ORCA-Flash4.0

Changing the Game

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In early 2001, the scientific imaging community turned to electron multiplying CCDs—the new superstars of detector technology—to realize breakthroughs in low light imaging.

This method worked brilliantly…until now.

ORCA-Flash4.0

Changing the Game

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We are rocketing into a new era of life science imaging that is powered by revolutionary advances in optics and sensors occurring alongside rapid improvements in computational power, super resolution methods and fluorescent markers. Less than 10 years ago, it was unimaginable that we could image live cells with the speed, resolution and sensitivity provided by today's detectors. We did not get here by accident. Each stage of detector development has spurred new frontiers in life science imaging: the quality and reliability of Sony ICX interline sensors providing our first high resolution live cell images; the breakthrough of on-chip gain in electron multiplying CCDs (EM-CCDs) allowing real time imaging of single molecules; and the incredible high speed, low noise performance of the first generation of scientific CMOS (sCMOS) offering temporal resolution without sacrificing spatial resolution. Each of these technologies was an awesome advancement at the time. Our new ORCA-Flash4.0 redefines sCMOS, adding high quantum efficiency (QE) to an already long list of achievements: low noise, high speed, and high resolution.

Until now, we've never had a camera that achieves low noise and high QE without the confounding contribution of EM-CCD multiplicative noise (Robbins and Hadwen, 2003). This level of sensitivity changes the game; we cannot rely on our past considerations of what makes a good camera, a good image, or a good system. Furthermore, if we want to take advantage of new detector technology, we need to understand camera technology in the context of the sample. We must recognize that a detector is just one part of the imaging equation: the sample and optical system complete (and often confound) every imaging experiment. In most camera comparison discussions, parameters such as quantum efficiency, background levels, multiplicative noise, and spatial pixel averaging are ignored. Such an approach offers a simple argument for choosing one camera over another, yet it is an unrealistic presentation. With research dollars dwindling and more pressure to produce quantitative imaging results, choosing the right camera is critical. We have deeply considered all of these parameters and present the following conclusions and supporting evidence regarding sample dependent camera selection:

- The ORCA-Flash4.0, because of a combination of high QE and low read noise, without multiplicative noise, is capable of replacing traditional interline CCDs and EM-CCDs for most fluorescent imaging. In addition to having equal or greater sensitivity as EM-CCDs in demanding low light applications ($\geq$ 4 photons/pixel), the ORCA-Flash4.0 also offers larger field of view and faster frame rates than EM-CCDs.
- EM-CCDs are still the best choice for extremely low light applications (< 4 photons/pixel) that have no background.
- Traditional interline CCD cameras, because of low dark current, will only be used for long (minutes) exposures.
- Background from the sample must be considered and may

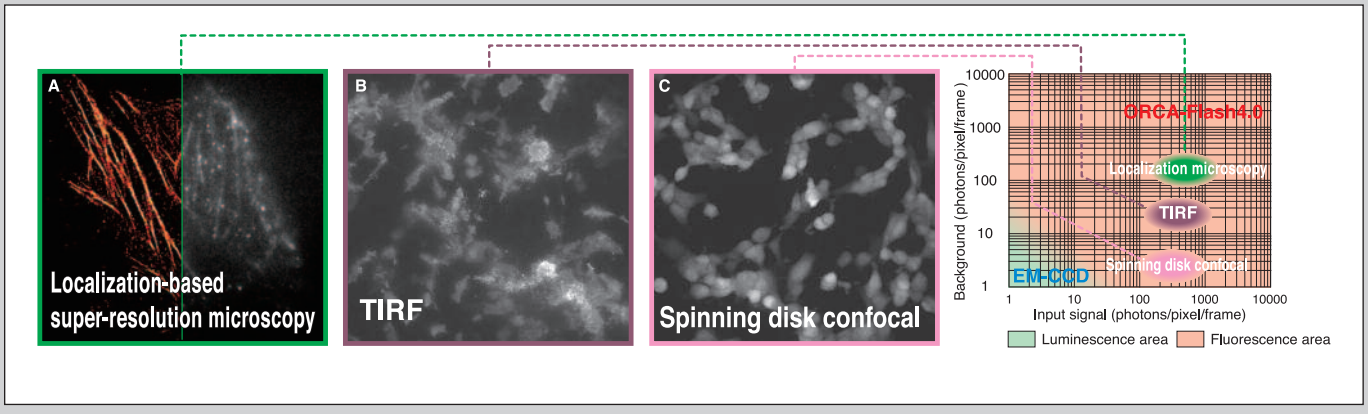


Figure 1. Signal photon and background photon levels in typical fluorescent microscopy applications. **(A)** HeLa cells labeled with d2EosFP. At left: reconstructed super resolution image. At right: single TIRF image from data used for reconstruction. **(B)** TIRF images of Ins-1 Cell MARCS-DsRed. **(C)** Wide-field confocal images of HEK293 cells stained with Fluo8-AM (Olympus DSU Spinning Disk Confocal). **(D)** Our data and experience suggest that most common fluorescent applications have between 10 to 1000 photons/pixel of signal at typical exposures times. In addition to signal photons, many applications also have background that exceeds 10 photons/pixel. At these signal and background levels, the ORCA-Flash4.0 provides higher SNR than either EM-CCDs or interline CCDs. EM-CCD performance is best suited to extremely low light applications with little or no background, such as luminescence. (Images graciously provided by (A) Prof. Zhen-li Huang, Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, and (B) Dr. Hideo Mogami, Hamamatsu University.)

become the defining factor in application dependent camera selection.

1. How do we compare CCD, EM-CCD, and sCMOS technologies?

With careful consideration, what first appears as a daunting task, in reality comes down to a few terms in an equation and some discoverable knowledge of your sample. Not only do we want to capture an image, we also want to understand the quality of the image (Fig. 1). In very general terms we are dealing with the following:

SAMPLE (Signal Photons + Background Photons) ⇨ Optics + Camera ⇨ Image

The goal is to have an image of the sample that most closely represents the reality of the sample. The sample, the optics and the camera are the three pieces that allow us to produce the image. The job of camera and optical engineers is to preserve the fidelity of the image by minimizing the effects of these pieces on the output. For cameras, this means keeping the noise of the camera as low as possible. Before adding the complexity of signal (with and without background) and optical systems, let's just look at the camera piece for now. And furthermore, let's assume we have a perfect detector (in this case, CCD or sCMOS). The perfect detector is noiseless, there is no variability; every photon is converted to a photoelectron (quantum efficiency, QE = 100%), every pixel voltage is digitized exactly the same (read noise, N_r = 0), and there are no sources of multiplicative noise (noise factor, F_n = 1). Of course, there are other features (pixel size, read out speed, etc.) that may be appealing, but at the core, if we could eliminate noise, we would have the perfect detector. Detector and camera engineers have been pursuing perfection since the first CCD in 1970. We are not there yet, but we are getting closer.

