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The double-helix point spread function enables precise and accurate measurement of 3D single-molecule localization and orientation

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Abstract

Single-molecule-based super-resolution fluorescence microscopy has recently been developed to surpass the diffraction limit by roughly an order of magnitude. These methods depend on the ability to precisely and accurately measure the position of a single-molecule emitter, typically by fitting its emission pattern to a symmetric estimator (e.g. centroid or 2D Gaussian). However, single-molecule emission patterns are not isotropic, and depend highly on the orientation of the molecule's transition dipole moment, as well as its z -position. Failure to account for this fact can result in localization errors on the order of tens of nm for in-focus images, and ~50–200 nm for molecules at modest defocus. The latter range becomes especially important for three-dimensional (3D) single-molecule super-resolution techniques, which typically employ depths-of-field of up to ~2 μ m. To address this issue we report the simultaneous measurement of precise and accurate 3D single-molecule position and 3D dipole orientation using the Double-Helix Point Spread Function (DH-PSF) microscope. We are thus able to significantly improve dipole-induced position errors, reducing standard deviations in lateral localization from ~2x worse than photon-limited precision (48 nm vs. 25 nm) to within 5 nm of photon-limited precision. Furthermore, by averaging many estimations of orientation we are able to improve from a lateral standard deviation of 116 nm (~4x worse than the precision, 28 nm) to 34 nm (within 6 nm).

Keywords

super resolution; fluorescence microscopy; three-dimensional microscopy; single molecule; dipole orientation; mislocalization; polarization microscopy

1. INTRODUCTION

1.1 Super-resolution fluorescence microscopy

Fluorescence microscopy has long been a useful tool in biological imaging due to the capacity for site-specific labeling and its relatively non-invasive nature. Until recently, however, the spatial resolution of fluorescence microscopy has been limited by the diffraction of light to ~250 nm in the lateral dimensions and ~500 nm in the axial dimension in the visible spectrum. The advent of super-resolution microscopy has enabled researchers to surpass this limit and push resolutions down to the order of 10 nm via a variety of methods.^{1, 2} Techniques like Structured Illumination Microscopy (SIM)³ and Stimulated Emission Depletion Microscopy (STED)⁴ rely on spatial manipulation of the illumination light beam(s) to achieve super resolution. Another major class of super-resolution techniques, known collectively as Single Molecule Active Control Microscopy (SMACM), relies on actively controlling and detecting emission from single molecules. SMACM includes the methods of (f)PALM^{5, 6} and STORM.⁷ All SMACM techniques rely on two major principles to break the diffraction limit. The first is active control of the concentration of emitters by taking advantage of the photophysics or photochemistry of the fluorophore. Properties like photoactivation or photoswitching are used to ensure that in any given image frame, only a sparse subset of fluorophores are emitting such that the diffraction-limited spot due to one emitter does not overlap spatially with that of another. The second major principle of SMACM, super localization, is then used to localize the isolated emitters to precisions on the order of ~10 nm. Even though a single molecule appears on a camera as a spot blurred by diffraction, one can find the center of the spot and thus, presumably the position of the molecule to much better precision by fitting to a two-dimensional (2D) Gaussian function and extracting the center of the resulting distribution, calculating the centroid position, or using a more sophisticated estimator. After this sparse subset of emitters is bleached, a new subset is activated and the process is repeated until a super-resolution pointillist reconstruction can be formed in post-processing.

