

Six-dimensional single-molecule imaging with isotropic resolution using a multi-view reflector microscope

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Code and data underlying this study are openly available from OSF at osf.io/s8m4x/ and by request.

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1 Dimensions and alignment of the pyramid mirrors

The detailed dimensions of the custom pyramidal mirrors and their mounts are shown in [Extended Data Figure 1](#). The air ([Extended Data Figure 1b,c](#)) and glass ([Extended Data Figure 1e](#)) pyramids were obtained from AVR Optics (Rochester, New York). The air pyramid is glued onto a custom stainless steel mount ([Extended Data Figure 1d](#)) using optical cement (Thorlabs NOA63). Using ray simulations, we designed the mirror dimensions to guarantee that no rays are clipped originating from the IIP (Figure S1, diameter of 4.7 mm equivalent to $48.3\text{ }\mu\text{m}$ in sample space) with angles corresponding to a pupil diameter of 4.7 mm (demagnified from 5.5 mm within the objective lens). Visualizations of the MVR mirrors are shown in Figure S1.

To place the pyramidal mirrors in the correct position in the imaging system, we first coalign them on a translation stage (Figure S1) using a laser. The mirrors are positioned and tilted so that we observe 4 symmetric laser spots after each reflection. The assembled mirror pair is then placed near the BFP of the second 4f system. We construct an imaging path to switch between observing the radially and azimuthally polarized BFPs versus the IIPs using folding mirrors (FMs, [Extended Data Figure 3](#)) and an additional mirror (M3, [Extended Data Figure 3](#)). We adjust the position of the pyramidal mirror pair so that we observe sharp edges of the glass pyramid and sharp edges of the pupil aperture simultaneously,

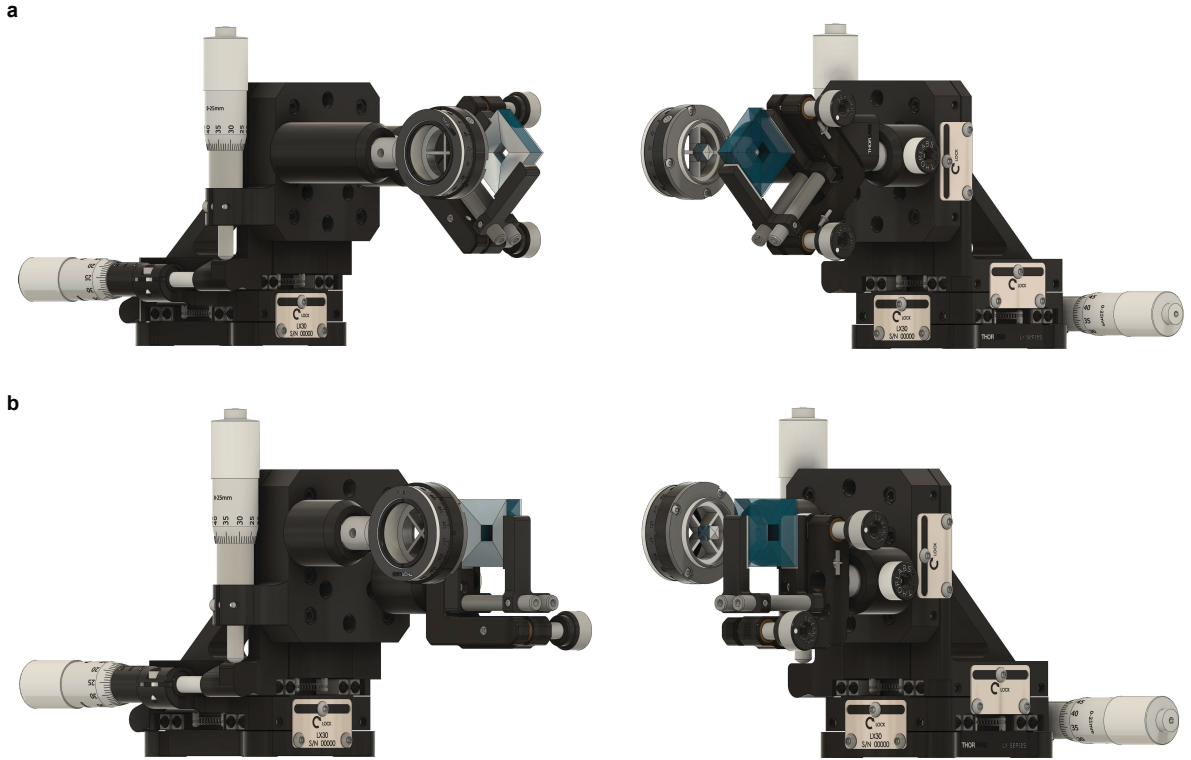


Figure S1: Assembled pyramidal mirrors on translational stages in the (a) radially and (b) azimuthally polarized imaging channels.

as well as four equally-sized partitions [[Extended Data Figure 3b\(i,ii\)](#)]. Note that depending on the illumination pattern and polarization, as well as the composition of the sample, the brightness of the fluorescence collected from each partition could be non-uniform.

2 Forward imaging model and detection and estimation performance

2.1 Forward imaging model

We first define the orientational second moments¹⁻⁴ of a molecule's transition dipole moment $\boldsymbol{\mu}(t) = [\mu_x(t), \mu_y(t), \mu_z(t)]^\top$ at time t as

$$m_{ij} = \int_0^t \mu_i(t) \mu_j(t) dt, \quad i, j \in \{x, y, z\} \quad (\text{S1})$$

where T is the camera integration time. We assume that the fluorescent molecule of interest exhibits uniform rotational diffusion within a symmetric cone, parameterized by an average orientation $\boldsymbol{\mu} = [\mu_x, \mu_y, \mu_z]^\top$ and “wobble” solid angle Ω such that⁵

$$\begin{aligned} m_{ii} &= \gamma \mu_i + \frac{1 - \gamma}{3} & i \in \{x, y, z\} \\ m_{ij} &= \gamma \mu_i \mu_j & i, j \in \{x, y, z\}, i \neq j, \text{ and} \\ \gamma &= 1 - \frac{3\Omega}{4\pi} + \frac{\Omega^2}{8\pi^2}. \end{aligned} \quad (\text{S2})$$

To compute the dipole spread function (DSF) at the image plane for a molecule located at $[x, y, z]$, we first write the optical field at the BFP,

$$\mathbf{E}_{\text{BFP}}(\xi, \eta) = \begin{bmatrix} E_{\text{BFP}}^{(r)}(\xi, \eta) \\ E_{\text{BFP}}^{(a)}(\xi, \eta) \end{bmatrix} = \begin{bmatrix} G_x^{(r)}(\xi, \eta) & G_y^{(r)}(\xi, \eta) & G_z^{(r)}(\xi, \eta) \\ G_x^{(a)}(\xi, \eta) & G_y^{(a)}(\xi, \eta) & G_z^{(a)}(\xi, \eta) \end{bmatrix} \boldsymbol{\mu}, \quad (\text{S3})$$

where (ξ, η) represents the BFP coordinates. The radially $G_i^{(r)}(\xi, \eta)$ and azimuthally $G_i^{(a)}(\xi, \eta)$ polarized basis fields are given by

$$\begin{aligned} \begin{bmatrix} G_i^{(r)}(\xi, \eta) \\ G_i^{(a)}(\xi, \eta) \end{bmatrix} &= \begin{bmatrix} \cos \phi_{\text{BFP}}(\xi, \eta) & \sin \phi_{\text{BFP}}(\xi, \eta) \\ -\sin \phi_{\text{BFP}}(\xi, \eta) & \cos \phi_{\text{BFP}}(\xi, \eta) \end{bmatrix} \begin{bmatrix} G_{i,0}^{(x)}(\xi, \eta) \\ G_{i,0}^{(y)}(\xi, \eta) \end{bmatrix} \\ &\exp \left\{ j \frac{2\pi n_1}{\lambda} \left[x\xi + y\eta - z_f \sqrt{1 - \xi^2 - \eta^2} + z \sqrt{\left(\frac{n_2}{n_1} \right)^2 - \xi^2 - \eta^2} \right] \right\}, \quad i \in \{x, y, z\}, \end{aligned} \quad (\text{S4})$$

where $\phi_{\text{BFP}}(\xi, \eta) = \arctan 2(\eta, \xi)$ (two-argument arctangent) is the polar coordinate at the BFP, z_f represents the position of the nominal focal plane above the RI interface, and $G_{i,0}^{(x)}(\xi, \eta)$ and $G_{i,0}^{(y)}(\xi, \eta)$ represent the x and y -polarized basis fields for $[x, y, z] = [0, 0, 0]$ and $z_f = 0$, respectively.^{3,4,6-13} This vectorial model accounts for the exact dipole emission and wave propagation in the imaging system,

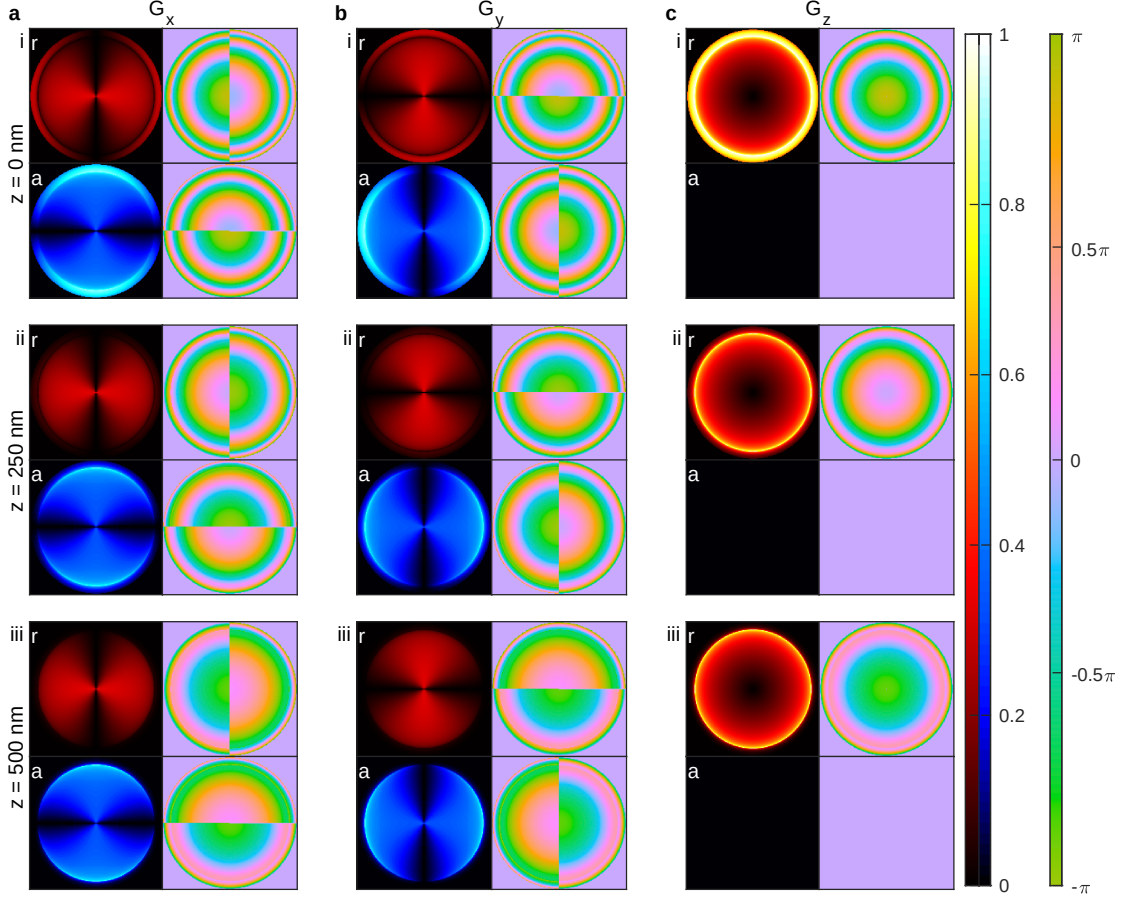


Figure S2: Radially and azimuthally polarized basis fields (a) G_x , (b) G_y , and (c) G_z at the back focal plane for molecules located at (i) $z = 0$ nm, (ii) $z = 250$ nm, and (iii) $z = 500$ nm with nominal focal plane position $z_f = 1200$ nm. Red: radial polarization; blue: azimuthal polarization. Colorbar: normalized amplitude (left) and phase (right).

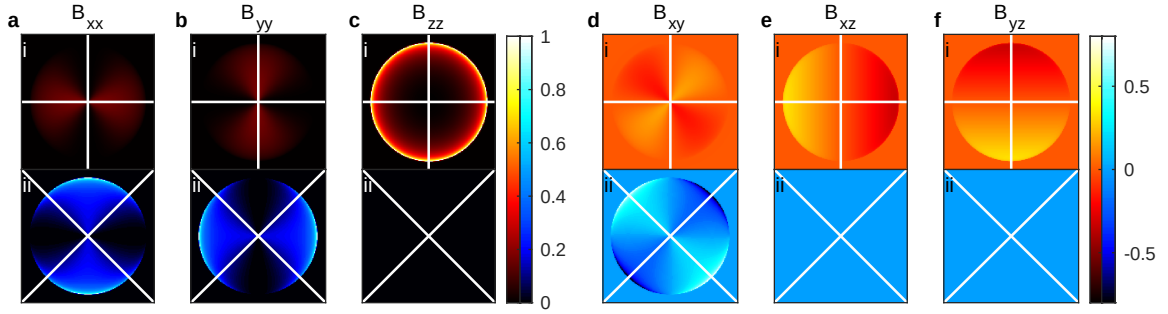


Figure S3: Radially and azimuthally polarized basis images (a) B_{xx} , (b) B_{yy} , (c) B_{zz} , (d) B_{xy} , (e) B_{xz} , and (f) B_{yz} at the back focal plane for molecules located at $z = 750$ nm with nominal focal plane position $z_f = 1200$ nm. (i) Red: radial polarization; (ii) blue: azimuthal polarization. Colorbar: normalized intensity.

including the refractive index mismatch interface between the sample and the lens immersion medium; more supercritical fluorescence is collected [Figure S2(i)] for molecules located closer to the coverslip ($z = 0$).

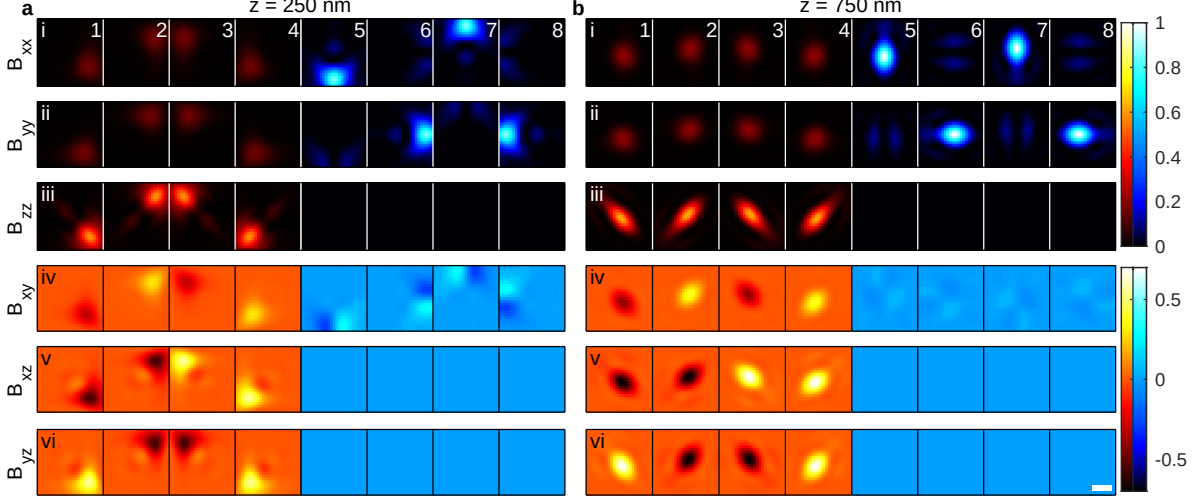


Figure S4: Basis images (i) B_{xx} , (ii) B_{yy} , (iii) B_{zz} , (iv) B_{xy} , (v) B_{xz} , and (vi) B_{yz} of the raMVR microscope for molecules located at (a) $z = 250$ nm and (b) $z = 750$ nm with nominal focal plane position $z_f = 1200$ nm. The sample refractive index $n = 1.33$ and imaging medium refractive index $n = 1.515$. Colorbar: normalized intensity; scale bar: 500 nm.

The image plane basis fields in the k th imaging channel are calculated using a Fourier transform,

$$g_i^{(k)}(u, v) = \begin{cases} \mathcal{F}\{G_i^{(r)}(\xi, \eta)A_k[\phi_{\text{BFP}}(\xi, \eta)]\} & k \in \{1, 2, 3, 4\} \\ \mathcal{F}\{G_i^{(a)}(\xi, \eta)A_k[\phi_{\text{BFP}}(\xi, \eta)]\} & k \in \{5, 6, 7, 8\} \end{cases}, \quad (\text{S5})$$

where (u, v) represent coordinates on the detector. The indicator function $A_k[\phi_{\text{BFP}}(\xi, \eta)] = 1$ selects the appropriate pupil segment for each imaging channel according to

$$\phi_{\text{BFP}}(\xi, \eta) \in [(k-1)\pi/2, k\pi/2), \quad k \in \{1, 2, 3, 4\} \quad (\text{S6})$$

$$\phi_{\text{BFP}}(\xi, \eta) \in [\pi/4 + (k-1)\pi/2, \pi/4 + k\pi/2), \quad k \in \{5, 6, 7, 8\} \quad (\text{S7})$$

and 0 otherwise (see Figure S3 for a visualization of each pupil segment).

The image formed by a microscope is a linear combination of the basis images weighted by the orientational second moments [Equation (1) in main text]. These basis images (Figure S4) can be calculated as the product between basis fields,

$$B_{ii}^{(k)} = |g_i^{(k)}|^2 \quad i, j \in \{x, y, z\} \quad (\text{S8})$$

$$B_{ij}^{(k)} = 2\text{Re}\{g_i^{(k)*}g_j^{(k)}\} \quad i, j \in \{x, y, z\}, i \neq j. \quad (\text{S9})$$

Their spatial gradients are shown in Figure S5.

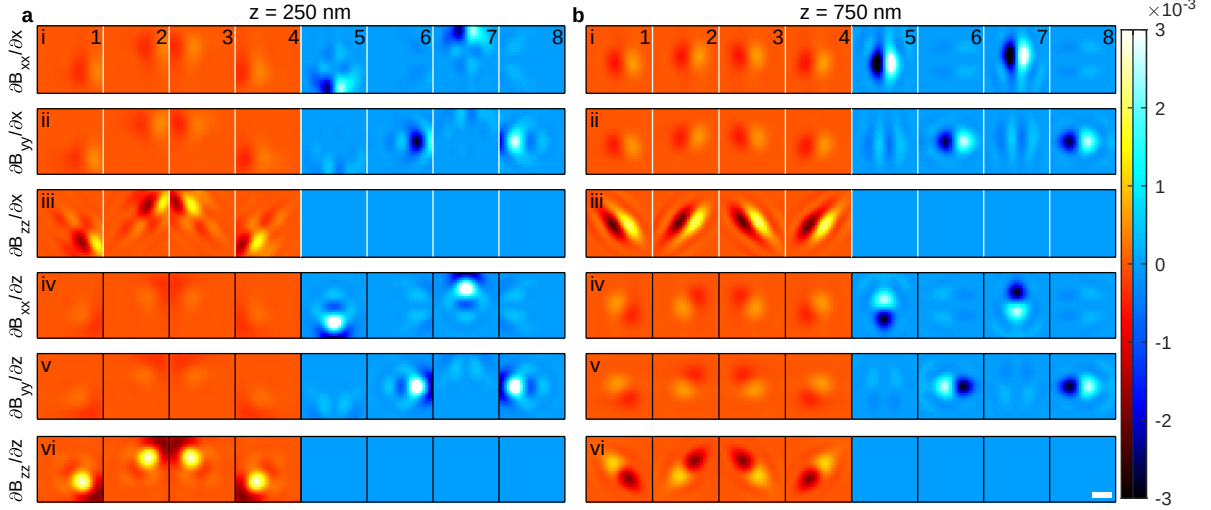


Figure S5: Spatial gradient of basis images (i) $\partial B_{xx}/\partial x$, (ii) $\partial B_{yy}/\partial x$, (iii) $\partial B_{zz}/\partial x$, (iv) $\partial B_{xx}/\partial z$, (v) $\partial B_{yy}/\partial z$, and (vi) $\partial B_{zz}/\partial z$ of the raMVR microscope for molecules located at (a) $z = 250$ nm and (b) $z = 750$ nm with nominal focal plane position $z_f = 1200$ nm. The sample refractive index $n = 1.33$ and imaging medium refractive index $n = 1.515$. Colorbar: normalized intensity/nm; scale bar: 500 nm.

