Localization-based super-resolution microscopy with an sCMOS camera Part II: Experimental methodology for comparing sCMOS with EMCCD cameras

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Abstract: Nowadays, there is a hot debate among industry and academic researchers that whether the newly developed scientific-grade Complementary Metal Oxide Semiconductor (sCMOS) cameras could become the image sensors of choice in localization-based super-resolution microscopy. To help researchers find answers to this question, here we reported an experimental methodology for quantitatively comparing the performance of low-light cameras in single molecule detection (characterized via image SNR) and localization (via localization accuracy). We found that a newly launched sCMOS camera can present superior imaging performance than a popular Electron Multiplying Charge Coupled Device (EMCCD) camera in a signal range (15-12000 photon/pixel) more than enough for typical localization-based super-resolution microscopy.

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1. Introduction

Localization-based super-resolution microscopy (or called localization microscopy) has been
recognized as one of the most promising tools for biology by offering unprecedented spatial
resolution from relative simple setup [1]. The selection of an appropriate low-light camera is
critical in realizing localization microscopy, since this kind of microscopy is based on repeated
cycles of imaging and localization of single fluorescence molecules to reconstruct a final
super-resolution image [2].

Currently, Electron Multiplying Charge Coupled Device (EMCCD) cameras are widely
accepted as the image sensors of choice for localization microscopy [2, 3], because EMCCD
cameras utilize electron multiplication processes to effectively eliminate camera read noise and
thus are capable of detecting the weak fluorescence signal from single molecules [4]. Unfortunately,
the use of electron multiplication also introduces an additional noise (called
excess noise) and thus effectively halves the quantum efficiency of EMCCD cameras [5]. And,
the maximum available data read-out rate of EMCCD camera (~35 MHz), which is
fundamentally limited by the serial-readout nature of CCD technology [4], obstructs the
versatility and power of super-resolution localization microscopy in applications where both
resolution and throughput are desired, for example, visualizing the connectivity of neural
circuits extended over vast volumes [6].

On the other hand, with the rapid advancements in complementary metal oxide
semiconductor (CMOS) technology [7], a new generation of scientific-grade CMOS (sCMOS)
camera has been developed into a truly low-light detector, whose imaging performance
approaches the key requirements crucial for single molecule detection. Taking advantages of
the parallel-readout nature of CMOS technology, sCMOS camera offers low read noise (1~2 e-) at extremely rapid readout rate (up to 560 MHz) [8]. Therefore, ever since its launch in late 2010, sCMOS has been expected to be used in a wide range of application fields which are currently dominant by EMCCD. In fact, several trade articles or white papers from major camera manufacturers used technical values from marketing literature and tried to estimate when will sCMOS begin to provide better imaging performance (characterized by a key parameter, signal-noise-ratio, SNR) than EMCCD. Although the crossover point varies significantly (4~48 photon/pixel) among these estimations, commercial sCMOS cameras are expected to become the cameras of choice when the signal is > 50 photon/pixel [9–12].

Considering the potentials of sCMOS cameras in localization microscopy, recently we performed an experimental investigation on the applicability of commercial sCMOS camera in localization microscopy. We found that, with the use of bright fluorescence probes such as d2Eos, it is possible to use a commercial sCMOS camera in the detection of single molecules [13]. Later, M. P. Bruchez et al also proved that commercial sCMOS cameras are capable of detection and localization of single Cy5 molecules, although the EMCCD cameras used in their study exhibit better imaging performance [14]. However, these findings are dependent on the experimental conditions used, and there is still no quantitative and precise description of the overall imaging performance of sCMOS cameras.

In this paper, we developed an experimental methodology, which is based on two key parameters including image SNR and localization accuracy, to characterize the performance of low-light cameras in single molecule detection and localization. Quantitative comparisons on three commercial low-light cameras, including a Hamamatsu Gen I Flash 2.8 sCMOS, a Hamamatsu Gen II Flash 4.0 sCMOS, and an Andor iXon 897 EMCCD, were performed. The contributions of different camera noises to the image SNR were also investigated.

2. Methods

We adopted a well-established technique called photon transfer curve (PTC) measurements to characterize many important features of a camera's response to illumination [15]. From the PTC measurement results, we calculated the dependence of image SNR on signal intensity, since the latter is better understandable to researchers in the field of image processing and, more importantly, is a key parameter that describes a camera's overall performance. We derived an experimental methodology, which is based on repeated imaging of fixed point-like emitters (fluorescent beads with diameter < 200 nm), to quantitatively evaluate the performance of a camera in single molecule detection (characterized by image SNR) and localization (via localization accuracy).

2.1 PTC measurement

The PTC measurement is one of the most valuable tools to quantify the imaging ability of cameras by measuring the response of camera at different illumination intensity, which can be used to extract many useful camera specifications including shot noise, read noise, fixed pattern noise, full well capacity, dynamic range [15]. In principle, these parameters are obtained from image frames at different illumination intensities, which start with no light and end with the intensity that makes sensor saturation became barely evident.

