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# HOW ACCURATELY CAN A SINGLE MOLECULE BE LOCALIZED WHEN IMAGED THROUGH AN OPTICAL MICROSCOPE?

*Sripad Ram*<sup>1,2</sup>, *E. Sally Ward*<sup>1,3</sup> and *Raimund J. Ober*<sup>3,4\*</sup>

<sup>1</sup>Center for Immunology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX,

<sup>2</sup>Joint Biomedical Engineering Graduate Program, University of Texas at Arlington, Arlington, TX  
and University of Texas Southwestern Medical Center at Dallas, Dallas, TX,

<sup>3</sup>Cancer Immunobiology Center, University of Texas Southwestern Medical Center at Dallas, Dallas, TX,

<sup>4</sup>Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX.

## ABSTRACT

We present a simple analytical expression for the fundamental limit to the accuracy with which the location of a single molecule can be determined that is imaged through an optical microscope. This expression depends on the optical properties of the microscope and the photophysical properties of the single molecule. We also show how the fundamental limit is deteriorated by factors like pixelation of the detector and noise sources in the detection system. The present results gives an experimenter insight into what is achievable in an optical microscope and provide guidelines for experimental design.

## 1. INTRODUCTION

One of the most important questions that is central to single molecule detection concerns the accuracy with which the location of a single molecule can be determined. Quantifying the localization accuracy of a single molecule is important, since it not only provides insight into the type of studies that can be carried out, but it is also of relevance in the analysis of single molecule data. For example, it has been recently shown [1] that the accuracy of the location estimates has to be taken into account when analyzing the diffusion behavior of single molecules, since noisy measurement of single molecule locations could lead to the erroneous interpretation that subdiffusional behavior is present when it is not the case.

Earlier approaches to quantifying the localization accuracy have mainly relied on the least squares criterion [2, 3, 4], which is problematic when applied to data that arise from non-Gaussian probability distributions. Aside from this, other approximations have been made that are often difficult to verify in the case of single molecule data. Moreover, the image of a single molecule predicted by standard

diffraction theory [5] is often replaced by a Gaussian profile. Importantly, our results do not rely on any of the above approximations and assumptions.

Our present approach is based on statistical estimation theory. Due to the random nature of photon detection, the data acquisition process is modeled as a stochastic data generation process. A general expression for the Fisher information matrix corresponding to the above stochastic process is derived and the Cramer-Rao lower bound (CRLB) [6] is computed. Since the CRLB is a minimum variance bound for any unbiased estimator [6, 7], we define the limit of the localization accuracy as the square root of the CRLB.

## 2. FUNDAMENTAL LIMIT

We consider a basic optical setup in which a single molecule in the object plane is placed at the focus of an objective lens and the image of the single molecule is captured by a detector. The position of the single molecule in the object plane is  $\theta := (u, v) \in \mathbb{R}^2$  and in the detector plane is  $M\theta \in \mathbb{R}^2$ , where  $0 < M < \infty$  denotes the magnification of the lens.

The stochastic data generation process comprises of a temporal and a spatial component. The temporal part describes the time points of emission of photons from the single molecule and is modeled by a counting process  $\{N(t); t \geq t_0\}$ . The spatial part describes the coordinates of the point of detection of each photon (emitted by the single molecule) on the detector and is modeled as independent and identically distributed random variables  $U_k : \Omega \rightarrow \mathbb{R}^2$ ,  $k = 1, 2, \dots$ , with density function

$$f_\theta(r) := \frac{1}{(M\theta)^2} q\left(\frac{r}{M} - \theta\right), \quad r \in \mathbb{R}^2, \quad (1)$$

where  $q$  denotes an image function and  $\Omega$  denotes the sample space. An image function describes the image of a single molecule that is located at the center of the coordinate

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system and is imaged through a lens with unit magnification. We assume that  $f_\theta$  satisfies the regularity conditions [6]. Due to the finite transmission efficiencies of the optical components, some of the photons are randomly deleted with probability  $1 - \gamma$ , where  $\gamma$  denotes the overall efficiency of the detection system.

We assume that the temporal component does not depend on  $\theta$  and is mutually independent of the spatial component. Let  $t$  denote the acquisition time,  $L$  denote the total number of detected photons and  $\mathcal{Z} := \{z_1, \dots, z_L\}$  denote the observed data, where  $z_k := (t_k, r_k)$ ,  $t_0 < t_1 \leq \dots \leq t_L \leq t$  denote the arrival times of the photons on the detector and  $r_k := (x_k, y_k) \in \mathbb{R}^2$  denotes the spatial coordinate of the  $k^{\text{th}}$  detected photon on the detector,  $k = 1, \dots, L$ .

