDs (EMCCDs)
- Simulations comparing perfect to product by spec
- All pixels are not created equal
- Actual product measurements
- Camera noise & visualization

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Why is a
camera manufacturer
proclaiming
that
cameras are not perfect?

Because NO camera is perfect

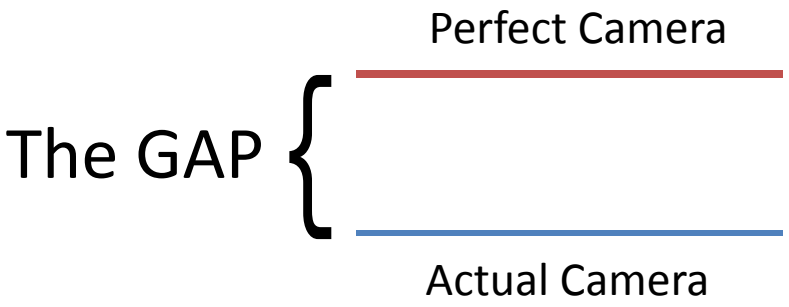
&

Because understanding why

matters to your science

{3} WHAT IS THE GAP?

The difference between the performance of an actual camera and a theoretically perfect camera



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{3} UNDERSTANDING **WHY** THERE IS A GAP ENABLES:

- Appropriate camera selection
- Optimized camera usage
- Optimized experimental design
- More reliable data analysis

} Better Results

{3} The Gap Depends on:

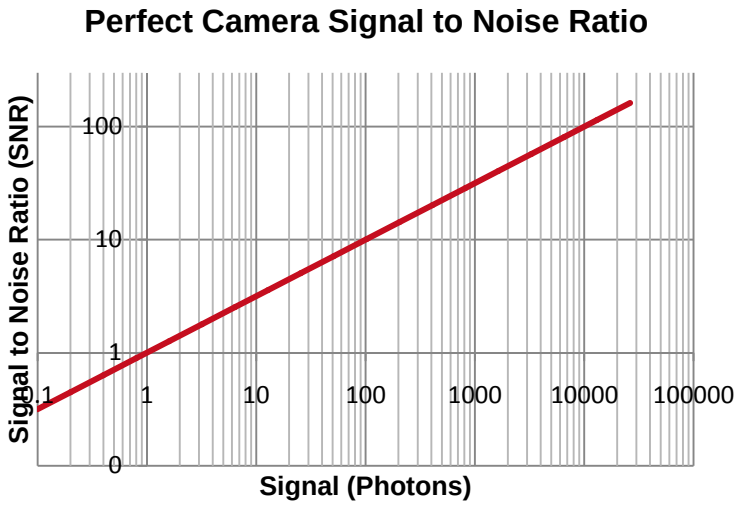
1. Sensor technology
   - CCD
   - EMCCD
   - sCMOS
2. Camera specs
   - Quantum Efficiency
   - Camera Noise
     - Read noise
     - Excess noise
     - Photo-response non-uniformity (PRNU)
3. Input photon level
   - Ultra low light
   - Low Light
   - Intermediate
   - High

{3} The (Hypothetical) Perfect Camera

100% QE	Every photon is converted into one electron
0 e- read noise	Every electron is digitized exactly as expected every time
0% fixed pattern noise	Every pixel and amplifier perform identically and predictably

In a perfect camera, the SNR of a single pixel is limited only by the physics of photon statistics… i.e. shot noise.

$$SNR = \sqrt{S}$$



{3}

REAL CAMERAS ARE NOT PERFECT

ImagEM X2
EMCCD: Electron Multiplying CCD

ORCA-Flash4.0 V2
Scientific CMOS Camera

ORCA-R2
Cooled Interline CCD

{3} BASIC SPECS: COMPARED

	CCD	EMCCD	CMOS
Camera Name	ORCA-R2	ImagEM x2	ORCA Flash4.0 V2
QE (550 nm)	70 %	90 %	72 %
Read Noise Single Frame rms (e-)	6	< 0.5 (M = 200)	1.5
Full Well Capacity (e-)	18,000	Gain dependent	30,000
Dynamic Range	3000:1	Gain dependent	20,000:1
Bit Depth	16	16	16
Max pixel rate (Mps)	13	18	420
Pixel Size (μm)	6.45 x 6.45	16 x 16	6.5 x 6.5
Pixel Number	1024 x 1344	512 x 512	2048 x 2048

{3} AMPLIFIERS

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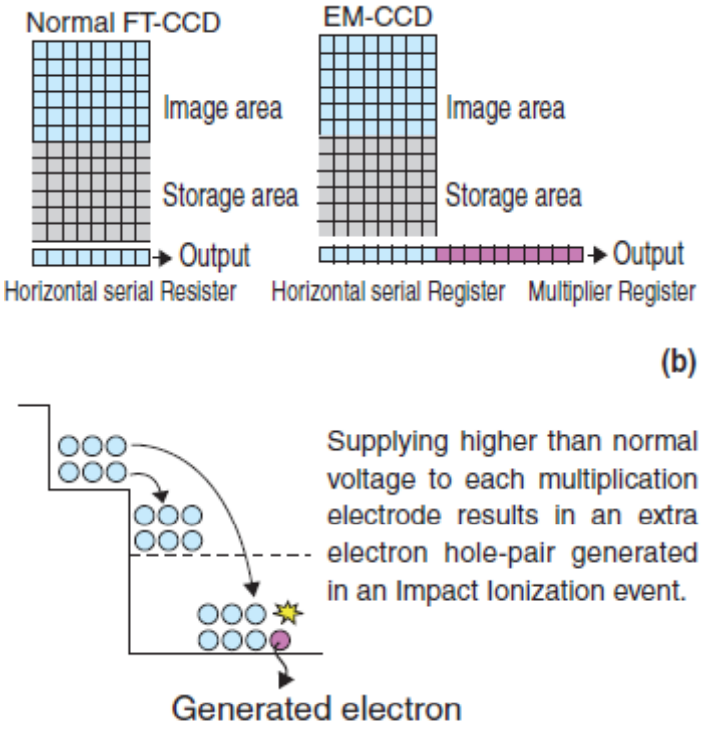
Important differences { CCD and sCMOS
EMCCD

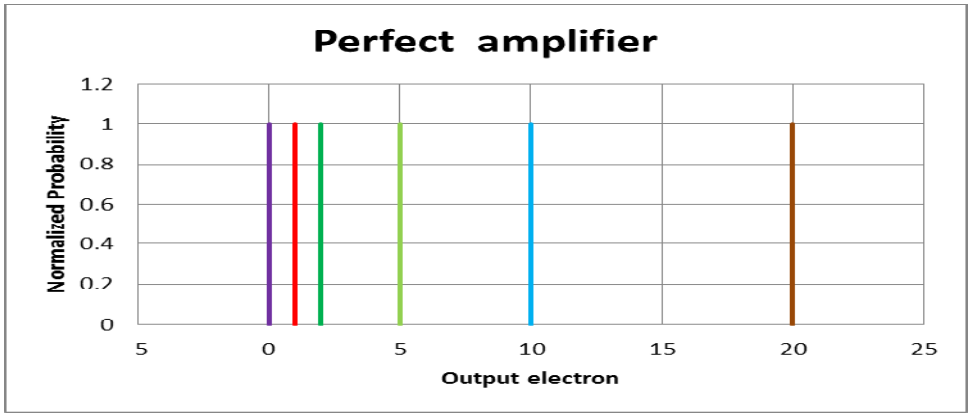
{3} Electron Multiplying CCDs (EMCCDs)

- A type of CCD: Frame transfer and back-thinned for increased QE
- Frame transfer requires ~ 100μs
- Serial devices where each pixel's charge is read out one at a time
- High voltage gain register on sensor for on-chip amplification.
- Option to read out through EM circuitry or non-EM circuit (normal CCD mode)



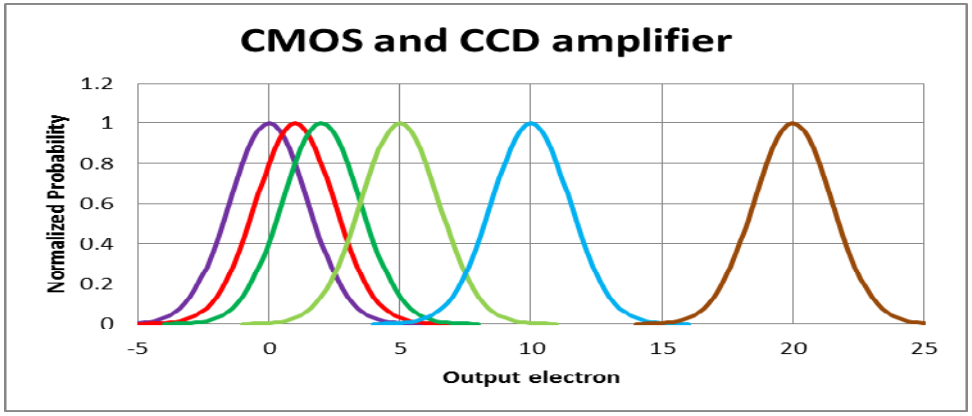
{ EMCCD architecture }

{3} CMOS AND CCD AMPLIFIER NOISE



Output an exact multiple of the input

No noise broadening



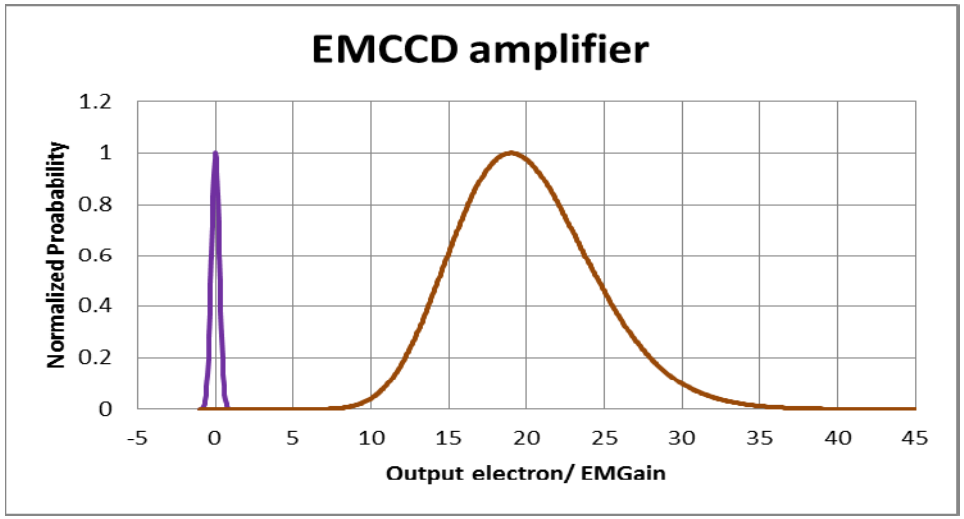
Output is a multiple of the input

“Read noise” broadening

Width independent of signal level

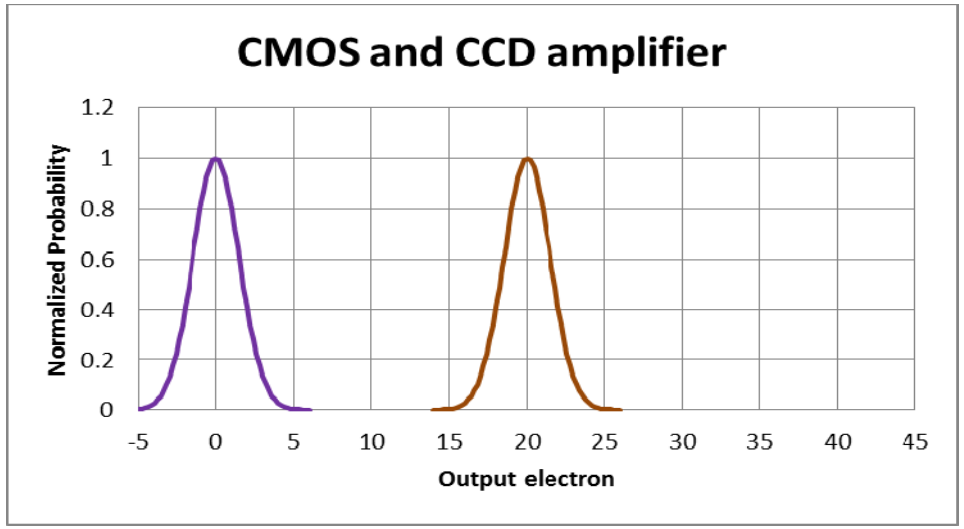
CMOS read noise: 1.5 e- rms

{3} EMCCD AMPLIFIER NOISE DEPENDS ON SIGNAL



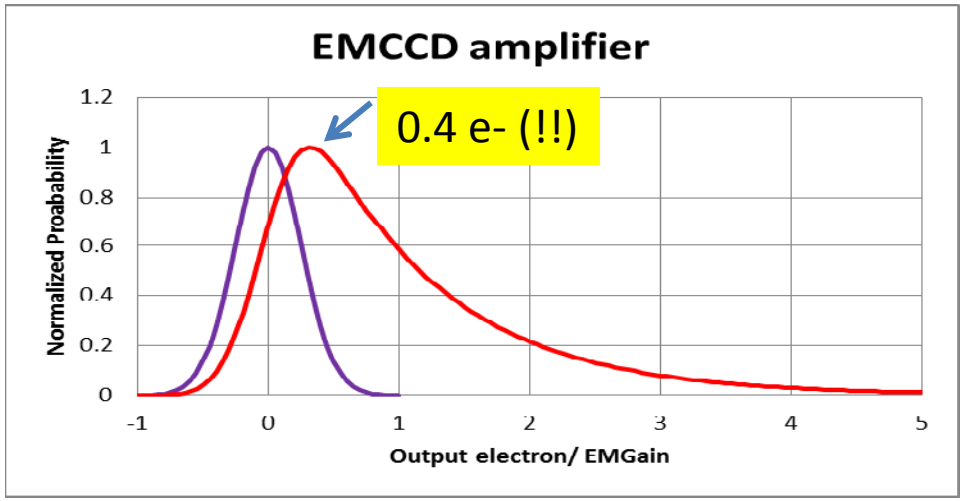
No electron:
- **Very small noise**
- **beautiful blacks**

Signal:
- **Broad (excess noise)**
- **Long tail: larger apparent contrast**



Signal independent
- **No excess noise**
- **Short tail**

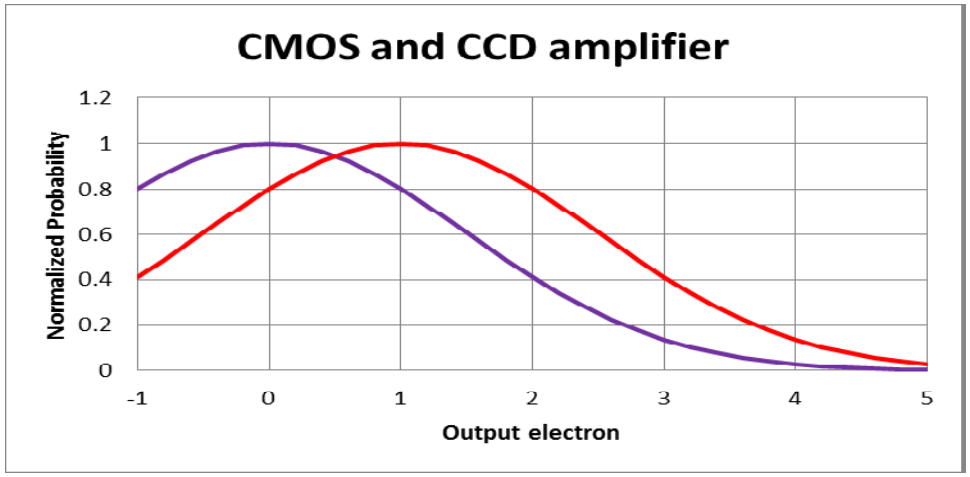
{3} EMCCDs "DETECT" SINGLE PHOTONS, BUT



Peak of 1e- output is ~0.4e-!