1.2 Localization error due to molecular dipole orientation

The super-localization process described above equates the center of the recorded emitted photon distribution from a single molecule with the true position of the molecule. The underlying assumption here is that the molecule emits isotropically, which is not generally true for a rotationally immobile single molecule. Instead, single molecules are known to exhibit detectable dipole emission patterns due to the interaction of the electromagnetic field with the molecule's transition dipole moment,⁸ especially since most single-molecule fluorophores emit via an electric-dipole-allowed electronic transition. The dipole radiation pattern in the far-field is inherently anisotropic, and the corresponding image recorded depends heavily on the molecule's rotational mobility and orientation (θ, ϕ) (Figure 1). For SMACM techniques, this effect implies that the positions extracted in super localizing molecules can be skewed from the true positions of the molecules. For fixed dipoles located within the focal plane of the microscope, this can result in errors on the order of ~10 nm.⁹ The mislocalization is a function of the z position of the emitter, and can exceed 100 nm at modest defocus ($\sim \pm 300$ nm).¹⁰ The z dependence of the mislocalization causes the 3D PSF to incur tilt in the lateral directions (Figure 1). While this mislocalization is potentially

problematic for standard 2D widefield super-resolution imaging, it is especially so for 3D techniques such as astigmatism,¹¹ multi-plane,¹² and Double-Helix Point Spread Function (DH-PSF)¹³ imaging, which operate over an extended depth of field.

Rotational lifetimes of fluorescent dyes and proteins are typically much faster (~ 0.1 – 10 ns) than camera integration times used in a super-resolution experiment (~ 10 – 100 ms), and so if rotational mobility is unconstrained, an isotropic emission pattern will be recovered and the error fully mitigated.¹⁴ In real biological systems, however, it may often be the case that rotational mobility is at least partially constrained,¹⁵ and so the error may be non-negligible. Recently, several groups have proposed methods to simultaneously estimate orientation and position (and thus account for the mislocalization) of rotationally immobile fluorophores,^{16–18} but it is unclear that these would work in a real SMACM experiment under realistic signal-to-noise conditions since they rely on sensing subtle features in the standard point spread function or weak rings and asymmetry of the single-molecule image. Here we present and demonstrate a powerful solution for the worst-case scenario of rotationally immobile fluorophores using the DH-PSF microscope¹⁹. Our method shows promise for incorporation into super-resolution measurements since the DH-PSF has previously been shown to be an effective method for 3D super-resolution imaging.^{20, 21} We experimentally demonstrate the ability to correct mislocalizations as large as 100 nm, improving to within a few nm of the shot noise-limited precision.

2. DH-PSF Microscope

2.1 General principle

The DH-PSF microscope has been described in detail elsewhere.¹³ Briefly, the DH-PSF microscope allows for 3D super-resolution imaging over an extended $2\text{-}\mu\text{m}$ depth of field by replacing the standard PSF with one that appears as two bright lobes on the camera, which revolve around one another as a function of z . An in-focus emitter will appear as two vertically displaced lobes, while an emitter placed above or below the focal plane will appear as two lobes laying along either a forward- or backward-slanting diagonal. The emitter image can be fit using a double-Gaussian (i.e. the sum of two 2D Gaussian functions) estimator, and the precise (x,y) position of the emitter is found by finding the midpoint of the two Gaussian centers; the precise z position is determined by calculating the angle between the line containing the two Gaussian centers and the vertical. It is the goal of this paper to extract not only precise, but also accurate (x,y) position.

The DH-PSF is created by adding a $4f$ optical processing system immediately after the image plane of a standard inverted widefield microscope. A lens placed one focal length (f) from this input image plane produces the Fourier transform of the electric field at its back focal plane. At this position a phase-only spatial light modulator (SLM) encoding the DH-PSF phase mask is placed, in order to multiply the Fourier-transformed field by the appropriate phase pattern. The resulting field is then Fourier transformed again by a second lens placed a distance f from the SLM, and the final image is produced at the back focal plane of the second lens. In effect the $4f$ system convolves the image of each emitter with the DH-PSF shape.

