Over the last decades, super-resolution techniques have revolutionized the field of fluorescence microscopy. Among them, interferometric or 4Pi microscopy methods exhibit supreme resolving power in the axial dimension. Combined with single-molecule detection/localization and adaptive optics, current 4Pi microscopy methods enabled 10–15 nm isotropic 3D resolution throughout whole cells. However, further improving the achieved 3D resolution poses challenges arising from the complexity of single-molecule emission patterns generated by these coherent single-molecule imaging systems. These complex emission patterns render a large portion of information carrying photons unusable. Here, we introduce a localization algorithm that achieves the theoretical precision limit for a 4Pi based single-molecule switching nanoscopy (4Pi-SMSN) system, and demonstrate improvements in localization precision, accuracy as well as stability comparing with state-of-the-art 4Pi-SMSN methods.
The achievable resolution of a far-field fluorescence microscope was constrained by the diffraction limit of light, approximately 200–300 nm in the lateral direction and 500–700 nm in the axial direction. Over the last decades, substantial efforts were made to overcome this resolution limit. Based on confocal and widefield microscopy geometries respectively, 4Pi (type A-C)\(^1\)–\(^3\) and IM4\(^6\) methods use coherent illumination and/or detection based on two opposing objectives to improve the resolution by 3–7 fold in the axial dimension\(^7\). Combining coherent detection and the stochastic switching of single molecules with high-precision localization, 4Pi (or interferometric) based single-molecule switching nanoscopy (SMSN) techniques, such as iPALM\(^8\),\(^9\) and 4Pi-SMSN\(^10\), allow another 5–10 fold improvement in the axial resolution\(^8\),\(^11\) in comparison with conventional 3D SMSN. Further incorporating adaptive optics and interferometry-specific algorithm design, W-4PiSMSN\(^12\) allowed high-resolution reconstruction of the whole mammalian cell (up to \(9\mu m\)) without deterioration of resolution throughout the imaging depth.

The resolution enhancing capacity of the interferometric systems comes from their complex point spread function (PSF) patterns, thereafter, referred as 4PiPSF. One of its distinct features is the rapid intensity modulation within the pattern center along the depth (axial) direction which, in turn, improves the axial resolution when combined with confocal, stimulated emission depletion microscopy and single-molecule localization microscopy methods. Aside from the center peak, the complexities of 4PiPSF majorly arise from the features such as rings and lobes at its periphery. Depending on the axial position of the single emitter, these features contain up to 80% of the entire 4PiPSF energy (Methods). Theoretical precision calculations based on the Fisher information theory suggested that better localization precision can be obtained in both axial and lateral directions\(^11\),\(^13\) when compared with other incoherent dual-objective systems\(^14\).

To date, these complex features remain largely unexplored when localizing single-molecule emission patterns from 4Pi systems, due to the challenge in modeling this complex pattern with high accuracy. An accurate 4PiPSF model must be able to take into account of the static imperfections of the interferometric single-molecule imaging system, such as aberrations and transmission variances in both interferometric arms, partial coherence due to the broadband emission spectra and the dynamic changes of the system, such as the temperature-dependent changes of the interferometric cavity length.

To overcome those difficulties, we introduce a method based on coherent pupil functions to allow accurate modeling of 4PiPSFs and develop an localization algorithm to extract the position information content at the theoretical information limit while, at the same time, dynamically compensates the temperature-induced cavity drift. The confluence of these new analytical methods improves both localization precision and bias in all three dimensions compared with existing 4Pi-SMSN systems\(^10\),\(^12\) and at the same time extends imaging volume in the axial direction.

**Results**

**4PiPSF modeling with coherent phase-retrieved pupils.** The hallmark of a 4PiPSF generated by an interferometric microscopy system is the distinct multi-lobe PSF in both axial and lateral dimensions. Currently, extracting single-molecule axial positions from the center lobes (including center peak intensity and intensities falls within a empirically defined ring region) has been the major focus in 4Pi single-molecule localization analysis\(^8\),\(^10\),\(^12\), discarding or underutilizing substantial amount of information-carrying photons among the lateral side lobes. At the same time, when pin-pointing molecular centers at the lateral dimension (i.e., localization in \(x\), \(y\)) the interferometric features of the 4PiPSFs have been, to a large extent, ignored\(^8\),\(^12\). As a result, no lateral resolution enhancement is reported comparing with conventional PSFs\(^8\),\(^12\).

