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Single-molecule 3D orientation imaging reveals nanoscale compositional heterogeneity in lipid membranes

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Abstract: In soft matter, thermal energy causes molecules to continuously translate and rotate, even in crowded environments, thereby impacting the spatial organization and function of most molecular assemblies, such as lipid membranes. Directly measuring the orientation and spatial organization of large collections (>3000 molecules/ μm^2) of single molecules with nanoscale resolution remains elusive. In this paper, we utilize SMOLM, single-molecule orientation localization microscopy, to directly measure the orientation spectra (3D orientation plus “wobble”) of lipophilic probes transiently bound to lipid membranes, revealing that Nile red’s (NR) orientation spectra are extremely sensitive to membrane chemical composition. SMOLM images resolve nanodomains and enzyme-induced compositional heterogeneity within membranes, where NR within liquid-ordered vs. liquid-disordered domains shows a $\sim 4^\circ$ difference in polar angle and a $\sim 0.3\pi$ sr difference in wobble angle. As a new type of imaging spectroscopy, SMOLM exposes the organizational and functional dynamics of lipid-lipid, lipid-protein, and lipid-dye interactions with single-molecule, nanoscale resolution.

Introduction

Tracking a molecule’s 3D position and orientation (and associated translational and rotational motions) within soft matter is critical for understanding the intrinsically heterogeneous and complex interactions of its various components across length scales. In living cells, the organization of many biomolecular assemblies, such as lipid membranes, chromosomes, and cytoskeletal proteins,^[1–4] ensure the proper functioning of all cellular compartments. Molecular organization also significantly impacts the nanoscale morphology of supramolecular structures,^[5] the physical and mechanical properties of polymers,^[6] and carrier mobility in light-emitting diodes.^[7]

Molecular orientations are commonly inferred from an order parameter determined via X-ray diffraction,^[8] infrared spectroscopy,^[9] nuclear magnetic resonance (NMR),^[10] Raman spectroscopy,^[11] sum frequency generation spectroscopy,^[12] and fluorescence microscopy.^[13] However, the order parameter is an ensemble average and cannot unambiguously determine the 3D orientation of a single molecule (SM).^[14] Spectrally-resolved SM localization microscopy (SMLM)^[15–17] maps the local polarity or hydrophobicity of protein aggregates and subcellular structures,^[17,18] and fluorescence lifetime imaging identifies sub-resolution lipid domains in the plasma membrane.^[19] However, these approaches require specific environment-sensitive fluorescent probes (e.g., Nile red,^[20] Laurdan,^[21] and 3-

hydroxyflavone derivatives^[22]) whose fluorescence spectra (intensities) or lifetimes are sensitive to their local environment.

Alternatively, the orientation and motion of any fluorescent probe are influenced by intermolecular forces from surrounding molecules; these interactions can be strengthened by exploiting specific dye-binding modes^[23] or bifunctional linkages^[24,25]. Therefore, imaging an SM’s 3D orientation and wobble, which we term as single-molecule orientation localization microscopy (SMOLM),^[26] offers an alternative strategy for sensing molecular interactions using any SMLM-compatible fluorescent dye. Numerous technologies can characterize SM orientation with varying degrees of sensitivity and resolution, e.g., by varying the polarization of excitation light,^[27–29] calculating the ratio of photons detected between multiple polarization channels,^[30,31] defocusing and matching image patterns,^[32,33] and implementing engineered PSFs.^[34,35] However to our knowledge, no demonstrated technique is capable of imaging the positions and 3D orientations of large collections of molecules (>3000 molecules/ μm^2) with single-molecule sensitivity and sufficient spatiotemporal resolution to visualize, for example, dynamic remodeling of a lipid bilayer over minutes to hours. To address these limitations, we implement SMOLM by combining 1) an engineered point spread function (PSF) that efficiently encodes the 3D orientation and wobble, termed “orientation spectra” in this work, of dipole-like emitters^[35] into fluorescence images, 2) a polarization-sensitive fluorescence microscope, and 3) a maximum likelihood estimator with joint-sparse regularization for estimating molecular position, orientation, and wobble from those images.^[36,37] This combination of hardware and software is critical for resolving molecular positions and orientations robustly; otherwise, neighboring molecules, wobbling molecules, and translationally diffusing molecules could be confused with one another.

