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Exploring the Spatiotemporal Organization of Membrane Proteins in Living Plant Cells

Li Wang,^{1,2,4} Yiqun Xue,² Jingjing Xing,² Kai Song,²
and Jinxing Lin^{1,2,3}

¹Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, Beijing 100083, China; linjx@bjfu.edu.cn

²Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

³College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China

⁴College of Horticulture and Plant Protection, Yangzhou University, Yangzhou 225009, China

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Abstract

Plasma membrane proteins have important roles in transport and signal transduction. Deciphering the spatiotemporal organization of these proteins provides crucial information for elucidating the links between the behaviors of different molecules. However, monitoring membrane proteins without disrupting their membrane environment remains difficult. Over the past decade, many studies have developed single-molecule techniques, opening avenues for probing the stoichiometry and interactions of membrane proteins in their native environment by providing nanometer-scale spatial information and nanosecond-scale temporal information. In this review, we assess recent progress in the development of labeling and imaging technology for membrane protein analysis. We focus in particular on several single-molecule techniques for quantifying the dynamics and assembly of membrane proteins. Finally, we provide examples of how these new techniques are advancing our understanding of the complex biological functions of membrane proteins.



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INTRODUCTION

The plasma membrane forms the limiting boundary of eukaryotic cells and has a crucial regulatory role in the exchange of nutrients and metabolites and in the transduction of external signals. The plasma membrane is a phospholipid bilayer, and different phospholipids are asymmetrically distributed between the two membrane layers. In addition to phospholipids, sterol, a major constituent found in both halves of the plasma membrane, helps maintain membrane structure (46, 98). Plasma membrane proteins, which are present in the phospholipid bilayer, have key roles in transport across the membrane and in the perception and transmission of signals (87). Lipids also function as key determinants in protein–membrane interactions, with many proteins using specific lipids as major adaptors for their membrane attachment (93).

Membrane proteins and lipids are not homogeneously distributed in the membrane but are instead segregated into discrete regions. These microdomains (also known as lipid rafts) can be visualized as sterol- and sphingolipid-enriched regions in the plasma membrane (42). Studies using plasma membrane isolation and lipid analysis have demonstrated that membrane microdomains form via preferential interactions between sterols, sphingolipids, and specific proteins and that these microdomains act as signaling platforms that regulate protein sorting and membrane signaling in a variety of contexts (82, 103).

Plasma membrane proteins are not static; instead, they exhibit dynamic behaviors such as diffusion within the plasma membrane, recycling from the plasma membrane to sorting endosomes, and interconversion between multiple conformations in equilibrium (101). The dynamics

Membrane microdomains:

a lateral compartment within the plane of the plasma membrane that exhibits a distinct composition, structure, and biological function

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of membrane proteins are thought to be an important determinant that controls lipid–protein and protein–protein interactions (107). Owing to the small size of membrane proteins and the short timescale of these dynamic events, it is notoriously challenging to analyze membrane protein behavior and function. Previous studies used detergent treatment to solubilize and purify membrane proteins. However, detergent-solubilized proteins are often unstable and tend to aggregate, and they may not be present in their native conformation. Therefore, the development of methods that allow membrane proteins to be assessed in the native membrane remains an active area of research.

Single-molecule methods are new approaches that allow detection and manipulation of individual biological molecules in order to investigate those molecules' conformations and dynamics at the nanoscale level (4). Single-molecule methods can generally be divided into two categories: The first is a force-based approach and the second uses optical detection, which requires optical microscopes to collect information about single molecules. In this review, we focus mainly on recent advances in single-molecule optical detection. After individual molecules are labeled by fluorescent probes and imaged through optical fluorescent microscopy at high or super resolution, we can image a single molecule—as opposed to an ensemble—by means of stochastic blinking or photo-bleaching, or by comparing it with the intensity of single fluorescent protein molecules during imaging and tracking processes. Not only do these methods reveal dynamic, nanometer-scale spatial information about single biomolecules in their native, complex environments, they also enable investigations of individual proteins to be conducted in greater detail than would be possible with bulk methods (33, 71, 78, 147). Special emphasis is placed on membrane protein stoichiometry and protein clustering, which are implicated in signal transduction.

Stoichiometry: the number of subunits in a molecular complex

Quantum dots (QDs): nanosized particles of semiconductor materials used for labeling

LABELING TECHNIQUES FOR MEMBRANE PROTEIN IMAGING

Labeling of membrane proteins is a crucial step in single-molecule analysis because the fluorescent signals from single molecules are extremely weak and easily drowned out by background noise. Therefore, it is essential to use bright fluorophores and to minimize the competing noise from the background. Three types of labels, each of which has advantages and disadvantages, are frequently used in plant cells: small-molecule dyes, nanosized fluorescent particles, and fluorescent proteins and their variants. (Table 1).

Small-Molecule Dyes

Many small-molecule dyes, such as fluorescein, rhodamine, and boron-dipyrromethene BODIPY, are used to label organelles for long periods (23, 70). Because of their small size, wide spectral range, and high photostability, these dyes are also used to label membrane proteins for single-molecule detection in animal cells. For example, fluorescein isothiocyanate, Alexa488/555, and Cy3/5 are commonly used in single-molecule experiments to investigate subunit interactions within a complex (44). However, it is difficult to load intact plant cells with these dyes because some of the components of the cell wall render the membrane impermeable to the dye (149). Even when labeling is achieved, certain chemical coupling reactions may occur with unspecific results, and the incomplete removal of dyes from samples may generate unreliable images. Although the use of a binary probe (consisting of two single-stranded fluorescent-labeled oligonucleotides) has been reported (23), the application of this approach to plant cells remains at an early stage of development.