Signal < (some) noise

Long tail



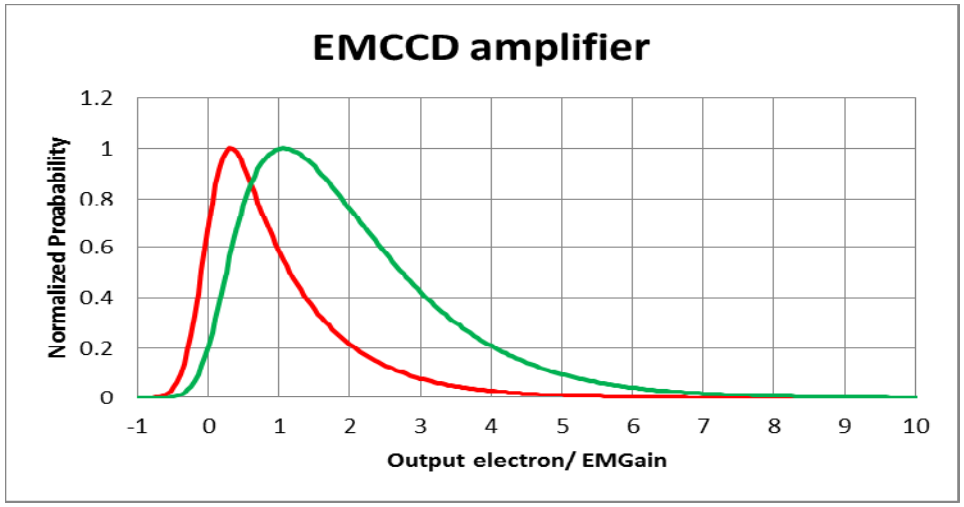
Symmetric distribution, with noise extending ~±2 σ (3 e-) from mean.

Significant overlap

Quantization of ADC not included

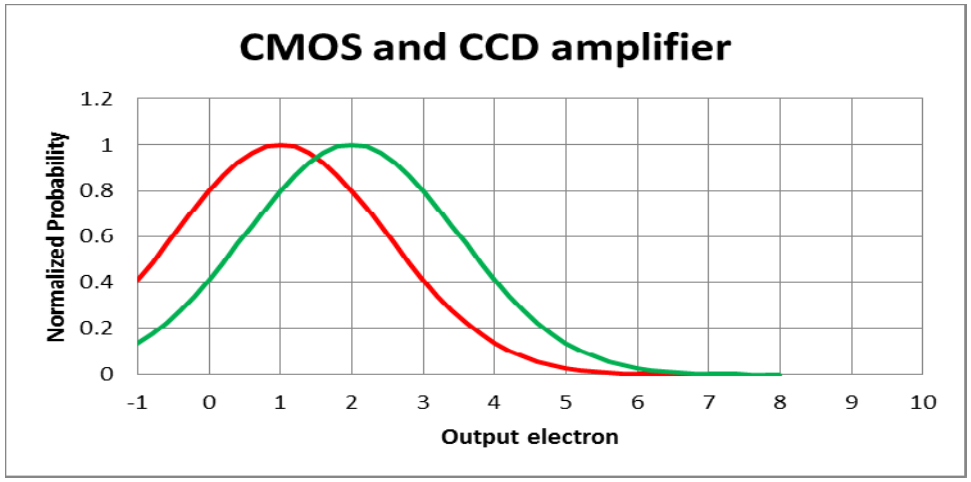
{3}

EMCCDs CAN'T COUNT



Outputs from $1e^-$ and $2e^-$ overlap.

Peak output of $2e^-$ input is ~ $1e^-$



CMOS not so good either at very low light

$2e^-$ input, CMOS tail is shorter than EMCCD

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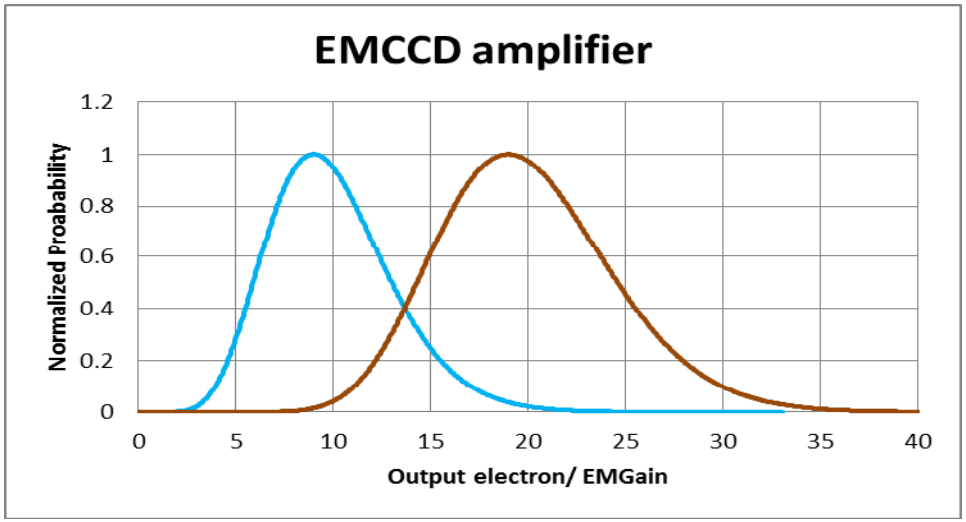
{3} EMCCD: SIGNAL DEPENDENT NOISE

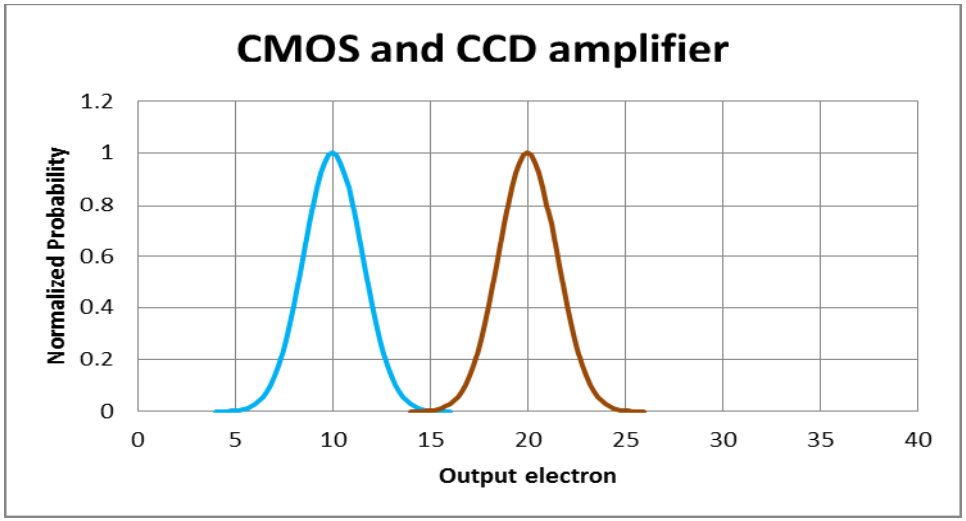


Most probable output < mean.

Very long tail

σ^2 = signal

Lots of overlap: $10e^-$ & $20e^-$



Most probable output = mean

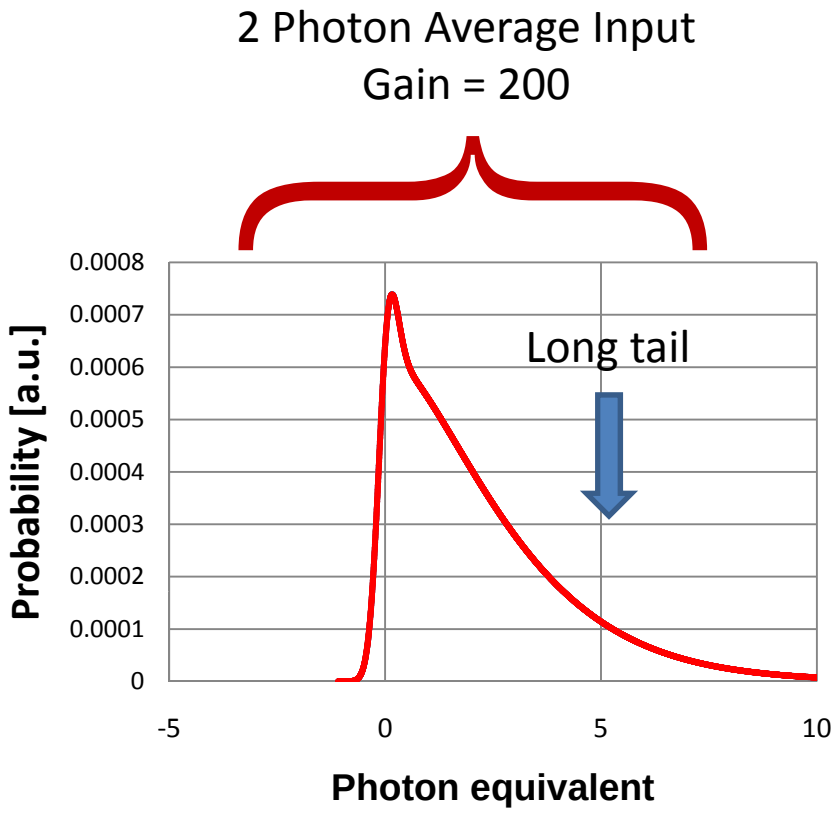
Short tail

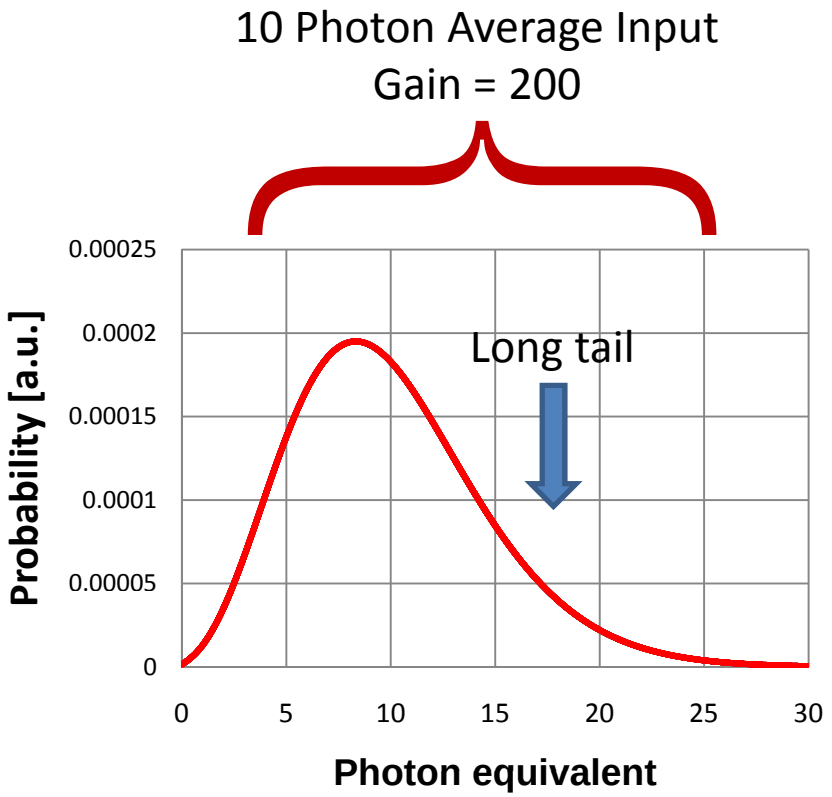
σ^2 = 1.5 e-

CMOS clearly better

{3} EMCCD OUTPUT INCLUDING PHOTON SHOT NOISE

In simulated probability distribution functions for EMCCD, the output at high gain is **not** Poisson due to the electron multiplication process!





{3} EMCCD vs. CMOS amplifiers

- *Stochastic* EM amplification:
  - Very low noise without input
  - Excess noise effectively doubles photoelectron shot noise ($F_n^2 = 2$)
  - Asymmetric output distribution
    - At low light, peak output is much below mean
    - Long tail
- CMOS
  - Noisier with no or very low input
  - Noise independent of signal

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ELECTRON MULTIPLYING CCDS

Are they
really what
you
thought?

{3}

Simple (Pixel) SNR Equation

$$SNR = \frac{QE * S}{\sqrt{F_n^2 * QE * (S + I_b) + (N_r / M)^2}}$$

Terms included:

QE: Quantum Efficiency

S: Input Signal Photon Number (photon/pixel)

F_n: Noise Factor

(= 1 for CCD/sCMOS and √2 for EM-CCD)

N_r: Readout Noise

M: EM Gain (=1 for CCD / CMOS)

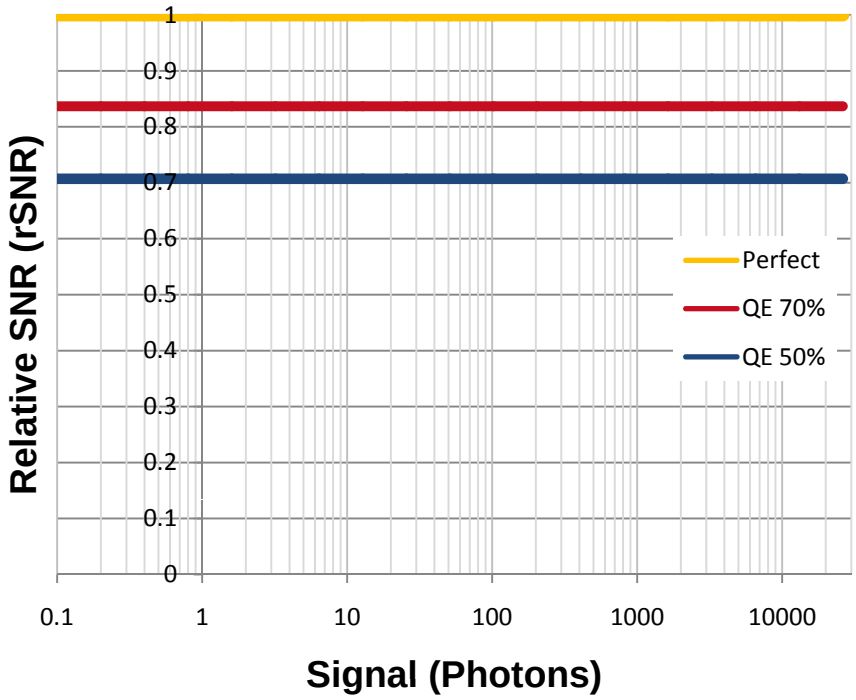
I_b: Background

Not included:

Dark Noise: Dark current X time; considered negligible

Photo response non uniformity: necessary for *image* SNR

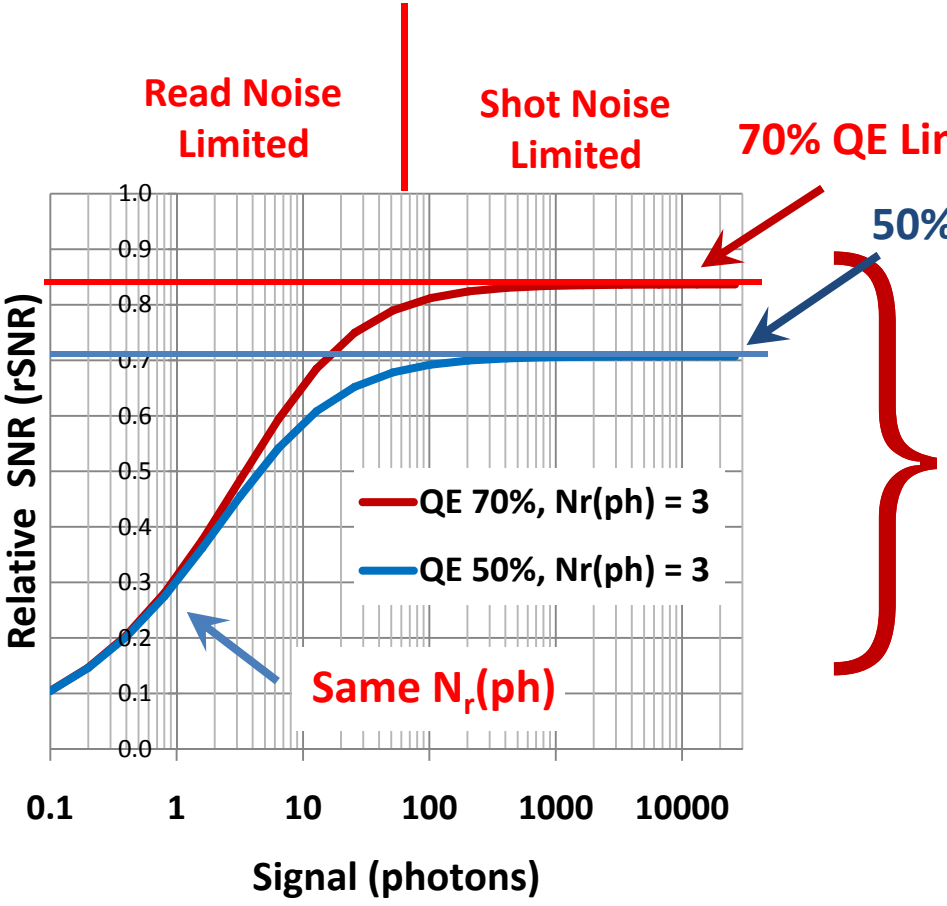
{3} RELATIVE SNR: DISPLAYS IMPERFECTIONS PERFECTLY



rSNR is the SNR for a camera plotted relative to the perfect camera

rSNR shows differences among cameras over full range of signal level

{3} READ NOISE REDUCES rSNR ONLY AT LOW LIGHT



- **N_r(ph): Read noise in photons is the key low light parameter**
  $N_r(ph) = N_r(e-)/QE$
- **QE: always important**

