Single-image axial localization precision analysis for individual fluorophores

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Abstract: Bio-mechanism investigations demand single particle tracking with high spatial and temporal resolutions which require single fluorophore 3D localization measurements with matching precision and speed. Although the precision for lateral-localization measurements is well described by an analytical expression, for the axial direction, it is often obtained by repeating location measurements or by estimating a lower bound. Here, we report a precision expression for an axial-localization method that analyzes the standard deviations of single fluorophores’ intensity profiles. Like the lateral-localization precision, this expression includes all relevant experimental effects measurable from a Gaussian intensity profile of the fluorophore. This expression completes the precision analysis for single-image 3D localization of individual fluorophores and lifts the temporal resolution to the typical exposure timescales of milliseconds.

OCIS codes: (100.6640) Superresolution; (180.2520) Fluorescence microscopy; (180.6900) Three-dimensional microscopy; (100.6890) Three-dimensional image processing; (110.2960) Image analysis.

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1. Introduction

Single particle tracking (SPT), whose building blocks are consecutive 3D localization measurements of the investigated particle, is important for many biological investigations [1–3]. To achieve SPT studies with high spatial and temporal resolutions, it is essential to determine the precisions to the 3D localization measurements of the investigated particle in an accurate and timely manner [2].

In current single molecule localization measurements using standard imaging setups, such as in epifluorescence and total internal reflection fluorescence (TIRF) imaging measurements, the intensity profile of a stationary or a slowly moving molecule (relative to the imaging timescale) is called a point spread function (PSF) and can be approximated by a Gaussian function. The centroid of the Gaussian fit to the PSF yields the lateral location of the molecule, and the standard deviation (SD) can be used to determine the molecule’s axial position or defocusing distance (relative to the focal plane) [4] as well as the precision to the lateral location measurements [5, 6]. Although the lateral-localization precision can be expressed by fitting parameters of an individual fluorophore’s single image (PSF and background intensities) [5, 6], thus enabling lateral localization with lateral precision in the typical single-image exposure timescales of milliseconds, the axial-localization precision awaits an analogous expression to complete 3D localization with 3D precision in milliseconds.

Current single fluorophore axial-localization methods – which include methods that analyze PSFs obtained using standard imaging setups, such as inferring the axial location from the measured PSF SD [4, 7–10] and using novel algorithms [11–14], and methods that require instrumentation modifications [4, 14–17] – use two approaches in quantifying precision: (i) experimentally repeating the axial-location measurement and using the SD of the axial-location distribution for precision [4, 11, 12, 15–17], and (ii) using an estimation, either the SD of the calculated axial locations according to novel algorithms [12, 14] or a Cramér-Rao lower bound that predicts the lowest possible error to the axial location measurements [9, 12, 13]. For the first approach, although the results include the instrumentation and fluorophore effects (camera’s
readout noise and pixelation effect; PSF’s photon noise, respectively), it decreases the temporal resolution of SPT by at least 20-fold due to repeated imaging; for the second approach, (i) the Cramér-Rao calculation is an estimation of the lower bound, rather than a precise description of the axial-localization precision, and (ii) it does not consider the instrumentation and fluorophore effects, which can affect the precision determination considerably [5, 6]. Furthermore, due to the lack of an expression that includes all experimental noise parameters, neither method can be used to assess axial-localization precisions in alternative experimental settings.

Here we report a precision expression for a fluorophore’s single-image axial location measurement that includes the instrumentation and fluorophore’s noise effects, based on the axial-localization method that measures PSF’s SDs. This axial-localization method involves minimal modifications to standard single-molecule imaging setups, and all the parameters in the axial precision expression can be obtained from a single Gaussian fit to the PSF of the fluorophore: SD, photon count, pixel size, and background noise values. This axial-precision expression is a new addition to our single-image molecular analysis (SIMA) studies [19]; it completes individual fluorophores’ single-image 3D localization measurements by providing the axial-direction precision, and lifts the temporal resolution of SPT to the typical single-image exposure timescales of milliseconds.

