A bisected pupil for studying single-molecule orientational dynamics and its application to three-dimensional super-resolution microscopy

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A phase mask design that we term a “bisected pupil” (BSP) provides several advantages for single-molecule optical imaging. When using the BSP with a dual-polarization optical Fourier processing system, both the position and dipole orientation of individual fluorescent molecules may be measured from a single camera image. In the context of single-molecule super-resolution microscopy, this technique permits one to diagnose, and subsequently to remove imaging artifacts resulting from orientation-induced localization errors. If the molecules labeling a structure are rotationally mobile, thus mitigating dipole orientation errors, this technique enables super-resolution imaging in three dimensions. We present simulations and experimental verification. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4876440>]

The field of fluorescence microscopy has made remarkable progress in recent years, benefitting from new techniques that resolve structures much smaller than the wavelength of light, thus achieving “super-resolution.”¹ One family of methods including [fluorescence] photoactivated localization microscopy ([F]PALM)^{2,3} and stochastic optical reconstruction microscopy (STORM)⁴ obtains resolution enhancement using the following procedure: (1) By optical or chemical means, the concentration of actively fluorescing molecules labeling a structure of interest is reduced to such an extent that their individual emission patterns, approximations of the microscope’s point-spread function (PSF), become distinguishable in the image plane. (2) Single molecules are then localized to within a few tens of nanometers by fitting a model function to their PSFs. (3) Steps 1 and 2 are repeated many times such that the labeled structure is fully sampled. (4) The underlying structure is then reconstructed by plotting the positions of all the molecules detected. In this Letter, we will refer to methods employing this strategy as “Single-Molecule Active Control Microscopy” (SMACM). When analyzing data from SMACM experiments, a Gaussian function is typically used to model the single-molecule PSF, and the positions of molecules are estimated to lie at the center of the fitted Gaussian. However, single-molecule fluorescence does not arise from a point emitter, but rather resembles the inherently asymmetric emission pattern of an oscillating electric dipole.⁵ This implies that systematic errors are incurred when estimating molecule positions with simple model functions that do not match experimental data.^{6,7} This problem is compounded by the effects of slight microscope defocus ($|\Delta z| \leq 250$ nm), which enhances the asymmetric features of the PSF, and may lead to x - y errors of up to ~ 200 nm.⁸ To address this source of error, one approach involves simultaneously estimating both the position and orientation of the dipole moment with respect to the microscope objective for every molecule in a SMACM experiment.⁹ In previous work, we demonstrated that the three-dimensional (3D) positions of molecules immobilized in a polymer could be accurately inferred by first

estimating their dipole orientation, then subtracting the respective systematic localization error using a lookup-table.¹⁰ However, it may not always be necessary to go to such lengths. In biological specimens, molecules labeling structures often undergo some degree of rotational motion if the tether is floppy. Our calculations¹¹ indicate that as a fluorophore’s rotational mobility increases, its PSF becomes more isotropic, thus reducing any localization error introduced by orientation.

Here, we apply the bisected pupil (BSP) phase mask to two distinct imaging regimes: First, in the case of rotationally immobilized fluorophores, we demonstrate that by modulating single-molecule PSFs in the Fourier plane with the BSP, acquired images can be fit with increased (x - y) accuracy for many orientations. For orientations that persist in causing pronounced localization error, we propose a simple strategy for identifying such molecules in a typical SMACM experiment and pruning these spurious localizations from a final super-resolved image. Second, by analyzing features of BSP-modulated single-molecule PSFs along with polarization data, it is possible to determine whether labels within a sample are sufficiently mobile such that orientational artifacts are mitigated. In an experiment imaging microtubules immunolabeled with Alexa-647 in fixed cells, we demonstrate that rotational mobility is indeed great enough to guarantee localization accuracy. Furthermore, since emitters are isotropic in this case, the BSP permits acquisition of 3D localization data.

