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# Optimization of Precision Localization Microscopy using CMOS Camera Technology

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## ABSTRACT

Light microscopy imaging is being transformed by the application of computational methods that permit the detection of spatial features below the optical diffraction limit. Successful localization microscopy (STORM, dSTORM, PALM, PhILM, etc.) relies on the precise position detection of fluorescence emitted by single molecules using highly sensitive cameras with rapid acquisition speeds. Electron multiplying CCD (EM-CCD) cameras are the current standard detector for these applications. Here, we challenge the notion that EM-CCD cameras are the best choice for precision localization microscopy and demonstrate, through simulated and experimental data, that certain CMOS detector technology achieves better localization precision of single molecule fluorophores. It is well-established that localization precision is limited by system noise. Our findings show that the two overlooked noise sources relevant for precision localization microscopy are the shot noise of the background light in the sample and the excess noise from electron multiplication in EM-CCD cameras. At low light conditions ( $< 200$  photons/fluorophore) with no optical background, EM-CCD cameras are the preferred detector. However, in practical applications, optical background noise is significant, creating conditions where CMOS performs better than EM-CCD. Furthermore, the excess noise of EM-CCD is equivalent to reducing the information content of each photon detected which, in localization microscopy, reduces the precision of the localization. Thus, new CMOS technology with 100fps,  $< 1.3 e^-$  read noise and high  $QE$  is the best detector choice for super resolution precision localization microscopy.

**Keywords:** Scientific-CMOS, sCMOS, EM-CCD, localization microscopy, STORM, PALM, excess noise, super resolution

## 1. INTRODUCTION

Light microscopy imaging is being transformed by the application of computational methods that permit the detection of spatial features below the optical diffraction limit. Successful localization microscopy (STORM, dSTORM, PALM, etc.) relies on the precise position detection of fluorescence emitted by single molecules using highly sensitive cameras with rapid acquisition speeds. The precision of localization is dependent on the entire system, everything from the properties of the fluorescent molecules to the alignment of the optical system to the amount of noise in the detector. Thus, simply by understanding detector noise and making choices to reduce the noise and noise sources in the detector, it is possible to increase the precision of localization. This paper presents evidence that sCMOS cameras, especially Gen II versions, due to fewer noise sources and therefore lower total noise offer better precision localization performance when compared to electron multiplying CCDs (EM-CCDs). In addition, Gen II sCMOS also provides fast frame rates, large field of view and high dynamic range, making Gen II sCMOS a new and highly valuable option for precision localization microscopy.

### 1.1 Camera Noise Sources

Noise in the measurement of the optical intensity is a significant factor determining the localization precision. Although EM-CCD cameras have traditionally been used for localization microscopy due to their very low read noise ( $N_r$ ,  $< 1 e^-$ ) and high apparent quantum efficiency ( $QE$ ), excess noise ( $F_n$ ) arising from the electron multiplication process broadens the statistical distribution of the camera digital output<sup>1</sup> significantly reducing localization precision. The effect of  $F_n$  in EM-CCDs is roughly equivalent to reducing the quantum efficiency by a factor of 2.

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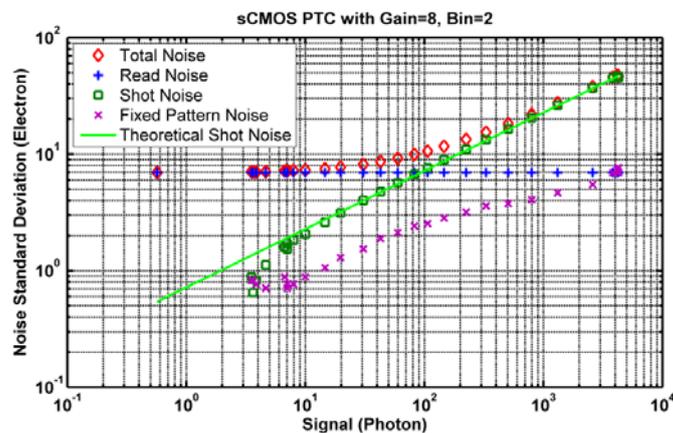
Noise in the measurement of optical intensity arises from two distinct sources:

