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A multi-emitter localization comparison of 3D superresolution imaging modalities

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Abstract

Single-molecule localization-based superresolution imaging is complicated by emission from multiple emitters overlapping at the detector. The potential for overlapping emitters is even greater for 3D imaging than for 2D imaging due to the large effective ‘volume’ of the 3D point spread function (PSF). Overlapping emission can be accounted for in the estimation model, recovering the ability to localize the emitters, but with the caveat that the localization precision has a dependence on the amount of overlap from other emitters. We investigate if a particular 3D imaging modality has a significant advantage in facilitating the position estimation of overlapping emitters. We compare variants of two commonly used and easily implemented imaging modalities for 3D single-molecule imaging: astigmatic imaging; dual focal plane imaging; and the combination of the two approaches- dual focal plane imaging with astigmatism. We use the Cramér-Rao lower bound (CRLB) to quantify the multi-emitter estimation performance by calculating the theoretical best localization precision under a multi-emitter estimation model. We investigate the performance of these 3D modalities under a wide range of conditions including various distributions of collected photons per emitter, background counts, pixel sizes, and camera readout noise values. Differences between modalities were small and we therefore conclude that multi-emitter fitting performance should not be a primary factor in selecting between these modalities.

Keywords

fluorescence microscopy; superresolution imaging; Cramér-Rao lower bound

2 Introduction

Single-molecule localization-based superresolution [1, 2, 3, 4] relies upon finding the position of individual fluorophores with precision and accuracy better than the diffraction limit. Due to the slowly changing shape of the microscope point spread function (PSF) near the focus, a 3D application of this technique relies upon some type of PSF modification for adequate axial localization precision. This modification has been implemented using various methods such as simultaneously imaging two focal planes [5, 6], by introducing axial astigmatism with a cylindrical lens [7], and with more sophisticated engineered ‘double-helix’ PSFs [8].

The most straightforward and commonly implemented method to analyze the data collected from this imaging method is to 1) identify regions of the images that could possibly represent light originating from single emitters; 2) perform some type of PSF fitting to the region; and 3) evaluate the quality of the fit to accept or reject the localization. In this approach, single molecule fits to overlapping emitters will be rejected by step 3) and not included in the analysis, degrading the final reconstructed image. However, significantly overlapping fluorophores can be localized with little loss in precision if the proper multi-emitter estimation is used [9]. Several analysis techniques have been described that make use of this concept to deal with overlapping emitters in 2D imaging [10, 11, 12, 13, 14, 15]. The concept can be extended to 3D multi-emitter fitting [16] and a multi-fitter algorithm for 3D superresolution using astigmatic imaging has been recently described [17].

Several comparisons of single emitter fitting precision for various 3D imaging geometries have already been published [16, 18, 19, 20]. However, potentially important in practice is the ability to resolve several overlapping emitters. Fig. 1 illustrates the increased potential of overlapping emitters as compared with 2D imaging using the 3D modalities compared here. An active emitter density that would contain predominantly isolated individual emitters produces highly overlapping images when the emitters are not located near the focus. Since commonly used organic fluorophores have a minimum achievable active duty percentage [21], it is often not possible to reduce active emitter density in a densely labeled sample such that single emitter fitting is sufficient, particularly in densely labeled regions.

Presented in this manuscript is the theoretical estimation precision of multi-emitter fitting for several 3D imaging geometries. We compare the dual focal plane, astigmatism and combined (dual focal plane with astigmatism) geometries since they are relatively simple and inexpensive to implement as a modification to a typical epi-fluorescence microscope.

3 Results and Discussion

We first introduce a few concepts that will be used to evaluate the performance of the imaging modalities. One complication for evaluating fit precision is that multi-emitter parameter estimation can lead to spatial covariance in the localization precision (see section 5.1), meaning that not only can the position uncertainly vary with the different Cartesian axes, but the principal axes of the error ellipsoid can be oriented in any direction. This is accounted for by calculating an 'error volume', σ_{3D} , for the fit position of each emitter, which is presented in a more intuitive fashion as $\sqrt[3]{\sigma_{3D}}$. For a cubic error volume, $\sqrt[3]{\sigma_{3D}}$ would be the standard deviation of the position estimation for each dimension. All results are presented as distributions of $\sqrt[3]{\sigma_{3D}}$, where small values indicate better performance.