Figure 1(a) depicts our experimental apparatus. Emitted fluorescence exiting the microscope and passing through the image plane a Fourier transforming lens and is then separated into two imaging channels termed T and R for light transmitted and reflected by a polarizing beamsplitter (containing p- and s-polarized emission, respectively). Figure 1(b) shows the coordinate system used for reporting position and orientation. Both polarization channels are relayed onto a liquid crystal spatial light modulator (SLM) programmed with the BSP phase mask (Figures 1(c) and 1(d)). Note that the use of a pyramidal mirror ensures that the polarization of

FIG. 1. (a) BSP Experimental setup. $E^{T,R}$ denotes transmitted and reflected portions of the electric field, with polarization directions indicated. (b) Coordinate system for reporting molecule position and orientation. (c) The BSP phase mask. Arrows depict direction of polarization with respect to mask. (d) Side-view illustrations of portion of experimental setup. (e) High-resolution simulation of isotropic emitter demonstrates depth-determination by lobe separation. (f) Sample raw data for single Alexa-647 molecules in BSC-1 cells.

both channels is identical with respect to the orientation of the phase mask. After phase modulation, both polarization channels are Fourier transformed by another lens and projected onto separate regions of an electron multiplying charge coupled device (EMCCD) image sensor. The phase modulation, $\psi(\xi, \eta)$, imparted by the bisected pupil consists of two linear phase ramps and may be expressed as

$$\psi(\xi, \eta) = C_0 - C(|\eta|), \quad (1)$$

where (ξ, η) are spatial coordinates at the Fourier plane, and C_0 and C are empirically adjustable constants. The purpose of this phase mask is to separate each object point into two “lobes” within each polarization channel of the image plane, each composed of light impinging upon the top or bottom half of the Fourier plane. If an emitter in the object plane is isotropic, the two lobes will have equal intensity. However, if the angular distribution of intensity varies, the two lobes will not be of equal brightness. Analyzing the brightness of each of the lobes in both of the two polarization channels allows one to determine the dipole orientation of a fixed single molecule. In previous work, we introduced a quadrated phase mask that separated intensity into four lobes as opposed to two,¹² permitting a higher precision orientation measurement. Our current approach facilitates collection of position data in addition to orientation. If an emitter is isotropic, its distance from the microscope’s focal plane, Δz , may also be gauged: If an emitter is closer to ($\Delta z < 0$) or further from ($\Delta z > 0$) the objective than the focal plane, the separation distance of the centers of the two lobes will shrink or grow respectively, permitting a depth estimate to be inferred (Figures 1(e) and 1(f)).

We use the following methodology to localize single molecules from BSP images: For a given polarization channel, we first use a template matching¹³ procedure to identify candidate molecules within a frame of raw data. Next, we fit a Gaussian function to each lobe in the PSF and calculate the x - y position of the molecule as the midpoint between the centers of the two lobes. We then infer z -position from the distance between the two lobe centers (a calibration z -scan using a fluorescent bead is a convenient means of generating a lookup-table relating lobe spacing to depth). We refer to this localization method as “double-Gaussian” fitting. For each

molecule, we also calculate two more parameters—the lobe asymmetry (LA)¹⁰ and linear dichroism (LD)¹⁴—defined as

$$LA_{T,R} = \frac{L_1 - L_2}{L_1 + L_2}, \quad LD = \frac{A_T - A_R}{A_T + A_R}, \quad (2)$$

where $A_{T,R}$ is the number of background-subtracted photons contained in each polarization channel attributed to a given molecule and $L_{1,2}$ is the number of photons contained in one lobe of the PSF in a given polarization channel (different lobe asymmetries may be calculated for the T and R polarization channels). Together, $LA_{T,R}$ and LD may be compared to simulated images to determine a molecule’s orientation. Additionally, by simulating a library of molecules at different orientations and Δz values, (see supplementary Figures S1–S3¹⁵), we observe that molecules with high LA are tilted significantly away from $\theta = 0^\circ$ or 90° and are therefore more susceptible to mislocalization on account of their asymmetrical PSF. From our simulations, a good rule of thumb is that a localization in a given polarization channel is inaccurate beyond the photon-limited precision if the LA exceeds 0.5. By rejecting images of molecules with an LA exceeding this threshold, we can “prune” spurious localizations and recover an underlying structure with better fidelity.