2.2 Detectability analysis using the peak signal-to-background ratio

It is cumbersome to compare the detection sensitivity of the raMVR hardware and software to other techniques since it uses a bespoke localization algorithm (Section 3.2). However, we can use the peak signal-to-background ratio (SBR), i.e., the peak intensity divided by the background, as a proxy for single-molecule detection rate.¹⁴ The raMVR system has a greater depth of focus over other non aperture-splitting techniques due to the reduced effective aperture of each individual imaging channel. Moreover, with the splitting of background photons across the 8 channels, the raMVR DSF exhibits better peak SBR compared to raPol,¹⁴ CHIDO,¹⁵ and both vortex DSFs^{16,17} when the SM is located away from the focal plane [Figure S6(i,iii), $3.34\times$, $3.39\times$, $3.73\times$, and $6.10\times$ larger than those of raPol, CHIDO, unpolarized and polarized vortex DSFs, respectively on average, with ± 500 nm defocus]. For molecules close to the focal plane, raMVR exhibits slightly worse peak SBR compared to raPol [Figure S6a,b(ii), 1.1% smaller averaged across all possible orientations] due to its larger size caused by pupil segmentation [Extended Data Figure 4c,d(ii)]. However, the size of the raMVR DSF is comparable to those of CHIDO and the vortex DSF when in focus [Extended Data Figure 4a,b,d(ii)] and more compact when out of focus [Extended Data Figure 4(i,iii)].

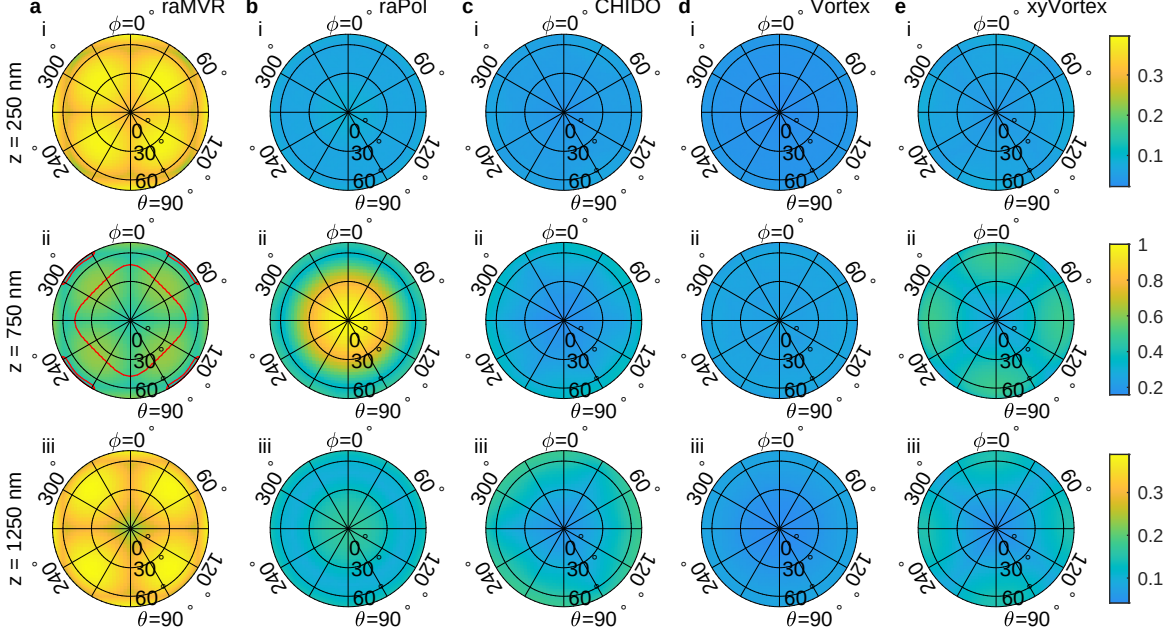


Figure S6: Peak signal-to-background ratio (SBR) of (a) the raMVR microscope, (b) the raPol standard DSF, (c) CHIDO, (d) unpolarized vortex DSF, and (e) x - and y -polarized vortex DSF for SMs located at (i) $z = 250$ nm, (ii) $z = 750$ nm (best focus), and (iii) $z = 1250$ nm as a function of molecular orientation. The SBRs are normalized to the best SBR using raPol. Red lines indicate orientations where raMVR exhibits the same SBR as raPol. The nominal focal plane position $z_f = 1200$ nm; a total of 2456 orientations are evaluated with wobble angle $\Omega = 0.28\pi$ sr.

2.3 Cramér-Rao lower bound for estimating 3D position and 3D orientation

We parameterize the orientation and position of an SM using $(\mu_x, \mu_y, \Omega, x, y, z)$ or the equivalent $(\theta, \phi, \Omega, x, y, z)$ in spherical orientation coordinates and express the Fisher information (FI) matrix as³

$$\mathcal{J}_{jk} = \sum_i \frac{1}{I_i} \frac{\partial I_i}{\partial \xi_j} \frac{\partial I_i}{\partial \xi_k}, \quad (\text{S10})$$

where $\xi_j, \xi_k \in \{\mu_x, \mu_y, \Omega, x, y, z\}$ or $\xi_j, \xi_k \in \{\theta, \phi, \Omega, x, y, z\}$. The Cramér-Rao bound (CRB)¹⁸ gives the best-possible precision of any unbiased estimator and is given by either

$$\boldsymbol{\tau} = \boldsymbol{\mathcal{J}}^{-1} = \begin{bmatrix} \mathcal{T}_{\mu_x \mu_x} & \mathcal{T}_{\mu_x \mu_y} & \mathcal{T}_{\mu_x \Omega} & \mathcal{T}_{\mu_x x} & \mathcal{T}_{\mu_x y} & \mathcal{T}_{\mu_x z} \\ \mathcal{T}_{\mu_y \mu_x} & \mathcal{T}_{\mu_y \mu_y} & \mathcal{T}_{\mu_y \Omega} & \mathcal{T}_{\mu_y x} & \mathcal{T}_{\mu_y y} & \mathcal{T}_{\mu_y z} \\ \mathcal{T}_{\Omega \mu_x} & \mathcal{T}_{\Omega \mu_y} & \mathcal{T}_{\Omega \Omega} & \mathcal{T}_{\Omega x} & \mathcal{T}_{\Omega y} & \mathcal{T}_{\Omega z} \\ \mathcal{T}_{x \mu_x} & \mathcal{T}_{x \mu_y} & \mathcal{T}_{x \Omega} & \mathcal{T}_{xx} & \mathcal{T}_{xy} & \mathcal{T}_{xz} \\ \mathcal{T}_{y \mu_x} & \mathcal{T}_{y \mu_y} & \mathcal{T}_{y \Omega} & \mathcal{T}_{yx} & \mathcal{T}_{yy} & \mathcal{T}_{yz} \\ \mathcal{T}_{z \mu_x} & \mathcal{T}_{z \mu_y} & \mathcal{T}_{z \Omega} & \mathcal{T}_{zx} & \mathcal{T}_{zy} & \mathcal{T}_{zz} \end{bmatrix} \quad \text{or} \quad (\text{S11})$$

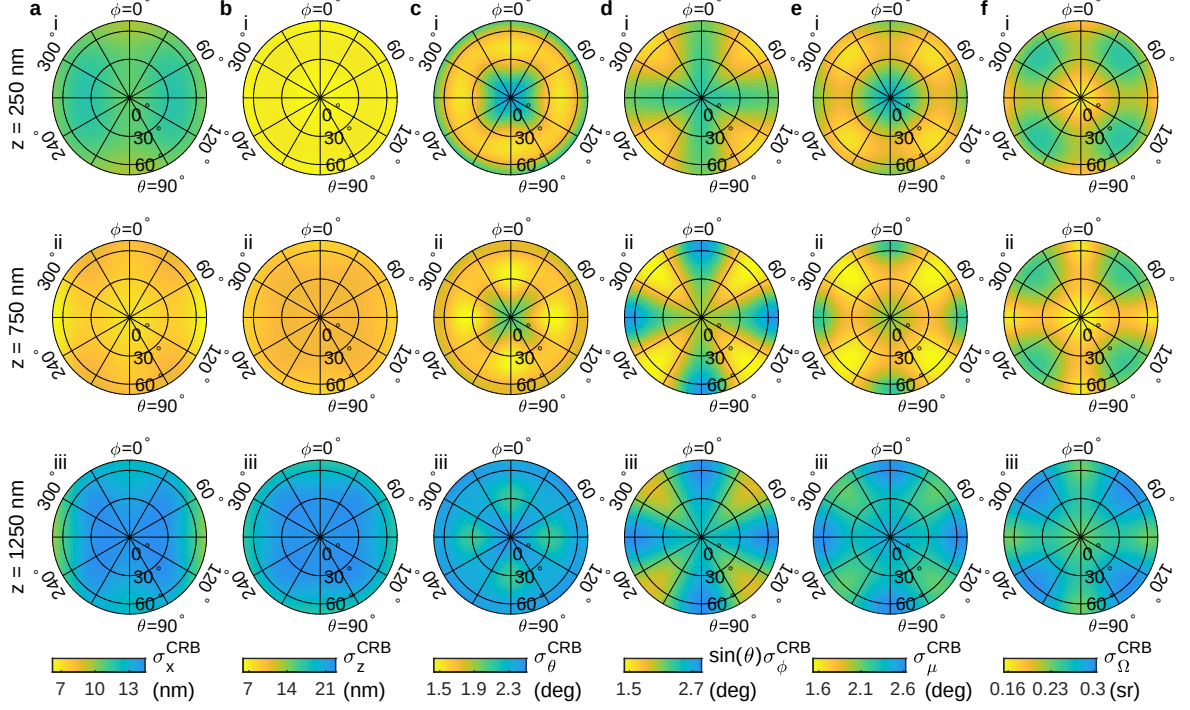


Figure S7: Cramér-Rao lower bound (CRB) for estimating (a) lateral position x (nm), (b) axial position z (nm), (c) polar angle θ (deg), (d) $\sin\theta$ -weighted azimuthal angle ϕ (deg), (e) average orientation μ (deg), and (f) wobble Ω (sr) of SMs located at (i) $z = 250$ nm, (ii) $z = 750$ nm (best focus), and (iii) $z = 1250$ nm as a function of molecular orientation. A total of 5000 signal photons (for in-plane molecules at $z = 0$ nm) and 40 photons per pixel background in all channels are detected. The nominal focal plane position $z_f = 1200$ nm; a total of 2456 orientations are evaluated; the wobble angle is $\Omega = 0.28\pi$ sr.

$$\mathcal{T} = \begin{bmatrix} \mathcal{T}_{\theta\theta} & \mathcal{T}_{\theta\phi} & \mathcal{T}_{\theta\Omega} & \mathcal{T}_{\theta x} & \mathcal{T}_{\theta y} & \mathcal{T}_{\theta z} \\ \mathcal{T}_{\phi\theta} & \mathcal{T}_{\phi\phi} & \mathcal{T}_{\phi\Omega} & \mathcal{T}_{\phi x} & \mathcal{T}_{\phi y} & \mathcal{T}_{\phi z} \\ \mathcal{T}_{\Omega\theta} & \mathcal{T}_{\Omega\phi} & \mathcal{T}_{\Omega\Omega} & \mathcal{T}_{\Omega x} & \mathcal{T}_{\Omega y} & \mathcal{T}_{\Omega z} \\ \mathcal{T}_{x\theta} & \mathcal{T}_{x\phi} & \mathcal{T}_{x\Omega} & \mathcal{T}_{xx} & \mathcal{T}_{xy} & \mathcal{T}_{xz} \\ \mathcal{T}_{y\theta} & \mathcal{T}_{y\phi} & \mathcal{T}_{y\Omega} & \mathcal{T}_{yx} & \mathcal{T}_{yy} & \mathcal{T}_{yz} \\ \mathcal{T}_{z\theta} & \mathcal{T}_{z\phi} & \mathcal{T}_{z\Omega} & \mathcal{T}_{zx} & \mathcal{T}_{zy} & \mathcal{T}_{zz} \end{bmatrix}. \quad (\text{S12})$$

The precision bound for estimating the lateral position x (Figure S7a), axial position z (Figure S7b), polar angle θ (Figure S7c), azimuthal angle ϕ (Figure S7d, scaled by the spherical projection factor $\sin\theta$), and wobble angle Ω (Figure S7f) are defined as

$$\sigma_\xi = \mathcal{T}_{\xi\xi}^{1/2}, \quad \xi \in \{\theta, \phi, \Omega, x, z\}. \quad (\text{S13})$$

We also define the precision bound for estimating the average orientation μ as

$$\sigma_\mu = \mu_z^{-1/2} \det \left(\begin{bmatrix} \mathcal{T}_{\mu_x \mu_x} & \mathcal{T}_{\mu_x \mu_y} \\ \mathcal{T}_{\mu_y \mu_x} & \mathcal{T}_{\mu_y \mu_y} \end{bmatrix} \right)^{1/4}, \quad (\text{S14})$$

which represents the square root of the area of the error ellipse for measuring the average orientation μ on the unit hemisphere (Figure S7e).¹⁴

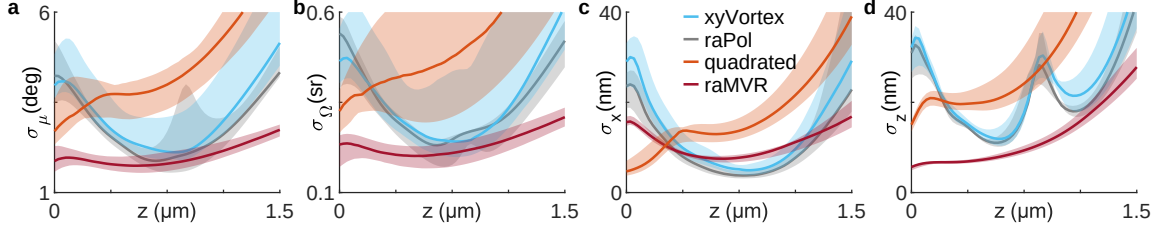


Figure S8: Measurement precision of the (a) average orientation, (b) wobble angle, (c) lateral position and (d) axial position using the radially and azimuthally polarized MVR (raMVR) microscope compared to other state-of-the-art methods with 5000 signal photons and 40 background photons per pixel across all imaging channels. Lines represent the median, and shaded areas represent the 10th and 90th percentiles across all possible orientations. Blue: x - and y -polarized vortex DSF (xy Vortex); gray: radially and azimuthally polarized (raPol) standard DSF; orange: quadrated DSF; red: raMVR.

Table S1: Average Cramér-Rao bound for estimating the lateral position x (nm), axial position z (nm), polar angle θ (deg), $\sin \theta$ -weighted azimuthal angle ϕ (deg), average orientation μ (deg), and wobble Ω (sr) with 5,000 signal photons for in-plane molecules located at the water-glass interface and 40 photons per pixel across all imaging channels background detected. The nominal focal plane position $z_f = 1200$ nm.

z (nm)		σ_x^{CRB} (nm)	σ_z^{CRB} (nm)	$\sigma_\theta^{\text{CRB}}$ (deg)	$\sin(\theta)\sigma_\phi^{\text{CRB}}$ (deg)	σ_μ^{CRB} (deg)	$\sigma_\Omega^{\text{CRB}}$ (sr)
250	raMVR	10.6	6.7	1.9	1.9	1.9	0.21
	raPol	11.2	16.1	2.9	2.7	2.8	0.32
	CHIDO	15.8	19.4	4.7	3.5	4.0	0.43
	Vortex	19.2	21.8	6.3	5.3	5.8	0.64
	xy Vortex	13.8	17.1	3.3	3.2	3.2	0.36
750 (best focus)	raMVR	7.7	9.5	1.7	2.0	1.8	0.21
	raPol	3.6	17.2	2.3	1.7	1.9	0.21
	CHIDO	4.8	17.8	2.7	1.9	2.2	0.24
	Vortex	6.3	22.9	3.0	4.8	3.7	0.38
	xy Vortex	4.7	17.5	2.2	2.4	2.2	0.26
1250	raMVR	12.7	19.7	2.4	2.4	2.3	0.27
	raPol	11.6	20.8	3.4	2.6	3.0	0.34
	CHIDO	14.8	33.3	6.1	3.1	4.2	0.44
	Vortex	20.0	33.9	6.3	7.7	6.9	0.79
	xy Vortex	14.8	25.1	4.0	3.9	3.7	0.46

Besides recently developed methods (Tables S1 and S2), we also compare the precision of raMVR to another pupil-segmentation method, the quadrated DSF.¹⁹ Combining radial and azimuthal polarization separation with splitting background fluorescence across 8 channels greatly improves raMVR's precision

Table S2: Average Cramér-Rao bound for estimating the lateral position x (nm), axial position z (nm), polar angle θ (deg), $\sin \theta$ -weighted azimuthal angle ϕ (deg), average orientation μ (deg), and wobble Ω (sr) with 1,000 signal photons for in-plane molecules located at the water-glass interface and 10 photons per pixel across all imaging channels background detected. The nominal focal plane position $z_f = 1200$ nm.

z (nm)		σ_x^{CRB} (nm)	σ_z^{CRB} (nm)	$\sigma_\theta^{\text{CRB}}$ (deg)	$\sin(\theta)\sigma_\phi^{\text{CRB}}$ (deg)	σ_μ^{CRB} (deg)	$\sigma_\Omega^{\text{CRB}}$ (sr)
250	raMVR	25.7	16.3	4.5	4.7	4.6	0.52
	raPol	27.7	39.6	7.2	6.7	6.9	0.80
	CHIDO	39.1	48.1	11.6	8.6	9.9	1.06
	Vortex	47.5	53.9	15.6	13.2	14.3	1.59
	<i>xy</i> Vortex	34.1	42.2	8.0	7.9	7.8	0.90
750 (best focus)	raMVR	18.5	22.8	4.1	4.8	4.4	0.50
	raPol	8.6	41.6	5.6	4.1	4.6	0.52
	CHIDO	11.4	42.9	6.5	4.5	5.3	0.57
	Vortex	15.2	55.8	7.2	11.4	8.9	0.92
	<i>xy</i> Vortex	11.2	42.5	5.3	5.7	5.2	0.62
1250	raMVR	30.8	47.8	5.7	5.7	5.7	0.65
	raPol	28.3	50.5	8.3	6.5	7.3	0.84
	CHIDO	36.1	81.5	15.0	7.4	10.3	1.07
	Vortex	49.0	83.4	15.5	18.9	16.9	1.93
	<i>xy</i> Vortex	36.2	61.3	9.8	9.6	9.2	1.13

over that of the quadrated DSF (Figure S8). Note that raMVR also reduces DSF overlap for imaging high emitter densities since fluorescence spots are separated across different imaging channels (Figure S9a,b).

