Removing orientation-induced localization biases in single-molecule microscopy using a broadband metasurface mask

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Nanoscale localization of single molecules is a crucial function in several advanced microscopy techniques, including single-molecule tracking and wide-field super-resolution imaging1. Until now, a central consideration of such techniques is how to optimize the precision of molecular localization. However, as these methods continue to push towards the nanometre size scale, an increasingly important concern is the localization accuracy. In particular, single fluorescent molecules emit with an anisotropic radiation pattern of an oscillating electric dipole, which can cause significant localization biases using common estimators2-5. Here we present the theory and experimental demonstration of a solution to this problem based on azimuthal filtering in the Fourier plane of the microscope. We do so using a high-efficiency dielectric metasurface polarization/phase device composed of nanoposts with subwavelength spacing6. The method is demonstrated both on fluorophores embedded in a polymer matrix and in dLS protein complexes that bind malachite green6,8.

High-precision (~10 nm) molecular localization typically relies on estimators that assume isotropy of the collected fluorescence about the true position of the emitter, such as fitting the image to a two-dimensional (2D) Gaussian function1. However, molecules radiate via their transition electric dipole moments (μ) and so produce the characteristic anisotropic radiation distribution of an oscillating electric dipole5. Thus, the shape of the image that appears in a microscope is highly dependent on the orientation of the molecule relative to the microscope optics10. Figure 1a shows a simplified microscope schematic. The dipole emitter’s orientation is defined by two angles (Fig. 1a): the polar angle θ2, made between μ and z (the optical axis), and the azimuthal angle φ2 about z. Throughout this letter we consider the distribution of light in both the microscope’s image and the Fourier plane, the latter of which is located at the back focal plane (BFP) of the objective. The anisotropic intensity distribution at the BFP for an example dipole with orientation (θ2 = 45°, φ2 = 0°) is shown in Fig. 1b. This asymmetric illumination of the BFP gives rise to an asymmetric image (Fig. 1g). The centroid of the image and the true underlying molecular position in Fig. 1g are offset by 70 nm, which reveals the degree of rotational freedom depends on the labelling scheme and warrants careful characterization. In addition, the rotational freedom may be purposely restricted for certain applications, such as biological motor tracking3,14.

For the case of a rotationally fixed dipole emitter, one approach is to use a sophisticated image model that takes orientation into account15,16, or to estimate directly the bias and correct it computationally11,17. Such techniques have been shown to work in principle, but they are limited by the need to model the imaging system accurately and are computationally expensive or require a relatively high signal-to-noise ratio. A more direct removal of the bias is desirable.

A recent theoretical study found that an azimuthal polarization filter located at the BFP would provide such a bias removal18 (the details are given in the Supplementary Information). Radially polarized light contains contributions from a basis dipole oriented along z, as well as ones along x and y. This superposition induces the asymmetry. By contrast, the azimuthally polarized portion contains only contributions from the x and y basis dipoles. The (inversion-) symmetric intensity distribution of the azimuthally polarized component of the light for the same example orientation is shown in Fig. 1c. The corresponding point spread function (‘phi-PSF’) is also symmetric, and the localization bias is completely expunged (Fig. 1h). The rejected radially polarized light carries the entirety of the asymmetry that produces the bias (Fig. 1d,i).

We report the first (to our knowledge) experimental demonstration of single-molecule localization bias removal via azimuthal filtering. Segmented polarizers are often used in laser science to produce azimuthally polarized beams19, but the inherent macroscopic approximation of the filter pattern is not ideal for photon-limited single-molecule imaging. Other more-sophisticated schemes (for example, using two spatial light modulators and appropriate wave plates20) could work, but generally require additional optics to compensate for an incurred spiral-phase modulation21. Instead, we designed and employed a mask based on a novel type of high-efficiency dielectric metasurface that provides complete control of phase/polarization with subwavelength spatial resolution6. The metasurface consists of an array of amorphous silicon (a-Si) nanoposts with elliptical cross-sections arranged on a hexagonal lattice of subwavelength period (Fig. 2a,b). The nanoposts act as short waveguides with elliptical cross-section and a strong birefringence. They behave as uncoupled scatterers and can locally modify both the polarization and phase of transmitted light to any desired form4.