For low light detectors with proper cooling accessories, the total noise of an image frame includes mainly read noise, shot noise and fixed pattern noise. These noises can be calculated from the following steps: **Step 1:** The read noise can be obtained through the distribution of pixel values of the image frame under no illumination light, since this kind of noise is signal independent. **Step 2:** Notice that the fixed pattern noise results from sensitivity difference between pixels. Therefore, subtracting two image frames taken successively at the same illumination intensity effectively eliminates the fixed pattern noise and leaves the read noise (known from Step 1) and shot noise. **Step 3:** The fixed pattern noise can be calculated by subtracting the shot noise and read noise (both known from previous steps) from the total noise.
Plotting the noises as a function of illumination intensity generates a PTC, which can be used to obtain the full well capacity or dynamic range. Specific optical setup and data analysis method for PTC measurements were reported in detail in our previous paper [13]. The calculation of column fixed pattern noise and pixel fixed pattern noise were according to the method reported by A. El Gamal et al [16].

2.2 Experimental methodology

In the field of optical microscopy, fluorescent beads whose diameter is smaller than the diffraction limit of visible light (~200 nm) are often regarded as point-light emitters, and thus are widely used to measure the point spread function (PSF) of an optical microscope [17]. Based on this concept, we derived an experimental methodology to quantify the performance of a camera in single molecule detection and localization. This methodology is based on repeated imaging of fixed point-like emitters whose fluorescence intensity is controlled to match that from single molecules typically used in localization microscopy. In this methodology, the performance of a camera in single molecule detection and localization is characterized by image SNR and localization accuracy, respectively.

2.2.1 Defining image SNR

In image processing, SNR is the most important parameter for quantifying the visibility of the features of an image to the observer. To quantitatively evaluate the capability of a camera in detecting weak fluorescence emission from single molecules, here we calculate the SNR of the peak pixels in the fluorescence images from point-like fluorescence emitters.

For a fluorescence image from single point-like emitter, the peak signal \( N_{\text{sig}} \) locates in the peak pixel (the pixel with highest intensity value) of the PSF. According to the original definition of SNR (that is, signal/noise), the SNR of the peak pixel can be defined as

\[
SNR_{\text{theory}} = \frac{N_{\text{sig}} \times QE}{\sqrt{\left( N_{\text{sig}} + N_{\text{bkg}} \right) \times QE \times F_n^2 + N_{\text{camera}}^2}}
\]

(1)

where QE is the quantum efficiency of the camera, \( N_{\text{bkg}} \) is the average photon background, and \( N_{\text{camera}} \) includes mainly read noise and fixed pattern noise. The excess noise factor of the camera (\( F_n \)) is 1 for sCMOS (or CCD) and \( \sqrt{2} \) for EMCCD, respectively. Note that this equation assumes that the noise originated from photon background follows a Poisson distribution. Thereafter we call this image SNR “Theoretical Image SNR”.

If there is no photon background in the image, a camera is working with “Optimal Image SNR” and Eq. (2) can be modified to

\[
SNR_{\text{optimal}} = \frac{N_{\text{sig}} \times QE}{\sqrt{N_{\text{sig}} \times QE \times F_n^2 + N_{\text{camera}}^2}}
\]

(2)

In some trade articles and white papers from major camera manufacturers [9–12], the total noise is assumed to include only shot noise, read noise (RN), and excess noise, while photon background noise and fixed pattern noise are both ignored. This kind of image SNR, thereafter called “Simplified Image SNR”, can be calculated as

\[
SNR_{\text{simplified}} = \frac{N_{\text{sig}} \times QE}{\sqrt{N_{\text{sig}} \times QE \times F_n^2 + RN^2}}
\]

(3)

Experimentally, it is difficult to separate different noise sources from images. Therefore it would be convenient to use a new parameter, \( \sigma_{\text{bkg}} \), to represent the total background noise per pixel, which mainly consists of read noise, fixed pattern noise, and the noise associated with background fluorescence and/or residual excitation light. Thus Eq. (1) can be rewritten as
which we call “Experimental Image SNR” thereafter.

2.2.2 Defining localization accuracy

In the community of localization microscopy, the localization accuracy of an imaging system under a certain experimental condition is mainly estimated from single fluorescence images using a proper equation, which takes into account the photon noise from signal, pixelation noise, and background noise [18–20]. W. E. Moerner et al [21] and X. W. Zhuang et al [22] pointed out separately that it is better to measure localization accuracy from the distribution of the center positions of individual point-like emitter, which can be obtained by repeated imaging and localization of the same emitter. However, detailed description and discussions on this methodology were not reported in the literature.

The methodology originates from the following considerations. For a stationary point-like emitter visualized by an imaging system, due to the fluctuations in signal and photon background and/or pixelation noise, its center positions from repeated imaging are usually found to spread across a 2D plane. If stage drift is efficiently eliminated, such variation in the center positions reflects solely the measurement uncertainty, called localization accuracy, of the imaging system. Therefore, if the fluorescence emission from a fixed point-like emitter is captured by a 2D array of pixels (Fig. 1(a)), the center position of the emitter can thus be calculated by fitting the image to a 2D Gaussian function (see the red cross in Fig. 1(a)). Repeated imaging and localization of the emitter presents a distribution of the center positions (Fig. 1(b) and 1(c)). Thus the localization accuracy (σ) of the imaging system can be obtained by averaging the standard deviation of the center positions in x and y dimension, that is, $\sigma = \sqrt{\sigma_x^2 + \sigma_y^2}$ . It is worthy of note that in practical experimental design, it is necessary to control tightly on the fluorescence intensity of the emitter and the stage drift (see Section 3.2).