2. Pixel performance of the perfect camera

Let's consider the implications of achieving perfection on the question of pixel sensitivity. The current standard for discussion of sensitivity and quality of an image is to plot the signal to noise ratio (SNR) versus input photon number (Fig. 2a). An SNR can be calculated for a single pixel or for the average of a region of pixels. In most imaging we are interested in the latter but for now let's consider single pixel SNR at various light intensities. The formula for calculating a single coordinate of the SNR graph is:

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

QE: quantum efficiency

S: input signal (photon/pixel)

F_n: noise factor

N_r: readout noise

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

I_b: background

N_d: dark noise (not included, assumed to be negligible)

In our hypothetical perfect detector with an ideal signal (i.e., no background), this equation is now:

$$SNR = \frac{1 * S}{\sqrt{1^2 * 1 \times S + (0/1)^2}}$$

$$SNR = \frac{S}{\sqrt{(S)}}$$

$$SNR = \sqrt{S}$$

An important point to note is that even with a perfect detector, there is noise in the signal. This noise is a function of photon statistics and is called photon shot noise. Without negating fundamental properties of quantum physics, namely that we cannot have fractional photons, we are stuck with photon shot noise and it is the reason that we always want to collect more photons to improve our SNR.

Let's now consider two extreme cases of light levels using our ideal detector: a high input photon signal (1000 photons/pixel) level and a low input photon (10 photons/pixel) signal level.

Case 1. Perfect camera, high input photon signal level:

S=1000, I_b=0, *M*=1, *QE*=1, N_r=0

$$SNR = \frac{1 * 1000}{\sqrt{1 * 1 * (1000 + 0) + (0/1)^2}}$$

$$SNR = \frac{1000}{\sqrt{(1000)}}$$

$$SNR = 31.6$$

Case 2. Perfect camera, low input photon number:

S=10, I_b=0, *M*=1, *QE*=1, N_r=0

$$SNR = \frac{1 * 10}{\sqrt{1 * 1 * (10 + 0) + (0/1)^2}}$$

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

Signal & Background

S = signal, I_b = background. Photons falling on the sensor have an average photon flux. The fluctuations in this rate are governed by Poisson statistics and therefore have a standard deviation that is the square root of the number of photons (i.e. photon shot noise). In imaging, there are two sources of photons (and photon shot noise): the signal of interest (S) and the signal from the background (I_b). Limiting the amount of I_b and increasing S is critical to getting images with high SNR.

Quantum Efficiency

The QE of a camera is the wavelength dependent probability that photon is converted to a photoelectron. High QE is a fundamental attribute for obtaining high SNR, since QE is a predominant factor in the SNR equation.

EM-CCD only

M = EM gain, F_n = noise factor. EM gain occurs in a voltage dependent, step-wise manner and the total amount is a combination of the voltage applied and number of steps in EM register. EM gain has a statistical distribution and an associated variance, which is accounted for by F_n. At typical EM-CCD gains, $F_n = \sqrt{2} \cong 1.4$. All signal in an EM-CCD is subject to this additional noise. Since CCD and CMOS do not have EM gain, F_n = 1 in these cameras.

Camera Noise

N_r = read noise (e-). This is a statistical expression of the variability within the electronics that convert the charge of the photoelectrons in each pixel to a digital number expressing intensity.

N_d = dark noise (e-) (not shown above). This is camera noise that comes from thermally generated electrons and is time and sensor temperature dependent. N_d is not presented as a factor here because it is low and exposure times are short enough that it does not contribute significantly to the total noise.

Adding Noise Sources

Uncorrelated noise is added in quadrature. This means that each noise term must first be squared, then added to other terms, before the total noise can be calculated by taking the square root. The effect is meaningful: a read noise of 2 e- contributes 4 e- of noise to the total noise, while a read noise of 4 e- contributes 16 e-.

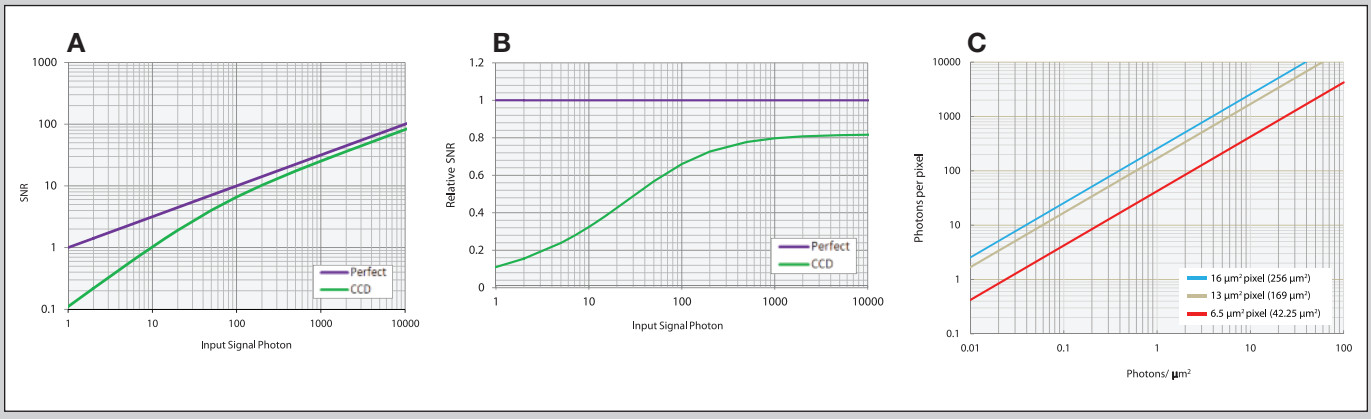


Figure2. The SNR for a hypothetical perfect camera compared to standard interline CCD camera. **(A)** Because there is no contribution from camera noise, the perfect camera SNR is a graph of $\sqrt{QE*S}$ where S = the number of input signal photons per pixel. For the CCD camera, at low input photon numbers, the SNR plot is influenced by read noise (N_r) until the signal reaches 1000 photons/pixel, then shot noise ($\sqrt{QE*S}$) from the signal dominates. **(B)** The graphs shown in (A) are converted to relative plots where the perfect camera is defined as 1. This presentation of the relative SNR clearly shows the difference between the two graphs. Even at high input photon numbers, the CCD can only achieve 0.85 SNR relative to the perfect camera. **(C)** Reference for converting photons per µm² to input photon number depending on pixel size. In SNR graphs that are presented without converting to input photon number per pixel, the primary factor in the comparison is pixel size. (For perfect camera: 100% QE, N_r = 0 e- rms, and F_n = 1; for CCD camera: 72% QE, N_r = 6 e- rms, and F_n = 1.)