2.2 DH-PSF response to molecular orientation

While the two lobes of the DH-PSF for a theoretical isotropic emitter have equal intensity, the same is not generally true for an oriented single molecule. Instead, single molecules may exhibit nonzero lobe asymmetry (LA), as defined in equation 1:

$$LA = \frac{A_{L1} - A_{L2}}{A_{L1} + A_{L2}}, \quad (1)$$

where A_{L1} and A_{L2} refer to the amplitudes of the two fitted Gaussians. Qualitatively this is explained by the fact that oriented single molecules are known to exhibit asymmetric emission patterns in the back focal plane of the microscope objective.²² This pattern is precisely what is multiplied by the DH-PSF phase mask, and so various spatial frequencies of the DH-PSF are attenuated as a result of molecular orientation, allowing for asymmetry in the two lobes. LA is a complicated function of (θ, ϕ, z) and polarization, and thus measuring LA provides information about the orientation of a molecule when it is not known *a priori*. Furthermore, it is well-known that recording images of a single molecule in orthogonal polarization channels provides information about its orientation. Roughly, a molecule's linear dichroism (LD) is related to ϕ via equation 2:

$$LD = \frac{N_T - N_R}{N_T + N_R} \approx \cos(2\phi), \quad (2)$$

where N_T and N_R are the number of photons detected in the transmitted and reflected polarization channels, respectively. Here “transmitted” and “reflected” refer to the polarizations which are separated by an ordinary polarizing beam splitter. Splitting signal into two polarization channels is particularly compatible with the DH-PSF microscope since the use of a liquid crystal-based SLM requires polarization considerations anyway as only one polarization incident on the SLM is phase-modulated. These facts suggest that by monitoring (LA, LD, z) given by the DH-PSF one should be able to determine (θ, ϕ) and in turn be able to extract the expected size and direction of the lateral mislocalization (x, y) .

3. Simulations

To demonstrate the above, we performed full vectorial diffraction calculations which take into account molecular orientation and polarization of the electric field based on methods described elsewhere.^{19, 23} The resulting simulated electric fields were convolved with the DH-PSF and the final simulated images were fit with a double Gaussian estimator. An example set of images and fits is shown in Figure 2A–D. From the fits we mapped out the behavior of the functions $LA(\theta, \phi, z)$ (Figure 2E–F) and $LD(\theta, \phi, z)$, as well as the mislocalizations $x(\theta, \phi, z)$ and $y(\theta, \phi, z)$ (Figure 2G–J). Interestingly, our simulations show that the same molecule of a given orientation appears with different LA in the two polarization channels. This property is related to the orientation of the polarization vector of a given channel relative to the unique axis of phase discontinuities in the DH-PSF mask (Figure 2K). Thus the separation of polarizations provides more information about ϕ than a typical SM polarization experiment.

These simulations provided a look-up table to be used in correcting mislocalizations in experimental data in the following way: first we extract a molecule's $(LA, LD, x_{apparent}, y_{apparent}, z)$ from the double-Gaussian fit to its recorded image. We then reference our look-up table to provide a minimum least squares estimation of (θ, ϕ) based on the determined (LA, LD, z) . Finally, we use this (θ, ϕ) to predict the localization error (x, y) and subtract these values from $(x_{apparent}, y_{apparent})$ to yield the corrected lateral position.

4. Experimental Validation

4.1 Setup

To perform a proof-of-principle experiment we built the polarization-sensitive DH-PSF setup shown in Figure 3. In this setup, the emission is split into orthogonal polarizations by a polarizing beam splitter (PBS) placed after the first $4f$ lens. Each polarization channel is then directed out of the plane parallel to the table (into the direction $\mathbf{w}$ marked in the figure) by a square pyramidal mirror, onto the face of the SLM which is mounted from above facing toward the table. The two channels are then reflected off the opposite sides of the pyramidal mirror and back into the plane parallel to the table. They both then travel through a second set of $4f$ lenses and onto the EMCCD camera. Due to the unique geometry of this “pyramid” setup, the polarization vector in each channel is projected onto the SLM with the same orientation relative to the phase mask (Figure 3C). Hence, in order to take advantage of the full simulated behavior of the DH-PSF (i.e. to capture both “perpendicular-” and “parallel-” type behavior as defined above), we found it useful to acquire images both with the phase mask oriented upright and with the phase mask rotated 90° . Thus, a single estimation of (θ, ϕ) required two successive camera acquisitions, resulting in four total images (2 acquisitions $\times$ 2 polarization channels) per “snapshot”. We assign color names to each of the four mask orientation/polarization channel combinations for ease of reference: mask parallel/transmitted polarization is “red”, mask perpendicular/transmitted is “gold”, mask parallel/reflected polarization is “green”, and mask perpendicular/reflected is “blue”. Despite the identical projections of the polarization vectors of the two channels, the images in each channel purvey unique LA values due to the fact that the molecular coordinates as defined at the intermediate image plane (IIP) are transformed differently in the two channels (see the transformation of (x_{input}, y_{input}) in Figure 3). This results in different “effective ϕ ” orientations in each channel as defined relative to the coordinates of the phase mask, given by the inverse transform relations of equation 3:

$$\begin{aligned} \phi_{red} &\mapsto \phi_{IIP} \\ \phi_{gold} &\mapsto \phi_{IIP} + 90^\circ \\ \phi_{green} &\mapsto 270^\circ - \phi_{IIP} \\ \phi_{blue} &\mapsto -\phi_{IIP} \end{aligned} \quad (3)$$

4.2 Experimental procedure

As a proof of principle, we measured the orientations and corrected the mislocalizations of single DCDHF-N-6 fluorophores²⁴ spun in a matrix of poly(methyl methacrylate) (PMMA) polymer. Nanomolar concentrations of DCDHF-N-6 were dissolved in 1% (by mass) PMMA/toluene solution and spin-coated onto glass slides, forming a thin (30–35 nm by

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ellipsometry) polymer layer. Samples were allowed to dry overnight in order to ensure that molecular orientation was stable on a timescale much longer than imaging. An aqueous solution containing fluorescent beads (FluoSpheres, 100-nm diameter, 580/605; Invitrogen) was spun on top of the polymer layer and allowed to dry, leaving a sparse field of beads to be used as both DH-PSF calibration standards and fiducial markers to compensate for stage drift. The sample was pumped with a roughly circularly polarized ($\sim 1.4:1$) 514 nm Ar-ion laser at intensity ~ 0.1 kW/cm². Fluorescence was collected through a 100x 1.4 NA oil-immersion objective (Olympus UPlanSApo 100x/1.4) and filtered using a Chroma Z514RDC dichroic and a 590/60 band pass. Images were recorded with an Andor iXon+ DU897-E EMCCD camera operating at an EM gain setting of 300. Images were recorded with camera exposures of 500 ms.

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Each field of view (FOV) was imaged many times at many different z positions in order to produce many estimations of (θ, ϕ, x, y) for each molecule. For each FOV, we scanned our microscope objective in z in 50-nm steps (at 5 acquisitions per step) over a range of 2 μm using a motorized z positioner (C-Focus; Mad City Labs). Scanning in z allowed us to test our estimation algorithm over a range of z , while at the same time allowing us to record experimental (x, y) vs. z curves to be compared with simulation. For each molecule we analyzed, a total of four scans were taken—two with the mask in its perpendicular orientation, and two with the parallel mask orientation. Raw images for example molecule 1 are shown in Figure 4 as they appear in each mask orientation/polarization channel over a range of z .

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As a separate test of the validity of our method, we independently measured orientation of each molecule using the established method of defocused widefield imaging.^{23, 25} To do so, in between each z scan we defocused our optics by 1.00 ± 0.15 μm , turned off the DH-PSF phase pattern on the SLM, and recorded between ten and thirty 1-s exposure images. The patterns were then used to generate an independent estimation of orientation by template matching to simulated images. Interestingly, we found the need to account for some primary aberrations in this independent estimation. We corrected primary astigmatism and coma by loading the appropriate Zernike polynomial²⁶ phase masks on our SLM and varying their amplitudes until appropriate symmetry was recovered. We also found the need to account for spherical aberration by applying the appropriate Zernike polynomial phase mask to our simulated image templates in order to achieve reasonable agreement. Such aberration correction did not significantly improve our results obtained via the DH-PSF orientation method, and so no aberration correction was included for the DH-PSF imaging. A likely explanation is that the phase changes applied by the DH-PSF mask itself are sufficiently large such that the PSF becomes relatively insensitive to minor changes in phase front incurred from aberration.²⁷