- 1) Photon physics: photon shot noise, a Poisson distribution
- 2) Camera characteristics
  - a)  $QE$ : photons incident on the camera are converted into photoelectrons. As both photons and electrons are quantized, the conversion process is characterized by a binomial distribution
  - b)  $N_r$ : electronics that convert photoelectrons into digital signals adds noise, usually Gaussian
  - c)  $F_n$ : EM-CCDs use a many ( $> 100$ ) stage multiplication process with a small ( $g-1 \ll 1$ ) gain ( $g$ ) at each stage to multiply the number of photoelectrons. This process is stochastic, and characterized by a multi-stage binomial distribution, which adds noise, termed “excess noise.” In EM-CCDs operating at gains typically used, the excess noise broadens the standard deviation of the output signal by the  $\sqrt{2}$ , which has effectively the same effect on the pixel signal to noise ratio as reducing  $QE$  by 50%. CCD and sCMOS detectors have no excess noise ( $F_n=1$ ).

## 1.2 Photon Transfer Curves (PTC) as Method for Measuring Noise in sCMOS and EM-CCD

As extensively documented by Janesick<sup>2</sup>, photon transfer curves are a method of evaluating the performance of cameras. In Fig 1, the PTC for ORCA-Flash2.8, a Gen I sCMOS, is paired with a PTC for an EM-CCD. The mismatch between the theoretical photon shot noise and the actual measured standard deviation in the number of electrons for a given signal in the EM-CCD plot shows the excess noise of EM-CCDs.

A.



B.

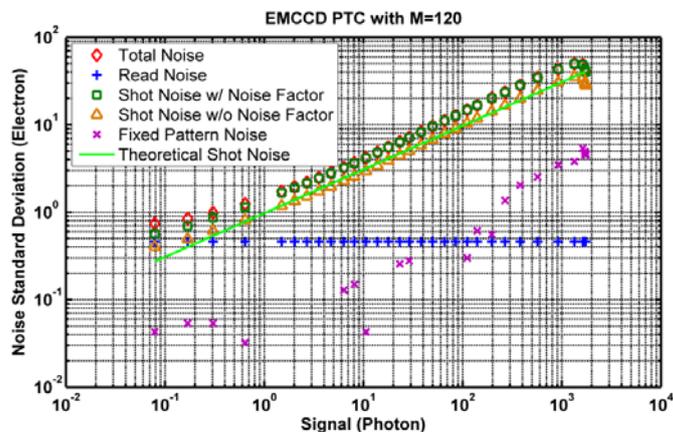


Figure 1. Photon transfer curves (PTCs) are a recognized method of testing camera performance. By carefully measuring camera response through range of photon levels, it is possible to plot values for various noise sources. Unlike the sCMOS, the curve for the EM-CCD does not match the theoretical photon shot noise curve. The reason for this mismatch is the excess noise factor due to electron multiplication noise. Data measured and provided by Prof. Zheng-li Huang<sup>3</sup>.

### 1.3 Probability Distribution Function (PDF) of Camera Output

Another way to examine the noise in camera is to plot the probability distribution function of a specific signal. The camera pixel output is the convolution of the probability distributions of each of the noise sources: Poisson distribution of the input photons, binomial distribution of the conversion from photons to photoelectrons,  $N_r$  and  $F_n$ . Statistically, this is described by a probability distribution function: the probability of a specific output signal for a given input (average photon flux). In Fig. 2, we show the PDF for the cameras presented in Table 1.