$$SNR = \frac{10}{\sqrt{(10)}}$$

$$SNR = 3.16$$

Because we've calculated these using the perfect detector, 31.6 and 3.16 are the the absolute best possible SNR we can ever achieve with 1000 and 10 input photons numbers respectively. The full perfect SNR curve is plotted in Figure 2a and defines the upper limit of SNR across the range of input photon levels. A standard CCD is also show for comparison.

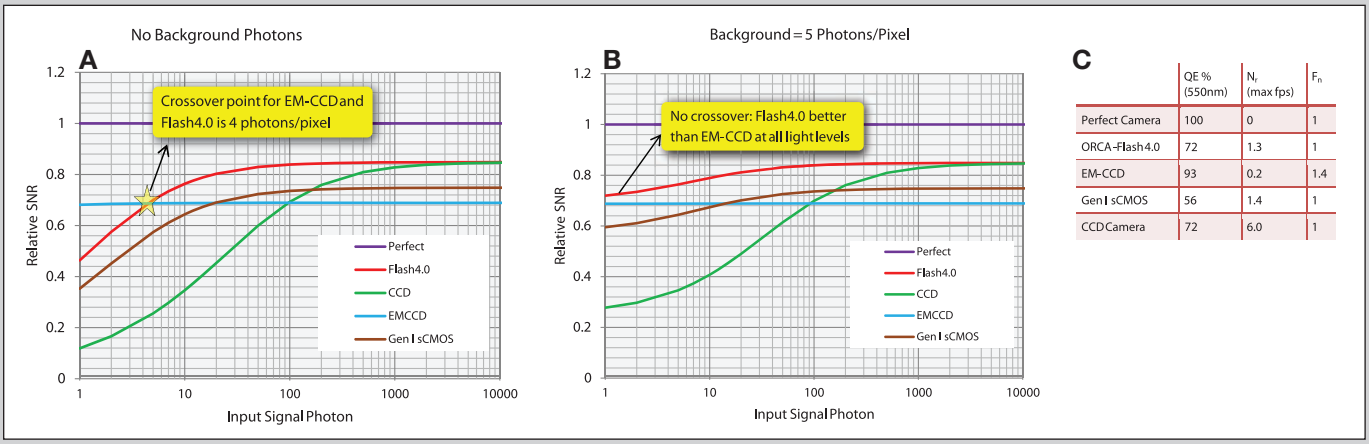
Throughout the rest of this article we will frequently normalize data collected under a variety of realistic conditions to our "perfect" camera. Our purpose in using this method is to provide a simple and visually accessible presentation of the effects of noise, including N_r, F_n, signal, and background on image data parameters such as SNR.

3. The effects of EM-CCD multiplicative noise (F_n) on a perfect camera

Our concept of a perfect camera is: 100% QE, 0 N_r and F_n of 1. However, we have not fully addressed the concept of F_n nor have we defined M. F_n and gain (M) are two factors that specifically apply to EM-CCD technology. EM-CCD sensors, introduced in 2001 by E2V (Jerram et al., 2001) and Texas Instruments (Hynecek, 2001), promised near camera perfection. In an EM-CCD, on-chip impact ionization amplifies the signal x M. The importance of this gain was that it allowed for a calculation of relative read noise as N_r/M. By applying a low EM gain setting (typically 100x – 200x), N_r becomes < 1. With almost no N_r and high QE achieved through back-thinning, EM-CCDs brought us a step closer than interline CCDs to the perfect camera. For the first time, we did not have to sacrifice speed for sensitivity, enabling high speed imaging of even single molecules. Up until now, EM-CCDs were a good choice for many demanding low light fluorescent applications including TIRF, wide-field confocal, Ca^{2+} imaging, single molecule imaging and super resolution precision localization microscopy. The release of our ORCA-Flash4.0 camera changes this game.

EM-CCD gain comes with a high price in performance that is often overlooked. The same mechanism (impact ionization) that achieves gain and effectively lowers N_r, also introduces additional statistical variation, i.e. noise. EM gain is a stochastic process and is characterized by a multistage binomial distribution. This means that any signal passing through the EM register, including sample signal, non-specific background signal and dark current signal are all subject to a multiplicative noise factor, F_n, which has been calculated to be $\sqrt{2} \cong 1.4$ (Robbins and Hadwen, 2003).

If we return to our perfect detector, we assumed an F_n of 1. To see how F_n affects SNR, let's redo our calculations of the perfect camera ($N_r = 0$, QE = 100%) but now include $F_n = 1.4$. For our high light condition the best possible SNR with an ideal EM-CCD is 22.36, and at low light it is 2.23 (0.7 relative to ideal). It's worth noting that current EM-CCD detector technology, with QEs exceeding 90%, is essentially the best we can achieve with EM-CCD; EM-CCDs have reached their full potential.