N_r (e-) = 2.1 e-
N_r (ph) = 3

N_r (e-) = 1.5 e-
N_r (ph) = 3

{3} EMCCDs: Excess Noise Creates a Gap

SNR for CCD / CMOS

$$SNR = \frac{QE \times P}{\sqrt{QE \times P}} = \sqrt{QE \times P}$$

SNR for EM-CCD

$$SNR = \frac{M \times QE \times P}{F_n \times M \times \sqrt{QE \times P}} = \sqrt{\frac{QE \times P}{F_n^2}} = \sqrt{QE_{eff} \times P}$$

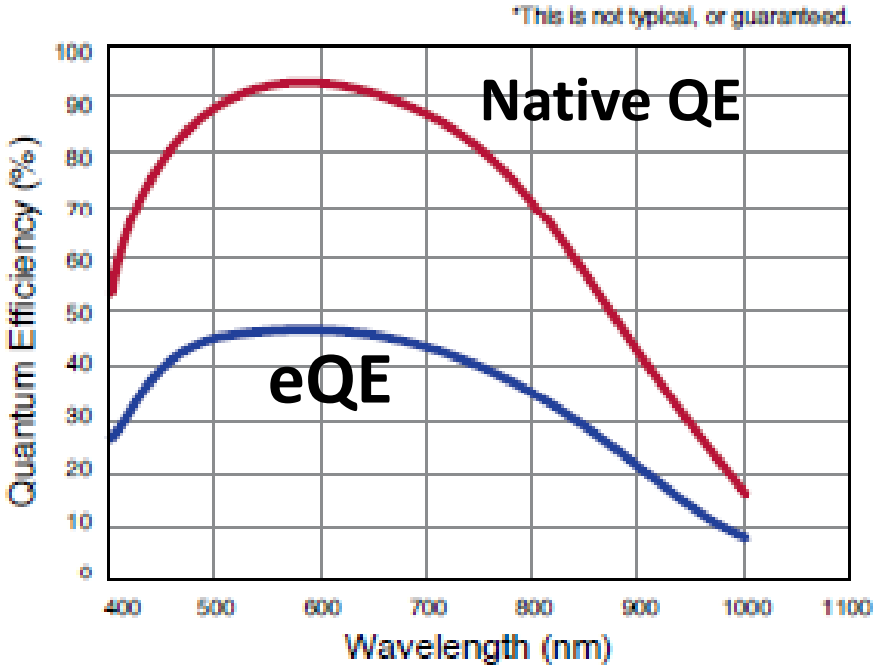
$$QE_{eff} = \frac{QE}{F_n^2} = \frac{QE}{2}$$

QE: Quantum Efficiency,
P: Input Signal Photon Number,
M: EM Gain
F_n: Noise Factor
(assumes dark current and read noise are negligible)

{3}

EMCCDs

- Stochastic EM amplification **adds excess noise**
- Excess noise effectively lowers the SNR to a detector with **½ the QE**

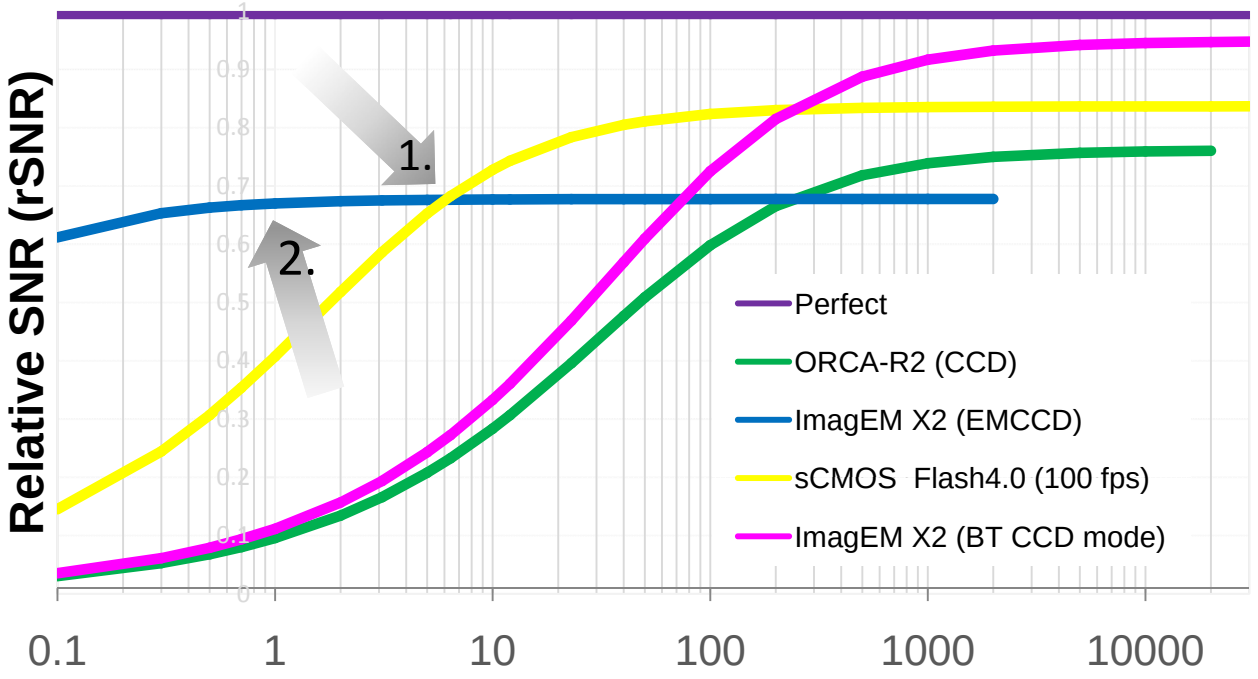


{ Effective QE in EMCCDs }

{3} MIND THE GAP: PREDICTED PIXEL rSNR PERFORMANCE FOR COMMON CAMERAS

1. A camera with the highest SNR at the lowest light level may not be the best at higher light levels

2. The SNR of an EMCCD above 1 electron/pixel is comparable to a camera with QE_{eff} =QE/2 due to excess noise from EM gain.



λ = 650 nm

ARE ALL PIXELS THE SAME?

- Offset non-uniformity
- Photo response non-uniformity (PRNU)
- Dark signal non-uniformity (DSNU)
- Read noise distribution

{3} ACCURATE MEASUREMENT OF THE NUMBER OF PHOTONS

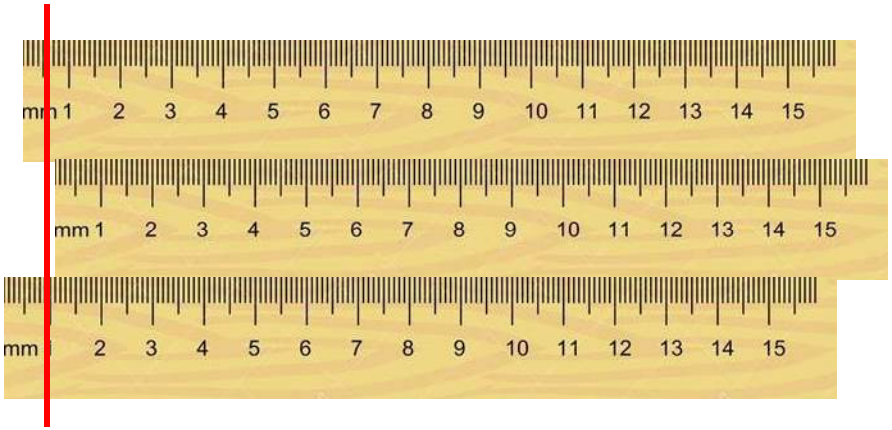
OFFSET NON-UNIFORMITY

Pixel to pixel variation of readings in the dark

+0.6

-0.2

+1.0



If the **zero is incorrect, then absolute measurement is** also **incorrect.**

- Most noticeable in dark or low light conditions.
- Usually expressed as DN or e-, rms.
- For scientific cameras, should be less than read noise.

{3} ACCURATE MEASUREMENT OF THE NUMBER OF PHOTONS

PHOTO RESPONSE NON-UNIFORMTIY

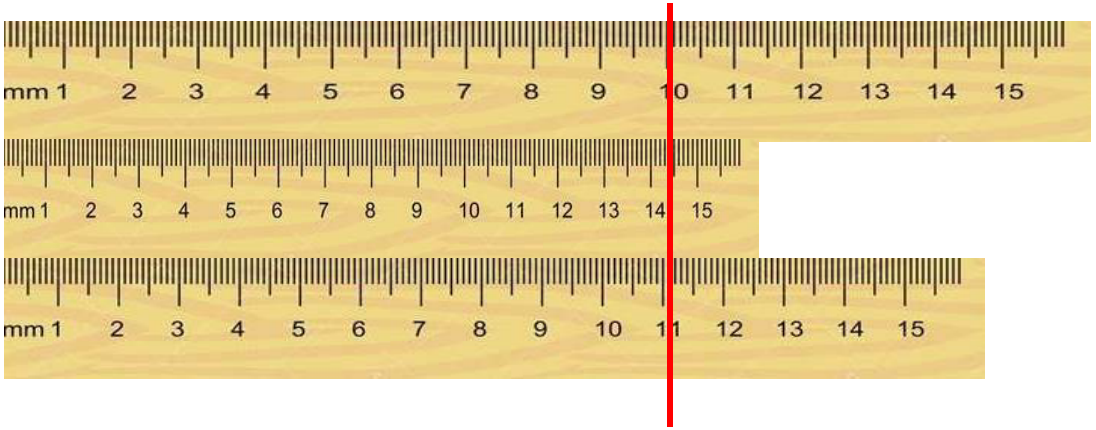
PRNU: pixel to pixel variation of the response to light (DN / photon)

- QE variation : conversion rate of photon to e^-
  (may be spectrum dependent)
- Electronic gain variation: Conversion factor from e^- to DN

-15%

+22%

-6.5%



Mean: 11.9

If the **unit length incorrect, then absolute measurement is** also **incorrect.**

- Most noticeable in higher light conditions.
- May have spatial pattern, stable over time.
- Usually expressed as % maximum.

{3} ACCURATE MEASUREMENT OF THE NUMBER OF PHOTONS

TOTAL FIXED PATTERN NOISE

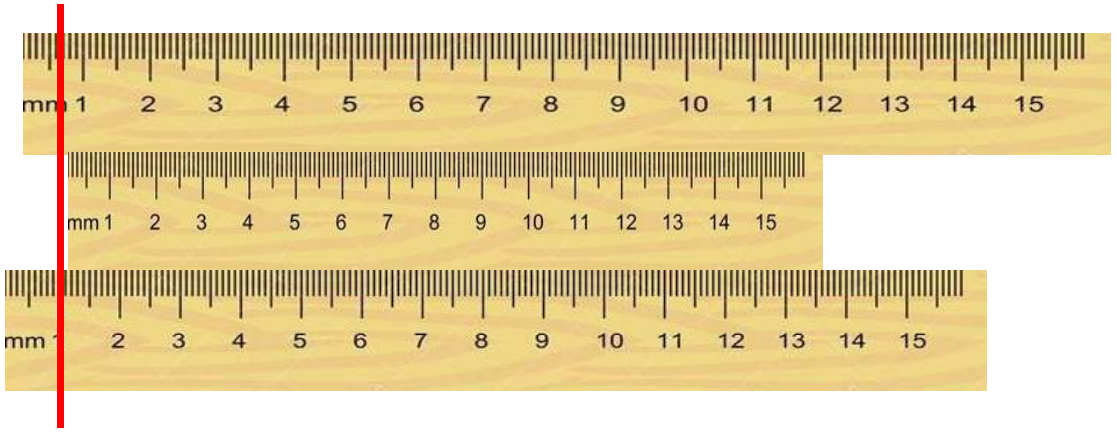
Total **pixel-to-pixel** variation in the accuracy of the measurement of the number of photons. Includes

- Offset non-uniformity
- Photo-response non-uniformity

-15%

+22%

-6.5%



Overall specification of the non-uniformity measurement across the image sensor

Does not include:

- Errors in average QE
- Temporal noise (excess noise, read noise)
- Dark current and dark current shot

{3} DARK SIGNAL NON-UNIFORMITY (DSNU)

Pixel-to-pixel variation in dark current

Offset : dark signal x exposure time.

Noise : √(offset in e-)

How big?

- Proportional to **exposure time**.
- Can **be >100 e- / sec** for a few pixels, especially for sensors > 0C
- For a given image sensor, a multiple of average dark current
- Doubles for each ~8C increase **in sensor temperature**
- Higher noise for high dark current pixels due to **dark shot noise**.

Which technologies?

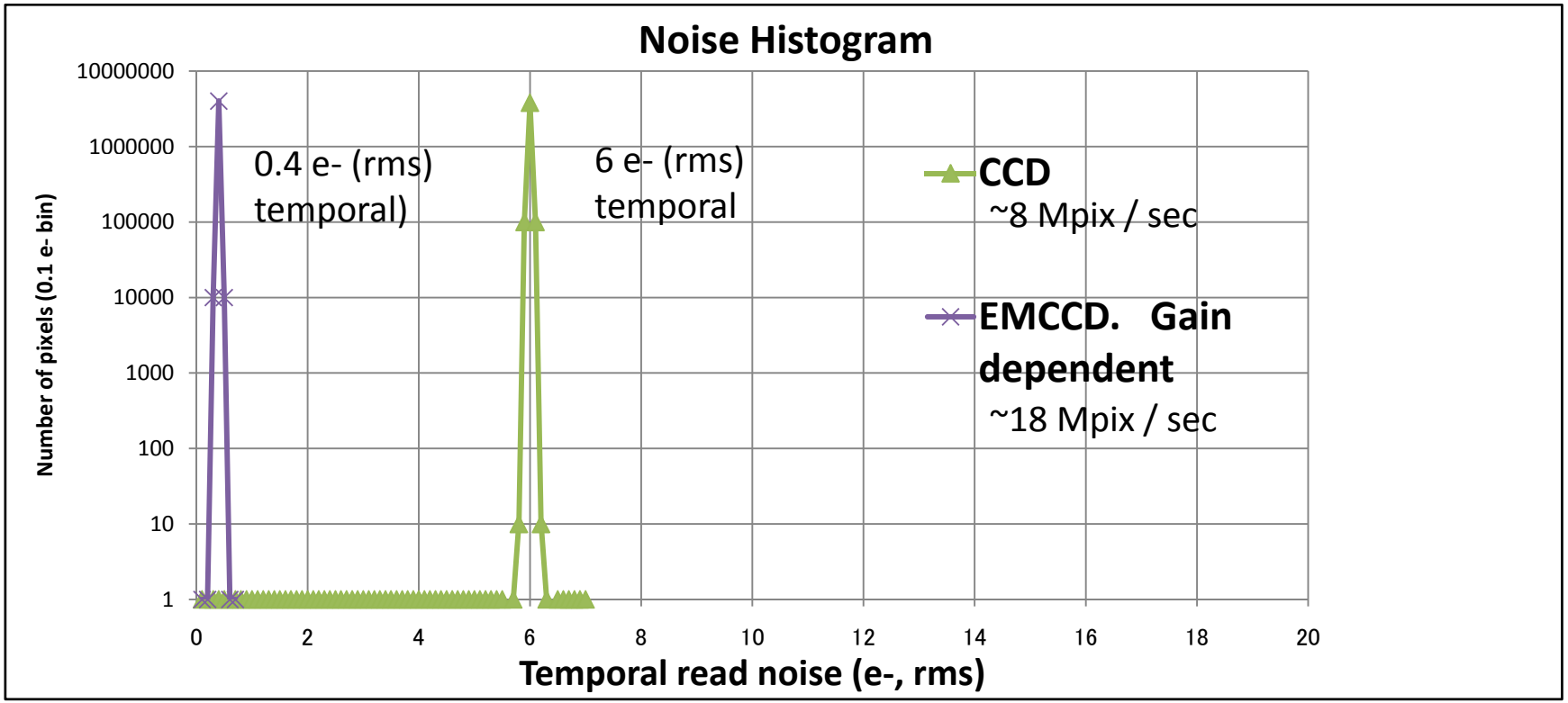
- Mainly sCMOS

Correction

- Identify high noise pixels and correct in image
- Dark shot noise can NOT be corrected.