The log likelihood function for the observed data is denoted by  $\mathcal{L}(\theta|\mathcal{Z})$  and the general expression for the Fisher information matrix is given by [6, 7]

$$\mathbf{I}(\theta) = E \left[ \left( \frac{\partial \mathcal{L}(\theta|\mathcal{Z})}{\partial \theta} \right) \left( \frac{\partial \mathcal{L}(\theta|\mathcal{Z})}{\partial \theta} \right)^T \right] \quad (2)$$

$$= \gamma E[N(t)] \int_{\mathbb{R}^2} \frac{1}{q(x, y)} \begin{bmatrix} \frac{\partial q(x, y)}{\partial x} \\ \frac{\partial q(x, y)}{\partial y} \end{bmatrix} \begin{bmatrix} \frac{\partial q(x, y)}{\partial x} \\ \frac{\partial q(x, y)}{\partial y} \end{bmatrix}^T dx dy \quad (3)$$

where  $E[\cdot]$  denotes the expectation operation and the above expression was derived by taking conditional expectation. By inverting the above matrix and taking the square root of the leading diagonal elements we obtain a general expression for the limit of the localization accuracy for a single molecule.

If we assume that the image function  $q$  is rotationally symmetric, then the off-diagonal terms of the Fisher information matrix given in eq. 3 are zero, since the integrand is a product of an odd and an even function. Thus the general expression for the limit of the localization accuracy for the  $u$  coordinate of the single molecule with a symmetric image function is given by (the expression for the  $v$  coordinate is analogous)

$$\delta_u = \left[ \gamma E[N(t)] \int_{\mathbb{R}^2} \frac{1}{q(x, y)} \left( \frac{\partial q(x, y)}{\partial x} \right)^2 dx dy \right]^{-\frac{1}{2}}. \quad (4)$$

According to diffraction theory [5] the image of a point source is described by the classical point spread function given by

$$q(x, y) = \frac{1}{\pi} \frac{J_1^2(\alpha \sqrt{x^2 + y^2})}{x^2 + y^2}, \quad (x, y) \in \mathbb{R}^2, \quad (5)$$

where  $J_1$  denotes the first order Bessel function of the first kind,  $\alpha := 2\pi n_a / \lambda_{em}$ ,  $n_a$  denotes the numerical aperture of the objective lens,  $\lambda_{em}$  denotes the wavelength of the

photons emitted by the single molecule and the constant  $1/\pi$  is a normalization constant.

We can easily verify that the above expression of  $q$  is symmetric. For simplicity, we assume that the counting process  $N(t)$  is a homogeneous Poisson process with intensity  $A$ ,  $0 < A < \infty$ . Hence we have  $E[N(t)] = At$ . Substituting for  $q$  in eq. 4 we obtain the *fundamental limit* to the localization accuracy of the  $u$  ( $v$ ) coordinate of the single molecule, which is given by

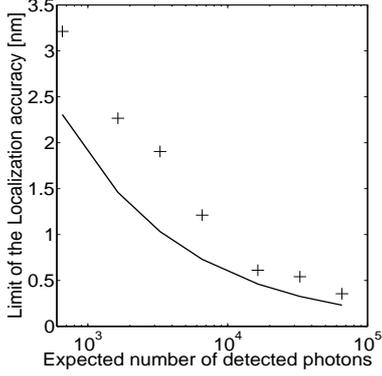
$$\delta_u^{fun} = \frac{\lambda_{em}}{2\pi n_a \sqrt{\gamma A t}}. \quad (6)$$

In deriving the above result, the partial derivative of the image function  $q$  with respect to  $x$  was calculated by using the recurrence relations for Bessel functions [8, pg. 18] and the resulting integral expression was evaluated by using an integral identity for Bessel functions [8, pg. 405].

The above expression is referred as ‘fundamental’, since the underlying model does not take into account any deteriorating effects such as pixelation of the detector and noise sources that are present in the experimental data (in the next section we show how these factors deteriorate the fundamental limit). From eq. 6 we see that the limit of the localization accuracy depends on the optical properties of the microscope (i.e. numerical aperture of the objective lens and optical efficiency of the detection system) and the photophysical properties of the single molecule (i.e. emission wavelength and photon emission rate of the single molecule). The fundamental limit exhibits an inverse square root dependence on the expected number of detected photons ( $\gamma A t$ ), which is in agreement with previously published results [3, 4] (see Fig. 1).

To improve the limit of the localization accuracy by a factor of two (i.e. halve the value of  $\delta_u^{fun}$ ), we either need to double the numerical aperture of the objective lens, or increase the photon emission rate / the optical efficiency by a factor of four, or halve the emission wavelength of the single molecule. This means that the location of a single molecule emitting blue light can be more accurately determined than one that is emitting red light, provided all other factors remain the same. Note that the fundamental limit is independent of the magnification  $M$  of the optical system.