Nanosized Fluorescent Particles

Several nanosized particles have been developed into fluorescent probes, including quantum dots (QDs), nanodiamonds, and noble-metal nanoclusters. Among these, QDs, which are inorganic

Table 1 Techniques for quantifying the stoichiometry and interactions of membrane proteins

Technique	Labeling	Imaging	Principle	Functions	Comments	References
PCH/FIDA	FPs, SMDs	Confocal microscopy	Fluorescence intensity distribution	Numbers and oligomerization states of proteins	Requires high photon counts for accurate analysis	24, 53
SpIDA	FPs, SMDs, QDs	Confocal microscopy	Fluorescence intensity	Density and ratio of different oligomerization states	Not limited by protein expression or antibody labeling but cannot detect protein interactions	40, 134
smSubunit-counting	FPs	TIRFM, super-resolution microscopy	Single-molecule imaging and photobleaching	Stoichiometry of proteins	Not limited by the number of subunits but restricted to low membrane protein density and photobleaching efficiency	5, 28, 61, 97, 136, 144
smFRAP	FPs	TIRFM, super-resolution microscopy	Single-molecule recovery imaging and precise localization	Protein dynamics and interactions	Requires super-resolution microscopy and multi-object tracking algorithms	19, 104
FCS	FPs, SMDs, QDs	Confocal microscopy	Fluorescence intensity fluctuation	Density, dynamics, oligomerization	Not suitable for proteins with slow motion and limited by the region imaged	2, 6
FCCS	FPs, QDs, SMDs	Confocal microscopy	Fluorescence intensity fluctuation	Protein interactions, ratio of monomers to dimers	Limited by its low spatial resolution and detects only small molecules with high dynamics	80, 91, 116
FRET	FPs, SMDs, QDs	Confocal microscopy	FRET reaction	Protein interactions, conformational changes	Limited by fluorophore concentration and location and cannot detect multimeric organization	105, 108
FRET-FLIM	FPs, SMDs, QDs	Confocal microscopy	FRET reaction + FLIM	Protein interactions, ratio of monomers to dimers	Internally calibrated to alleviate many shortcomings of intensity-based FRET measurements, but still cannot detect multimeric organization	15, 16, 34, 138
smFRET	FPs, SMDs, QDs	TIRFM, super-resolution microscopy	Single-molecule imaging + FRET	Protein interactions, conformational changes	Requires sophisticated design and rigorous labeling of proteins	41, 120, 123, 132
smPPI	FPs, SMDs, QDs	TIRFM, super-resolution microscopy	Single-molecule imaging + colocalization	Protein interactions	Free from image heterogeneity-induced false identification but cannot detect direct protein interactions	141, 150, 151
SiMPull	FPs, SMDs	TIRFM	Single-molecule imaging + pull down	Protein interactions, stoichiometry of proteins	Directly determines the stoichiometry and interactions of proteins; newly applied to plant cells	1, 55

Abbreviations: FCCS, fluorescence cross-correlation spectroscopy; FCS, fluorescence correlation spectroscopy; FIDA, fluorescence intensity distribution analysis; FLIM, fluorescence lifetime imaging microscopy; FPs, fluorescent proteins; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; PCH, photon-counting histogram; QDs, quantum dots; SiMPull, single-molecule pull-down; SMDs, small-molecule dyes; smFRET, single-molecule FRET; smPPI, single-molecule protein proximity index; smSubunit, single-molecule subunit; SpIDA, spatial intensity distribution analysis; TIRFM, total internal reflection fluorescence microscopy.

nanocrystals, have been successfully used in plant cells, representing a promising method for protein labeling. After the conjugation of specific proteins with streptavidin or antibody coat, QDs can be used to label target proteins for long-term imaging or in samples with poor signal-to-noise ratios owing to their substantially improved photostability, higher extinction coefficients, and stronger brightness than small-molecule dyes (114). QDs have been successfully used to detect the localization of membrane proteins or binding sites in living plant cells (142, 146). However, the limitations of QDs are also worth noting. In general, QDs (10–30 nm) are much larger than fluorescent proteins (3 nm) and small-molecule dyes (<1 nm). In large part, this is due to their coupling to the protein of interest via a streptavidin or an antibody and this is also because QDs self-assemble into larger structures. They may therefore slow the diffusion of target proteins and alter the mode of diffusion (106). In addition, photoblinking of QDs is difficult to eliminate. Finally, we lack an effective method for introducing QDs across the plant cell wall. Nevertheless, QDs are good candidate labels for single-molecule tracking in living cells (113, 145).

FCCS: fluorescence cross correlation spectroscopy

Photoswitchable fluorescent proteins: the fluorescent proteins whose fluorescence can be reversibly switched on and off upon excitation at a certain wavelength

Fluorescent Proteins and Their Variants

Labeling with genetically encoded fusions to a fluorescent protein is the most natural method for labeling proteins for single-molecule analysis. In this technique, the target protein and the fluorescent protein are produced as a translational fusion and thus are covalently linked. Because the labeling occurs at the genetic level, nonspecific labeling can be intrinsically removed. In particular, it is valuable to generate transgenic plants fused with fluorescent proteins in the genome-editing mutant background, such as mutants created by CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9). This technique is therefore highly specific. Furthermore, this type of label is more biocompatible and less toxic than organic dyes and nanosized particles for use in living cells (12).

The discovery of green fluorescent protein (GFP) and the resulting development of a large family of homologous fluorescent proteins based on GFP revolutionized the visualization of proteins in plant systems. A new generation of fluorescent proteins with various colors, brightness levels, and folding efficiencies has been widely used in single-molecule studies of living cells. These next-generation fluorescent proteins include the GFP structural variants enhanced GFP (EGFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP), as well as the red-shifted intrinsic fluorescent proteins mStrawberry and mCherry (12, 39). Among these, EGFP and mCherry are widely used in single-molecule imaging and fluorescence intensity analyses to investigate the stoichiometry and composition of membrane protein complexes. Similarly, CFP/YFP and GFP/mCherry pairs have been extensively used in Förster resonance energy transfer/fluorescence cross-correlation spectroscopy (FRET/FCCS)-based investigations to detect protein interactions in living cells (reviewed in 23). In addition, pH-sensitive GFPs modified to target the cell surface or endosomes are suitable for monitoring pH changes in exo- and endocytotic vesicles (86, 99). Interestingly, the fluorescent protein DsRed-E5, whose fluorescence emissions progressively shift from green to red, has also been used as a fluorescent timer to visualize the dynamics of proteins and vesicles in plants (100, 140).

In addition to the above single-colored fluorescent proteins, optical highlighters, including photoactivatable (such as PA-GFP), photoswitchable (such as monomeric Eos fluorescent protein), and photoconvertible (such as Dronpa) fluorescent proteins (22, 111), have been created. When irradiated at a particular wavelength, these proteins undergo structural changes and switch from one color to another or switch from dark to bright (85). These proteins are usually used in super-resolution microscopy and are suitable for the kinetic visualization of highly specific localization and transient changes in membrane proteins in living cells (36, 51).

