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PERSPECTIVE

Tracking Single Molecules in Biomembranes: Is Seeing Always Believing?

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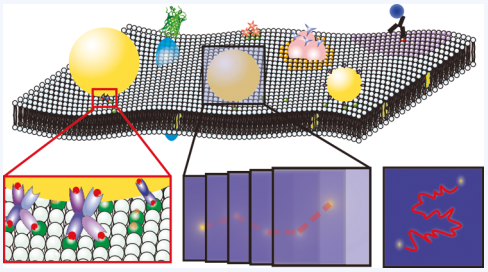
ABSTRACT: **The spatial organization of molecules in cell membranes and their dynamic interactions play a central role in regulating cell functions. Single-particle tracking (SPT), a technique in which single molecules are imaged and tracked in real time, has led to breakthrough discoveries regarding these spatiotemporal complexities of cell membranes. There are, however, emerging concerns about factors that might produce misleading interpretations of SPT results. Here, we briefly review the application of SPT to understanding the nanoscale heterogeneities of plasma membranes, with a focus on the unique challenges, pitfalls, and limitations that confront the use of nanoparticles as imaging probes for tracking the dynamics of single molecules in cell membranes.**



In an old Chinese story, a group of blind men were asked to touch an elephant and describe its shape. One concluded, after petting the trunk, that the elephant had the shape of a pestle. After feeling its head, another argued that it was more like a giant rock. Yet a third, after feeling the elephant's flank, compared it to a wall. All of the descriptions were misleading and only partially accurate, because each blind man was limited by the narrow scope of the information they could obtain from their portion of the elephant. Our understanding of the structure of cell membranes has undergone a similar evolution. Based on information that cell membranes were composed mostly of lipids and proteins, an early model depicted them as lipid bilayers studded randomly with proteins.[1] This concept was later replaced by the "fluid mosaic" model, which described the plasma membrane as a two-dimensional solution in which lipids and integral proteins diffuse freely.[2] In recent decades, research has shown that the "fluid mosaic" model is also an oversimplified view of the complexities of the membrane. It is now known that lipids and membrane proteins often exist in clusters on the nanometer scale and that the temporal dynamics of their movements are heterogeneous.[3−6] This heterogeneity is a consequence of the constraints imposed by interactions between proteins and lipids,[7−9] molecular crowding,[10,11] and other factors. This newfound structural and dynamical complexity plays a key role in regulating cell signaling and various cellular functions.[12−15]

The complex nanoscale organization of the cell membrane and the functional significance of that organization were revealed by methods that involved the direct imaging and tracking of single molecules. By optically labeling single membrane molecules and tracking their motion, it is possible to resolve the heterogeneous dynamics of different membrane species, their non-uniform organization, and the transient interactions between them.[16,17] The high spatiotemporal resolution and quantitative precision of the data obtained from single-molecule tracking are unattainable by any other method. This approach, nonetheless, has important limitations and pitfalls. It is increasingly recognized that results from single-molecule tracking can be skewed by various factors, particularly the properties of the imaging probes used. If these confounding influences and limitations are not properly taken into account, results can be misinterpreted, much as the blind men misinterpreted their tactile observations of the elephant. With these limitations in mind, we will provide here a brief overview of the single-molecule imaging and tracking methods used to quantify organization and interactions in biological membranes. We will focus on some important, but often overlooked, pitfalls of these methods. In particular, we highlight the relevant work by Liao *et al.* published in this issue of *ACS Nano*, which systematically examines how the molecular design of cross-linkers on nanoparticle probes impacts the dynamics of the target membrane molecules.[18]

THE ENSEMBLE-AVERAGE MEASUREMENTS OF DIFFUSION IN BIOMEMBRANES

Before we consider single-molecule tracking, it is important to review two techniques that have been widely used to measure the dynamics of biomembranes by averaging over the behavior of ensembles of biomolecules. The first technique is fluorescence recovery after photobleaching (FRAP). In FRAP measurements, fluorescently labeled lipids or proteins in a small area of the biomembrane are selectively photobleached by strong laser illumination. The fluorescence emission in this area recovers with time as nonphotobleached fluorescent molecules diffuse into it. The recovery of fluorescence as a function of time can be used to calculate the diffusion