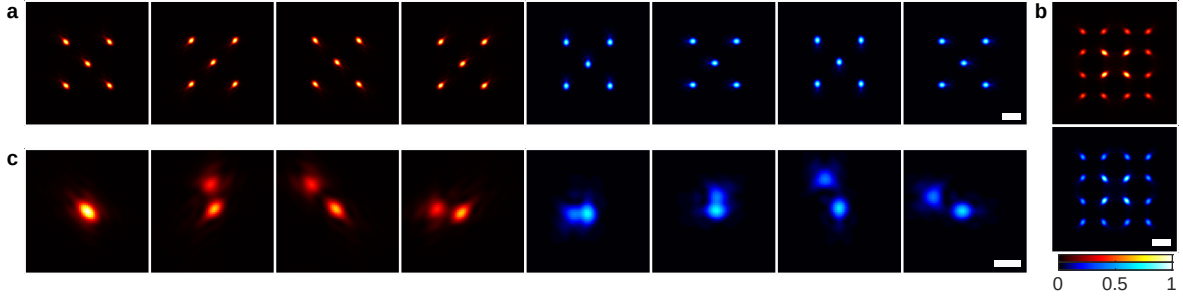


Figure S9: Example images of five emitters using the (a) raMVR system and (b) quadrated DSF. The quadrated DSF suffers from severe DSF overlaps since 4 spots are created in each imaging channel, whereas the raMVR system clearly resolves 5 laterally separated emitters. (c) Example image of two emitters $[(x_1, y_1, z_1) = (0, 0, 750) \text{ nm}, (x_2, y_2, z_2) = (-535, 535, 250) \text{ nm}]$. The nominal focal plane position $z_f = 1200$ nm. While overlapping caused by defocus-induced lateral displacement makes it difficult to resolve the two emitters in one channel (far left), they can be clearly resolved in the other channels. Colorbar: normalized intensity; scale bars: 2 μm in (a,b), 1 μm in (c).

Since the raMVR microscope exhibits a larger DSF than a standard epifluorescence microscope and does not rely on subtle changes in DSF shape to encode molecular orientation, its optimal camera pixel size could be larger than those of other SMLM and SMOLM techniques. To find an optimal camera pixel size, we compute the best-possible localization precision for isotropic emitters in the presence of detector

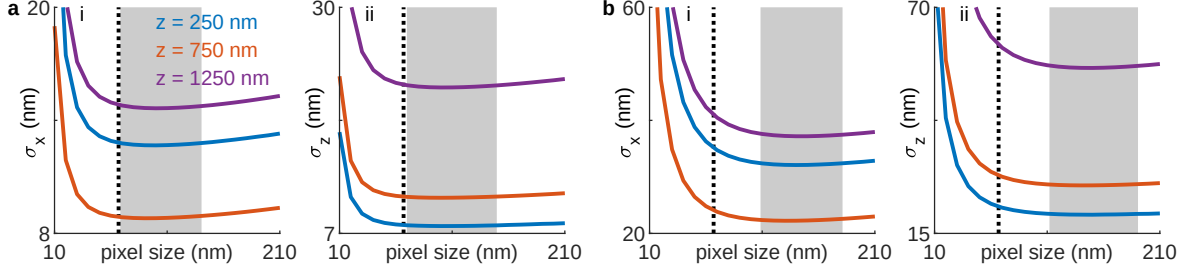


Figure S10: Cramér-Rao bound for localizing molecules along the (i) lateral and (ii) axial directions as a function of object-space pixel size under (a) high and (b) low SBR with a detector noise of 1 photoelectron per pixel. Gray areas represent pixel sizes within 1% of the optimal localization precision. Dotted lines represent an object-space pixel size of 66.8 nm. Blue: molecule located at $z = 250$ nm; orange: $z = 750$ nm; purple: $z = 1250$ nm. (a) High SBR with a total of 5000 signal photons (for in-plane molecules at $z = 0$ nm) and 40 background photons per $(66.8 \text{ nm})^2$ background across all channels. (b) Low SBR with a total of 1000 signal photons (for in-plane molecules at $z = 0$ nm) and 10 background photons per $(66.8 \text{ nm})^2$ across all channels.

readout noise. For simplicity, we use a Gaussian noise model. The entries of the FI matrix are given by

$$\mathcal{J}_{jk} = \sum_i \frac{1}{I_i + \sigma_{\text{rn}}^2} \frac{\partial I_i}{\partial \xi_j} \frac{\partial I_i}{\partial \xi_k} \left(1 + \frac{1}{2I_i + 2\sigma_{\text{rn}}^2} \right), \quad (\text{S15})$$

where $\sigma_{\text{rn}} = 1$ is a typical readout noise in SMLM and matches the cameras utilized in this work. For the experimental SBR in this work, the raMVR's pixel size of 66.8 nm (see Methods) is close to optimal (Figure S10a). The optimal pixel size increases as the signal-to-background ratio decreases (Figure S10b).

3 Detection and estimation algorithms for raMVR imaging

3.1 Registering the eight imaging channels in the raMVR system

To register the 8 imaging channels of the raMVR microscope, we image in-focus fluorescent beads (Thermo Fisher F8800, 0.1- μm diameter, embedded in lens immersion oil) to determine corresponding positions within each channel. For small fields of view, e.g., lipid-coated silica sphere imaging (Figure 3), a single fluorescent bead is moved to the position of the silica sphere to ensure the most accurate registration. ThunderSTORM²⁰ is used to localize the spot in each imaging channel with a localization precision of ~ 2 nm. For larger fields of view, e.g., HEK-293T cell imaging (Figure 5, [Extended Data Figure 5](#) and [Extended Data Figure 6](#)), the entire field of view is scanned using a fluorescent bead with 6 μm separation. A total of 16 points (Figure S11) are used to generate the registration map and calibrate field-dependent aberrations within each channel.

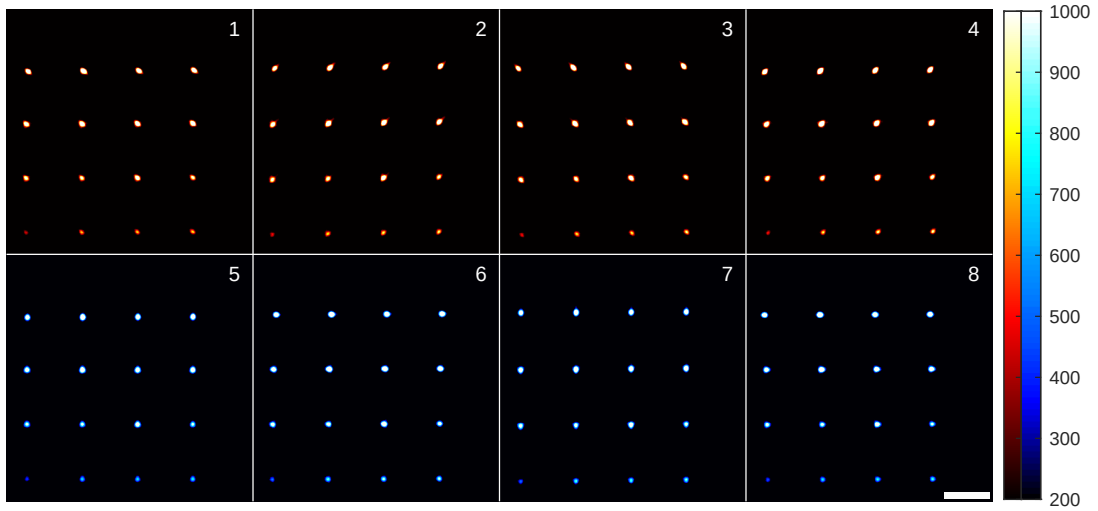


Figure S11: Composite fluorescent bead images for channel registration. A single bead is scanned across the field of view at 6 μm intervals, and these images are combined into the composites shown above. Colorbar: intensity; scale bar: 5 μm .

3.2 Detection and initial estimate of 3D position

We first use our Robust Statistical Estimation algorithm (RoSE),²¹ a regularized maximum-likelihood estimator, to detect and localize the spots independently within each channel (Figure S12a) with pixel-scale precision. We approximate the raMVR DSF by computing images of isotropic emitters and averaging them over a certain z range depending on the expected thickness of the sample of interest; DSFs at each z position are re-centered laterally to compensate for the defocus-induced lateral shift (Figure S13). Fitting the peak intensity of the defocused spots to a linear function, we find that the relationship between the apparent lateral shift and the physical shift of emitters in z direction is approximately given

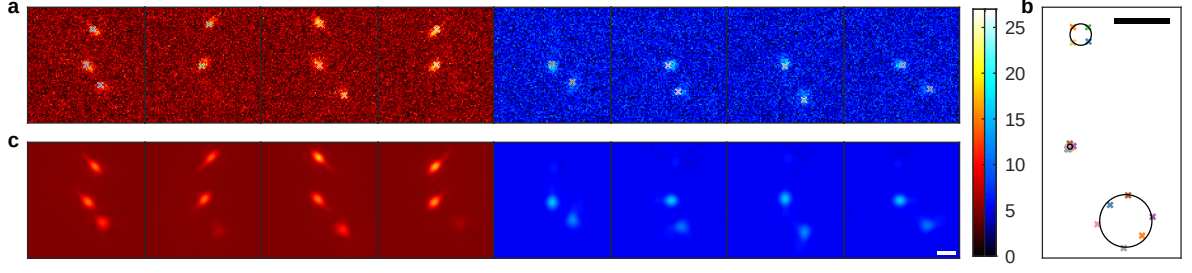


Figure S12: Detection and estimation algorithm for MVR images. (a) Simulated noisy image of 3 molecules with 3D position, orientation, and signal $[x, y, z, \theta, \phi, \Omega, s]$ of $[0 \text{ nm}, 0 \text{ nm}, 750 \text{ nm}, 0^\circ, 0^\circ, 2\pi \text{ sr}, 7000 \text{ photons}]$, $[1003 \text{ nm}, 1337 \text{ nm}, 1250 \text{ nm}, 90^\circ, 45^\circ, 1.35 \text{ sr}, 5000 \text{ photons}]$, and $[201 \text{ nm}, -2006 \text{ nm}, 550 \text{ nm}, 10^\circ, 20^\circ, 0.43 \text{ sr}, 4000 \text{ photons}]$, respectively. The background is 40 photons per pixel across all channels. The nominal focal plane position $z_f = 1200 \text{ nm}$. Crosses represent the localized spots in each channel using RoSE. (b) The detected spots across the 8 imaging channels (crosses with unique colors) are coregistered to a common (x, y) coordinate system. Fitted circles to the detected spots provide initial guess of the 3D position of the SMs. (c) Reconstructed images based on the estimated second-order orientational moments and 3D position using a FISTA-based MLE. Colorbar: photons; scale bar: $1 \mu\text{m}$.

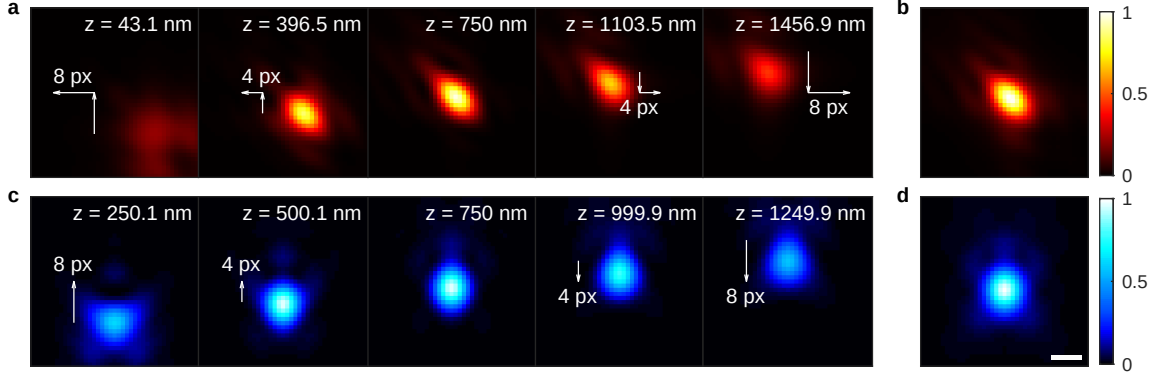


Figure S13: Apparent lateral shift of MVR DSFs and DSF model for spot detection in the (a,b) radially and (c,d) azimuthally polarized imaging channels using RoSE. After shifting (as depicted by the arrows) the (a,c) images of isotropic emitters located at various z positions, we obtain (b,d) the average DSF used in the spot detection using RoSE. Colorbar: normalized intensity; scale bar: 500 nm .

by (Figure S13)

$$\sqrt{\Delta x_{\text{apparent}}^2 + \Delta y_{\text{apparent}}^2} \approx 0.91 \Delta z. \quad (\text{S16})$$

Next, we calculate the coarse 3D position of each SM by constructing a list of all possible pairwise associations of spots across imaging channels. This list can then be used to compute a list of all possible SM positions (Algorithm S1). We then group pairs of spots together using a scoring algorithm (Algorithm S2, Figure S12b). The results are initial guesses of each molecule's 3D position to be refined in the next stage of the estimator.

Algorithm S1: Localize molecules based on detected DSFs paired across two channels

```
input : list of DSF positions  $[x, y]$  from RoSE and localization precision  $\sigma$  in 8 channels from CRB
 $k \leftarrow 1$  //  $k$  is an index representing each molecule
// define vectors the connecting position of the SM and the apparent position of the spot
for each channel  $i$  do
    //  $\alpha_i$  represents the apparent lateral shift direction of the spot in channel  $i$  when the molecule move towards  $+z$  direction, e.g.,  $135^\circ$  for  $i \leftarrow 1$ 
    if  $i < 5$  then
        |  $\alpha_i \leftarrow i \times 45^\circ + 90^\circ$ 
    else
        |  $\alpha_i \leftarrow i \times 90^\circ$ 
    end
     $\mathbf{v}_i \leftarrow [\sin \alpha_i, \cos \alpha_i]$ 
end
// each detected DSF  $m$  is represented by parameters  $[x_m, y_m, s_m, \sigma_m]$ 
for each spot  $m$  in channel  $i$  and each spot  $n$  in channel  $j$ ,  $i < j$  do
     $\Delta \mathbf{r} \leftarrow [x_m - x_n, y_m - y_n]$ 
     $\mathbf{v}_{\parallel, ij} \leftarrow (\mathbf{v}_i - \mathbf{v}_j) / |\mathbf{v}_i - \mathbf{v}_j|^2$ 
     $\mathbf{v}_{\perp, ij} \leftarrow [\cos \alpha_j - \cos \alpha_i, \sin \alpha_i - \sin \alpha_j] / |\cos \alpha_j - \cos \alpha_i, \sin \alpha_i - \sin \alpha_j|$ 
    // if the DSFs between spots  $m$  and  $n$  are separated by the expected distance on the camera, then add the spot pair to our list
    // possible registration errors are corrected using  $2 \times$  pixel size: default  $\sigma_{\text{tol}} = 5$ ,  $z_{\text{tol}} = 2 \mu\text{m}$ 
    if  $|\Delta \mathbf{r} \cdot \mathbf{v}_{\perp, ij}| < 2 \times \text{pixel size} + \sigma_{\text{tol}}(\sigma_m + \sigma_n)$  and  $|\Delta \mathbf{r} \cdot \mathbf{v}_{\parallel, ij}| \leq z_{\text{tol}}$  then
        // compute 3D position
         $z_k \leftarrow \Delta \mathbf{r} \cdot \mathbf{v}_{\parallel, ij}$ 
         $[x_k, y_k] \leftarrow (\sigma_n([x_m, y_m] - z_k \mathbf{v}_i) + \sigma_m([x_n, y_n] - z_k \mathbf{v}_j)) / (\sigma_m + \sigma_n)$ 
         $\sigma_k \leftarrow \sqrt{2} \sigma_m \sigma_n / (\sigma_m + \sigma_n)$  // expected localization precision
         $p_k \leftarrow -0.5 i_k^2 + 7.5 i_k - 8 + j_k$  // channel pair index
        // With 8 channels, there are 28 possible pairwise combinations. There is a one-to-one mapping between  $p_k \in \{1, 2, \dots, 28\}$  and the channels  $[i_k, j_k]$  that form the spot pairing.
         $c_k \leftarrow [x_k, y_k, z_k, \sigma_k, p_k, i_k, j_k, m_k, n_k]$  // center list
         $k \leftarrow k + 1$  // increment number of molecules
    end
end
output: list of molecules' 3D positions as calculated by all DSF pairs  $c_k$ 
```

Algorithm S2: Group localizations across multiple channels for estimation initialization

input : list of molecules' 3D positions as calculated from DSFs paired across two channels (Algorithm S1)

$q \leftarrow 0$

for each channel index pair η from 1 to 28 **do**

for each center c_{k1} in this channel pair ($p_{k1} = \eta$) **do**

$q \leftarrow q + 1$

$C_q \leftarrow [x_{k1}, y_{k1}, z_{k1}, \sigma_{k1}, 0, 0, 0, 0, 0, 0, 0, 0, \text{score} = 1]$ // assign molecule position

 // Score represents the number of molecules localized from different channel pairs using Algorithm S1 whose 3D positions are close to C_q . The maximum possible score for a molecule is 28.

$C_q(i_{k1} + 4) \leftarrow m_{k1}$, $C_q(j_{k1} + 4) \leftarrow n_{k1}$ // m_{k1} and n_{k1} represent the spot index corresponding to the molecule in channels i and j

 // associate more DSF spots with this molecule

for each channel index pair ζ from $\eta + 1$ to 28 **do**

 // If a channel in pair ζ is the same as one of the two channels in pair η , find the closest molecule from channel pair ζ with the same spot whose index matches that from the same channel in pair channel η . Otherwise, find the closest molecule.

if channel index $i(\eta) = i(\zeta)$ **then**

$k2 \leftarrow \text{argmin}_{k'} |[x_{k1} - x_{k'}, y_{k1} - y_{k'}, z_{k1} - z_{k'}]|_{p_k=\zeta, m_k=m_{k1}}$

else if channel index $j(\eta) = i(\zeta)$ **then**

$k2 \leftarrow \text{argmin}_{k'} |[x_{k1} - x_{k'}, y_{k1} - y_{k'}, z_{k1} - z_{k'}]|_{p_k=\zeta, m_{k'}=n_{k1}}$

else if channel index $j(\eta) = j(\zeta)$ **then**

$k2 \leftarrow \text{argmin}_{k'} |[x_{k1} - x_{k'}, y_{k1} - y_{k'}, z_{k1} - z_{k'}]|_{p_k=\zeta, n_{k'}=n_{k1}}$

else

$k2 \leftarrow \text{argmin}_{k'} |[x_{k1} - x_{k'}, y_{k1} - y_{k'}, z_{k1} - z_{k'}]|_{p_k=\zeta}$

end

 // If the 3D distance between computed positions between the pairs is within tolerance, then update the molecule position.

if $|[x_{k1} - x_{k2}, y_{k1} - y_{k2}, z_{k1} - z_{k2}]| < 2 \times \text{pixel size} + \sigma_{\text{tol}}(\sigma_{k1} + \sigma_{k2})$ **then**

$C_q(1, 2, 3) \leftarrow (\sigma_{k2}[x_{k1}, y_{k1}, z_{k1}] + \sigma_{k1}[x_{k2}, y_{k2}, z_{k2}]) / (\sigma_{k1} + \sigma_{k2})$

$C_q(4) \leftarrow \sqrt{2}\sigma_{k1}\sigma_{k2} / (\sigma_{k1} + \sigma_{k2})$

 score $\leftarrow$ score + 1

else

$q \leftarrow q + 1$

$C_q \leftarrow [x_{k2}, y_{k2}, z_{k2}, \sigma_{k2}, 0, 0, 0, 0, 0, 0, 0, 0, \text{score} = 1]$ // obtain a new molecule position

end

$C_q(i_{k2} + 4) \leftarrow m_{k2}$, $C_q(j_{k2} + 4) \leftarrow n_{k2}$ // record spot index

end

end

end

Algorithm S2: Group localizations across multiple channels for estimation initialization (continued)

Sort all positions C_q first by their score from high to low, then by the precision from small to large

for all positions C_{q1} **do**

for all positions C_{q2} , $q2 > q1$ **do**

if any entry of $C_{q2}(5 : 12)$ is equal to the corresponding entry of $C_{q1}(5 : 12)$ **then**

 mark C_{q2} as a duplicate 3D position

end

end

end

Remove all duplicate 3D positions from list C_q

output: list of molecules' 3D positions

3.3 Estimating the 3D positions and 3D orientations of single molecules

To estimate the 3D position and orientation of molecules from the MVR image, we construct a dictionary of basis images and their partial derivatives at grid points regularly spaced throughout the 3D volume of interest. Since the image is shift-invariant in the x and y directions, the spacing between grid points is chosen to be equal to the camera pixel size (66.8 nm), thereby reducing computation cost. The distance between adjacent grid points in the z direction is chosen to be 50 nm. Note that these choices place an upper limit on the density of localized single molecules in any given image; each molecule must be assigned to a unique nearest grid point.