FCS: fluorescence correlation spectroscopy

Despite their high specificity, fluorescent protein-labeling techniques for single-molecule analysis have some shortcomings. Fluorescent proteins are not as bright as small-molecule fluorophores because their extinction coefficients are generally smaller than those of small-molecule dyes, and they are also less photostable and become photobleached much more easily than small-molecule dyes. In some cases, their bulky size may perturb the structure or function being probed. Therefore, control experiments should be performed prior to single-molecule analysis. Particularly for stoichiometry analysis, a standard monomer fluorescent protein control is essential to rule out the effects of inherent dimer or even oligomer formation (128).

INSTRUMENTATIONS FOR BIOMOLECULE IMAGING AND DETECTION

In recent decades, advanced instrumentations have revolutionized cell biology, allowing us to decipher biological mechanisms in increasing molecular detail within their native environments. Newly equipped with these instrumentations, the conventional optical microscope has become a powerful tool for quantitative studies of dynamic processes. Moreover, high- and super-resolution microscopy have opened exciting new avenues for exploring the organization, dynamics, and functions of biomolecules at the nanosecond and nanometer scales. Dynamic analysis of these molecules as individual entities will shed light on the underlying mechanisms of cell signal transduction.

Confocal Microscopy

Initially, conventional confocal microscopy involved the use of a focused laser beam to scan across a sample followed by spatial filtration of the excited fluorescence through a pinhole to remove out-of-focus signals. A benefit of confocal microscopy is that it offers the ability to minimize background signals and to image deeply into a sample along the z -direction to generate high-contrast three-dimensional (3D) images (20). However, because of the lack of intrinsic optical sectioning, the photons emitted from out-of-focus molecules contribute to background, lowering the signal-to-background ratio of single-molecule detection and reducing the precision of localization (83). Thus, the use of this type of microscopy for direct single-molecule imaging and detection in living cells has been met with much difficulty.

Confocal microscopy coupled with multiple detectors and relevant analysis systems has partly overcome the limitations of conventional confocal microscopy and has allowed researchers to track the mobility, diffusion, concentration, and aggregation of single molecules. For instance, fluorescence correlation spectroscopy (FCS) and FCCS, with a confocal-based setup for detection with highly sensitive avalanche photo diodes and the calculation of autocorrelation functions, were used to analyze events occurring at the single-molecule level and on nanosecond-to-second timescales (**Figures 1a–c**) (**Table 1**) (6). In addition, the FRET technique, when applied to confocal microscopy, allows the interactions between two molecules to be detected when the molecules are sufficiently close for molecular interactions to occur (**Table 1**) (105). In short, when coupled with high-sensitivity detectors, confocal microscopy can be used to explore the assembly and dynamics of membrane proteins in living cells.

Super-Resolution Microscopy

The Abbe diffraction limit ($d = 0.61\lambda/\text{NA}$, where λ is the emission wavelength and NA is the numerical aperture of the objective) is the main limitation to increasing the resolution of conventional microscopy. Specifically, the image of an infinitely small molecule in optical microscopy