For this application, a specific nanopost array was designed to rotate the local polarization of the light at the BFP such that radially (ρ see coordinate definitions in Fig. 1b)) polarized light is converted into x, and azimuthally (φ) polarized light is converted into –y.

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(Fig. 2c) with a high experimental efficiency of 86.5% (see the Supplementary Information). We refer to the mask as the ‘y-phi’ (as in ‘WiFi’) mask. To remove \( \rho \)-polarized light effectively, we can place the y-phi mask at the BFP followed by a y-oriented linear polarizer (LP). In the Fourier plane this produces light resembling that shown in Fig. 1e—the intensity distribution is the same as in Fig. 1c, but the polarization is rotated. The resulting y-phi-PSF (Fig. 1j) is symmetric, but inequivalent to the phi-PSF (Fig. 1h). Instead, the additional polarization rotation renders the light concentrated into two bright lobes, with a line of symmetry.
and LP in place. Numbers above and below the grey bars indicate the maximum number of localizations per bin in 200 nm (bin size, 20 nm), as obtained with the standard PSF (that is, y-phi mask and LP removed).

When \( \theta \) images. As expected, the standard PSF shows large position biases that result from simple Gaussian-based estimators of the \( \phi \)-, y-phi and standard PSFs. Figure 1l analogously depicts the localization histograms obtained with the y-phi mask and polarizer in place. The bias removal results in localization clusters that are dramatically more symmetric and concentrated. Figure 4d depicts the distance between the apparent lateral positions at either end of the depth range for both PSFs. We see improvement by as large as a factor of \( \sim 7 \) (from \( 80 \pm 2 \) nm to \( 11 \pm 4 \) nm) such that the bias disappears into the photon-limited localization precision.

As a final biological demonstration of our method we imaged dL5, a fluorogen-activating protein that consists of a tandem dimer of antibody variable light chain domains that binds malachite green (MG) (Fig. 5a inset)\(^{7,8} \). This protein is a recently developed genetically directed label for use in cells. As the fluorogen is bound strongly, the orientation of the emission dipole is restrained relative to the protein, and thus would benefit from azimuthal filtering in localization microscopy. The dL5 complexes were cast on a poly-L-lysine layer on a glass coverslip and the same basic imaging procedure was repeated. Figure 5a shows the successful removal of localization bias in the lateral direction \( w \) along the estimated in-plane orientation for two such dL5 complexes (raw images shown in Fig. 5b).

Figure 3 | Experimental set-up. The sample consists of dyes spun in a layer of PMMA (inset). The fluorescence was collected with the OL, and filtered through a dichroic mirror (DM) and band-pass (BP) filter. Within the body of the commercial inverted microscope the TL focuses the collected light onto the intermediate image plane (IIP). Between the TL and the IIP there is a reflecting glass prism (GP). A 4f optical processing unit that consists of two lenses (L1, L2) was built outside the microscope. The y-phi mask was placed at the conjugate Fourier plane. A linear polarizer (LP) was placed immediately after the y-phi mask to pass only y polarized light. A phase-compensating element (PCE) had to be placed before the y-phi mask to compensate for the reflection from the GP and birefringence of the DM (see the Supplementary Information). Images were recorded on an EMCCD camera.

The response of the y-phi-PSF as a function of \( (\theta_D, \phi_D, z_D) \), where \( z_D \) is the \( z \) position of the dipole emitter, is shown in Supplementary Fig. 1. The y-phi mask acts correspondingly on radially polarized light (Fig. 1f.k).