We first rewrite the forward imaging model [Equation (1)] using a first-order approximation for the image $\mathbf{I}$ with respect to the position of the molecule, given by

$$\begin{aligned} \mathbf{I} &= \left[B_{xx}, B_{yy}, B_{zz}, B_{xy}, B_{xz}, B_{yz}, \frac{\partial B_{xx}}{\partial x}, \frac{\partial B_{yy}}{\partial x}, \frac{\partial B_{zz}}{\partial x}, \frac{\partial B_{xx}}{\partial y}, \frac{\partial B_{yy}}{\partial y}, \frac{\partial B_{zz}}{\partial y}, \frac{\partial B_{xx}}{\partial z}, \frac{\partial B_{yy}}{\partial z}, \frac{\partial B_{zz}}{\partial z} \right] \times \\ &\quad [sm_{xx}, sm_{yy}, sm_{zz}, sm_{xy}, sm_{xz}, sm_{yz}, sm_{xx}\Delta x, sm_{yy}\Delta x, sm_{zz}\Delta x, \\ &\quad sm_{xx}\Delta y, sm_{yy}\Delta y, sm_{zz}\Delta y, sm_{xx}\Delta z, sm_{yy}\Delta z, sm_{zz}\Delta z]^\top + \mathbf{b} \\ &= [B_1(x, y, z), \dots, B_{15}(x, y, z)][M_1, \dots, M_{15}]^\top + \mathbf{b}. \end{aligned} \quad (\text{S17})$$

Here, we omit $\partial B_{ij}/\partial k$, $i, j, k \in \{x, y, z\}$ with $i \neq j$ to improve the convexity of the optimization.²² For N molecules, the image captured by the camera is a mixture of their individual DSFs, given by

$$\begin{aligned} \mathbf{I} &= \left[B_1^{(1)}(x_1, y_1, z_1), \dots, B_{15}^{(1)}(x_1, y_1, z_1), \dots, B_1^{(N)}(x_{15}, y_{15}, z_{15}), \dots, B_{15}^{(N)}(x_{15}, y_{15}, z_{15}) \right] \times \\ &\quad [M_1^{(1)}, \dots, M_{15}^{(1)}, \dots, M_1^{(N)}, \dots, M_{15}^{(N)}]^\top + \mathbf{b}, \end{aligned} \quad (\text{S18})$$

where the superscripts $(1), \dots, (N)$ represent each of the localizations detected by Algorithm S2. We note that the 3D position (x_j, y_j, z_j) of each molecule is represented by a unique nearest 3D grid point

(x_{jg}, y_{jg}, z_{jg}) and a distance $(\Delta x_j, \Delta y_j, \Delta z_j)$ from that grid point such that

$$(x_j, y_j, z_j) = (x_{jg}, y_{jg}, z_{jg}) + (\Delta x_j, \Delta y_j, \Delta z_j). \quad (\text{S19})$$

Each of the basis images $B_1^{(j)}(x_j, y_j, z_j), \dots, B_{15}^{(j)}(x_j, y_j, z_j)$ are calculated with respect to the nearest grid point (x_{jg}, y_{jg}, z_{jg}) .

Algorithm S3: Measuring the 3D position and orientation of SMs with nanoscale accuracy and precision

```

input : List of probable of 3D positions  $(x_j, y_j, z_j)$  and their closest grid points  $(x_{jg}, y_{jg}, z_{jg})$ 
        of  $N$  detected molecules, image  $\hat{\mathbf{I}}$ , background  $\mathbf{b}$ , basis image dictionary  $\mathbf{B}_{\text{dictionary}}$ 
// assemble basis matrix  $\mathbf{B}$  according to the initial guess  $(x_{jg}, y_{jg}, z_{jg})$ 
for every molecule  $j \in \{1, 2, \dots, N\}$  do
     $\mathbf{B}^{(j)} \leftarrow \mathbf{B}_{\text{dictionary}}(z_{jg})$ 
    shift basis images  $B_1^{(j)}, \dots, B_{15}^{(j)}$  along the  $(x, y)$  directions by  $(x_{jg}, y_{jg})/\delta_{xy}$  pixels
     $\mathbf{B}(15j - 14, \dots, 15j) \leftarrow \mathbf{B}^{(j)}$ 
end
 $\Delta x \leftarrow \infty, \Delta y \leftarrow \infty, \Delta z \leftarrow \infty$ 
iter  $\leftarrow 1$ 
// 10 iterations of fast basis pseudo inverse (LMSE) estimation
// break if all sub-grid positions  $|\Delta x|, |\Delta y|, |\Delta z|$  are smaller than half the grid size
while  $\max(|\Delta x|/\delta_{xy}, |\Delta y|/\delta_{xy}, |\Delta z|/\delta_z) > 0.5$  and iter  $< 10$  do
     $\mathbf{M} \leftarrow \mathbf{B}^{-1}(\hat{\mathbf{I}} - \mathbf{b})$ 
    for every molecule  $j \in \{1, 2, \dots, N\}$  do
        //  $(sm_{xx}\Delta x + sm_{yy}\Delta y + sm_{zz}\Delta z)/(sm_{xx} + sm_{yy} + sm_{zz})$ 
         $\Delta x_j \leftarrow [M(15j-8) + M(15j-7) + M(15j-6)]/[M(15j-14) + M(15j-13) + M(15j-12)]$ 
        //  $(sm_{xx}\Delta y + sm_{yy}\Delta y + sm_{zz}\Delta z)/(sm_{xx} + sm_{yy} + sm_{zz})$ 
         $\Delta y_j \leftarrow [M(15j-5) + M(15j-4) + M(15j-3)]/[M(15j-14) + M(15j-13) + M(15j-12)]$ 
        //  $(sm_{xx}\Delta z + sm_{yy}\Delta z + sm_{zz}\Delta z)/(sm_{xx} + sm_{yy} + sm_{zz})$ 
         $\Delta z_j \leftarrow [M(15j-2) + M(15j-1) + M(15j)]/[M(15j-14) + M(15j-13) + M(15j-12)]$ 
        //  $\lfloor \cdot \rfloor$  represents rounding to the nearest integer
         $[x_{jg}, y_{jg}, z_{jg}] \leftarrow [x_{jg}, y_{jg}, z_{jg}] + [\lfloor \Delta x_j/\delta_{xy} \rfloor \delta_{xy}, \lfloor \Delta y_j/\delta_{xy} \rfloor \delta_{xy}, \lfloor \Delta z_j/\delta_z \rfloor \delta_z]$ 
         $\mathbf{B}^{(j)} \leftarrow \mathbf{B}_{\text{dictionary}}(z_{jg})$  // update basis matrix  $\mathbf{B}$ 
        shift basis images  $B_1^{(j)}, \dots, B_{15}^{(j)}$  along the  $(x, y)$  directions by  $[(x_{jg}, y_{jg})/\delta_{xy}]$  pixels
         $\mathbf{B}(15j - 14, \dots, 15j) \leftarrow \mathbf{B}^{(j)}$ 
    end
    iter  $\leftarrow$  iter + 1
end
// FISTA-based MLE with backtracking
 $\mathbf{M} \leftarrow \text{argmin}_{\mathbf{M}'} \Lambda(\hat{\mathbf{I}}, \mathbf{B}, \mathbf{M}', \mathbf{b})$ 
output: 3D position, second-order orientational moments, signal of the SMs

```

Assuming that camera read noise is negligible, the i th pixel in the detected image is modeled as $\hat{I}_i \sim \text{Poisson}(I_i)$. The negative log likelihood (NLL) function is thus given by¹⁴

$$\Lambda(\hat{\mathbf{I}}, \mathbf{B}, \mathbf{M}, \mathbf{b}) = \sum_i \left[\hat{I}_i \log(I_i/b_i) - I_i \right]. \quad (\text{S20})$$

The estimation process is detailed in Algorithm S3. We first use a fast least mean-squared error (LMSE) estimator to calculate $\mathbf{M}$, shifting the basis images $\mathbf{B} = [B_{xx}, \dots, \partial B_{zz}/\partial z]$ to ensure that the estimated

sub-grid distances Δx , Δy and Δz are less than half the grid size. After a maximum of 10 iterations, we use a fast iterative shrinkage-thresholding algorithm²³ (FISTA)-based maximum likelihood estimator (MLE) to minimize the Poisson NLL [Equation (S20)]. Finally, we map the measured second-order orientational moments $[m_{xx}, \dots, m_{yz}]$ of each molecule to orientation parameters $[\theta, \phi, \Omega]$ by minimizing an FI-weighted least mean squared cost function²⁴

$$(\mu_x, \mu_y, \gamma) = \underset{\mu'_x, \mu'_y, \gamma'}{\operatorname{argmin}} (\Delta \mathbf{m}^\top \mathcal{J}_{m_{xx}, \dots, m_{yz}} \Delta \mathbf{m}), \text{ where} \quad (\text{S21})$$

$$\Delta \mathbf{m} = \left[\gamma' \mu_x'^2 + \frac{1 - \gamma'}{3}, \gamma' \mu_y'^2 + \frac{1 - \gamma'}{3}, \gamma' \mu_z'^2 + \frac{1 - \gamma'}{3}, \gamma' \mu'_x \mu'_y, \gamma' \mu'_x \mu'_z, \gamma' \mu'_y \mu'_z \right]^\top$$

$$- [m_{xx}, m_{yy}, m_{zz}, m_{xy}, m_{xz}, m_{yz}]^\top \text{ and} \quad (\text{S22})$$

$$\mathcal{J}_{m_{xx}, \dots, m_{yz}} = \left(\begin{bmatrix} \mathcal{T}_{m_{xx}m_{xx}} & \mathcal{T}_{m_{xx}m_{yy}} & \mathcal{T}_{m_{xx}m_{zz}} & \mathcal{T}_{m_{xx}m_{xy}} & \mathcal{T}_{m_{xx}m_{xz}} & \mathcal{T}_{m_{xx}m_{yz}} \\ \mathcal{T}_{m_{yy}m_{xx}} & \mathcal{T}_{m_{yy}m_{yy}} & \mathcal{T}_{m_{yy}m_{zz}} & \mathcal{T}_{m_{yy}m_{xy}} & \mathcal{T}_{m_{yy}m_{xz}} & \mathcal{T}_{m_{yy}m_{yz}} \\ \mathcal{T}_{m_{zz}m_{xx}} & \mathcal{T}_{m_{zz}m_{yy}} & \mathcal{T}_{m_{zz}m_{zz}} & \mathcal{T}_{m_{zz}m_{xy}} & \mathcal{T}_{m_{zz}m_{xz}} & \mathcal{T}_{m_{zz}m_{yz}} \\ \mathcal{T}_{m_{xy}m_{xx}} & \mathcal{T}_{m_{xy}m_{yy}} & \mathcal{T}_{m_{xy}m_{zz}} & \mathcal{T}_{m_{xy}m_{xy}} & \mathcal{T}_{m_{xy}m_{xz}} & \mathcal{T}_{m_{xy}m_{yz}} \\ \mathcal{T}_{m_{xz}m_{xx}} & \mathcal{T}_{m_{xz}m_{yy}} & \mathcal{T}_{m_{xz}m_{zz}} & \mathcal{T}_{m_{xz}m_{xy}} & \mathcal{T}_{m_{xz}m_{xz}} & \mathcal{T}_{m_{xz}m_{yz}} \\ \mathcal{T}_{m_{yz}m_{xx}} & \mathcal{T}_{m_{yz}m_{yy}} & \mathcal{T}_{m_{yz}m_{zz}} & \mathcal{T}_{m_{yz}m_{xy}} & \mathcal{T}_{m_{yz}m_{xz}} & \mathcal{T}_{m_{yz}m_{yz}} \end{bmatrix} \right)^{-1} \quad (\text{S23})$$

A representative reconstructed image is shown in Figure S12c.

3.4 Precision and accuracy of the detection and estimation algorithm

The precision [Figure S14a-f, Figure S15(ii)] and accuracy (Figure S14g-n, Figure S15(iii)) of our SM detection and orientation-position estimation algorithm are shown in Table S3, relative to CRB [Figure S7, Figure S15(i)]. The precision and accuracy are evaluated using 96 noisy images per orientation with $s = 5000$ signal photons (for in-plane molecules located at the water-glass RI interface) and $b = 40$ total background photons per pixel across all channels detected (Figure S14), in addition to a lower SBR more typical of SM imaging, $[s, b] = [1000, 10]$ (Figure S15). Estimation bias is defined as the average of the estimation minus the ground truth for x , z , θ , ϕ , and Ω , while

$$\boldsymbol{\mu}_{\text{bias}}^{\text{est}} = \cos^{-1}(\bar{\boldsymbol{\mu}}_{\text{est}} \cdot \boldsymbol{\mu}_{\text{true}}) \quad (\text{S24})$$

for $\boldsymbol{\mu}$. The algorithm performs at a precision close to the CRB and is unbiased for x , z , θ , and ϕ . We only see a small negative bias in Ω as expected from the finite SBR.⁵

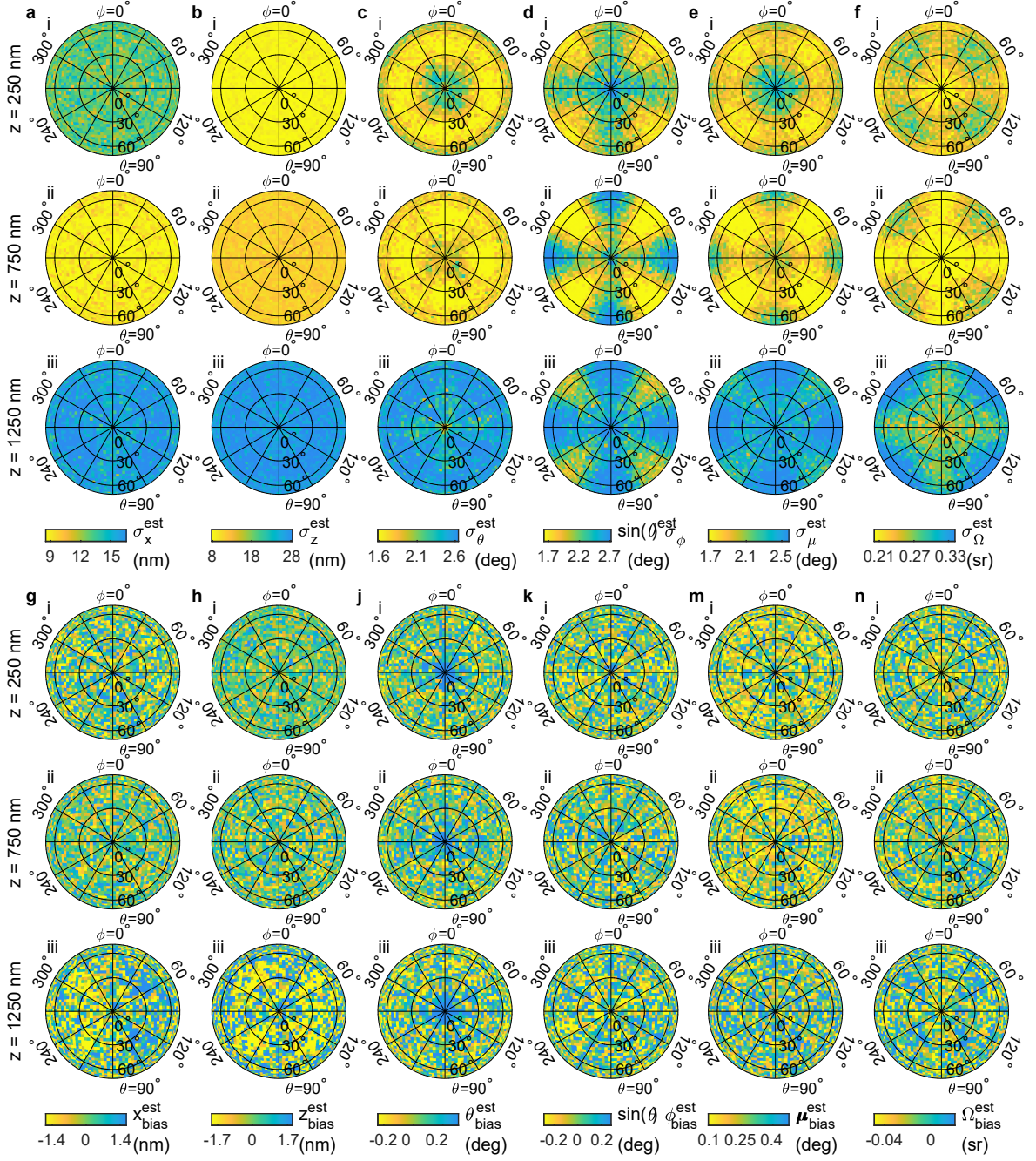


Figure S14: (a-f) Precision and (g-n) accuracy for estimating (a,g) lateral position x (nm), (b,h) axial position z (nm), (c,j) polar angle θ (deg), (d,k) azimuthal angle ϕ (deg), (e,m) average orientation μ (deg), and (f,n) wobble Ω (sr) of SMs located at (i) $z = 250$ nm, (ii) $z = 750$ nm (best focus), and (iii) $z = 1250$ nm as a function of molecular orientation using the FISTA-based MLE. A total of 5000 signal photons (for in-plane molecules at $z = 0$ nm) and 40 per pixel background in all channels are detected. The nominal focal plane position $z_f = 1200$ nm; a total of 2456 orientations with 96 noisy realizations per orientation are evaluated; the wobble angle is $\Omega = 0.28\pi$ sr.