![Fig. 1. Illustration for determining the localization accuracy from repeated single molecule imaging. (a) PSF of a point-like emitter and its center position (shown by the red cross) found by 2D Gaussian fitting. (b) Distribution of the center positions of the emitter from repeated imaging. (c) A representative histogram showing the center positions projected in x dimension. Note that the localization accuracy is averaged from the standard deviation of the histograms in x and y dimensions.](image)

2.2.3 Imaging experiment

Fluorescent beads (170 nm in diameter, FluoSpheres, Invitrogen) in distilled water were deposited on a specimen slide, allowed to air dry, covered with a cover slip on top and then sealed with nail polish for further use.

The optical setup for localization microscopy imaging is similar to the one reported previously [13], except that in this study it is not necessary to use the 405 nm photoactivation laser. Fluorescent beads were excited with a 561 nm diode-pumped solid-state laser, and the fluorescence was filtered with a narrow band-pass filter (FF01-580/14-25, Semrock). Because
the cameras used in this study have different pixel sizes, particular considerations on the total magnification of the microscope system were made. For comparing the Flash 4.0 and the Flash 2.8 sCMOS cameras, fluorescence was collected with a 60x/NA1.42 oil immersion objective (PLAPON60XO, Olympus), and a 0.5X C-mount adapter was placed before the Flash 2.8. In this case, the pixel size at sample plane is 108 nm for the Flash 4.0, and 121 nm for the Flash 2.8, respectively. For the comparison of the Flash 4.0 sCMOS and the iXon 897 EMCCD, fluorescence was collected with a 100x/NA1.49 oil immersion objective (UAPON 100XOTIRF, Olympus), and a 0.35X C-mount adapter was placed before the Flash 4.0, corresponding to the pixel size at sample plane of 186 nm for the Flash 4.0, and 160 nm for the iXon 897, respectively. The image acquisition time for all the measurements was 30 ms and no adapter was placed before cameras unless specified otherwise.

In the comparison, the illumination intensities from the 561 nm laser were varied to allow desired number of detected photons from fluorescent beads. The photon background levels were controlled by adjusting the voltage of the halogen lamp, which is typically used as the light source for bright field observation. Under certain illumination intensity, a set of 500 successive image frames was captured under each photon background level.

2.2.4 Calculating image SNR
As mentioned in Section 2.2.1, image SNR is a good indicator for comparing the performance of different cameras in single molecule detection. For experimental images without prior knowledge on camera noise, it is effective to use $SNR_{\text{experiment}}$ for the comparison. However, before calculating $SNR_{\text{experiment}}$ using Eq. (4), it is necessary to use the camera conversion factor provided by camera manufacturer to convert the signal in the peak pixels ($N_{\text{sig}}$) from grey value to photon. Then, the $SNR_{\text{experiment}}$ was calculated for individual images in a set of 500 successive image frames, and then averaged to minimize the effect from associated noise.

If the prior knowledge on camera noise is known (typically by PTC measurement), it is beneficial to calculate $SNR_{\text{theory}}$ and evaluate the contribution of different noise sources to single molecule detection. Here in calculating $SNR_{\text{theory}}$ with Eq. (1), $N_{\text{sig}}$ was averaged from all sets of image frames with the same illumination intensity.

2.2.5 Calculating localization accuracy
Localization accuracy is used to compare the performance of different cameras in single molecule localization. For individual fluorescence image, the center position of the emitters was determined by the previously reported MaLiang method [23], which fits pixelated fluorescence images to a 2D Gaussian function using maximum likelihood estimator. The localization accuracy of the imaging system under certain photon background intensity was estimated from a set of 500 successive image frames. To minimize the effect from stage drift, these image frames were divided into 10 groups equally according to the capture time, and each group provided localization accuracy by the statistics method described in Section 2.2.2. The localization accuracy was obtained by averaging all the localization accuracy values from the 10 groups.

3. Results and discussions
3.1 Low-light cameras for localization microscopy
To evaluate relative sensitivity performance of popular low-light cameras on the market today, image SNR was calculated for a popular EMCCD camera (Andor iXon DU 897 BV) [24], a representative cooled CCD camera (Photometrics CoolSNAP HQ2) [25], a first generation sCMOS camera (Hamamatsu ORCA-Flash 2.8) [26] and an improved (second generation) sCMOS camera (Hamamatsu ORCA-Flash 4.0) [27]. Several important parameters of these
cameras are showed in Table 1. To keep consistence with trade articles and white papers from major camera manufacturers [9–12], here we calculated the “Simplified Image SNR” \( (SNR_{\text{simplified}}) \), see Eq. (3)) for these low-light cameras.

From the \( SNR_{\text{simplified}} \) curves, the iXon 897 EMCCD exhibits superior sensitivity than other cameras when signal is < 4 photon/pixel, while the Flash 4.0 sCMOS is the best choice when signal is higher than this threshold (Fig. 2). The sensitivity advantage of the iXon 897 EMCCD is even overtaken by the HQ2 CCD when signal is > 110 photon/pixel, and by the Flash 2.8 sCMOS when signal is > 180 photon/pixel, respectively (see the inset in Fig. 2). Interestingly, the HQ2 CCD is better than the Flash 2.8 sCMOS when signal is > 65 photon/pixel and becomes the second best cameras when signal is > 110 photon/pixel. However, in practical experiments, the relative low full frame rate of HQ2 camera limits its versatility in localization microscopy. On the other hand, considering the magnification (typically 60x or 100x) of the objectives with high numerical aperture, when an iXon897 or a Flash 2.8 is used in a localization microscope, secondary magnification is usually required to meet the Nyquist sampling criterion (that is, 4 to 5 pixels to cover a PSF) [17].