We simulated noisy images to compare the behaviour of the phi-, y-phi and standard PSFs. Figure 11-o depicts the localization biases that result from simple Gaussian-based estimators of the images. As expected, the standard PSF shows large position biases when \( \theta_D < 90^\circ \) and \( |z_D| > 0 \), which result in an apparent lateral shift of the molecule as \( z_D \) is varied—the phi-PSF and y-phi-PSF both completely remove this bias.

Although the phi-PSF removes localization bias, it worsens the localization precision relative to that of the standard PSF, largely because the azimuthal polarization filter reduces the number of detected signal photons\(^{18} \) (Supplementary Fig. 2). Surprisingly, both simulated Gaussian fitting and the computed Cramer–Rao lower bound (CRLB)\(^{21,22} \) show that the y-phi-PSF recovers much of this precision, even though the number of detected photons is the same between the phi-PSF and y-phi-PSF (Supplementary Fig. 3).

Evidently, splitting the light into two lobes (Fig. 1j) is more than offset by the suppression of the side lobes of the phi-PSF (Fig. 1h). Furthermore, the lobes of the y-phi-PSF rotate as a function of \( \phi_D \) to yield precise estimations of \( \phi_D \) (Supplementary Fig. 3).

Figure 3 sketches the set-up used for the experimental demonstration. Our first sample consisted of DCDHFA-6 fluorophores\(^{23} \) immobilized in a thin layer of poly(methylmethacrylate) (PMMA) polymer (Fig. 3 inset). Each field-of-view (FOV) was imaged continuously as the objective was scanned in \( z \) from \(-200 \) to \( 200 \) nm. Figure 4a shows in-focus y-phi-PSF images of ten example molecules. The various orientations of the y-phi-PSF indicate the different underlying \( \phi_D \) values. Each molecule was also imaged with the y-phi mask and polarizer removed: Fig. 4b shows 2D histograms of the lateral positions estimated using the standard PSF as the objective was scanned in \( z \). Owing to the \( z \)-dependent localization bias, each of these molecules appears to shift in the direction of the estimated \( \phi_D \), to yield severely elongated localization clusters.

Figure 4 shows similar results for 15 additional example molecules.

Figure 4 | Experimental results. a. Experimental images of ten in-focus example molecules with different orientations, as obtained using the y-phi mask (pixel size, 160 nm). Images are averages of ten 300 ms exposures. b. 2D histograms of \( (x, y) \) localizations of each molecule over \( z \) scan from \(-200 \) to \( 200 \) nm (bin size, 20 nm), as obtained with the standard PSF (that is, y-phi mask and LP removed). c. Localization histograms obtained with the y-phi mask and LP in place. Numbers above and below the grey bars indicate the maximum number of localizations per bin in b and c, respectively. Mean \( \phi_D \) is estimated from the y-phi-PSF marked in b by magenta bars. d. Mean distance between \( (x, y) \) localizations determined at each edge of the \( z \) scan, from the first scan of each molecule. Black, standard PSF; blue, y-phi-PSF. Filled portions give the uncertainty range (± s.e.m.).
We experimentally demonstrated an all-optical direct removal of the orientation-induced lateral mislocalization of single molecules based on an azimuthal polarization filter placed in the microscope’s Fourier plane. The y-phi mask is compatible with typical wide-field microscopes because the parts can be added to the exterior. In many biological samples, fluorophore labels display intermediate rotational mobility. This is compatible with our scheme because the method ensures an inversion-symmetric image for all degrees of floppiness. Only the estimator needs to be adjusted. We reserve a more thorough investigation of rotational mobility for future work.

One constraint is that silicon absorbs towards the visible range, requiring redder fluorophores. Ongoing work should lift this limitation. We hope this work inspires future collaborations between the metamaterials and single-molecule communities because, although Fourier-plane engineering with more conventional optics has already been proved extremely valuable in single-molecule imaging, the exquisite control provided by metasurfaces will lead to even more dramatic advances in the future.