5. Results and Discussion

5.1 Estimation of orientation

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In order to produce the desired groups of four images needed to estimate orientations, we grouped each pair of images (from each polarization channel) recorded simultaneously while the mask was perpendicular with a corresponding pair recorded with the mask parallel based

on z proximity. Each image was fit in MATLAB using a least-squares fit to a double-Gaussian function and the background was estimated by selecting a nearby region by hand and calculating the average photon counts. If the automatic double-Gaussian fit failed due to the presence of one sufficiently dim lobe, a single Gaussian fit was applied to each lobe by selecting their locations by hand. The background was subtracted from each image and the total signal photon count in each channel was used to calculate LD (two calculations per snapshot to be averaged together) according to equation 2. The amplitudes of each Gaussian fit were used to calculate four measurements of LA (according to equation 1) per snapshot. Measurements of (z, LA, LD) were then used to estimate (θ, ϕ) by minimizing the cost function $H(\theta, \phi)$ given by equation 4:

$$H(\theta, \phi) = \sum_{i=1}^4 \left\{ W(LA_{sim}(z_i, \theta, \phi)) [LA_{meas,i} - LA_{sim}(z_i, \theta, \phi)]^2 + [LD_{meas,i} - LD_{sim}(z_i, \theta, \phi)]^2 \right\}, \quad (4)$$

where i indexes each mask orientation/polarization channel combination, the subscripts “*sim*” and “*meas*” denote simulated and experimentally measured quantities, respectively, and the function $w(\cdot)$ is a weighting factor defined in equation 5:

$$w(x) = \begin{cases} 1, & |x| \leq 2/3 \\ 3(1-|x|), & |x| \geq 2/3 \end{cases} \quad (5)$$

This weighting factor reduces noise in the estimation by deemphasizing measurements of LA exceeding 2/3 in absolute value since LA values above this threshold tend to require the presence of one very dim lobe for which the amplitude above background is more difficult to determine precisely.

Figure 5 shows results of the DH-PSF based estimation of orientation for six molecules. The top two rows show each measurement of LD and LA recorded over the full z range, as well as the corresponding curves predicted from simulations for the mean estimated orientation (solid overlays). The histograms show distributions of estimated θ and ϕ values. At the bottom of each channel we list the mean ($\pm$ s. d.) orientation obtained from the DH-PSF method and the mean ($\pm$ s. d.) orientation obtained from the defocus/template matching method. Molecules 1–4 show excellent agreement between the two methods, with each DH-PSF measurement coming to within a few degrees of the defocus measurements. Molecules 5 and 6 show worse agreement. This can be justified by noting that for molecules 1–4 we have $\theta_{defocus} < 75^\circ$, while $\theta_{defocus} > 75^\circ$ for molecules 5 and 6. More inclined molecules (i.e. those for which $\theta_{defocus} > 75^\circ$) tend to exhibit LA values closer to zero, and thus the map to θ becomes ambiguous. However, this has minimal effect on the ultimate goal of correcting large mislocalizations since the same more inclined molecules also tend to exhibit less severe shifts, as demonstrated in the next section.

5.2 Estimation and correction of lateral mislocalization

Rows A and B of Figure 6 show the measured mislocalization vs. z curves of each of the six molecules examined in the last section. The solid overlaid curves are the mislocalizations predicted from simulation based on the mean orientation measurement for each molecule.