| Camera     | Pixel number | Pixel size (µm) | QE \(^a\) | Read noise at full speed | Fast frame rate at full field (frame/s) |
|------------|--------------|-----------------|-----------|-------------------------|-------------------------------------|
| HQ2        | 1392 × 1040  | 6.45            | 0.62      | 4.5 e- @ 10 MHz         | 11                                  |
| iXon 897   | 512 × 512    | 16              | 0.95      | < 1 e- @ 10 MHz (with EM gain) | 35                                  |
| Flash 2.8  | 1920 × 1440  | 3.63            | 0.52      | 3 e- (with 8x gain)     | 45                                  |
| Flash 4.0  | 2048 × 2048  | 6.5             | 0.72      | 1.3 e-                  | 100                                 |

\(^a\)Quantum efficiency at 580 nm.

Fig. 2. Simplified Image SNR for representative cameras. The read noise of the iXon 897 was set to be 0.5 e-, corresponding to a EM gain of 120 [24]. The inset in the right corner is an enlarged view of the region marked by the red rectangle. The signal values for the crossovers were shown by the numbers nearby.
3.2 Experimental methodology for camera comparison

Recently, two papers were reported to evaluate experimentally the performance of sCMOS cameras in single molecule detection and localization [13, 14]. These papers adopted almost the same methodology, which is mainly based on statistical differences on image SNR and localization accuracy, to compare the newly developed sCMOS camera with other popular low-light cameras (especially EMCCD). The reliability of this experimental methodology is limited by (1) the stability of fluorescence emission from point-like emitters, which could not be obtained satisfactorily from either chemical probes or fluorescence proteins because these probes emit fluorescence with a broad range of intensity, and (2) the suitability of the methods used to calculate localization accuracy, which are not unified in the literatures [18, 28, 29].

Accordingly, in this paper the experimental methodology has two major modifications: (1) the point-like emitters are changed to fixed fluorescent beads with diameter smaller than the diffraction limit of visible light (~200 nm), and (2) the localization accuracy is calculated as the distribution of the center positions of individual fixed point-like emitters (see Section 2.2.2).

The use of fluorescent beads is based on the following considerations. First of all, fluorescent beads with diameter smaller than Airy disk size are frequently used as point-like emitters in single molecule imaging [21, 22]. Secondly, fluorescent beads allows a long imaging time with narrow emission intensity distribution and negligible photobleaching. We found that, in 5000 successive image frames under appropriate illumination intensity, the fluctuation is 3% in the total number of detected signal from fluorescent beads, and 5% in photon background, respectively. Thirdly, because fluorescent beads emit very strong fluorescence, the total number of detected signal photons from fluorescent beads can be adjusted continuously by a careful control on the illumination intensity, thus allowing for easier matching the detected signal photons from fluorescent beads with those from fluorophores typically used in localization microscopy.

In addition, the stage drift of our optical setup for 500 successive image frames was estimated to be less than 5 nm over the entire image acquisition time. Therefore, it is not necessary to correct stage drift when calculating the localization accuracy from only 50 successive image frames.

3.3 Comparing camera noises

3.3.1 PTC measurements

Camera noise can be well quantified by photon transfer curve (PTC) measurements [15]. Here representative PTC results for the Flash 4.0 and the iXon 897 are shown in Fig. 3. It was found the read noise is 1.6 e- for the Flash 4.0, and 0.5 e- for the iXon 897 with EM gain 120, respectively, which are consistent with the data reported by the corresponding manufacturers.

When taking a close look at the PTC of the iXon 897, we found that the read noise and fixed pattern noise are relatively negligible to the total noise in almost the entire dynamic range; however, the excess noise contributes significantly to the total noise by multiplying the shot noise with a constant value of $\sqrt{2}$ (Fig. 3(b)). The PTC of the Flash 4.0 shows three distinct detection regions: read noise limited region (< 4 photon/pixel), shot noise limited region (4-3500 photon/pixel), and fixed pattern noise limited region (3500-40000 photon/pixel). For localization microscopy where the signal is typically within 50-2000 photon/pixel (marked with the red rectangular in Fig. 3, see Section 3.3.2 for explanations), the both cameras are working in the shot noise limited region.

In addition, we found that the full well capacity of the iXon 897 with EM gain of 120 (~2000 photon/pixel) is much lower than that of the Flash 4.0 (40000 photon/pixel) (Fig. 3). Notice that the maximum full well capacity ($\text{FWC}_{\text{max}}$) of the iXon 897 is ~220000 electron when no EM gain (M) is applied, and the effective full well capacity of EMCCD is inversely proportional to the EM gain setting ($\text{FWC}_{\text{eff}} = \text{FWC}_{\text{max}}/M$). On the other hand, according to the dependence of effective read noise on EM gain [24], for the iXon 897 a read noise of 1.6 e- (the
same as that in the Flash 4.0) can be obtained when the EM gain is reduced to 30. In this case, the effective full well of the iXon 897 increases to ~8000 photon/pixel, but is still significantly lower than that from the Flash 4.0.

