Ram, S.; Chao, J.; Prabhat, P.; Abraham, A.V.; Ward, E.S.; Ober, R.J., "Breaking resolution limits: advances and challenges in single molecule microscopy," in *Signals, Systems and Computers, 2007. ACSSC 2007. Conference Record of the Forty-First Asilomar Conference on* , vol., no., pp.1284-1287, 4-7 Nov. 2007
doi: 10.1109/ACSSC.2007.4487433
keywords: {fluorescence;information theory;matrix algebra;optical microscopy;optimisation;2D localization;3D localization;Fisher information matrix;Rayleigh's criterion;deterministic framework;fluorescent single molecules;heuristic notions;information theory;microscopes resolution;optical microscopy;photon emission;single molecule microscopy;Biomedical imaging;Biomedical optical imaging;Chaos;Fluorescence;Immune system;Molecular biophysics;Noise level;Optical imaging;Optical microscopy;Optical noise},
URL: http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=4487433&isnumber=4487143

Breaking resolution limits: advances and challenges in single molecule microscopy

Sripad Ram, Jerry Chao, Prashant Prabhat, Anish V. Abraham, E. Sally Ward and Raimund J. Ober

Abstract— **The resolution of an optical system is a measure of its ability to distinguish two closely spaced point sources. In optical microscopy, Rayleigh's criterion has been extensively used to determine the resolution of microscopes. Despite its widespread use, it is well known that this criterion is based on heuristic notions that are not suited to modern imaging approaches. Formulated within a deterministic framework, this criterion neglects the stochastic nature of photon emission and therefore does not take into account the total number of detected photons. In fact, recent single molecule experiments have shown that this criterion can be surpassed in a regular optical microscope thereby illustrating that Rayleigh's criterion is inadequate for current microscopy techniques. This inadequacy of Rayleigh's criterion has, in turn, necessitated a reassessment of the resolution limits of optical microscopes. By adopting an information-theoretic framework and using the theory concerning the Fisher information matrix, we recently proposed a new resolution measure that overcomes the limitations of Rayleigh's criterion. Here, we provide a review of this and other related results. The new resolution measure predicts that distances well below Rayleigh's limit can be resolved in an optical microscope. The effect of deteriorating experimental factors on the new resolution measure is also investigated. Further, it is experimentally verified that distances well below Rayleigh's limit can be measured from images of closely spaced fluorescent single molecules with an accuracy as predicted by the new resolution measure. We have also addressed an important problem in single molecule microscopy that concerns the accuracy with which the location of a single molecule can be determined. In particular, by using the theory concerning the Fisher information matrix we have derived analytical expressions for the limit to the 2D/3D localization accuracy of a single molecule.**

I. Introduction

Single molecule microscopy is a relatively new imaging technique that enables the study of individual biomolecules in real time within a cellular environment ([1]). The study of cellular processes at the single molecule level is motivated by several considerations. Single molecule experiments overcome the averaging effect that is characteristic of bulk measurements and provide information on the behavior of individual molecules. Single molecule studies also remove the need for synchronization of many copies of individual biomolecules that are involved in a time dependent process. Thus it is believed that imaging biomolecular interactions at the single molecule level holds the promise that significant new insights can be gained.

While the promises of this technique are great, so are its challenges. Single molecule experiments place extraordinary demands on the capabilities of current microscope setups and data analysis tools. The detection of single molecules requires a very careful design of the experimental setup. The signal from the single molecule is obscured by high levels of noise, which arises from a variety of sources such as autofluorescence of the cell and/or buffer, scattered photons that randomly reflect off optical components, the readout noise that originates from the detector ([2]), etc. Even without any noise sources the analysis of single molecule images would not be devoid of problems, since the acquired data is stochastic due to photon emission being a random process. However, even under the best circumstances the signal to noise ratio is very unfavorable ([3]). Therefore there is a considerable need for advanced signal and image processing methods that allow the available information to be extracted from the experimental data as accurately as possible. This is particularly so if quantitative results are to be obtained. In this regard, it is it is important for an experimenter to know the capabilities of the instrument. This not only provides insight into determining the feasibility of a particular experiment, but it also helps in designing an optimal experimental setup.

