Evaluation of sCMOS cameras for detection and localization of single Cy5 molecules

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Abstract: The ability to detect single molecules over the electronic noise requires high performance detector systems. Electron Multiplying Charge-Coupled Device (EMCCD) cameras have been employed successfully to image single molecules. Recently, scientific Complementary Metal Oxide Semiconductor (sCMOS) based cameras have been introduced with very low read noise at faster read out rates, smaller pixel sizes and a lower price compared to EMCCD cameras. In this study, we have compared the two technologies using two EMCCD and three sCMOS cameras to detect single Cy5 molecules. Our findings indicate that the sCMOS cameras perform similar to EMCCD cameras for detecting and localizing single Cy5 molecules.

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1. Introduction

The rapid development of single molecule (SM) microscopy techniques has significantly improved our understanding of biophysical systems through various methods, including diffusion analysis [1], energy transfer measurements [2] and more recently, super resolution microscopy [3–5]. SM methods are advantageous because they measure the distribution of molecular properties rather than ensemble averages. Observing single fluorescent molecules, however, requires careful optimization of imaging conditions and sample preparation [6,7].

Most single molecule methodologies now rely on sensitive charge coupled device (CCD) based detectors to convert photons into readable signal from a wide-field imaging system, allowing interrogation of many molecules simultaneously. The availability of highly sensitive and robust camera systems has allowed this approach to be employed as a practical tool to study chemical and biophysical systems in many labs [8].

Most wide-field single molecule experiments currently employ Electron-Multiplication Charge Coupled Device (EMCCD) based cameras. EMCCD detectors amplify the signal from a detected photon through collisional amplification of the photoelectron, a process also used in avalanche diodes [9]. Single molecule detection typically requires exposure times of 10—100 ms. Such short exposure times require fast read-out rates with very low read noise. EMCCDs typically have a read noise of 30-40 electrons (RMS value without EM gain) at fast frame rates. Under optimal settings, however, EMCCDs can provide an effective read noise substantially lower than the signal generated from a single photon. Since read noise depends on the square root of the readout speed and the area of the capacitor (detector) storing the charge, reducing the pixel size can reduce the read noise while improving spatial sampling [9,10]. Importantly, the typical 13 µm, 16 µm or 24 µm pixel sizes of EMCCD sensors are incapable of providing diffraction-limited Nyquist sampling using standard, 60x or 100x high-NA objectives without intermediate magnifying lenses between the microscope and the camera. Very recently, scientific imaging sensors based on a Complementary Metal Oxide Semiconductor (CMOS) technology have been introduced with low read-noise, high read out rates, and small pixels, that may significantly improve and simplify single molecule detection systems [11].

In an EMCCD, photoelectrons are collected at each sensor and then transferred to the readout register where they are converted to current, multiplied, and digitized by chip-wide electronics. In contrast, CMOS sensors convert photoelectrons to voltage on the pixel. These pixel voltage values are then loaded into column-level amplifiers and analog-to-digital converters. The per-pixel and column-wide gain and digitization can result in more substantial fixed pattern noise in CMOS sensors [12], but allows for very fast operation. CMOS cameras do not have electron multiplication, so they have higher read noise. However, these sensors have no multiplication noise, a characteristic of EMCCD cameras that reduces the effective quantum yield of the sensor [10]. These properties suggest that the performance of scientific-grade CMOS (sCMOS) cameras, where engineering approaches have reduced the read noise
and fixed pattern noise to levels acceptable for quantitative imaging, may perform well for single molecule detection. Recently, three scientific CMOS (sCMOS) cameras have been introduced that have attractive properties for single molecule imaging such as low read noise at high frame rates (less than 3 electrons RMS at 30 frames per second) and small pixel size (3.6 – 6.5 µm). A recent study has shown the applicability of an sCMOS camera (Flash2.8 sCMOS) for localization microscopy [13]. Here, we compare the quantitative performance of three sCMOS and two EMCCD camera systems for detection and localization of single Cy5 molecules under typical objective-type TIRF imaging conditions for the first time. Our study shows that the three sCMOS cameras evaluated are close in performance to the EMCCD cameras and thus can be used effectively for single molecule detection.