Note added in proof: A commercial retarder implementing a vortex half-wave plate functionality has recently appeared (Thorlabs).

Methods
Methods and any associated references are available in the online version of the paper.

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Author contributions
M.P.B. and W.E.M. conceived and designed the experiments. M.P.B. did the simulations and CRLB calculations. A.A., E.A. and A.F. designed, fabricated and characterized the mask. M.P.B. and P.N.P. performed experiments and analysed the data. S.S. prepared the dLS samples. All the authors contributed to writing the paper.

Additional information
Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence should be directed to W.E.M. for questions regarding the single-molecule experiments and to A.F. for questions regarding the metasurface mask.

Competing financial interests
The authors declare no competing financial interests.

Figure 5 Biological demonstration. a. Dipole-induced lateral shift of dL5 complexes (blue) and (red) along the direction of estimated in-plane orientation, \( \mathbf{w} = \mathbf{w}_x \cos \phi_0 + \mathbf{w}_y \sin \phi_0 \). Estimates of \( \phi_0 \) were 34 ± 4° and 9 ± 2° (± s.d.) for dL5 complexes 1 and 2, respectively. Solid lines show the shift with standard PSF and dashed lines show the results from y-phi-PSF. Data depict the average of two scans for each dL5 complex. (Inset) Structure of the dLS fluorophore-activating protein shows the tandem dimer variable light chain domains (purple) and bound MG (green). b. Y-phi-PSF images of dL5 complexes (top) and (bottom).
Methods

Simulations. Simulated distributions of light at the BFP were produced by propagating dipole emission fields using the appropriate Green’s tensor.24,25 The mathematical formalism, including symmetry considerations for the current application, is provided in the Supplementary Information. We assumed a numerical aperture of 1.4 and emission wavelength of 686 nm, consistent with the peak emission of DCDHF-A-6.24 Figure 1 and Supplementary Fig. 3 assume the indices of refraction of both the sample and imaging medium are matched to that of immersion oil (n = 1.518). In addition, Supplementary Fig. 5 considers an emitter placed in water 1 µm above a water–glass interface (for example, as in a cell-imaging measurement), whereas Supplementary Fig. 6 corresponds to the molecule embedded at the top of a 1.3 µm layer of immersion oil, as in our first demonstration. To simulate the action of the y-phi mask we effectively multiplied the field computed at the BFP by the spatially varying Jones matrix \( J = \begin{pmatrix} \cos \varphi & \sin \varphi \\ -\sin \varphi & \cos \varphi \end{pmatrix} \).

Simulated images were produced by taking the appropriately scaled Fourier transform of the field at the BFP (details are given in the Supplementary Information) with \( x \times 100 \) magnification. High-resolution images (20 nm sampling) were first produced and then binned into pixels of side length 160 nm to match the conditions of our imaging set-up. For simulated data we realized 10^6 noisy images for each data point. We assumed Poisson noise with the photon numbers and background as stated in Supplementary Fig. 3. Mean signal photons were scaled as necessary for polarization, relative detection efficiency, and bandwidth. Using this approach, optimum values of 365 nm and 325 nm were computed for the uniform array shown in Supplementary Fig. 7a using a Gaussian function centred at 686 nm and with 60 nm full-width at half-maximum.

CRLB calculations. CRLB data are presented in Supplementary Figs 3, 5 and 6. To compute the CRLB of \( \sigma_e \) and \( \sigma_\varphi \), we assumed Poisson noise and constructed the Fisher Information (FI) matrix as outlined in previous work.22 The square roots of the diagonal elements of the FI matrix give the CRLB for each parameter. To compute \( \sigma_e \), we constructed a 2 \times 2 FI matrix with parameters (x, y); to compute \( \sigma_\varphi \), the FI matrix was 3 \times 3 with parameters (x, y, \varphi). This distinction was made because we noted that \( \sigma_\varphi \) actually worsens when \( \varphi_0 \) is included because off-diagonal elements appear in the FI matrix, which represents a propensity to confuse the estimated parameters. We chose to report the CRLB of \( \sigma_e \) in this way because typical position estimators of the standard PSF do not fit \( \varphi_0 \).