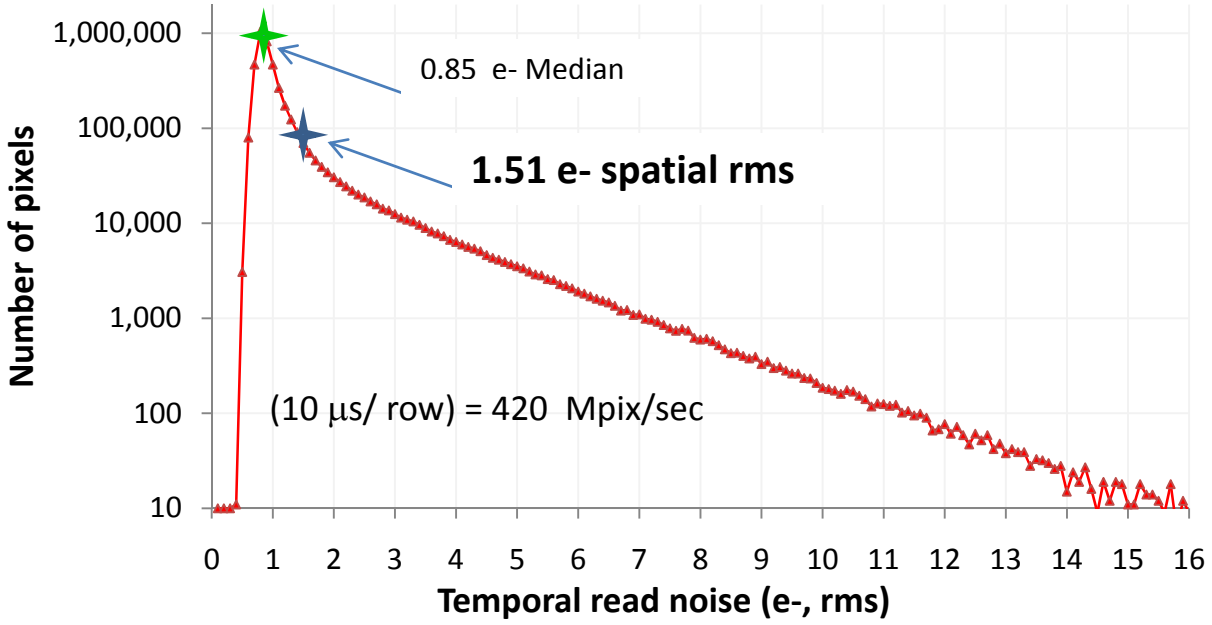
{3} READ NOISE UNIFORMITY: CCD & EMCCD

CCDs and EMCCDs: All pixels are readout through the same amplifier and digitization circuits and therefore read noise is very uniform.
Median = spatial rms



READ NOISE UNIFORMITY: CMOS

CMOS: Each pixel has an independent amplifier and each column has an independent amplifier. Read noise is pixel dependent
"Median" < spatial rms.



{3} READ NOISE

- **CCDs:** Uniform, readout speed dependent, relatively high.
- **EMCCDs**: Uniform, gain and readout speed dependent, very low with EM gain > ~50, but relatively high in “normal CCD” mode.
- **sCMOS:** pixel dependent, little dependence on readout speed for a particular camera.

Things to keep in mind

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MEASURING THE REAL GAP

An in-depth look at noise in CCD, EMCCD and CMOS cameras

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{3} A CLEARER WAY TO COMBINE CAMERA SPECIFICATIONS

- Single Frame rSNR
  Summarizes whole sensor performance
  - QE
  - Gain
  - Noise: including spatial rms read noise, excess noise, dark shot noise
  - Fixed pattern noises, including offset non-uniformity and PRNU
  - Saturation

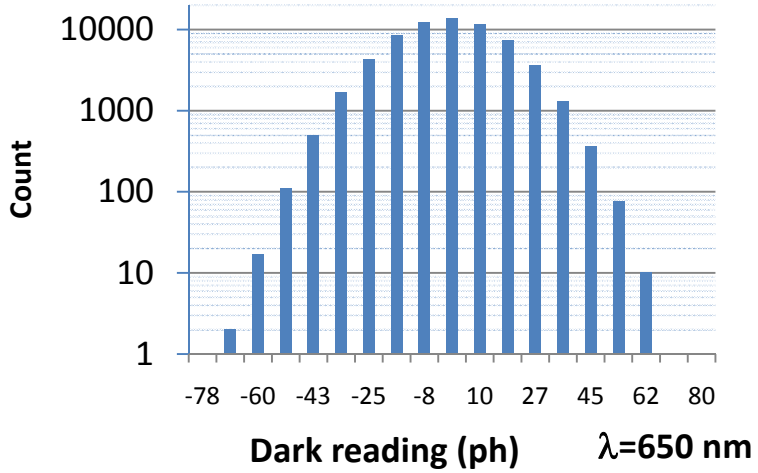
{3} ORCA-R2 INTERLINE CCD: PREDICTABLE AND ROBUST

1. PRNU is insignificant

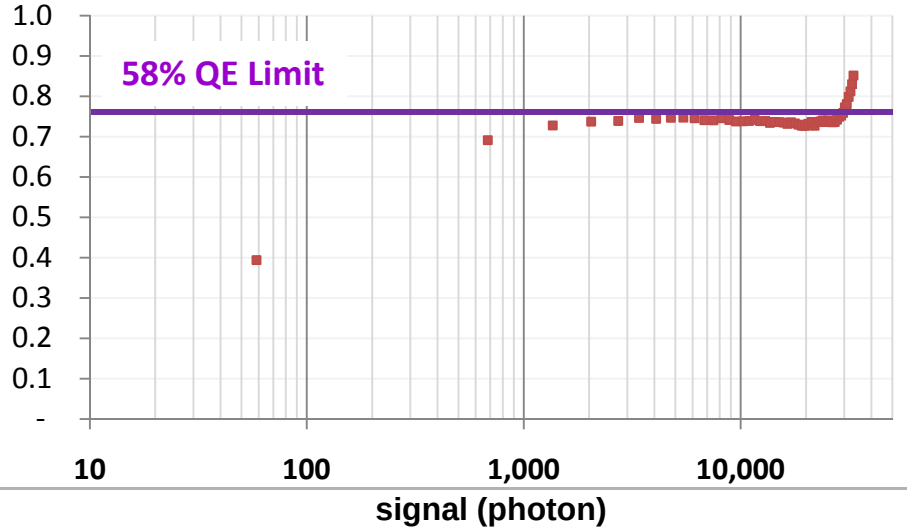
2. Single frame read noise histogram has a Gaussian distribution

Bright Image:
shot noise limited

Mean intensity: 17,300 e-
σ: 130.5e-
PRNU: not measurable



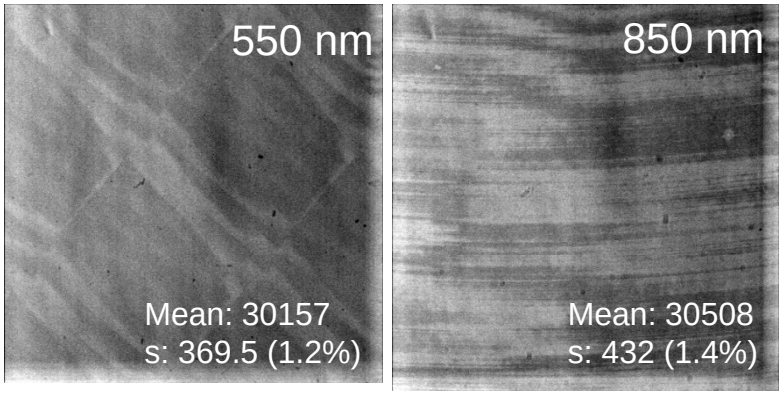
Read Noise (N_r/QE) | **Shot Noise & QE**

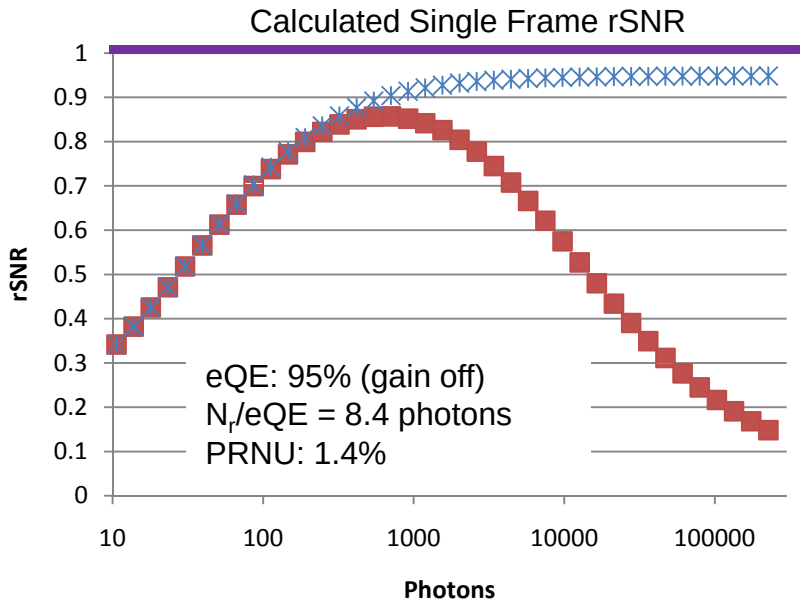


{3} EMCCD: SOME SURPRISING RESULTS

1. Thickness variations from back-thinning process causes spectrally-dependent PRNU
   - Cannot be removed during manufacturing
   - Must be calibrated by users for *their specific spectrum*.
   - Individual pixel map required for correction

2. The Gap for EMCCD in CCD mode becomes very wide due to PRNU

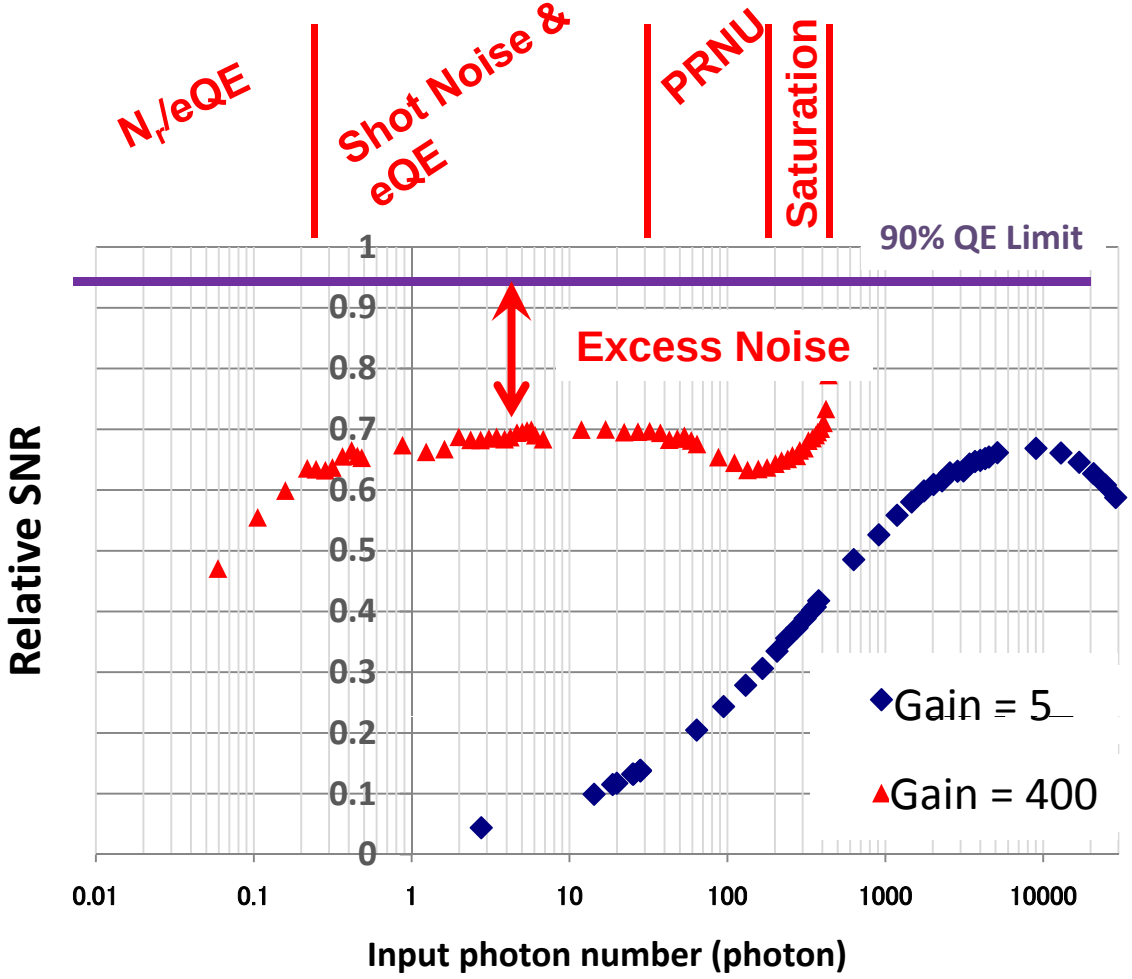




{3} COMPLEX BEHAVIOR: A CLOSER LOOK AT EMCCD SNR WITH HIGH AND LOW GAIN

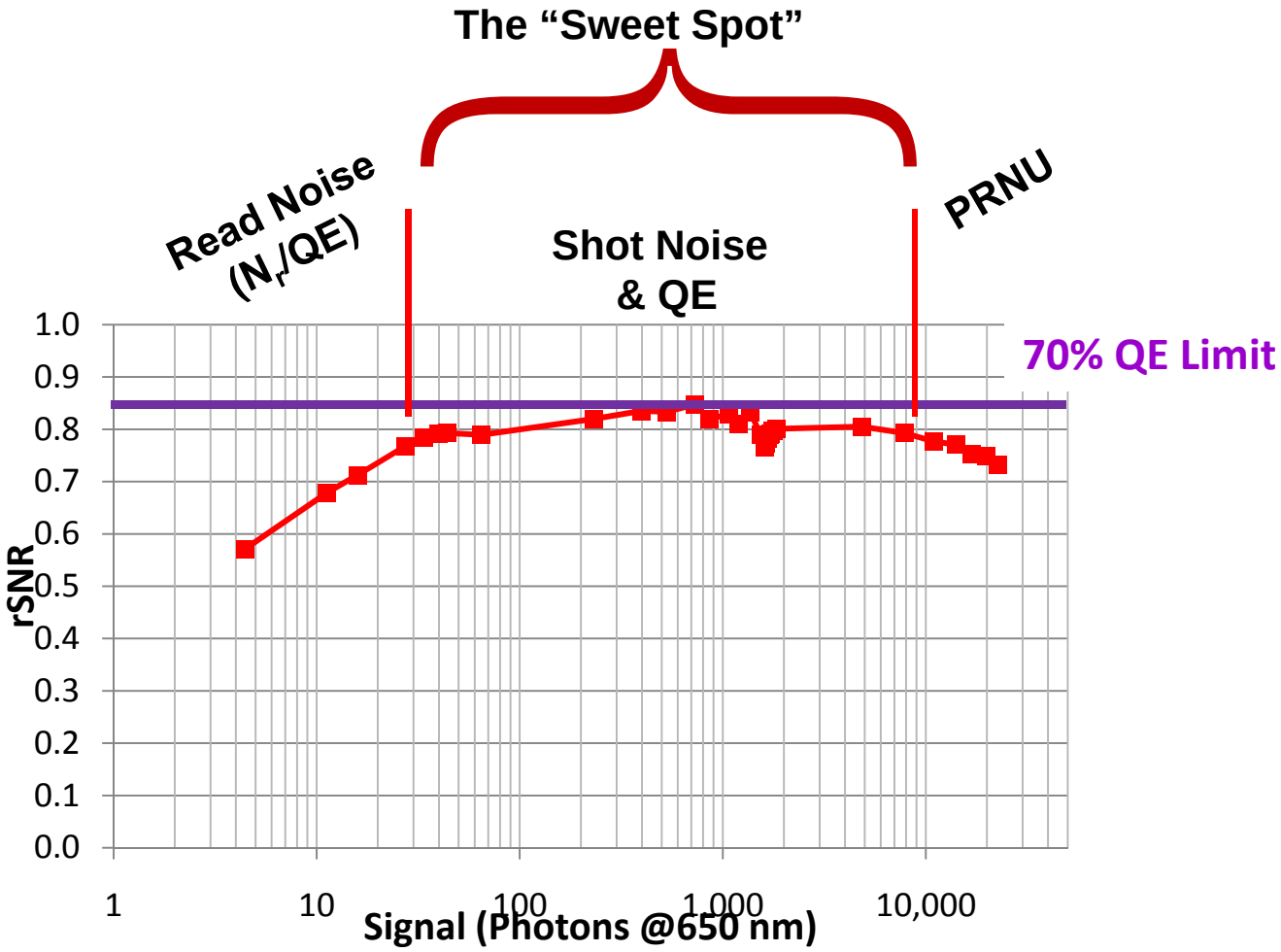
Complex Behavior

- Excess noise (eQE)
- PRNU
- Saturation
- High read noise
  (34 e- @ M=5, 70 fps)
- Gain hard to measure