![Graph](image)

**Fig. 3.** Representative PTCs for the Flash 4.0 (a) and the iXon 897 with EM gain 120 (b). Note that: (i) Due to insufficient illumination intensity, image frames close to the Flash 4.0 camera's saturation (signal > 7600 photon/pixel) were obtained by increasing exposure time from 30 ms to 200 ms. (ii) These are representative curves from three independent measurements which presented almost identical results. (iii) The PTC for the iXon 897 is similar to that reported in our previous paper [13]. (iv) Theoretical Shot Noise was calculated by the square root of Signal*QE. The QE (quantum efficiency) in 580 nm was set to be 0.72 for the Flash 4.0, and 0.95 for the iXon 897, respectively. (v) The excess noise factor is \( \sqrt{2} \) for the iXon 897. (vi) The red rectangles highlight typical signal ranges in localization microscopy.

### 3.3.2 Total noise vs. different noise sources

It would be beneficial to evaluate the contributions of different noise sources to the total noise in these low-light cameras, especially in the peak signal intensity range from typical fluorescence emitters used in localization microscopy. To accomplish this task, we firstly examined the reported signal intensity values in literatures and found out that the total detected signal is 500-1200 photons for fluorescence proteins, and 1000-6000 photons for chemical probes [22, 30, 31]. Then we assumed that the PSF of these emitters has a Gaussian profile, and
that the PSF was covered by different pixel array sizes (sampling densities). Finally, through simple calculations this peak signal range was found to be 50-2000 photon/pixel (Table 2).

Since the read noise (RN), shot noise (SN) and fixed pattern noise (FPN) are independent of each other, the square of total noise (TN) equals to the sum of squares of all the noise sources (that is, \( TN^2 = RN^2 + SN^2 + FPN^2 \), see Eq. (6) in our previous paper [13]). This equation is valid for sCMOS cameras, but needs to be modified to include the excess noise (EN) in EMCCD cameras. According the relationship between shot noise and excess noise, the equation for EMCCD can be obtained as \( TN^2 = RN^2 + SN^2 + EN^2 + FPN^2 \), where the SN refers to the \textit{Shot Noise w/o Noise Factor} in Fig. 3(b). Basing on the considerations shown above, we calculated the percentage contribution of different noise sources to total noise from the PTC measurement results shown in Fig. 3 and in our previous paper (see Fig. 4 in the previous paper, Gain = 8, Bin = 1 for the Flash 2.8) [13]. Here the percentage was defined as the square of individual noise divided by the square of total noise.

The contribution of different noise sources to total noise under different incident signal intensities are shown in Fig. 4. In the signal range between 70 and 2000 photon/pixel, the shot noise of the Flash 4.0 is the dominant noise source, verifying by the finding that the percentage of shot noise to total noise is > 90% (Fig. 4(a)). For the Flash 2.8, the contribution of shot noise to total noise increases gradually and becomes dominant (> 90%) when the signal intensity is > 300 photon/pixel (Fig. 4(b)). Surprisingly, for the iXon 897, excess noise is equal to shot noise and contributes ~50% to total noise. Therefore, it is clear that for the peak signal range typically used in localization microscopy (the red rectangles in Fig. 4), the Flash 4.0 is the best camera of choice. It is worthy of note that this camera is closest to perform shot noise limited imaging.

The read noise has a fixed value for individual cameras, and thus would become significant only when the incident signal is very weak. In fact, for the signal intensity < 50 photon/pixel, the contribution of the read noise to the total noise could not be neglected for the sCMOS cameras. On the other hand, the fixed pattern noise depends on the signal intensity and will become evident especially in an sCMOS camera, which has an additional column fixed pattern noise than an EMCCD camera (see Section 3.3.3 for details). For example, in the Flash 4.0, the contribution from the fixed pattern noise to the total noise increases gradually when the signal intensity is > 2000 photon/pixel, and becomes larger than that from the shot noise when the signal intensity is > 18000 photon/pixel.

Table 2. Peak signal intensity of individual fluorescence emitters under different sampling densities

| Total emitted signal (photon) | Peak signal intensity (photon) |
|------------------------------|--------------------------------|
|                              | 3 x 3 | 5 x 5 | 7 x 7 |
| 500                          | 160   | 80    | 50   |
| 1200                         | 400   | 200   | 120  |
| 6000                         | 2000  | 1000  | 550  |

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3.3.3 Fixed pattern noise and image uniformity

Fixed pattern noise (FPN) has been a huge limitation for CMOS-based cameras [7], since this kind of camera suffers not only from pixel FPN (pFPN) which is related to pixel-to-pixel sensitivity differences, but also column FPN (cFPN) which originates from column-to-column
differences in voltage column readout circuits and results in obvious strip bands in images [32]. As a product manufactured from CMOS technology, sCMOS cameras intrinsically also face these drawbacks. With these considerations, researchers who are interested in integrating sCMOS cameras into their localization microscopy systems would naturally worry that sCMOS cameras may have poor performance in image uniformity. Therefore it would be beneficial to compare quantitatively the image uniformity of the Flash 4.0 sCMOS camera with the popular Andor iXon 897 EMCCD camera, which is manufactured from CCD technology and has only pixel FPN.

We investigated the image uniformity of the Flash 4.0 sCMOS and the iXon 897 EMCCD at different illumination intensities (Fig. 5). Under the same uniformly illumination intensity, a set of 100 successive image frames was captured by each camera and then averaged. Random noise (such as read noise and shot noise) is reduced by the averaging, thus the remaining pixel intensity fluctuation (or imaging uniformity) is mainly cause by the FPN [16]. Representative images under different intensities of uniform illumination are shown in Fig. 5. Here the pixel intensity is converted from grey value to electron using the camera conversion factors obtained by PTC measurements (see Section 3.3.1).