To allow extracting information contained within the complex interferometric PSF, we describe here a PSF model based on two coherent pupil functions for 4Pi single-molecule imaging systems\(^10\),\(^12\). This method allowed us to create a realistic model of the interferometric emission patterns taking into account of the independent wave front distortions from the two interference arms, the wavelength-dependent coherence modulation and the spontaneous interferometric path length drift.

Let \(h_x\) and \(h_y\) be a pair of pupil functions of the 4Pi system, representing the wave fields at the pupil planes of the upper and lower interference paths respectively (Fig. 1). The two pupil functions, retrieved independently through a phase retrieval (PR)
algorithm\textsuperscript{15-17}, allowed us to incorporate aberrations for both interference paths. Interferometric PSFs (termed as 4PiPSF) are generated from the superposition of the wave fields, \( h_A \) and \( h_B \). Four superposed wave fields with different polarizations and phase shifts can be written as,

\[
\begin{align*}
    h_{s1} &= I_A h_A D(-z) e^{i\phi} + h_B D(z) e^{i (\phi_0 + \phi)}, \\
    h_{s2} &= I_A h_A D(-z) + h_B D(z) e^{i (\phi_0 + \phi)}, \\
    h_{p1} &= I_A h_A D(-z) + h_B D(z) e^{i \phi}, \\
    h_{p2} &= I_A h_A D(-z) + h_B D(z) e^{i \phi_0}.
\end{align*}
\]

Here, we assume \( h_A \) and \( h_B \) are independent against polarization directions, where \( \phi_0 = 2 \pi \delta L (n_1 - n_2) / \lambda \), with \( n_1 \) and \( n_2 \) the refractive indices of s- and p-polarized light in quartz, and \( \delta L \) the quartz thickness difference between the two interference paths (Supplementary Note 1). \( \phi_0 \) is the phase difference between the two interference arms when the single emitter is in the common focus of the two objective lenses. A factor \( I \) the Fourier transform of each superposed wave.

We can then write our the coherent PSF, \( \mu_{lm} \), in terms of Fourier transform of each superposed wave field, \( \mu_{lm} (x,y,z) = |\mathcal{F}[h_{s1}(k_x, k_y)]|^2 \), \( m \in \{S1, S2, P1, P2\} \),

\[
\mu_{lm} (x,y,z) = \left| F[h_A(k_x, k_y)D(-z)] + F[h_B(k_x, k_y)D(z)] \right|^2,
\]

with subscript W indicates wide-field in the sense of conventional microscope. Therefore, the final PSF is a combination of the interferometric PSF and the conventional PSF with an empirical factor \( a \in [0,1] \), describing the coherence strength (Supplementary Note 2 and Supplementary Fig. 2),

\[
\mu_{m0}(x,y,z) = a \mu_{lm}(x,y,z) + (1-a) \mu_{lw}(x,y,z),
\]

where \( \mu_B \) represents the normalized PSF (sum of the PSF intensities in s- or p-polarization equals to 1). By adding a total photon count of \( I \) and a background of \( b \), the PR-4PiPSF model is,

\[
\mu_m(x,y,z) = I_m \mu_{m0}(x,y,z) + b_m,
\]

which includes quadruple PSFs for each emitter (Fig. 1). Here all \( I_m \) and \( b_m \) were considered as independent to account for the difference in transmission efficiency between the two polarizations and the emission paths after the beam splitter\textsuperscript{12}. We found that PR-4PiPSFs can produce relatively uniform resolutions within a large \( a \) range (0.5–1), an attractive feature for thicker specimens, where \( a \) is depth dependent (Supplementary Fig. 2 and Supplementary Note 2).