{3}

ORCA-FLASH4.0 V2 (SCMOS): A VERY COMFORTABLE SWEET SPOT



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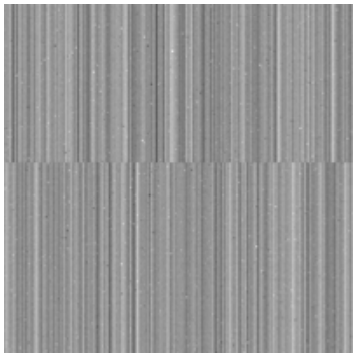
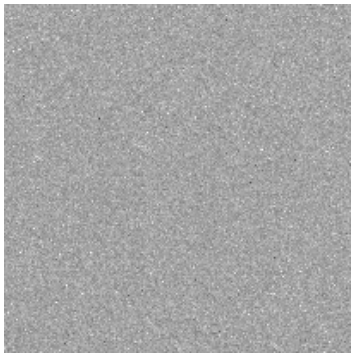
{3} THE IMAGE SENSOR IS NOT THE CAMERA: PRNU IS SIGNIFICANT IN "SCIENTIFIC" CMOS IMAGE SENSORS

Raw Data
Bright Image



PRNU: ~2%

Corrected Data
Bright Image

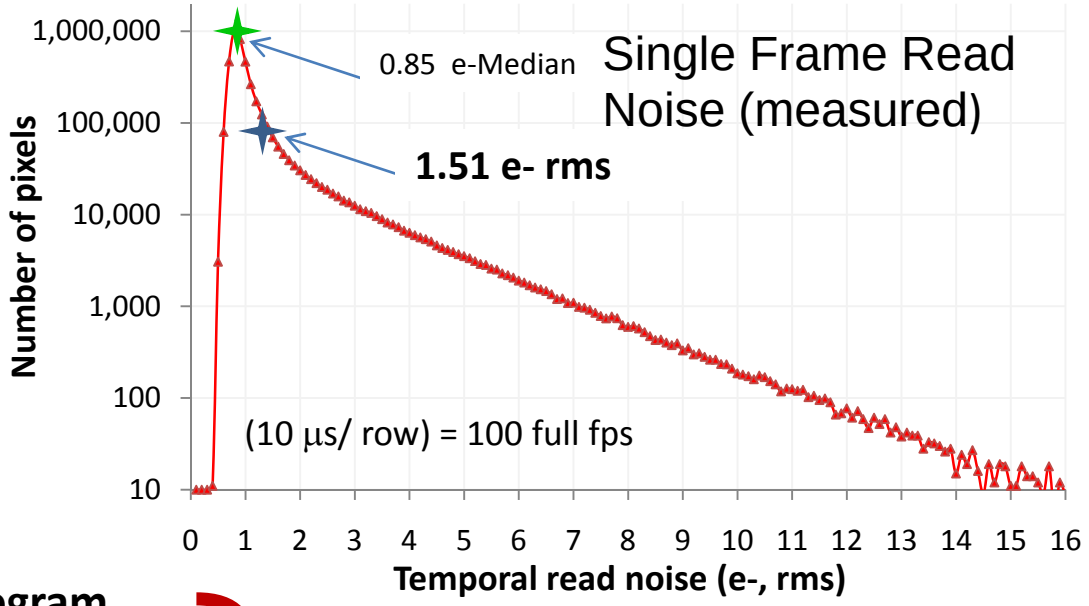


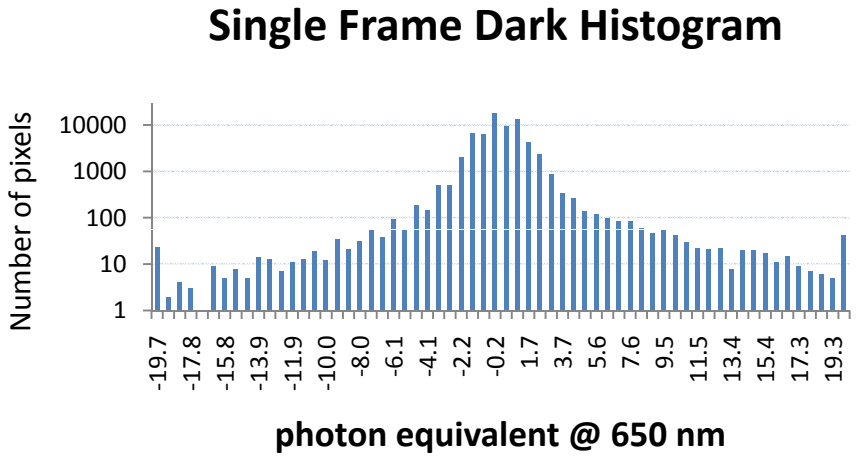
PRNU: ~0.5%

Signal amplified and digitized in column-parallel ADC. FPGA provides offset and gain correction to the raw digitized signal.

{3} sCMOS: PIXEL-DEPENDENT READ NOISE

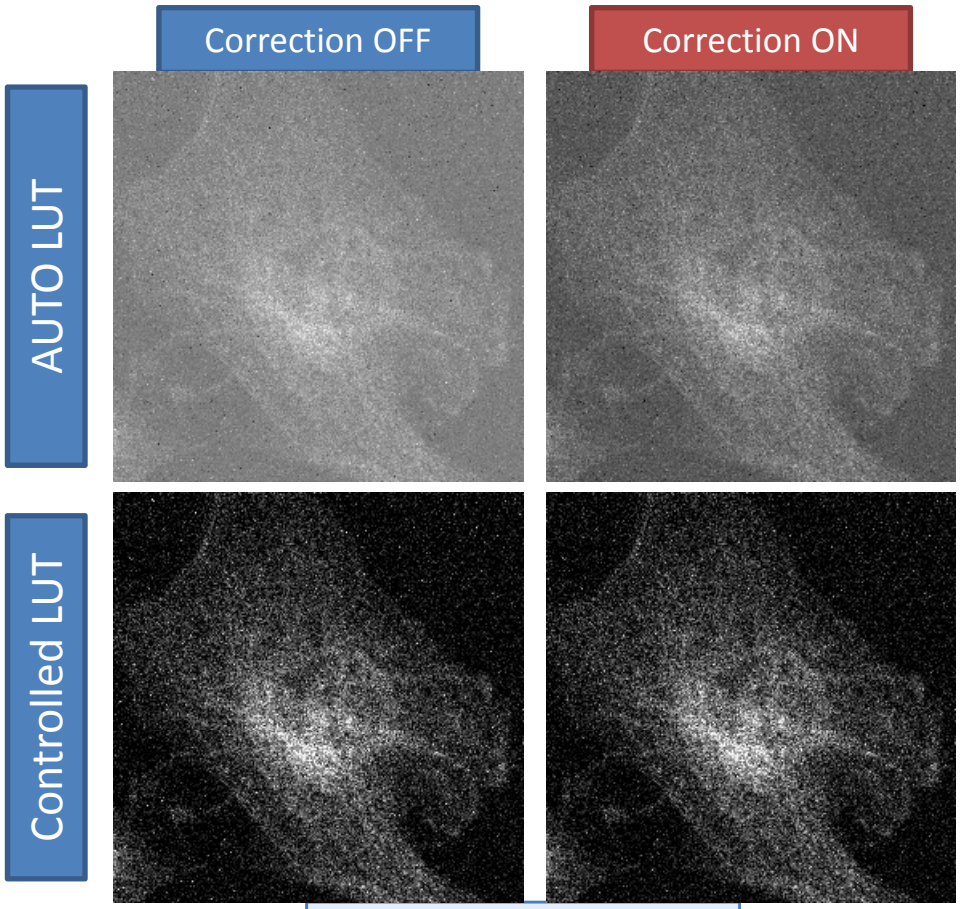
Rms read noise matches single frame rSNR.





Does not fit a Gaussian distribution, i.e. is not completely modeled by a single “read noise.”

{3} sCMOS: Improving visual image quality "Noisy" Pixel Filtering



Map high noise pixels and selectively replace value with the average of the surrounding pixels.

- Improves contrast & "flicker" with "auto" LUT.
- Small difference with controlled LUT
- Affects only a very small number of pixels in frame

{3} MANAGING READ NOISE

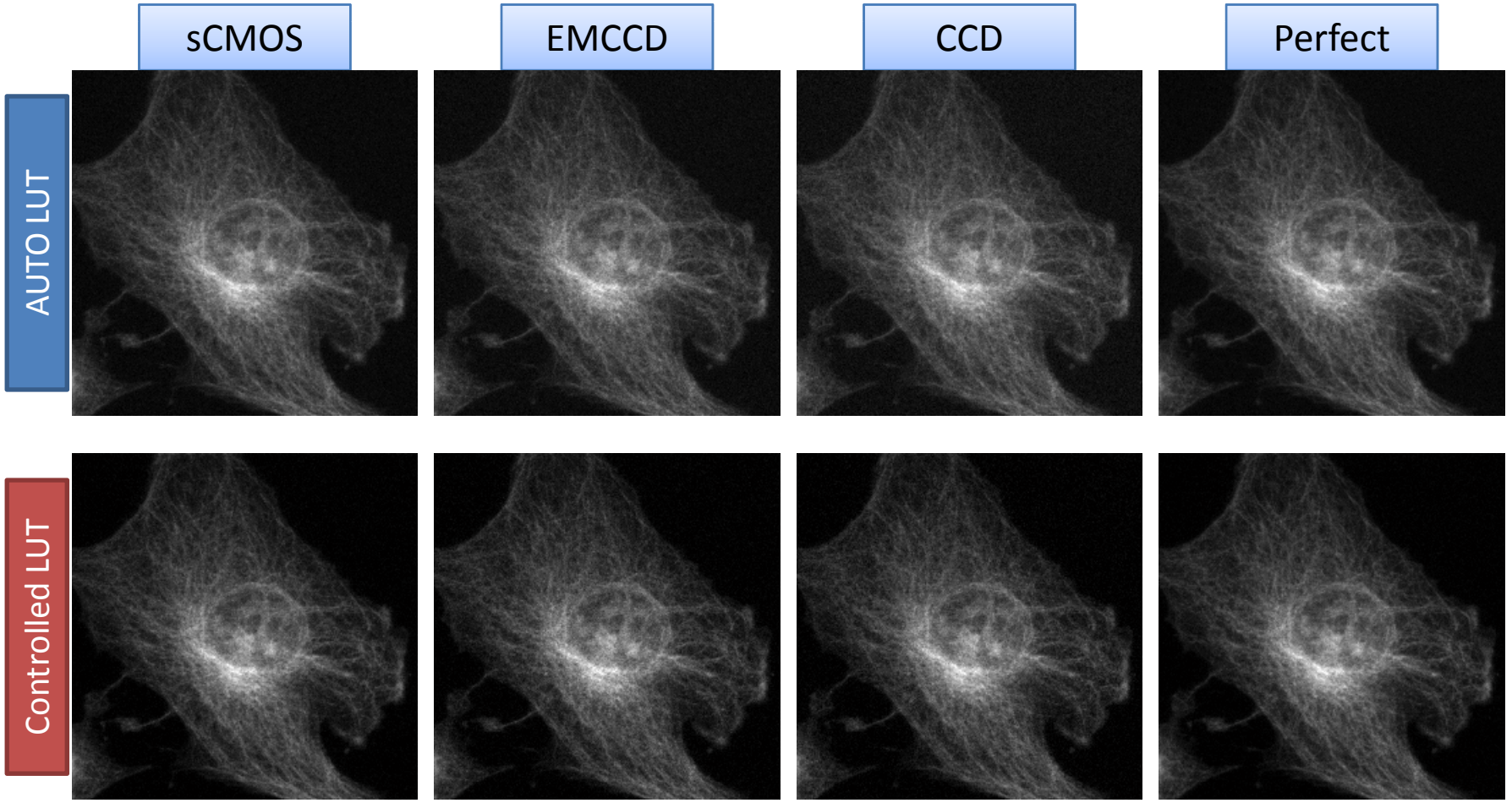
	CCD	EMCCD	CMOS
Specs	Read noise expressed in photons is the key specification. N_r (ph) = N_r (e-)/QE		
			Distribution Use spatial or single frame rms, not median rms
Data collection	Analog binning, optical matching Use slowest clock speed possible		Optical matching Use pixel noise filter when possible
Visualization	Set lower threshold to a minimum of offset plus 0 to 3 noise standard deviations		
Statistical noise model	Poisson + uniform Gaussian	Complicated, gain dependent	Poisson + pixel-dependent Gaussian

WHAT ABOUT IMAGES?

- Perfect and real cameras
- Visualization
- Histograms
- How many photons do you need?