It is important to determine whether an estimator exists whose performance comes close to the fundamental limit. Here we consider the maximum likelihood estimator and show that (see Fig. 1) under typical experimental conditions as the expected number of detected photons increases the standard deviation of the maximum likelihood estimator comes close to the fundamental result.



**Fig. 1.** Fundamental limit to the localization accuracy of a GFP single molecule under typical experimental conditions (—), (i.e.  $A = 2 \times 10^6$  photons/s,  $n_a = 1.4$ ,  $\lambda_{em} = 520$  nm,  $\gamma = 0.033$  and  $t := 0.01 - 1$  s). (+) denotes the standard deviation of the maximum likelihood estimator.

### 3. INFLUENCE OF PIXELATION AND NOISE

We next consider the effect of pixelation and the presence of noise sources and show how the fundamental limit is affected by these factors. In deriving the fundamental limit it was assumed that the time points of photon detection and the precise coordinates of the detected photons were known. Current imaging detectors have (finite sized) pixels and provide the coordinates of the detected photons only upto a pixel. Moreover, the time points of detected photons are generally not available. Hence the number of detected photons (that come from the single molecule) at each pixel is modeled as a Poisson random variable  $S_{\theta,k}$  with mean  $\mu_{\theta}(k) := \gamma A t \int_{C_k} f_{\theta}(r) dr$ , where  $f_{\theta}$  is given in eq. 1,  $C_k$  denotes the  $k^{th}$  pixel for  $k = 1, \dots, K$  and  $K$  denotes the total number of pixels on the detector. We assume no specific shape, size or orientation for these pixels and that no two pixels on the detector share a common region.

In addition, we consider additive Poisson and Gaussian noise sources that are commonly encountered in experimental data. Poisson noise is used to model the effects of auto-fluorescence, background and dark current and Gaussian noise is used to model the effect of measurement noise that arise in the detector [10]. Thus at each pixel the observed photon count  $Z_k$  is given by

$$Z_k = S_{\theta,k} + B_k + W_k, \quad k = 1, \dots, K,$$

where  $B_k$  is a Poisson random variable with mean  $\beta(k)$  that denotes the Poisson noise component at the  $k^{th}$  pixel and  $W_k$  is a Gaussian random variable with mean  $\eta_k$  and variance  $\sigma_k^2$  that denotes the Gaussian noise component at the  $k^{th}$  pixel,  $k = 1, \dots, K$ . We assume that  $S_{\theta,k}$ ,  $B_k$  and  $W_k$  are mutually independent of each other and  $\eta_k$ ,  $\sigma_k$  and  $\beta(k)$  are independent of  $\theta$  for  $k = 1, \dots, K$ .

We consider two different scenarios and derive the limit of the localization accuracy in each case. For all the cases, we assume the image function to be the classical point spread function given in eq. 5 and the derivation is analogous to that of the fundamental limit.

First we consider the case where only the Poisson noise component is present, i.e.  $W_k = 0$ ,  $k = 1, \dots, K$  and we set  $\beta(k) := b_k t$ , where  $b_k$  denotes the rate of the Poisson noise component. Using the fact that the sum of two independent Poisson random variables is also Poisson distributed, the Fisher information matrix can be easily derived and the limit of the localization accuracy for the  $u$  coordinate of the single molecule (the expression for the  $v$  coordinate is analogous) is given by

$$\delta_u^{fun} \left( \frac{1}{2} \left[ \sum_{k=1}^K \frac{\mathcal{J}_x^2(k)}{h_{\theta}(k) + \frac{b_k}{\gamma A}} - \frac{\left( \sum_{k=1}^K \frac{\mathcal{J}_x(k)\mathcal{J}_y(k)}{h_{\theta}(k) + \frac{b_k}{\gamma A}} \right)^2}{\sum_{k=1}^K \frac{\mathcal{J}_y^2(k)}{h_{\theta}(k) + \frac{b_k}{\gamma A}}} \right]^{-\frac{1}{2}} \right), \quad (7)$$

where  $\delta_u^{fun}$  is given in eq. 6,  $\mathcal{J}_x(k)$ ,  $\mathcal{J}_y(k)$  are given by

$$\mathcal{J}_x(k) := \int_{C_k} (x - Mu) \frac{J_1(a||r - r_0||)}{\pi||r - r_0||} \frac{J_2(a||r - r_0||)}{||r - r_0||^2} dr,$$

$$\mathcal{J}_y(k) := \int_{C_k} (y - Mv) \frac{J_1(a||r - r_0||)}{\pi||r - r_0||} \frac{J_2(a||r - r_0||)}{||r - r_0||^2} dr,$$

$J_2$  denotes the second order Bessel function of the first kind,  $a = 2\pi n_a / (\lambda_{em} M)$  and  $h_{\theta}(k) = \int_{C_k} f_{\theta}(r) dr$  for  $k = 1, \dots, K$ . Note that setting  $b_k = 0$  in eq. 7 gives an expression for the limit of the localization accuracy for a pixelated finite sized detector in the absence of any noise sources.