As a demonstration of the accuracy of the PR-4PiPSF model, we tested it with the experimental PSFs obtained by imaging 40 nm beads attached on the coverslip surface. We pin-pointed 3D positions of isolated beads with various photon counts and compared with the position readout from a high-precision piezo stage (20.5 nm close-loop) (Fig. 2, Supplementary Figs. 3–5). We found that comparing with ideal-4PiPSF model (where \( h_A \) and \( h_B \) are unaberrated pupil functions, with \( I \) and \( a \) equal to 1, Supplementary Fig. 3), PR-4PiPSF model based localization results in comparable localization accuracy (bias) in the lateral dimension and improved localization accuracy in the axial dimension (Supplementary Fig. 4). When comparing with contrast-based method (referring to the method using incoherent PSF models for lateral localization and PSF’s central moment for axial localization\textsuperscript{12}), both ideal- and PR-4PiPSF models show improved localization accuracies in both lateral and axial directions (Supplementary Fig. 4). We also notice that the localization deviations (bias) caused by contrast-based method are bead specific (Fig. 2, Supplementary Figs. 4–5), all bead data were collected from the same sample), especially in the lateral dimension, making it difficult to perform post acquisition correction due to the random nature of such deviations.

**Information content within a 4PiPSF.** Given the ability to generate a realistic 4PiPSF model, we are able to quantitatively investigate the information content within the 4PiPSF pattern recorded on our microscope using Fisher information matrix\textsuperscript{18,19}. By quantifying information content by pixel within the 4PiPSF (as similarly shown previously\textsuperscript{11} for ideal PSFs), we found previous 4Pi-SMSN localization analysis either discarded the additional information from interferometric detection (as for lateral localization, Supplementary Note 3 includes derivation of a general case for such information gain) or incorporated only part of it (as for the axial localization that uses 6th and/or 3rd central moments of the PSFs, Supplementary Figs. 6–7). Incorporating a comprehensive and accurate PSF model allows us to further improve localization accuracy and precision in three dimensions.

To quantify the information content of 4PiPSFs, we calculated the Fisher information matrix, which quantifies the lower bound of the localization precision for an unbiased estimator: the Cramér–Rao lower bound (CRLB)\textsuperscript{19},

\[
\text{var}(\theta) \geq [F(\theta)^{-1}]_{ii},
\]

where \( F \) is the Fisher information matrix, \( \theta \) is a vector of estimation parameters, \( i \) denotes the index of each parameter.

Incorporating Poisson noise and the readout noise from an sCMOS camera, a numerical calculation of each element in Fisher information matrix is\textsuperscript{20},

\[
F_{ij} = \sum_m \sum_q \frac{1}{\mu_{mq} + \gamma_{mq}} \frac{\Delta \mu_{mq} \Delta \mu_{mq}}{\Delta \theta_m \Delta \theta_q} (\theta_1, \theta_2) \in (x,y,z,I_m,b_m),
\]

where \( q \) is the pixel index and \( m \) is the index of the quadruple 4PiPSFs, the factory equals to \( \sigma^2 / g^2 \), where \( \sigma^2 \) is the variance of the readout noise per pixel and \( g \) is the gain of each pixel. In comparison with incoherent dual-objective PSFs\textsuperscript{14}, we observed the predicted information in a 4PiPSF increase significantly along the axial dimension (17–160 folds at 1000 total emitted photon and 10 background photons) and a relatively
large information gain at the out-of-focus regions along the lateral dimension (5–10 folds at 1000 total emitted photon and 10 background photon) (Supplementary Figs. 8–9). Comparing to an ideal-4PiPSF (Supplementary Fig. 3), the information contained in a realistic PSF model as PR-4PiPSF, is slightly lower (Supplementary Fig. 9).

This observed information-content boost predicts and quantifies the improvement in the achievable localization precisions in all three dimensions by using 4PiPSFs. Existing 4Pi localization methods obtain the lateral position estimation by either merging all three dimensions by using 4PiPSFs. Existing 4Pi localization methods obtain the lateral position estimation by either merging all three dimensions by using 4PiPSFs. Existing 4Pi localization methods obtain the lateral position estimation by either merging all three dimensions by using 4PiPSFs.