In this paper, we report the practical application of SMOLM to measure the orientation spectra of single lipophilic dyes in lipid membranes and observe that their characteristic orientation spectra are determined by their chemical structures and surrounding lipid environment. In particular, we discover that the orientation spectra of Nile red (NR), a classic solvatochromic dye,^[38] are extremely sensitive to the composition and packing of lipid membranes. To achieve high sampling density for SMOLM, we apply the PAINT (points accumulation for imaging in nanoscale topography) blinking mechanism,^[39] in which certain lipophilic dyes transiently attach to lipid bilayers and exhibit fluorescence solely while in a non-polar environment. Here, we image supported lipid bilayers (SLBs) containing various well-characterized membrane model components, including DPPC (di(16:0) PC), DOPC (di(18:1) PC), POPC (16:0/18:1 PC), SPM

RESEARCH ARTICLE

(palmitoyl sphingomyelin) and cholesterol. SMOLM resolves nanoscale lipid domains in lipid mixtures and monitors *in-situ* lipid compositional changes induced by low doses of sphingomyelinase with resolution beyond the diffraction limit. SMOLM imaging clearly resolves interactions between various lipid molecules, enzymes, and fluorescent probes with detail that has never been achieved previously.

Results and Discussion

Figure 1. SMOLM imaging of the 3D orientations and wobbling of single fluorescent molecules. (a) Schematic of the 3D orientation and wobble of a single dipole, parameterized by polar angle (θ), azimuthal angle (ϕ), and wobble solid angle (Ω , modeling rotational diffusion within a cone). (b) Schematic of the

orientation and wobble of Dil and MC540 within a gel and fluid supported lipid bilayer respectively. (c,d) Representative microscope images of Dil and MC540 in (i) x-polarized and (ii) y-polarized fluorescence channels using the Tri-spot point spread function (PSF). Yellow crosses represent the recovered position of each molecule. Insets: Magnified images of a single molecule. (iii) Orientation (polar angle θ) and wobble (solid angle Ω) measurements of single Dil and MC540 molecules. The thick solid lines show the first to third quartile range of measured polar and solid angles; their intersection indicates the median values. The ends of the dashed lines indicate the 9th and 91st percentiles. The red triangle indicates the orientation spectra of the molecule shown in insets i and ii. (e,f) Orientation (polar angle θ) and wobble (solid angle Ω) of Nile red in (e) DPPC with different cholesterol levels and in (f) DOPC, POPC, DPPC with various acyl chain structures. Insets: median polar angle and solid angle over different lipid conditions. Scale bar: 2 μ m in c,d.

SMOLM imaging principle

Most organic fluorescent probes are well-approximated as oscillating electric dipoles.^[13] We model the orientation of each molecule using an average polar angle (θ) and azimuthal angle (ϕ), plus a uniform wobble within a hard-edged cone (solid angle Ω corresponding to the cone's half-angle, Scheme S1) in 3D (Fig. 1a). These parameters can be readily adapted for other types of rotational diffusion.^[40,41]

Our SMOLM imaging system (Scheme S2) includes a polarization beam splitter to separate emission light into x-polarized and y-polarized imaging channels. The orientation-sensitive phase mask (Tri-spot^[35] or Duo-spot, Scheme S2) is loaded by a spatial light modulator placed in the back focal plane. The Tri-spot PSF^[35] was designed to redistribute the photons from a single molecule into three spots and provide highly accurate and precise measurements of orientation and wobbling without angular degeneracy. However, this splitting of photons hampers SM detection in low signal-to-background (SBR) conditions. We therefore designed another orientation-sensitive PSF, called the Duo-spot PSF, that redistributes photons into two spots for sensitive orientation measurements of dim molecules oriented out of the xy plane (Supplementary Note 1.1).