Table 1: Camera Specifications

Camera (Hamamatsu Corporation)	Status	Technology	QE @560 nm	Read Noise $N_r/M$	Noise Factor $F_n$
ImagEM	Existing	EMCCD	92%	< 0.1 e-	$\sqrt{2}$
ORCA-Flash4.0	New	Gen II sCMOS	72%	1.3 e-	1

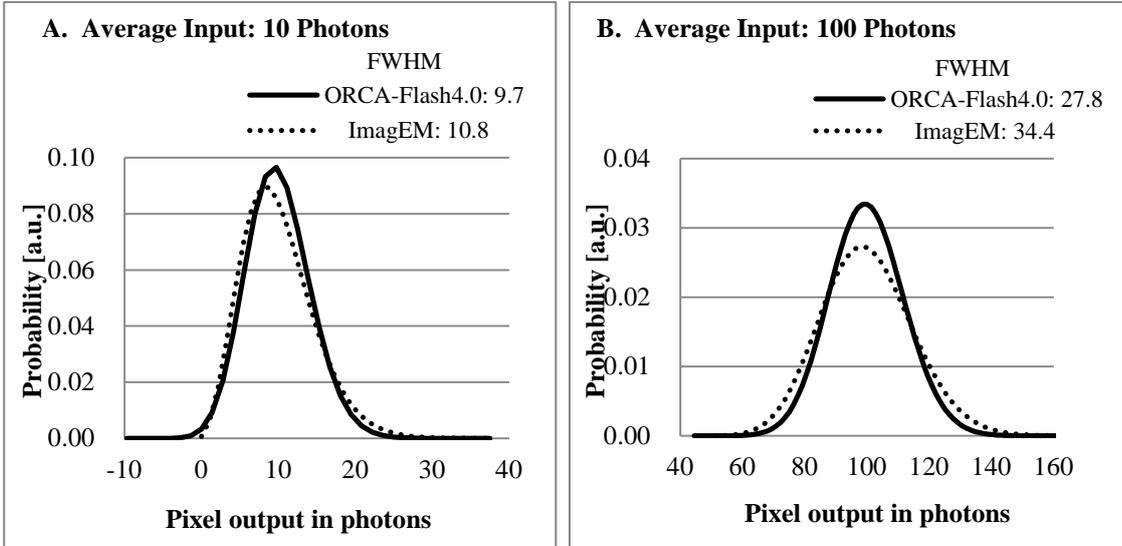


Figure 2: Simulated PDFs of current ImagEM EM-CCD camera and new ORCA-Flash4.0 Gen II CMOS camera at (A) 10 photons average/pixel/frame, typical of the intensity of optical background in localization microscopy and (B) 100 photons average/pixel/frame, typical of the collected intensity levels for single molecules fluorescent proteins. At 100 photons average pixel/frame, the full width at half maximum (FWHM) of the ORCA-Flash4.0 camera is 20% less than an EM-CCD.

### 1.4 Signal to Noise Ratio (SNR) of Camera Pixels

The variance of the camera pixel output is given by the sum of the variance of each noise source. The SNR, which is the ratio of the output signal to the standard deviation of the signal, is given by:

$$SNR = \frac{QE \times S}{\sqrt{F_n^2 \times QE \times (S + I_b) + (N_r / M)^2}} \quad (1)$$

$QE$ : Quantum Efficiency

$S$ : Input Signal (mean photons / pixel)

$F_n$ : Noise Factor (=1 for CCD/sCMOS and  $\sqrt{2}$  for EM-CCD)

$N_r$ : Readout Noise ( $e^-$ )

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

$I_b$ : Optical Background (mean photons / pixel)

Figure 3 shows the comparison of the SNR of an EM-CCD and the ORCA-Flash4.0 Gen II sCMOS at 600nm. The SNR for the ORCA-Flash4.0 is greater than that of an EM-CCD for a photon flux of more than 5 photons per pixel. This crossover value is wavelength dependent and from 450nm-900nm is  $\leq 10$  photons per pixel for these two cameras.

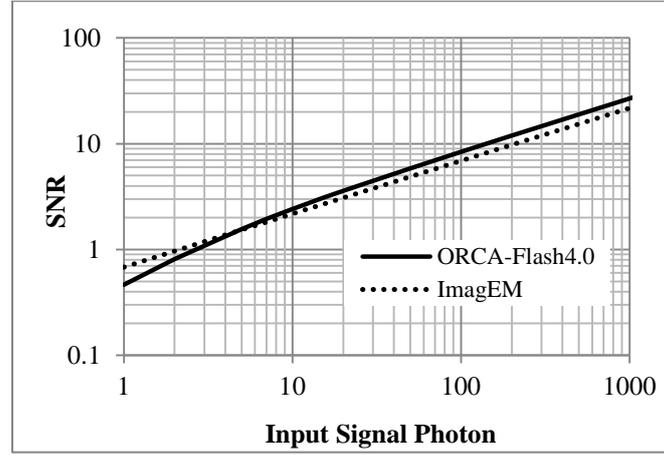


Figure 3: SNR curves for the ORCA-Flash4.0 versus ImageEM EM-CCD at 600 nm. Above 5 mean photons/pixel (@ 600 nm wavelength) the SNR of the ORCA-Flash4.0 exceeds the SNR of the EM-CCD.