Because of the many possible ways two or more emitters can be placed within a volume, most of the results in this paper are shown using the cumulative distribution of $\sqrt[3]{\sigma_{3D}}$, where $\sqrt[3]{\sigma_{3D}}$ is calculated for each emitter over many, randomly selected sets of emitter positions. The cumulative distribution is the integral of the probability distribution of a random variable and gives the probability of the random variable to have a value less than that given on x -axis. The cumulative distribution is convenient in presenting and comparing the overall performance of various simulation conditions since it includes all calculation results and

makes it easy to tell the percentage of $\sqrt[3]{\sigma_{3D}}$ less than a given value. For example, if in a particular application all localizations with $\sqrt[3]{\sigma_{3D}}$ better than a certain value were equally useful, the modality with the highest cumulative distribution at that value of $\sqrt[3]{\sigma_{3D}}$ would be the most desirable.

3.1 Localization precision for two-emitter estimation

When the sample contains a low active emitter density, the required multi-emitter fit model will be predominantly the two-emitter model and therefore we first compared the performance of two-emitter estimation. Since the parameter space for emitter positions is large, we restricted the analysis to comparing average localization precision under three conditions: 1) Emitter one was positioned such that $x = x_0$, $y = y_0$ and randomly in z , where (x_0, y_0) was the center of a 2D fitting region. Emitter two was positioned randomly within a specific 3D volume (see section 5.3). 2) Emitter one was positioned at x_0, y_0, z_1 , where z_1 was a randomly selected z position. Emitter two is positioned at $z = z_1$, and randomly in x and y (same with condition 1)). 3) Emitter one was positioned at x_0, y_0 and randomly in z . Emitter two was positioned at $x = x_0$, $y = y_0$ and randomly in z . These situations are illustrated in Fig. 2 (bottom row) where the z position of emitter one happened to be $z = 0$.

Fig. 2 shows the average localization precision, as well as the cumulative distributions of localization precision for each geometry under conditions 1), 2) and 3) described above. The average localization precision was calculated by taking the either the mean value of $\sqrt[3]{\sigma_{3D}}$ over all emitters with the same z position (Fig. 2 top row) or the mean value over all conditions with the same emitter separation (Fig. 2 second row). Note that averaging over different parameters can result in different minimum values.

When comparing $\sqrt[3]{\sigma_{3D}}$ versus z for the three imaging modalities, there is not a clear advantage for any modality for any of the fluorophore separation conditions. It is interesting to note that there are absolute estimation error differences for the various separation conditions. There is a small increase under condition 2) (Fig. 2b) as compared with condition 1) (Fig. 2a) and a much larger increase under condition 3) (Fig. 2c) as compared with the other two conditions. Condition 1) benefits from larger possible separations given the possible values of z , whereas condition 3) suffers from the fact that the images of emitters always overlap directly on top of each other at the detector.

When averaging over z and plotting $\sqrt[3]{\sigma_{3D}}$ versus the separation between two emitters, the combined modality shows a small advantage over dual focal plane and astigmatism. At small separations, the precision diverges as the separation goes to zero, as expected [9, 16]. As shown in Fig. 2d and Fig. 2f, for conditions 1) and 3) the error also increases with large separations for all imaging modalities. This is because large separations are only possible at the more extreme (out of focus) z positions where all modalities have worse estimation error. Under condition 2) where both emitters are confined to the same focal plane, $\sqrt[3]{\sigma_{3D}}$ always decreases with larger separation (Fig. 2e). The maximum separation in x - y plane is $0.9 \mu\text{m}$ and at this separation out of focus emitters could still have overlap depending on the z position. However, as separation increases, $\sqrt[3]{\sigma_{3D}}$ would converge to that expected from the z -position average of a single emitter (not shown).