When illumination intensity is low, the Flash 4.0 provides similar imaging uniformity with the iXon 897 (see Fig. 5(a)-5(b) and Fig. 5(e)-5(f)); however, unlike the iXon 897 whose image is uniform across the entire signal intensities, images from the Flash 4.0 exhibits obvious stripes in a certain signal intensity range (Fig. 5(c)) and become uniform again when the signal intensity is higher (Fig. 5(d)).

To find some clues to reveal the changing image uniformity in the Flash 4.0, it is necessary to separate the FPN into pFPN and cFPN. According to the method reported by A. El Gamal et al [16], we calculated the dependence of cFPN and pFPN on signal intensity (Fig. 6(a)). The pFPN was found to decrease gradually with increasing signal intensity, while the cFPN has a big jump in the signal range centered at ~1500 photon/pixel. It was reported that the cFPN is much more visible than pFPN because of its spatial correlation, and that the strips will become invisible to the human eye only when the following criteria are both met: (1) the magnitude of the cFPN is < 1% of the corresponding signal, and (2) the pFPN/cFPN ratio is > 5 [32]. Therefore, we also calculated the dependence of pFPN/cFPN ratio on the signal intensity (Fig. 6(b)). Two regions were found to meet such criteria. The first region, which locates between 80 and 1000 photon/pixel, is within the peak signal intensity range of individual fluorescence emitters typically used in localization microscopy (see Table 2). The second region locates > 12000 photon/pixel and is beyond reach in current localization microscopy. Obviously, to enhance the versatility of Flash 4.0, more efforts are required to reduce the magnitude of the cFPN, especially for the signal range of 1000-2000 photon/pixel, which is critical for imaging chemical probes.

Finally, it is worthy of note that the fixed pattern noise of the Flash 4.0 contributes a small portion to the total noise (< 10% in the signal range of 50-2000 photon/pixel, see Fig. 5(a)), and the stripes will appear only when a large number of images are stacked together (for example, overlaying hundreds or even thousands of image frames). For single image frame, the stripes are still invisible even when the Flash 4.0 is working in fixed pattern noise limited regions (3500-40000 photon/pixel, see Section 3.3.1).
Fig. 5. Imaging uniformity of the Flash 4.0 (a-d) and the iXon 897 (e-h) under different intensities of uniform illumination (shown by the values on the top of the images). Each image was overlaid from a set of 100 successive image frames. The mean values and standard deviations (S.D.) of individual images are shown in the right corner of the corresponding figures.

Fig. 6. Dependence of different fixed pattern noises (a) and the ratio of pixel fixed pattern noise (pFPN) to column fixed pattern noise (cFPN) (b) on signal intensity in the Flash 4.0. In the cFPN curve in (a), the marked positions (1-4) correspond to the images in Fig. 5(a)-5(d). Note that the red rectangles highlight typical signal ranges in localization microscopy.
3.3.4 The excess noise of EMCCD

Integrating electron multiplication (EM) processes with charge coupled devices (CCD) creates a most successful low-light camera, EMCCD, by offering simultaneously ultra-high sensitivity and high imaging speed. As the EM gain is increased, read noise becomes insignificant. However, the EM processes also result in an increase in shot noise from signal. The increase is described by the excess noise factor, which is \( \sim \sqrt{2} \) when the number of multiplication stages is reasonably high (for example, \( > 20 \)) [4, 33].

In the field of localization microscopy, the advantage of the EM processes has always been emphasized, but their drawback (that is, bringing in the excess noise) to single molecule detection is seldom studied. Here we investigated how the excess noise deteriorates the imaging performance of EMCCD in detecting point-like emitters.

We used fixed fluorescent beads with diameter of 170 nm to represent point-like emitters. After proper experimental control, constant fluorescence emission from these beads can be obtained (See Section 3.2 for details), which can be used to compare the imaging performance of the Flash 4.0 and the iXon 897. To make the comparison more precise, a set of 500 successive image frames was detected by the Flash 4.0, followed by another set of 500 frames by the iXon 897 using the same fluorescent beads under the same experimental conditions. The variations of the peak signal intensity of a representative bead over time detected by the Flash 4.0 and the iXon 897 are shown in Fig. 7(a) and 7(c), respectively. It was found that, although the mean values of the peak signal intensities are almost the same, the signal fluctuation detected by the iXon 897 (Fig. 7(d)) is much greater than that by the Flash 4.0 (Fig. 7(b)).

A further investigation on the origins of such signal fluctuation would be beneficial to reveal the impact of the excess noise to the image quality. It is well-known that if an optical detection is performed in shot noise limited region, the detected signal is governed by Poisson distribution, and thus the standard deviation of the signal equals to the square root of its mean intensity. For the signal detected by the Flash 4.0, the square root of the mean signal (\( \sqrt{206} \approx 14.4 \)) is almost identical to the signal fluctuation (14.5) found by statistics (see Fig. 7(b)). However, for the iXon 897, the experimental signal fluctuation (20.7) is much higher than the predicted fluctuation (\( \sqrt{210} \approx 14.5 \)), indicating that additional noise source is presented in the images. Interestingly, if we take the excess noise factor into consideration, a predicted fluctuation of 20.5 (\( \sqrt{210 \times 2} \approx 20.5 \)) can be nicely consistent with the experimental value of 20.7.