Mask design and fabrication. Post height, lattice constant and ellipse diameters were used as design parameters for the optimization of the metasurface y-phi mask shown in Fig. 2a. For each set of the design parameters, transmission coefficients \( t_x \) and \( t_\varphi \) were computed for the uniform array shown in Supplementary Fig. 7a using the rigorous coupled-wave analysis technique with a freely available software package.26 These transmission coefficients were computed at eight wavelengths, 650 to 720 nm, in steps of 10 nm. Wavefront dependence of the measured index and extinction coefficient of the Si was considered in these simulations. The a-Si refractive index and extinction coefficient values used in the simulations were measured using a variable-angle spectroscopic ellipsometer, and are shown in Supplementary Fig. 7b. For each design, the x- and y-convolution conversion efficiency \( (\eta) \) was obtained at each wavelength using the simulated transmission coefficients \( t_x \) and \( t_\varphi \). A weighted average of the convolution-efﬁciency values was used to compare different metasurfaces with different design parameters. The conversion efﬁciency values were weighted by the relative power density emitted from the ﬂuorescent dye at each wavelength. The emission spectrum of DCDHF-A-6 was approximated by a Gaussian function centred at 686 nm and with 60 nm full-width at half-maximum bandwidth. Using this approach, optimum values of 365 nm and 325 nm were found for the post height and lattice constant, respectively. Supplementary Fig. 7c shows the average conversion efﬁciency values for an array with the optimum lattice constant and post height as a function of the elliptical post diameters. Diameters of \( D_x = 180 \) nm and \( D_y = 110 \) nm (indicated by the black dot in Supplementary Fig. 7c) were selected to reduce the sensitivity to fabrication-induced errors in the diameters of the elliptical posts, and to achieve an average conversion efﬁciency of 88%. The simulated conversion efficiency of the optimized metasurface mask as a function of wavelength is presented in Supplementary Fig. 7d.

A metasurface y-phi mask with a diameter of 4.2 mm was fabricated on a 500 µm thick fused silica substrate using standard nanofabrication techniques. First, a 365 nm thick layer of hydrogenated a-Si was deposited on the substrate using a 5% mixture of silane in argon at 200 °C with the plasma-enhanced chemical vapour deposition technique. Then, a ~300 nm thick positive electron beam resist (ZEP 520-A, Zeon Chemicals) was spin-coated on the a-Si layer and baked at 180 °C for three minutes. To avoid static charging effects during the electron-beam lithography, a ~60 nm thick layer of a water-soluble charge-dissipative conductive polymer (AquaSAVE, Mitsubishi Rayon) was spin-coated on the resist and baked at 70 °C for five minutes. After writing the nanopost pattern on the resist using electron-beam lithography, the charge-dissipative polymer was dissolved in water, and the resist was developed by immersing the sample in a solvent (ZED-N50, Zeon Chemicals) for three minutes followed by a 30 s second rinse in methyl-1,4-dibutyl ketone. The pattern was subsequently transferred through a lift-off process to a 70 nm thick layer of aluminum oxide, which was evaporated on the resist after its development. The patterned aluminum oxide layer was used as a hard mask for inductively coupled plasma reactive ion etching of the a-Si layer in a mixture of SF₆ and C₄F₆ gases. Finally, the aluminum oxide mask was removed in a 1:1 mixture of ammonium hydroxide and hydrogen peroxide at 80 °C. Scanning electron microscope images of the top and tilted views of the final device are shown in Fig. 2b.

Additional characterization of the y-phi mask is described in the Supplementary Information and Supplementary Fig. 8.