The relative brightness of each lobe of the Tri-spot or Duo-spot PSF can be used to infer both the mean orientation and wobble of a SM emission dipole via a two-step estimation method. In the first step, we estimated the positions and six orientational second-order moments (Supplementary Note 1.1) of SMs using a regularized maximum likelihood estimator.^[36,37] In the second step, we projected the second moments into angular (first-moment) orientation space, thereby obtaining measurements of polar angle θ , azimuthal angle ϕ , and wobbling area Ω (Supplementary Note 1.3).

Resolving the orientation spectra of single molecules

We first validate the SMOLM concept by imaging lipophilic dyes with known orientation spectra within supported lipid bilayers (SLBs). In SLBs, an SM's orientation and wobble is affected by how its molecular structure interacts with its local environment. Dil, for example, bears two long hydrocarbon chains that incorporate into the nonpolar core of a lipid bilayer, while its chromophore headgroup resides in the charged polar region of the bilayer (Fig. 1b). We captured images of single Dil molecules in x- and y-polarized emission channels using the orientation-sensitive Tri-spot PSF^[35] (Fig. 1c(i)(ii)) and measured their orientations and wobble. Our results (Fig. 1c(iii)) indicate that in DPPC (di(16:0) PC) SLBs, most Dil molecules exhibit large polar angles ($\theta=73.6\pm21.2^\circ$, median $\pm$ std) corresponding to an orientation approximately parallel to the plane of the coverslip,^[42] which is

RESEARCH ARTICLE

corroborated by molecular dynamics simulations.^[43] The solid “wobble” angles of DiI are small ($\Omega=0.21\pi\pm0.41\pi$ sr), implying that the long hydrocarbon chains of DiI tightly embed into the nonpolar core of the SLB and limit its rotational diffusion. SMOLM imaging of another lipophilic dye, Merocyanine 540 (MC540), shows that it binds perpendicularly to a fluid (DOPC, di(18:1) PC) lipid membrane (Fig. 1b) with an orientation of $\theta=17.5\pm14.2^\circ$ (Fig. 1d) and a narrower distribution of large solid angles ($\Omega=0.71\pi\pm0.22\pi$ sr), which agree with ensemble measurements.^[44,45] These observations confirm that SMOLM is capable of resolving both in-plane and out-of-plane molecules, as well as fixed and freely rotating dyes, within lipid membranes.

Orientation spectra reveal lipid composition and packing

Within cell membranes, cholesterol (chol) plays a vital role in ordering and condensing lipid acyl chains, stabilizing lipid membranes, and forming nanoscale membrane domains.^[1,46] We discovered that the orientation spectra of single NR molecules are remarkably sensitive to the composition and packing of lipids influenced by chol. In DPPC without chol, NR exhibits a tilted out-of-plane orientation ($\theta=26.0\pm19.2^\circ$) and relatively large wobble ($\Omega=0.96\pi\pm0.31\pi$ sr). As the chol concentration increases to 40%, both polar and solid angles decrease drastically ($\theta=8.7\pm7.7^\circ$, $\Omega=0.26\pi\pm0.18\pi$ sr, Fig. 1e). These data suggest that chol strongly orders and condenses NR within the membrane in addition to the lipids themselves. Alternatively, we applied cholesterol-loaded methyl- β -cyclodextrin (10–400 μ M, Supplementary Note 2) to elevate chol concentration *in-situ* within DPPC SLBs. The tilt and wobble of NR decrease to a level (Fig. S3a,b) commensurate with 40% chol. We observed the opposite effects on the orientation spectra of NR by adding melatonin (4%–30%, Supplementary Note 2, Fig. S3c), which is known to increase the disorder of lipid acyl chains and alleviate cholesterol's effects.^[47]

Our observations of NR's orientational dynamics are remarkably consistent with the “umbrella model” of a lipid bilayer. In this model, the large hydrophilic phosphocholine headgroups form a cover, shielding cholesterol's hydrocarbon steroid rings from the surrounding solvent while its hydroxyl group lies in close proximity to the lipid-water interface (Supplementary Note 3.1).^[48] Chol tends to align and condense lipid acyl chains, thereby restricting translational and rotational movements of molecules within the bilayer. Based on our observations, we infer that NR primarily resides in the non-polar region of the bilayer surrounded by acyl chains. Chol-induced ordering orients NR parallel to neighboring acyl chains and perpendicular to the plane of the bilayer, and chol-induced condensation crowds molecules within the bilayer and thus decreases NR wobbling.