	QE % (550nm)	N_r (max fps)	F_n
Perfect Camera	100	0	1
ORCA-Flash 4.0	72	1.3	1
EM-CCD	93	0.2	1.4
Gen I sCMOS	56	1.4	1
CCD Camera	72	6.0	1

Figure 3. SNR for four cameras: the ORCA-Flash 4.0, an EM-CCD, a Gen I sCMOS, and standard CCD camera, relative to a perfect camera. **(A)** Relative SNR curves show the crossover point for the ORCA-Flash4.0 and EM-CCD is 4 photons/pixel at 550 nm. Thus for imaging extremely low light levels with no background, the EM-CCD is still the better choice. At all light levels above 4 photons per pixel, which is the case in most fluorescent applications, the ORCA-Flash4.0 provides the best SNR. **(B)** When the calculation for SNR includes background, the relative SNR plot shows that the ORCA-Flash4.0 outperforms all other camera at all input light levels. **(C)** Table showing specifications of each camera graphed in Fig. 3 (A) and (B). Specifications for Gen I sCMOS come from published specifications. Specification of EM-CCD is published for Hamamatsu ImagEM 512 x 512 EM-CCD camera and for CCD is best case scenario for the Sony ICX 285 sensor. All data presented assume optical adjustments are made to account for pixel size differences and that dark current contributions to the noise are negligible.

There is an easy way to think about F_n in an SNR calculation: for most microscopy applications, it effectively lowers the QE across the entire spectra by 50%. An EM-CCD camera that has a maximum physical QE of 90%, has an *effective* QE (eQE) of $QE/F_n^2 = 90\% / 2 = 45\%$. In light of this, it seems remarkable that EM-CCDs garnered such wide acceptance. It is important to keep in mind that when introduced, maximum frame rates of CCDs were about 10 fps with $N_r = 6 – 8$ e- rms. Thus, EM-CCDs were truly revolutionary and enabled tremendous advances in imaging that were not feasible with conventional CCDs.

Similar to the way EM-CCDs eventually challenged our notions of sensitivity and reset the lower limits of imaging, Gen II sCMOS technology requires us to revisit these concepts again. The ORCA-Flash4.0 achieves over 70% max QE, 1.3 e- N_r and inherently has an F_n of 1. At 550 nm and assuming each pixel images the same area in the sample, our ORCA-Flash4.0 has higher SNR than an EM-CCD with input photon numbers of 4 (background + signal) photons/pixel (Fig. 3a) or higher. At less than 4 photons/pixel, the lower relative read noise of EM-CCD gives it an advantage over the ORCA-Flash4.0. It's worth noting that the crossover point of the ORCA-Flash4.0 with the EM-CCD is wavelength dependent, but from 450 – 750 nm this value is always ≤ 10 input photons.

Such direct camera comparisons assume that appropriate optical adjustments are made to project equivalent photons per pixel on sensors with different size pixels. Obviously, if this is not done, then cameras with bigger pixels will collect more photons and show higher SNRs than cameras with smaller pixels. Bigger pixels also reduce spatial resolution. The best way to truly compare cameras directly on a microscope is to use optics to generate equivalent input photons. For a comparison of the ORCA-Flash4.0 to an EM-CCD with 16 μm^2 pixels this would require using a 0.40x demagnifying relay lens on the ORCA-Flash4.0 or a 2.5x magnifying lens on the EM-CCD. If these optical adapters are not used when direct camera comparison are made, then each 16 μm^2 pixel of the EM-CCD has 6.1x the photon detection area compared to the 6.5 μm^2 pixels of ORCA-Flash4.0. Just like a bigger bucket collects more rain, a bigger pixel collects more photons. If it is not possible to reduce the pixel size mismatch optically, it can be managed through digitally binning in the sCMOS. There is a 2x increase in N_r with 2 x 2 binning, but it allows for direct (1x relay lens) comparison to a 13 μm^2 pixel EM-CCD. Even under these suboptimal comparison conditions, the crossover point at which the ORCA-Flash4.0 exceeds the SNR of EM-CCD is 30 photons/pixel.

4. How many photons of signal do I have?

A very common and significant problem is that it is not possible to provide details about photon flux for a given sample. Since these numbers are key to choosing a camera, this presents a bit of a chicken and egg problem: camera selection is dependent on these parameters but one needs a camera to measure them. (A full description of quantifying photons in an image is beyond the scope of this presentation but such details are often covered extensively in scientific imaging workshops). As first approximation, we present images from three common microscopy methods and show both signal photon number and background photon number. Our experience and data suggest that most fluorescent applications have a photon flux of several hundreds of photons/pixel (Fig. 1). Even precision localization super resolution microscopy, a demanding single molecule application, often has 50 – 100 photons/pixel. Clearly this value is highly application and system dependent, but we feel confident that very few microscopy applications have fewer than 4 photons per pixel per frame.

5. Effects of optical background on SNR

While it's instructive to look at a single pixel in an ideal scenario our reality is not so simple. Images are composed of millions of pixels; some that have features/labeling of interest and some without. When we measure intensity we want to know the intensity over a region of pixels. Furthermore, we want to compare our signal of interest to other "blank" regions, so that we can understand how much the "signal" is composed of real sample signal versus background. It is very common for samples and optical systems to contribute unwanted background to our image and detectors cannot distinguish background from signal of interest. If we refer to our SNR equation, the total signal in the denominator only is the sum of the signal of interest and background (I_b). It's clear that background photons will reduce the SNR and this effect is multiplied by F_n in EM-CCDs.

Figure 3b shows the effect of having 5 background photons on an SNR plot. With increasing background photons, the signal photon level at which the ORCA-Flash4.0 SNR crosses over EM-CCD SNR is shifted towards fewer input photons. In other words, at 0 background, the ORCA-Flash4.0 SNR exceeds the EM-CCD at 4 input photons; at 5 photons of background, the ORCA-Flash4.0 is better than any EM-CCD or CCD at any input signal photon level.

Several trade articles have compared EM-CCD to Gen I sCMOS. These presentations have ignored the contribution of background in SNR calculations. Since we expect that most fluorescent imaging has meaningful background, we want to emphasize the importance

of considering background on SNR since it could have significant implications for selecting an application appropriate camera.

The logical next question is "What are my background photon levels?" Again, the answer is system dependent, but our data and conversations with researchers suggest that most "low light" fluorescent applications routinely have between 10 – 50 photons/pixel of background signal and occasionally are as low as 5 and more than 100 photons/pixel (Fig. 1). Many aspects of a system can contribute to optical background including the microscope optics and light scatter, the light-tightness of the fluorescent light path, non-specific labeling, the choice of dye or buffer, out of focus fluorescence or nearby fluorescence, etc. Optical techniques such as TIRF and spinning disk confocal that avoid imaging out of focus light help reduce background, but, especially in the case of spinning disk confocal, these techniques significantly reduce signal as well.