Previously, we had derived results to calculate the performance limits that quantify the capabilities of an optical microscope. The detailed derivation of these results are given in [4], [5]. Here, we present a review of this and other related results that were recently reported by our group ([6], [7], [8], [9], [10]). Due to the random nature of the acquired data, we adopted a stochastic framework and used the statistical theory concerning the Fisher information matrix ([11], [12]) to determine the performance limit. We considered a data model in which the photon emission (detection) process is described as a random process (shot noise process). We have taken into account the pixelation of the detector and extraneous, additive noise sources, such as Poisson and Gaussian noise, that are typically present in the acquired data ([13]). We note that our results are applicable to several microscopic techniques such as fluorescence microscopy,

This work was supported in part by the National Institutes of Health (RO1 GM071048).

S. Ram is in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

P. Prabhat and J. Chao are in the Department of Electrical Engineering, UT Dallas, Richardson, TX and in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

A. V. Abraham is in the Department of Computer Science, UT Dallas, Richardson, TX and in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

E. S. Ward is in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

R. J. Ober is in the Department of Electrical Engineering, UT Dallas, Richardson, TX and in the Department of Immunology at UT Southwestern Medical Center, Dallas, TX; Corresponding Author, Email: ober@utdallas.edu.

978-1-4244-2110-7/08/$25.00 ©2007 IEEE

bright-field microscopy, etc.

We have investigated the resolution problem ([6]), which is an important problem in optical microscopy. The classical resolution criterion of Rayleigh, although widely used, is well known to be based on heuristic notions that are incompatible with current imaging approaches. By using the above stochastic framework, we have derived a new resolution measure that overcomes the limitations of Rayleigh's criterion. According to our new resolution measure, the resolution of an optical microscope is not limited and it can be improved by increasing the expected number of detected photons. By imaging closely spaced single molecules and estimating their distance of separation, it was verified that distances well below Rayleigh's resolution limit can be resolved in an optical microscope setup with an accuracy as predicted by the new resolution measure ([6]).

We have also addressed an important problem in single molecule microscopy that concerns the accuracy with which the location of a single molecule can be determined ([7], [8]). We considered both in focus and out of focus scenarios and derived analytical expressions for the limit to the localization accuracy of a single molecule. A major drawback of the current microscope design is that one only focal plane can be imaged at a time. As a result 3D tracking of fast moving components is problematic with conventional microscopy techniques. Moreover regular optical microscopes also suffer from poor depth discrimination which results in significant uncertainty in determining the axial location of objects when they are especially close to the plane of focus ([8], [9]). To overcomes these shortcoming we had developed a new imaging modality called multifocal plane microscopy (MUM) ([14], [15]). By using the stochastic framework, we have shown that the MUM exhibits significantly improved depth discrimination and that the axial location of objects can be determined with high accuracy ([9]).

II. General Stochastic Framework

In a typical quantitative imaging experiment, attributes of the object of interest such as location, distance of separation from other objects, orientation, photon count etc., are determined from the acquired data by using a specific estimation procedure. In any estimation problem, it is important to know whether the specific estimation technique used to estimate the desired attribute indeed comes close to the best possible accuracy. This can be determined by calculating the Fisher information matrix for the underlying parameter estimation problem. The Fisher information matrix $\mathbf{I}(\theta)$ plays a central role in the theory of parameter estimation algorithms. Its inverse provides, through the classical Cramer-Rao inequality ([11]), a lower bound to the variance of any unbiased estimator $\hat{\theta}$ of an unknown parameter θ, i.e., $\mathrm{var}(\hat{\theta}) \geq \mathbf{I}^{-1}(\theta)$. Because the performance of estimation algorithms is typically specified in terms of its standard deviation, the above inequality implies that the square root (of the corresponding leading diagonal entry) of the inverse Fisher information matrix provides a lower bound to the performance of any unbiased estimator of θ. Stated otherwise, the square root of the inverse Fisher information matrix provides a *limit* to the accuracy with which a specific object attribute can be determined.