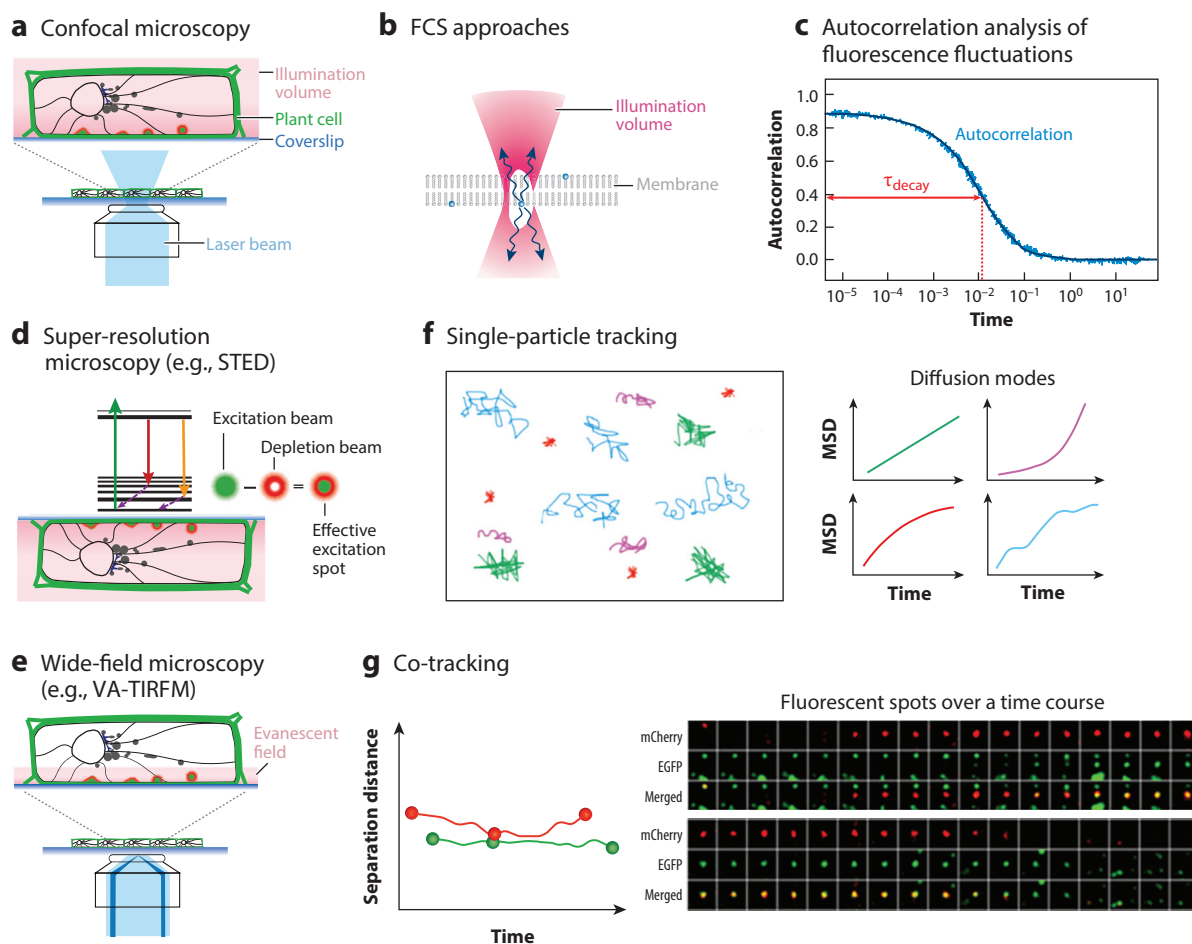


Figure 1

Main principles related to the imaging and detection of membrane proteins in plant cells. (a) In confocal microscopy, the laser beam is focused on the plane of interest, reducing fluorescence excitation in other planes, and scanned across the sample. (b) FCS, based on a confocal setup, is a quantitative method for detecting the motion of fluorescently labeled molecules in and out of the illumination volume. (c) Autocorrelation curves represent the decay of temporal correlation over time in an FCS experiment. (d) STED, as an example of super-resolution microscopy, provides a much smaller effective excitation beam to excite the fluorescence in the cell. In STED, the excitation beam of the first pulse overlaps with a doughnut-shaped beam from the second one, which achieves high-resolution fluorescence images. (e) VA-TIRFM, as an example of wide-field microscopy, takes advantage of the evanescent field induced by TIR and involves tuning the incident excitation angle to achieve images with good signal-to-noise ratios. (f) Single-particle tracking shows the trajectories of membrane proteins in real time (*left*). MSD analysis for various trajectories and classification into diffusion modes. Green, purple, red, and blue represent Brownian, directed, restricted, and multimodal diffusion models, respectively (*right*). (g) Different fluorescently labeled particles on the living cell surface are simultaneously tracked and imaged. By evaluations of spatial separations between nearby fluorophores, particle trajectories can be obtained and interacting molecules can be identified (*left*). Representative images over a time course show the codiffusion of different fluorescently labeled proteins. Top, middle, and bottom panels represent proteins labeled by mCherry, EGFP, and merged images, respectively (*right*). Abbreviations: EGFP, enhanced green fluorescent protein; FCS, fluorescence correlation spectroscopy; MSD, mean square displacement; STED, stimulated emission depletion microscopy; TIR, total internal reflection; VA-TIRFM, variable angle–total internal reflection fluorescence microscopy. Panel g reproduced from Reference 35.

Variable angle-TIRFM (VA-TIRFM):

adjusts the incident angle of excitation light, which makes total internal reflection occur at the cell wall-cytosol interface

Single-particle tracking (SPT):

traces the movements of single particles from sequences of images

would appear as a finite-size diffraction pattern including a central spot (200–300 nm) and a series of higher-order diffraction rings; thus, it is difficult for conventional microscopy to resolve objects closer than this distance. In the past decade, newly emerging advanced microscopy techniques have been developed to break the Abbe diffraction limit, leading to super-resolution (20–100 nm) optical microscopy using either patterned light to spatially modulate fluorescence emissions or single-molecule localization (83). Among these advanced techniques, stimulated emission depletion microscopy (STED) surpasses the Abbe diffraction limit by spatially modulating fluorescence emissions through patterned light. Using a doughnut shaped beam to de-excite the fluorescent molecules around a central excitation peak for reducing the point-spread function of the microscope, STED enables molecule localization with nanometer accuracy in the far field (**Figure 1d**) (112, 125). In addition, single-molecule localization microscopy techniques have been developed, including photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). Making use of the stochastic blinking of individual fluorophores, PALM and STORM provide the localization of single molecules with nanometer precision by fitting their intensity profiles with a Gaussian function (112). Nevertheless, owing to the presence of the cell wall and unspecific labeling with dyes, the full potential of STORM for imaging plasma membrane proteins in plant cells has not been explored (63). This section is intentionally brief, as several recent reviews have explored this topic in great detail (33, 63, 124).

Wide-Field Microscopy

Wide-field microscopy is based on the imaging principle in which a laser illuminates the sample, exciting all fluorophores in a near-cylindrical volume. Because the excitation volume is very large in the *z*-direction in epi-illumination and the residual fluorescence from emitters out of the focal plane is collected as well, this microscopy leads to low contrast in the resulting image. To overcome this shortcoming, researchers developed a form of wide-field microscopy, total internal reflection (TIR) fluorescence microscopy (TIRFM). TIRFM takes advantage of the evanescent field produced by TIR, resulting in sectioning excitation with a typical probing depth of 100–160 nm along the *z*-direction from the interface for visible light (37). Use of this TIR illumination technique confines the excitation of TIRFM to a region near the cover glass and greatly improves contrast, providing a powerful approach for single-molecule studies.

However, the presence of the plant cell wall (on the order of 200 nm thick) poses additional challenges for TIRFM, making TIRFM without modifications unsuitable for detecting single molecules in plant tissues with cell walls (139). Moreover, autofluorescence produced by the cytoplasm in plant cells reduces excitation profiles and detection efficiency. Recently, Konopka and colleagues (64, 65) developed and successfully used variable-angle epifluorescence microscopy, which is similar to variable angle-TIRFM (VA-TIRFM), to analyze the dynamics of fluorescently labeled molecules on the plasma membrane surface in plant cells by tuning the incident angle of excitation light (**Figure 1e**) (131, 137). This approach has greatly improved the level of image contrast compared with epi-illumination; a good signal-to-noise ratio can be achieved even when less emissive probes are used. Despite the higher contrast and improved images, the sectioning effect inherent in this technique confines the imaging depth. As a consequence, only molecular events that occur on the plasma membrane or on the isolated organelle membranes of plant cells can be analyzed (64, 89).

On the basis of fluorescence imaging with a high signal-to-noise ratio, the dynamical behaviors of individual molecules over time and space can be obtained and analyzed. Single-particle tracking (SPT) is a valuable analytical method for unraveling the involvement of the diffusion dynamics of membrane proteins in the regulation of signal transduction. By detecting single

membrane particles imaged by VA-TIRFM in a time series to obtain individual molecular trajectories (**Figure 1f**), investigators have characterized and quantified various diffusion properties, including velocity, trajectory, diffusion coefficient, and mean square displacement (26, 54, 58, 79). Single-molecule co-tracking (smCo-tracking) involving the simultaneous tracking and imaging of different molecule species labeled with spectrally separate fluorophores, has also been used to obtain molecule trajectories and to analyze their interactions in a spatiotemporally organized manner (**Figure 1g**) (31, 35). Furthermore, based on the real-time imaging of membrane protein, two-dimensional (2D) kymographs (a spatiotemporal map with the x-axis representing distance and the y-axis representing time over a specific period) over a specific period can be generated to describe the dynamics of proteins. With these kymographs, it is possible to further dissect the time-normalized intensity profiles and dwell times of different proteins from individual endocytic events; this technique has great potential for analyzing key parameters of endocytosis in relation to signal transduction (35, 59).