6. Making sense of SNRs.

Even with all of this discussion, it is not readily apparent what SNRs really means when translated into an actual image. One significance of the SNR is that it provides the limit of precision for intensity measurements in a given scenario. The SNR is not what we "see" when we look at an image; it is a measurement made on an image that can be used to make quantitative analysis of an image. A higher SNR yields better results after any computational imaging analysis. These considerations are particularly relevant to demanding applications such as precision localization microscopy (super resolution), where the noise of the camera directly affects the precision of the result (Quan et al., 2010; Mortensen et al., 2010).

SNR curves have often been used to demonstrate how camera specifications contribute to sensitivity. Traditionally, "low light" meant the region where camera read noise (N_r), not shot noise,

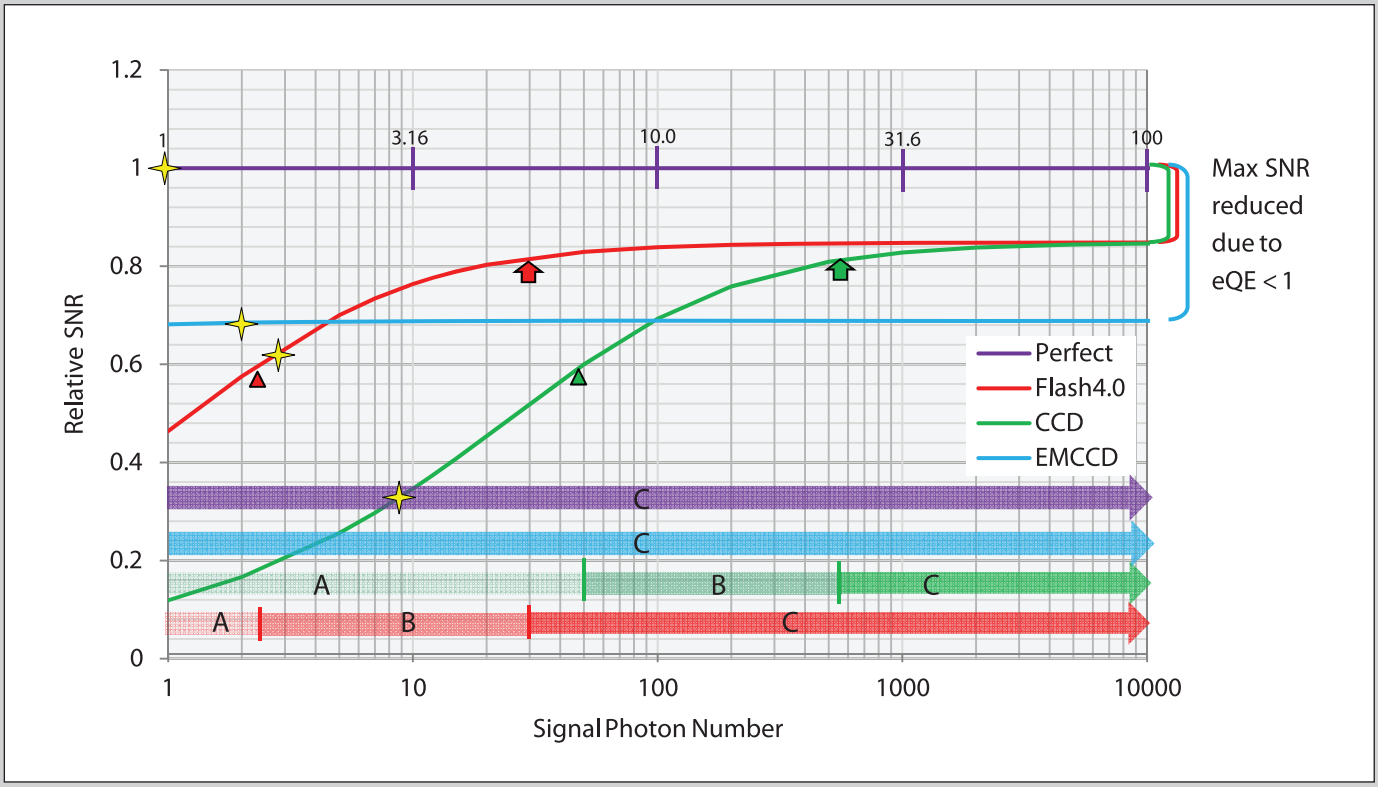


Figure 4. Effects of Camera Specifications on Relative SNR. Historically, N_r has been the primary camera spec used to define sensitivity. With the performance of Gen II sCMOS, it is crucial to understand how QE, N_r and F_n all affect SNR. The purple line is the relative SNR for a perfect camera. The numbers above this line indicates the SNR ratio at a series of intensities and SNR = 1 is indicated by a star on each curve. Because this is a perfect camera, these SNRs are only limited by photon shot noise. For each of the three real cameras on this graph, there are bars below that represent regions of each curve. At lowest light level, shown in region (A), N_r dominates relative SNR calculations ($S < N_r^2/(QE * F_n^2)$) and the crossover into shot noise dominated regions is the upper boundary of this low light region (triangle, 2.3 photons for ORCA-Flash4.0 and 50 photons for CCD). The (B) region is the intermediate zone, where N_r, eQE and F_n all contribute to the relative SNR. We define the upper boundary of this region as the point at which the curve is 95% of the maximum relative SNR for that camera (arrow, 20 photons for ORCA-Flash4.0 versus 550 photons for CCD). The (C) region is the high light region where eQE is the only camera parameter that matters (SNR loss shown by vertical brackets). These three regions are easily defined for the ORCA-Flash4.0 and for an interline CCD, both of which have $F_n = 1$. For EM-CCD the curve is flat. Except at the very lowest light levels, the EM-CCD curve mirrors the shape of the perfect camera almost exactly, except that SNR reduced to 0.68 of the value of the perfect camera. Thus, it is clear that in spite of low N_r and high apparent QE, the SNR of the EM-CCD is greatly affected by $F_n = 1.4$, and all input light levels in the EM-CCD reside in the region where eQE dominates. All parameters are the same as shown in Fig. 3.

dominated the SNR, for $S < N_r^2/(QE * F_n^2)$ (Fig. 4). Above this value, there are two distinct regions of a relative SNR curve, the region of increasing relative SNR, which depends upon QE, N_r and F_n, and the plateau region, where the SNR is only dependent on eQE. Every detector, for a given set of specs, has a very low light region (A), an intermediate zone (B), and a high light, eQE plateau (C). A crucial point is the concept that the camera parameters that determine "sensitivity" differ with increasing light levels. Understanding the three regions of an SNR plot gets to the core of understanding why the ORCA-Flash4.0 is likely the best choice for most fluorescent applications, when compared to current CCDs and EM-CCDs.