A. Fisher information matrix

The data acquired in an optical microscope setup is modeled as a spatio-temporal random process, which we refer to as the image detection process $\mathcal{G}$ ([4]). The temporal part is an inhomogeneous Poisson process that models the time points of the detected photons and the spatial part is a sequence of independent random variables that models the location coordinates at which the photons hit the detector. The general expression of the Fisher information matrix for the problem of estimating an unknown parameter θ from the acquired data is given by ([4], [12])

$$\mathbf{I}(\theta) = \int_{t_0}^{t}\int_{\mathcal{C}} \frac{1}{\Lambda_\theta(\tau) f_{\theta,\tau}(r)} \left(\frac{\partial[\Lambda_\theta(\tau) f_{\theta,\tau}(r)]}{\partial\theta}\right)^T \times \frac{\partial[\Lambda_\theta(\tau) f_{\theta,\tau}(r)]}{\partial\theta} dr\, d\tau, \quad \theta \in \Theta. \tag{1}$$

In the above expression, Λ_θ denotes the intensity function of the Poisson process, $\{f_{\theta,\tau}\}_{\tau \geq t_0}$ denotes the density function of the independent random variables and $\mathcal{C}$ denotes the detector. It is assumed that the spatial and the temporal components are mutually independent of each other. In deriving eq. 1 no specific assumptions have been made regarding the functional form of $f_{\theta,\tau}$ or Λ_θ. Therefore, the above expression of $\mathbf{I}(\theta)$ is applicable to a wide variety of imaging conditions, such as (in)coherent/polarized illumination and detection, etc. We note that an implication of the time dependence of the density function $f_{\theta,\tau}$ is that the above equation is applicable to moving objects.

III. Results

A. New resolution measure

The advent of single molecule microscopy has generated significant interest in studying nano-scale interactions within a cellular environment. It is widely believed that Rayleigh's criterion impedes the study of single molecular interactions at distances below 200 nm. Fluorescence resonance energy transfer based techniques are typically used to probe biomolecular interactions up to a distance of 10 nm. This, however, leaves a gap in the distance range of 10 - 200 nm which is important for the study of many biological processes in an optical microscope. It has been suggested that Rayleigh's resolution limit can be overcome when apriori information in conjunction with parameter estimation approaches are used to analyze the acquired data ([16]). In fact by using such approaches, several groups have shown that Rayleigh's limit can be surpassed in an optical microscope setup ([17], [18], [19], [6]).

By using the methodology based on the Fisher information matrix laid out above, we have derived a new resolution measure that overcomes the limitations of Rayleigh's criterion ([6]). Known as the fundamental resolution measure FREM, the new resolution measure predicts that distances well below

Rayleigh's limit can be resolved in an optical microscope. An analytical formula for the FREM has been derived and is given by ([6])

$$\delta_d := \frac{1}{\sqrt{4\pi \cdot \Lambda_0 \cdot (t-t_0) \cdot \Gamma_0(d)}} \cdot \frac{\lambda}{n_a}, \qquad (2)$$

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

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

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 $r_{02} := \sqrt{(x-d/2)^2 + y^2}$. From eq. 2 we see that the resolution measure is given in terms of quantities such as the expected number of detected photons, numerical aperture of the objective lens, and wavelength of the detected photons. The above expression was derived for imaging conditions analogous to those of Rayleigh's criterion, i.e., two equal intensity, in-focus point sources that emit incoherent, unpolarized light. In many applications, however, these conditions are not satisfied. Hence a general analytical expression for the resolution measure has been derived that is applicable to a variety of imaging conditions ([6]). The FREM provides the best-case scenario for a microscope setup, where experimental factors that potentially deteriorate the acquired data are not taken into account. This was done to obtain an expression for the best possible resolution in the absence of deteriorating factors due to specific experimental settings. Current imaging detectors have pixels and therefore the acquired data is a discretized version of the original image. Aside from this, the acquired data contains extraneous noise sources. Here two independent, additive noise sources are considered, i.e., Poisson noise and Gaussian noise. Poisson noise can be used to model, for example, the effect of scattered photons on the measured data and Gaussian noise characterizes, for example, measurement noise in the CCD detector ([2]). Analytical expressions for the resolution measure that take into account these deteriorating factors have also been obtained ([6]). The latter result is referred to as the practical resolution measure PREM.

By definition, the new resolution measure is a bound to the accuracy with which the distance between two point sources can be resolved. To verify if the resolution measure can be attained in a practical experimental setup, images of closely spaced single molecules were acquired and their distance of separation were estimated by using the maximum likelihood estimator (see [6] for details). It was found that distances well below Rayleigh's resolution limit can be determined from the acquired data with an accuracy as predicted by the new resolution measure.