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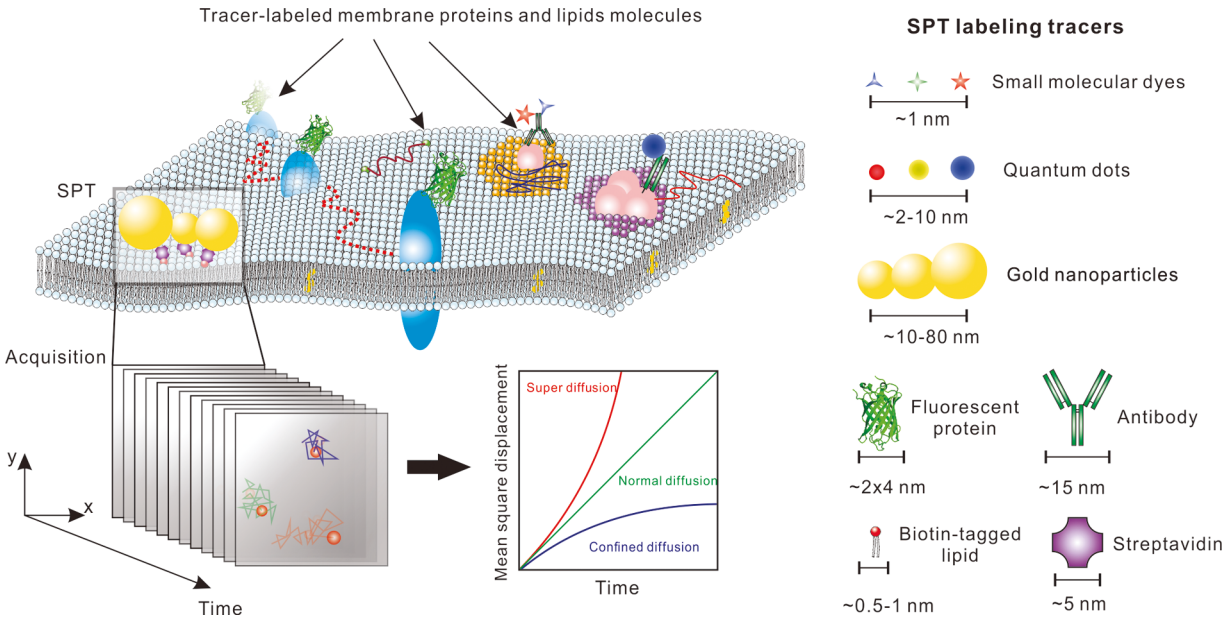

Figure 1. A simplified schematic illustration of the plasma membrane with lipids and transmembrane proteins. To quantify the dynamics of single membrane molecules, they are labeled with optical probes (commonly used ones shown), and their trajectories are determined in fluorescence time-series images by using single-particle tracking (SPT, shown in inset). Their dynamics, such as the different modes of diffusion, reflect the interactions they experience and the organization in the plasma membrane.

coefficient of the unbleached fluorescent molecules.[19,20] The second widely used ensemble method is fluorescence correlation spectroscopy (FCS). This method records temporal changes in the fluorescence emission intensity in a well-defined observation volume as fluorophores pass through. A small detection volume and a low fluorophore concentration are necessary to ensure sufficient detection sensitivity.[21,22] Both FRAP and FCS have been extensively used to measure the lateral diffusion of lipids and membrane-associated proteins.[23−29] The temporal resolution of both techniques can be as fast as milliseconds, which is potentially sufficient to resolve transient heterogeneity in biomembranes. However, both techniques share some major limitations. First, the spatial resolutions of FRAP and FCS are limited by the photobleached or illuminated volume, respectively, which is constrained by the diffraction of light.[30] By their nature, these techniques can only provide ensemble time-averaged dynamic information about the molecules of interest. Some new variants of the techniques have been developed to overcome these limitations. The most notable one is the combination of FCS with stimulated emission depletion microscopy (STED). In this technique, the illuminated volume is reduced to contain only a single fluorophore molecule at a time.[31,32] Another limitation of both FRAP and FCS is that the interpretation of the results depends on the choice of models used for data fitting. Although data fitting is straightforward for normal diffusion of a single membrane species, it becomes challenging and sometimes impossible when unknown subpopulations are present or molecules exhibit multiple modes of dynamics. For this reason, the accurate interpretation of FRAP and FCS results from cell membranes can be challenging.[33] In contrast, single-molecule tracking provides direct visualization of the heterogeneous dynamics in cell membranes, sidestepping these difficulties.