If we look at Figure 4, we can see that the specifications of the ORCA-Flash4.0 have significantly reset the locations of the three light levels relative to CCDs. First, the low light (A) region for the ORCA-Flash4.0 is very small, and ends at just 2.3 photons/pixel. The ORCA-Flash4.0 intermediate region (B) extends from 2.3 to 30 photons/pixel and (C) begins at 30 photons/pixel. For a CCD, the low light region (A) extends to 50 photons/pixel and the high light region (C) begins at 550 photons/pixel. From this perspective, it makes sense that the camera spec that defined low light sensitivity for CCDs was N_r and it's also easy to see why the ORCA-Flash4.0 hands-down outperforms the CCD.

EM-CCDs broke the barrier to low light sensitivity with major improvements in both QE and N_r compared to CCDs, but this was diminished by F_n reducing the QE to eQE. If you look at the relative SNR for an EM-CCD it is essentially flat and all input photon levels reside in the "high light" plateau region (Fig. 4). At low input levels it is flat because of the extremely low N_r. At higher photon levels there is no increase in the relative SNR because F_n cuts the QE by half (eQE). The importance of eQE on the upper limits of SNR should not be overlooked. This point explains why EM-CCDs and Gen I sCMOS, which both have low N_r, still failed to completely satisfy our imaging needs: they did not deliver high enough eQE.

Another way to look at the meaning of relative SNR curves is to define the input light level at which a pixel achieves an SNR of 1. In the absence of optical background, this value is the lowest light level at which a camera can make a barely visible image in a single frame at the full spatial resolution of the camera. What has happened with the release of the ORCA-Flash4.0, is that we've reduced this value to ~3 photons/pixel versus the ~9 photons/pixel required with an interline CCD, and have come very close to matching an EM-CCD at 2 photon/pixel.

Since most fluorescent microscopy applications are likely to have at least 5 photons per pixel and typically have 50 – 100 photons/pixel, the ORCA-Flash4.0 offers not only excellent low light capabilities but also extreme versatility over a range of signal levels. The performance of the ORCA-Flash4.0 also opens up the possibility to utilize higher frame rates, since the low light sensitivity allows for detection of very few photons. With regions of interest applied to the sensor, the ORCA-Flash4.0 is capable of over 25,000 fps.

7. When SNR is not enough

For quantifying the ability of a camera to detect a signal (i.e., "Can I see my signal?") and for defining the precision of the

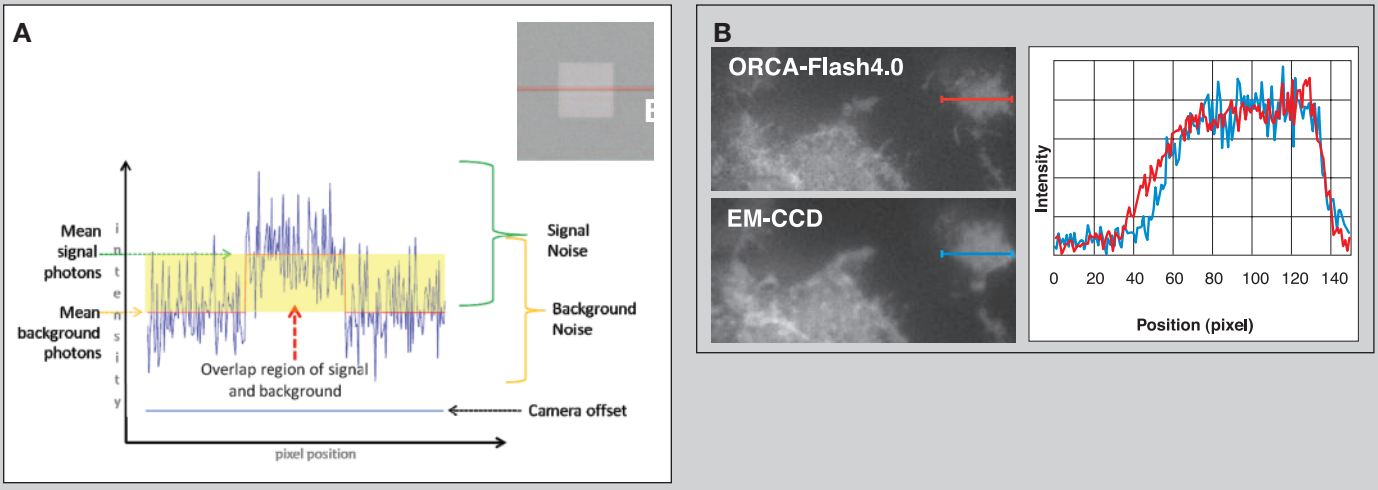


Figure 5. Contrast and noise. **(A)** The graph depicts the signal intensity and noise of the line through the inset gray squares and demonstrates the problem with background in the context of contrast. Contrast in an image is the perceived ability to distinguish between the background and the signal of interest. If both were noiseless, this would not be too difficult even if the signal was nearly identical to the background. However, camera noise and photon shot noise create an overlap in the signal and background regions with similar intensity, making it difficult to separate signal from background. **(B)** Because of F_n, the noise in images taken with an EM-CCD is greater than those from an ORCA-Flash4.0. Thus, when background is high, separation of signal from background in an EM-CCD image will be more difficult.

measurements made with a detector, the SNR of a camera is an extremely powerful tool. Yet there are cases when SNR cannot provide the most meaningful data. Imagine a photograph of a cityscape on a foggy day. The image is bright and would likely have a high SNR, but our ability to "see" the skyline is impaired because of the lack of contrast. Contrast is a general term that gets to concept of "visibility": Can I distinguish the signal of interest from the background signal? When we have an abundance of photons, i.e. in the shot noise regime, sensitivity becomes the ability to discriminate between two different, distinct levels of signal and in many cases the most important two levels are signal and background.