2. Methods

Single Molecule (SM) measurements utilize TIRF Microscopy. By selectively exciting the fluorophores at the glass-imaging solution interface, a significant reduction in fluorescence from the background is attained. A key requirement for making an effective SM measurement is immobilizing the fluorophores on the glass surface. For this to be possible, a glass surface free from any fluorescent impurities is strictly required. This is achieved by aggressive chemical treatment to remove fluorescent impurities and surface derivatization of the glass to ensure that fluorophores are non-covalently tethered to glass.

2.1 Surface preparation

For SM measurements, we cleaned glass coverslips as follows: Ten glass coverslips (25 mm round, #1.5, Warner Instruments) were subjected to a Piranha solution (100 mL, 70% H₂SO₄ + 30% H₂O₂) treatment for 30 min with agitations at 10 and 20 min. The coverslips were washed with ultra-pure water (Fisher) after Piranha solution treatment and sonicated in HPLC grade methanol (Fisher) for 20 min. The coverslips were let dry and were immersed in a solution containing 0.1 mL (3-aminopropyl)trimethoxysilane, 0.5 mL glacial acetic acid and 10 mL methanol (HPLC Grade) for 1 hour. The coverslips were washed again using ultra pure water and dried using argon gas. After this the coverslips were kept on a flat surface. 210 µL of a solution made from 0.4 mg biotin-polyethylene glycol (PEG)-succinimidyl carboxymethyl ester (MW = 5000), 7 mg methoxy-PEG-succinimidyl carboxymethyl ester (MW = 5000), and 210 µL of 0.1 M NaHCO₃ was added to each coverslip. The coverslips were incubated in a dark and humid environment overnight.

The coverslips were washed with ultra-pure water, dried and stored at ~20°C. The prepared coverslips were assembled in an imaging chamber and imaged with 200 µL PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH7.4, filtered using a 0.22 µm filter and irradiated with light from a Mercury arc lamp for 45 minutes) to detect any impurities on the coverslip or in the buffer. Coverslips showing less than 20-30 objects in a 512×512 pixel region (pixel size = 16 µm×16 µm) were used for further experiments. The coverslips were then incubated with 100 µL of 0.1 mg/mL streptavidin for 1 minute and imaged to check for impurities in the streptavidin solution. After satisfying the same criterion for the number of objects, the coverslips were washed again with PBS. Finally, the coverslips were incubated in 200 µL of a 30 nM solution of biotin-Cy5 for 1 minute. The excess solution was washed off and replaced with PBS. Sample density was observed using the Evolve 512 EMCCD camera. The TIRF angle and the sample were not changed once the expected sample density was obtained. Different cameras were mounted on the microscope stand using a C-mount adapter, one at a time, and measurements were initiated at a new field-of-view of the same prepared sample.
2.2 Cameras used in the study

Five cameras were evaluated in this study: the Evolve 512 EMCCD (Photometrics), 887 EMCCD (Andor), pco.Edge sCMOS (Cooke Corp.), Neo sCMOS (Andor) and ORCA Flash 2.8 CMOS (Hamamatsu). Huang et al. have recently reviewed the ORCA Flash 2.8 CMOS for single molecule localization microscopy [13]. The sensor in the pco.Edge and Neo sCMOS cameras is manufactured by Fairchild Imaging while the sensor in the ORCA Flash 2.8 camera is manufactured by Hamamatsu Photonics. Both EMCCD systems are based on the e2v L3Vision sensor. Apart from electronics and chip properties, pixel size was the main distinguishing feature of these two technologies. While the two EMCCD cameras used in the study had a pixel size of 16µm × 16µm, the sCMOS cameras had smaller pixel sizes (6.5µm × 6.5µm for pco.Edge and Andor Neo; and 3.63µm × 3.63µm for the ORCA Flash 2.8) (Supplemental Table 1). Smaller pixels may improve resolution at lower magnifications by preserving Nyquist sampling for high N.A. 60x and 40x objectives. This may be an improvement over EMCCD cameras, where high N.A., high magnification lenses and intermediate magnifying lenses are required for Nyquist-sampled objective-type TIRF.