Interestingly, SMOLM reveals that the orientation spectra (polar angle and wobble) of NR are more sensitive to the identity of lipid acyl chains than headgroups. We compared SLBs containing DPPC with two fully saturated acyl chains, POPC (16:0/18:1 PC) with one saturated and one unsaturated acyl chain, and DOPC with two unsaturated acyl chains. In the presence of 40% chol (Fig. 1f), NR shows the largest solid ($\Omega=0.73\pi\pm0.21\pi$ sr) and polar ($\theta=15.1\pm11.8^\circ$) angles in disordered DOPC, compared to $\Omega=0.26\pi\pm0.18\pi$ sr and $\theta=8.7\pm7.7^\circ$ in ordered DPPC. The disordered acyl chains likely counter chol's ordering effect, thereby increasing the solid and polar angles of embedded fluorescent probes (Fig. S4a,b). In contrast, SPM has the same acyl chains as DPPC but different

headgroups. Interestingly, the orientation spectra of NR within SPM and DPPC are virtually indistinguishable with increasing chol concentration (Fig. 1e, S4c).

These SMOLM observations provide powerful insight into fluorophore interactions with lipid structures; in contrast to its structural analog Nile blue (Supplementary Note 3.2); NR emits fluorescence while inhabiting the non-polar region of a lipid bilayer, and its rotational dynamics are dictated by the specific environment “underneath the umbrellas”. Thus, SMOLM imaging reveals a molecule's precise spatial positioning (<1 nm) within the lipid membrane, i.e., near headgroups vs. acyl chains, in addition to measuring the chemical environment surrounding each SM. Conventional SMLM does not have sufficient spatial resolution nor chemical sensitivity to visualize these properties.

SMOLM imaging resolves lipid domains

Lipophilic probes that are sensitive to lipid packing enable SMOLM to map compositional and structural heterogeneities within lipid membranes, such as lipid domains. We carried out SMOLM imaging on a lipid mixture of DOPC/DPPC/chol. This mixture forms liquid-ordered (Lo) and liquid-disordered (Ld) phases as shown in conventional PAINT SMLM, where Lo/Ld domains are revealed by densities of MC540 localizations (Fig. 2a(i), dark: Lo, bright: Ld, Supplementary Note 4).^[49] SMOLM imaging, on the other hand, captures sensitive maps of chol concentration and acyl chain structure using the orientation spectra of NR (Fig. 2a(ii)(iii)). The Lo phases consist of ordered DPPC with chol,^[1,50] resulting in small NR solid and polar angles, compared to Ld phases formed by disordered DOPC (Fig. 2c). Since both orientation and wobble carry information useful for resolving Lo/Ld domains, we use principal component analysis (PCA, Experimental section) to combine the polar and solid angle data into a scalar PCA score (which we term “phase index” in this work) map that discriminates Lo and Ld domains (Fig. 2a(iv), -0.28 arb. unit threshold).

SMOLM imaging shows Lo domains of various sizes both above (~ 500 nm) and below (<200 nm) the diffraction limit (green lines in Fig. 2b(ii)). In large Lo domains, the cross-sectional profile of phase index (small values) matches well with the SMLM data (few localizations, Fig. 2d(ii)). However, SMLM imaging is susceptible to contrast fluctuations from stochastic probe binding times, as shown by the local spikes (bins with dramatically more localizations) within Lo domains (Fig. 2d(i), marked by asterisks). These spikes are generated by MC540 with long binding times and confined lateral diffusion in Lo domains (Movie S1). On the other hand, SMOLM measures the orientation spectra of every probe molecule independent of the probe's binding time to the membrane and is more robust to nonuniform localization densities over different lipid phases. SMOLM also shows good performance for resolving Lo domains below the diffraction limit. The cross-sectional profile of phase index within these small Lo domains is noisier but still consistent with the SMLM profile (Fig. 2d(ii)).