More than any other noise factor, background in the sample has been overlooked as a relevant term in considering sensitivity. This consideration is especially relevant for biological samples (Murray et al., 2007). We know that background affects SNR, but it also affects contrast. Fundamentally, because of photon shot noise, wherever there is background signal there is also background noise, yet this also has a camera component. Similar to SNR equations, it's possible to have contrast to noise (CNR) equations: $CNR = QE * S / [N_b + N_s]$ where N_b is noise of the background and N_s is noise of the signal. An easy way to visualize the difficulty that background and background noise poses in imaging is depicted in Figure 5. This figure shows that CNR is especially relevant when considering background in the context of choosing between an EM-CCD and the ORCA-Flash4.0. We know that the cross-over intensity into the shot noise regime is a function of the N_r of camera. Due to the great reduction in N_r with Gen II sCMOS cameras, in most fluorescence microscopy, both the signal and the background now reside in the shot noise (or eQE) domain. In this regime, because of F_n, the noise of the signal and background detected with an EM-CCD will be higher than with the ORCA-Flash4.0 resulting in reduced CNRs.

CNR also describes how we perceive the quality of the image. A good rule of thumb is that a pixel with a CNR of 2 can be detected by eye. On the low side, a pixel with a CNR of 1 can be just barely detected. However, this is a CNR for a single pixel of signal relative to background. Images with a CNR < 1 can show structures at reduced spatial or temporal resolution. When pixels of much lower CNR are grouped together, there is an effect called spatial pixel averaging (Fig. 6). When we look at images our brain performs complex functions including integrating large areas of similar signal, looking for patterns, symmetries and edges. For this reason, if we have a collection of adjacent pixels even with a very poor CNR (< 1), we may still be able to detect them visually. Mathematically, visibility is improved by the square root of the number of pixels averaged (Thompson, 2003). In a quantitative imaging experiment, measurements are made by well-defined algorithms, not by eye. But we can only view images in any publication or presentation with our eyes and therefore we must be aware of the spatial averaging or integration that is happening automatically in our brain. Along with this automatic visual processing, images that are displayed are subject to many variables intrinsic to the display format (e.g., quality of the monitor, intensity scaling of the image data, printing technique, etc.) that can affect the perceived contrast. For these reasons, determinations of the quality of an image from a given camera should never be assessed exclusively by eye or on image files that have been subject to lossy compression, such as jpeg.

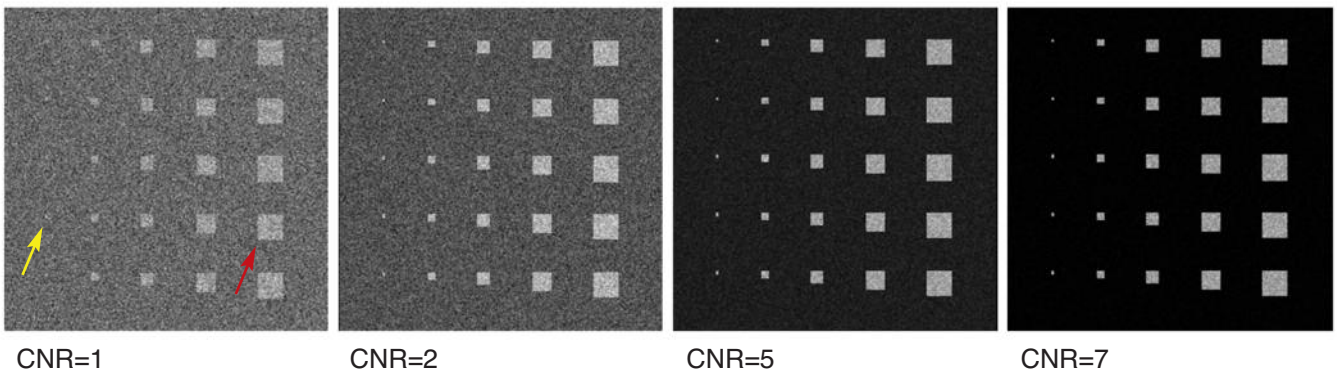


Figure 6. Demonstration of CNR and spatial pixel averaging using simulated images with "perfect" camera specs of N_r= 0 and QE = 100%. Each image is 256 x 256 pixels. Within each image there are five columns, each with five square spots. From left to right the spots in each column are 2, 4, 10, 15, 20 pixels square. **(A)** Each signal pixel has a CNR of 1. Without knowledge of the pattern in this image, the 2 x 2 pixel spots in the first column are just barely recognizable as signal (yellow arrow). The effects of spatial pixel averaging are readily apparent since the spots in all the other columns can be visualized. Because of spatial pixel averaging, the spots in the last column (red arrow), which are 20 x 20 pixels, have a $\sqrt{400}$ = 20x the visibility of a single pixel (Thompson, 2003). **(B, C, D)** These images show increasing CNR. By CNR of 2, even the first column is readily visible.

8. Special considerations about CMOS technology

As with any new technology, there are differences in the underlying structure that can require a deeper understanding to create a truly revolutionary product. This is true for sCMOS and we must take a minute to examine some of these distinctions so that our audience can be certain that we've done due diligence in the design of our camera. The three often expressed concerns about sCMOS involve the following: image uniformity, read out noise distributions, and rolling shutter.

One notable difference is sCMOS technology compared to CCD is the method of pixel readout. In CCDs, the pixels are all read out through a single amplifier and A/D converter. In a CMOS, each pixel acts as its own detector and has the circuitry to convert the photoelectron to a voltage. This voltage is then converted to a digital number though a column level amplifier and A/D. Because of this column level structure in sCMOS, image uniformity must be examined at all light levels. Images that are uniform at low light may show vertical stripes at middle or higher light conditions, and furthermore, these stripes may change with light intensity. Our Gen II sCMOS achieves new levels of image uniformity when compared to Gen I sCMOS images (Fig. 7). This excellent performance allows for quantitative imaging throughout the dynamic range of the camera.

Because there are more electronics involved in the formation of the image in sCMOS, the read noise of sCMOS has a wider distribution including a small percentage (< 1%) of outliers that occasionally show high read noise. Our read noise specification includes all outliers which is essential for the specification to be meaningful. In addition to careful design to minimize the presence of these "hot pixels" we have also implemented an on-board, real-time method to provide images without the visual distraction of "hot pixels," while maintaining the scientific integrity of the image. This filter is optional and can be turned on and off by user via software.