2. Materials and methods

2.1. Sample preparation and imaging

In this study, we used phycobilisome (PBS; Synechosystis PCC 6803) protein complexes for our \( z \) and \( \Delta z \) investigations. PBSs are large hemidiscoidal-shaped light harvesting antenna protein complexes in cyanobacteria; the molecular weight is \( \approx 10 \text{MDa} \) and it measures \( 60 \times 30 \times 20 \text{nm} \) in width, height, and length [20]. A PBS molecule contains 144 fluorophores distributed throughout the complex; consequently, it serves as an ideal emitter to meet the demands of our study due to its brightness and long fluorescence lifetime. PBSs were purified following the method described in Ref. [21]; the purified PBSs were then crosslinked according to a protocol from the Noam Adir group [22]. The PBSs were diluted in 20 mM Tris-HCl buffer (pH 8.0) to approximately 0.1 nM. Manufacturer pre-cleaned fused-silica chips (6W675-575 20C, Hoya Corporation USA, San Jose, CA) were used, where isolated PBS molecules were adsorbed to surfaces at low concentration. A PBS solution of 5 \( \mu \text{L} \) was sandwiched between the fused-silica surface and an oxygen-plasma-cleaned coverslip (2.2 \( \times \) 2.2 cm\(^2\)), resulting in a 10.5 \( \mu \text{m} \) thick water layer. Because of the prism TIRF imaging setup, our sample on the fused-silica surface is 10.5 \( \mu \text{m} \) away from the coverslip surface; therefore, refractive index mismatch will affect the PBS SD versus \( z \) relation [17, 18].

Single-molecule imaging was performed using a Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY) in combination with a Nikon 100X objective (Nikon, 1.49 N.A., oil immersion). Samples were excited by prism-type TIRF microscopy with a linearly polarized 568 nm laser line (I70C-SPECTRUM Argon/Krypton laser, Coherent Inc., Santa Clara, CA) focused on a \( 40 \times 20 \mu \text{m}^2 \) region. The incident angle at the fused-silica water interface was 70° with respect to the normal. The 568 nm line was filtered from the multiline laser emission using a polychromatic acousto-optic filter (48062 PCAOM model, NEOS Technologies, Melbourne, FL). The laser excitation was pulsed with illumination intervals of 10 ms; the excitation intensity was 5.2 kW/cm\(^2\). Images were captured by an iXon back-illuminated electron multiplying charge coupled device (EMCCD) camera (DV897ECS-BV, Andor Technology, Belfast, Northern Ireland). An additional 2X expansion lens was placed before the EMCCD, producing a pixel size of 79 nm. The excitation filter was 568/20 nm, and the emission filter was a 580 nm long pass filter.
2.2. Data acquisition and selection

PBS movies were obtained by synchronizing the onset of camera exposure with laser illumination. The maximum gain level of the camera was used and the data acquisition rate was 1 MHz pixels/sec (≈3.3 frames/sec). Single-molecule images were checked such that there were no saturations in the intensity profiles. The axial position of the objective was controlled by a focus drive (H122, Prior Scientific Inc., Rockland, MA), moving one-way from \( z \approx 700 \) nm to \(-350 \) nm in 50 nm increments and 1 s intervals while a PBS movie was recorded, taking individual snapshots at every axial location; then, 50-image movies were acquired at the next three consecutive axial locations (0.3 s intervals), before a last movie was recorded for \( z \approx -500 \) nm to \(-750 \) nm. Here, positive defocusing distance \( z \) is defined as when the surface-adsorbed PBS molecules are on the side of the focal plane closer to the glass coverslip. For PBS analyses, 35 × 35 pixel boxes centered at the molecule were selected by hand using IMAGEJ (NIH, Bethesda, MD); the center 25 × 25 pixels containing the PSF were used for 2D Gaussian fitting, and the peripheral pixels were used for background analysis. Only PBS molecules with signal-to-noise ratios (SNR) ≥ 2.5 were used for analysis.