2.3 Single molecule imaging

Single molecule imaging was performed using objective-type TIRF on a Nikon Ti inverted microscope stand (Nikon Instruments) equipped with a 100x 1.45 N.A. oil immersion TIRF objective. For imaging Cy5, a filter set containing a 630/23 – 532/12 dual band pass excitation filter, 532/638 (ZT) RPC dichroic mirror, and a 685/70 (ET) emission filter was used (all from Chroma Technologies). An additional 685/70 (ET) filter (Chroma Technologies) was used in the emission filter wheel to minimize the stray reflected light getting to the detector. These filters are slightly tilted to provide optimal blocking of the reflected light. We used a 639 nm diode pumped solid-state laser for excitation. The measured power at the back of the objective was 3.8mW. The TIRF angle was optimized using the motorized TIRF mirror attached to the stand. For the Hamamatsu ORCA Flash 2.8, we used a 0.7x relay lens, between the stand and the camera to roughly match pixel sizes of the sCMOS cameras (Andor Neo and pco.Edge ~6.5 µm; Hamamatsu ORCA Flash2.8 (post demagnification) ~5.2 µm). The exposure time used for acquisition was 100 ms at a frame rate of 10 frames per second. Electron multiplication slider values were 300 for the Evolve 512 EMCCD and 250 for the Andor 887 EMCCD cameras. Data acquisition and component control was handled using Micro-manager [14]. Sample single Cy5 images acquired using various detectors are shown in Fig. 7. A in the supplemental information. Objects that were between 8 and 14 pixels in size (imaged through Evolve 512 EMCCD camera) and above 3000 counts in peak intensity were defined as the objects of interest. Sample density was visually observed in at least five 50 × 50 pixel regions to ensure that we are obtaining the expected number of objects over a 512 × 512 pixel region (1023±150) (Fig. 7B, supplemental information).

2.4 Single molecule data analysis

2.4.1 Object identification

All analysis and comparisons were made for single 100 millisecond acquisitions for each camera. We used an adaptive local thresholding algorithm to detect the objects with intensities that span the full dynamic range in an image frame. Local thresholding also takes into account the fluctuations in intensity or differences in properties across all pixels. We have employed the empirical ‘sigma’ rule of statistics for finding the signal pixels. We calculated the mean value and standard deviation of the intensities inside a square block of pixels. We used a block size of 8 × 8 pixels for EMCCD images and a block size of 32×32 pixels for the sCMOS images. We set the signal detection threshold as two standard deviations above the mean of the block for EMCCD cameras. The same threshold applied to sCMOS camera data sets detected only 70-75% of the objects. EMCCD cameras showed high peak object
intensities and low read noise compared to the sCMOS cameras owing to decreased pixelation and electron multiplication. Therefore the detection criterion was set to be one standard deviation for all sCMOS cameras. For EMCCDs the full 512×512 pixel frame was analyzed, while for the sCMOS detectors, each image was cropped to a 1024×1024 pixel centered region. Cropping the sCMOS images was essential because the field of view of these cameras is large enough to image the entire back aperture of the objective, which results in vignetting and significant aberrations at the image periphery (not shown). A centered 52µm×52µm area in the sample space was analyzed for all cameras to ensure that the analysis was free of any heterogeneity in the illumination area. After thresholding we also applied an object size filter to remove clusters of objects (if any) so as to retain the expected number of connected pixels defining an object. We were able to detect 90-95% of the objects across all images using this thresholding method.

2.4.2 Signal to noise ratio calculation

For each of the detected objects, a rectangular area of interest (AOI) just enclosing the object pixels was determined. The AOI was extended by 3 pixels on each side. The peak intensity \( I_0 \) for the object was the maximum value of the thresholded pixels and the mean background intensity \( I_{B_{\text{avg}}} \) and standard deviation \( \sigma_B \) was calculated for the background region within the extended AOI excluding the object pixels. The peak signal to noise ratio (SNR) for each of such AOI, was then calculated according to Eq. (1).

\[
SNR = \frac{I_0 - I_{B_{\text{avg}}}}{\sigma_B}
\]

2.4.3 Localization of single molecules

One of the key advantages of single molecule imaging studies lies in the precise localization of the centroids of the molecules. Localization techniques typically involve centroid estimation by fitting the intensity profile to a theoretical point spread function (PSF) such as a two dimensional Gaussian function [15]. We localized the centroids of single Cy5 molecules by fitting the intensity profile of each object using a nonlinear least squares fitting. For comparison, we have also used a maximum likelihood estimation (MLE) method to determine the positions of the same objects.

