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A novel stochastic resolution criterion for fluorescence microscopes

Sripad Ram[1,2], E. Sally Ward[1] and Raimund J. Ober[1,3*]

[1]Center for Immunology NB9.106, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Boulevard, Dallas, TX 75235-8576, USA.
[2]Joint Biomedical Engineering Graduate Program, University of Texas at Arlington/University of Texas Southwestern Medical Center at Dallas, TX, USA.
[3]Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX 75083-0688, USA.

ABSTRACT

Rayleigh's criterion is widely in optical microscopy to determine the resolution of microscopes. Despite its widespread use, it is well known that this criterion is heuristic and does not consider the actual measurement process. For instance, it has been pointed out that the resolution of a fluorescence microscope can, in principle, exceed Rayleigh's criterion when distance determination between two point sources is postulated as a parameter estimation problem. In fact, recent results from single molecule fluorescence experiments show that the location of two closely spaced point sources with distance of separation well below Rayleigh's criterion can be accurately estimated. These results suggest that Rayleigh's criterion is inadequate for current microscope techniques. Here, by adopting an information-theoretic stochastic framework, we re-visit the resolution problem and derive a new stochastic resolution criterion that provides a limit to the accuracy with which the distance between two point sources can be determined. Our results predict that the distance between two point sources can be determined to an arbitrary accuracy provided a sufficient number of photons are detected. The new criterion is given in terms of quantities such as the expected number of detected photons, the numerical aperture of the objective lens and the wavelength of the detected photons. We also investigate how the new resolution measure is influenced by deteriorating experimental factors such as pixelation of the detector and additive noise sources.

1. INTRODUCTION

The resolution of an optical system is a measure of the system's ability to distinguish two closely spaced point sources. Rayleigh's resolution criterion, although widely used in optical microscopy, is based on heuristic notions.[1] For instance, Rayleigh's criterion neglects the stochastic nature of the photon detection process and hence does not consider the total photon count in the acquired data. Rayleigh's criterion was developed at a time when the unaided human eye was used as the detector. Therefore, this criterion is not well adapted to current microscope setups, in which highly sensitive photon counting cameras are used. Not surprisingly, recent single molecule experiments have shown that Rayleigh's criterion can be surpassed in an optical microscope setup.[2–4] Classically, Rayleigh's criterion has been used as a tool to evaluate the feasibility of carrying out experiments that involve distance determination between two identical point sources. However, the inadequacy of Rayleigh's criterion, especially in the context of quantitative imaging techniques such as single molecule microscopy, suggests the need for a new resolution measure that is adapted to modern imaging approaches. In particular, for proper planning of an experiment, it is important to have a methodology available to be able to assess with what accuracy the distance between two single molecules can be determined.

In this paper we propose a novel resolution measure that overcomes the shortcomings of Rayleigh's criterion and provides a quantitative measure of a microscope's ability to determine the distance between two point sources. This resolution measure is obtained by using a stochastic framework and is based on the theory concerning the Cramer-Rao inequality.[5,6] The new resolution measure, unlike Rayleigh's criterion, predicts

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that the resolution of a microscope can be improved by increasing the number of photons collected from the point sources. The resolution measure is given in terms of quantities such as the expected number of detected photons, the numerical aperture of the objective lens and the wavelength of the detected photons. We also investigate how the resolution measure is influenced by various experimental factors that affect the acquired data such as pixelation of the detector. Our results provide novel tools for the design and analysis of single molecule experiments.