B. Single molecule localization accuracy

One of the central problems in single molecule data analysis concerns the accuracy with which the location of a single molecule can be determined. By using the methodology based on the Fisher information matrix, we have investigated the single molecule localization accuracy problem. We derived a simple analytical formula that provides the fundamental limit to the accuracy with which the 2D location coordinates (x_0, y_0) of a single molecule can be determined ([7], [4]), which is given by

$$\delta_{x_0}^{2d} = \delta_{y_0}^{2d} = \frac{\lambda}{2\pi n_a \sqrt{\Lambda_0 (t-t_0)}}, \qquad (3)$$

where λ denotes the wavelength of the detected photons, n_a denotes the numerical aperture of the objective lens, Λ_0 denotes the photon detection rate of the single molecule and $[t_0, t]$ denotes the acquisition time interval. The importance of this result lies in the fact that it shows with an unexpectedly simple expression how fundamental properties of the single molecule (emission wavelength, photon-detection rate) and of the detection system (numerical aperture, acquisition time) influence the localization accuracy of the single molecule. The above result is referred as 'fundamental', since the model that underlies the derivation of the result does not take into account deteriorating experimental factors such as pixelation of the detector or extraneous noise sources in the acquired data. Therefore, the above result pertains to the best-case scenario for a given imaging configuration.

Eq. 3 provides an expression for the 2D fundamental limit to the localization accuracy of a single molecule, where it is assumed that the single molecule lies in the focal plane of the objective lens (in-focus scenario). However, in a cellular environment a single molecule can move in all three dimensions. Therefore, for such cases it is important to know the 3D limit of the localization accuracy. In [8] we have addressed this problem and have obtained analytical expressions for the 3D fundamental limit to the localization accuracy of a single molecule.

The derivation of the fundamental limit given in eq. 3 assumes the best case scenario for the acquisition system. This was done to obtain an expression for the best possible localization accuracy in the absence of deteriorating factors due to specific experimental settings. Analytical expressions for the 2D and the 3D limit of the localization accuracy have been obtained that take into account deteriorating factors ([7], [8]).

It should be pointed out that the stochastic framework described in Section II allows for both stationary and time varying image profiles. Therefore analytical expressions for the limit of the localization accuracy of moving objects can also be obtained.

C. Multifocal plane microscopy and 3D single-molecule tracking

One of the shortcomings of conventional wide-field optical microscopes is their poor depth discrimination capability. Due to this, there exists significant uncertainty in determining

the axial location of point objects (e.g., single molecules), especially when they are close to the plane of focus. Previously we showed that the limit of the 3D localization accuracy of a single molecule can significantly vary depending upon the defocus level ([8]). For instance, for small defocus values ($\leq$ 200 nm), it was predicted that the x_0/y_0 coordinate of the single molecule can be determined with relatively high accuracy whereas the z_0 coordinate can be determined with poor accuracy. On the other hand, for large defocus values (200 - 700 nm), it was predicted that the x_0/y_0 coordinate of the single molecule can be determined with poor accuracy whereas the z_0 coordinate can be determined with high accuracy. Due to this mismatch in the limit of the localization accuracy between the x_0/y_0 coordinate and the z_0 coordinate, it is difficult to determine all three coordinates with the same level of accuracy.

To overcome this problem, we have proposed to use the multifocal plane microscope that was previously developed by our group ([14], [15]). Here we simultaneously image two distinct focal planes within the specimen. For example, one of the focal planes could correspond to the standard focal plane that is imaged in a conventional widefield microscope, while the other focal plane could correspond to a plane that is shifted away from the standard focal plane. If single molecules are imaged in the above setup, then the image of the shifted focal plane provides additional information regarding the single molecule location. By making use of this additional data, we have shown that the axial (z_0) location of the single molecule can be determined with very high accuracy. A detailed derivation of the performance limits of the multifocal plane microscope has been carried out and is reported in [9]. An alternative to the multifocal plane imaging technique is to use a focusing device, which *sequentially* moves the objective lens to acquire images of the different focal planes. A drawback of this approach is that focusing devices are typically slow and moreover, suffer from the lack of synchrony between their movement and the movement of the single molecules in the specimen. Thus when the specimen is being imaged at one focal plane important events can be missed in the other planes. With the multifocal plane imaging approach these problems are avoided, since there is no movement of the objective lens and more importantly the specimen is simultaneously imaged at multiple planes.