To demonstrate the utility of the “pruning” strategy, we generated simulated images (100×1.4 NA objective, matched imaging media $n = 1.518$) of single molecules labeling two parallel, infinitely thin sheets (Figure 2). The sheets were specified to be $10 \mu m$ long and to extend 500 nm in z . The focal plane of the simulated microscope was placed at the z -midpoint of the sheets such that their far edges were just at the ends of the depth of focus of the standard, clear-aperture (CA) microscope without a BSP phase mask. The sheets were labeled stochastically with a total of 90 000 molecules resulting in a mean density of ~ 0.02 molecules/ nm^2 . Each molecule was assigned a random, fixed orientation drawn uniformly from the unit sphere.

For our first simulation, we varied sheet separation distance and compared the resolving capabilities of the standard CA and pruned BSP imaging modalities. For sheet separations $d \in \{20, 25, 30, 35, 40, 45, 50, 55, 60\} \text{ nm}$, we mimicked a SMACM experiment by generating stacks of molecular images using vectorial optical calculations.^{16,17}

FIG. 2. Simulated super-resolution reconstructions of thin parallel sheets. (a) Resolution ratio χ as a function of separation of the sheets for both the BSP (red) and CA (black) reconstructions. The dashed blue line shows the value at which the sheets cease to be resolved. Inset: The 2D reconstruction for the 60-nm separation case for both the CA (top) and BSP (bottom). Scale bar: 100 nm, bin size: 10 nm $\times$ 10 nm. (b) χ as a function of varying number of maximum signal photons. (c) Projected 1D histogram of localizations from the simulation of thin sheets of molecules separated by 60 nm and 1000 signal photons. (d) Projected 1D histogram of localizations from the simulation involving a separation distance of 75 nm and 2500 signal photons. The histogram bin width is 2.5 nm.

One stack consisted of CA images and the other pair of stacks comprised the polarized BSP images. Each image frame contained exactly one emitting molecule in order to avoid overlapping PSFs—a complication which is otherwise present in any real SMACM experiment, but is tangent to our purposes here. We first fixed the maximum signal level at $S_{max} = 1000$ photons (corresponding to a molecule oriented perpendicular to the optical axis, with maximum pumping/collection efficiency) and background at $B = 0$ photons/pixel (relaxed below). For the BSP images, signal was split between the polarized images, in the required ratio determined from the orientation of the molecule. Excess noise was added to each image so as to mimic an EMCCD camera with high electron-multiplication (EM) gain. The resulting combination of EM noise and photon shot noise can be approximated by allowing the intensity in each pixel to be Gaussian-distributed with variance equal to twice its mean.¹⁸ Each CA image was fit with a Gaussian and each BSP image with a double-Gaussian. For each molecule, the localization corresponding to the polarized BSP image with lower intensity was discarded (a method which is photon-inefficient but results in a conservative comparison between BSP and CA imaging modalities). BSP localizations were further pruned by removing those with $|LA| > 0.5$. Resulting reconstructions for $d = 60$ nm are inset in Figure 2(a). A qualitative inspection shows that the pruned BSP method results in better-resolved structures.

To quantify resolution enhancement, we define the resolution ratio, χ , as follows: For a given simulated image, we histogram all localizations along the axis perpendicular to the

two planes of molecules. These histograms will feature two “peaks” at the locations of the two molecule planes, in addition to a central “valley” (examples in Figures 2(c) and 2(d)). We compute χ as the ratio of the height of the two peaks divided by the height of the valley. The plot of $\chi(d)$ in Figure 2(a) shows that the pruned BSP method yields superior resolution to CA imaging at all d . We did not estimate emitter depth in these simulations, since the lobe separation distance varies significantly as a function of orientation (see supplementary Figure S4). (In this case of rotationally fixed molecules, it is necessary to simultaneously estimate orientation using a maximum likelihood estimation (MLE) based fitting method if Δz must be determined.) To evaluate the BSP method when the signal-to-background ratio is low, we simulated parallel sheets with $d = 75$ nm, $B = 40$ photons/pixel on average, and varied S_{max} between 500 and 2500 photons. The resulting plot of $\chi(S_{max})$ (Figure 2(b)) shows that the BSP enhances resolving capability over the CA approach, so long as $S_{max} \geq 1000$ photons.