SINGLE-MOLECULE (PARTICLE) TRACKING

Direct evidence that demonstrates the heterogeneous structural and functional organization of cell membranes has mostly been obtained by imaging and tracking single molecules in these membranes.[34−37] Typically, proteins or lipids of interest are labeled with fluorescent probes (molecules or particles) and imaged using light microscopy. Their trajectories and dynamics are then determined using image-processing algorithms that locate the centroids of single molecules or particles and connect their locations across a time series of images.[30,38,39] Because the working principles for tracking single fluorophores and single nanoparticles are similar, we include both techniques under the rubric of "single-particle tracking" (SPT) for simplicity. Unlike ensemble-averaging methods, SPT can reveal nanoscale structural heterogeneity in membrane organization and dynamics. It enables direct visualization and quantification of the pathways of travel and dynamics of individual membrane molecules with high spatiotemporal resolution. With SPT, it is possible, for example, to distinguish different types of motion. Normal (or Brownian) diffusion can be distinguished from subdiffusion and superdiffusion using mean-square displacement plots (Figure 1).[40−42] These various modes of motion reflect the different environments in which membrane molecules can travel. When such information is collected simultaneously from tens or hundreds of molecules that are spread out over a large membrane region, one can map the local structural and dynamic heterogeneities in the plasma membrane with spatial resolutions comparable to the size of nanoscale membrane domains and temporal resolutions shorter than the lifetime of transient membrane clusters.

In order to visualize and to track single molecules in the plasma membrane, an optical probe, such as a fluorophore, is conjugated to the molecule of interest. The SPT probes commonly used for membrane studies include organic dye molecules, fluorescent proteins, and nanoparticles, as listed in

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Single-particle tracking enables direct visualization and quantification of the pathways of travel and dynamics of individual membrane molecules with high spatiotemporal resolution.

Figure 1. Each type of optical probe has distinctive properties that can influence the SPT results.

Molecule-Based Probes: Organic Fluorescent Dyes and Fluorescent Proteins. Organic fluorescent dyes are the smallest possible SPT probes. Their typical hydrodynamic radius is 1−2 nm.[33] A major advantage of the small sizes of such probes is that it minimizes their potential for perturbing the diffusion of the membrane molecules one wishes to observe.[43,44] Therefore, small fluorescent dyes are widely used for imaging and tracking the dynamics of lipids[32,45,46] and transient interactions and binding between membrane receptors.[47−51] Organic dye molecules can be conjugated to a membrane protein *via* nonspecific methods such as reaction with primary amines on proteins, but more specific and controlled conjugation methods are usually preferable. In many studies, this control is achieved by conjugating organic fluorescent dyes to antibodies, antibody Fab fragments, streptavidin, or polypeptide tags. Although this method can result in high specificity toward the target membrane proteins, it also inevitably increases the size of the entire fluorescent probe and consequently its potential for perturbing the dynamical behavior of the membrane proteins of interest. Moreover, the stoichiometry of such labeling cannot be controlled, which may lead to inaccurate interpretation of molecular clustering in the plasma membrane.[52] Fluorescent proteins are another type of molecule-based probe. They are larger than organic dye molecules, with a typical monomeric molecular weight of 25−30 kDa and a hydrodynamic radius of *ca.* 2−4 nm.[53,54] A major advantage of these probes is that, because fluorescent proteins are created by engineering the gene that encodes the protein of interest, it is possible to choose to label different cytoplasmic domains of transmembrane proteins. Absolute monovalency and control of the exact labeling site in the protein of interest can be attained.[54] Along with their benefits, however, using either organic dyes or fluorescent proteins for SPT studies also involves major challenges due to their limited photostability and the finite number (10^5-10^7) of photons they emit before photobleaching.[34,55−58] Molecule-based fluorescent probes are also less bright than larger optical probes such as fluorescent beads, which limits both the spatial resolution and the temporal sampling rate. These probes are also less photostable, which limits the duration of imaging. As a result, some important details, such as the rapid transient dynamics of membrane molecules, may not be resolved with molecule-based imaging probes. In addition, the nonspecific interaction of organic fluorophores with lipid membranes[59,60] and dimerization of fluorescent proteins[61] may influence the accuracy of SPT results.

Particle-Based Probes: Quantum Dots and Gold Nanoparticles. Compared to dyes and fluorescent proteins, nanoparticle-based imaging probes often are brighter and offer better photostability. Many exciting new types of particle-imaging probes have been developed,[62−66] but we limit our brief discussion here to the two types that are most widely used: quantum dots and gold nanoparticles.