Before analysis, the camera’s intensity count at each pixel in an image was converted into photon count by using the camera-to-photon count conversion factor calibrated the same day of the measurement as described in our previous article [5]. The number of detected photons in an image was obtained by subtracting the total photon count of the background from the total photon count of the image. The PBS intensity profiles were fit to a 2D Gaussian function using a least squares curve fitting algorithm (lsqcurvefit) provided by MATLAB (The Mathworks, Natick, MA) in order to obtain the SD values of the molecule:

\[
f(x, y) = f_0 \exp \left[ -\frac{(x - x_0)^2}{2\sigma^2_x} - \frac{(y - y_0)^2}{2\sigma^2_y} \right] + \langle b \rangle,
\]

where \( f_0 \) is the multiplication factor, \( \sigma_x \) and \( \sigma_y \) are SDs in the \( x \)- and \( y \)-directions, respectively, \( x_0 \) and \( y_0 \) are the centroid location of the molecule, and \( \langle b \rangle \) is the mean background offset in photons.

3. Results

In the PSF SD-based axial-localization method, the measured fluorophore’s PSF SD in the \( x \)- or \( y \)-direction, \( \sigma_{x,y} \), is frequently described by a symmetric [7–9] or an asymmetric function of the defocusing distance \( z \) [4, 10]. Since \( z \) is a function of \( s_{x,y} \), by using a single image of the fluorophore, we should be able to represent the precision in \( z \), \( \Delta z \), by the precision in \( s_{x,y} \), \( \Delta s_{x,y} \), via error propagation calculation. Since we have recently obtained an expression for a PSF’s root mean square (rms) \( \Delta s_{x,y} \) as a function of \( s_{x,y} = \sqrt{s_{0x,0y}^2 + a^2/12} \) where \( s_{0x,0y} \) is the PSF SD without the pixelation effect, pixel size \( a \), number of detected photons \( N \), and the mean \( \langle b \rangle \) and variance \( \sigma^2_b \) of the background’s photon noise [5]

\[
\Delta s_{x,y,\text{rms}} = \sqrt{\frac{s_{0x}^2 + a^2/12}{N} + \frac{16\pi(s_{0x}^2 + a^2/12)^{3/2}(s_{0y}^2 + a^2/12)^{1/2}(\sigma^2_b + \langle b \rangle)}{3a^2N^2}},
\]

we can obtain \( \Delta z \) by studying a single image of a fluorophore. (Note that for the remainder of this article, we use \( i \) to denote \( x \) or \( y \) and \( \Delta s_i \) to denote \( \Delta s_{x,y,\text{rms}} \).)

In Fig. 1(A), we show images of a single PBS molecule at different defocusing distances \( |z| \), separated by 350 nm; it is obvious that the size of the image (or \( s_i \)) increases with \( |z| \). Figure 1(B) plots the mean \( s_x \) values of 6 simultaneously imaged PBS molecules for \( z \) moving from
The data fits well to the following expression
\[
s_i(z) = s'_i \sqrt{1 + Az^2 + Bz^4},
\]
where \(s'_i = \sqrt{s_{i0}^2(z = 0) + a^2/12}\) is the PBS SD at focus including the pixelation effect, \(A\) and \(B\) are fitting parameters which can be related to the focus depth of the microscope, \(d\), as \(A = 1/d^2\) and \(B = B'/d^4\) with \(B'\) being the coefficient of a higher order term to correct for the refractive index mismatch effect and the non-ideality of an imaging system [4, 17].

Having obtained \(s_i\) as a function of \(z\), we proceed to express \(z\) as a function of \(s_i\)
\[
z(s_i) = \pm \sqrt{\frac{C - A}{2B}}
\]
where \(C = \sqrt{A^2 - 4B[1 - (s_i/s'_{i0})^2]}\), and then to calculate \(\Delta z\) by using the error propagation calculation of \(\Delta z = (\partial z/\partial s_i)\Delta s_i\):
\[
\Delta z = \frac{s_i\Delta s_i}{s'_{i0}C} \sqrt{\frac{2B}{C - A}}.
\]
Now we have a \(\Delta z\) expression as a function of experimental parameters of a single PSF.