The cumulative distribution of $\sqrt[3]{\sigma_{3D}}$ shows similar relative performance between the three imaging modalities across the separation conditions (Fig. 2g,h,i). Both the dual focal plane and combined modalities perform slightly better than astigmatism. In each condition, if the best 25% of fits were used, the dual focal plane would have a slight advantage, whereas if the best 80% of fits were used the combined method would have a small advantage. Although there are small differences, the results do not dramatically favor any modality.

We note that the results of $\sqrt[3]{\sigma_{3D}}$ versus z -positions and separations used a large, but somewhat arbitrary rejection threshold of 173 nm for $\sqrt[3]{\sigma_{3D}}$. Random occurrences of very small separations leading to very large values of $\sqrt[3]{\sigma_{3D}}$ can greatly influence the average values. In practice, fits with large uncertainties would typically be discarded, so that excluding large $\sqrt[3]{\sigma_{3D}}$ gives a realistic expectation of the results. As shown in the cumulative distribution (Fig. 2g,h,i), with a threshold at 173 nm, the acceptance of total calculation results is nearly 100%, so that there are only a few $\sqrt[3]{\sigma_{3D}}$ with large values. Increasing or decreasing the threshold value produced similar relative results between modalities, but with a shift of the absolute value (data not shown).

3.2 Localization precision for multi-emitter fitting

With high active-emitter density, a fitting model with more than two emitters may be required. For multiple emitters it is not constructive to evaluate performance with respect to two-emitter inter-particle spacing. In this case we plot only the cumulative distribution of volume error under one and four-emitter estimation. For four-emitter estimation, the model simulation is similar to condition 1) above, in which emitter one is positioned such that $x = x_0$, $y = y_0$ and randomly in z , and the other emitters are positioned randomly. For the one-emitter model, a single emitter is positioned at $x = x_0$, $y = y_0$ and randomly in z . The cumulative distribution of volume error was calculated for each 3D geometry under various permutations of experimental conditions and the results are shown in Fig. 3 through Fig. 7. It is clear from a cursory inspection of the cumulative distribution plots that the relative performance of the three modalities is very similar under all conditions evaluated and that the dual focal plane and combined modalities have a small advantage over astigmatism.

Fig. 3 shows a comparison of cumulative distributions calculated using two different sized fitting regions for the estimation process. One region is large enough to be effectively infinite (large) whereas the other corresponds to a 24 pixels $\times$ 24 pixels (2.56 $\mu\text{m} \times$ 2.56 μm) fitting region (small). The small fit region is representative of a finite fit region that would be used in practice. The small subregion has the same size used for the PSF representations in Fig. 1(e,f,g) and will somewhat truncate off-center fluorophores with large amounts of defocus. Fig. 3 shows a slight deviation of the small box size from the large box size at large values of $\sqrt[3]{\sigma_{3D}}$, however the relative results between the imaging modalities are consistent between large and small box sizes. Using a large fitting region could provide a small increase in fit precision, however, the differences are small enough that using a small fitting region size will result in the same conclusions as using a large fitting region size. The following results are calculated using the small fitting region size.

To represent the variation of fluorophore intensities observed in practice, each emitter has an expected photon count drawn from either a normal distribution or an exponential distribution. Fig. 4(a,b) shows a comparison of modalities where fluorophore intensities are drawn from a normal distribution with a mean of 800 photons and a standard deviation of 200 photons. This represents the situation where the fluorescent to dark state transition rate is smaller than 1 frame^{-1} . Fig. 4a includes a background of 2 photons/pixel, whereas Fig. 4b includes a background of 16 photons per pixel. For the dual focal plane method, both the photons from the emitters and from background fluorescence are split evenly between focal planes. Fig. 4(c,d) shows the results of exponentially distributed fluorophore intensities with a mean of 800 photons. This represents the situation where the fluorescent to dark state transition rate is much larger than 1 frame^{-1} . Fig. 4c and Fig. 4d includes a background of 2 and 16 photons/pixel respectively. For four-emitter estimation, astigmatism has the largest estimation error. If any particular threshold in $\sqrt[3]{\sigma_{3D}}$ is used for accepting and using emitter locations, the astigmatism method would always have the lowest acceptance percentage. With normally distributed expected photon counts, the combined method and dual focal plane method intersect at a $\sqrt[3]{\sigma_{3D}}$ value of approximately 20 nm, for low background (Fig. 4a), and 40 nm for high background (Fig. 4b). Thus, for a cut-off threshold below the intersection point, the dual focal plane method will accept more fits, otherwise the combined method will accept more, although differences were small.