Quantum dots are one of the most popular probes in fluorescence imaging, owing to their immense brightness, photostability, narrow fluorescence emission spectra, and wide range of emission colors.[67−71] Their extinction coefficient is at least 10 times larger than that of the best organic dyes, so they are extremely bright compared to dyes and fluorescent proteins, which is critical for achieving high spatial and temporal resolution in SPT studies.[69,70,72] They also have exceptional photostability, which enables long periods of imaging.[72] Using quantum dots as probes, the dynamics of membrane molecules can be imaged and tracked with high frame rates for extended durations. For example, the dynamics of lipids and membrane receptors, all labeled with quantum dots, have been imaged in living cells at sampling frequencies of 1.75 kHz for several thousand frames.[73] Analysis of long single-particle trajectories revealed that a majority of the tracked molecules are transiently confined to membrane domains of 100−150 nm in size for tens of milliseconds a time. This is one of the many examples in which nanoscopic heterogeneity and transient interactions in the plasma membrane were revealed by SPT using quantum-dot-labeled molecules.[74−77] There are, of course, some significant limitations on the use of quantum dots for SPT. Some of these are applicable to all nanoparticle-based imaging probes, and this will be discussed further below. One shortcoming unique to quantum dots is their intermittent fluorescence emission, also known as blinking, which can lead to inaccurate SPT tracking.[68,78,79] Some strategies have been developed to reduce this blinking by tuning the properties of the inorganic shell.[80−82] If quantum dot blinking is unavoidable, then specific tracking algorithms can be applied in addition to traditional SPT analysis to reduce the impact of blinking on the accurate tracking of single particles.[83] In fact, the blinking of quantum dots has been used to achieve super-resolution imaging in cells, following a similar principle to that of photoactivatable localization microscopy.[84,85]

Gold nanoparticles are another type of imaging probe widely used for tracking single molecules in the plasma membrane. Because the imaging of gold nanoparticle probes is based on light scattering rather than fluorescence, there are no concerns about photobleaching. The amount of scattered light is strongly dependent on the size of the gold nanoparticle, thus gold nanoparticles larger than 20 nm are typically used to label lipids and membrane proteins to provide a sufficiently large optical signal for high-speed imaging (*i.e.*, 40 kHz frame rate) and nanometer tracking precision in living cells.[86−89] A wide range of lipids and membrane proteins in different cell lines have been found to exhibit surprisingly similar dynamical behaviors when labeled with gold nanoparticles. They undergo transient confinement within nanoscopic compartments for tens to hundreds of milliseconds before hopping to an adjacent compartment.[86,90,91] This confined diffusion of lipids was abolished when cortical actin filaments were removed.[86,91] Based on these findings, the anchored picket fence model was proposed. This model depicts the plasma membrane as compartmentalized by the actin cytoskeleton acting as "fences" and transmembrane proteins as anchored "pickets".[86] However, the large size of gold nanoparticle probes casts a cloud of doubt over such observations. A recent study using super-resolution STED-FCS reported a consistent finding of compartmentalized diffusion of lipids, but with much weaker

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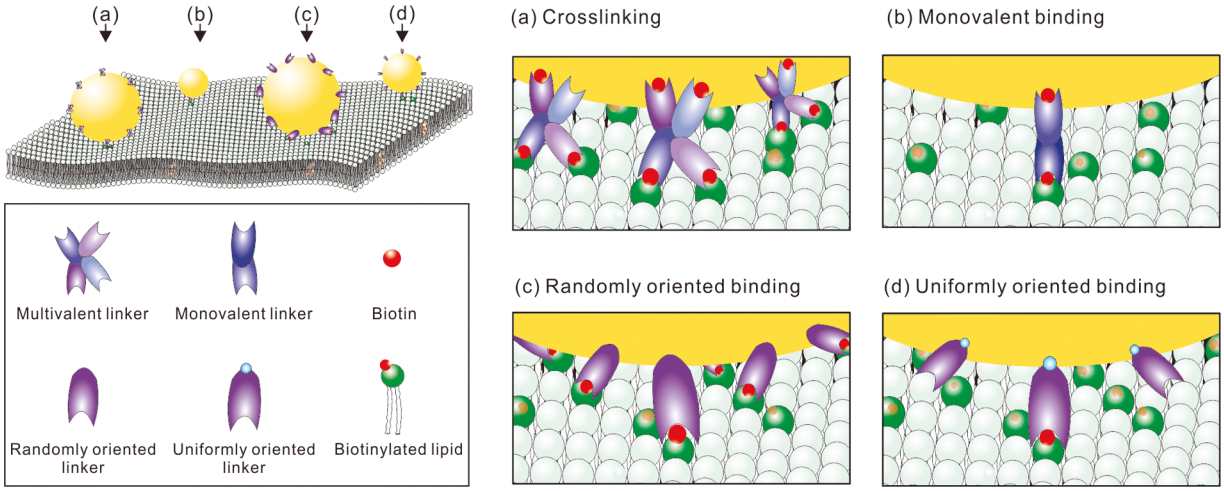