Fig. 2 Comparison of localization results of bead data using PR-4Pi and contrast methods. a Examples of experimental bead images with relatively low detected photons at various axial positions in b, each contains PSF patterns from four channels (P1, S2, P2, and S1). The pixel values represent the number of photon-electrons corrupted with sCMOS readout noise. b Axial localization results of the corresponding bead data in a. Notice that in spite of the low photon budget, PR-4Pi localization method achieves a precision ~5 nm. The estimation results fluctuated around the mean (solid black line) with a standard deviation of σ. c Localization deviations in x, y, and z, mean (n = 39). d Localization precisions in x, y, and z, s.d. (n = 39). e Scatter plots of lateral localization results from different bead data. The PR-4Pi method shows obvious improvement in both localization accuracy and precision. Contrast method in this work refers to the algorithm developed in ref. 12 that extracts the axial positions using the Gaussian-weighted 0th central moment (M0, Supplementary Note 4 and Supplementary Table 1). Scale bar: 1 μm in a. Sample: 40 nm dark red bead, photon: median total photon per objective (n = 819), bg: median background photon per pixel (n = 819). Photon and background were estimated from PR-4Pi method.

reduction of information since the interference pattern is not fully considered leading to up to twofold worse localization precisions when compared with the information limit, of which the deterioration is increasingly pronounced when emitters are located away from the common focus of the two objective lenses (Supplementary Figs. 6–7). As for axial localization, previously, position estimations were extracted from intensity contrast calculated from PSFs at different detection channels and such calculation often involves summing over all or partial pixel values (e.g., using the central moments, Supplementary Table 1) within the PSF of each channel. We found that these methods perform well within a small distance (±300 nm) from the common focus region but result in partial information loss, where the achieved precision (uncertainty value) and bias of these methods increase.
rapidly with the axial position of a single emitter (Supplementary Figs. 6–7).

In order to utilize the complete information contained within the interferometric PSF pattern and minimize localization biases, we combined our coherent pupil based 4PiPSFs model with a maximum likelihood estimator with the appropriate noise model10 (sCMOS noise model in our case) for three dimensional localization of 4PiPSF patterns (termed as PR-4Pi algorithm, Supplementary Notes 4–5, Supplementary Table 2). We demonstrated in the following sections that the PR-4Pi algorithm achieve the theoretical precision limit (defined by CRLB) of our 4Pi-SMSN system in both axial and lateral dimensions.

As a demonstration of PR-4Pi localization algorithm, first we tested the algorithm through a set of simulated 4PiPSFs. While the localization precisions using conventional intensity-contrast based methods varies drastically in different axial position, we found PR-4Pi algorithm provides localization precisions consistently approaching the theoretical information limit calculated by CRLB. A detailed characterization of PR-4Pi algorithm and other existing 4Pi localization algorithms are shown in Supplementary Fig. 6 and Supplementary Table 1. For fair comparison with existing 4Pi algorithms based on Gaussian mask for lateral and central moments for axial localizations10, we generate 4PiPSFs without astigmatism modification (Supplementary Figs. 6, 8, 10; astigmatism modification9,12 were used to reduce localization artifacts). We found that for lateral localization, Gaussian mask method performs worse than using incoherent PSFs (contrast-based method, Supplementary Fig. 6). And for axial localization, combining 0th and 3rd central moments10 achieves better precision at out-of-focus region than using only the 0th moment (contrast-based method), but still fail to achieve the theoretical limit (CRLB) when considering equal split of photon energy through the four detected channels (five estimation parameters include: x, y, z, I, and b, Supplementary Fig. 6 and Supplementary Note 4). Interestingly, when considering independent intensity and background for each channel (11 estimation parameters), the CRLB for axial estimation increases, which results from an anticorrelation between estimations on intensity/background and axial positions with astigmatism modification (Supplementary Note 4 and Supplementary Fig. 7). Nonetheless, in both cases, PR-4Pi algorithm achieves CRLB within the entire tested range (see Supplementary Note 6, Supplementary Figs. 11–15 for robustness test of PR-4Pi algorithm on various conditions).