It is well known that SMLM image resolution improves with both better localization precision and higher sampling (localization) density.^[51,52] One advantage of SMOLM is that lipid composition and packing information are inferred from orientation measurements; if the position and orientation of each molecule are accurately estimated, only one SMOLM localization is required to distinguish Lo vs. Ld phase in a given pixel. In contrast, SMLM imaging requires 1) consistently few localizations within Lo

RESEARCH ARTICLE

domains and 2) reliably many localizations within Ld domains to create a high-contrast reconstruction. Since this contrast relies upon high localization densities, SMLM image quality suffers drastically when fewer localizations are collected (Fig. 2a(i) inset). Therefore, when SM localization densities are comparable, SMOLM maps contain superior contrast between Lo and Ld domains compared to SMLM images (Fig. 2a insets, Supplementary Note 5, Fig. S6).

To achieve optimal SMOLM imaging with high spatiotemporal resolution, one must select the best combination of orientation-sensitive probes and PSFs. First, we choose the probe whose orientation spectra are most separable between various single lipid phases. Between DOPC and DPPC, MC540 shows a larger separation in polar angle ($\Delta\theta_{\text{DPPC-DOPC}}=55.6^\circ$, Fig.

S7b) than that of NR ($\Delta\theta_{\text{DPPC-DOPC}}=16.4^\circ$, Fig. S7f). Therefore, MC540 better discriminates gel versus liquid domains (Supplementary Note 6.1) in SMOLM. However, in the presence of chol, NR has superior performance to MC540 in distinguishing Lo and Ld domains (Supplementary Note 6.2). Next, one must choose a PSF that balances SBR, and therefore SM detection, with orientation sensitivity, i.e., the ability to resolve various orientational motions unambiguously.^[35] Due to varying measurement sensitivities, low SBRs, and tuning of analysis algorithms, different PSFs (Tri-spot and Duo-spot) may perceive identical orientation spectra differently. However, these effects may be mitigated via instrument calibration (Supplementary Notes 7-9).

Figure 2. SMOLM imaging of Lo and Ld domains within a ternary SLB of DOPC/DPPC/chol (35:35:30, molar ratio). (a) (i) Conventional MC540 SMLM image and (ii-iv) Nile red (NR) SMOLM images depicting (ii) solid angle (Ω), (iii) polar angle (θ), and (iv) combined phase index of a ternary lipid mixture of DOPC/DPPC/chol. Inset, lower-left: SMLM and SMOLM images filtered to show identical SM localization densities of 900 molecules/ μm^2 . (b) Magnified view of (i) SMLM and (ii-iv) SMOLM images from boxed region in a. (c) Histogram and median $\pm$ std of (i) solid angles and (ii) polar angles of Nile red in Lo and Ld domains in b. Gray pixels in SMOLM images represent bins with zero localizations. (d) Cross-sectional profiles of SMLM localizations, solid angle (Ω), polar angle (θ), and phase index along the green lines (1,2) in b. Gray shaded regions represent Lo domains. Scale bar: 2 μm in a, 500 nm in b. Bin size: 28 nm in SMLM, 40 nm in SMOLM.

Imaging enzyme-mediated changes to lipid composition

We next extend SMOLM to monitor *in situ* enzyme-mediated lipid compositional dynamics. In the plasma membrane, the hydrolysis of SPM via sphingomyelinase (SMase) generates a bioactive lipid, ceramide (cer), which selectively displaces chol from Lo domains at a 1:1 molar ratio,^[53] promotes lipid phase reorganization, forms a ceramide-rich ordered phase,^[54] and

impacts cellular signaling and other vital processes.^[55] Most of these nanoscopic structural details were first observed by atomic force microscopy (AFM),^[54] which however is mostly limited to planar and static lipid samples and often requires complementary fluorescence imaging for visualizing lipid dynamics on faster timescales.^[56,57]

RESEARCH ARTICLE

Conventional SMLM imaging shows that SMase causes extensive changes in the morphology of ordered domains in DOPC/SPM/cholesterol SLBs (Supplementary Note 10). However, for low SMase concentrations, the morphology of the *Lo* domains are mostly conserved and limited information on enzyme activity can be obtained. We instead applied SMOLM to monitor the underlying lipid compositional changes and resolve the spatial redistribution of newly generated ceramide and displaced cholesterol within individual *Lo* domains.

