MEASURING SINGLE-MOLECULE ORIENTATIONS USING A RADIALLY AND AZIMUTHALLY POLARIZED EPIFLUORESCENCE MICROSCOPE

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Single-molecule orientation localization microscopy (SMOLM) has been recently developed to measure the orientations of single molecules (SMs) in addition to their positions. In typical SM experiments, a camera detects multiple photons during each acquisition frame. Therefore, the rotational dynamics of SMs are inherently encoded in the detected images, i.e., a rotating molecule can be parameterized by its average orientation $\bar{\boldsymbol{\mu}} = [\bar{\mu}_x, \bar{\mu}_y, \bar{\mu}_z]$, where $\bar{\mu}_z$ represents the direction parallel to the optical axis, and wobble solid angle Ω over a unit sphere.

Since the polarization of light emitted by SMs depends on their orientations, a widely used method to measure molecular orientation is to add a polarizing beam splitter to a standard microscope to create *x*- and *y*-polarized (*xy*Pol) imaging channels, which can sensitively differentiate *x*- and *y*-oriented molecules. However, it is difficult to measure the orientation of molecules with non-zero $\bar{\mu}_z$ components since the photons emitted by *z*-oriented molecules are evenly distributed between the *x*- and *y*-polarization channels. Here, we propose using a vortex half-wave plate placed at the back focal plane of the imaging system to convert radially and azimuthally polarized light to *x*- and *y*-polarized light, creating a radially and azimuthally polarized (raPol) standard point spread function (PSF). This method measures the orientations of *z*-oriented molecules more precisely than *xy*Pol since light emitted by *z*-oriented molecules is radially polarized.

To demonstrate raPol imaging, we imaged Nile Red (NR) molecules in DPPC lipid bilayers with 40% cholesterol; molecular crowding causes the NR to be mostly parallel to the optical axis (*z*-oriented) [1]. The raw raPol fluorescence images [Fig. 1(a)] exhibit higher contrast than those of *xy*Pol [Fig. 1(b)]. Further Cramér-Rao bound analyses show that the precision for measuring both the average orientation $\bar{\boldsymbol{\mu}}$ [Fig. 1(c)] and wobble angle Ω using raPol [Fig. 1(d)] is superior to those using *xy*Pol. Surprisingly, our analysis shows that raPol also outperforms many engineered PSFs.

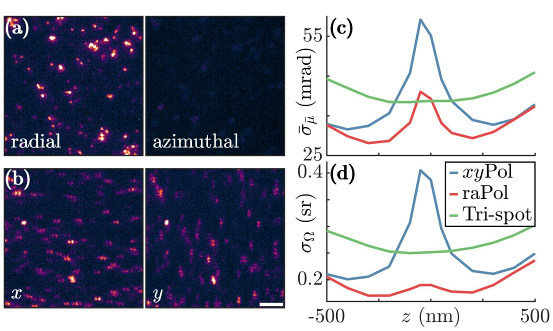

Figure 1. Images of Nile Red on DPPC with 40% cholesterol using (a) raPol and (b) *xy*Pol standard PSFs. Scale bar: 2 µm. Precisions for measuring the (c) average orientation $\bar{\boldsymbol{\mu}}$ and (d) wobble solid angle Ω of a molecule located at a water-glass interface at various defocus values z.

[1] J. Lu, H. Mazidi, T. Ding, O. Zhang, and M. D. Lew, "Single-molecule 3d orientation imaging reveals nanoscale compositional heterogeneity in lipid membranes," *Angew. Chem. Int. Ed.*, **59**, 17572–17579 (2020).