THE EFFECT OF BACKGROUND ON LOCALIZATION UNCERTAINTY IN SINGLE EMITTER IMAGING

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ABSTRACT

We analyze singe emitter localization at high background levels. Maximum Likelihood Estimation closely follows the Cramer-Rao lower bound for a wide range of signal photon counts and up to very large background levels. The use of an idealized Gaussian to fit measured spots that originate from freely and rapidly rotating dipole emitters appears to work well for all parameters considered. We also present a very practical closed-form expression that approximates the minimum achievable localization uncertainty excellently for all signal and background levels.

Index Terms— super-resolution microscopy, single emitter imaging, localization uncertainty, Cramer-Rao lower bound, Maximum Likelihood Estimation

1. INTRODUCTION

The breakthroughs in super-resolution microscopy (STED, PALM, STORM) are based on manipulating the emission characteristics of fluorophores over the course of time. The localization microscopy flavors (PALM, STORM) use localization of single emitters with an uncertainty well below the classical resolution limit of optical microscopy. Inevitable background photons corrupt the certainty of localization, an effect made worse by the transition from imaging in a 2D-TIRF context to full 3D widefield imaging. Several papers have appeared on the fundamental limits to localization uncertainty and the effect of background photons on it [1, 2, 3, 4, 5]. Currently it is unclear which of these different theoretical results best describes the minimum localization uncertainty as a function of background. Additional confusion arises from different assumptions concerning the computational method used, either Least Mean Squares (LMS) or Maximum Likelihood Estimation (MLE), and from differences between the use of a realistic Point Spread Function (PSF) for freely and rapidly rotating dipole emitters and an idealized Gaussian PSF in the fitting procedure [6]. In previous work [4] we showed that an MLE-fit of Gaussian PSFs corrupted by shot noise achieves a localization uncertainty equal to the Cramer-Rao lower bound (CRlb) for backgrounds up to 10 photons per pixel. Furthermore, it was shown that LMS-fitting gives an inferior localization uncertainty compared to MLE-fitting, especially with relatively low photon counts. Those results are extended here with the goal to clarify the above mentioned background related issues. We present a concise formula for the minimum localization uncertainty that we propose be used instead of the popular formula of Thompson, Larson and Webb [3], which is too optimistic for any non-zero background. Finally we show simulation results on the difference between the minimum (CRlb) and practically realized localization uncertainty over a large range of background values, as well as on the effect of fitting realistic dipole PSFs with a Gaussian.

2. ANALYTIC EXPRESSIONS FOR THE MINIMUM LOCALIZATION UNCERTAINTY

In 2002, Thompson, Larson and Webb published a formula [3], that is widely used and quoted in the literature ever since, for the variance of the emitter position when using LMS-fitting:

$$\left\langle (\Delta x)^2 \right\rangle = \frac{\sigma_a^2}{N} \left(1 + \frac{8\pi\sigma^2 b}{N a^2} \right), \tag{1}$$

with $\sigma_a^2 = \sigma^2 + a^2/12$. Here σ is the width of the Gaussian that is used to fit the PSF, a is the pixel size, N is the number of signal photons, and b is the number of background photons per pixel. Mortensen and co-workers [5] claim that such a LMS-fit should give rise to a localization uncertainty given by:

$$\left\langle (\Delta x)^2 \right\rangle = \frac{\sigma_a^2}{N} \left(\frac{16}{9} + \frac{8\pi\sigma_a^2 b}{N a^2} \right), \tag{2}$$

which is slightly different from Eq. (1). In addition they present a formula for the localization uncertainty for Maximum Likelihood Estimation (MLE) based fitting:

$$\left\langle (\Delta x)^2 \right\rangle = \frac{\sigma_a^2}{N} \left(1 + \int_0^1 dt \, \frac{\ln t}{1 + t/\tau} \right)^{-1}. \tag{3}$$