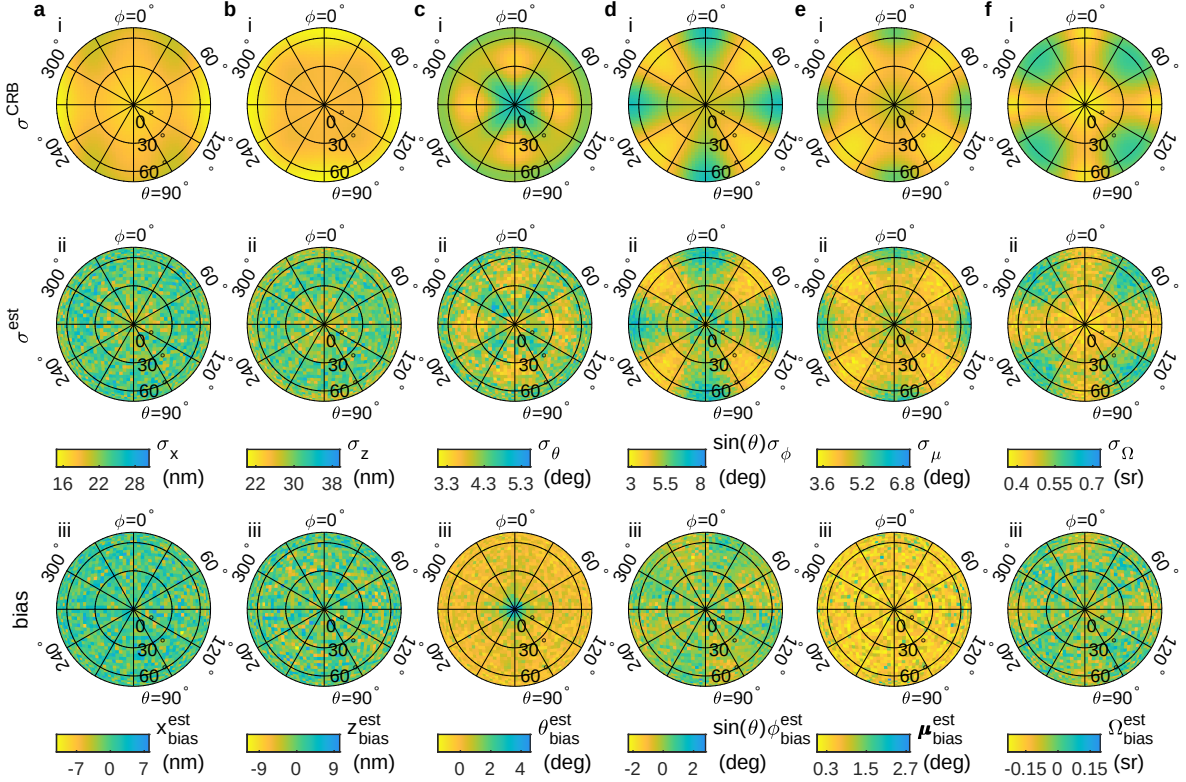


Figure S15: (i) Cramér-Rao bound (CRB), (ii) precision and (iii) accuracy using the FISTA-based MLE for estimating (a) lateral position x (nm), (b) axial position z (nm), (c) polar angle θ (deg), (d) azimuthal angle ϕ (deg), (e) average orientation μ (deg), and (f) wobble Ω (sr) of SMs located at $z = 750$ nm (best focus) as a function of molecular orientation. A total of 1000 signal photons (for in-plane molecules at $z = 0$ nm) and 10 per pixel background in all channels are detected. The nominal focal plane position $z_f = 1200$ nm; a total of 2456 orientations with 96 noisy realizations per orientation are evaluated; the wobble angle is $\Omega = 0.28\pi$ sr.

Table S3: Estimation precision and accuracy for the lateral position x , axial position z , polar angle θ , $\sin \theta$ -weighted azimuthal angle ϕ , average orientation μ , and wobble Ω relative to the Cramér-Rao lower bound averaged over the orientation hemisphere. The nominal focal plane position $z_f = 1200$ nm.

			x	z	θ	ϕ	μ	Ω
$s = 5000$ $b = 40$	$z = 250$ nm	$\sigma^{\text{est}}/\sigma^{\text{CRB}}$	1.22	1.22	1.01	1.03	1.01	1.12
		$\text{bias}/\sigma^{\text{CRB}}$	0.00	0.01	0.02	0.00	0.13	-0.07
	$z = 750$ nm (best focus)	$\sigma^{\text{est}}/\sigma^{\text{CRB}}$	1.16	1.17	1.01	1.01	1.01	1.09
		$\text{bias}/\sigma^{\text{CRB}}$	0.00	0.01	0.02	0.00	0.14	-0.08
	$z = 1250$ nm	$\sigma^{\text{est}}/\sigma^{\text{CRB}}$	1.28	1.40	1.08	1.04	1.06	1.19
		$\text{bias}/\sigma^{\text{CRB}}$	0.00	0.00	0.01	0.00	0.14	-0.05
$s = 1000$ $b = 10$	$z = 750$ nm (best focus)	$\sigma^{\text{est}}/\sigma^{\text{CRB}}$	1.26	1.30	1.03	1.06	1.05	1.04
		$\text{bias}/\sigma^{\text{CRB}}$	0.00	0.01	0.04	0.00	0.14	-0.06

3.5 Detection and estimation performance at high emitter densities

To evaluate the performance of the detection and estimation algorithm at a high density of SM flashes, we simulate images with 1-10 molecules randomly positioned within $6.75 \times 6.75 \times 1.5 \mu\text{m}^3$ with random average orientation and wobble angle. The density in our experiments is less than 3 molecules per $6.75 \times 6.75 \times 1.5 \mu\text{m}^3$ on average. The misdetection rate remains under 3.4% (Figure S16a) for the highest density tested, where misdetection typically occurs when the distance between two molecules is below the diffraction limit (Figure S16e).

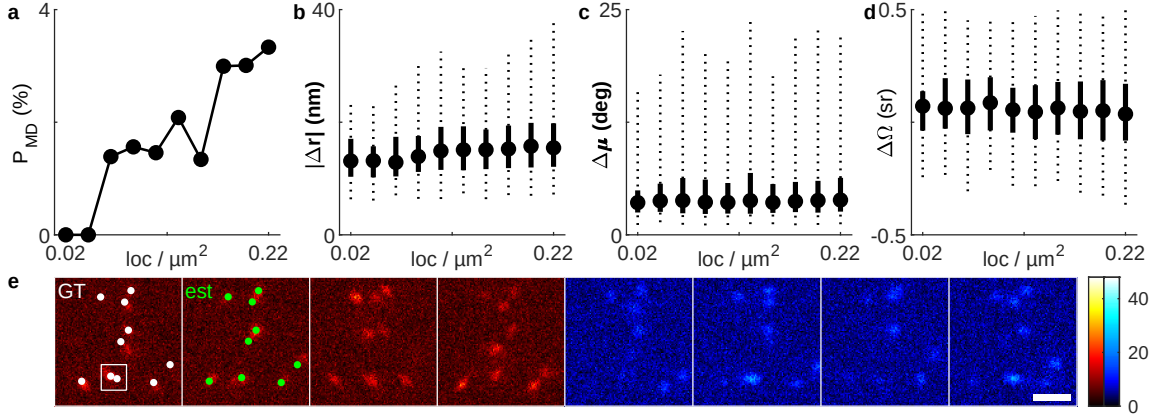


Figure S16: Performance of the detection and estimation algorithm as a function of molecular density (field of view: $6.75 \times 6.75 \mu\text{m}^2$). (a) Misdetection rate. (b) Distance between estimated position $\mathbf{r}_{\text{est}}$ and the ground truth $\mathbf{r}_{\text{true}}$. (c) Angle between estimated orientation μ_{est} and the ground truth μ_{true} . (d) Difference between estimated wobble cone angle Ω_{est} and the ground truth Ω_{true} . Dots, solid lines and dotted lines represent the median, 33rd and 67th percentile, and 10th and 90th percentile, respectively. A total of 5000 signal photons (for in-plane molecules at $z = 0$ nm) for each molecule and 40 per pixel background in all channels are detected. A total of 96 noisy realizations per molecular density are evaluated. (e) Representative frame of a misdetected molecule. White dots: ground truth position; green dots: estimated position. Colorbar: photons. Scale bar: 2 μm .

The estimation performance is evaluated by calculating

$$|\Delta \mathbf{r}| = |\mathbf{r}_{\text{est}} - \mathbf{r}_{\text{true}}| \quad (\text{S25a})$$

$$|\Delta \boldsymbol{\mu}| = \cos^{-1}(\boldsymbol{\mu}_{\text{est}} \cdot \boldsymbol{\mu}_{\text{true}}) \quad (\text{S25b})$$

$$\Delta \Omega = \Omega_{\text{est}} - \Omega_{\text{true}}. \quad (\text{S25c})$$

The medians of $|\Delta \mathbf{r}|$ and $|\Delta \boldsymbol{\mu}|$ with 10 molecules per image increase by 18% and 8%, respectively, compared to 1 molecule per image. The median of $\Delta \Omega$ does not increase as the density increases. These results show that the algorithm is capable of accurately and precisely measuring the position and orientation of the molecules under high density of SM flashes.

3.6 Pixel-wise basis inversion estimator for fast MVR image analysis

The MVR microscope encodes molecular orientation information into varying brightnesses within each imaging channel, making it capable of measuring the collective orientations of an ensemble of molecules within planar samples. Here, we demonstrate orientation-resolved diffraction-limited imaging of A β 42 fibrils. We first assemble an 8×6 basis matrix

$$\begin{aligned} \mathbf{B}_{\text{ens}} &= \begin{bmatrix} \mathbf{B}_{\text{ens},xx} & \mathbf{B}_{\text{ens},yy} & \mathbf{B}_{\text{ens},zz} & \mathbf{B}_{\text{ens},xy} & \mathbf{B}_{\text{ens},xz} & \mathbf{B}_{\text{ens},yz} \end{bmatrix} \\ &= \begin{bmatrix} 0.05 & 0.05 & 0.35 & -0.07 & -0.12 & 0.12 \\ 0.05 & 0.05 & 0.35 & 0.07 & -0.12 & -0.12 \\ 0.05 & 0.05 & 0.35 & -0.07 & 0.12 & -0.12 \\ 0.05 & 0.05 & 0.35 & 0.07 & 0.12 & 0.12 \\ 0.33 & 0.07 & 0 & 0 & 0 & 0 \\ 0.07 & 0.33 & 0 & 0 & 0 & 0 \\ 0.33 & 0.07 & 0 & 0 & 0 & 0 \\ 0.07 & 0.33 & 0 & 0 & 0 & 0 \end{bmatrix}, \end{aligned} \quad (\text{S26})$$

where

$$\mathbf{B}_{\text{ens},ij} = \begin{bmatrix} \sum_u \sum_v B_{ij}^{(1)}(u, v) \\ \sum_u \sum_v B_{ij}^{(2)}(u, v) \\ \vdots \\ \sum_u \sum_v B_{ij}^{(8)}(u, v) \end{bmatrix}, \quad (\text{S27})$$

$B_{ij}^{(k)}(u, v)$ represents the basis image B_{ij} in the k th imaging channel (Figure S4), the sums over u and v are taken over a 51×51 pixel region surrounding the DSF in each channel, and $i, j \in \{x, y, z\}$. We next

assemble an 8×1 vector $\mathbf{I}_{\text{ens},i} = [I^{(1)}(u,v), \dots, I^{(8)}(u,v)]^\top$ for each corresponding camera pixel (u,v) across the 8 MVR channels. The effective second-order orientational moments $\mathbf{m}_{\text{ens},i}$ associated with the corresponding (x,y) position in the sample can be computed from $\mathbf{I}_{\text{ens},i}$ as

$$\mathbf{m}_{\text{ens},i} = \mathbf{B}_{\text{ens}}^{-1} \mathbf{I}_{\text{ens},i}. \quad (\text{S28})$$

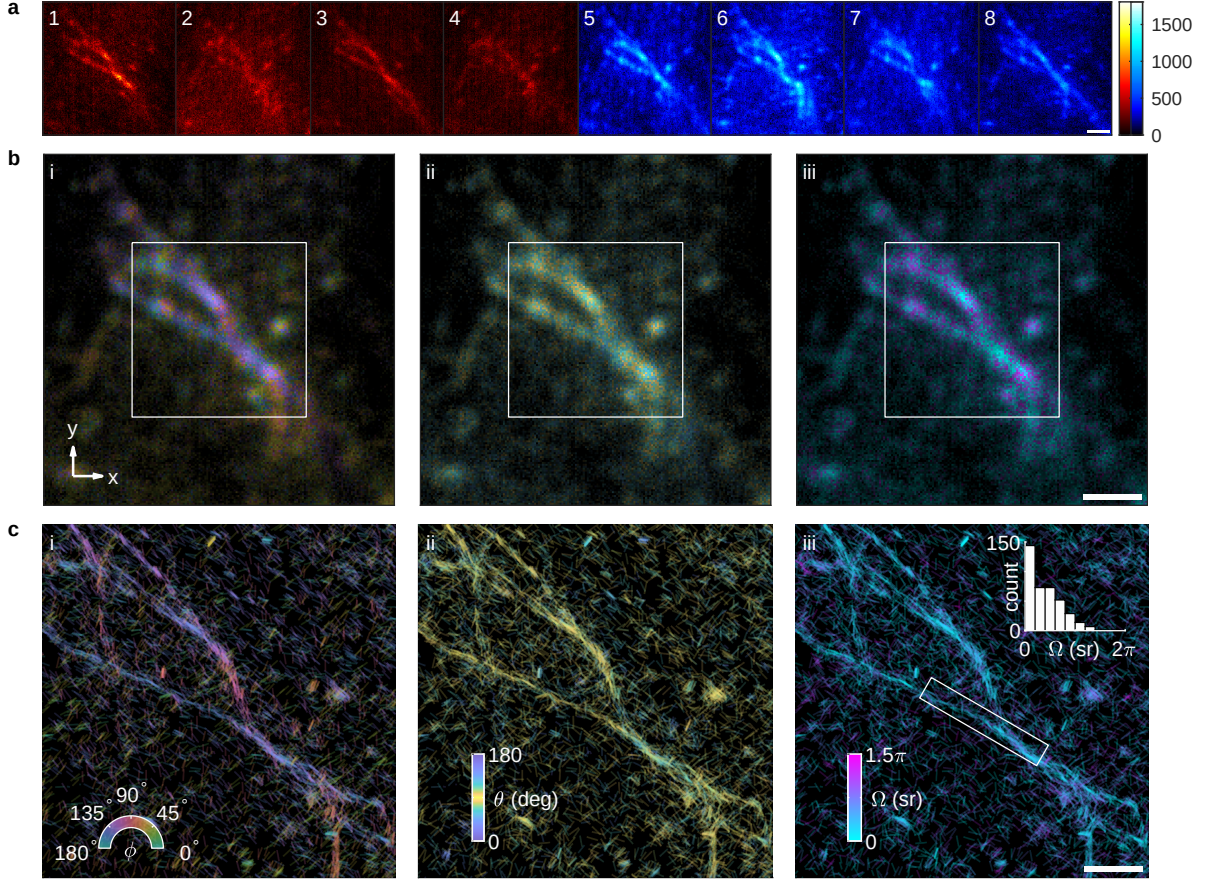


Figure S17: (a) Diffraction-limited raw raMVR images of Aβ42 fibrils. (b-c) The estimated (i) polar angle θ , (ii) azimuthal angle ϕ , and (iii) wobble angle Ω using (b) diffraction-limited ensemble imaging and (c) single-molecule orientation-localization microscopy (SMOLM). Lines are oriented according to the measured azimuthal angle ϕ and color-coded according to (i) ϕ , (ii) the measured polar angle θ and (iii) the measured wobble cone angle Ω . Their lengths are proportional to $\sin \theta$. Scale bars: $2 \mu\text{m}$ in (a,b), $1 \mu\text{m}$ in (c).

We then assemble a 3×3 matrix of these second moments and use eigendecomposition to compute λ_j, ν_j as

$$\begin{bmatrix} m_{\text{ens},i,xx} & m_{\text{ens},i,xy} & m_{\text{ens},i,xz} \\ m_{\text{ens},i,xy} & m_{\text{ens},i,yy} & m_{\text{ens},i,yz} \\ m_{\text{ens},i,xz} & m_{\text{ens},i,yz} & m_{\text{ens},i,zz} \end{bmatrix} = \sum_{j=1}^3 \lambda_j \nu_j \nu_j^\top. \quad (\text{S29})$$

The effective rotational constraint and average orientation for pixel i is therefore given by²

$$\gamma_i = \frac{2\lambda_1 - \lambda_2 - \lambda_3}{2(\lambda_1 + \lambda_2 + \lambda_3)} \quad (\text{S30})$$

$$\boldsymbol{\mu}_i = \boldsymbol{\nu}_1 \quad (\text{S31})$$

where λ_1 and $\boldsymbol{\nu}_1$ are the largest eigenvalue and its corresponding eigenvector.

The fast pixel-wise basis inversion estimator thusly directly utilizes the raw camera images (Figure S17a, a total of 301×301 pixels across 8 imaging channels corresponding to a $20 \times 20 \mu\text{m}^2$ field of view) to estimate the azimuthal angle ϕ [Figure S17b(i)], polar angle θ [Figure S17b(ii)], and wobble cone angle Ω [Figure S17b(iii)] of the ensemble of molecules in each pixel. The analysis runs at ~ 1.15 frames per second using a 4-core, 3.5 GHz CPU. Both the azimuthal and polar angles match those measured using SMOLM [Figure S17c(i,ii)]. However, since the diffraction-limited DSF mixes the fluorescence from all nearby SMs into the signal collected by each camera pixel, the effective wobble angle Ω of the ensemble is significantly larger than the wobble of each individual molecule [Figure S17c(iii)].

3.7 Robustness against optical aberrations and misalignment

To test the robustness of the raMVR system and detection and estimation algorithm against aberrations, we simulate ideal images with fluorescence wavelength of $\lambda = 633 \text{ nm}$ and sample refractive index of $n_2 = 1.33$. Aberrated images are compared to ideal images by calculating the difference in the brightness of each spot (Figure S18a-f), given by

$$|\Delta \mathbf{I}_{\text{ens}}| = |\Delta \mathbf{I}_{\text{ens,aberr}} - \Delta \mathbf{I}_{\text{ens,ideal}}|, \quad (\text{S32})$$

which indicates the amount of bias in the orientation estimation. The bias and precision for estimated orientation and axial position are evaluated by fitting the aberrated images with the ideal model using the detection and estimation algorithm introduced in Sections 3.2 and 3.3. For molecular orientation, x -oriented [Figure S18g(i)], y -oriented [Figure S18g(ii)], and z -oriented [Figure S18g(iii)] molecules and molecules with an orientation of $\boldsymbol{\mu}_{\text{true}} = [1, 1, 1]/\sqrt{3}$ [Figure S18g(iv)] are evaluated. For axial position, isotropic emitters located at $z = 1250 \text{ nm}$ [Figure S18h(i)], $z = 750 \text{ nm}$ [Figure S18h(ii)], and $z = 250 \text{ nm}$ [Figure S18h(iii)] are evaluated.