Figure 2. Schematic illustration of the potential effects of nanoparticle probes on single-particle tracking. (a, b) Linkers with multiple binding sites can cross-link multiple target membrane molecules onto the surface of a single particle probe. In contrast, monovalent conjugation linkers are preferred. (c, d) Orientation of the linkers can also influence the diffusion of target molecules in the membrane. Particle probes with randomly oriented linkers perturb the lipid diffusion more than ones with uniformly oriented linkers, as demonstrated by Liao *et al.* in ref 18.

confinement strength when lipids were labeled with smaller fluorescent dyes instead of gold nanoparticles.[35,45,92] In another study, no hop diffusion was observed for membrane receptors that were labeled with fluorescent Fab antibody fragments instead of gold nanoparticles.[93] Such findings led to the argument that the observation of nanoconfinement and hop diffusion for the receptors was skewed by the large size of gold nanoparticle probes.

A general question for fluorescent imaging studies is that of how the fluorescent probes might themselves affect the dynamics and structural arrangements of the molecules under study. Whenever lipids and membrane proteins are labeled with nanoparticles, the relatively large size of the nanoparticle probes is the foremost factor to consider. The gold nanoparticles used in SPT studies typically have a diameter of 20 nm or larger. Quantum dots are slightly smaller, but still fall in the size range of 15−30 nm in diameter, because their structure includes a 2−10 nm core and extra surface coatings needed for stabilization and bioconjugation functionalities.[68,94] Protein linkers, including antibodies, antibody Fab fragments, and streptavidin, are often added to the surfaces of gold nanoparticles and quantum dots to ensure their chemical specificity toward the target membrane molecules, and this further increases their size (Figure 2). As a result, nanoparticle probes are often comparable in size to, or larger than, the molecules they are intended to label. Fortunately, the hydrodynamic loading of the large probes on the diffusion of target molecules may be insignificant. According to the Saffman−Delbrück theory, diffusion in a viscoelastic membrane is largely determined by the transmembrane portion of the diffusing molecule and has a much weaker dependence on its radius than it would in a three-dimensional solution.[95] However, this prediction only applies to cases where the probe size is not much larger than that of the target molecule to which it is attached and the nanoparticle probe has no direct contact or interaction with the plasma membrane. The bad news, however, is that the large size of nanoparticle probes produces other effects that can have a profound influence on SPT results. One of these outcomes is the steric effect, in which large probes hinder or limit the target membrane molecules from diffusing into a spatially crowded or confined area. For instance, labeling membrane receptors with quantum dots of 20−30 nm was shown to hinder the receptors from reaching the neuronal synaptic cleft.[96,97] Another effect is the cross-linking of target molecules by single multivalent probes (Figure 2a,b). Some studies have shown that although large nanoparticle probes result in artificially reduced diffusion of lipids[98,99] and membrane receptors,[100−102] the main cause of such problematic effects was the cross-linking of lipids and receptors by way of the probes rather than any hydrodynamic effect.

Many SPT studies have provided evidence for nanoscopic heterogeneous dynamics and organization in membranes. But some of these findings have been called into question because it is not clear whether the observations truly reflect a heterogeneous membrane environment or are instead merely artifacts due to probe effects.[35,93,103] The extent to which the findings were shaped by probe effects remains unclear. As the application of SPT in biology continuously expands, it is becoming increasingly critical to establish a systematic understanding of the potential artifacts introduced by the use of imaging probes and to develop strategies to minimize them. Efforts on this front are emerging. For example, strategies have been reported to reduce the size of quantum dots[104−107] and the multivalency of nanoparticles, for more reliable imaging of living cells.[108−112] Nevertheless, many problems remain unresolved.

As the application of single-particle tracking in biology continuously expands, it is becoming increasingly critical to establish a systematic understanding of the potential artifacts introduced by the use of imaging probes and to develop strategies to minimize them.