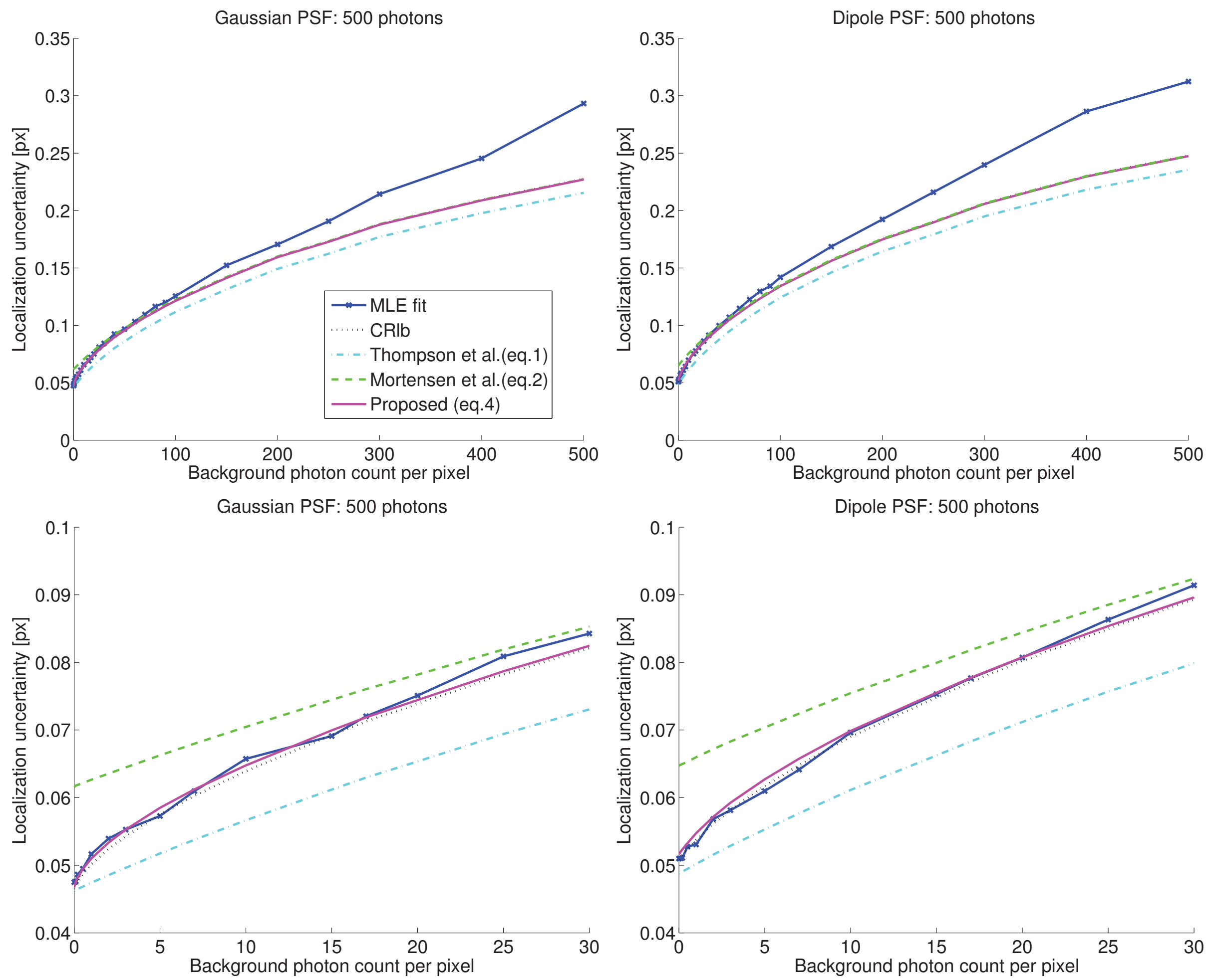

Fig. 1. Localization uncertainty, CRlb and three theoretical formulas for the expected localization uncertainty as a function of background for $N = 500$ signal photons for a Gaussian ground truth PSF (left column) and for a dipole PSF ground truth (right column). The top row shows the entire range of background levels (top: CRlb, Mortensen and proposed lie on top each other), the bottom row zooms into relatively low background levels. One pixel represents 90 nm.

where τ is a normalized dimensionless background parameter $\tau = 2\pi\sigma_a^2 b/(Na^2)$.

For large values of the background the MLE and LMS-results converge to the same value. Here we propose a closed form analytical approximation to Mortensen's MLE-formula:

$$\left\langle (\Delta x)^2 \right\rangle = \frac{\sigma_a^2}{N}\left(1 + 4\tau + \sqrt{\frac{2\tau}{1+4\tau}}\right). \quad (4)$$

It turns out that the difference between our analytical approximation and the exact MLE-formula is just a few percent for a wide range of parameters. We therefore will use the analytical formula Eq. (4) instead of the integral expression Eq. (3) from now on. Summarizing, there are four contenders for the formula that best describes the localization uncertainty as a function of background of which two give essentially the same result (Eq. (4) and (3)).