BSP imaging directly ameliorates mislocalizations for certain fixed molecular orientations. To demonstrate this feature experimentally, single dicyanomethylenedihydrofuran-N-6 (DCDHF-N-6) molecules¹⁹ were immobilized in a thin 1% polymethyl methacrylate (PMMA) film and excited with 514 nm light at ~ 0.1 kW/cm². The objective lens (100 $\times$ 1.4NA, Olympus) was translated in 50-nm steps over a $\Delta z = \pm 400$ nm range (Mad City Labs, C-Focus), with a 5-s exposure at high EM gain (Andor iXon) at each Δz -step. For all experiments, a 512 $\times$ 512 pixel Boulder Nonlinear xy

FIG. 3. (a) Estimated x - y positions of an immobilized DCDHF-N-6 molecule as a function of defocus (both experimental and simulated data shown). Orientation estimate also noted. (b) Comparison of experimentally acquired image and simulation at $\Delta z = 300$ nm. This molecule emitted an average of 7886 and 5057 photons per frame in the T- and R-channels, respectively. For this molecule: $\{LA_T = -0.32; LA_R = 0.36; LD = 0.22\}$.

FIG. 4. (a) 3D-super-resolved image of microtubules in a BSC-1 cell using the BSP. (i) Comparison of boxed region with a conventional diffraction-limited image. (ii) Lateral and axial resolving capability demonstrated by binning localizations along representative 1- μm segments perpendicular to the microtubule axis and parallel to the z-axis. (iii) 2D super-resolved images are color-coded by (T-channel) LA and LD—insignificant heterogeneity is evident. (b) LA and LD histograms for entire field of view.

phase series SLM with 92% reflectivity was used. The pyramidal mirror used to relay fluorescence to and from the SLM was custom fabricated from off-the-shelf aluminum mirrors (metal mirrors were used in order to better preserve polarization). Imaging was performed with the SLM enabled and repeated for the same field of view with the SLM off (clear aperture). Figure 3(a) plots the drift-corrected¹⁰ x - y positions of a sample molecule inferred from CA and BSP images as a function of Δz . By least-squares fitting of the total number of photons contained in each lobe of the bisected pupil image at $\Delta z = 0$ nm for simulations of a dipole at an air-glass interface, we estimated the molecule's orientation. For comparison, the expected x - y shift introduced by orientation was calculated from a set of simulated images. Sample simulated and experimentally acquired images are shown in Figure 3(b). These plots demonstrate that for molecules moderately inclined towards the optical axis, a considerably reduced x - y shift error is observed with BSP imaging.

Finally, we demonstrated super-resolution imaging in three dimensions. Microtubules immunolabeled with Alexa Fluor 647 (Invitrogen) were imaged in fixed BSC-1 cells^{20,21}

in a blinking buffer, with 641 nm excitation at $\sim 10\text{ kW/cm}^2$. A mean of ~ 2000 photons per molecule in each polarization channel was detected. Since the BSP imaging scheme increases the overall size of the PSF by roughly a factor of two, the overall density of fluorophores that may be actively emitting in a single frame of data must be decreased accordingly to prevent PSF overlap. More sophisticated multi-emitter image fitting schemes^{22–24} may be explored in the future in order to handle higher emitter densities. The LD of each detected molecule was computed, along with the T-channel LA. Supplementary Figure S5 contains information regarding calibration of our depth estimation method and localization precision data. Our image processing pipeline is detailed in supplementary Table ST1. Figure 4(a) depicts a super-resolution BSP 3D reconstruction, generated by binning localizations into 25-nm pixels, and color-coding by depth. This image shows localizations from the T-channel of the detector. In Figure 4(b), histograms of the LA and LD of all molecules detected in the T-channel are plotted, demonstrating that $|LA| \ll 0.5$ for the vast majority of molecules. Therefore, few localizations are likely to be skewed by orientation in this sample. Fig. 4(a-iii) depicts a super-resolved image, color coded according to the LA and LD of the individual molecules detected, confirming low-magnitude measurements for both of these parameters. We thus conclude that rotational mobility is sufficient to ensure localization accuracy for these antibodies. See supplementary Figure S6 for a super-resolved reconstruction of the entire field of view used to generate LA/LD histograms.

While our analysis of LA and LD statistics confirmed that orientation caused minimal degradation of localization accuracy for a specific SMACM application, we discourage drawing conclusions about other samples and fluorescent labels. For example, researchers have reported significant anisotropies in actin specimens.^{25,26} The bisected pupil will thus serve as a useful tool for future super-resolution imaging.

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