## 2. LOCALIZATION PRECISION

### 2.1 Localization Precision for CMOS and CCD Cameras

In 2002, Webb *et al.*<sup>4</sup> examined the factors that limit the precision of single molecule localization and presented an equation to describe these precision limits over a range of condition. This paper estimated the variance ( $\Delta x^2$ ) of the position using Gaussian least squares fitting. This formula has been re-written to explicitly show the effect of camera  $QE$ , and to separate the contributions of  $I_b$  and camera  $N_r$ :

$$\text{CMOS or CCD } \langle \Delta x^2 \rangle = \frac{s^2 + a^2 / 12}{QE \times P} + \frac{8\pi s^4 (QE \times I_b + N_r^2)}{a^2 (QE \times P)^2} \quad (2)$$

$s$ : standard deviation of point spread function (Gaussian)

$a$ : size of camera pixel referred to sample (pixel size/magnification)

$QE$ : Quantum Efficiency

$P$ : total number of signal photons collected

$I_b$ : background photon in a pixel

$N_r$ : Readout Noise

## 2.2 Localization Precision for EM-CCD Cameras

Webb's limit of precision localization equation (2) did not account for EM-CCD excess noise due to multiplicative gain. More recently, several authors have considered the impact of excess EM-CCD noise on localization precision<sup>3,5,6,7</sup>. Presenting Quan's<sup>6</sup> results to properly account for the complete effect of excess noise in the statistics obtained from the optical background and writing to explicitly separate the contribution from EM-CCD excess noise yields:

$$\text{EM-CCD } \langle \Delta x^2 \rangle = \underbrace{\left[ \frac{s^2 + a^2/12}{QE \times P} + \frac{8\pi s^4(QE \times I_b)}{a^2(QE \times P)^2} \right]}_{\text{Noise equivalent for CCD or CMOS}} + \underbrace{\left[ \frac{s^2}{QE \times P} + \frac{8\pi s^4(QE \times I_b)}{a^2(QE \times P)^2} \right]}_{\text{Excess noise } (F_n^2 - 1) \text{ from electron multiplication}} \quad (3)$$

## 3. RESULTS

Our first evidence showing the performance advantage of the ORCA-Flash4.0 over EM-CCDs for precision localization was calculated using equations (2) and (3) with values for the ORCA-Flash4.0 and EM-CCD from Table 2. These data show that for a given number of photons, the localization precision of the ORCA-Flash4.0 is better than the EM-CCD.

Table 2. Camera settings and specifications used for calculations and simulations.

Camera	Gen II sCMOS (ORCA-Flash4.0)	EM-CCD (ImagEM)
Pixel size ( $\mu\text{m} \times \mu\text{m}$ )	6.5 x 6.5	16 x 16
$QE$ @ 550 nm	72%	92%
Read noise, $N_r$ , e-	1.3	100
EM Gain	-	500
$F_n$	1	1.4
Optical Magnification	60 x 1.2 (with relay lens)	60 x 2.96 (with relay lens)
Field of view in pixel (nm)	90	90