Further, we tested localizations on 40-nm bead data with a photon range of 470 to 5700 per objective lens and a background range of 0–4 photons per pixel (see Supplementary Fig. 16 for theoretical precisions at various photon and background levels). Being consistent with our simulated results, PR-4Pi pin-points the 3D position of a single bead with improved precision (1–3.5 fold within 2-µm axial range, quantified from localization results of 17 bead datasets) and bias than contrast-based method (Supplementary Figs. 17–18). We found the bias caused by inaccurate PSF model is rather random between different beads instead of consistent, and therefore impedes the possibility of calibrate a systematic bias for post-analysis correction (Fig. 2E and Supplementary Figs. 4–5). Agreeing with simulation results, PR-4Pi with 11 estimation parameters (independent background and intensity estimations for each quadrant, Supplementary Note 4) worsens the axial localization precision at near focus region, while, PR-4Pi with five estimation parameters (single background and single intensity estimations for all quadrants, Supplementary Note 4) worsens the axial localization bias at out-of-focus region (Supplementary Figs. 17–18). Therefore, we propose to use PR-4Pi with five parameters when the axial localization range is less than 1.2 µm.

Continuous cavity phase measurement. Another key feature in 4Pi/interferometric systems is the cavity phase drift. The cavity phase describes the OPD between the two interference arms when the emitter is in focus at the common focal plane of the two objectives. The cavity phase, φ0, modulates with path length fluctuations and the changes of refractive indices of materials which photons travel through along the interference arms (~600 nm for each arm in our system). In turn, cavity phase drift changes 4PiPSF from one pattern to another rapidly. We found the fluctuation of the room temperature (RT) lead to obvious drift in φ0 (Supplementary Note 7 and Supplementary Fig. 19). The cavity phase drift (i.e., φ0 drift) is different from sample drift. It is a unique challenge for interferometric systems. In comparison with commonly observed sample axial drift which would make in-focused object out of focus, the cavity phase drift simply shifts the energy distribution between the four detected quadrants while an in-focus emitter remains in-focus. Here we examine the properties of cavity phase and propose a cavity-phase drift-correction method.

The cavity phase drift, left uncorrected, degrades the axial resolution achievable in a 4Pi systems and creates image artifacts in the final reconstruction. We found a 0.04 rad drift per second will cause noticeable axial-resolution deterioration in 10 s (equivalent to ~18 nm axial drift, Supplementary Fig. 20), mandating a correction per 10 s to avoid resolution deterioration. This is especially challenging for the previous W-4PiSMSN method as the number of emitters collected within 10 s is not sufficient to correctly perform, ridge-finding, phase unwrapping and subsequent drift correction12. In contrast, pupil-based method allows bypassing these problems by first, no longer relying on phase unwrapping to recover axial positions and second, automatically compensating the cavity phase drift during the regression step (Supplementary Note 4 and Supplementary Figs. 1, 21).

We are able to obtain reliable calibration of φ0 from each 10-s data batch, so that a unique PR-4PiPSF model for a specific segment of time was generated to account for the φ0 drift. This constricted the axial-resolution deterioration within 5 nm on average (Supplementary Fig. 20). In short, within a short segment of blinking dataset, total (or interference) phase φ and shape metric σφ of each emitter were measured. Assuming linear dependences of φ with the axial position of the molecule as well as the shape metric σφ within a 2π range, the cavity phase φ0 was estimated as the averaged interference phase φ at the common focus plane of the two objectives. For single-section imaging, assuming a smooth change of the cavity phase during imaging, the estimated φ0 over time were interpolated with a smooth spline to eliminate abrupt transitions generated by estimation uncertainties and the interpolated values were incorporated into the PSF model during the regression step (Supplementary Note 4 and Supplementary Fig. 1).