Experimental. DCDHF-A-6 was dissolved in 1% PMMA in toluene to a final concentration of ~2 nM. The solution was spin-coated on ozone-etched glass coverslips, then samples were mounted on an inverted Olympus IX71 microscope and pumped with a 561 nm laser (CrystaLaser) in the epi-illumination mode (peak intensity ~125–400 W cm⁻²) after reflection from a 561 nm dichroic beam splitter (Semrock). Fluorescence was collected through a 470/40 filter (Chroma Technology). The 4f system of Fig. 3 consists of two achromatic doublet lenses (Edmund Optics, 50 mm diameter × 150 mm focal length, VIS-NIR Coated). The LP (Newport 20LP-VIDS-B) was placed just after the y-phi mask. A broadband UL2 wave plate (B. Halle 508–990 nm achromatic version 1) was placed just before the mask to act as a phase-compensating element (PCE (Fig. 3)) to offset partially the phase incurred from reflection off the glass prism (GP) within the Olympus IX71 plus the birefringence of the dichroic (see Supplementary Information). Fluorescence was recorded on an EMCCD (electron-multiplying charge-coupled device) camera (Andor iXon+) that operated with an EM gain of either 100 or 300, at 3.33 Hz frame rate. Each FOV was imaged with the y-phi mask and LP in place as the objective was scanned in z using a piezo (Mad City Labs C-Focus) from ~300 to ~300 nm (only the central 400 nm was analysed) in 100 nm steps, with ten camera acquisitions per step. The LP and y-phi mask were then removed and a z scan was taken of the FOV in a clear aperture. Finally, a set of defocused (~1 µm towards the air) images was recorded (Supplementary Fig. 9) to check for consistency in the lobe orientation. The whole procedure was repeated five more times per FOV. Data were analysed using custom MATLAB software. Molecules were hand selected. The background was estimated from a nearby region of the image and subtracted. The mean signal and background levels for the molecules depicted in Fig. 4 are given in Table 1. A small amount of asymmetry was noted in the intensity of the lobes of the y-phi mask, which may be a result of small residual aberrations (for example, coma) in the imaging system.27 Supplementary Fig. 10 and accompanying text), or from imperfections in the mask itself. Future applications may require further correction of these residual aberrations.

To visualize single dL5-MG complexes, we prepared the recombinant dL5 protein using a PET21A expression vector in Rosetta gamI DE3 cells (Novagen). Protein expression and purification were performed as in a previously reported protocol.28 This protocol stock was diluted larger in (pH 7.4) and supplemented with MG ester such that the final concentrations of dL5 and MG in the solution were 10 nM and 100 pM, respectively. An aliquot of this solution (50 µl) was incubated on etched glass coverslips (FisherFinest, No. 1.5, 22 × 22 mm) that were coated with 0.01% (w/v) poly-L-lysine. After 15 minutes of incubation, the solution was washed twice using PBS and dried for two hours at room temperature.

Imaging experiments with the dL5 samples were conducted in the same manner as for DCDHF-A-6, with minor differences. The spectrum of the labelled protein required a 647 nm laser pump (Coherent OBIIS), which required a different dichroic beam splitter (Semrock D0101/R405/488/561/635). This dichroic induced a different relative phase lag between the x and y polarizations, so to compensate a second long-pass dichroic (Semrock FF650-D010) was added to the emission path in addition to the λ/2 wave plate from the DCDHF-A-6 measurements. The fluorescence passed through a 650 long-pass filter (Omega Optical) and a 700/75 band-pass filter (Chroma Technology). Signals were recorded at 150 nm intervals. The background was computed using MATLAB software. Images were acquired in the epi-illumination mode (peak intensity ~125–400 W cm⁻²) after reflection from a 561 nm dichroic beam splitter (Semrock). Fluorescence was collected through a 470/40 filter (Chroma Technology). The lobe asymmetry was larger in these experiments than in those described in the Supplementary Information and Supplementary Fig. 8.

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