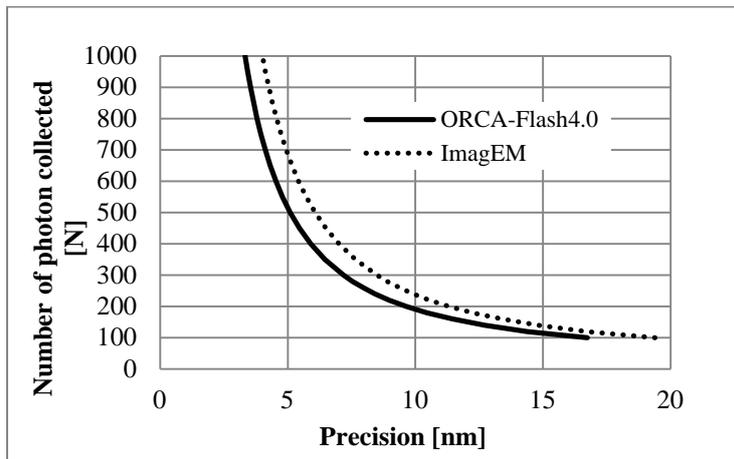


Figure 4: The theoretical number of collected photons required to achieve a given localization precision. Using equation (2) for the ORCA-Flash4.0 and equation (3) for the EM-CCD, and assuming a background of 10 photons/pixel, the ORCA-Flash4.0 sCMOS camera achieves better precision localization for a given number of photons collected.

### 3.1. Simulation of localization images

To go beyond theoretical calculations and to simplify camera and algorithm performance evaluations for localization precision microscopy, we developed a Camera Simulation Engine (CSE) using MATLAB. In this simulation code, a set of camera specifications are evaluated for the quality of localization precision using a stack of simulated images and a standard precision localization algorithm (MaLiang<sup>7</sup>). Figure 5 shows the simulated image structure: 4 line pairs with intervals of 30, 35, 40, and 45 nm oriented along a diagonal relative to the pixel array in the camera. The areal intensity profile of each fluorophore is modeled as a Gaussian and the emission intensity is set to 500 collected photons per molecule (consistent with a weak fluorescent molecule) and was simulated to avoid any overlap of single molecules in any given frame. The optical background, which in biological samples typically comes from non-specific fluorescence, was assumed to be uniform with a temporal mean in each pixel of 44 photons/frame. For details of CSE please see Ref. [8].