Fig. 5 shows the results for the same conditions as Fig. 4, but where the mean value of the expected photon counts is increased to 5000 photons and with a standard deviation of 1250 photons for the normally distributed emitter intensities. Again, the relative performance between modalities remains consistent. Fig. 6 shows the same conditions as Fig. 4 but with the focal plane separations increased to 600 nm for all modalities. With normally distributed emitter intensities, the larger focal plane separation somewhat increased the differences between dual focal plane and combined modalities giving a increased preference for the combined method when greater than 70% of fits are accepted.

The results shown in Fig. 2 through Fig. 6 assumed that the camera readout noise was zero, which is a good approximation for the widely used electron multiplying CCD camera. However, scientific CMOS (sCMOS) cameras are now becoming an attractive option for superresolution imaging [22]. We repeated the calculation of Fig. 4 taking into account finite camera readout noise. We calculated the results for two values of readout noise. First, $2 \text{ e}^-/\text{pixel}$ representing a state-of-the-art sCMOS camera and second, $6 \text{ e}^-/\text{pixel}$ representing a low-noise, well-cooled CCD camera. We calculated only the condition of normally distributed emitter intensities and low-background photon counts where differences with the zero readout noise case are the most pronounced. In the presence of camera readout noise, pixel size also plays an important role in affecting fit precision and we therefore made the calculation with two different pixel sizes. As shown in Fig. 7e, under the same fit region size ($2.56 \mu\text{m} \times 2.56 \mu\text{m}$), background counts ($312 \text{ photons}/\mu\text{m}^2$), and with a standard deviation of readout noise of $\sigma_s = 2 \text{ e}^-/\text{pixel}$, the pixel size of 128 nm produced better fit precision than the pixel size of 80 nm. This was also true for larger σ_s (Fig. 7f). With $\sigma_s = 6 \text{ e}^-/\text{pixel}$, the astigmatism geometry begins to outperform the other two geometries for single emitter fitting (Fig. 7b and Fig. 7d), which is due to a smaller number of total pixels for the

astigmatism geometry as compared to the dual focal plane geometries. However, in four-emitter fitting, astigmatism still performed slightly worse than the other modalities.

4 Conclusion

We have compared three easily implemented imaging modalities used for 3D single-molecule localization-based superresolution imaging to determine if any of the modalities have a significant advantage for multi-emitter fitting. Although there were small differences, no modality had a dramatic advantage over the others under all conditions, however, the astigmatism method did perform slightly worse under most conditions as compared to the dual focal plane techniques. Despite the small differences found with the three modalities investigated here, it is possible that a 3D PSF could be engineered specifically for good performance under multi-emitter estimation and could therefore substantially out-perform the geometries evaluated here. We conclude that in practice, multi-emitter fitting performance should take a secondary role in choosing a 3D imaging modality to other considerations such as ease of implementation, the facilitation of data analysis, potential for misalignment (dual focal plane methods), field of view, and tolerance to aberrations.

5 Computational Methods

5.1 Theory of Localization Precision

For each of the imaging geometries the localization precision is calculated from the Cramér-Rao lower bound (CRLB), which is a lower bound for the variance of any unbiased estimator and has been used previously in similar contexts to calculate localization precisions for 2D and 3D single molecule position estimation [6, 11, 23]. The CRLB is given by the inverse of the Fisher information matrix

$$\text{var}(\theta_i) \geq F_{ii}^{-1} \quad (1)$$

where the Fisher information matrix is

$$F_{ij} = E \left[\frac{\Delta \ln(L(\theta))}{\Delta \theta_i} \frac{\Delta \ln(L(\theta))}{\Delta \theta_j} \right] \quad (2)$$