QUANTIFYING THE ASSEMBLY AND DYNAMICS OF MEMBRANE PROTEINS

The assembly of membrane proteins determines their fundamental properties. Thus, accurately determining the self-assembly and clustering of membrane proteins *in vivo* is important for determining their effect on signal transduction. Several approaches, based on the labeling and imaging techniques described above, have been developed to facilitate the systematic, accurate analysis of the spatiotemporal organization of membrane proteins (**Figure 2**) (**Table 1**).

Techniques Based on Fluorescence Brightness

The brightness of any fluorophore reflects its intrinsic molecular properties. The total brightness of any group of fluorophores is the sum of the individual molecular brightness levels in the absence of any electronic interactions among the fluorophores. Thus, brightness can be used to quantify the number of molecules moving together in a group. Brightness analysis is commonly performed on the basis of the analysis of the moments of fluorescence intensity. Photon-counting histogram (PCH) and fluorescence intensity distribution analysis (FIDA), which are based on intensity histogram analysis of the distribution of detected fluorophores, were developed to detect the densities and oligomerization states of membrane proteins. PCH can be directly performed by means of curve-fitting approaches with one or multiple brightness levels as the free parameters. Through PCH analysis, the average number and diffusion times of fluorophores in a region of interest can be calculated (24). FIDA uses inverse transformations to directly obtain the distribution of brightness levels based on the distribution of photon counts. However, both PCH and FIDA require a large set of free parameters for fitting, which in turn necessitate the use of high photon counts for accurate analysis. Thus, the application of these brightness analysis techniques to live cells has been limited owing to the damage caused by the high laser intensities required to generate sufficiently high photon counts (**Table 1**).

Spatial intensity distribution analysis (SpIDA), a method based on spatial histogram analysis of fluorescence intensities from conventional laser-scanning confocal images, can be used to accurately extract information about protein densities and aggregation states (40, 134). In practice, because SpIDA is applicable to single images, it can be used to measure protein interactions and distribution not only in living cells (by means of fluorescent protein expression systems) but also in fixed cells and tissues (by means of fluorescent antibody labeling) (**Table 1**). SpIDA has been successfully used for sampling within small areas (6 μm^2) to analyze the presence of monomers

Single-molecule co-tracking (smCo-tracking):

simultaneously tracks and images different particles at the single-molecule level

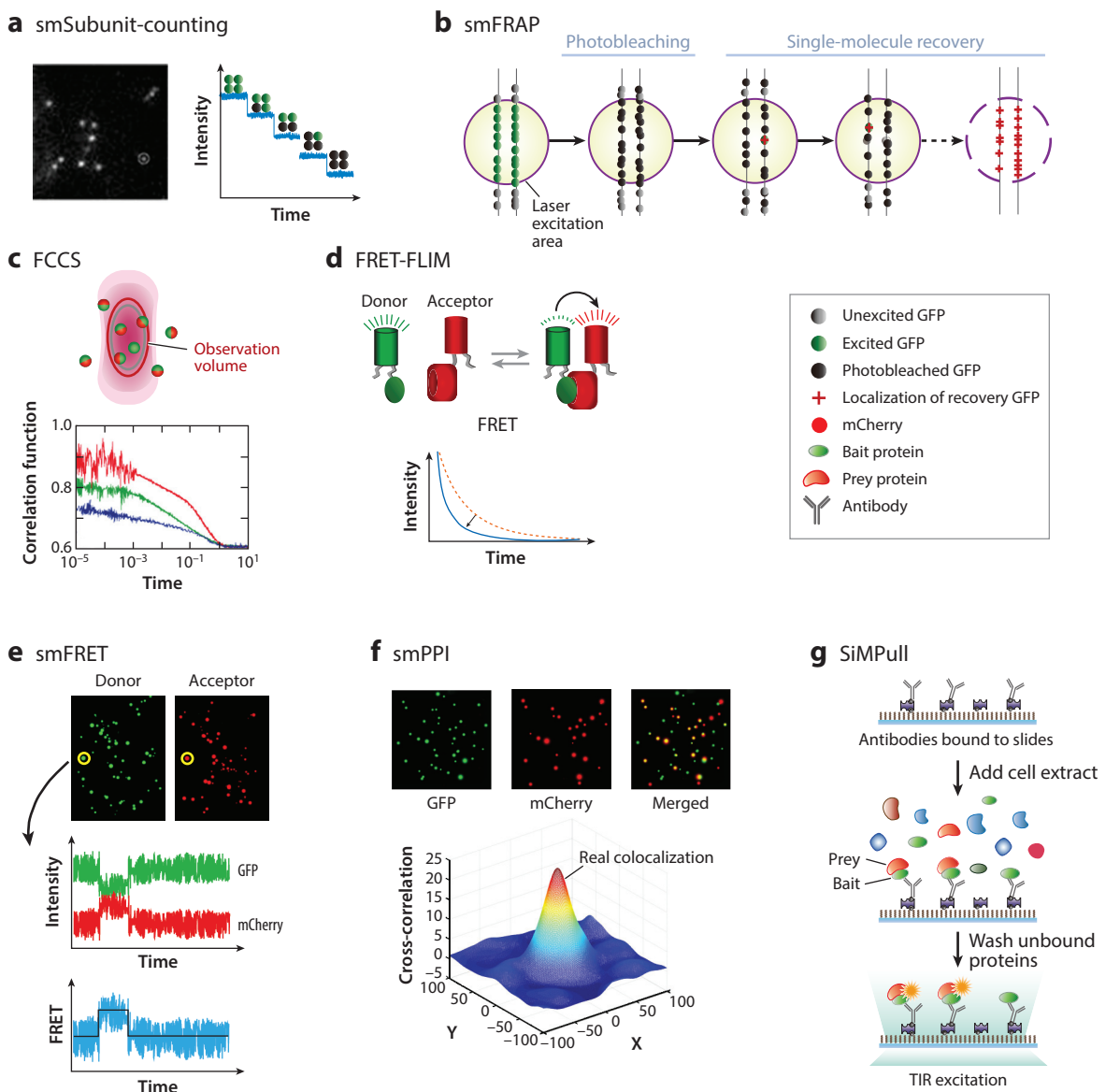
Photon-counting histogram (PCH):

calculates the average number and diffusion time of the fluorophores

Spatial intensity distribution analysis (SpIDA):

based on fitting super Poissonian distributions to intensity histograms

and dimers via single-dye labeling. For example, a study used SpIDA to quantify the dimerization of the epidermal growth factor receptor, providing direct evidence for dynamic changes in receptor-tyrosine kinase dimerization and trafficking in response to activation (134). Y. Xue & J. Lin (manuscript in preparation) used SpIDA to accurately calculate the distribution of oligomers in membrane proteins and the ratio of monomer and dimer density of phot1-GFP in the plant plasma membrane, indicating that this approach has great potential for use in determining the stoichiometry of membrane proteins in plant cells.