Using Eqs. (2), (3), and (5), we calculated and plotted the average \(\Delta s_i\) and \(\Delta z\) values for PBS molecules in Fig. 1 (including modification to the \(\Delta s_i\) values, and consequently the \(\Delta z\) values by the appropriate scaling factors; see Sec. 3.1) as the y- and x-axis error bars in Fig. 1(B), respectively. Since \(\Delta z\) diverges at low \(|z|\), only \(\Delta z\) at \(|z| \geq \Delta z(z)\) are shown.

In order to validate our method in obtaining \(\Delta z\) by error propagation calculation of \(z(s_i)\), we have performed repeated measurements of a single PBS molecule at three axial locations near \(z = -400\) nm separated by 50 nm. Figures 1(C) and 1(D) show the \(s_i\) and the corresponding \(z\) values [calculated from \(s_i\) using Eq. (4)], respectively, at the three axial locations. The two error bars at each axial location compare the calculated with the experimentally determined \(\Delta s_i\) and \(\Delta z\) [insets to Figs. 1(C) and 1(D)], showing agreement.

3.1. Theoretical \(\Delta s_i\) scaling factor calculations

As shown in our previous article on \(\Delta s_i\) of single fluorophore images [5], the theoretical \(\Delta s_i\) values [Eq. (2)] underreports the true experimental \(\Delta s_i\) value. A scaling factor that depends on \(a/s_{0i}\) should be multiplied to the theoretical \(\Delta s_i\) in order to obtain the expected experimental \(\Delta s_i\). Although we have studied the \(a/s_{0i}\) dependence of the scaling factor by varying \(a\) [5], in this article, it is \(s_{0i}\) that changes at different \(z\) and the \(a/s_{0i}\) dependence may vary. We have performed \(\Delta s_i\) simulations for \(a/s_{0i} = 0.20\) to 0.76, which covers our measured PBS \(a/s_{0i}\) range of 0.20 to 0.55 for \(a = 79\) nm, \(s_{0i,max} = 404\) nm, and \(s_{0i,min} = 142\) nm. The simulation results are shown in Fig. 2, where each \(\Delta s_i\) datum is the SD of the \(s_i\) distribution of 2000 simulated PSFs using the average PBS parameters of \(a = 79\) nm, \(N = 3000\), \(\langle b \rangle = 4\), and \(s_{0i} = 1\) photons. The simulated \(\Delta s_i\) results were compared to the calculated \(\Delta s_i\) results, yielding a scaling factor expression of \(1.51 + 0.17a/s_{0i}\). The higher limit of \(a/s_{0i}\) was chosen to be 0.76, which is up to where the scaling factor expression remains valid.

4. Discussion

Unlike the lateral-localization precision, which is independent of the lateral position (\(\Delta x\), Refs. [5,6]), the axial-localization precision varies with \(z\). In Fig. 3, we plot \(\Delta z\) versus \(|z|\) for PBS PSFs
Fig. 1. PBS axial-localization precision studies. (A) Snapshots of a PBS molecule separated by 350 nm along $z$ (the middle image is at $z \approx -50$ nm). Scale bar, 500 nm. (B) Mean $s_x$ versus $z$ for 6 simultaneously imaged PBS molecules. The solid line is a fit to the data according to Eq. (3), yielding $s_{x0} = 144.1$ nm, $A = 2.91 \times 10^{-7}$ nm$^{-2}$, and $B = 1.87 \times 10^{-11}$ nm$^{-4}$. The $y$- and $x$-axis error bars are the average $\Delta s_y$ and $\Delta z$ values of the 6 PBS molecules. Note that the errors increase as $z$ decreases because the PBS molecules gradually bleached with imaging time from 4800 to 1400 mean photons per PSF. (C) 50 consecutive $s_x$ measurements for the PBS molecule in (A) at each of the three $z$ locations in the blue circle in (B) (gray lines). The mean photon counts per image is $\approx 3000$. (D) The corresponding $z$ values to $s_x$ values in (C) (gray lines). At each axial location in (C) and (D), the black horizontal lines outline the average $s_x$ and $z$ values, and the left (black) and right (red) error bars represent the respective experimental and theoretical $\Delta s_x$ and $\Delta z$ values. Insets to (C) and (D) show Gaussian fits to the distributions of the experimental $s_x$ and $z$ data for the middle axial location: the SDs of the fits (experimental error bars) are $\Delta s_x = 6.3$ nm and $\Delta z = 21.5$ nm, in good agreement with the theoretical values of $\Delta s_x = 6.0$ nm and $\Delta z = 20.0$ nm. Note that $\Delta z$ is clearly less than the $z$ increment size of 50 nm.
for a range of photon counts, providing a direct guideline for the axial-localization precisions that can be achieved by using single fluorophore images at specific axial locations and photon counts.