$L(\theta)$ is the likelihood function, θ is the set of estimated parameters and E stands for expectation. For independent pixels and Poisson distributed data the likelihood is

$$L(\theta) = \prod_q \frac{\mu_q^{N_q} e^{-\mu_q}}{N_q!} \quad (3)$$

where N is the observed data, μ is the expectation model and q is the pixel index. In this case, the Fisher information can be written as

$$F_{ij} = \sum_q \frac{1}{\mu_q} \frac{\Delta \mu_q}{\Delta \theta_i} \frac{\Delta \mu_q}{\Delta \theta_j} \quad (4)$$

In multi-emitter fitting, the model μ is the sum over all emitters in a selected fitting region

$$\mu = bg + \sum_{i=1}^m I_i \cdot \text{PSF}_0(x_i, y_i, z_i) \quad (5)$$

where I_i is the expected number of collected photons of the i th emitter, PSF_0 is the normalized point spread function, m is number of emitters, and bg is the background. The background is modeled as uniform across the fitting region but with independent values for each of the focal planes in the dual focal plane and combined geometries.

For the single plane astigmatism geometry, the estimated parameters are x , y , z , I of each emitter and the uniform bg , so the number of parameters to be estimated is $4m + 1$. For the dual focal plane geometries we note that since all pixels are treated independently, likelihood and Fisher information can be written as

$$L(\theta) = \prod_q \frac{\mu_{aq}^{N_{aq}} e^{-\mu_{aq}}}{N_{aq}!} \prod_q \frac{\mu_{bq}^{N_{bq}} e^{-\mu_{bq}}}{N_{bq}!} \quad (6)$$

and

$$F_{ij} = \sum_q \frac{1}{\mu_{aq}} \frac{\Delta \mu_{aq}}{\Delta \theta_i} \frac{\Delta \mu_{aq}}{\Delta \theta_j} + \sum_q \frac{1}{\mu_{bq}} \frac{\Delta \mu_{bq}}{\Delta \theta_i} \frac{\Delta \mu_{bq}}{\Delta \theta_j} \quad (7)$$

respectively, where the additional indexes a and b indicate the focal plane. The number of parameters to be estimated for this geometry is $4m + 2$.

In the presence of camera readout noise, the Fisher information matrix in Eq. 4 and Eq. 7 become [24, 25],

$$F_{ij} = \sum_q \frac{\Delta \mu_q}{\Delta \theta_i} \frac{\Delta \mu_q}{\Delta \theta_j} \gamma \quad (8)$$

$$F_{ij} = \sum_q \frac{\Delta \mu_{aq}}{\Delta \theta_i} \frac{\Delta \mu_{aq}}{\Delta \theta_j} \gamma_a + \sum_q \frac{\Delta \mu_{bq}}{\Delta \theta_i} \frac{\Delta \mu_{bq}}{\Delta \theta_j} \gamma_b \quad (9)$$

where γ is defined by,

$$\gamma = \int_{\Re} \frac{\left(\sum_{l=1}^{\infty} \frac{\mu_q^{l-1} e^{-\mu_q}}{(l-1)!} \times \frac{1}{\sqrt{2\pi}\sigma_s} e^{-\frac{1}{2}\left(\frac{q-l-\mu_s}{\sigma_s}\right)^2} \right)^2}{\sum_{l=0}^{\infty} \frac{\mu_q^l e^{-\mu_q}}{l!} \times \frac{1}{\sqrt{2\pi}\sigma_s} e^{-\frac{1}{2}\left(\frac{q-l-\mu_s}{\sigma_s}\right)^2}} dg - 1 \quad (10)$$

where σ_s, μ_s are standard deviation and mean of readout noise respectively.