(Caption appears on following page)

Figure 2 (Figure appears on preceding page)

Schematic representations of some new techniques to quantify the oligomeric states of membrane proteins in plant cells. (a) smSubunit-counting reveals stoichiometry by imaging and counting photobleaching steps in fluorescence intensity. (b) smFRAP enables single-molecule recovery imaging and precise localization after photobleaching the region of interest, which is used to analyze the distribution of membrane proteins and their interactions at the single-molecule level. (c) FCCS detects the fluctuating fluorescence signals in dual-color channels (*red* and *green*) and estimates the cross-correlation (*blue*). (d) FRET-FLIM, a combination of FRET and FLIM, is used to analyze protein–protein interactions by determining the donor fluorescence lifetime. (e) smFRET can track the visible interaction of a donor molecule with an acceptor fluorophore at the single-molecule level on the basis of an increase in acceptor fluorescence. (f) smPPI analysis, based on high- and super-resolution imaging, is used to quantitatively analyze the colocalization of membrane proteins with higher spatial and temporal accuracy. (g) SiMPull, which combines conventional coimmunoprecipitation with single-molecule fluorescence microscopy, can be used to directly visualize individual protein complexes in vitro. Panel *a* adapted from Reference 144. Abbreviations: FCCS, fluorescence cross-correlation spectroscopy; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; SiMPull, single-molecule pull-down; smFRAP, single-molecule fluorescence recovery after photobleaching; smFRET, single-molecule FRET; smPPI, single-molecule protein proximity index; smSubunit-counting, single-molecule subunit counting; TIR, total internal reflection.

Techniques Based on Photobleaching

Photobleaching is the irreversible loss of fluorescence in a molecule due to changes in its structure following a light-induced chemical reaction (28). Single-molecule subunit (smSubunit)-counting, a technique based on single-molecule imaging and photobleaching of fluorescently labeled fusion proteins, was developed to analyze the number of subunits and the stoichiometry of membrane proteins on the surfaces of living cells (136). Upon fusion of a monomeric fluorescent protein to a membrane protein, some isolated fluorescent spots on the cell surface can be imaged and recorded via TIRFM and VA-TIRFM (**Figure 2a**). Under continuous excitation, the fluorescence intensity in regions of interest enclosing putative single molecules decreases in steps of approximately equal amplitude, which is consistent with the bleaching of single fluorophore molecules (61, 79). Because the photobleaching of a single fluorescent protein is a discrete process, the fluorescence intensity of a protein complex with one or several fluorescent protein molecules drops in a stepwise fashion, with the number of steps reflecting the number of fluorescent-protein-tagged subunits in the complex (**Figure 2a**). The number of steps required to bleach the regions of interest to background levels provides an estimate of the stoichiometry of the molecule (97). By way of smSubunit-counting, the stoichiometry of the subunits of over 40 proteins in plant and animal cells has been analyzed (5, 144). Nevertheless, smSubunit-counting is restricted to the analysis of proteins with low densities due to the diffraction limit of light. In practice, GFP and YFP variants are frequently used because they undergo clear bleaching steps, whereas blue and red fluorescent proteins are not suitable because they bleach too rapidly to enable individual steps to be distinguished. For multimerization states, the noise is generally excessive, which impedes the discernment of individual steps. In the future, as tracking algorithms improve, higher-order oligomeric complexes should be solved, leading to more accurate analysis.

Fluorescence recovery after photobleaching (FRAP) is based on the irreversible bleaching of a pool of fluorescent probes and monitoring the recovery of fluorescence due to the movement of the surrounding intact probes into the bleached spot. FRAP was originally established to detect protein mobility in living cells by measuring the rate of fluorescence recovery at a previously bleached site. Subsequently, the scope of these studies has expanded to include assessing protein dynamics and interactions with other cellular components (56). However, FRAP data analysis is usually performed on a large number of molecules and provides measurements of population mobility; additionally, this technique is also limited to the measurement of rapidly moving molecules. Single-molecule FRAP (smFRAP) was developed by integrating single-molecule imaging and FRAP to overcome the need for averaging that is intrinsic to bulk measurement methods. Through single-point

Photobleaching: the irreversible conversion of a fluorescent molecule into a nonfluorescent entity induced by optical excitation

Single-molecule subunit (smSubunit)-counting: analyzes the subunit number and stoichiometry of membrane proteins at the single-molecule level

Single-molecule FRAP (smFRAP): single-molecule fluorescence recovery after photobleaching

illumination, the photobleached area can be restricted to 0.5 μm . Once the area is completely photobleached, diffusion events of incoming fluorescently tagged membrane proteins occur at the single-molecule level and can be precisely localized (**Figure 2b**) (19, 104). By way of smFRAP, the distribution and translocation rates of some nuclear envelope transmembrane proteins in live HeLa cells were determined (104). With the development of super-resolution microscopy and multi-object tracking algorithms, there is no doubt that the simple smFRAP approach will help determine the distribution of membrane proteins and their turnover rates on the plasma membrane in plant cells at the single-molecule level.

Techniques Based on Fluorescence Fluctuations

FCS, a powerful method involving the measurement of fluorescence fluctuations, is used to decipher with high accuracy the heterogeneous motion of proteins and the organization of membranes (6). During confocal single-photon FCS microscopy, fluorescently labeled molecules are detected with avalanche photo diodes with single-molecule sensitivity when passed through a minute detection volume (10^{-15} L) (**Figure 1b**). Subsequently, the FCS data are subjected to an autocorrelation function to obtain temporal fluorescence intensity data (**Figure 1c**), which are further analyzed by specific diffusion models to explore the concentrations, mobility, and diffusion of molecules (83). In addition, FCS provides insights into molecular aggregation in the plant plasma membrane. For instance, FCS analysis of ATPase CDC48A oligomerization revealed that active CDC48A hexamers form part of a much larger complex in vivo and that inhibiting ATP binding also inhibits the formation of complexes of CDC48A with other proteins (2). Recently, combined scanning FCS comprising raster image correlation spectroscopy, pair correlation function, and number and brightness were used to analyze the mobility and interactions of SHORTROOT (SHR) with its downstream target SCARECROW (SCR) during *Arabidopsis thaliana* development. Examination of SHR using number and brightness alone and in combination with cross-correlation analysis revealed the oligomeric state of SHR and the stoichiometry of the SHR-SCR complex, respectively (25).