2. RESULTS

2.1. Fundamental resolution measure

The derivation of the resolution measure is based on the theory concerning the Cramer-Rao inequality. The task of determining the distance of separation between two point sources is formulated as a parameter estimation problem. A stochastic framework is adopted to obtain a bound/limit to the accuracy with which the distance between two point sources can be estimated based on the acquired data. The detailed description of the stochastic framework for a general parameter estimation problem can be found elsewhere.[7] Analogous to Rayleigh's criterion, we consider two identical, self-luminous, in-focus point sources emitting unpolarized, incoherent light. For this imaging condition, the fundamental resolution measure (FREM) is given by

$$\delta_d := \frac{1}{\sqrt{4\pi \cdot \Lambda_0 \cdot (t - t_0) \cdot \Gamma_0(d)}} \cdot \frac{\lambda}{n_a}, \tag{1}$$

where λ denotes the emission wavelength of the detected photons, n_a denotes the numerical aperture of the objective lens, Λ_0 denotes the photon detection rate (intensity) per point source, $[t_0, t]$ denotes the acquisition time interval, and $\Gamma_0(d)$ is given by

$$\Gamma_0(d) := \int_{\mathbb{R}^2} \frac{1}{\frac{J_1^2(\alpha r_{01})}{r_{01}^2} + \frac{J_1^2(\alpha r_{02})}{r_{02}^2}} \left((x + \frac{d}{2}) \frac{J_1(\alpha r_{01}) J_2(\alpha r_{01})}{r_{01}^3} - (x - \frac{d}{2}) \frac{J_1(\alpha r_{02}) J_2(\alpha r_{02})}{r_{02}^3} \right)^2 dxdy, \tag{2}$$

with J_n denoting the n^{th} order Bessel function of the first kind, $\alpha := 2\pi n_a/\lambda$, $r_{01} := \sqrt{(x + d/2)^2 + y^2}$ and $r_{02} := \sqrt{(x - d/2)^2 + y^2}$. In contrast to Rayleigh's criterion that states that the minimum resolvable distance between two point sources is $0.61\lambda/n_a$, the FREM provides a more complex expression, which, in addition to the dependence on the ratio λ/n_a, exhibits an inverse square root dependence on other factors, i.e., the expected number of detected photons ($\Lambda_0 \cdot (t - t_0)$) and the term $\Gamma_0(d)$ given by eq. 2. Note that the FREM exhibits a non-linear dependence on λ/n_a due to its presence in $\Gamma_0(d)$ through the term α ($= 2\pi n_a/\lambda$).

The derivation of the FREM is based on a stochastic framework in which the acquired data consists of the time points and the spatial coordinates of every detected photon without adding any extraneous noise. For any imaging condition, this can be thought of as an idealization of current imaging detectors, since the presence of finite-size pixels and measurement noise deteriorates the acquired data. Thus the resolution measure derived within this framework provides a result that is *fundamental* for the given imaging condition. In the present context, the FREM is obtained for imaging conditions analogous to those of Rayleigh's criterion. Hence the spatial distribution of the detected photons from each point source is described by the Airy profile.[8]

The new resolution measure FREM predicts how accurately the distance d between two point sources can be resolved. A small numerical value for the FREM predicts a high accuracy in determining d, while a large numerical value of the FREM predicts a low accuracy in determining d. Fig. 1 shows the behavior of the FREM as a function of the distance of separation between a pair of TRITC molecules ($\lambda = 600$ nm) that are imaged with an objective lens of numerical aperture 1.45. For the TRITC molecules, Rayleigh's criterion predicts the smallest resolvable distance to be about 252 nm ($\approx 0.61\lambda/n_a$). In contrast, Fig. 1A shows that the FREM has a small numerical value for distances in the range of 50 - 300 nm, which are well below Rayleigh's resolution limit. For distances less than 50 nm, however, the FREM deteriorates (i.e. increases) significantly with decreasing distance of separation (see Fig. 1B).