The estimation precision in each dimension is given by $\sigma_i = \sqrt{(F^{-1})_{ii}}$, where i is the parameter index for x, y , or z . We compute the volume estimation error, σ_{3D} , which is expressed as the volume of the ellipsoid,

$$\sigma_{3D} = \frac{4\pi}{3} \sqrt{\det(V)} \quad (11)$$

in which matrix V describes an ellipsoid centered at the origin of Cartesian coordinate system,

$$V = \begin{bmatrix} \sigma_x^2 & \sigma_{xy}^2 & \sigma_{xz}^2 \\ \sigma_{xy}^2 & \sigma_y^2 & \sigma_{yz}^2 \\ \sigma_{xz}^2 & \sigma_{yz}^2 & \sigma_z^2 \end{bmatrix} \quad (12)$$

and is built from the corresponding elements of F^{-1} . For single-emitter fitting, the off-diagonal elements are close to zero, making V a diagonal matrix, and therefore $\sigma_x, \sigma_y, \sigma_z$ are the lengths of principle axes of the ellipsoid. For multi-emitter position estimation, the estimation of x, y and z are not necessarily independent with each other, giving off diagonal elements in V and therefore the ellipsoid is tilted from the Cartesian axes.

5.2 PSF generation

The PSF used in μ is calculated from the Fourier transform of the pupil function,

$$\text{PSF} = |\mathcal{F}[P(k_r, \theta) e^{i2\pi k_z z}]|^2 \quad (13)$$

where $P(k_r, \theta)$ is the pupil function in $\mathbf{k}$ space, with length $k = n/\lambda$, n is the refractive index of immersion medium, k_r is the projection of $\mathbf{k}$ in x - y plane, $\exp(i2\pi k_z z)$ describes defocus, with $k_z = (k^2 - k_r^2)^{1/2}$. With a numerical aperture (NA), the pupil function takes the form

$$P(k_r, \theta) = A \cdot \text{circ}\left(\frac{k_r}{\lambda/\text{NA}}\right) e^{i\phi} \quad (14)$$

for ideal optical system, the magnitude is $A = 1$, and the aberration phase is $\phi = 0$. In the astigmatism method, the aberration phase becomes [26],

$$\phi - C \left(\frac{k_r}{\lambda/NA} \right)^2 \cos(2\theta) \quad (15)$$

where C is a positive number and describes the strength of the astigmatism aberration. We used either $C = 2$ or $C = 3$, which results in ~ 400 nm and ~ 600 nm separation between the two foci in the object space.

The PSF calculated from Eq. 13 is based on scalar diffraction theory [27] with the emitter as an ideal point source. To account for the broadening of the PSF due to dipole radiation combined with large numerical apertures [28, 29, 30] we modified the PSF using a method adopted from Hanser *et al* [31]. An empirical function was applied to the PSF after the calculation of Eq. 13. The empirical function was a 2D Gaussian fitting to the optical transfer function (OTF) ratio, which was calculated from the OTF of a measured PSF divided by the OTF of the PSF calculated from Eq. 13. The OTF was calculated from the Fourier transform of the corresponding in-focus PSF. It is assumed that both the bead used for the PSF measurement and the fluorophores imaged in the sample are well modeled by a collection of randomly oriented dipoles or a freely rotating dipole. This is true for most fluorophore labeled samples [29]. After applying the empirical function, Eq. 13 becomes,

$$\text{PSF} = |\mathcal{F}[[P(k_r, \theta)e^{i2\pi k_z z}]]^2 \otimes \mathcal{F}[G(k_r, \theta)]| \quad (16)$$

where $\otimes$ denotes convolution and G is the 2D gaussian fit to the OTF ratio. The fitting parameters for G are the magnitude I , and the width in the x , y dimensions, σ_x and σ_y respectively. The values used were $1, 2 \mu\text{m}^{-1}, 2 \mu\text{m}^{-1}$, respectively, which are typical for that found using 40 nm diameter bead (Fluo-Spheres F-8789, dark red, Invitrogen) for PSF measurement in our laboratory.