3. MAXIMUM LIKELIHOOD ESTIMATION SIMULATION

Simulated images are generated using the finite pixel approximation, including background as described in Ref. [4] for the Gaussian PSF model or in Ref. [6] for the realistic PSF model for free dipole emitters. The center coordinate of each simulated emitter is randomly shifted within a uniform area of two pixels from the central pixel to prevent a biased result. After the generation of the data stack, the images are corrupted with Poisson noise. We simulate an area of 7×7 pixels and a Gaussian width $\sigma = 1.0$. For the realistic dipole PSF we assume water immersion (NA $= 1.25$ focusing into a medium with refractive index $n = 1.33$), a wavelength $\lambda = 500$ nm and a pixel size in object space equal to $0.22\ \lambda/\mathrm{NA}$ which gives rise to a PSF for which the best-fit Gaussian has a width equal to

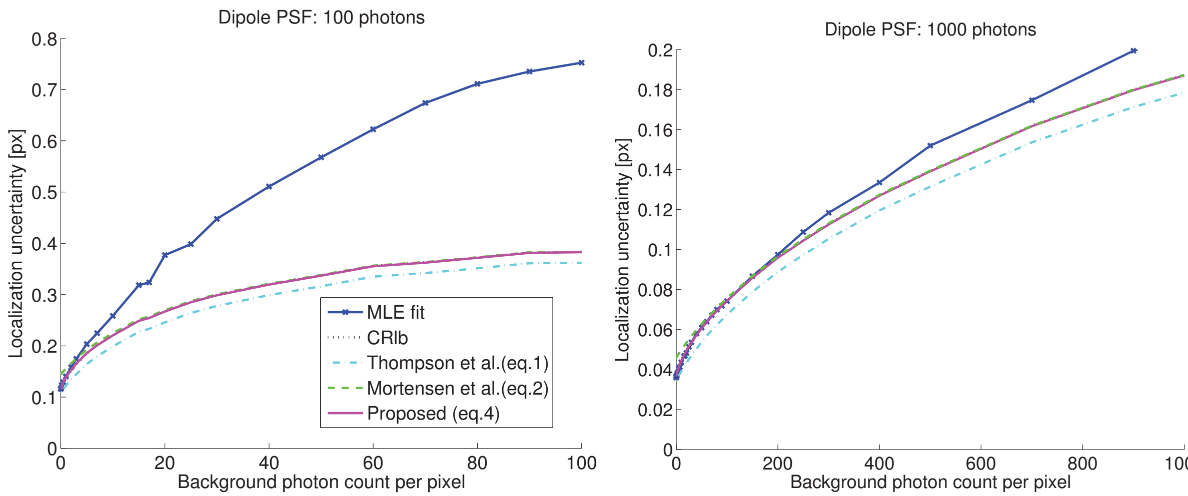

Fig. 2. Localization uncertainty, CRlb and three theoretical formulas for the expected localization uncertainty as a function of background for $N = 100$ signal photons (left) and for $N = 1000$ photons (right). One pixel represents 90 nm.

one pixel, thus making a fair comparison between an assumed Gaussian ground truth PSF and a free dipole ground truth PSF. The pixel size is 90 nm (slight oversampling, Nyquist sampling would be 100 nm).

For each pair of signal and background photon counts 5000 images are simulated and fitted by an earlier described MLE routine that achieves theoretically minimum uncertainty [4]. The routine was implemented on a graphics card for parallel processing, thus giving a significant speed advantage over conventional CPU implementations. The routine returns the estimated position, signal and background photon counts, width of the fitted Gaussian and an estimate for the covariance between the fitted parameters (bounded from below by the CRlb). The Fisher matrix and the attendant CRlb can be readily calculated at the found parameter values.

The choice for the different parameters of signal and background counts is directly related to typical experimental situations. In 2D-TIRF imaging a very low background count is common (1 to 5 photons/pixel/frame) due to the small imaging volume whereas in out-of-TIRF conditions 10-30 background photons/pixel/frame can be encountered depending on the sample. Typical signal levels depend of course on the exposure time and fluorophore, but values between 500-5000 are reported commonly.

4. RESULTS AND DISCUSSION

Fig. 1 shows the realized localization uncertainty as a function of the number of background photons per pixel for both a dipole PSF ground truth and a Gaussian PSF ground truth, the CRlb, our approximation Eq. (4) to the CRlb given by Eq. (3) and for reference, the two LMS-based formulas, Eqs. (1) and (2). Several conclusions can be drawn form these graphs. First of all, it appears that the realized localization uncertainty closely follows the CRlb up to very large background photon counts. The localization uncertainty remains within 10% of the CRlb for background photon counts of up 150-200 photons per pixel (depending on the assumed ground truth PSF), for only 500 signal photons originating from the emitter. For a 7×7 pixel large area of interest this means that 10% localization uncertainty is lost only at a signal-to-background ratio of about 1:3! It is not strange that the actual computations deviates from the theoretical CRlb at some point due to numerical reasons if the signal to background and therefore the SNR becomes too low. Our second conclusion is that our approximation formula for the CRlb works very well for all background photon counts involved. Third, it appears that the Thompson-Larson-Webb formula works well for low backgrounds and is too optimistic for high backgrounds, whereas Mortensen et al.'s formula works well for high backgrounds and is too pessimistic for low backgrounds. Fourth, it turns out that there is hardly an effect of the assumed ground truth PSF on the localization uncertainty. For very large background values it appears that the achieved uncertainty for the dipole PSF ground truth is a bit worse than for the Gaussian PSF ground truth. The practical implication is that fitting the PSF with an idealized Gaussian model PSF works fine for free dipole emitters, even though the real PSF is not a Gaussian.