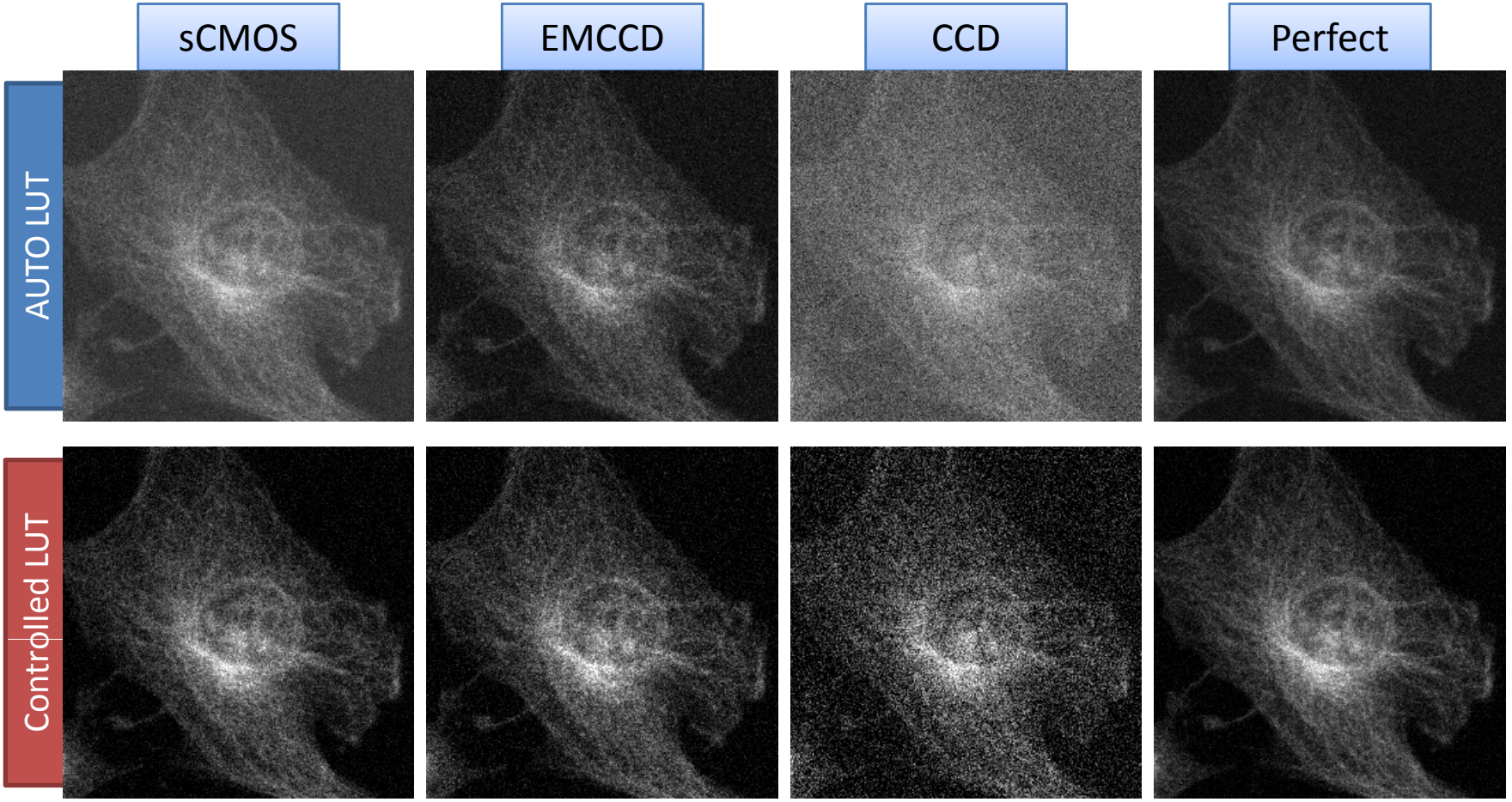
{3}

COMPARING CAMERAS: 1000 PHOTON PEAK VISUALLY SIMILAR



sCMOS:
Noise Correction ON

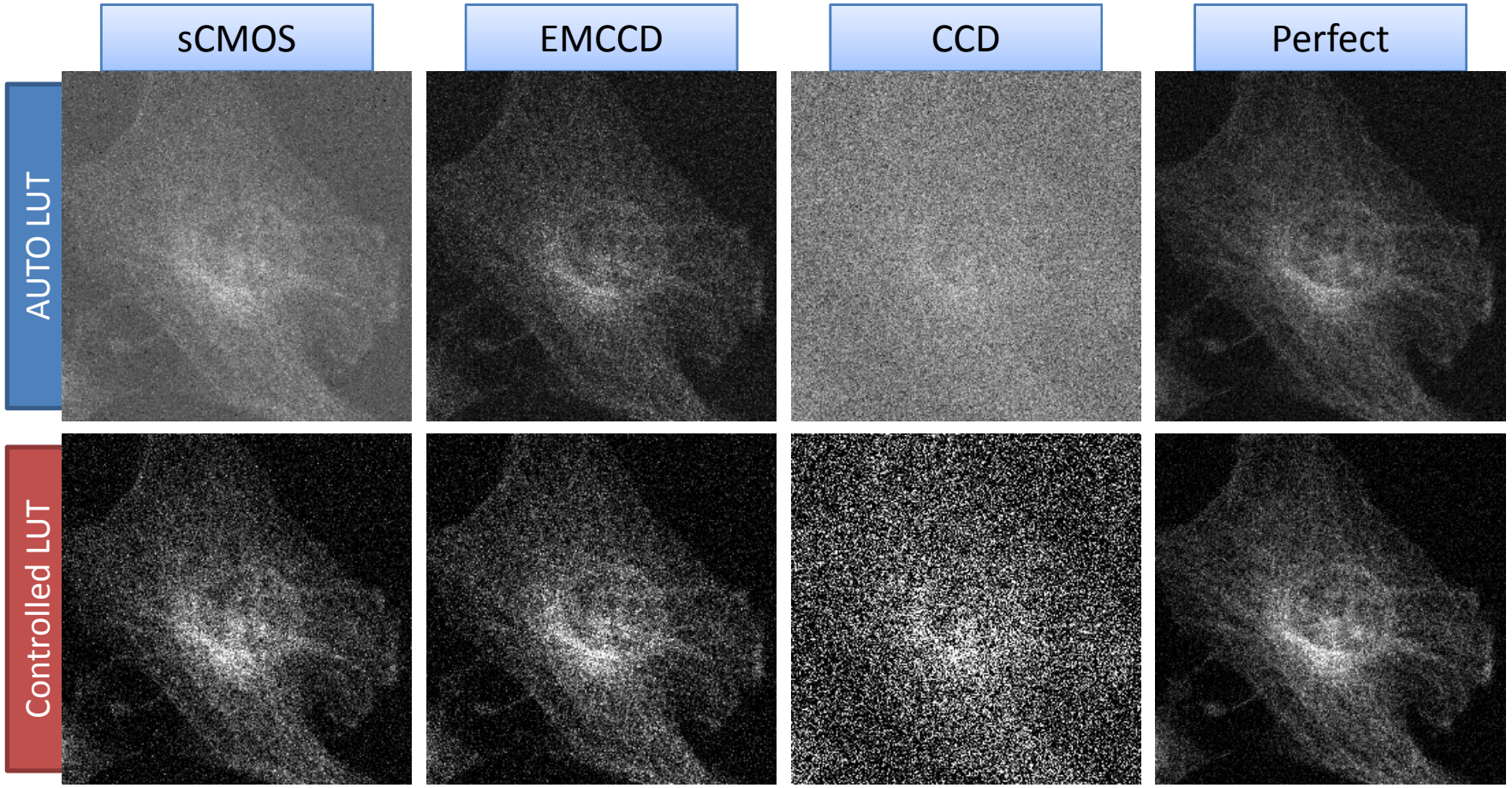
{3} Comparing cameras: 100 photon peak
Camera noise and / or visualization matter



sCMOS:
Noise Correction ON

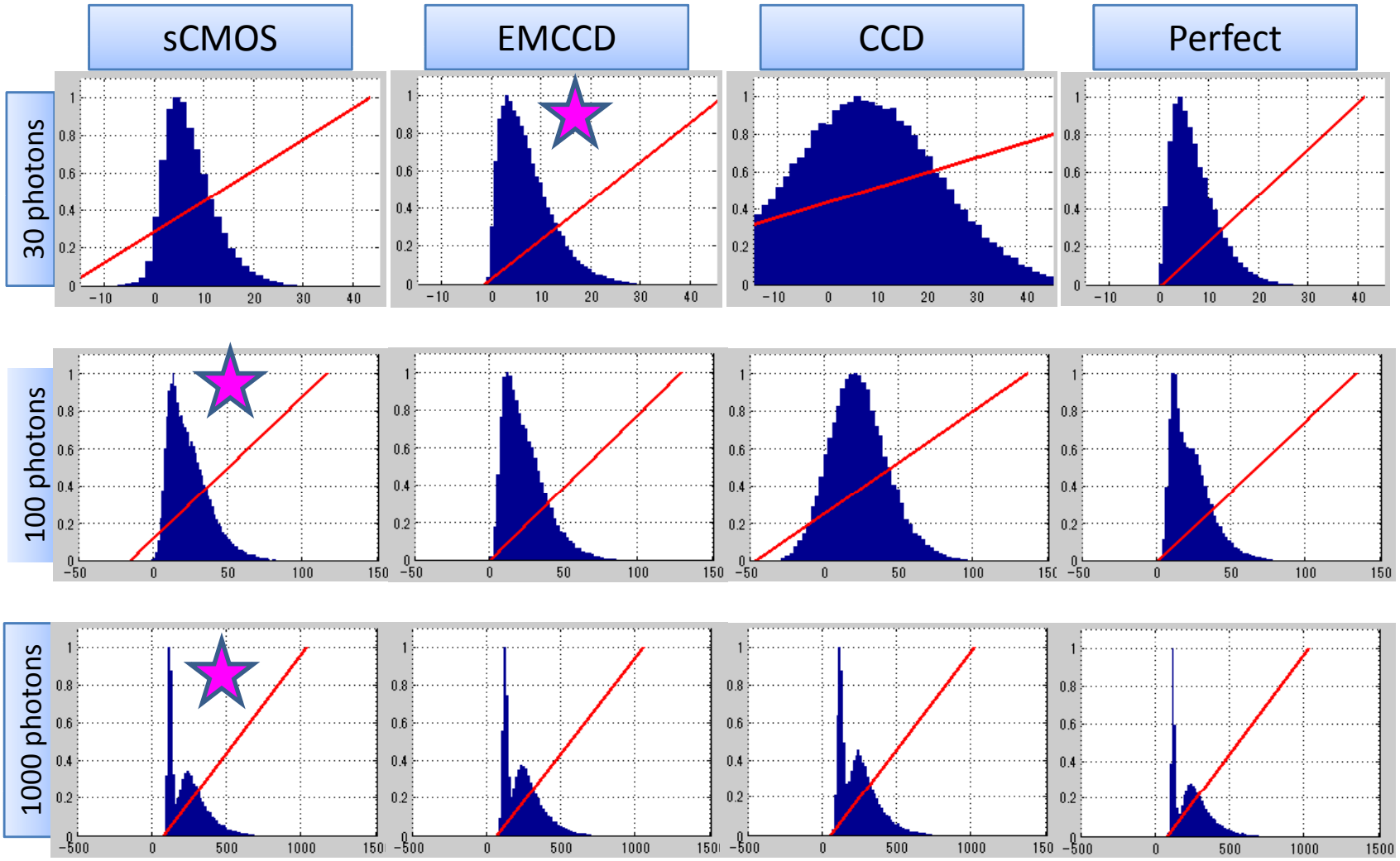
{3}

HARDER TO SEE IN THE DARK: 30 PHOTON PEAK
CAMERA & VISUALIZATION CRITICAL



sCMOS:
Noise Correction ON

{3} HISTOGRAMS: MOST SIMILAR TO THE PERFECT CAMERA



Mean photons: ~35% of peak

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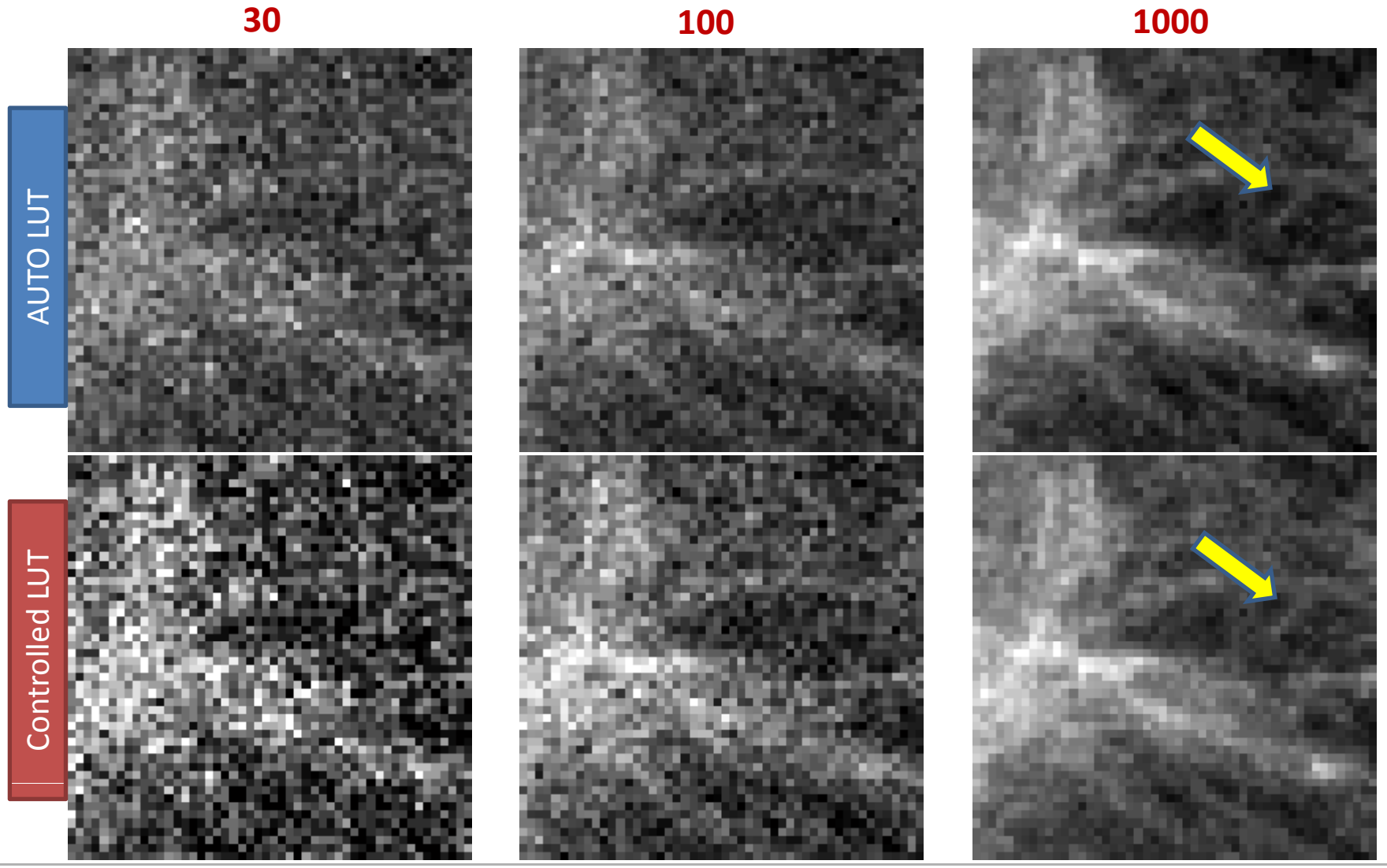
{3} How many photons do I need with a **PERFECT** camera?



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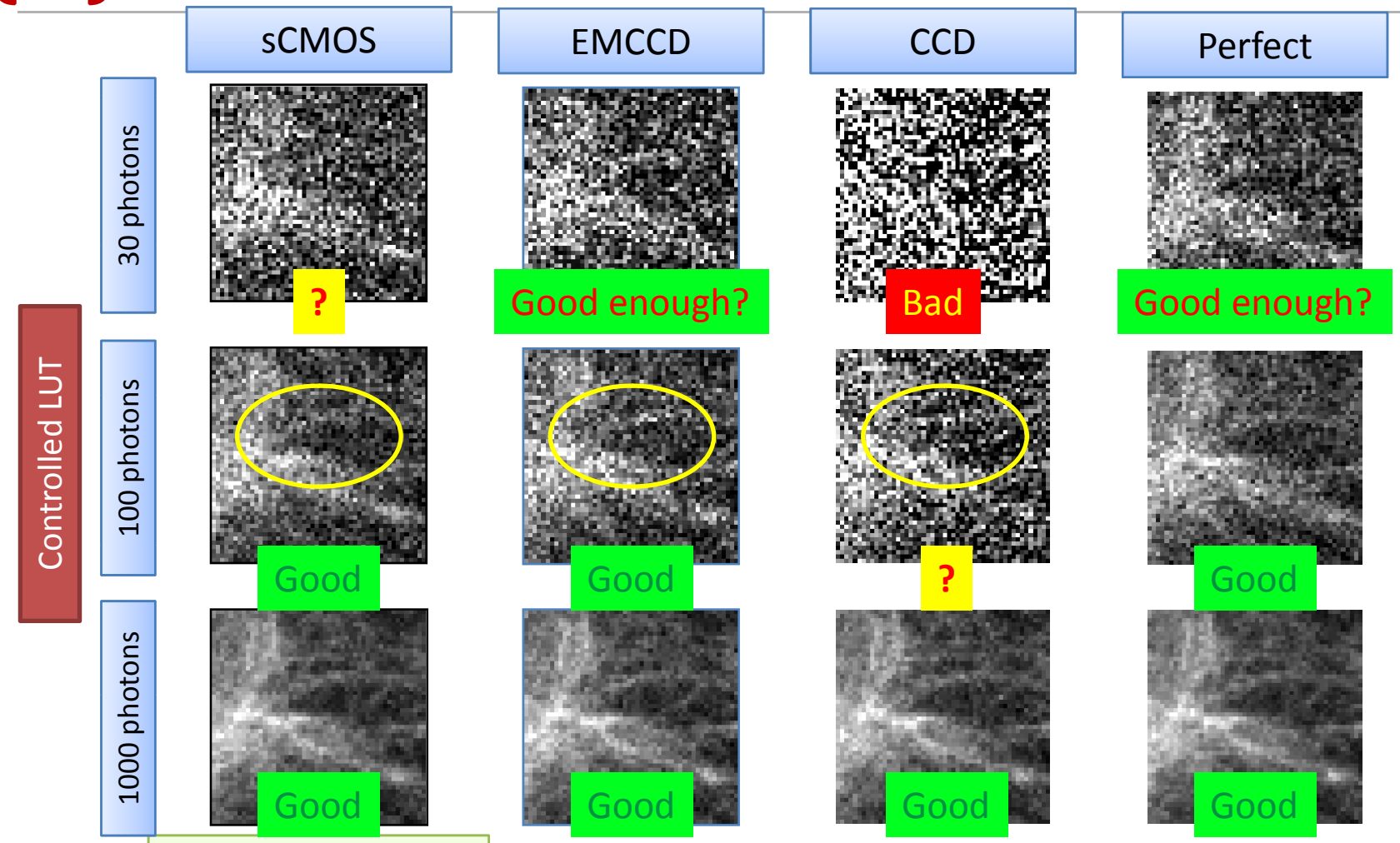
{3} How many photons do I need with a **REAL** camera?