5.3 Multi-emitter calculation details

The parameters used in the PSF calculations were numerical aperture $NA = 1.46$, wavelength $\lambda = 0.67 \mu\text{m}$, and refractive index of immersion medium $n = 1.52$. The wavelength, λ , was selected according to the nominal emission wavelength of emission spectra of bead (FluoSpheres F-8789, dark red, Invitrogen) and Alexa Fluor 647 (A-20006, Invitrogen). For the astigmatism geometry, the expected collected photon count of each emitter and the background count rate were doubled compared with dual focal plane geometry, giving a equal total photon count between two imaging geometries. The plane separations of 400 nm and 600 nm were used for dual focal plane geometry, the relative magnification of plane b to plane a was 1 and background at plane a and b were equal. All photon count values given indicate that collected on a single plane of the dual focal plane or combined geometry. The foci separations of ~ 400 nm and ~ 600 nm were used for the astigmatism geometry, which was obtained by setting the value of C in Eq. 15 to 2 and 3 respectively. The parameters for the combined model were set to the same values as with dual focal plane method but included the astigmatism aberration in the PSF model. The pixel size, fitting region size, expected photon counts of emitters and background count were specified in each result.

For multi-emitter calculations, emitter one is always positioned at the fitting region center (x_0, y_0), and randomly distributed in z . The other emitters are uniformly distributed in a region bounded by a 3D volume, with x and y dimensions equal to $1.28 \mu\text{m}$. The z range was fixed at -1 to $1 \mu\text{m}$. The z positions of the dual focal plane and combined geometries were relative to the middle plane of two focal planes. For CRLB calculations including camera readout noise, the standard deviation of readout noise was set to $2 e^-/\text{pixel}$ and $6 e^-/\text{pixel}$ and did not include any offset.

In the simulation of top four images in Fig. 1, we used the OTF re-scaled PSF model for all three imaging geometries. 40 single emitter models were generated with assigned x, y, z positions, each measuring $20 \text{ pixels} \times 20 \text{ pixels}$, equivalent to $2.13 \mu\text{m} \times 2.13 \mu\text{m}$. Second, all emitter images were placed in the same image according to their x, y coordinates of the upper left pixel, which was equal to emitter's x, y position subtracted by its relative x, y position to the upper left pixel. For dual focal plane geometry, the x, y positions at each plane were related by a translation matrix. The PSF model calculations used the same parameter settings as described above.

The PSF and CRLB calculations were implemented on graphic processing unit (GPU) hardware for parallel computation. Calculation of the CRLB for 1000 sets of emitter models required between 1 and 10 s depending on number of emitters, image modality and inclusion of readout noise.

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Figure 1.

A conceptual illustration comparing the potential for overlapping emitters in 2D and 3D. (a) All emitters are in focus. (b,c,d) Same emitters in (a) with randomly assigned axial position between $[-1, 1]$ μm . (e,f,g) PSF model at various z positions. (b,e) Dual focal plane. (c,f) Astigmatism. (d,g) Dual focal plane with astigmatism (combined geometry). Axial positions of dual focal plane and combined methods are relative to the middle plane of two focal planes. All images are independently contrast enhanced for display purposes. Scale bar is 1 μm .

Figure 2.

A comparison of two-emitter localization precision for three types of 3D imaging geometries. Emitter 1 is always fixed at the center of a 2D fitting region and randomly placed in z . Emitter 2 is randomly positioned in the 3D space (condition 1). Emitter 2 has the same z position as emitter 1, but randomly positioned in x , y (condition 2). Emitter 2 is placed in the center of the fit region in x , y , but randomly positioned in z (condition 3). (j,k,l) Position relationship of emitter 1 and emitter 2 under different conditions for the case of emitter 1 at $z = 0$. The focal plane separation (dual focal plane geometries) and foci separation (astigmatism geometries) are 400 nm and ~ 400 nm respectively. The number of simulated two-emitter models used are 10^5 . Expected photon counts of all emitters are 800 photons. The fit region size is 60 pixels $\times$ 60 pixels, measuring $6.4 \mu\text{m} \times 6.4 \mu\text{m}$ at the sample plane. The results for $\sqrt[3]{\sigma_{3D}}$ versus z and separation only include values of $\sqrt[3]{\sigma_{3D}}$ less than 173 nm.

Figure 3.