In the past few years, several extensions of FCS have been implemented to go beyond the optical diffraction limit (approximately 200 nm), such as STED-FCS, TIR-FCS, and spot variation FCS (32, 48, 49). These new approaches provide superior resolution for cell surface imaging and have been used to monitor the interactions of a variety of ligand-receptor pairs in the membrane and to investigate the binding between cell membrane and membrane lipid-binding proteins in living cells.

FCCS is an extension of FCS in which two colored labels are simultaneously excited by two excitation lasers at different wavelengths (91). In dual-color FCCS, the binding and colocalization of proteins are detected with fluorescent proteins labeled in different colors (such as red and green), and the fluctuating fluorescence signals are detected in each channel (**Figure 2c**). As a result, the cross-correlation function between the different labeling traces can be calculated, providing information about the degree of binding or colocalization (**Figure 2c**) (116). More importantly, FCCS signals are unaffected by the distance and orientation of the two fluorophores, making FCCS superior to other techniques for quantifying the interactions between two molecules (80). Dual-color FCCS and its derivatives are widely used to quantify the interactions between many protein pairs in the plasma membrane. For example, dual-color FCCS was used to monitor complex formation by fluorescently tagged BRASSINOSTEROID INSENSITIVE1 (BRI1) and *A. thaliana* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 (AtSERK1) in protoplasts (53). However, it is difficult to use conventional FCCS to determine the diffusion rates of slowly diffusing molecules, and the use of a confocal setup often disturbs FCCS in a heterogeneous background (116). To compensate for the disadvantages of FCCS, researchers developed TIR-FCCS.

This technique uses TIRFM imaging to increase the z -axis resolution, which facilitates the probing of molecular interactions in the plant membrane with higher collection efficiencies and signal-to-noise ratios.

Techniques Based on Förster Resonance Energy Transfer

FRET is a distance-dependent physical phenomenon that occurs when two fluorescent molecules are near each other (between 2 and 10 nm) and energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor). FRET microscopy based on this principle has been developed as a quantitative measure for distances between single donor and acceptor molecules to investigate the dynamic interactions between proteins (**Table 1**) (105). For hetero-FRET, energy is transferred between distinct fluorophores, and two tags can be used to label either two proteins (to analyze protein–protein interactions) or the same protein (to monitor conformational changes and dimerization). Frequently, hetero-FRET is used as a biosensor for calcium measurements and phosphorylation analysis (108). By contrast, homo-FRET was developed to investigate the self-assembly of proteins in living cells. Homo-FRET involves the transfer of excited-state energy between the same fluorophore and thus requires only a single fluorophore moiety for labeling; this transfer does not change fluorescence lifetime properties. Consequently, homo-FRET is only detectable by measuring the loss of anisotropy upon excitation with polarized light via fluorescence anisotropy imaging microscopy, and it is more suitable in the analysis of molecular self-assembly than hetero-FRET (21). Nevertheless, because all these FRET methods are based on signal intensity, they are limited by spectral bleed-through and fluorophore concentration (34). It is also challenging to obtain quantitative information owing to the difficulty in interpreting energy transfer efficiency in terms of 2D distributions. Moreover, FRET does not provide information about protein densities or multimeric organization beyond dimers.

Fluorescence lifetime is a measurement of the rate of decay of the emission of a fluorescent molecule, which is independent of fluorophore concentration and excitation intensity (**Figure 2d**) (138). Combining FRET and fluorescence lifetime imaging microscopy (FLIM) makes it possible to provide high spatiotemporal resolution to analyze protein–protein interactions. Importantly, because the lifetime of only one protein partner, the donor fluorophore, is measured, it is not necessary to use spectral bleed-through correction in FRET-FLIM imaging. FRET-FLIM has been extensively used to analyze protein–protein interactions in living plant cells; Bücherl et al. (15) describe this protocol in detail. Examples of these studies include investigations of the dynamics of the interaction between MLO (plasma membrane–localized mildew resistance locus O) and calmodulin in epidermal cells (13), the interaction of PIP1s and PIP2s (10, 148), changes in BRI1 and AtSERK3 receptor homodimerization or heterodimerization during active brassinosteroid signaling at the plasma membrane (18), and ARABIDOPSIS CRINKLY4 and CLAVATA1 homomeric and heteromeric complexes (130). Compared with intensity-based methods, FRET-FLIM measurements require the analysis of a greater number of photons, and the longevity of the fluorophore is sacrificed in favor of better temporal resolution (16). Even though this technique is somewhat complex, it provides information about protein interactions under physiological conditions at very high spatiotemporal resolution.

Single-molecule FRET (smFRET) is based on TIRFM combined with electron-multiplying charge-coupled device camera detection or confocal detection using fast single-photon-counting point detectors. Thus, smFRET can be used to directly characterize the heterogeneity of biomolecules on the microsecond time scale at the single-molecule level (**Figure 2e**). smFRET has been used to analyze the conformational changes and intermolecular interactions between two or more molecules in animal cells (41, 120). To date, smFRET has been performed mainly to

Hetero-FRET:

FRET between distinct fluorophores

Homo-FRET: FRET between fluorophores of a single type

Fluorescence

lifetime: an average period of time a fluorophore spends in an excited state before undergoing relaxation

Single-molecule

FRET (smFRET): detects fluorescence resonance energy transfer at the single-molecule level

Single-molecule PPI (smPPI): calculates protein proximity index at the single-molecule level

SiMPull: single-molecule pull-down

analyze the interactions between organic dye-labeled ligands or between a ligand and fluorescent protein-tagged protein delivered by microinjection. smFRET requires sophisticated experimental design and rigorous labeling of proteins (123, 132), likely hindering its application to plants. Although the use of this approach in plant cells has not been reported, smFRET will undoubtedly be used in the near future to detect biomolecular interactions in living plant cells.