A new orientation-sensitive Duo-spot PSF was developed for improved SBR over the Tri-spot PSF (Supplementary Note 1.1). We confirmed that NR SMOLM imaging using the Duo-spot PSF has excellent sensitivity for distinguishing newly generated ceramide domains vs. cholesterol-rich *Lo* domains in static single-phase lipid samples (Supplementary Note 11.2). We next conducted SMOLM imaging of mixed DOPC/SPM/cholesterol SLBs with successive 5 min SMase treatments of increasing dosage. Low SMase doses were chosen to test SMOLM sensitivity for detecting subtle enzyme activity within *Lo* domains (Fig. 3a, SMLM images at t_0, t_3). The SMOLM maps (Fig. 3b) indicate a dose-dependent disappearance of cholesterol-rich *Lo* domains. Before treatment (t_0), the shapes and positions of cholesterol-rich *Lo* domains (small polar angle, solid angle, and phase index) imaged by SMOLM match the *Lo* domains mapped by SMLM. SMase treatment (16 mU/mL) induced insignificant changes in the SMOLM maps (Fig. 3b, t_1), while more regions within the *Lo* domains begin to lose their cholesterol-rich signature at a larger dose (50 mU/mL SMase, t_2). After a 250 mU/mL dose, almost all the cholesterol-rich *Lo* domains disappeared (Fig. 3b, t_3); however, SMLM only reveals very minor changes in the size and shape of *Lo* domains (Fig. 3a, t_3). The changes in orientation spectra agree well with those of NR within SPM+cholesterol and SPM+ceramide lipid samples and strongly indicate the generation of ceramide-rich, cholesterol-poor *Lo* domains (Fig. S16b, Table S3).^[53]

We focus our analysis on one particular *Lo* domain (red box in Fig. 3a,b). The boundary of the *Lo* domain before (solid black line in Fig. 3c) and after (dotted black line in Fig. 3c) SMase treatment was determined by the SMLM images. At a dose of 50 mU/mL (t_2), the phase indices in several regions increase (arrows in Fig. 3c(ii)), indicating that these *Lo* regions are beginning to lose cholesterol. This process is always localized in the interior of the domain (arrow 1, Fig. 3c(ii)) or at the intersection of two domains (arrow 2, Fig. 3c(ii)), which agrees with previous nanoscopic AFM observations.^[54,56] As the dose of SMase increases to 250 mU/mL (Fig. 3c(iii)), the cholesterol-rich phase continues to shrink and is replaced by ceramide-rich domains. This compositional transition is also clearly illustrated by the change in orientation spectra from t_0 to t_3 (Fig. 3d).

To clearly visualize the lipid composition distribution, we designate the region outside of the domain boundary as the *Ld* phase and use the phase index threshold of -0.014 (arb. Units, Supplementary Note 11.2) to separate the cholesterol-rich phase from the ceramide-rich phase (Fig. 3c(iv)). We also identified newly formed *Lo* domains (orange box in Fig. 3a,b), which are composed of well-separated cholesterol-rich and ceramide-rich phases (Fig. 3e(ii)(iii)). The lipid composition maps among different *Lo* domains (Fig. 3c, 3e, Supplementary Note 13) reveal spatially heterogeneous nanoscale SMase activity, and also suggest that both SMase-generated ceramide and ceramide-displaced cholesterol rapidly (~minutes) condense into respectively enriched *Lo* domains.

Discussion

It has long been observed, using fluorescence polarization imaging of giant vesicles, that NR or Laurdan derivatives exhibit preferentially perpendicular orientations relative to the membrane surface in *Lo* phases due to constrained lipid packing and no preferential orientation in loosely packed *Ld* phases.^[20–22] Our SMOLM images provide the first quantitative measurements of this phenomena at the SM level and confirm that both polar angle and wobbling are increased in the *Ld* phase (Table S3). Leveraging this effect, SMOLM images (i.e., phase-index maps) can be used to discriminate between types of lipid domains (Supplementary Note 11). Compared to SMLM, SMOLM requires fewer total localizations and is less affected by localization density fluctuations when used for classifying *Lo/Ld* domains.