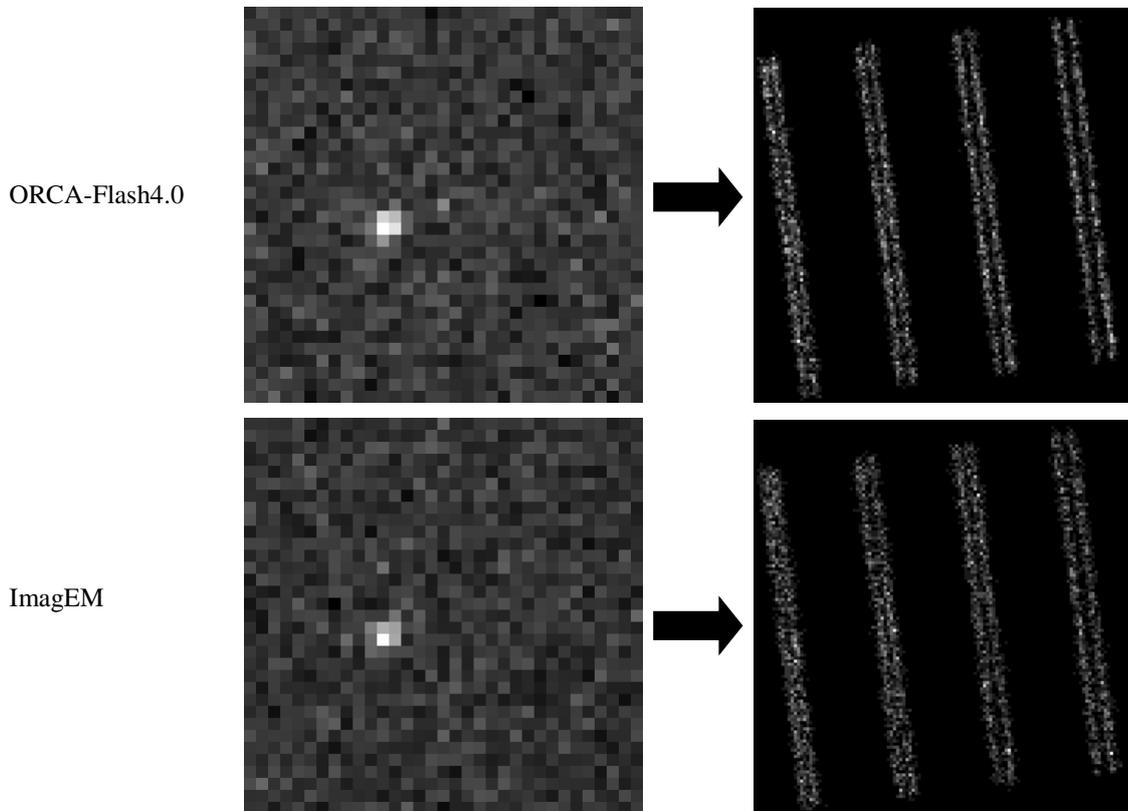


Figure 5: Simulation image of single fluorophore (left) and reconstruction image (right). Using our CSE, we defined the conditions to simulate raw images of non-overlapping single molecules and used the MaLiang method to build reconstructed super resolution images. The variables entered in the CSE include camera specifications such as  $QE$ ,  $N_r$ ,  $F_p$ , and pixel size and optical system magnification, along with fluorescent molecule properties. The camera specifications used to create these images are shown in Table 1 and the simulations are made using 500 photons collected from each molecule. The 4 pairs of diagonal lines in the reconstructed image are spaced at 30, 35, 40 and 45 nm to allow for easy visualization of image quality. Our reconstructed images show the improvement in precision localization using the ORCA-Flash4.0 versus the EM-CCD.

### 3.2 Precision localization from theoretical calculations and simulated images

Equations (2) and (3) represent one method (Gaussian least squares) of determining precision localization. The MaLiang method used to analyze our CSE generated single-molecule images is a maximum likelihood method.

Other algorithms that have been developed. See for example, Mortensen<sup>5</sup> and the references in Quan<sup>6</sup>. Each may provide slightly different results or may be more or less sensitive to certain types of noise. In Fig 6A we present data comparing the EM-CCD and ORCA-Flash4.0 sCMOS using the localization precision algorithms in equations (2) and (3) over a range of background levels. Based on this theoretical model of fitting precision, the ORCA-Flash4.0 offers better performance. In Fig 6B we use the MaLiang method combined with our CSE to show that our precision localization simulation data supports the results that would be expected from the higher SNR of the ORCA-Flash4.0: the ORCA-Flash4.0 offers better precision localization performance. Furthermore, as expected, our MaLiang results present better precision than theoretical models of localization precision based on Gaussian least squares fitting.