2.4.4 2D Gaussian fitting

A nonlinear least squares fitting algorithm was implemented in MATLAB to fit the intensity profiles of the objects to a two-dimensional elliptical Gaussian point spread function

\[
I(x, y) = I_B + I_o \exp \left[ \frac{-(x - \mu_x)^2}{2\sigma_x^2} - \frac{(y - \mu_y)^2}{2\sigma_y^2} \right]
\]

Traditionally, a symmetric 2D PSF (\( \sigma_x = \sigma_y \)) is used to fit objects for images acquired using EMCCD cameras. However, we observed ~6% objects with small asymmetries of the order of ~5% relative to \( \sigma_x \). Therefore, we chose to perform the fitting using a general form, keeping the standard deviations in x and y directions as parameters for fitting across all cameras for consistency. We also calculated the standard error in the fitted centroid positions as an indicator of the localization performance of the Gaussian least square-fitting algorithm on the different cameras.

2.4.5 Maximum likelihood estimation

Localization using least squares fitting with a Gaussian point spread function works very well for sufficiently high photon counts where Poisson noise can be approximated by Gaussian noise. However, at relatively low photon counts, this approximation does not hold true and the
failing performs poorly. Localization obtained by least squares fitting under low-photon conditions may not be accurate. Statistical estimation methods that are based on theoretical image formation models, such as maximum likelihood estimation (MLE) [16,17], are more suited to estimate the location of single molecules under photon-limited conditions. The MLE approach uses the finite pixel size and all of the noise contributions (read out noise, shot noise, background signal) in the image model and is capable of accurately determining the centroid of the objects even at low photon count. The Cramér–Rao Lower Bound (CRLB), gives the theoretical limit for the variance of an estimator. This is a well-established statistical method for determining the lower bound on the variance of unbiased estimators of deterministic parameters [18]. The variance of any unbiased estimator \( \hat{\theta} \) of the parameter \( \theta \) is bounded by the inverse of the Fisher information matrix \( I(\theta) \):

\[
\text{var}(\hat{\theta}) \geq I(\theta)^{-1}
\]

where, the Fisher information matrix is defined by \( I(\theta) \):

\[
I(\theta) = E \left[ \frac{\partial \ln(L(\hat{x}|\theta))}{\partial \theta_i} \frac{\partial \ln(L(\hat{x}|\theta))}{\partial \theta_j} \right]
\]

Here \( \theta \) is the set of parameters and the \( L(\hat{x}|\theta) \) is the likelihood function of the data set \( \hat{x} \).

For single molecule images the parameters are \( \theta = \{\theta_x, \theta_y, \theta_{\text{peak}}, \theta_{\text{bg}}\} \), where the parameters are for centroid coordinates \( x, y \), peak intensity and background respectively. We have used an iterative GPU implementation in Nvidia Compute Unified Device Architecture (CUDA) for performing the MLE localization of single molecules. The localizations calculated using this approach have previously been shown to achieve the CRLB [19].

3. Results

A single molecule is characterized in the image plane as a point source of fluorescence signal with an intensity spread around the peak. Figure 1 shows the distribution of peak intensity of single Cy5 molecules detected by each camera. We generated histograms of peak object intensity and applied Gaussian fitting on these histograms. We obtained reasonably good fitting parameters (\( R^2 \) and sum of residuals) for all cameras except for the Andor Neo sCMOS, which had a broader distribution of peak intensities across a set of fluorophores compared to the other sCMOS cameras, pco.Edge and Hamamatsu Flash 2.8 sCMOS cameras.
Fig. 1. Distribution of peak intensity for single Cy5 molecules detected using, (A) Evolve 512 EMCCD, (B) Andor 887 EMCCD, (C) pco.Edge sCMOS, (D) Andor Neo sCMOS, and (E) Hamamatsu ORCA Flash 2.8.

have coefficients of variation (C.V.) comparable to EMCCD cameras, a feature that may be useful for single molecule measurements such as FRET that sometimes require differentiating small differences in fluorescence intensity.

Fig. 2. Distribution of peak signal-to-noise ratio for single Cy5 molecules imaged using (A) Evolve 512 EMCCD, (B) Andor 887 EMCCD, (C) pco.Edge sCMOS, (D) Andor Neo sCMOS, and (E) Hamamatsu ORCA Flash 2.8.