For data acquired at multiple optical sections for large volumetric 3D imaging, the scanning of the specimen to image different optical sections in the axial direction also changes the cavity phase. Assuming the scanning step size d is a constant during the data acquisition, this additional phase can be calculated from,

$$\phi_d = \frac{4\pi n_{\text{imm}} d N_{\text{step}}}{\lambda_0} \left(1 - \frac{n_{\text{med}}^2}{n_{\text{imm}}^2}\right),$$  

where λ0 is the emission wavelength in air, and N_{step} is the step number, which is indexed from 0. We would like to note that when the refractive index of the immersion media n_{imm} matches the one of the sample media n_{med}, sample scanning in axial direction no longer causes cavity phase change. Equation 8 allows...
us to quantitatively connect the cavity phases obtained at different optical sections and therefore, results in a continuous phase curve which improves the robustness of cavity phase calibration (Supplementary Fig. 1). Together, we are able to calibrate cavity phase based on short single-molecule blinking dataset (~10 s) with high consistency across multiple optical sections throughout a cell. We found that for both single-section and multi-section imaging the calibrated curves match well with the estimated \( \phi_0 \), and are robust to the estimation noise (Supplementary Note 4 and Supplementary Fig. 1).

We tested the reliability of our cavity phase measurement by localizing bead images at various \( z \) positions (Supplementary Fig. 22). An incorrect cavity phase can result in nearly 50% ghost images and when using an ideal-4PiPSF model (Supplementary Fig. 3), in spite of cavity phase values, the smallest ghost-image percentage is nearly 20% of the entire localizations. In contrast, we found that using a correctly retrieved cavity phase together with PR-4Pi algorithm, the percentage of ghost images is limited to 5–10% for both bead and cell imaging. For imaging in biological specimens, additional ghost reduction procedures further reduce it to ~2% (Supplementary Note 4, Supplementary Fig. 1 and Supplementary Table 1).

**Imaging of various cellular structures.** Next, we applied the developed PR-4Pi localization algorithm to resolve a variety of cellular structures and in vitro specimens using fluorescent labels with different photon budgets. For single optical-section data, a total of 40–360 distinct PR-4PiPSFs, each representing the concurrent state during a 10 s imaging period, were generated for localization analysis (Supplementary Fig. 1). For multi-section volumetric 4Pi-SMSN imaging, 120–320 distinct PR-4PiPSFs (10 s per PSF) were used to obtain 3–4 optical volumes spaced 500–800 nm apart (Supplementary Fig. 1). These time dependent 4PiPSFs allow capturing the dynamic states of the interferometric system with high accuracy, and therefore pin-pointing single-molecule interferometric patterns with high precision and fidelity.

We imaged immune-labeled Nup98 protein at the top nucleus envelope in COS-7 cells. From profiles of multiple nucleus pore complexes (NPC), we found that the PR-4Pi improves the sharpness of resolved structure in both lateral and axial directions (Fig. 3). We observe this improvement is more obvious with increased axial distance from the focal plane (e.g., localizations with blue color, Fig. 3) especially along the lateral dimension. This improved precision as well as its dependence on the axial position of a single molecule is in agreement with our simulation results and experimental results based on beads (Fig. 2, Supplementary Figs. 4–7). Similarly, the super-resolution reconstruction of immune-labeled TOM20 protein in COS-7 cells also shows improved lateral sharpness of resolved clusters using PR-4Pi (example profiles and quantifications are shown in Fig. 4). In comparison, contrast-based method shows increased blurriness in one of the lateral dimensions both above and below the focal plane (\( z = 0 \) nm), which is caused by the lateral stretching of the incoherent astigmatism PSFs at out-of-focus regions (validations of drift-correction performance is shown in Supplementary Fig. 23). Importantly, PR-4Pi also results in higher number of localizations at the out-of-focus region because of its realistic modeling of the 4Pi emission patterns (~80% more localizations for Nup98 within the axial range of −700 to −300 nm, blue color in Fig. 3).

To evaluate the performance of PR-4Pi for low photon budget emitters such as photo-switchable/convertible proteins, we imaged purified mEos3.2 proteins on coverslips (Supplementary Fig. 24). The median photon count of localized mEos3.2 emission events is 358 ± 140 (median ± s.t.d.) per objective with a median background count of 1.6 ± 0.5 photon (median ± s.t.d.) per pixel. At such low photon condition, we found that even though most localized emitters are in focus, PR-4Pi still shows improved localization precision in both axial and lateral dimensions (10% \((n = 28)\) and 22% \((n = 40)\), Supplementary Fig. 25, narrower profiles in lateral and axial direction, examples are shown in Supplementary Fig. 24). This suggests PR-4Pi is robust for imaging single-molecule probes with low photon budget.