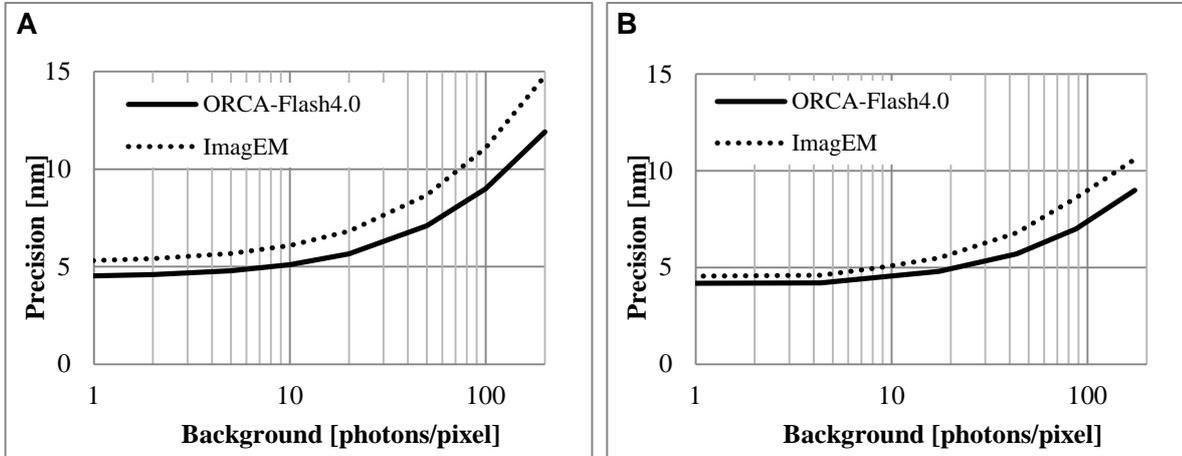


Figure 6: (A) Precision of localization using equation (2) and equation (3), and (B) results from the CSE combined with MaLiang reconstruction for the simulated images of Fig. 5. These graphs show the precision of localization for our simulated images at various background photon levels assuming 500 collected photons from a single molecule emission. Under these conditions, our ORCA-Flash4.0 provides more precise localization at all background photon levels.

### 3.2 Demonstration of Photo-Activated Localization Microscopy (PALM) using an sCMOS camera

Prof. Zhen-li Huang published a comparison of PALM images of d2EosFP fluorescent protein (560 – 700 nm emission wavelength, imaged with a 580 nm long pass filter) labeled actin filaments obtained with an Andor iXON EM-CCD camera and a Hamamatsu ORCA-Flash2.8 sCMOS camera<sup>3</sup>. As the sCMOS camera had much smaller pixels (3.6  $\mu\text{m}$ ) than the EM-CCD (16  $\mu\text{m}$ ), an optical reduction lens of 0.5X and digital 2 x 2 binning of the sCMOS data was used prior to the localization computation, which resulted in an effective read noise of  $\sim 7 e^-$ , or twice the read noise that would be expected with a properly matched optical system. Even so, images obtained with the ORCA-Flash2.8 are nearly the same quality as obtained with an EM-CCD camera.

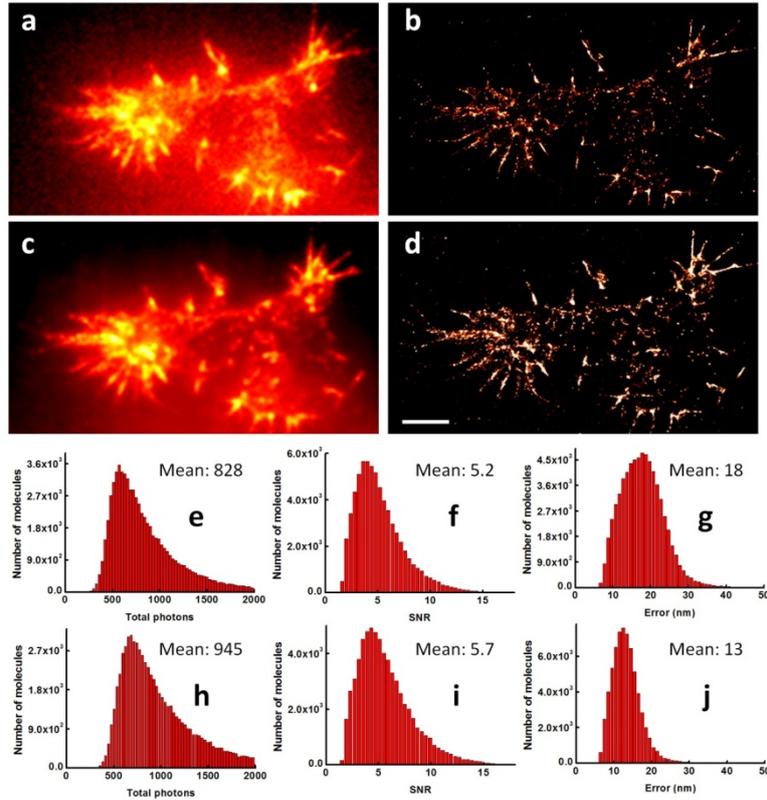


Figure 7: Comparison of the performance of the Flash 2.8 sCMOS camera (subset 1 in a, b, e, f and g) and the iXon 897 EMCCD camera (subset 2 in c, d, h, i and j) in TIRF microscopy (a, c) and localization microscopy (b, d) imaging of actin bundles in fixed HEK293T cells labeled with d2EosFP. A stack of 2000 image frames was firstly captured with the Flash2.8 sCMOS.