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To understand the effects of probes in SPT studies, it is necessary to deconvolute the influences of multiple effects, such as those of size and of cross-linking. This process is challenging, since those effects often appear simultaneously. Liao *et al*. demonstrated a feasible approach to this challenge, as described in this issue of *ACS Nano*.[18] Their study focused on the diffusion of lipids that were labeled with gold nanoparticles in model membranes. The authors identified the individual effects of particle size, multivalent cross-linking, and oriented labeling on the measured lipid diffusion as they systematically varied those parameters independently. They demonstrated that lipids diffuse more slowly when labeled with gold nanoparticle probes (within the size range of 10−40 nm) compared to organic dyes, mostly due to cross-linking effects from multivalent streptavidin−biotin binding. This finding was not surprising, as it confirmed a notion previously reported.[113,114] However, the authors went a step further to show how to create monovalent gold nanoparticle probes that exert negligible disruption on lipid diffusion. A second, but perhaps more notable, finding from the study is that lipid diffusion is also influenced by the orientation of the streptavidin−biotin linker between the lipid and its nanoparticle probe (schematic illustration in Figure 2c,d). Particle probes with randomly oriented linkers were shown to perturb the lipid diffusion more than ones with uniformly oriented linkers. Although the cause for this effect remains unclear, this observation calls attention to a seemingly trivial yet important factor that may bias SPT results obtained from biomembrane studies. The study by Liao *et al*. was done with model membranes, but it forms the basis for further investigations in the more complex environment of plasma membranes.

To understand the effects of probes in single-particle tracking studies, it is necessary to dissect apart the influences of multiple effects, such as those of size and crosslinking. Liao *et al*. demonstrated a feasible approach to this challenge, as described in this issue of *ACS Nano*.

CONCLUSIONS AND PERSPECTIVES

The development of SPT for imaging individual membrane molecules has revolutionized our understanding of the organization of plasma membranes. It enables us to visualize and to track the different modes of diffusion of single membrane molecules directly, to detect the transient dynamics and interactions between these molecules, and to reveal nanoscale heterogeneities in the plasma membrane. As membrane heterogeneity has increasingly been found to play a key role in regulating protein signaling and various cellular processes,[34,35,115,116] the application of SPT to plasma membrane studies can be expected to continue to expand. However, as powerful as SPT is, one must bear in mind the potential limitations and pitfalls of this technique when interpreting imaging results. As briefly summarized in this Perspective, the quality of SPT results and the interpretation of the data deriving from such experiments are critically dependent on the choice of imaging probes. From organic dye molecules, to fluorescent proteins, quantum dots, gold nanoparticles, and beyond, each category of imaging probes offers unique advantages and limitations. Evidence that imaging probes may influence SPT results is not grounds for rejecting the technique as a whole. Instead, knowing the potential pitfalls enables us to make informed decisions about experimental design and to interpret results appropriately. As optical imaging and SPT are applied to even more complex biological systems, this awareness becomes increasingly important.

The nanoscale organization and functions of the plasma membrane are still poorly understood, and many key questions remain unresolved. Contradictory results have been reported regarding the exact nature of the nanoscale membrane domains that are sometimes called lipid rafts.[117] The function of these nanodomains remains poorly understood and is likely to be different depending on the cell function involved and the type of cells.[118,119] The intrinsic complexity of such questions warrants the development of more sophisticated SPT techniques. Efforts are underway to develop new imaging probes that are small, bright, photostable, and nondisruptive to the native dynamical behavior of membrane molecules. New imaging techniques that push the boundaries of optical imaging by using existing imaging probes will also be important. The integration of super-resolution microscopy with SPT represents a promising approach. For example, photoactivated localization microscopy is being combined with SPT to study protein dynamics in the plasma membrane where the density of labeled proteins cannot be feasibly controlled.[120] Another example is the integration of STED with FCS (STED-FCS) for detecting the diffusion of a single molecule within a focal spot as small as 30 nm across in the plasma membrane.[16,32] Meanwhile, investigations that systematically examine the potential artifacts in SPT studies are critical. Some studies, such as the one by Liao *et al*., have started to educate the field about the effects of imaging probes on SPT results.[18] Those studies have mostly been done in model membranes, in which lipids and transmembrane proteins experience far simpler interactions than in a cell membrane. Therefore, many more factors that might bias SPT results remain to be explored. For example, the three-dimensional membrane topology should be taken into consideration when one analyzes SPT results from two-dimensional projection imaging. Protein diffusion on bleb-like cell membrane protrusions may artificially appear as nanoconfined motion.[35,121,122] With proper understanding of these effects, one can employ SPT to achieve an unprecedented level of understanding of the plasma membrane.

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The authors declare no competing financial interest.

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