The results and discussions shown above clearly point out the negative impact of the excess noise on imaging performance of EMCCD cameras, which is supported by the finding reported in Section 3.3.2 (that is, the excess noise equals to the shot noise and contributes \( \sim 50\% \) to the total noise). In other words, it is essential to take the excess noise factor into consideration when analyzing images from EMCCD cameras. In fact, this consideration is exactly reflected in the equations we used to calculate the image SNR for experimental images (Section 2.2.1).
Fig. 7. The variation of the peak signal intensity of the same fluorescent bead over time detected by the Flash 4.0 (a) and the iXon 897 (c). The data in (a) and (c) were further analyzed, and the results are presented in (b) and (d), respectively.

3.4 Comparing cameras with image SNR

3.4.1 Optimal image SNR without photon background

With the PTC measurement results shown in Section 3.3.1, we characterized the optimal imaging performance of the cameras in single molecule detection by comparing the image SNR without photon background (Optimal Image SNR, $SNR_{\text{optimal}}$, see Section 2.2.1). The $SNR_{\text{optimal}}$ was calculated from Eq. (2) where $N_{\text{camera}}$ was assumed to include read noise and fixed pattern noise. Results show that the Flash 4.0 provides the highest $SNR_{\text{optimal}}$ when the incident signal is $>15$ photon/pixel, and the Flash 2.8 achieves higher $SNR_{\text{optimal}}$ than the iXon 897 when incident signal is $> 200$ photon/pixel. Note that the threshold values are both higher than the results in Section 3.1 due to the influences from fixed pattern noise. In the peak signal range of typical fluorescence emitters used in localization microscopy (marked with the red rectangle in Fig. 8), the Flash 4.0 was found to be the best camera of choice (that is, closest to the perfect camera).

It is interesting to evaluate the impact of the excess noise to the $SNR_{\text{optimal}}$. When read noise and fixed pattern noise are both ignorable, and there is no photon background, the equation used to calculate $SNR_{\text{optimal}}$ (Eq. (2)) can be rewrite as

$$SNR_{\text{optimal}} \approx \frac{N_{\text{sig}} \times QE}{\sqrt{N_{\text{sig}} \times QE \times F_n^2}} = \frac{N_{\text{sig}} \times QE}{\sqrt{N_{\text{sig}} \times QE \times F_n^2}} = \frac{N_{\text{sig}} \times QE_{\text{eff}}}{\sqrt{N_{\text{sig}} \times QE_{\text{eff}}}}$$

where the effective QE ($QE_{\text{eff}}$) equals to $QE / F_n^2$. Note that the excess noise factor equals to 1 for sCMOS and $\sqrt{2}$ for EMCCD, respectively. Apparently, the QE of EMCCD cameras is effectively halved due to the excess noise, while the QE of sCMOS cameras keeps unchanged. On the other hand, in the shot noise limited region where read noise and fixed pattern noise are both ignorable, the QE plays the determinant role for optimal image SNR. Since the effective QE of the iXon 897 is $0.95/2 = 0.475$, the optimal image SNR of this camera is no longer comparable to the Flash 4.0 which has an effective QE of 0.72. In fact, this estimation is confirmed by the results presented in Fig. 8.
It should be pointed out that, because the Simplified Image SNR curves shown in Fig. 2 did not take fixed pattern noise into consideration, the threshold values where the imaging performance of the iXon 897 is surpassed were not correctly predicted. For example, it was found from Fig. 2 that the Flash 4.0 has better image SNR than the iXon 897 when the signal is > 4 photon/pixel. However, the optimal image SNR from the PTC measurements shows that the Flash 4.0 is better than the iXon 897 in the signal range of 15-12000 photon/pixel. Note that the threshold in low signal range can be read from Fig. 8, while the threshold in high signal range (not shown in Fig. 8) was found when the iXon 897 is working with EM gain of 10 and read noise of 4.9 e-.

Fig. 8. Dependence of Optimal Image SNR on incident signal intensity. Note that no photon background is assumed to accompany with the signal. The red rectangles highlight typical signal range in localization microscopy. The inset in the right corner is an enlarged view of the region marked by the red rectangle. The QE of the perfect camera was set to 1, while the QE of other cameras was set according to Table 1. The perfect camera was assumed to have no camera noise. The signal values for the crossovers were shown by the numbers nearby.

3.4.2 Experimental SNR with photon background

Real single molecule imaging experiments are always accompanied with photon background which is originated from background fluorescence and/or residual excitation light. Therefore, we also investigated the dependence of experimental image SNR ($SNR_{\text{exp}}$) on photon background intensity (Fig. 9). The experiments and data analysis procedures were described in Section 2.2.3 and Section 2.2.4, respectively, and the results are shown in Fig. 9(a)-9(d).

It was found that the $SNR_{\text{exp}}$ matches nicely with the theoretical image SNR ($SNR_{\text{th}}$) for all the cameras, and that the Flash 4.0 presents much better image SNR than the Flash 2.8 (Fig. 9(a)-9(b)), and the iXon 897 (Fig. 9(c)-9(d)). The usefulness of the $SNR_{\text{optimal}}$ calculated from PTC measurements (Fig. 8) was shown by Fig. 9(e), evidencing by the finding that the isolated data points match nicely with the curves.
Fig. 9. The dependence of image SNR on different photon background intensity (a-d) and signal (e). Experimental Image SNR were determined from fluorescent beads with a total number of emitted photon of 1180 (a), 1550 (b), 1040 (c), and 2100 (d), respectively. But due to the pixel size difference of the cameras, the peak signal is 217 photon for the Flash 4.0 and 207 photon for the Flash 2.8 (a), 281 photon for the Flash 4.0 and 274 photon for the Flash 2.8 (b), 301 photon for the Flash 4.0 and 237 photon for the iXon 897 (c), and 611 photon for the Flash 4.0 and 477 photon for the iXon 897 (d), respectively. The theoretical curves in (a-d) are Theoretical Image SNR which was calculated from Eq. (1). The curves in (e) were reproduced from Fig. 8. The isolated data points in (e) were obtained from (a-d). Each of the data points in (a-d) was averaged from 500 independent measurements. The standard deviations of the measurements are indicated by the error bars in (a-d).