SMOLM relies on optimized orientation-sensitive PSFs to precisely measure orientation spectra and discover structural and chemical details of the sample. Monte Carlo studies indicate that photon shot noise, SMOLM analysis algorithms, and translational diffusion (when significant) account for ~26% up to over 50% of the observed variations (standard deviation) in polar and wobble angles of lipophilic dyes within SLBs (Supplementary Note 7.3); a significant portion of the remaining variations could be induced by the sample itself. Fundamentally, to measure orientation with high sensitivity, the photons from each SM must be spread across multiple snapshots or camera pixels, thereby lowering the SBR compared to SMLM. Furthermore, rotational motions of fluorescent molecules are often accompanied by translational motions (diffusion), all of which are critical parameters to disentangle when probing molecular interactions in complex soft matter systems. Therefore, designing compact PSFs that can discriminate between translational and rotational diffusion, combined with new image analysis algorithms based upon machine learning,^[58] could further improve SMOLM's spatiotemporal resolution for capturing faster biological processes.

Conclusion

A defining feature of soft matter is the impact of thermal fluctuations on the organization and self-assembly of molecules into mesoscopic structures like lipid membranes – processes that are notoriously difficult to observe directly. SMOLM extends conventional SMLM to measure both the positions and 3D orientations of single fluorescent molecules with high precision and sampling density (>3000 mol./ μm^2). We have utilized the orientation and wobble of fluorescent probes to reveal their interactions with the surrounding environment, such as the ordering of and condensation dynamics within lipid membranes. Our study demonstrates the feasibility of a new type of nanoscale imaging spectroscopy, namely measuring single-molecule orientation spectra,^[35,59] to resolve nanoscale chemical properties, similar to classic spectroscopies such as absorption, fluorescence emission, fluorescence lifetime,^[60] and NMR.

We anticipate that SMOLM will enable high throughput studies of both translational and orientational dynamics of single fluorescent probes within various soft matter systems and facilitate the discovery of mechanisms that control the orientation of individual molecules. The power of this imaging technology will likely promote the design of new probes whose orientations convey improved sensitivity and specificity for sensing various biophysical and biochemical phenomena.

RESEARCH ARTICLE

Figure 3. SMOLM imaging of SMase-induced alterations of lipid composition and domain reorganization in a ternary SLB of DOPC/SPM/chol (35:35:30, molar ratio). (a) Conventional MC540 SMLM image and schematic of a lipid mixture of DOPC/SPM/chol before (t_0) and after (t_3) three treatments of SMase. (b) SMOLM images (solid angle (Ω), polar angle (θ), and phase index) of Nile red before (t_0) and after successive 5 min SMase treatments of 16 mU/mL (t_1), 50 mU/mL (t_2), and 250 mU/mL (t_3). (c) Magnified view of the SMOLM phase-index map from the red boxed region in (a) and (b) at (i) t_0 , (ii) t_2 , and (iii) t_3 , and (iv) a lipid composition map at t_3 , indicating compositional changes within the Lo domain and minor changes in size and shape during SMase treatment. (d) Histogram and median $\pm$ std of (i) solid angle, (ii) polar angle, and (iii) phase index of the single Lo domain in (c) before (t_0) and after (t_3) SMase treatment. (e) Magnified view of the SMOLM phase-index map from the orange boxed region in (a) and (b) at (i) t_0 and (ii) t_3 and (iii) a lipid composition map at t_3 , indicating a newly generated Lo domain by SMase. The solid and dotted black lines in (c, e) represent the Lo domain boundary before and after SMase treatment, respectively. Scale bar: 2 μ m in (a, b), 200 nm in (c, e). Bin size: 28 nm in SMLM, 45 nm in SMOLM.

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RESEARCH ARTICLE

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See how single molecules point and wobble in 3D: A fluorescent molecule within soft matter itself acts as a nanoscale sensor, where intermolecular forces influence its orientation in 3D space. Imaging the position, orientation, and wobble of single molecules reveals a high-dimensional “fingerprint” of the chemical environment within lipid nanodomains and enzyme-induced compositional transformations within membranes.