#### 4. DISCUSSION

From the beginning of super resolution precision localization microscopy until now, the only cameras available that provided the speed along with the necessary sensitivity were EM-CCD cameras. The advances in sCMOS technology including low noise and high frame rates along with the absence of electron multiplication noise, suggested that this technology could provide a new camera solution for precision localization microscopy. Huang et al. (2010) tested this hypothesis and demonstrated localization microscopy with 18 nm localization standard deviation using d2EosFP fluorescent proteins and the ORCA-Flash2.8 sCMOS camera (Hamamatsu Photonics.) Even with optical conditions favoring EM-CCD performance, this Gen I sCMOS performed roughly equivalent to an EM-CCD<sup>3</sup>.

Subsequent to these results, the first Gen II sCMOS camera, the ORCA-Flash4.0 was released. Offering higher  $QE$  over a range of wavelengths, lower  $N_r$ , larger pixels, faster frame rates, without EM-CCD multiplicative noise, this camera has specifications particularly well suited for super resolution precision localization microscopy. We have tested this hypothesis using theoretical calculations based on well-established methods and through CSE simulated super resolution images reconstructed using the MaLiang algorithm. Both approaches have confirmed our hypothesis: the ORCA-Flash4.0, due to a combination of low noise, high  $QE$  without excess noise, provides better precision localization than EM-CCD technology. Specifically, obtaining the same localization precision with the ORCA-Flash4.0 Gen II sCMOS camera requires 20% fewer photons/molecule as would be required with an EM-CCD. There are numerous advantages to needing fewer photons including the ability to obtain better localization precision with weaker fluorophores, to collect better images in the presence of high optical background or to achieve better temporal resolution

for particle tracking. It is also important to note that at the photon levels typical (10 – 100 photons / pixel / frame) of localization microscopy, the noise of the ORCA-Flash4.0 is dominantly photoelectron shot noise<sup>9</sup>.

In our investigation of EM-CCD versus sCMOS for precision localization we observed that the excess noise due to EM-CCD multiplicative gain was frequently overlooked. Since the limit of precision localization is highly dependent on the noise in the signal and background of the raw images, it is not surprising that excess noise negatively affects the outcome of precision localization data. The effects of excess noise can be summarized as follows:

1. The effective  $QE$  ( $eQE$ ) of an EM-CCD is roughly  $\frac{1}{2}$  the “nominal”  $QE$ .
2. In general, for fluorescence microscopy, the SNR of EM-CCDs is significantly reduced compared to Gen II sCMOS cameras especially the presence of optical background.
3. The maximum SNR of EM-CCDs is 70% of the photoelectron shot noise limit.

## 5. FUTURE DIRECTIONS

This presentation provides the foundation for further investigation of the performance of sCMOS for precision localization microscopy. We have on-going efforts to directly compare the ORCA-Flash4.0 to EM-CCD cameras using a variety of localization precision optical and mathematical methods. Furthermore, we are investigating Fisher Information Theory so that we can establish the fundamental limit of the precision with which a molecule can be localized. The Fisher Information Theory limit of precision is computed for a particular measurement system and conditions (number of photons collected from the fluorophore, optical background, illumination, magnification, system model, camera, etc.) from the probability distribution functions of the measurements. This limit does not depend upon the reconstruction algorithm. In practice, however, the effect of noise on localization precision depends upon the algorithm used for reconstruction. We also plan to enhance our CSE to allow for easy simulations of precision localization results under a variety of conditions, with numerous different algorithms and with real and theoretical camera specifications. We encourage researchers interested in collaboration on these questions to contact us.

## 6. CONCLUSIONS

Fundamentally, localization precision is limited by noise including shot noise from the localized molecule and from background, read noise, and EM-CCD excess noise. Our results demonstrate that sCMOS is a valid option for precision localization and that the Gen II sCMOS camera, the ORCA-Flash4.0, has better performance than EM-CCDs. In addition to enabling more precise localization, the Gen II sCMOS also provides a larger field of view, faster frame rates and higher dynamic range than an EM-CCD making the ORCA-Flash4.0 an extremely versatile camera for a wide range of microscopy applications.

## ACKNOWLEDGEMENTS

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