The same conclusions as presented above apply to other values of the signal photon count. Fig. 2 shows the localization uncertainty as a function of the number of background photons per pixel for 100 and for 1000 signal photons. Fig. 3 shows the maximum background level as a function of signal photon count before the actual estimation is more than 10% above the CRlb. For the purpose of illustration we show in Fig. 4 simulated images for which the MLE-fit is on average within 10% of the CRlb. The noteworthy difference between these results and the results for 500 signal photons is that the background level at which 10% certainty loss is suffered does not linearly scale with the signal photon count. The found curve in Fig. 3 approximately follows a square dependency on the number of signal photons N; the best fit is found by a curve with a power of 2.2. This dependency can be qualitatively understood from Eqs. (2) and (4), which give a scaling of the localization variance proportional to b/N^2 in the high background regime. The realized localization uncertainty will approximately follow the same b/N^2 scaling. It follows that a relative failure threshold, such as the 10% above CRlb criterion, will lead to a relation that should be close to $b_{\max} \propto N^2$.

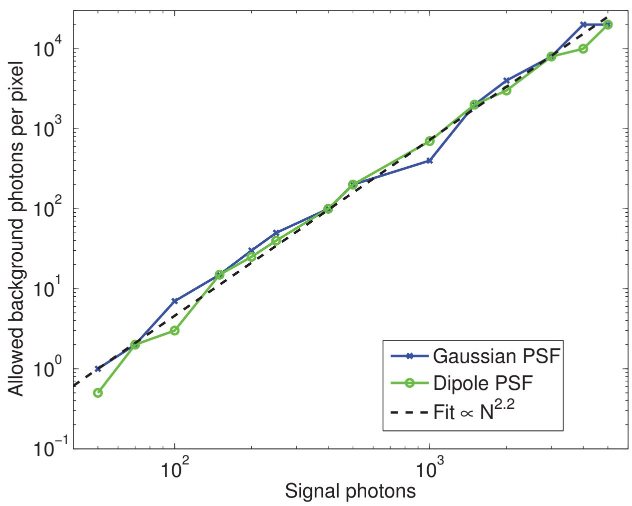

Fig. 3. Background level for which 10% loss in certainty is suffered compared to the CRlb as a function of signal photon count for an assumed ground truth PSF that is either Gaussian or dipole. The dashed line gives the power law fit.

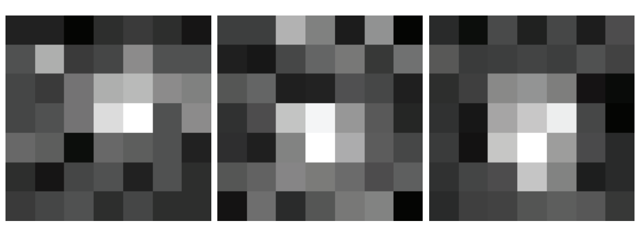

Fig. 4. Examples of images for which the estimation is on average within 10% of the estimated CRlb. Left: $N = 100, b = 5$ Middle: $N = 500, b = 200$ Right: $N = 1000, b = 300$

A number of issues must be taken into consideration with respect to comparisons with real experiments. First camera calibration (offset from dark image, ADU to photon conversion via gain measurement) must be done properly for accurate results, second the excess noise of the EMCCD's avalanche multiplication process needs to be taken into account. According to Mortensen and co-workers this boils down to doubling the variance of the Poisson noise only results, i.e. the right hand side of Eq. (3) or (4) times two [5]. Finally, for 2D-TIRF imaging the NA is usually larger than the medium refractive index in order to gain signal (evanescent waves are captured as well). We also have done simulations under TIRF imaging conditions. It turns out that there is hardly any change in the dipole PSF shape and the localization uncertainty; we found that Gaussian fitting works equally well as in the cases with lower NA considered previously in this paper.

In conclusion, we have demonstrated that Maximum Likelihood Estimation is the method of choice for single emitter localization, as it closely approaches the Cramer-Rao lower bound for a wide range of signal photon counts and up to excessive background levels. Fitting the realistic free dipole PSF with a Gaussian does not appear to introduce significant compromises in terms of uncertainty. Finally, we have presented a novel, concise, closed-form expression for the minimum achievable localization uncertainty. The formula is an excellent candidate for replacing earlier practical formulas that can be computed directly from easily accessible experimental parameters .

5. REFERENCES

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