When the wavelength of the fluorophore is different from the model, the Jones matrix representing the polarization modulation using the VWP is given by¹⁴

$$\mathbf{J}_{\text{BFP}} = \begin{bmatrix} \cos^2(\phi_{\text{BFP}}/2) + \exp(j\psi) \sin^2(\phi_{\text{BFP}}/2) & [1 - \exp(j\psi)] \cos(\phi_{\text{BFP}}/2) \sin(\phi_{\text{BFP}}/2) \\ [1 - \exp(j\psi)] \cos(\phi_{\text{BFP}}/2) \sin(\phi_{\text{BFP}}/2) & \sin^2(\phi_{\text{BFP}}/2) + \cos^2(\phi_{\text{BFP}}/2) \exp(j\psi) \end{bmatrix}, \quad (\text{S33})$$

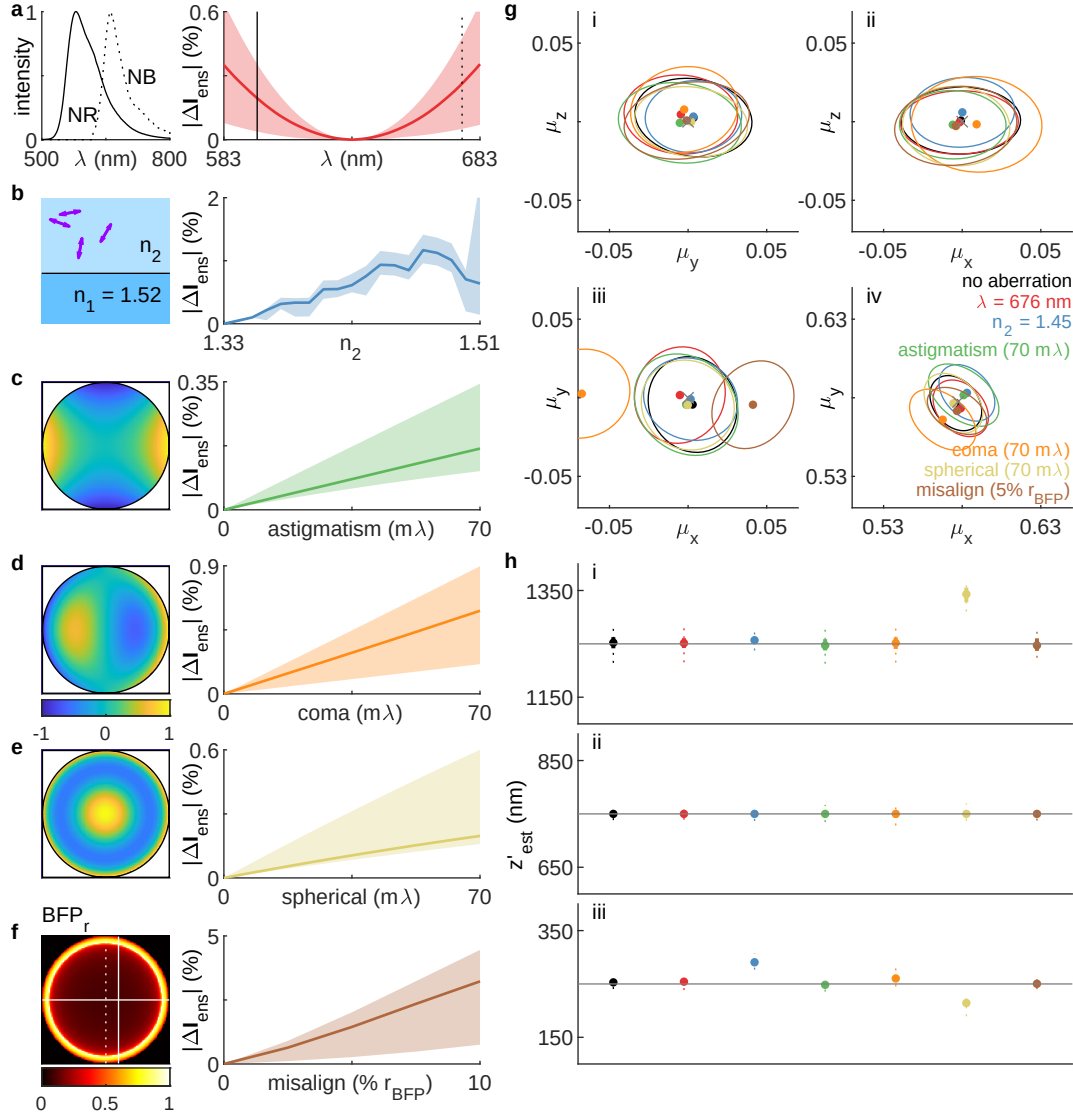


Figure S18: (a-f) Channel intensity change $|\Delta I_{\text{ens}}|$, relative to a perfect imaging system, as functions of (a) wavelength λ , (b) sample refractive index n_2 , (c) astigmatism, (d) coma, (e) spherical aberration, and (f) misalignment of the pyramid mirror. Lines represent the median; shaded areas represent the maximum and minimum. Colorbar: normalized phase in (c-e), normalized intensity in (f). (g) Error ellipses representing the estimated orientation for molecules with ground truth orientations μ_{true} (gray crosses) of (i) $[1, 0, 0]$, (ii) $[0, 1, 0]$, (iii) $[0, 0, 1]$, and (iv) $[1, 1, 1]/\sqrt{3}$ and axial positions $z = 750$ nm (best focus). Dots represent the average estimated orientation $\bar{\mu}_{\text{est}}$. (h) Shifted axial position estimates z'_{est} relative to ground truth (black line) for isotropic emitters ($\Omega = 2\pi$) located at (i) $z = 1250$ nm, (ii) $z = 750$ nm, and (iii) $z = 250$ nm. Dots, solid lines and dotted lines represent the median, 33rd and 67th percentile, and 10th and 90th percentile, respectively. Black: no aberration ($\lambda_{\text{ideal}} = 633$ nm), red: $\lambda = 676$ nm, blue: $n_2 = 1.45$, green: rms astigmatism of 70 m λ , orange: rms coma of 70 m λ , yellow: rms spherical aberration of 70 m λ , and brown: pyramid misalignment of 5% of the BFP radius. A total of 96 noisy realizations for each aberration scenario (5000 photons detected with a background of 40 photons/pixel total across all channels) are evaluated. Since axial positions are always measured relative to some reference point, we define the median measurement z'_{est} at $z = 750$ nm to be the reference for each condition.

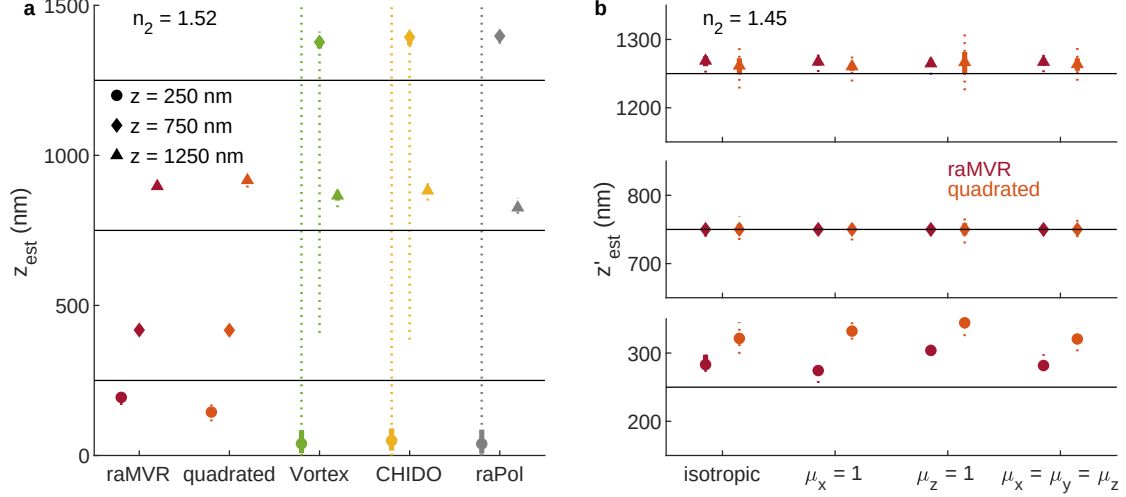


Figure S19: Measured axial position using various techniques when the sample refractive index is different from the model ($n_{2,\text{model}} = 1.33$). (a) Isotropic emitters ($\Omega = 2\pi$) with a sample refractive index of $n_2 = 1.52$. Red: raMVR; orange: quadrated DSF; green: Vortex DSF; yellow: CHIDO; gray: raPol standard DSF. (b) Isotropic emitters, x and z -oriented molecules and tilted molecules with a sample refractive index of $n_2 = 1.45$. Black lines represent ground-truth positions. Circles, diamonds and triangles represent the median for $z_{\text{true}} = 250$ nm, $z_{\text{true}} = 750$ nm, and $z_{\text{true}} = 1250$ nm, respectively. The measurements are shifted so that the median z'_{est} for $z = 750$ nm is aligned with the ground truth. Solid lines and dotted lines represent 33rd and 67th percentile, and 10th and 90th percentile, respectively. A total of 500 noisy realizations for each scenario are evaluated.

where $\psi = (\lambda_{\text{aberr}}/\lambda_{\text{ideal}})\pi$, instead of the matrix in Equation (S4). Since the pyramid mirrors are achromatic, the images can still be calculated using Equations (S5) and (S7). With 50 nm difference in wavelength, a peak difference of 0.6% is observed in measured channel intensity I_{ens} (Figure S18a). When using the ideal wavelength of 633 nm for imaging at 676 nm, we observe no bias or precision degradation for measuring the orientation and axial position of the molecule (Figure S18g,h, blue). The accurate and precise results show that the raMVR system can be used for multi-color imaging, e.g., imaging NR at 596 nm and Nile blue (NB) at 676 nm simultaneously by simply swapping fluorescence emission filters without changing the VWP or the pyramid mirrors.

When the sample refractive index is different from the model, the channel intensity change can be as large as 2% (Figure S18b). For $n_2 = 1.45$, a bias of 0.8° is observed for $\mu_{\text{true}} = [1, 1, 1]/\sqrt{3}$ [Figure S18g(iv)], and no bias is observed for other orientations [Figure S18g(i-iii)]. We next evaluate the axial localization bias (Figure S18h). Since the *relative* axial position reflects the shape deformation instead of the *absolute* axial position, we shift all estimates z_{est} so that the median of the shifted estimates z'_{est} for $z = 750$ nm is at the ground truth. We observe a bias up to 40 nm, which is consistent with the eccentricity of 0.28 (a difference of 38 nm between long and short axes for 350 nm spheres) and 0.20 (a difference of 40 nm between long and short axes for 1000-nm spheres) reported in the main text. Note that this simulation may overestimate the amount of bias since some of the collected fluorescence does not propagate through the spheres in these experiments. We also compare the axial localization bias

to those of other methods (Figure S19). For a fair comparison, we use the same maximum likelihood estimator for measuring z across all techniques; this estimator uses a full vectorial diffraction model of each DSF. The Vortex DSF, CHIDO and raPol standard DSF fail when the sample refractive index ($n_2 = 1.52$) is drastically different from the model ($n_{2,\text{model}} = 1.33$, Figure S19a), while raMVR and the quadrated DSF can still estimate z with a moderate bias. With a refractive index mismatch of $n_2 = 1.45$ (silica spheres) versus $n_{2,\text{model}} = 1.33$, the bias using raMVR is smaller than that of the quadrated DSF (Figure S19b).

We next simulate two typical aberration modes, astigmatism (Figure S18c) and coma (Figure S18d). With 70 mλ (rms) aberration, perturbations of 0.35% and 0.9% in the channel intensity are observed, respectively, relative to an aberration-free system. Besides a 3.9° bias for $\boldsymbol{\mu}_{\text{true}} = [0, 0, 1]$ under 70 mλ of coma [Figure S18g(iii)], no bias greater than $1 \times \sigma_{\boldsymbol{\mu}}^{\text{CRB}}$ (Table S1) is observed for either orientation [Figure S18g(i,ii)] or axial position measurements (Figure S18h). For 70 mλ (rms) spherical aberration (Figure S18e), the molecules’ apparent axial position is biased by up to 93 nm [Figure S18h(i)]. However, the orientation measurements are still accurate (Figure S18g).

We note that typical optical (phase) aberrations, e.g., defocus, astigmatism, and coma, only have a modest impact on the number of photons detected in each imaging channel. Thus, a glass pyramid mirror misalignment, i.e., a placement that does not split the BFP evenly, would affect the observed relative channel intensities to a greater degree. Here, we simulate the misalignment of the pyramid mirror in the radially-polarized imaging channel (Figure S18f). The maximum channel intensity difference is 5% for a misalignment of 10% of the radius of the BFP (0.24 mm in our setup). Despite the large change in the channel intensity, we only observe a bias of 2° for $\boldsymbol{\mu}_{\text{true}} = [0, 0, 1]$ in the orientation measurements [Figure S18g(iii)]. No bias is observed in axial localization (Figure S18h).

3.8 Limitations of the detection and estimation algorithm

Our single-molecule detection algorithm relies on first fitting DSF spots in each imaging channel. When molecules are further away from the focal plane, the shapes of their DSFs are significantly different from those of in-plane molecules; raMVR’s usable depth range is limited by the reduced detection rate. The algorithm is also computationally expensive; it takes ~5 hours to analyze 2500 frames with a field of view of $23 \times 23 \mu\text{m}^2$ (344×344 total pixels per frame) on a 32-core, 3.7 GHz CPU. Both these limitations can be resolved in the future using more advanced, e.g., deep-learning based, detection and estimation algorithms.^{25,26}

4 Sample preparation

The volume of each component and centrifuge speed for preparing lipid-coated silica spheres are listed in Table S4. The chemical structures of the fluorescent molecules are shown in Figure S20.

Table S4: Volume of each component and centrifugation speed for preparing lipid-coated silica spheres.

radius of sphere (nm)	volume of 40 $\mu\text{g/mL}$ sphere solution (μL)	volume of Tris Ca^{2+} buffer (μL)	volume of lipid LUV solution (μL)	centrifugation speed (rpm)
150	1.4	53.6	125.0	2000
350	3.0	57.0	120.0 (60.0*)	1300
1000	6.5	83.5	90.0	500

* reduce the amount of excessive lipid for A β 42 experiment

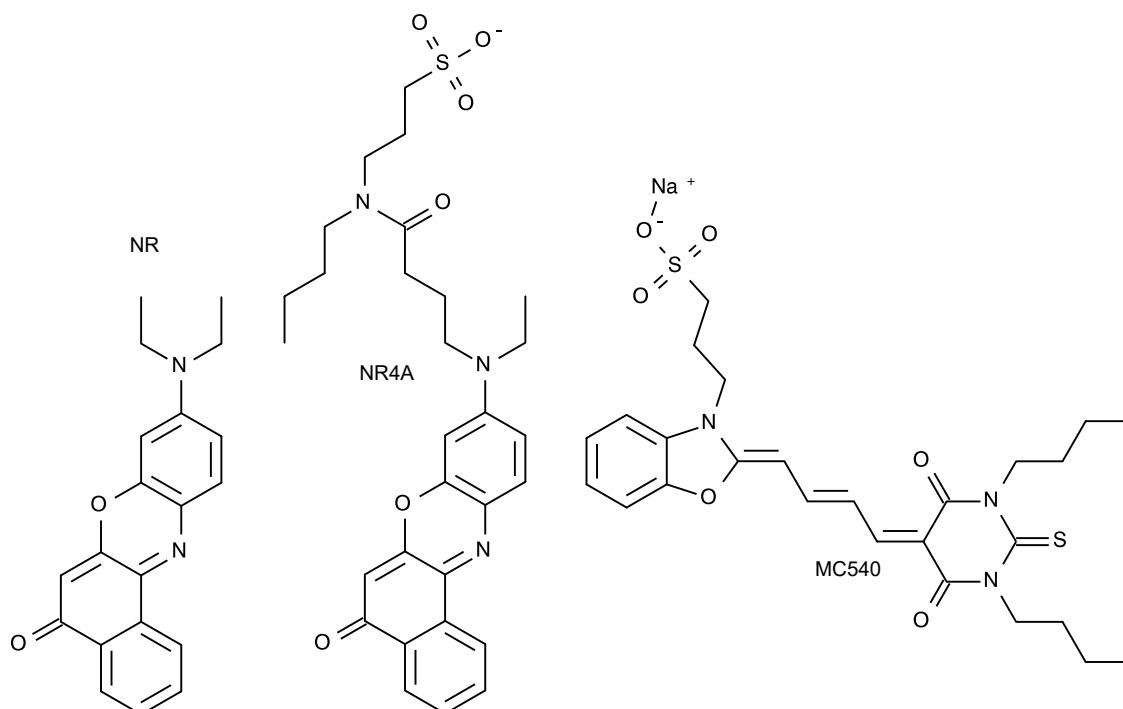


Figure S20: Chemical structures of fluorescent dyes Nile red (NR), NR4A, and merocyanine 540 (MC540).

5 Additional data analysis

5.1 Emission anisotropy of fluorescent beads

We captured a total of 15,632 images of 977 fluorescent beads (Thermo Fisher F8800, 0.1- μm diameter, embedded in lens immersion oil). The measured apparent wobble angle is 5.44 ± 0.35 sr [median $\pm$ standard deviation, Figure S21a(i)], which is smaller than simulated measurements of isotropic emitters [5.89 ± 0.02 sr, Figure S21b(i)]. However, the measured apparent wobble angle is consistent with previous observations.²⁷ The apparent average orientations are also more diverse [Figure S21a,b(ii)]. These effects likely stem from imperfections in the fluorescent beads; we have previously shown that emission anisotropy varies significantly across a population of fluorescent beads.⁵ For example, the raw image in Figure S21c shows a more isotropic emission pattern compared to that in Figure S21e.

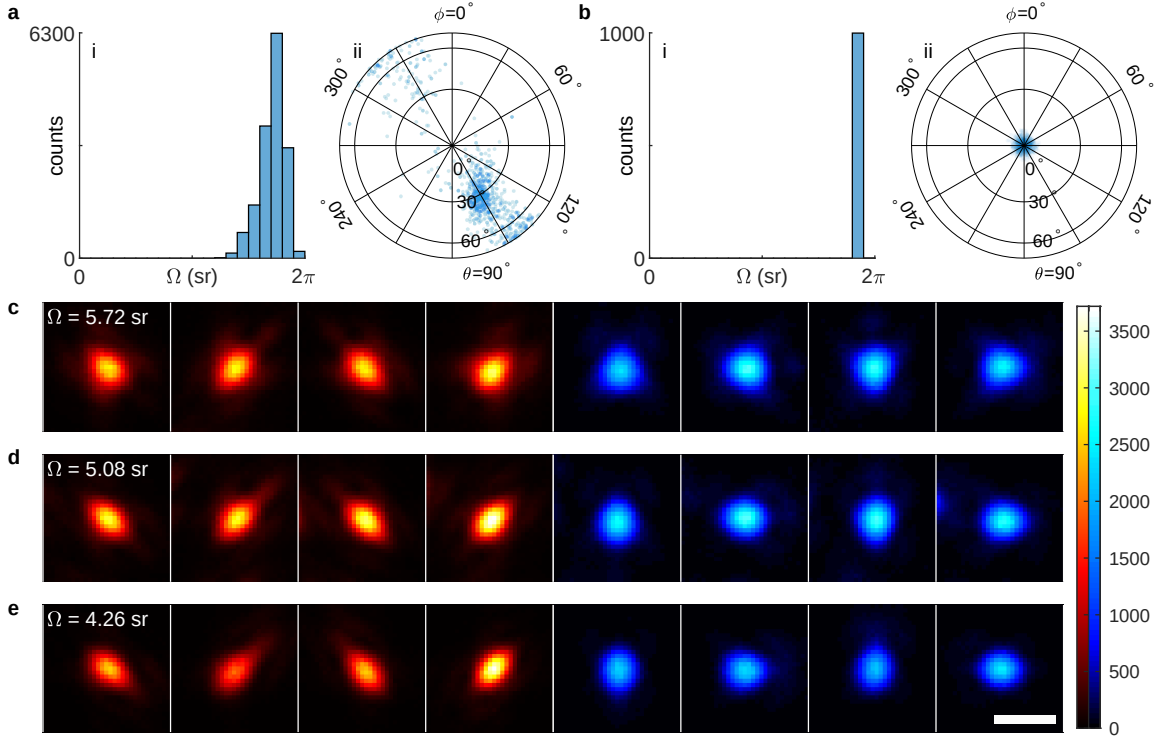


Figure S21: Estimated (i) wobble angles and (ii) orientations of (a) fluorescent beads and (b) simulated isotropic emitters with an SBR of 5×10^5 signal photons and 25 photons per pixel background across all channels. (c-e) Example images of fluorescent beads with apparent wobble angles of (c) $\Omega = 5.72$ sr, (d) $\Omega = 5.08$ sr, and (e) $\Omega = 4.26$ sr. Colorbar: photons; scale bar: 500 nm.

5.2 Additional data for Nile red molecules bound to lipid-coated spheres

We show additional cross-sectional views of 150-nm radius spheres (Figure S22), demonstrating that raMVR imaging accurately resolves the hollow spherical morphology and NR alignment within the SLB.