Photons : peak intensity in whole, not zoomed, image

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{3} How to **Narrow the Gap**

1 Know what you want to do

The number of photons required to “see” something depends upon what you want to see, and how clearly you want to see it, even with a perfect camera.

2 Turn up the light carefully

Real cameras reduce image quality, however when there is enough light, all scientific cameras work well

3 Visualization matters

Monitor choice, ambient light, LUT settings all make a difference

4 Use the right camera

Gen II sCMOS cameras have comparable or better image quality than EMCCDs at light levels typically required for visual imaging

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CHOOSING AND USING SCIENTIFIC CAMERAS

1 The image problem

2 Think in photons

3 Real cameras are not perfect

4 **Know thyself**

5 The Living Image: Case Studies

{4}

KNOW THYSELF

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WHAT IS MOST IMPORTANT FOR YOUR EXPERIMENT?

throughput

field of view

sample contrast

frame *rate*

resolution

accuracy

SAMPLE **BRIGHTNESS**

minimum bleaching rate

distance measurement

background

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{4} CONSIDER THE ENTIRE SYSTEM

TWO EXAMPLES

- Lightsheet microscopy (SPIM)
- Single molecule localization microscopy

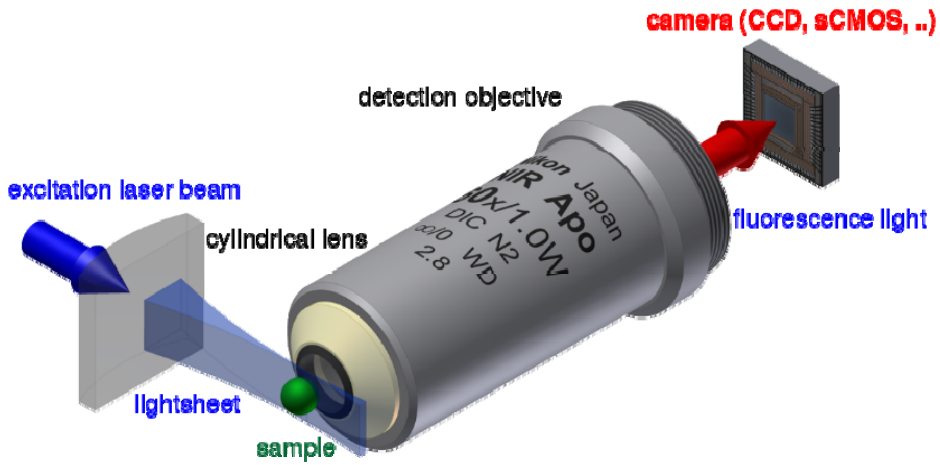
{4}

Light Sheet Micoscopy

- Just like Localization Microscopy LSM has many faces

LSFM - Light **S**heet **Fluorescence M**icroscope
SPIM - **S**ingle **P**lane **I**llumination **M**icroscope
OPM - **O**blique **P**lane **M**icroscopy
sTSLIM – **S**canning **T**hin **S**heet **L**aser **I**llumination **M**icroscopy
mSPIM – Multidirectional **SPIM**

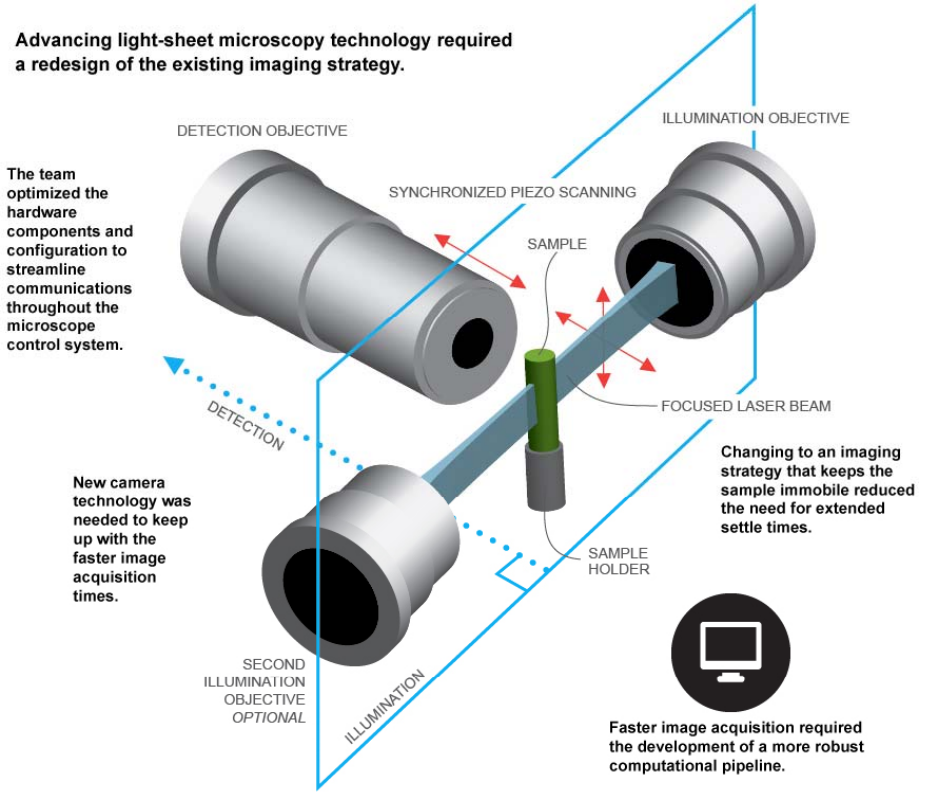
- Benefits
  - Better sectioning vs. widefield
  - Less photodamage vs. confocal
  - Fast acquisition of large samples



- New developments
  - Multiple Cameras
  - Structured Illumination

MuVi-SPIM and SIMView

- Multiple illumination beams and cameras
- Increased isotropy and axial resolution
- Faster scanning with phase or wavelength separation of **offset** beams



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{4} sCMOS is >20x faster than EMCCDs

SPEED!

A Drosophila embryo approximately 3 hours post fertilization (top: dorsal view, bottom: ventral view). The embryo, which expresses a genetically encoded marker labeling all cell nuclei, was recorded simultaneously from four different directions with a SiMView light-sheet microscope equipped with two Hamamatsu ORCA-Flash4.0 cameras. William Lemon and Philipp Keller, HHMI/Janelia Farm. http://www.janelia.org/lab/keller-lab

{4}

LIGHT SHEET MICROSCOPY

http://thelivingimage.hamamatsu.com/

http://player.vimeo.com/video/74253101

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{4} LIGHT SHEET MICROSCOPY

CRITICAL CAMERA CHARACTERISTICS

- Large field of view (high pixel number)
- High speed (data rate)
- Large dynamic range
- Reasonably low noise
- Rolling shutter synchronized to sample scanning with variable speed

Camera: ORCA Flash4.0 Scientific CMOS

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{4}

LIGHT SHEET MICROSCOPY

Light sheet microscopy – matching the camera and optical system

http://www.hamamatsu.com/sp/sys/en/promotion/mp4/s_Lightsheet_en.html

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{4} Optimally Using The Camera for the Task

CCD

Subnanometre single-molecule localization, registration and distance measurements

Alexandros Pertsinidis[1,2], Yunxiang Zhang[1,2] & Steven Chu[1,2,3,4]†

EMCCD

Ultrahigh accuracy imaging modality for super-localization microscopy

Jerry Chao[1–3], Sripad Ram[1–3], E Sally Ward[2] & Raimund J Ober[1,2]

sCMOS

Video-rate nanoscopy using sCMOS camera–specific single-molecule localization algorithms

Fang Huang[1], Tobias M P Hartwich[1–3,9], Felix E Rivera-Molina[1,9], Yu Lin[4,5], Whitney C Duim[1], Jane J Long[6], Pradeep D Uchil[7], Jordan R Myers[1], Michelle A Baird[8], Walther Mothes[7], Michael W Davidson[8], Derek Toomre[1] & Joerg Bewersdorf[1,4,5]

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{4} STANDARD PRACTICE IS NOT THE BEST PRACTICE: USING EMCCD WITH GAIN YIELDS LEAST ACCURATE RESULTS

Mean photon count	Ultimate accuracy limit (nm)	Conventional EMCCD accuracy limit (nm)	UAIM at 900× accuracy limit (nm)	UAIM at 4500× accuracy limit (nm)	CCD accuracy limit[a] (nm)
200	4.84	9.71 [100.6%]	5.41 [11.8%]	5.13 [6.0%]	7.94 [64.0%]
400	3.42	6.94 [102.9%]	3.92 [14.6%]	3.63 [6.1%]	5.26 [53.8%]
800	2.42	4.93 [103.7%]	2.85 [17.8%]	2.57 [6.2%]	3.45 [42.6%]
1600	1.71	3.49 [104.1%]	2.08 [21.6%]	1.82 [6.4%]	2.32 [35.7%]
3200	1.21	2.48 [105.0%]	1.52 [25.6%]	1.30 [7.4%]	1.57 [29.8%]

[a]Computed at near-optimal magnification (i.e., magnification that yields approximately the best localization accuracy limit) of 128.6×, 185.7×, 185.7×, 185.7×, and 242.9× for mean photon count of 200, 400, 800, 1600, and 3200, respectively.

CCD QE: 100%, read noise = 1.8 ph, no background; No fixed pattern noise.

… "the fact that the noise coefficient approaches 1 with increasing photon count demonstrates the suitability of the CCD (CMOS) detector when enough light is available…."

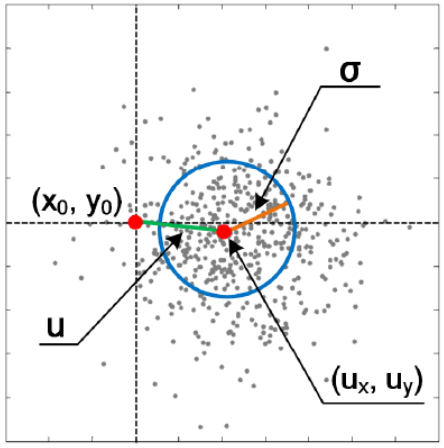
… "even with a low readout noise of σ=2 electrons, the CCD detector is unsuitable for extreme low light imaging and implementing UAIM."

Adapted from: J. Chao et al (Ober Lab), Nat. Meth10, 2013) doi:10.1038/nmeth.2396

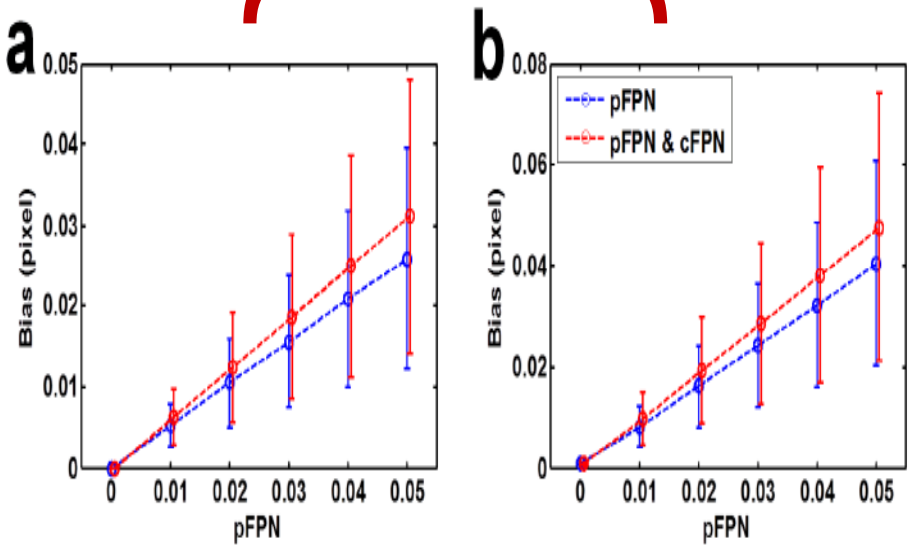
http://www.wardoberlab.com/

{4} Uncorrected PRNU can Lead to Localization Bias

Localization distribution & bias



Impact of PRNU on localization bias:



Alexa 647 simulation (3000 photons)

mEos2 simulation (750 photons)

0.5% PRNU: 1 – 2 nm @ 100 nm/ pixel

Courtesy: Zhen-li Huang, Huazhong University of Science and Technology, (unpublished)