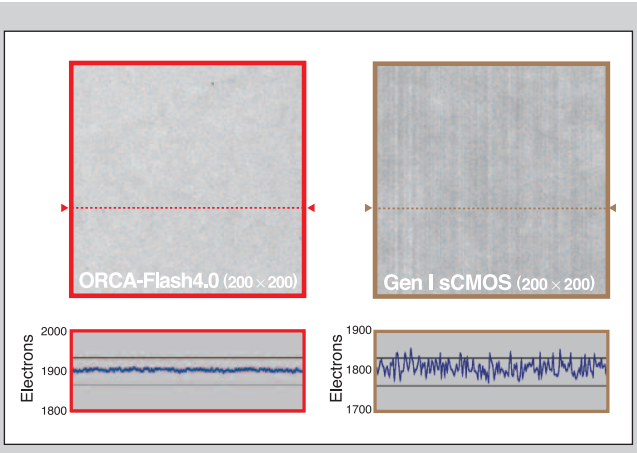


Figure 7. Comparison of image uniformity between the Gen II ORCA-Flash4.0 and a Gen I sCMOS camera. **(A)** sCMOS requires careful sensor and camera design to ensure uniform response for every pixel in the image. The ORCA-Flash4.0 has excellent image uniformity. **(B)** Gen I sCMOS shows vertical stripes. Because such patterns may not appear at low light levels it is important to look for these stripes at other intensities.

Another concern regarding sCMOS is the rolling shutter method of readout. Because of the readout structure, there is a temporal shift in the readout of each row of pixels, although each row is exposed for the same duration. This timing induces the concern is that moving objects cannot be imaged without distortion. Much of the general hesitancy about rolling shutter comes from consumer CMOS video camera discussions showing skewed horizontal lines. These cases, however, come about when a handheld video camera is moved much faster relative to the object, or in high speed industrial inspection and have little relevance to most scientific imaging. In the ORCA-Flash4.0, the row by row time differential of the rolling shutter is 10 µs resulting in a 10 ms (1024 x 10 µs) shift from first pixel to last for the full image. For high speed imaging, unless the sample is moving a distance of more than one pixel faster than the 10 µs per line temporal shift, and the exposure time is short, the ORCA-Flash4.0 will offer both improved temporal resolution and significantly less blurring than any CCD. Furthermore, when sCMOS is run in global shutter mode, the frame rates are reduced by half and there is an increase in N_r. The enhanced temporal resolution and low N_r mean that a rapid sequence of images collected in rolling shutter mode can be mathematically compiled to build a higher quality image than a single global shutter image of equivalent time. For these reasons and based on our two years of experience with the ORCA-Flash2.8 sCMOS camera, we see many advantages of rolling shutter and expect that it will work extremely well for the majority of microscopy applications.

Conclusions

Throughout this presentation, we've deconstructed the SNR equation into its component parts starting with a single pixel in a perfect camera and progressed to increasingly complex scenarios adding real-world camera specs such as read noise, F_n and QE and real-world sample challenges such as background. From SNR, we introduced the concept of CNR. The purpose of this process was to isolate the contribution of camera specs and photon shot noise to overall camera performance in a given application and to demonstrate that the specifications of the ORCA-Flash4.0 truly offer performance advantages over other cameras in the majority of microscopy applications.

The fundamental message of this paper is that the best possible images are captured with a camera that has both high eQE and low overall noise including N_r and F_n. With the advances offered first by EM-CCDs and now Gen II sCMOS, we cannot simply use N_r as a proxy for defining sensitivity; we must look carefully at all camera parameters. Interline CCDs provided high eQE but N_r was high. EM-CCDs seemingly offered sensitivity nirvana but the effect of F_n was either overlooked or deemed acceptable at the time. Gen I sCMOS delivered on noise but not on eQE. With the release of the the ORCA-Flash4.0, which has both high eQE and low N_r in the absence of F_n we are stepping closer to having a perfect camera. As discussed in Section 7, the ORCA-Flash4.0 lowers the crossover point into the shot noise regime, and this applies to both the signal and the background. This reduction in number of photons required to reach the shot noise domain means that the old "low light" has become the new "high light." In addition to signal levels, we also need to pay special attention to background photon levels since they likely no longer "hide" in the camera noise. For both EM-CCDs and sCMOS, background photons can significantly diminish the SNR and CNR of an image, but this is especially true with EM-CCDs since the background photons are subject to the same noise increasing effects of F_n as the signal photons.

We conclude that the ORCA-Flash4.0 is capable of providing the best SNRs for almost all life science imaging applications (Fig. 8). In addition to this quality of imaging, the ORCA-Flash4.0 has pixels that are ideally matched to typical micropscopy resolution needs, has many pixels to provide spatial resolution over a large field of view and with extremely fast readout offers excellent temporal resolution. We are confident that the combined features of the ORCA-Flash4.0 are changing the game of imaging, and we hope that you enjoy the results.

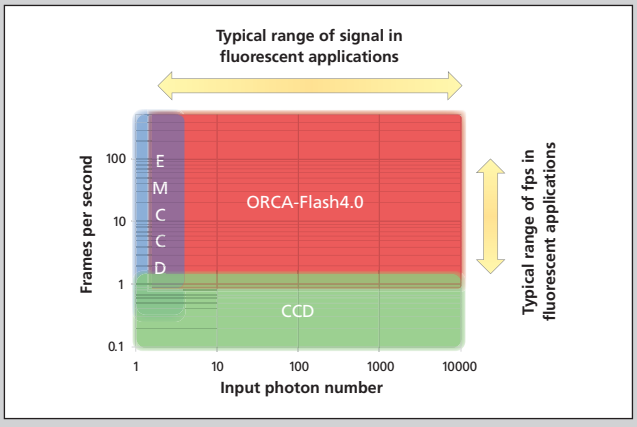


Figure 8. The ORCA-Flash4.0 offers extreme versatility. The specifications of the ORCA-Flash4.0 make it the ideal camera for a wide variety of fluorescent applications including those that have ≥ 4 photons/pixel to 1000s of photons/pixel and at full frame rates ranging from 1 to 100 fps (up to 25,600 fps with region of interest). These ranges correspond well with typical needs of fluorescent applications, including demanding single molecule fluorescence and precision localization microscopy. EM-CCDs, due to F_n, are most applicable at extreme low light (< 4 photons per pixel) with no background. CCDs, because of high N_r but low dark current, are most useful at slow frame rates across a range of input photon numbers.

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