3.5 Comparing cameras with localization accuracy

Localization accuracy is of particular importance in quantifying the performance of a camera in single molecule localization. In principle, the localization accuracy of a point-like emitter is mainly determined by a combination of three factors: photon shot noise, pixelation noise, and background noise [19, 20]. Basing on the well-established statistical theory concerning the Fisher information matrix, R. J. Ober et al [19] derived an analytical expression that shows fundamental limit of the localization accuracy for a single molecule (which is determined by photon shot noise). However, since other determinative factors are experimental dependent and
are hard to be modeled precisely, it is ineffective to derive an equation for assessing how well one can localize a molecule based on a single measurement [21]. Therefore, as suggested by W. E. Moerner et al [21], it is best to use repeated imaging to obtain the statistical positions of a fixed point-like emitter from which a standard deviation (localization accuracy) can be obtained for particular experimental condition (including signal intensity, background noise, pixel size, etc.). Interestingly, another paper presented by X. W. Zhuang et al used similar methodology to estimate the localization accuracy of their microscopy system [22].

Considering the effectiveness of this experimental methodology, here we characterized the localization accuracy of the cameras from repeated imaging of point-like emitters (fixed fluorescent beads) embedded in different photon background intensities. The experimental methods and data analysis procedures were described in Section 2.2.3 and Section 2.2.5, respectively. The results are shown in Fig. 10. It was found that the Flash 4.0 presents higher localization accuracy than the Flash 2.8 (Fig. 10(a)-10(b)) and the iXon 897 (Fig. 10(c)-10(d)), which are in agreement with the findings in Section 3.4.2.

It is beneficial to take a closer look at the data shown in Fig. 10 and assess roughly how the experimental conditions, including probe brightness and photon background, influence the localization accuracy of the camera. It was found that: (1) Localization accuracy increases with brighter probes (Fig. 10(a) vs. Figure 10(b), or Fig. 10(c) vs. Figure 10(d)), and (2) Localization accuracy decreases with increasing photon background (all data in Fig. 10), and decreases more rapidly in the EMCCD camera (Fig. 10(c) and Fig. 10(d)). The former finding is easy to understand: Image SNR increases with brighter signal and thus increases the accuracy in finding the locations of individual point-like emitters. That is to say, further development in the quantum efficiency of the Flash 4.0 is highly desirable to increase the localization accuracy of this camera. The latter finding is mainly a result from the photon background noise: when the photon background is reasonably high, the localization accuracy is dominant by the photon background noise. The excess noise factor in EMCCD cameras amplifies the photon background noise, and thus results in poorer localization accuracy in the iXon 897 than that in the Flash 4.0. Of course, more experimental results are required to address such influences, but are beyond the scope of the current research.

![Fig. 10. Dependence of localization accuracy on photon background intensity. Localization accuracy was determined from fluorescent beads with a total number of emitted photon of 1180 (a), 1550 (b), 1040 (c), and 2100 (d), respectively. To aid data observation, smoothing spline fitting was used to generate the dotted lines. Each of the data points was averaged from 10 independent measurements. The error bars indicate the standard deviation. The pixel size at sample plane: (a-b) 108 nm for the Flash 4.0, 121 nm for the Flash 2.8; (c-d) 186 nm for the Flash 4.0 and 160 nm for the iXon 897.](image-url)
4. Conclusion

We derived an experimental methodology for comparing the performance of low-light cameras in single molecule detection and localization. This methodology is based on the characterization of image SNR and localization accuracy using repeated imaging of point-like emitters (in our case, fluorescent beads with diameter < 200 nm). Photon transfer curve measurements were also performed to characterize the camera noise sources, which can be further used to calculate theoretical image SNR. In the typically signal intensity range of localization microscopy, the newly developed Hamamatsu Gen II Flash 4.0 sCMOS camera was found to exhibit the best performance than the Hamamatsu Gen I Flash 2.8 sCMOS camera and the popular Andor iXon 897 EMCCD camera.

Moreover, we found that the excess noise in the iXon 897 EMCCD contributes ~50% to the total noise, which effectively halves the quantum efficiency of the camera. While for applying the Flash 4.0 sCMOS in the field of localization microscopy, it is of great beneficial to increase the quantum efficiency of this camera and reduce the magnitude of the fixed pattern noise, especially for the high signal range which is critical for imaging chemical probes.

Since the excess noise originates intrinsically from the electron multiplication process, it is impossible to develop EMCCD cameras into a shot noise limited detector. However, for sCMOS cameras, it is still possible to further increase the quantum efficiency and/or reduce the fixed pattern noise via optimal hardware and/or software design [7]. In another words, with further improvements, sCMOS cameras will have great potentials to be developed into a perfect camera which would surely become the camera of choice in the field of localization microscopy.

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