The signal to noise ratio is a key parameter for any detection system. It provides us with a basis to compare data collected using different detection paradigms. For calculation of SNR, we have used the method discussed in the section 2.4.2. Figure 2 shows that the EMCCD cameras have a broader distribution of signal to noise and higher median SNR values compared to sCMOS cameras. With C.V. values comparable to EMCCD cameras and peak
SNR values of 4.8-6.4 for sCMOS cameras, it is evident that these cameras are usable for single molecule detection.

The standard error in the positions ($\Delta r$) of centroid fitting using a non-linear least squares fitting is shown in Fig. 3. The $\Delta r$ distribution is similar across all data sets and is spread around 16-24 nm. The median values are more precise by 4-7 nm for EMCCD cameras compared to sCMOS cameras, due to higher peak signal and SNR. The other factor that may explain the observed smaller $\Delta r$ values for EMCCD cameras compared to sCMOS cameras is the spread over fewer pixels in the image plane.

The results of localization using the MLE approach are shown in Fig. 4. The localization accuracy values ($\sigma_{\text{MLE}}$) using MLE are comparable for the sCMOS and EMCCD cameras, although the median values for the EMCCD cameras are slightly better compared to the sCMOS cameras. The $\sigma_{\text{MLE}}$ values are around 18 nm for the EMCCD cameras whereas the spread is 22-24 nm for the sCMOS cameras. One may expect improved localization accuracy for the sCMOS cameras under better pixel matching for the objective and using brighter emitters such as quantum dots, where the number of photons per pixel will be significantly higher.

Photon transfer curve measurements demonstrated that for bright probes such as d2EosFP [13,20] signal shot noise is more dominant compared to the read noise under TIRF imaging conditions. This should hold true for even brighter probes such as Cy5 [21] used in our study. Further, surface immobilization combined with TIRF microscopy ensures that the background noise is low, supporting Cy5 shot noise as the primary noise term. Based on these considerations we simulated single molecule data for three types of chips used in this study considering per pixel shot noise and read noise. The simulation results and their comparison with experimental data are shown in Fig. 5. The details of the simulation are provided in the supplemental information. Sample simulated objects are shown in Fig. 8 in the supplement.

We applied the non-linear least squares fitting algorithm on these simulated single objects and calculated the $\Delta r$ values for the fit. We computed these values for simulated objects with varying number of photons (100, 200, 500).
For a direct comparison with experimental data, the image intensities were scaled according to the gain factor for each camera to match the intensity range to the experimental data. The circular markers in Fig. 5 show corresponding Δr values for simulated data with the error bars showing the standard deviation of these values.

The experimental data is in close agreement with the simulated data for total photon counts per object of 100, 200 and 500 photons. These results are also in agreement with the findings from previous PTC measurements by Huang et al [13]. This also suggests that
sCMOS cameras may provide improved localization accuracy with brighter fluorophores. Analogous simulations were performed for different pixel sizes. A read noise of 0.78 electrons/pixel was used that corresponds to the measured read noise for pco.Edge sCMOS. The simulation results (Fig. 6) show that Nyquist matching of the pixel size (134 nm) for sCMOS cameras could improve the localization precision from 26 nm to 12.5 nm (median values).

Fig. 6. Simulation results showing expected standard error in position ($\Delta r$) for a sCMOS detector as a function of pixel size (Mean and median values) from nonlinear least squares fitting to a Gaussian PSF. The simulations were performed for 200 photons.

4. Conclusion

In this study we have compared three recently developed sCMOS cameras to two EMCCD cameras for detection and localization of single Cy5 molecules. We have demonstrated that single molecule detection is possible with the sCMOS cameras used in the study. EMCCD cameras have an advantage over the sCMOS cameras when it comes to peak signal to noise ratios. EMCCD cameras displayed 10-35% higher localization precision than sCMOS cameras shown by the $\Delta r$ and $\sigma_{MLE}$ values under low photon, over-sampled (for the sCMOS cameras) conditions. Both least-squares and MLE localization analyses show the same relative performance trends between sCMOS and EMCCD cameras. Simulations of localization with comparable image-space pixels show that the use of sCMOS cameras with a Nyquist matched pixel size (achieved using a demagnification lens or lower magnification objectives) could lead to a two-fold improvement in localization precision, equal to or better than levels achievable with EMCCD cameras. The ability of sCMOS cameras to accurately and quantitatively measure single molecule position and intensity may provide scientists engaged in such studies an attractive alternative to EMCCD cameras, one that can serve both as a high-resolution camera and a high sensitivity, high accuracy single molecule localization system.
Supplemental information

![Image: Figure 7. (A) Single Cy5 molecules imaged using the various sensors used in this study (scale bar = 200nm). (B) Single Cy5 molecules sample density imaged using Evolve 512 EMCCD camera (scale bar = 9µm).]