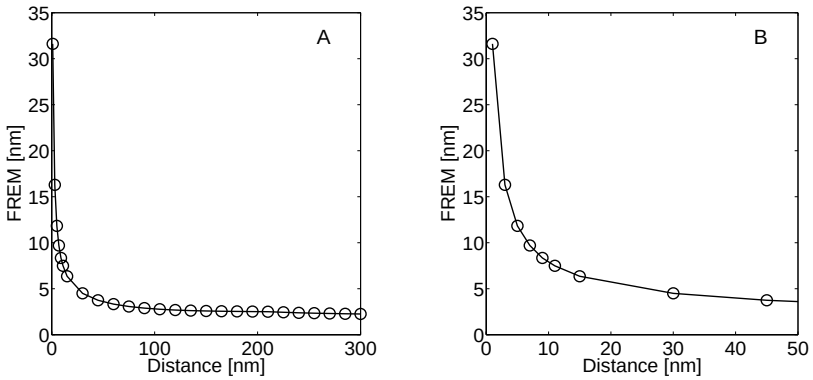

Figure 1. Panel A shows the FREM as a function of the distance of separation for a pair of TRITC molecules. Panel B shows the same for a distance range of $1 - 50$ nm. In all the plots, the numerical aperture is set to be $n_a = 1.45$, the wavelength of the detected photons from the TRITC molecule is set to be $\lambda = 600$ nm, the photon detection rate Λ_0 of each TRITC molecule is set to be $\Lambda_0 = 2500$ photons/s and the acquisition time is set to be 1 s.

2.2. Effects of pixelation and noise

In the previous section we saw that the FREM pertained to the best case scenario, where deteriorating experimental factors were not taken into account. We next investigate how the resolution measure is affected by such experimental factors. Here, we take into account the presence of additive noise sources, namely Poisson and Gaussian noise. For example, Poisson noise models the spurious photons that arise due to autofluorescence of the sample and dark current of the detector,[9] while Gaussian noise models the measurement noise that arises during the readout process in the detector.[10] It should be pointed out that the additive Poisson noise considered here is distinct from the shot noise, which describes the statistics of the photon detection process from the single molecules and this is already accounted for by the FREM. Aside from these extraneous noise sources, the effect of pixelation of the detector is also taken into account. We obtain an analytical expression for the resolution measure that takes into account these experimental factors and we refer to this result as the practical resolution measure (PREM). The PREM can be thought of as an extension to the FREM. Fig. 2A shows the behavior of the PREM as a function of the distance between two TRITC molecules in the presence and absence of noise sources for a pixelated detector. The figure also shows the FREM for reference. Note that even in the absence of extraneous noise sources the numerical value of the PREM is consistently greater than that of the FREM due to the pixelation of the detector. Moreover, in the presence of noise sources this behavior of the PREM becomes more pronounced. In particular, for very small distances (≤ 50 nm), the numerical value of the PREM is at least 3-5 times greater than that of the FREM (see Fig. 2B).

From eq. 1, we know that the resolution measure can be improved by increasing the expected number of detected photons from each of the point sources. In many fluorescence microscopy techniques such as single molecule microscopy this is not always possible, since the fluorescent point sources may photobleach. However, for a single-molecule pair that exhibits a double-step photobleaching behavior,[11] additional information can be obtained from the photons collected from the fluorophore that remains after the first photobleaching event (see also Ref. 2). Fig. 2C shows the behavior of the resolution measure for a pair of TRITC molecules spaced 10 nm apart by taking into account the number of photons collected before and after the first photobleaching event. Here the resolution measure is determined for an imaging condition with numerical values analogous to those used in Fig. 2A. From the figure we see that the resolution measure predicts an accuracy not smaller than ± 4.9 nm to determine a distance of 10 nm, when on average 5000 photons are collected from each fluorophore before and after the first photobleaching event. This is in contrast to the case when the additional information obtained from after the first photobleaching event is not used. In this case, the resolution measure predicts an accuracy not smaller than ± 50 nm to resolve a distance of 10 nm for the same photon count per fluorophore. Note that increasing the photon count collected from before and after the first photobleaching event results in the further