For each molecule, the channel with lowest localization precision is omitted from the analysis since the orientation-induced mislocalizations in these channels are obscured by noise resulting from a combination of low photon counts and high LA. It is important to note, however, that useful information was still gathered from these channels in the form of LA and LD measurements, which were in turn used to correct the mislocalizations in the channels which did not suffer from such low localization precision. Row C of Figure 6 gives the 2D histograms of the raw lateral localizations binned into 15-nm pixels (row (i)) and the corresponding corrected localizations (rows (ii) and (iii)). Row (ii) shows the corrected localizations based on individual estimations of (x, y) ; i.e. a separate (x, y) was estimated and subtracted for each snapshot of the molecule. Row (iii) shows corrected localizations based on an average estimated (x, y) ; i.e. the mean estimated (θ, φ) was used to produce an average (x, y) vector that was then subtracted from the apparent lateral positions. A molecule that suffers from a significant orientation-induced mislocalization appears as an elongated streak in the uncorrected histogram due to the fact that it appears to systematically shift laterally as it is moved in z . A successfully corrected histogram of such a molecule should be circularly symmetric and concentrated, with width solely dictated by shot noise-limited localization precision.

As can be predicted from the results described in the previous section, the corrected histograms of molecule 5 and 6 do not significantly improve the uncorrected histograms. However, in these cases the uncorrected histograms are relatively concentrated and do not exhibit significant smearing along any particular direction, corroborating the previous claim that these more inclined molecules tend not to require correction in the first place. This suggests that an efficient automated algorithm should not apply corrections to such more inclined molecules. By contrast, molecules 1–4 do show significant amelioration of mislocalizations. For example, the green channel of molecule 1 extends along the y direction by more than 200 nm in the uncorrected histogram. The individual correction is shown to give qualitative improvement and the mean correction gives even further improvement. This effect is quantified further for molecules 1 and 2 in Table 1, which gives the standard deviations along the labeled direction $\mathbf{a}$ corresponding to the predominant direction of apparent shift. These values are compared directly to the precision limit set by photon shot noise, below which one should not expect σ_a to dip. This precision limit was estimated by calculating standard deviations of lateral localizations within 100-nm z bins, over which apparent lateral shifts are negligible. The σ_a value of the green channel of molecule 1 highlighted above is shown to improve dramatically from 54 nm to 35 nm to 24 nm (within 6 nm of the precision). The gold channel of molecule 1 shows even more dramatic improvement in σ_a : from 116 nm ($>4\times$ the precision) to 55 nm to 34 nm (within 6 nm of the precision). The green and red channels of molecule 2 show similar improvements.

6. Conclusions

We have demonstrated the capability of the DH-PSF microscope to discern molecular orientation and correct the resulting lateral mislocalizations for single molecules fixed in orientation over a depth range of 2 μm . Our algorithm is particularly adept at estimating orientations of molecules with intermediate inclinations, for which mislocalizations are otherwise most severe. Because the DH-PSF microscope has been used successfully to

obtain 3D super-resolution images in biological samples, and because we rely on measuring LA and LD rather than fine details of the PSF, our method is a good candidate to be incorporated into SMACM experiments. While we here show corrections based on two sequential image acquisitions, the rate of toggling the phase mask orientations is limited only by the latency of the SLM. Typical SLMs can be toggled at rates better than 60 Hz. Furthermore, while we show two acquisitions to be sufficient to estimate orientation, it may not be necessary. In particular, a setup which uses separate SLMs for each polarization arm would likely be able to provide estimations based on a single acquisition. In order to make our method more directly applicable to biological imaging, we plan to modify the algorithm to allow for limited rotational mobility of fluorophores.

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Figure 1.

(left) Schematic of standard widefield microscope, imaging a single molecule whose dipole orientation is fixed. The molecule emits an anisotropic field (orange haze in object plane) such that its 3D PSF is tilted with respect to the optical axis (orange haze in image plane). The molecule is located below the object plane and so a lateral mislocalization is incurred (orange 'x'). (right) Coordinate definitions. θ is the polar angle relative to the optical axis and ϕ is the azimuthal angle about that axis. Adapted from Ref.,¹⁴ copyright American Chemical Society.

