Resolution beyond Rayleigh's criterion: a modern resolution measure with applications to single molecule imaging

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Abstract— Rayleigh's criterion, although extensively used, is well known to be based on heuristic notions that are inadequate for modern optical microscopy applications. This inadequacy has necessitated a reassessment of the resolution limits of optical microscopes. By adopting a stochastic framework and using the statistical theory concerning the Fisher information matrix, we have derived a new resolution measure that overcomes the limitations of Rayleigh's criterion. Here, we provide a brief overview of this and other related results published by our group. The new resolution measure predicts that there is no resolution limit, but that the resolvability depends on the number of detected photons. It has been experimentally verified that distances well below Rayleigh's limit can be measured from images of closely spaced single molecules with an accuracy as predicted by the new resolution measure. The stochastic framework used to obtain the new resolution measure is applicable to a wide variety of estimation problems encountered in optical microscopy. As an application, we have investigated the localization accuracy problem, which is concerned with how accurately the 2D/3D location of a microscopic object can determined from its image. One of the shortcomings of current microscopy techniques is that they suffer from poor depth discrimination and as a result they are not well adapted for 3D tracking of single molecules/particles. We have recently developed a novel imaging modality called multifocal plane microscopy (MUM) to overcome this limitation. Using the stochastic framework, we have shown that MUM has significantly improved depth discrimination, which in turn enables 3D single particle tracking at high axial localization accuracy.

I. INTRODUCTION

The optical microscope has been an invaluable tool for biological research. Recent technological advances have rendered optical microscopes to perform task ranging from long term (hours time scale) three dimensional imaging of live cells ([1]) to fast imaging (milliseconds time scale) of molecular interactions within a cellular environment even at the single molecule level ([2], [3]). In many biological applications the acquired microscopy data requires extensive quantitative analysis ([4]). For this purpose, 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 [5], [6]. Here, we present a review of this and other related results that were recently reported by our group ([7], [8], [9], [10], [11]). Due to the random nature of the acquired data, we adopted a stochastic framework and used the statistical theory concerning the Fisher information matrix ([12], [13]) 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 ([14]). We note that our results are applicable to several microscopic techniques such as fluorescence microscopy, bright-field microscopy, etc.

We have investigated the resolution problem ([7]), 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 ([7]).

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 ([8], [9]). 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 ([9], [10]). To overcomes these shortcoming we had developed a

new imaging modality called multifocal plane microscopy (MUM) ([15], [16]). 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 ([10]).

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 ([12]), a lower bound to the variance of any unbiased estimator $\hat{\theta}$ of an unknown parameter θ, i.e., 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 $\mathcal{G}$ ([5]). 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 ([5], [13])

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

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 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 ([17]). In fact by using such approaches, several groups have shown that Rayleigh's limit can be surpassed in an optical microscope setup ([18], [19], [20], [7]).

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 ([7]). 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 ([7])

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

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

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

with J_n denoting the nth 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 ([7]). 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 ([21]). Analytical expressions for the resolution measure that take into account these deteriorating factors have also been obtained ([7]). 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 [7] 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 ([8], [5]), 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 [9] 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 ([8], [9]).

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 ([9]). For instance, for small defocus values (≤ 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 ([15], [16]). 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 [10]. 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.

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