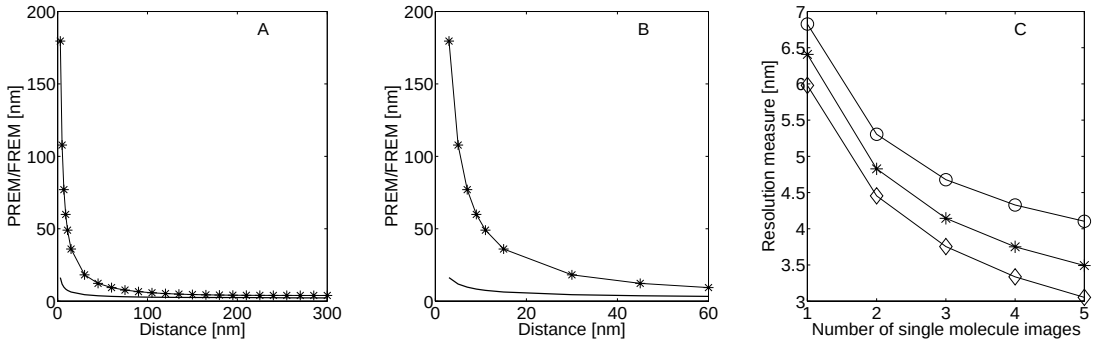

Figure 2. Panel A shows the PREM as a function of the distance of separation for a pair of TRITC molecules. The FREM given in eq. 1 is also shown for reference (—). Panel B shows the same for a distance range of $1 - 50$ nm. Panel C shows the effect of using additional spatial information on the resolution measure for a pair of TRITC molecules ($d = 10$ nm) that exhibits a double-step photobleaching behavior. The panel shows the resolution measure as a function of the expected photon count collected from the single molecule after the first photobleaching event for a pixelated detector in the presence of noise sources. The plots shown consider three scenarios, i.e., when the expected number of photons collected from each single molecule before the first photobleaching event are 2500 ($\circ$), 5000 ($*$) and 12500 ($\diamond$). In all panels, the pixel dimension is set to be 12.9 $\mu m \times 12.9$ μm, the pixel array size is set to be 13×13, the mean of the additive Poisson noise is set to be 80 photons/pixel/s, the mean and standard deviation of the additive Gaussian noise is set to be 0 e^-/pixel and 8 e^-/pixel, respectively, the noise statistics is assumed to be the same for all pixels, and one of the single molecules is assumed to be at the center of the pixel array. All other numerical parameters are analogous to those used in Fig. 1.

improvement of the resolution measure.

2.3. General imaging conditions

The FREM (eq. 1) was obtained for specific imaging conditions that were analogous to those assumed in the derivation of Rayleigh's criterion, i.e., two equal intensity, in-focus point sources that emit unpolarized, incoherent light. However, in many cases these conditions are not met, for instance when using polarized illumination and detection setups.[11] A generalization of the FREM can be obtained in which the point sources can have unequal intensities that vary as a function of time, and where the image profiles of the point sources can be distinct. The analytical expression for the 'generalized' resolution measure (**g-FREM**) is given by

$$\left[\frac{1}{4}\int_{t_0}^{t}\int_{\mathbb{R}^2}\frac{1}{\Lambda_1(\tau)q_1(x+\frac{d}{2},y)+\Lambda_2(\tau)q_2(x-\frac{d}{2},y)}\left(\Lambda_1(\tau)\frac{\partial q_1(x+\frac{d}{2},y)}{\partial x}-\Lambda_2(\tau)\frac{\partial q_2(x-\frac{d}{2},y)}{\partial x}\right)^2 dxdyd\tau\right]^{-\frac{1}{2}}, \qquad (3)$$