Figure 2.

Data for simulated DH-PSF images. Left side pertains to parallel polarization channel, right side to perpendicular polarization channel. (A-B) Simulated images for a single molecule with orientation $(\theta, \phi) = (47^\circ, -173^\circ)$ at various z . (C-D) Double-Gaussian fits to the images in A-B. (E-F) Calculated lobe asymmetry plotted over 2D-projected orientation space (see axes for coordinate definitions). The orientation corresponding to A–D is marked with $\diamond$. (G–H) Calculated apparent x mislocalization. (I–J) calculated apparent y mislocalization. (K) Perpendicular and parallel polarization channels are defined by the relative orientations of the polarization vectors (blue and red double-headed arrows) and the axis of the DH-PSF phase mask marked with the orange dashed line. Adapted from Ref.,¹⁹ copyright U. S. National Academy of Sciences.

Figure 3.

(A) Setup for experiment. Molecular coordinates which define orientation are marked throughout the setup as they are transformed by the various reflections. Polarization vectors are also marked. (B) Side-on view of transmitted polarization channel as it is reflected off of the square pyramidal mirror onto the SLM. (C) View from below facing the SLM shows how the polarization vector of each channel is projected onto the phase mask in both the perpendicular and parallel mask configuration. Adapted from Ref.,¹⁹ copyright U. S. National Academy of Sciences.

Figure 4. Sample of raw images of molecule 1 at various z positions in each of the four mask orientation/polarization channels (color code defined in text). Strong lobe asymmetry is especially noticeable in the red, gold, and blue channels. Scale bar $1\ \mu\text{m}$.

Figure 5.

Results of orientation estimation for molecules 1–6. Top two rows show measurements of LD and LA, with solid overlay corresponding to the simulated curves for the average estimated orientation. Histograms show distributions of estimated θ and ϕ values. Bottom row gives average estimated orientations ($\pm$ s. d.) given by the DH-PSF based method and those obtained through template matching to defocus images ($\pm$ s. d.). Adapted from Ref.¹⁹ copyright U. S. National Academy of Sciences.

Figure 6.

Results for lateral mislocalization and correction for molecules 1–6. (A–B) Measurements of x and y with solid overlay of simulated curves corresponding to average orientation estimations. (C) 2D histograms (bin size 15 nm) of lateral localizations in 3 channels for each molecule. Row (i) is the uncorrected localizations, row (ii) the corrected localizations based on individual estimations, and row (iii) the corrected localizations based on the average estimation. The directions of most prominent shift **a** are marked for molecules 1 and 2 (arrow length 100 nm). Adapted from Ref.¹⁹ copyright U. S. National Academy of Sciences.

Table 1

Standard deviations of localizations along directions of most prominent shift (σ_a) for molecules 1 and 2, and these values divided by the localization precisions in each channel. Adapted from Ref.,¹⁹ copyright U. S. National Academy of Sciences.

	<i>Green</i>		<i>Red</i>		<i>Gold</i>		<i>Blue</i>	
	σ_a (nm)	σ_a /Precision	σ_a (nm)	σ_a /Precision	σ_a (nm)	σ_a /Precision	σ_a (nm)	σ_a /Precision
<i>Molecule 1</i>								
Uncorrected	54	3	-	-	116	4.1	36	1.4
Individually	35	1.9	-	-	55	2	37	1.5
Corrected								
Mean Corrected	24	1.3	-	-	34	1.2	36	1.4
Precision	18	-	-	-	28	-	25	-
<i>Molecule 2</i>								
Uncorrected	48	1.9	35	2.1	26	0.96	-	-
Individually	30	1.2	21	1.2	28	1	-	-
Corrected								
Mean Corrected	28	1.1	18	1.1	27	1	-	-
Precision	25	-	17	-	27	-	-	-