REFERENCES

[1] W. E. Moerner and D. P. Fromm, "Methods of single-molecule fluorescence spectroscopy and microscopy," *Review of Scientific Instruments*, vol. 74, no. 8, pp. 3597–3619, 2003.

[2] G. C. Holst, *CCD arrays, cameras and displays*, SPIE Press, Bellingham, WA, 2nd edition, 1998.

[3] S. Inoue and K. R. Spring, *Video Microscopy: The fundamentals*, Plenum Press, 2nd edition, 1997.

[4] S. Ram, E. S. Ward, and R. J. Ober, "A stochastic analysis of performance limits for optical microscopes," *Multidimensional Systems and Signal Processing*, vol. 17, pp. 27–58, 2006.

[5] S. Ram, *resolution and localization in single molecule microscopy*, Ph.D. thesis, University of Texas at Arlington/University of Texas Southwestern Medical Center at Dallas, 2007.

[6] S. Ram, E. S. Ward, and R. J. Ober, "Beyond Rayleigh's criterion: A resolution measure with application to single-molecule microscopy," *Proceedings of the National Academy of Sciences USA*, vol. 103, pp. 4457–4462, 2006.

[7] R. J. Ober, S. Ram, and E. S. Ward, "Localization accuracy in single molecule microscopy," *Biophysical Journal*, vol. 86, pp. 1185–1200, 2004.

[8] S. Ram, E. S. Ward, and R. J. Ober, "How accurately can a single molecule be localized in three dimensions using a fluorescence microscope?," *Proceedings of the SPIE*, vol. 5699, pp. 426–435, 2005.

[9] S. Ram, J. Chao, P. Prabhat, E. S. Ward, and R. J. Ober, "A novel approach to determining the three-dimensional location of microscopic objects with applications to 3D particle tracking," *Proceedings of the SPIE*, vol. 6443, pp. D1–D7, 2007.

[10] S. Ram, A. V. Abraham, E. S. Ward, and R. J. Ober, "A novel 3D resolution measure for optical microscopes with applications to single molecule imaging," *Proceedings of the SPIE*, vol. 6444, pp. D1–D9, 2007.

[11] C. R. Rao, *Linear statistical inference and its applications.*, Wiley, New York, USA., 1965.

[12] D. L. Snyder and M. I. Miller, *Random point processes in time and space.*, Springer Verlag, New York, USA, 2nd edition, 1991.

[13] D. L. Snyder, C. W. Helstrom, A. D. Lanterman, and R. L. White, "Compensation for read out noise in charge coupled device images," *Journal of the Optical Society of America A*, vol. 12, no. 2, pp. 272–283, 1995.

[14] P. Prabhat, S. Ram, E. S. Ward, and R. J. Ober, "Simultaneous imaging of different focal planes in fluorescence microscopy for the study of cellular dynamics in three dimensions," *IEEE Transactions on Nanobioscience*, vol. 3, no. 4, pp. 237–242, 2004.

[15] P. Prabhat, S. Ram, E. S. Ward, and R. J. Ober, "Simultaneous imaging of several focal planes in fluorescence microscopy for the study of cellular dynamics in 3D," *Proceedings of the SPIE*, vol. 6090, pp. 115–121, 2006.

[16] P. C. D. Hobbs, *Building electro-optical systems*, John Wiley and Sons, 2000.

[17] M. P. Gordon, T. Ha, and P. R. Selvin, "Single molecule high resolution imaging with photobleaching," *Proceedings of the National Academy of Sciences USA*, vol. 101, pp. 6462–6465, 2004.

[18] X. Qu, D. Wu, L. Mets, and N. F. Scherer, "Nanometer-localized multiple single-molecule fluorescence microscopy," *Proceedings of the National Academy of Sciences USA*, vol. 101, no. 31, pp. 11298–11303, 2004.

[19] K. A. Lidke, B. Rieger, T. M. Jovin, and R. Heintzmann, "Superresolution by localization of quantum dots using blinking statistics," *Optics Express*, vol. 13, no. 18, pp. 7052–7062, 2005.