To provide quantitative measurements on these resolved volumes, we measured the full width at half maximum (FWHM) from Gaussian fit of 30 to 70 profiles of isolated clusters from each of the reconstructed images (Supplementary Fig. 25). We found that PR-4Pi localization of mEos3.2 on cover glass approaches the equivalent resolution predicted from the CRLB outputs of localized emission events. Assuming that each cluster represents a single mEos3.2 molecule with a diameter of ~5 nm, well below the CRLB predicted resolution of PR-4Pi results (xy: 27.7 ± 5 nm, z: 22.4 ± 5.6 nm, median ± s.d., \( n = 88056 \) (number of localizations)), the measured FWHM values (xy: 29.7 ± 3 nm \((n = 28)\), z: 24.7 ± 2.2 nm \((n = 40)\), median ± s.d.) of 4Pi-PR localization of mEos3.2 suggest that the achieved 3D resolution is mainly limited by the low photon count emitted by the fluorescent protein. For PR-4Pi reconstructions of TOM20 and Nup98, we found the measured FWHMs are much larger than the predicted resolutions (FWHMs) from CRLB (Supplementary Fig. 25). This discrepancy can be caused by two potential reasons: each cluster may contain multiple single molecules and the linker size using primary and secondary antibodies is larger than the achievable resolution. As for the TOM20 reconstruction, assuming a linker size \(^{22} \) of ~17.5 nm, we found that the FWHMs (xy: 17.1 ± 3.3 nm \((n = 58)\), z: 16.1 ± 2.5 nm \((n = 66)\), median ± s.d.) of individual clusters from PR-4Pi are close to the combined antibody size, suggesting that the 3D resolution of PR-4Pi achieved resolving TOM20 clusters is mainly limited by the size of labeling antibodies. We further quantified the resolution of PR-4Pi reconstructed images using the recently published method based on decorrelation analysis \(^{23} \) (Supplementary Fig. 26). The resolution is calculated by \( 2 \times \text{SMSN pixel size/maximum frequency} \) (where SMSN pixel size is the pixel size of the SMSN reconstructed images. We found that the resolution obtained from decorrelation analysis is slightly smaller than the FWHMs from CRLB (Supplementary Fig. 25), which may be caused by the difference in resolution definitions between the two methods. For all specimens quantified with decorrelation analysis, axial cross sections achieved 3–5 nm smaller resolution than that of the lateral cross sections (Supplementary Fig. 26).

**Discussion**

In this work, we developed a coherent, phase-retrieved pupil based single-molecule localization algorithm for 4Pi-SMSN systems. We demonstrated PR-4PiPSF allows us to accurately model the complex single-molecule emission patterns generated from 4Pi interferometric single-molecule imaging systems and subsequently allows us to fully extract the information carried by the detected photons. Comparing with existing 4Pi single-molecule localization algorithms, we demonstrated that PR-4Pi allows further improvement on both precision and bias in all three dimensions. We demonstrated the performance of PR-4Pi in a variety of cellular structures and in vitro specimens by resolving mitochondria networks and nuclear pore complexes in mammalian cells and individual fluorescent proteins in vitro.