We discuss two features of Fig. 3 that will aid in generalizing our method to all SD-based axial-location precision studies using different fluorophores and experimental settings. (i) At high $N$ and large $|z|$, the single-image determined $\Delta z$ is in the nanometer range, allowing 3D localization measurements with simultaneous high spatial and temporal resolutions. (ii) As seen
in Fig. 2, $\Delta z$ diverges as $|z|$ approaches 0 (this has been previously reported in Refs. [9, 12]). In order to obtain $\Delta z$ at low $|z|$, one solution is to use astigmatism by introducing a cylindrical lens [23] that shifts the foci of $s_x(z)$ and $s_y(z)$ to be on opposite sides of $z = 0$ [4]. In Fig. 4(A) we shift the PBS $s_x(z)$ and $s_y(z)$ foci by 300 nm, assuming that $s_x(z)$ and $s_y(z)$ remain the same shape and measurable range after the cylindrical lens modification. Figure 4(B) plots the corresponding $\Delta z(z)$ curves to the shifted $s_x(z)$ and $s_y(z)$ for a range of photon counts. At all measurable $z$ for PBS, appropriate $s_i(z)$ values can be used to obtain the minimum $\Delta z$, marked as bold lines, and nanometer precision for single-image axial localization measurements can be achieved at high photon counts.

The studies in this article are based on PBS molecules with a specific $z(s_i)$ relation [Eq. (4)]. Because PBS differs from a single fluorophore in that it is an aggregate of 144 fluorophores, and our TIRF imaging setup further introduces the additional refractive index mismatch effect,
our PBS $s_i(z)$ relation, and thus our $z(s_i)$ relation may differ slightly from those of single fluorophores at different depths in water (relative to the refractive index mismatch interface). Our group and two additional groups have observed that for single fluorophores in water, the $s_i(z)$ curve is asymmetric and the degree of asymmetry varies with their depths [4, 10, 18, 24]. As a consequence, single fluorophore studies with different refractive index mismatch effects may have different $z(s_i)$ and thus $\Delta z$ relations. What our study has shown is that regardless of the sample or imaging setup, when a $z(s_i)$ expression can be obtained for the molecule of interest, error propagation can be used to determine the axial-location precision from a single image of the fluorophore. In some complicated situations, such as when $s_i(z)$ is asymmetric [4, 10, 18], multiple $s_i(z)$ functions can be used to describe different regions of the $s_i(z)$ curve in order to obtain simple $z(s_i)$ expressions for $\Delta z$ calculation.

In summary, we have developed a precision expression for a fluorophore’s single-image axial localization measurement that includes all relevant experimental parameters. The improvement in temporal resolution will enable investigations of previously inaccessible SPT studies in the regime of milliseconds.

Acknowledgments

M. C. D. is supported by a National Institutes of Health predoctoral fellowship awarded under 5T90 DA022871. X. L. is supported by the Photosynthetic Antenna Research Center (PARC), an Energy Frontier Research Center funded by the US Department of Energy under Award Number DE-SC 0001035.