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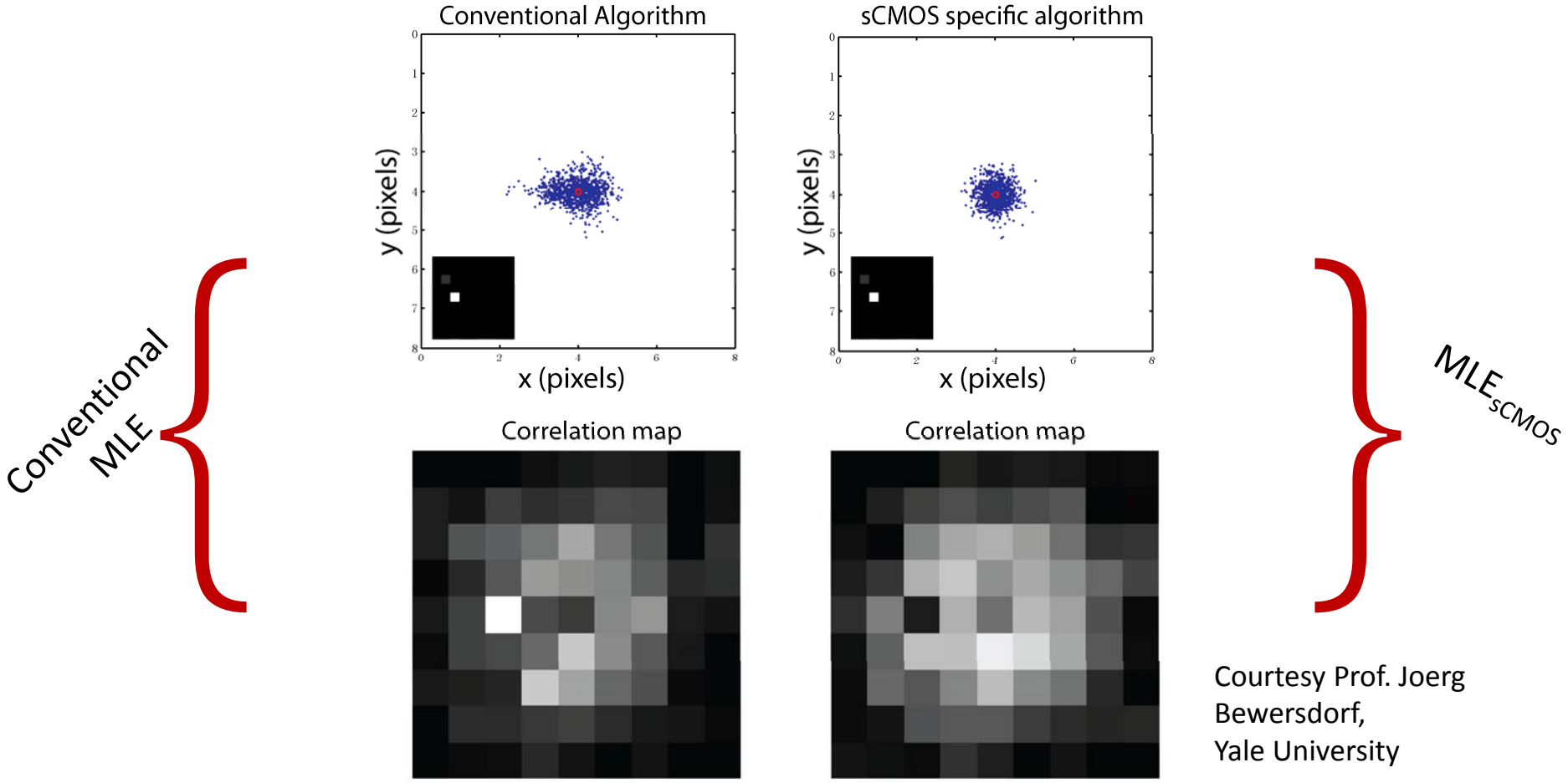
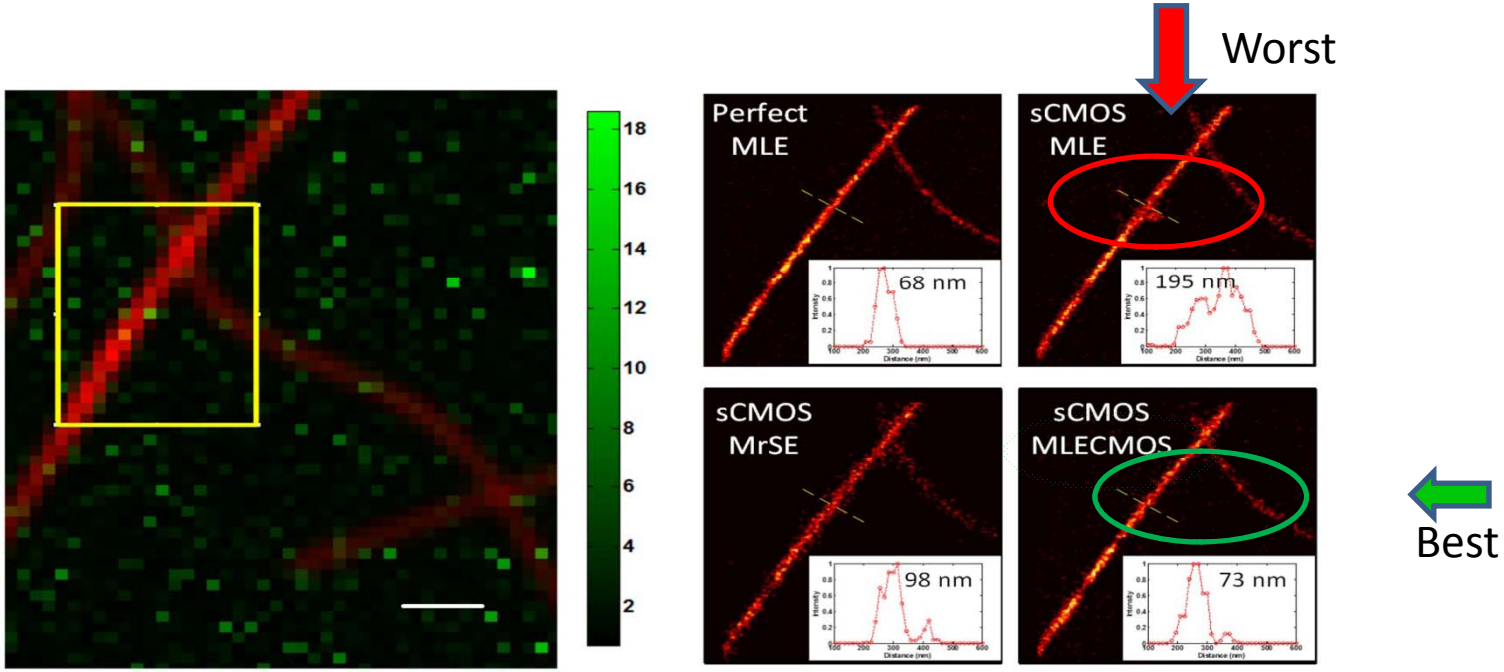
{4} Compensating Read Noise Variation



Courtesy Prof. Joerg Bewersdorf, Yale University

Incorporating pixel-specific read noise into the Maximum Likelihood Probability Model eliminates and narrows the asymmetric distribution of localized molecules caused by higher read noise pixels.
Courtesy F. Huang, Bewersdorf Lab

{4} MLE Reconstruction must use a noise model including camera noise



Simple and good

Note: MLE for EMCCDs are also difficult:

- Inaccurate gain
- Output PDF not Poisson
- Even at “high” light, the variance is 2X the mean signal (in photons).

Courtesy: Zhen-li Huang, Huazhong University of Science and Technology, (unpublished)

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{4} Selecting and Using Cameras: Case Studies

CCD

Subnanometre single-molecule localization, registration and distance measurements

Alexandros Pertsinidis[1,2], Yunxiang Zhang[1,2] & Steven Chu[1,2,3,4]†

EMCCD

Ultrahigh accuracy imaging modality for super-localization microscopy

Jerry Chao[1–3], Sripad Ram[1–3], E Sally Ward[2] & Raimund J Ober[1,2]

sCMOS

Video-rate nanoscopy using sCMOS camera–specific single-molecule localization algorithms

Fang Huang[1], Tobias M P Hartwich[1–3,9], Felix E Rivera-Molina[1,9], Yu Lin[4,5], Whitney C Duim[1], Jane J Long[6], Pradeep D Uchil[7], Jordan R Myers[1], Michelle A Baird[8], Walther Mothes[7], Michael W Davidson[8], Derek Toomre[1] & Joerg Bewersdorf[1,4,5]

{4} Subnanometre single-molecule localization, registration and distance measurements

Alexandros Pertsinidis[1,2], Yunxiang Zhang[1,2] & Steven Chu[1,2,3,4]†

Results

Accurate measurement of the *distance* between two fluorophores of different colors. $\sigma_{distance}$ ~0.77 nm using a dichroic beamsplitter to direct each color of light to separate halves of the CCD camera.

Camera Correction

Measured PRNU maps for each color. Improved localization relative accuracy by ~2– 4 nm.

Details

Speed: 5 – 50 s / measurement

Light: ~4,000 – 10,000 ph/ mol/frame

~10^5 ph / mol before bleaching

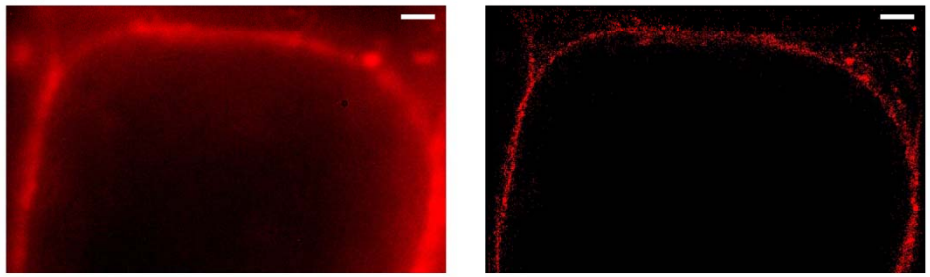
Imaging: Simultaneous 2 color

Camera: Back-thinned EM-CCD, gain off

Nature (2010)| doi:10.1038/nature09163

4 Ultrahigh accuracy imaging modality for super-localization microscopy

Jerry Chao[1–3], Sripad Ram[1–3], E Sally Ward[2] & Raimund J Ober[1,2]



Cholera toxin B subunit

scale bar: 1 µm

Results

Localization Microscopy with Minimal Bleaching. Plasma membrane dynamics for > 60 s (594 frames). 40% better localization precision than "conventional" EMCCD localization

Camera Correction

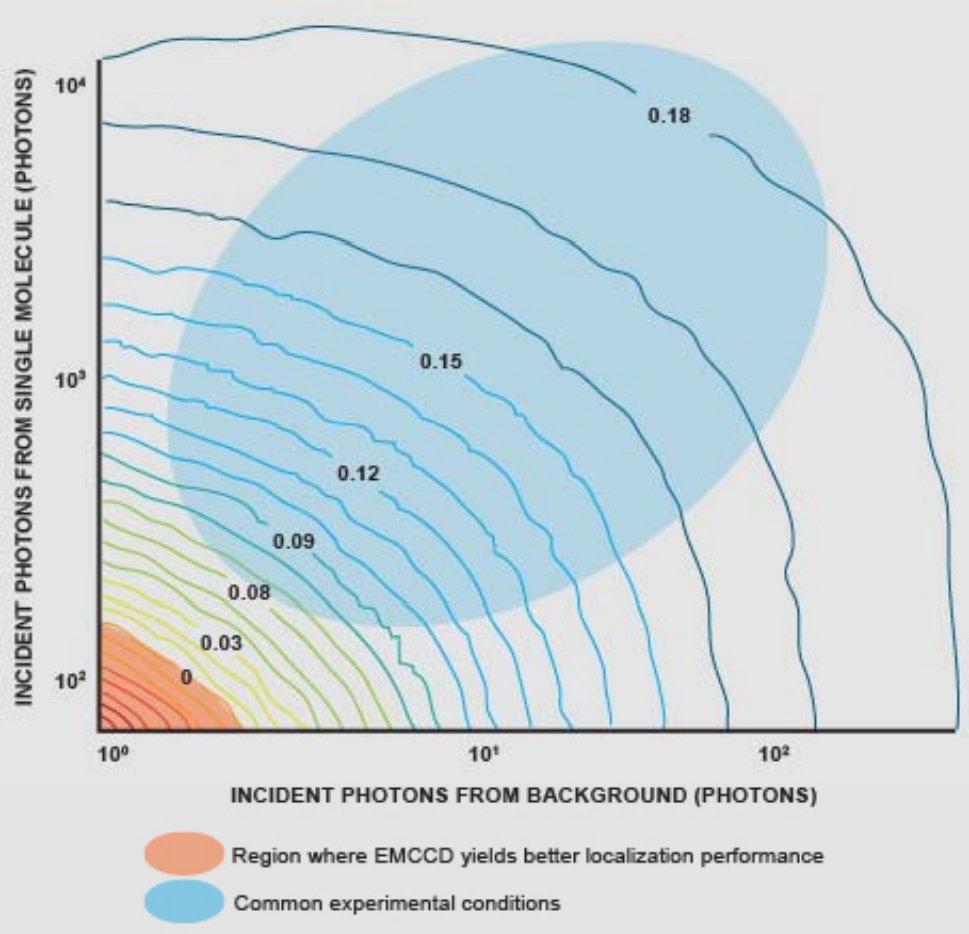
Implemented detailed statistical EM noise model into maximum likelihood reconstruction probability model.

Details

Speed: ~60s / reconstructed image
Light: ~100 photons /molecule frame
Mag: 630X
Camera: EM-CCD, Gain ~1000

Courtesy of J. Chao et al (Ober Lab)
Adapted from Nat Meth (2013) doi:10.1038/nmeth.2396

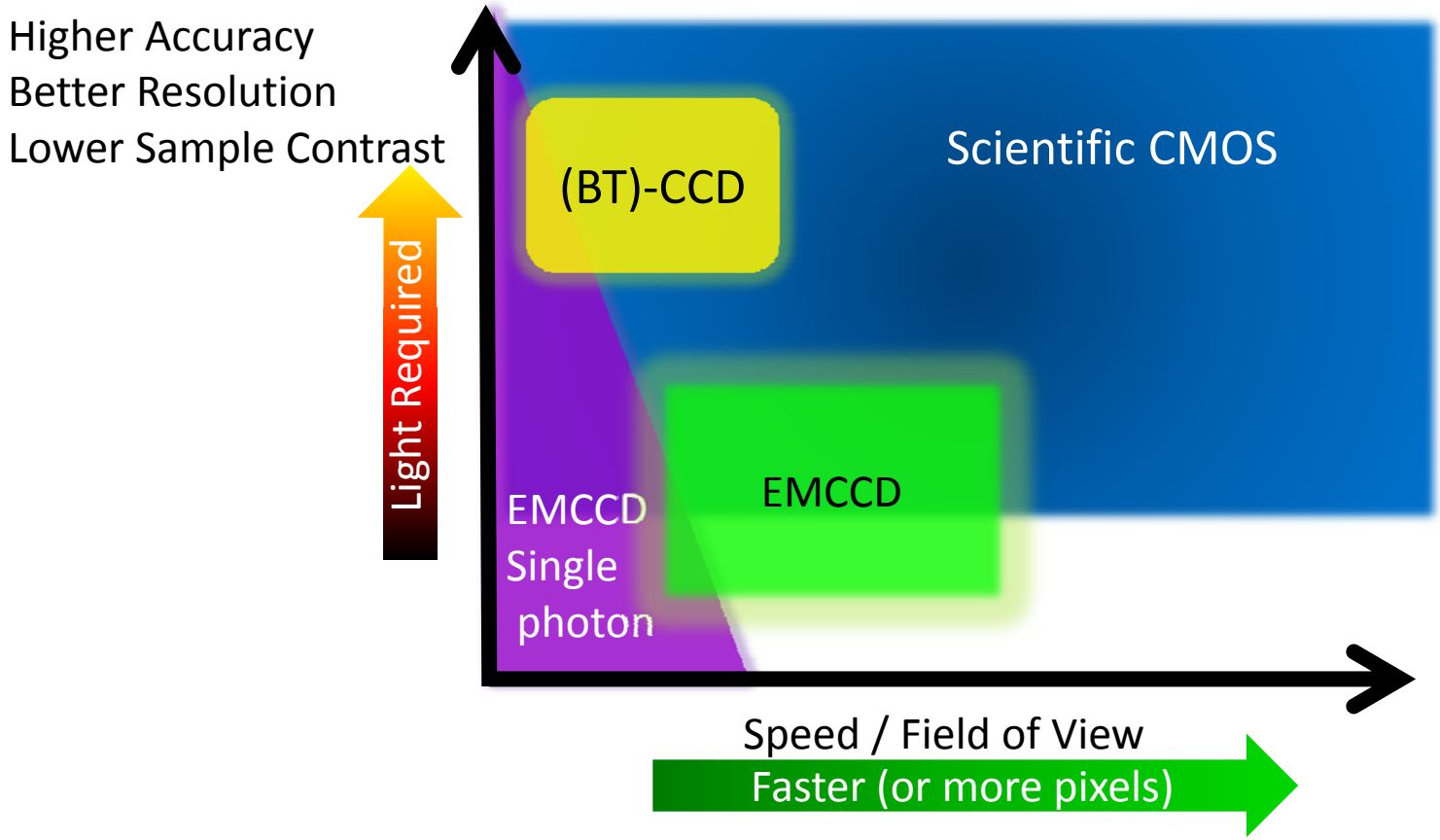
{4} Localization Precision “conventional” EMCCD vs. sCMOS



Courtesy of F. Huang. Bewersdorf Lab, Yale

Adapted from F. Huang *et al.*, Nature Methods 10(7): 653-658 (2013)

{4} Minimizing the Gap: Matching the Camera to Your Needs



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{5}

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Prof. Zhen-li Huang, Huazhong University of Science and Technology
F. Long et al, OPTICS EXPRESS 17741 (2012)

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F. Huang *et al.*, Nature Methods 10(7): 653-658 (2013)

Prof. Raimund Ober, Texas Southwestern University
J. Chao et al, Nat Meth (2013) doi:10.1038/nmeth.2396

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Hiroyuki Kawai: camera measurements
Stephanie Fullerton: presentation guidance
Katsuhide Ito: Lightsheet microscopy
Eiji Toda: budget

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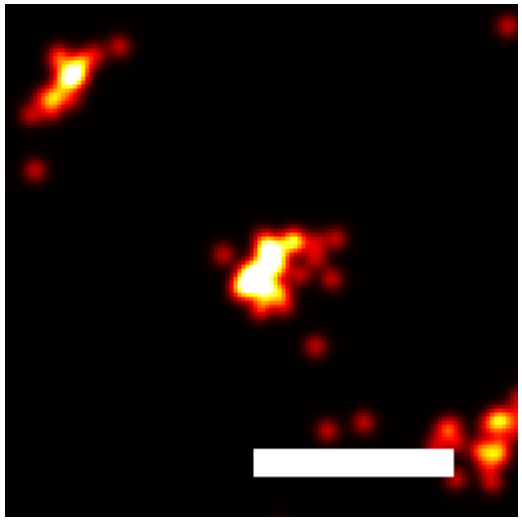

32 fps dynamics. 500 nm scale
Courtesy Vutara / Prof. Bewersdorf