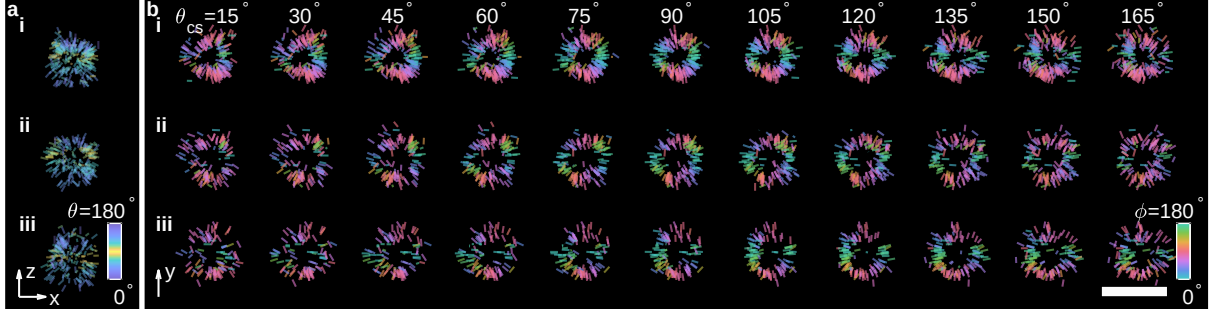


Figure S22: The (a) xz view and (b) cross-section views of three 150-nm lipid-coated spheres i-iii. Lines are oriented and color-coded according to the measured (a) polar angle θ and (b) azimuthal angle ϕ . Their lengths are proportional to $\sqrt{\mu_x^2 + \mu_z^2}$ in (a) and the length of the unit vector $\boldsymbol{\mu}$ projected onto the cross-section planes in (b). The data in (i) are duplicated from Figure 3 for ease of comparison. Scale bar: 500 nm.

The apparent thickness of the SLBs on the silica spheres are reported as the full width at half maximum (FWHM) of the distributions in Figure S23. The apparent thicknesses of the 150-nm and 350-nm spheres are consistent with raMVR's localization precision (Table S5, $\text{FWHM} \approx 2.36\sigma$) plus the thickness of ~ 5 nm for DPPC SLBs.²⁸ The 1000-nm spheres exhibit larger thicknesses due to the performance degradation caused by reduced signal collected from emitters far from the coverslip, i.e., less supercritical light is captured.

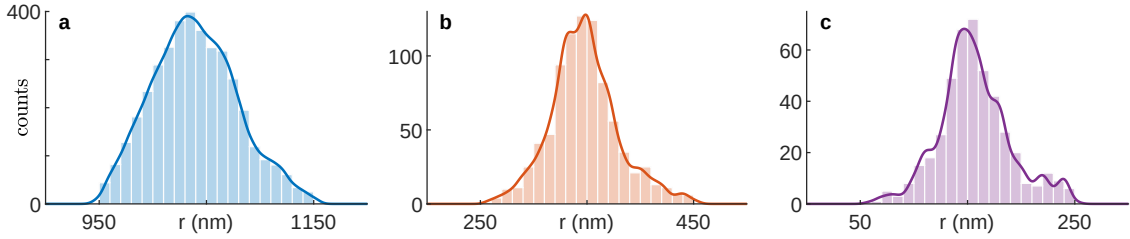


Figure S23: Distribution of sphere radii r , as measured by each NR localization, for (a) the 1000 nm-radius sphere, (b) the 350-nm sphere, and (c) the 150-nm sphere in Figure 3. Solid lines represent the smoothed curves for calculating the full width at half maximum (FWHM). Assuming a lipid bilayer thickness of 5 nm, these widths correspond to 3D localization precisions of 30.1 nm for 1000-nm spheres, 15.9 nm for 350-nm spheres, and 19.8 nm for 150-nm spheres. For comparison, our detection and estimation algorithm achieves a 3D localization precision of 13.6 nm (Tables S1 and S3) using simulated data.

5.3 Kolmogorov-Smirnov tests for comparing Nile red wobble-angle distributions

We apply two-sample Kolmogorov-Smirnov tests, using the MATLAB function `kstest2`, for comparing NR wobble within pure-lipid SLBs [Figure 4c(i)] to that within SLBs containing A β 42 [Figure 4c(ii-iv)]. The tests return p -values of 4×10^{-7} , 7×10^{-5} , and 3×10^{-3} for 1-day, 5-day, and 7-day incubation periods, respectively. In contrast, testing the localizations within slices z_1 and z_2 of two 350-nm pure-lipid spheres (Figure 4a,b vs. Figure 3) yields a p -value of 0.16 (Figure S24c,f). These tests suggest

that NR wobble distributions are significantly different when A β 42 is present in the SLB. Note that the spheres in Figure 4 (day 0) and Figure S24 are incubated for 7 days as a negative control to show that the changes in wobble angle are due to amyloid aggregates instead of lipid degradation.

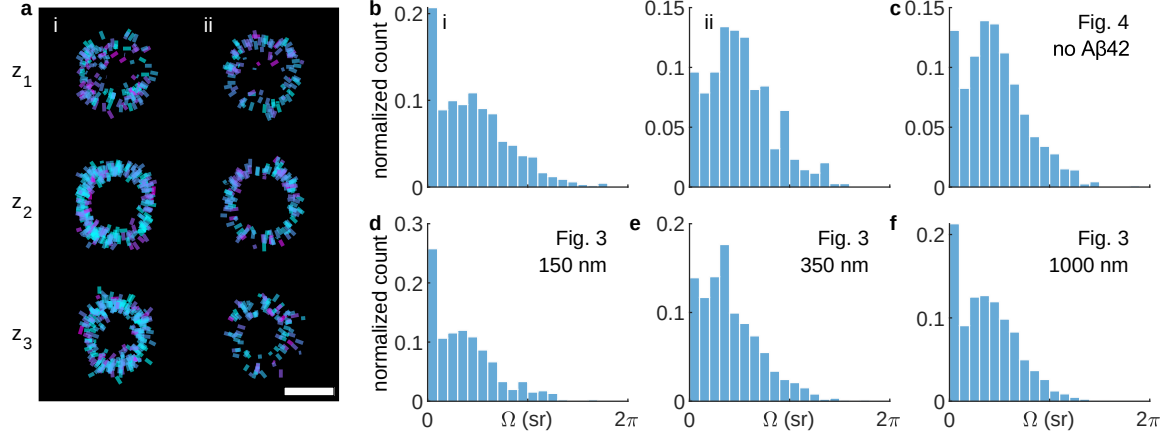


Figure S24: (a) Two additional 350-nm lipid-coated spheres i and ii incubated without A β 42 monomers after 7 days. Localizations are shown from the 150 nm-thick z slices marked in Figure 4(a) centered at ($z_1 = 600, z_2 = 400, z_3 = 200$) nm. Lines are oriented according to the measured azimuthal angle ϕ and color-coded according to the measured wobble cone angle Ω . Their lengths are proportional to $\sin \theta$. Scale bar: 500 nm. (b-f) Distribution of measured wobble cone angles for (b) the spheres in (a), (c) a sphere incubated without A β 42 (slices z_1 and z_2 in Figure 4), and (d-f) spheres of various sizes in Figure 3.

5.4 Principal component analysis for identifying amyloid intercalation within lipid bilayers

Here, we apply principal component analysis (PCA) to identify which areas on the lipid-coated spheres show the intercalation and aggregation of amyloid peptides based on the orientation and wobble of NR molecules. We first isolate all localizations within 100 nm of the surfaces of 350-nm spheres. The PCA projected value (principal component score, PCS) p for each localization is given by $p = [\theta_{\perp}, \Omega] \mathbf{v}_{\text{cov}}$, where $\mathbf{v}_{\text{cov}}$ (Figure S25) is the eigenvector corresponding to the largest eigenvalue of the covariance matrix $\text{cov}(\theta_{\perp}, \Omega)$ for all localizations on the 25 spheres we analyzed (Figures 4 and S26). Both the orientation distribution (Figure S25) and PCA (Figure S27) qualitatively show strong heterogeneity in the magnitude and spatial distribution of orientational disorder. The reconstruction of larger A β 42 aggregates linking two spheres is shown in Figure S28.

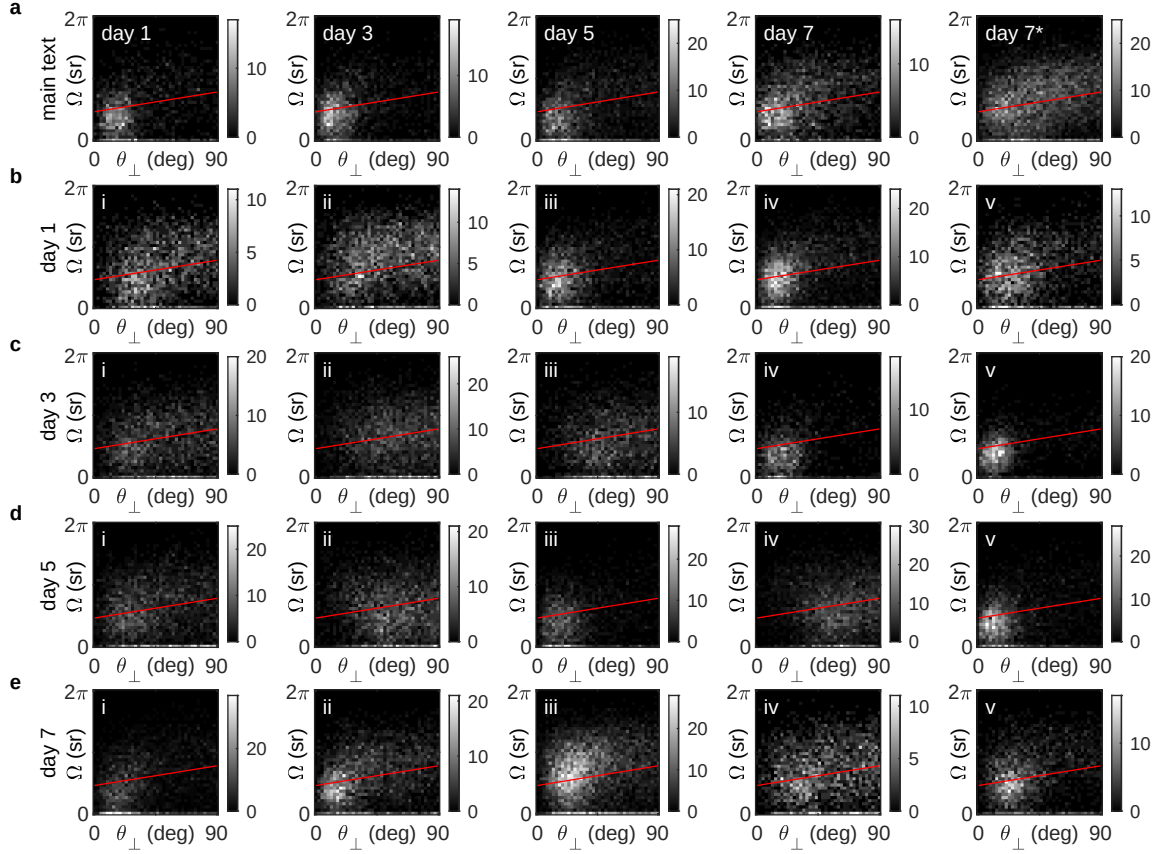


Figure S25: Orientation distribution of NR molecules on lipid-coated spheres after incubation with Aβ42 in the (a) Main text (Figure 3) and (b-d) Figure S26. The incubation times are (b) 1 day, (c) 3 days, (d) 5 days and (e) 7 days. Colorbar: localizations per bin. The orientation $\theta_{\perp}$ and wobble Ω of every localization is projected onto ν_{cov} (direction of the red line) to obtain a single a principal component score (PCS) p .

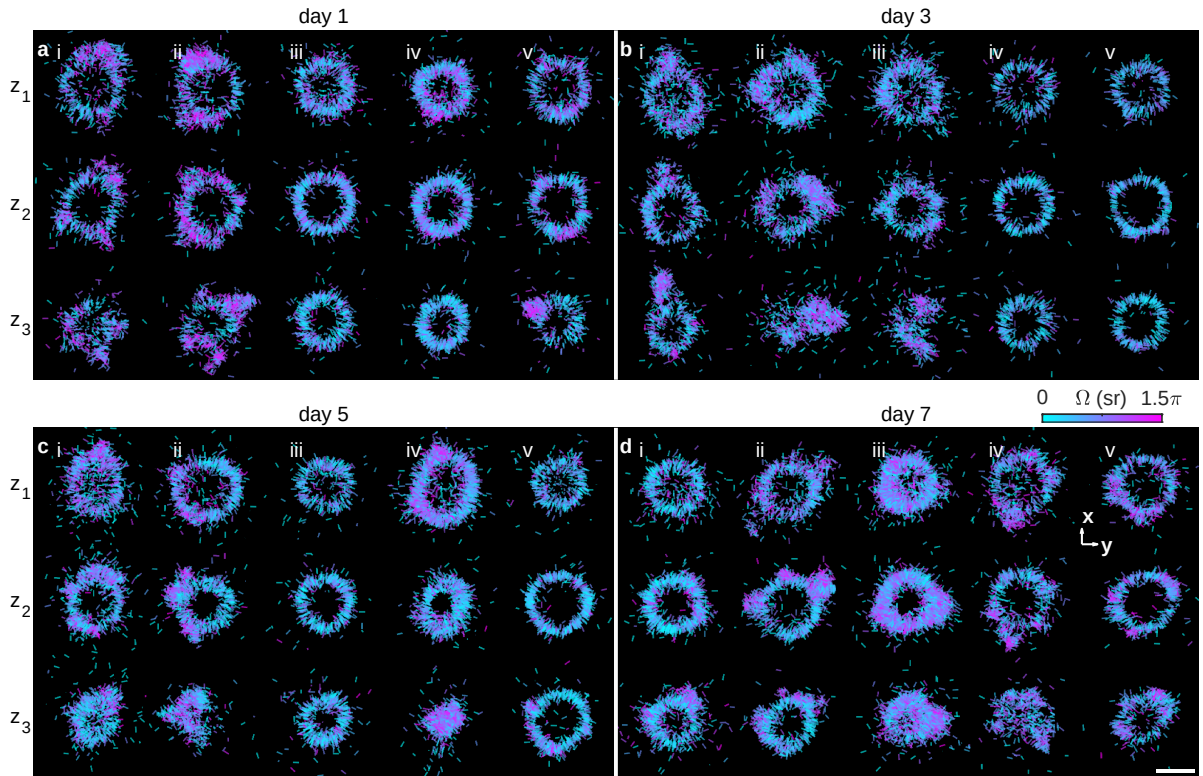


Figure S26: Additional 350-nm lipid-coated spheres incubated with A β 42 monomers after (a) 1 day, (b) 3 days, (c) 5 days and (d) 7 days. Localizations are shown from the 150 nm-thick z slices marked in Figure 4(a) centered at $(z_1 = 600, z_2 = 400, z_3 = 200)$ nm. Lines are oriented according to the measured azimuthal angle ϕ and color-coded according to the measured wobble cone angle Ω . Their lengths are proportional to $\sin \theta$. Scale bar: 500 nm.

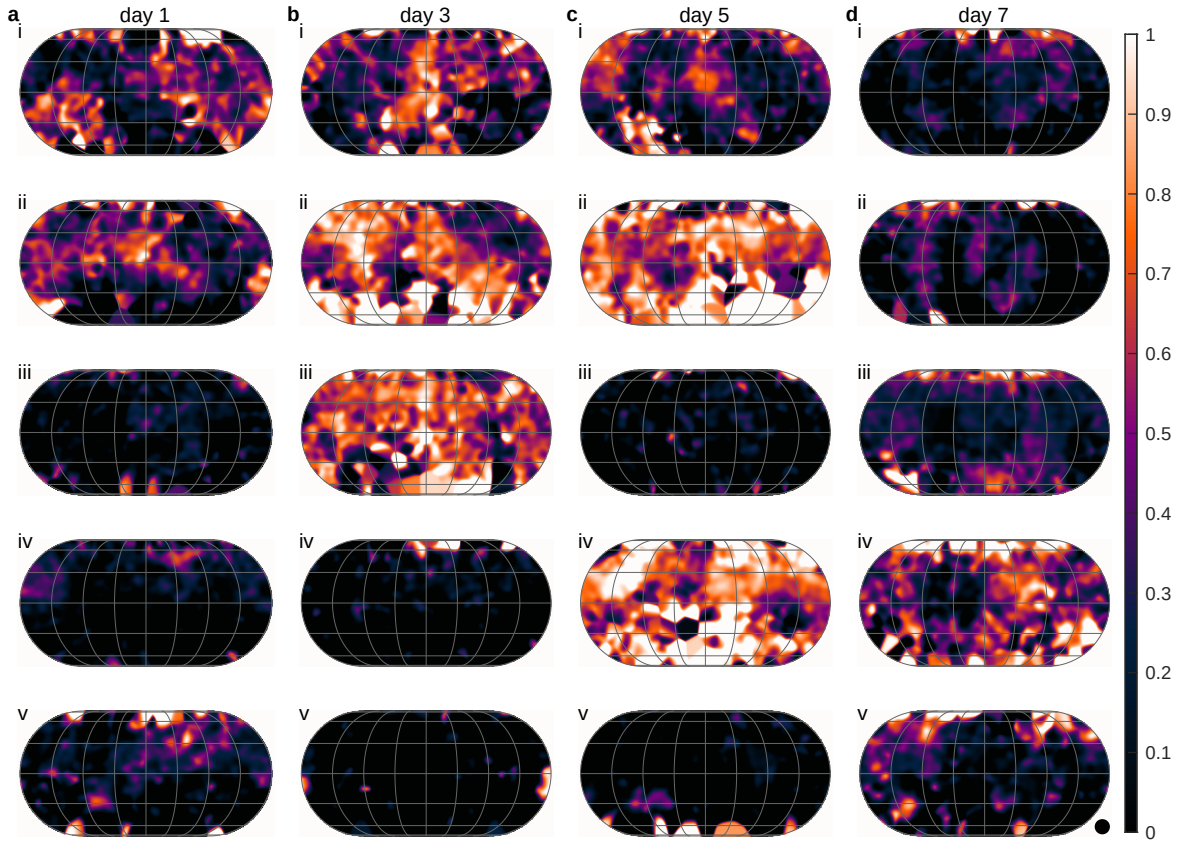


Figure S27: Amount of amyloid aggregates on the DPPC SLBs near the sphere surface according to principal component analysis for the spheres in Figure S26 plotted using the Eckert IV Projection. The incubation times are (b) 1 day, (c) 3 days, (d) 5 days and (e) 7 days. Colorbar: A β 42 index. Scale area: 10^4 nm^2 .

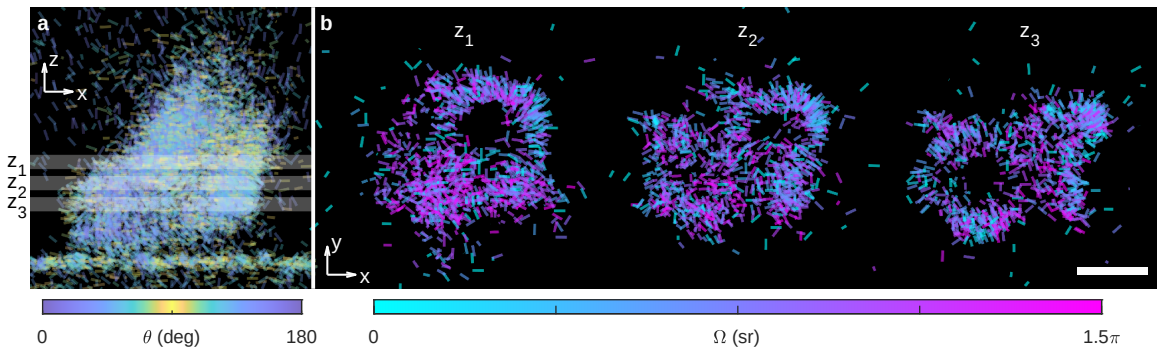


Figure S28: NR localizations on amyloid aggregates linking two lipid-coated spheres. (a) xz -view of all NR localizations after incubation with A β 42 monomers for 5 days. Lines are oriented and color-coded according to the measured polar angle θ . Their lengths are proportional to $\sqrt{\mu_x^2 + \mu_z^2}$. (b) Localizations within the 150 nm-thick z slices marked in (a) centered at ($z_1 = 700, z_2 = 550, z_3 = 400$) nm. Lines are oriented according to the measured azimuthal angle ϕ and color-coded according to the measured wobble cone angle Ω . Their lengths are proportional to $\sin \theta$. Scalebar: 500 nm.