Table 1. Key Characteristics of the Cameras used in the Study

|                  | Photometrics Evolve 512 | Andor 887 | Pco.Edge | Andor Neo | Hamamatsu ORCA Flash2.8 |
|------------------|-------------------------|-----------|----------|-----------|-------------------------|
| **Pixel Size**   | 16 µm                   | 16 µm     | 6.5 µm   | 6.5 µm    | 3.63 µm                 |
| **Measured Read Noise (e/pixel)** | 0.02*                     | 0.20*     | 0.78     | 0.80†       | 0.65§                   |
| **Frame Rate** (Full Frame) | 33.7                      | 35        | 100      | 100        | 45                      |
| **Q.E. 660**§§   | 95%                     | 90%       | 55%      | 54%       | 34%                     |

* Measured read noise with electron multiplication (slider value of 300 for Photometrics Evolve 512 and 250 for Andor 887 EMCCD).
† Measured for pre-amplification gain value of 4
§ Measured for a gain value of 255
§§ Q.E. 660 = Quantum Efficiency at 660 nm

Measurement of read noise

For measuring read noise, we subtracted two zero exposure frames (when no light was allowed to get to the sensor) from one another and then divided the resulting image by the \( \sqrt{2} \). The final value in counts was calculated from five such observations and then converted to electrons using the gain calibration on the EMCCD cameras. Since Cy5 molecules should emit the same number of photons on an average in a given exposure, we could estimate the gain on the sCMOS cameras and could convert the counts that we obtained from them to electrons. The gain factors for sCMOS cameras were normalized according to the relative quantum efficiency compared to the EMCCD camera.

Simulation details

Simulated SM data sets were generated for three pixel sizes 16µm, 6.5 µm and 5.2 µm that correspond to 160 nm, 65 nm and 51.9 nm in the image space with 100x magnification. The profile within the first minimum can be approximated by a two-dimensional Gaussian profile. The standard deviation of the point spread function is taken as \( \sigma_{PSF} = r_f/2 \). The photon count of each pixel was taken as the expected mean value for the Poisson distribution. The corresponding shot noise count generated by the distribution was then added to the pixel.
pixel sizes of 6.5 \( \mu m \) and 5.2 \( \mu m \), an additive Poisson read noise with the mean value taken as the measured experimental value was added to the pixels.

Fig. 8. Simulated single molecule images for (A) EMCCD sensor, (B) an sCMOS sensor used in pco.Edge and Andor Neo cameras, and (C) an sCMOS sensor used in the Hamamatsu ORCA Flash 2.8 camera (\( N = 100 \) photons). The lower panel shows experimental data for the same sensors respectively: (D) EMCCD image taken using Andor 887, (E) sCMOS image taken using pco.Edge, (F) Image taken using Hamamatsu ORCA Flash2.8. The scale bars are each 200 nm.

Rationale for histogram binning

Correct choice of bin width influences the representation of the actual data and sometimes can introduce bias. Therefore in order to have consistent criteria for binning we have used Scott’s formula for binning all of our data distributions [1]. It is based on the idea of minimizing the integrated mean square error (IMSE) in approximating the true density function \( f(x) \) with an estimate \( \hat{f}(x) \), where

\[
IMSE = \int \left[ E[\hat{f}(x) - f(x)] \right]^2 dx
\]

The asymptotically optimal choice of bin width as derived by Scott is given by

\[
h_n = (6^{1/3} \int_{-\infty}^{\infty} f(x)^{2} dx)^{-1/3} / \sqrt{n}
\]

where \( n \) is the sample size.

The optimal bin size for a data distribution \( D \) having an underlying Gaussian distribution is given by \( h_n = 3.49 \sigma / \sqrt{n} \) and so the number of bins is given by

\[
N_{bin} = \left\lceil \left( \max(D) - \min(D) \right) / h_n \right\rceil
\]

where, \( \lceil \cdot \rceil \) denotes the next nearest integer value to the number (for e.g. \( \lceil 5.4 \rceil = 6 \)).

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