Next we consider the case where both Poisson and Gaussian noise sources are present. The Fisher information matrix is given by

$$[\mathbf{I}(\theta)]_{ij} = \sum_{k=1}^K \frac{\partial \mu_{\theta}(k)}{\partial \theta_i} \frac{\partial \mu_{\theta}(k)}{\partial \theta_j} \Psi(k),$$

where  $i, j = 1, 2$ ,  $\Psi(k) := \Phi(k) - 1$  and  $\Phi(k)$  is given by

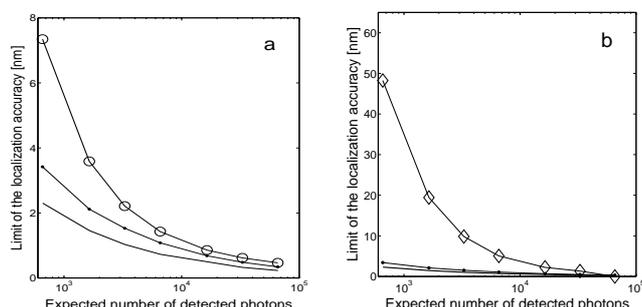
$$\Phi(k) = \frac{e^{-\nu_{\theta}(k)}}{\sqrt{2\pi}\sigma_k} \int_{\Re} \frac{\left( \sum_{n=1}^{\infty} \frac{(\nu_{\theta}(k))^{n-1}}{(n-1)!} e^{-\frac{1}{2\sigma_k^2}(z-\eta_k-n)^2} \right)^2}{\sum_{n=0}^{\infty} \frac{(\nu_{\theta}(k))^n}{n!} e^{-\frac{1}{2\sigma_k^2}(z-\eta_k-n)^2}} dz,$$

with  $\nu_{\theta}(k) := \gamma A t h_{\theta}(k) + b_k t$ ,  $k = 1, \dots, K$  and the limit of the localization accuracy for  $u$  coordinate of the single molecule is given by

$$\delta_u^{fun} \left( \frac{1}{2\sqrt{\gamma A t}} \left[ \sum_{k=1}^K \mathcal{J}_x^2(k) \Psi(k) - \frac{\left( \sum_{k=1}^K \mathcal{J}_x(k)\mathcal{J}_y(k)\Psi(k) \right)^2}{\sum_{k=1}^K \mathcal{J}_y^2(k)\Psi(k)} \right]^{-\frac{1}{2}} \right). \quad (8)$$

From eqs. 7 and 8 we see that the presence of pixelation and noise sources introduces a correction term to the fundamental limit that is given in parentheses. Note that the correction term introduces the dependence of the limit of the localization accuracy on various factors like magnification, pixel size, pixel shape, detector size and the relative position of the single molecule with respect to the center of the detector.

We now illustrate the above results by showing how noise levels deteriorate the limit of the localization accuracy. Fig. 2 shows the limit of the localization accuracy for a GFP single molecule that is imaged under typical experimental conditions. Fig. 2a shows the results for low noise levels ( $\eta_k = 0$ ,  $b_k = 660$  photons/pixel/s,  $\sigma_k = 7 e^-$  (rms)) and Fig. 2b shows the results for high noise levels ( $\eta_k = 0$ ,  $b_k = 6600$  photons/pixel/s,  $\sigma_k = 57 e^-$  (rms)). In both the figures the noise statistics for all the pixels is set to be the same. From Fig. 2 we see that in the presence of high noise levels the limit of the localization accuracy can be an order of magnitude higher than that of the noise free case especially for small photon count numbers. However, by increasing the number of detected photons it is possible to come close to the fundamental limit. Although not shown here, it can be verified that the standard deviation of the maximum likelihood estimator comes reasonably close to the limit of the localization accuracy for a pixelated detector for the different noise levels considered here.



**Fig. 2.** Limit of the localization accuracy for a GFP single molecule for different noise levels. Fig. 2a shows results for low noise levels ( $\circ$ ) and Fig. 2b shows results for high noise levels ( $\diamond$ ). In both figures, the limit of the localization accuracy in the noise free case ( $\bullet$ ) and the fundamental limit ( $\text{—}$ ) are also shown for reference. For all the plots, the pixel array size is fixed to be  $5 \times 5$  and the pixel dimension is fixed to be  $6.8 \times 6.8 \mu m$ .

The present results provide a framework to evaluate an experimental setting in the context of single molecule detection. Moreover, they can be used as a benchmark to compare the performance of different algorithms that are used to calculate the location of a single molecule from experimental data. In conclusion we note that our present formulation

can be extended to calculate the limit of the localization accuracy of any object with a known image function that is imaged by an optical microscope.

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