5.5 Fourier shell correlation analysis and additional cell imaging data

A diffraction-limited focal stack of the cell in Figure 5 is shown in Figure S29. To evaluate the resolution of the reconstructed super-resolution 3D image, we evaluate the Fourier shell correlation^{29,30} of $(2\text{ }\mu\text{m})^3$ cubic volumes (Figure 5a and Figure S30). We randomly assign all localizations into two groups and assemble two $100 \times 100 \times 100$ 3D images with a bin size of $(20\text{ nm})^3$ based on each group. We then compute the discrete Fourier transform of both images as $\mathbb{I}_1$ and $\mathbb{I}_2$ and calculate the correlation C_i and

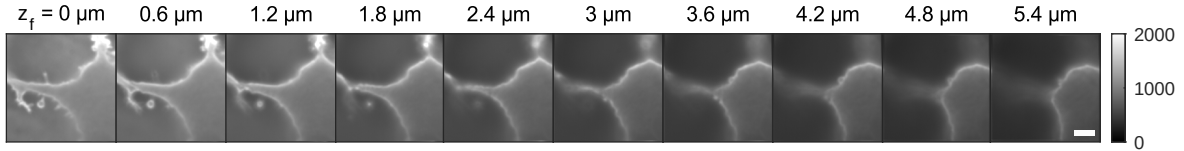


Figure S29: Diffraction-limited focal stack of the cell in Figure 5. Colorbar: photons; scale bar: 5 μm .

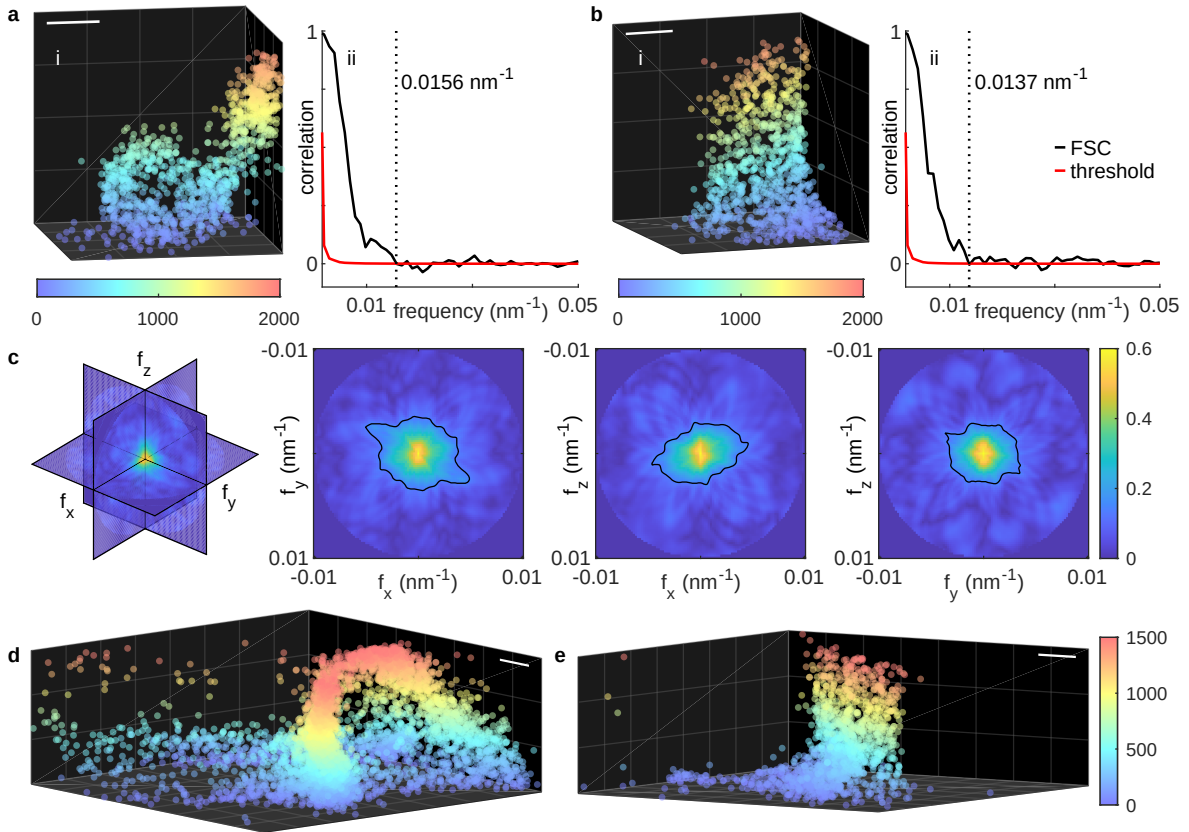


Figure S30: Image resolution analysis using Fourier shell correlation. (i) Three-dimensional localizations and (ii) Fourier shell correlation of the (a) yellow and (b) green boxed areas in Figure 5a. Black lines represent the correlation and red lines represent the threshold. (c) Cross-sections through the Fourier plane correlation cube corresponding to panel (a). Black lines represent the contours of $1/8$. Colorbar: correlation. (d,e) Three-dimensional views of the areas in Figure 5e and Extended Data Figure 5e. Colorbar: axial position z (nm); scale bar: 500 nm.

threshold T_i of the i th shell S_i as (Figure S30)

$$C_i = \frac{\sum_{S_i} \mathbb{I}_1 \mathbb{I}_2^*}{\sqrt{(\sum_{S_i} |\mathbb{I}_1|^2) (\sum_{S_i} |\mathbb{I}_2|^2)}} \quad (\text{S34a})$$

$$T_i = \sqrt{\frac{2}{N_i}}, \quad (\text{S34b})$$

where N_i is the number of pixels within shell S_i . The image resolution is determined by inverting the smallest spatial frequency where $T_i \geq S_i$. The cross-sections in the Fourier plane correlation cube (Figure S30c) show isotropic resolution along the lateral and axial directions, which justifies the usage of Fourier shell correlation.³¹

Figure S31a,b(i) show images of two single molecules with a brightness close to the median signal level in the cell imaging and the fitted image based on our model [Figure S31a,b(ii)]. The orientation estimates match our intuition. The spot in channel 3 is dimmer in Figure S31a, suggesting a positive m_{xy} according to the basis images [Figure S4(iv)], which agrees with the estimation of $\phi = 37.8^\circ$; the spot in channel 2 is dimmer in Figure S31b, suggesting a negative m_{xy} , which agrees with the estimation of $\phi = 138.3^\circ$. Figure S31c shows a frame with multiple overlapping DSFs that is successfully resolved using the algorithm in Section 3. We also show the average DSF within the boxed area in Extended Data Figure 5e(iv) (Figure S31d,e). Since the measured azimuthal orientations of most molecules are along the $+45^\circ$ direction ($\phi = 51.5^\circ \pm 42.0$), we see much stronger fluorescence in channels 2 and 4 compared to 1 and 3.

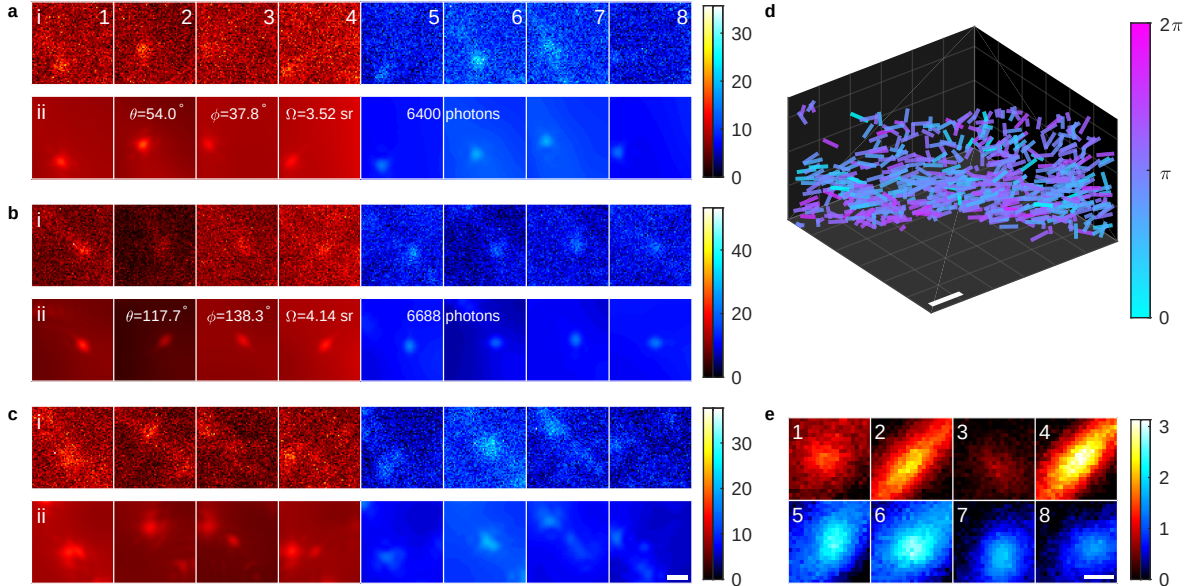


Figure S31: Comparing (a-c) (i) raw DSF images in cell imaging to (ii) fitted images reconstructed using the raMVR analysis algorithm in Section 3. (d) Three-dimensional localizations and (e) the average DSF of the boxed area in Extended Data Figure 5e(iv). Lines are oriented according to μ and color-coded according to the wobble angle Ω . Colorbar: photons in (a-c,e); wobble angle Ω (sr) in (d); scale bar: 1 μm in (a-c), 500 nm in (d,e).

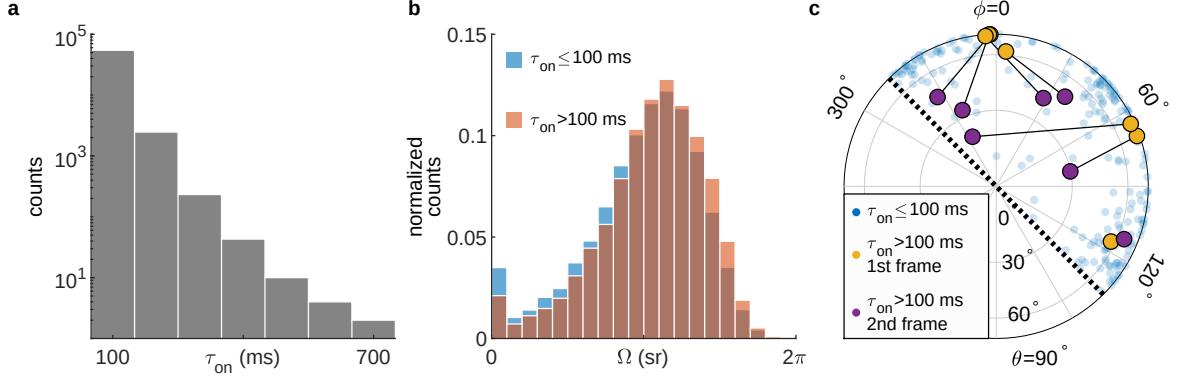


Figure S32: (a) Fluorescence burst lengths τ_{on} of MC540 molecules bound within the membranes of HEK-293T cells (57293 total bursts). (b) Distribution of measured wobble angle Ω . Blue: 54215 molecules with burst lengths shorter than one camera frame; orange: 3078 molecules with burst lengths longer than one camera frame. (c) Orientations of MC540 molecules bound within the membrane in [Extended Data Figure 5d\(i\)](#). Blue: molecules with burst lengths shorter than one camera frame; yellow: orientation of molecules during first frame with a burst length $\tau_{\text{on}} \in (100, 200]$ ms; purple: orientation of molecules during the second frame with a burst length longer than one camera frame.

The average fluorescence burst length $\bar{\tau}_{\text{on}} = 67$ ms of MC540 molecules bound within the membranes of HEK-293T cells is measured via exponential fit (Figure S32a). To check whether the burst length is sufficiently long for MC540 molecules to explore all possible orientations, we plot the distribution of measured wobble cone angle as a function of $\bar{\tau}_{\text{on}}$ (Figure S32b). A signal threshold of 3000 photons is applied to reduce the effect of SBR-induced measurement bias.² No correlation between measured wobble and burst length is observed, showing that the MC540 molecules sample their entire accessible orientation space within the membrane before unbinding. Examining the area in [Extended Data Figure 5d\(i\)](#) that contains both parallel and perpendicularly oriented molecules, we observe no obvious patterns in their orientation trajectories (Figure S32c).

Since NR easily penetrates cell membranes and binds to intracellular lipid structures, we instead image its variant, NR4A, bound within the membranes of fixed HEK-293T cells. We observe disordered areas where molecules' orientations exhibit a wider distribution [[Extended Data Figure 6e,g\(i\)](#), $\Delta\phi = 67.7^\circ \pm 36.7^\circ$], as well as ordered areas where molecules are close to perpendicular to the membrane [$\Delta\phi = 75.4^\circ \pm 28.0^\circ$ in [Extended Data Figure 6e,g\(ii\)](#), $\Delta\phi = 113.2^\circ \pm 28.4^\circ$ in [Extended Data Figure 6f,g\(iii\)](#), and $\Delta\phi = 84.7^\circ \pm 24.2^\circ$ in [Extended Data Figure 6h,j\(iv\)](#)]. Again, we observe changes in the orientation distributions on a continuous membrane, from $\Delta\phi = 62.0^\circ \pm 28.1^\circ$ [[Extended Data Figure 6h,j\(v\)](#)] to $\Delta\phi = 90.0^\circ \pm 24.4^\circ$ [[Extended Data Figure 6h,j\(vi\)](#)]. No parallel population is observed for NR4A.

5.6 Photon statistics

We excite the prepared DPPC SLBs using a 532-nm continuous-wave laser (Cobolt 0532-06-91-0200-100). The collected fluorescence is filtered by a dichroic mirror (DM, Semrock Di03-R532) and a bandpass filter (BP, Semrock FF01-596/83). For fixed HEK-293T imaging, we use a 561-nm continuous-wave laser (Coherent Sapphire 561-200 CW CDRH), a dichroic mirror (Di03-R488/561) and a bandpass filter (Semrock FF01-523/610). The excitation power, photon statistics, and measurement precision in the experiments are listed in Table S5. The three-dimensional localization precision $\sigma_{\mathbf{r}}$ is reported as the square root of the standardized generalized variance, given by

$$\sigma_{\mathbf{r}} = \left\{ \det \left(\begin{bmatrix} \mathcal{J}_{xx} & \mathcal{J}_{xy} & \mathcal{J}_{xz} \\ \mathcal{J}_{yx} & \mathcal{J}_{yy} & \mathcal{J}_{yz} \\ \mathcal{J}_{zx} & \mathcal{J}_{zy} & \mathcal{J}_{zz} \end{bmatrix} \right) \right\}^{-1/6}. \quad (\text{S35})$$

Table S5: Peak excitation intensity $I_{\text{ex,max}}$ (W/cm²), photon statistics [signal s (photons) and background b (photons/pixel)] and measurement precision (Cramér-Rao bound) of NR molecules. The precision σ of estimating lateral position x (nm), axial position z (nm), three-dimensional position $\mathbf{r}$ (nm), average orientation μ (deg), and wobble Ω (sr) are compared.

	Lipid-coated spheres with various radii, Figures 3 and S22			A β 42 incubated with lipid-coated spheres for multiple days, Figures 4 and S26				HEK-293T Figures 5, S30, Extended Data Figure 5	A β 42 fibrils, Figure S17
	1000 nm	350 nm	150 nm	1 day	3 days	5 days	7 days		
$I_{\text{ex,max}}$		1526			1129			1098	565
s	5045	5214	3564	4755	5501	6239	5524	5872	3326
b	36	46	41	34	36	51	37	67	44
σ_x	10.2	11.0	15.3	10.5	9.3	9.7	9.4	11.9	17.1
σ_z	11.5	12.5	17.3	11.9	10.6	11.1	10.7	13.4	19.4
$\sigma_{\mathbf{r}}$	10.2	11.1	15.4	10.6	9.4	9.8	9.5	11.9	17.2
σ_{μ}	1.9	2.1	2.9	2.0	1.8	1.8	1.8	2.2	3.2
σ_{Ω}	0.22	0.24	0.33	0.23	0.20	0.21	0.20	0.25	0.37

List of Supplementary Movies and Images

The movies and images listed below are available from OSF at osf.io/s8m4x/.

- Movie S1 Fluorescent beads on the surface of a glass coverslip ($z = 0$), embedded in lens immersion oil, axially scanned from $z_f = -500$ nm to $z_f = 500$ nm. Color scale: photons; scale bar: 2 μ m.
- Movie S2 Three-dimensional view and cross-sectional images of Nile red localizations on the 1000 nm-radius lipid-coated sphere in Figure 3. Localizations are depicted as points at left and as line segments matching the measured molecule orientations at right. Color scale: (left) polar angle θ in deg, (right) azimuthal angle ϕ in deg.
- Movie S3 Three-dimensional view of Nile red localizations on 350 nm-radius lipid-coated spheres incubated without (day 0) and with amyloid beta (A β 42, Figure 4). Color scale: azimuthal angle ϕ in deg.
- Movie S4 Two-dimensional (xy) view of merocyanine 540 localizations along the HEK-293T cell membrane in Extended Data Figure 5a, and a sliding window showing yz cross-sections. Localizations are depicted as points at left and as line segments matching the measured molecule orientations at right. Color scale: axial position z in nm.
- Movie S5 Three-dimensional view of merocyanine 540 localizations along the HEK-293T cell membrane in Figure 5c-e and Extended Data Figure 5c-e. Localizations are depicted as line segments matching the measured molecule orientations. Color scale: (left) polar angle ϕ in deg, (middle) wobble solid angle Ω in sr, (right) azimuthal angle ϕ in deg.
- Movie S6 Blinking merocyanine 540 molecules along the HEK-293T cell in Figure 5a. Dashed lines represent where the cell is in contact with coverslip (bottom-right area in each channel). The nominal focal plane is placed at $z_f = 1200$ nm. Color scale: photons; scale bar: 5 μ m.
- Image S1 High-resolution image of merocyanine 540 localizations on the HEK-293T cell in Figure 5. Lines are oriented and color-coded according to the azimuthal angle ϕ . Their lengths are proportional to the projection of the unit vector $\boldsymbol{\mu}$ into the xy plane and are equal to $100 \sin \theta$ nm.
- Image S2 High-resolution image of merocyanine 540 localizations on the HEK-293T cell in Extended Data Figure 5. Lines are oriented and color-coded according to the azimuthal angle ϕ . Their lengths are proportional to the projection of the unit vector $\boldsymbol{\mu}$ into the xy plane and are equal to $100 \sin \theta$ nm.

Image S3 High-resolution image of NR4A localizations on the HEK-293T cell in [Extended Data Figure 6a,b](#). Lines are oriented and color-coded according to the azimuthal angle ϕ . Their lengths are proportional to the projection of the unit vector $\boldsymbol{\mu}$ into the xy plane and are equal to $100 \sin \theta$ nm.

Image S4 High-resolution image of NR4A localizations on the HEK-293T cell in [Extended Data Figure 6c,d](#). Lines are oriented and color-coded according to the azimuthal angle ϕ . Their lengths are proportional to the projection of the unit vector $\boldsymbol{\mu}$ into the xy plane and are equal to $100 \sin \theta$ nm.

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