where q_1 and q_2 denote the image functions of the point sources and Λ_1 and Λ_2 denote the intensities of the point sources. An image function describes the image of an object at unit lateral magnification when the center of the object is located at the origin of the coordinate axes in the specimen plane. In many situations the image of the point source significantly differs from the Airy profile, for example, due to the aberrations present in the imaging setup[14,15] or due to the different orientations of the point-source emission dipole.[12,13] Moreover, depending upon the nature of illumination, the intensity of the point sources can be unequal when their emission dipole orientations are different.[11,12] If in the above equation we set the intensities to be constant and identical, i.e., $\Lambda_1(\tau) = \Lambda_2(\tau) = \Lambda_0$, $\tau \geq t_0$, and assume the image functions to be given by the Airy profile, then we immediately obtain the expression for the FREM given in eq. 1. Analogous to eq. 3, a 'generalized' version of the practical resolution measure can also be derived.

3. DISCUSSION

The advent of single molecule microscopy has generated significant interest in studying nanoscale biomolecular interactions. The understanding of the distance determination problem between two identical point sources is

important for the study of protein-protein and other molecular interactions with an optical microscope. Classically, fluorescence resonance energy transfer based methods have been used to probe interactions in the distance range of 1 - 10 nm. It is widely believed that Rayleigh's criterion precludes the resolution of two single molecules at distances less than 200 nm. This leaves a gap in the distance range of 10 - 200 nm that is vital for the study of many biological processes with an optical microscope. Rayleigh's criterion, although widely used in microscopy, is based on heuristic notions that are incompatible with modern imaging approaches. For instance, it has been suggested that Rayleigh's resolution limit can be superseded if the distance between two point sources is determined by curve fitting the image with the sum of two point-source image profiles.[16] In fact, by adopting this approach several groups have shown that Rayleigh's limit can be surpassed in experiments.[2–4] The inadequacy of Rayleigh's criterion, however, raises concerns over its use as a performance criterion of resolution for modern imaging techniques. This, in turn, emphasizes the need for a new resolution criterion, i.e., a modern version of Rayleigh's resolution criterion that is applicable to current imaging approaches. This paper presents a systematic investigation of the resolution problem to address the above concerns and provides a new resolution measure that overcomes the limitations of Rayleigh's criterion. The new resolution measure is based on the theory concerning the Cramer-Rao inequality and is derived within a stochastic framework.

The fundamental resolution measure (FREM) in eq. 1 gives a bound for the best resolution performance that can be attained in a given imaging condition when the acquired data is not affected by deteriorating experimental factors. The expression is a limit for the accuracy with which the distance between the point-sources/single-molecules can be estimated. An important property of the FREM is that it provides a quantitative assessment of how the optical characteristics of the experimental setup and the photon budget influence the resolution performance in determining a particular distance of separation. The practical resolution measure (PREM) derived here extends the results of the FREM by illustrating how the resolution measure is deteriorated by experimental factors such as pixelation of the detector and extraneous noise sources. In Figs. 2A-2B, it was shown that the numerical value of the PREM is consistently greater than that of the FREM. This behavior of the PREM can be attributed to the fact that the data acquired by a pixelated detector is a discretized version of the actual image, and the presence of extraneous noise sources corrupts the acquired data (e.g. scattered photons, noise in the acquisition electronics). Moreover, a comparison to the FREM illustrates that control of the noise sources is also of great importance to improve the accuracy of the estimated distance parameter in a practical scenario.

The FREM was derived for imaging conditions that were analogous to those assumed in Rayleigh's criterion. In some single molecule experiments, however, the conditions are different to those assumed in the derivation of Rayleigh's criterion and which formed the basis for the derivation of the FREM. Whereas the FREM assumes that the image of a point source is given by an Airy profile, the generalized FREM (g-FREM) was derived so that more complex image profiles can be analyzed. Such profiles could arise, for example, due to out of focus conditions,[12] the presence of aberrations,[14, 15] or the use of polarized illumination.[11, 17] We note that the g-FREM can also be used to calculate the resolution measure for determining the distance of separation between any two (distinct) objects such as cellular organelles, provided the intensities and the image functions of the objects are known.

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