Comparison of fit box size. The cumulative distribution of cube root of volume error ($\sqrt[3]{\sigma_{3D}}$) for single-emitter and four-emitter fitting is shown for a small fitting region size (S) and a large fitting region size (L). The small fitting region size is 24 pixels $\times$ 24 pixels, measuring 2.56 $\mu\text{m} \times$ 2.56 μm at the sample plane. The large fitting region size is 60 pixels $\times$ 60 pixels, measuring 6.4 $\mu\text{m} \times$ 6.4 μm at the sample plane. The expected numbers of photons from each emitter are normally distributed with a mean of 800 photons and a standard deviation of 200 photons. The focal plane separation (dual focal plane geometries) and foci separation (astigmatism geometries) are 400 nm and $\sim$ 400 nm respectively.

Figure 4.

Comparison of the cumulative distribution of cube root of volume error ($\sqrt[3]{\sigma_{3D}}$) with mean expected photon counts of 800. (a,b) The expected numbers of photons from each emitter are normally distributed with a mean of 800 photons and a standard deviation of 200 photons. (c,d) The expected numbers of photons from each emitter are exponentially distributed with a mean of 800 photons. (a,c) Background photon count is 2 photons/pixel. (b,d) Background photon count is 16 photons/pixel. The focal plane separation (dual focal plane geometries) and foci separation (astigmatism geometries) are 400 nm and ~400 nm respectively. The number of simulated single or four-emitter models for each case is 20000. The fitting region size is 24 pixels $\times$ 24 pixels, measuring $2.56 \mu\text{m} \times 2.56 \mu\text{m}$ at the sample plane.

Figure 5.

Comparison of the cumulative distribution of cube root of volume error ($\sqrt[3]{\sigma_{3D}}$) with mean expected photon counts of 5000. (a,b) The expected numbers of photons from each emitter are normally distributed with a mean of 5000 photons and a standard deviation of 1250 photons. (c,d) The expected numbers of photons from each emitter are exponentially distributed with a mean of 5000 photons. (a,c) Background photon count is 2 photons/pixel. (b,d) Background photon count is 16 photons/pixel. The focal plane separation (dual focal plane geometries) and foci separation (astigmatism geometries) are 400 nm and ~400 nm respectively. The number of simulated single or four-emitter models for each case is 20000. The fitting region size is 24 pixels $\times$ 24 pixels, measuring $2.56 \mu\text{m} \times 2.56 \mu\text{m}$ at the sample plane.

Figure 6.

Comparison of the cumulative distribution of cube root of volume error ($\sqrt[3]{\sigma_{3D}}$) with focal plane and foci separation of 600 nm. (a,b) The expected numbers of photons from each emitter are normally distributed with a mean of 800 photons and a standard deviation of 200 photons. (c,d) The expected numbers of photons from each emitter are exponentially distributed with a mean of 800 photons. (a,c) Background photon count is 2 photons/pixel. (b,d) Background photon count is 16 photons/pixel. The focal plane separation (dual focal plane geometries) and foci separation (astigmatism geometries) are 400 nm and ~400 nm respectively. The number of simulated single or four-emitter models for each case is 20000. The fitting region size is 24 pixels $\times$ 24 pixels, measuring $2.56 \mu\text{m} \times 2.56 \mu\text{m}$ at the sample plane.

Figure 7.

Cumulative distribution of $\sqrt[3]{\sigma_{3D}}$ of single-emitter and four-emitter fitting in the presence camera readout noise. The expected numbers of photons from each emitter are normally distributed with a mean of 800 photons and a standard deviation of 200 photons. The camera readout noise has a standard deviation of 2 e^-/pixel (a,c,e) and 6 e^-/pixel (b,d,f). (a,b) Pixel size is 80 nm at the sample plane. (c,d) Pixel size is 128 nm at the sample plane. (e,f) Comparison of four-emitter fitting under different pixel sizes. The fitting region size is fixed at $2.56 \mu\text{m} \times 2.56 \mu\text{m}$. Total number of simulated single or two-emitter models are 5000. Background counts are fixed at $312 \text{ photons}/\mu\text{m}^2$.