The demonstrated ability to fully extract molecular position information generated by 4Pi single-molecule systems could potentially enable high-precision localization with low photon budget (150–600 photons per emission per objective), a typical...
condition for live cell single-molecule experiments with fluorescent proteins and high speed acquisition. In synergy with the rapid development of novel probes, sample preparation techniques, labeling methods and PSF engineering methods, we hope this approach allows efficient use of detected photons to overcome the practical barriers of achievable resolution in super-resolution experiments in both fixed and living specimens.
Fig. 4 SMSN reconstruction of immune-labeled TOM20 protein in COS-7 cells. **a** Lateral view of SMSN reconstruction from PR-4Pi method. **b, c** 200-nm thick x–z and x–y slices of one mitochondria in **a** (white box), reconstructed from PR-4Pi and contrast methods. **d, e** Selected single clusters in the x–y plane and x–z plane and their profiles along the central line, obtained by averaging the pixel values within the white box along the orthogonal dimension of the central line. Each profile was fitted by a 1D Gaussian with a width of $\sigma$, and the FWHM was calculated from $2.355\sigma$. The sample was imaged at a single-optical section with 180,000 frames under epi illumination. Scale bars: 2 $\mu$m in **a**, 200 nm in **b–d**, 50 nm in **e, f**.
Sample preparation. Before single-molecule imaging, round coverslip (25-mm diameter) was incubated with 200 µL bead dilution, containing 40 nm dark red bead (F8799, Invitrogen) with blocking buffer (3% bovine serum albumin (BSA, 001-000-162, Jackson ImmunoResearch) and 0.2% Triton X-100 in PBS, at RT for 30 min, gently rocking at RT. After blocking, cells were incubated with anti-NUP98 primary antibody (2598, Cell Signaling Technology), diluted to 1:200 in 1% BSA and 0.2% Triton X-100 in PBS, at RT for 4–5 h. After incubation with secondary antibody, cells were washed three times each time for 10–15 min with wash buffer. And then cells were post-fixed with 4% PFA in PBS for 10 min. After post-fixation, cells were rinsed three times with PBS and stored in PBS at 4 °C until they were imaged.

Immunoﬂuorescence labeling of Nup98. COS-7 cell were seeded on 25-mm diameter coverslips 1–2 days before immunoﬂuorescence labeling. Cells were first rinsed one time with PBS and then fixed for 10 min at room temperature with 4% PFA in PBS. Cells were then incubated for 15 min at RT with pre-warmed (at 37 °C) 3% paraformaldehyde (PFA, 15710, Electron Microscopy Sciences, Hatﬁeld, PA) and 0.5% glutaraldehyde (GA, Electron Microscopy Sciences, 16019, Hatﬁeld, PA) in PBS. Cells were then washed twice with PBS and treated for 10 min in freshly prepared ﬂuorescence quenching buffer (sodium borohydride (NaBH₄, 452882-25 G, Sigma-Aldrich) in PBS). After ﬂuorescence quenching, cells were washed three times with PBS and treated for 10 min with 10 mM Tris (pH 7.3, JT4109-02, VWR). Cells were then rinsed three times with PBS and permeabilized with blocking buffer (3% bovine serum albumin (BSA, 001-000-162, Jackson ImmunoResearch) and 0.2% Triton X-100 (X100, Sigma-Aldrich) in PBS) for 30 min, gently rocking at RT. After blocking, cells were incubated with anti-NUP98 primary antibody (sc-11415, Santa Cruz Biotechnology), diluted to 1:500 in 1% BSA and 0.2% Triton X-100 in PBS, at 4 °C for overnight. Cells were then washed three times each time for 10–15 min with wash buffer (0.1% Tween 20 and 0.2% Triton X-100) and incubated with secondary antibody conjugated with Alexa Fluor 647 (A21245, Life Technologies, Grand Island, NY), diluted to 1:500 in 1% BSA and 0.2% Triton X-100 in PBS, at RT for 4–5 h. After incubation with secondary antibody, cells were washed three times each time for 10–15 min with wash buffer. And then cells were post-fixed with 4% PFA in PBS for 10 min. After post-fixation, cells were rinsed three times with PBS and stored in PBS at 4 °C until they were imaged.
Data availability
Data underlying the plots in Figs. 2b–e, 3d,e and Fig. 4e, f are available as Excel files in Supplementary Data and via Figshare at https://doi.org/10.6084/m9.figshare.11987448.v1. Example test data for using PR-4Pi algorithm are also available in the Supplementary Software. Other data that support the findings of this study are available from the corresponding author upon request.

Code availability
The analyzing algorithms for 4Pi-SMSN data are implemented as a MATLAB class, named SR4pi_demo, which provides localization methods using PR-4Pi algorithm and contrast algorithm. An example script on using SR4pi_demo class is available in Supplementary Software and further updates will be made available at https://github.com/HuanglabPurdue/PR-4Pi.

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Author contributions
S.L. and F.H. developed the algorithm. S.L. performed the experiments and analyzed the data. S.L. and F.H. constructed the imaging systems. S.L. and F.H. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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