Techniques Based on Colocalization and Interactions

Colocalization between two fluorescently labeled proteins in fluorescence microscopy images is widely used to map and envision the possibility of protein–protein interactions. A high level of colocalized signals generally indicates the two proteins of interest are near each other, suggesting possible interactions between them. The degree of this colocalization can be quantitatively estimated by various approaches, such as Pearson's correlation coefficient and Manders colocalization coefficient analyses (30). Zinchuk et al. (151) provided a protocol for calculating the protein proximity index (PPI) by combining the median filtering and advanced thresholding procedures to reduce background. This technique provides a universal approach for background reduction. PPI values yield good estimations of the percentage of colocalized molecules, which is especially useful for studying membrane proteins. Owing to the lack of a unified approach for interpreting PPI results, Zinchuk et al. (150) proposed linguistic variables related to the range of values in colocalization coefficients. However, colocalization values are frequently overestimated. Single-molecule PPI (smPPI) was recently developed on the basis of single-molecule images collected from VA-TIRFM (**Figure 2f**). Using smPPI calculation, investigators (47, 141) have performed quantitative colocalization analyses of several membrane proteins, including respiratory burst oxidase homolog D (RbohD) and BRI1. Through high- and super-resolution imaging, biomolecules can be precisely localized and analyzed. Compared with bulk measurement, this approach provides accurate estimations via quantitative colocalization analysis. smPPI can potentially be applied to other biological systems, but unified criteria for interpreting PPI results are needed.

Single-molecule pull-down (SiMPull) is a technique that integrates the principles of conventional coimmunoprecipitation with powerful single-molecule fluorescence microscopy. In this method, antibodies against a known protein are immobilized on a microscope slide and capture the protein together with its physiological interaction partners. After the unbound components are washed away, single-molecule fluorescence microscopy is used to probe the pulled-down proteins (**Figure 2g**). This technique mitigates the disadvantages of commonly used biochemical assays and is minimally invasive, sensitive, and rapid, allowing direct visualization of single macromolecule complexes at the single-molecule level (1). Husbands et al. (55) applied SiMPull to plants to analyze the colocalization of HOMEODOMAIN LEUCINE ZIPPERIII (HD-ZIPIII)-YFP and LITTLE ZIPPER (ZPR)-mCherry (**Figure 2g**), providing strong statistical evidence that they form a complex. When proteins are stoichiometrically labeled, SiMPull can be used to directly determine the stoichiometry of a complex. SiMPull, in conjunction with photobleaching assays of individual complexes, was used to determine that HD-ZIPIII:ZPR exists as a heterotetramer comprising two HD-ZIPIII and two ZPR molecules rather than heterodimers, as reported previously (55). Therefore, SiMPull is poised to make a unique contribution to studies of membrane protein assembly.

The techniques mentioned above compose a versatile toolbox for quantifying the dynamics and probing the stoichiometry of molecular complexes, which greatly improves our understanding of the roles proteins have in cellular processes in their native environments. The particular benefit of these techniques is to constrain the membrane plane in two dimensions. Furthermore, TIRFM significantly increases the signal-to-noise ratio for almost all measurements of molecular behaviors in living plant cells. Fluorescence microscopy with new modalities of sample illumination will

promote single-molecule imaging in a true 3D space in living plant cells. For instance, light sheet microscopy, which entails the use of a focused light sheet to illuminate the sample and the collection of the fluorescence signal via an orthogonally positioned imaging objective, significantly improves the signal-to-background ratio and is suitable for single-molecule imaging in a 3D space (117). With the low photobleaching and rapid optical sample sectioning, new combinations with light sheet microscopy will help researchers track and quantify the 3D distribution and movement of membrane and intracellular proteins (115). Moreover, super-resolution microscopy will facilitate the development of several new combined approaches, such as interferometric PALM and STED-FCS/FCCS, which further promote investigations of the spatiotemporal organization of protein complexes and interactions in plants.

BIOLOGICAL APPLICATIONS

Oligomerization and Activation for Signaling Initiation

Many membrane proteins, such as ion channels, transporters, receptors, and enzymes, are key regulators of critical cellular processes. For example, receptor-like kinases (RLKs) such as CLAVATA1, BRI1, Flagellin Insensitive 2, and S-locus receptor kinase control various processes in plants (76). Early studies suggested that the overall topology of RLKs and their intracellular signaling pathways were highly conserved during evolution. In general, ligands can induce dimerization or oligomerization of membrane proteins, which is often considered to be the first step in the activation of intracellular signals, leading to the regulation of signal transduction by phosphorylation, ubiquitination, or both (7). However, a subset of membrane proteins can also form dimers in the absence of extracellular stimulation. The use of single-molecule analysis may determine the equilibrium constant of monomers and dimers in living cells, allowing us to correctly predict time-dependent changes in the intensity and spatial spread of downstream signals.

Early experiments using PCH and FCCS to analyze the assembly state of BRI1 in plant cells showed that approximately 20% of BRI1 molecules in the plasma membrane are present in homo-dimerized form in the absence of exogenous brassinolide (53). Furthermore, an smSubunit-counting technique revealed that even at low brassinosteroid levels, some BRI1 receptors were still present as dimers on the plasma membrane, suggesting that BRI1 may exist in the form of inactive dimers in the absence of ligand (141). Furthermore, an analysis of the spatial correlation between BRI1 and BAK1 (SERK3) revealed that large populations of BRI1 and BAK1 colocalized independently of ligand, suggesting that preassembled BRI1–BAK1 hetero-oligomers may be involved in the initiation of downstream signal transduction (18). These findings led to the hypothesis that in plants, some RLKs may exist in preassembled dimers or hetero-oligomers in the absence of ligands, and although these forms are inactive, they are primed for ligand binding and signaling (**Figure 3a**). However, the equilibrium of monomers, predimers, and dimers of membrane proteins in plant cells is poorly understood. We still do not know how the interconversion between monomers and dimers occurs on a timescale of seconds and whether it affects downstream signal transduction. Furthermore, in most cases, multiprotein complexes act coordinately to regulate signal transduction. Therefore, it would be beneficial to develop multicolor labeling and detection techniques to analyze the dynamic assembly state of hetero-oligomerization at the single-molecule level in living cells.

Changes in Relation to Membrane Microdomains

Cell signaling relies on modular domains that generate protein interactions at the membrane (43). Biophysical evidence suggests that a variety of phenomena likely influence the clustering of

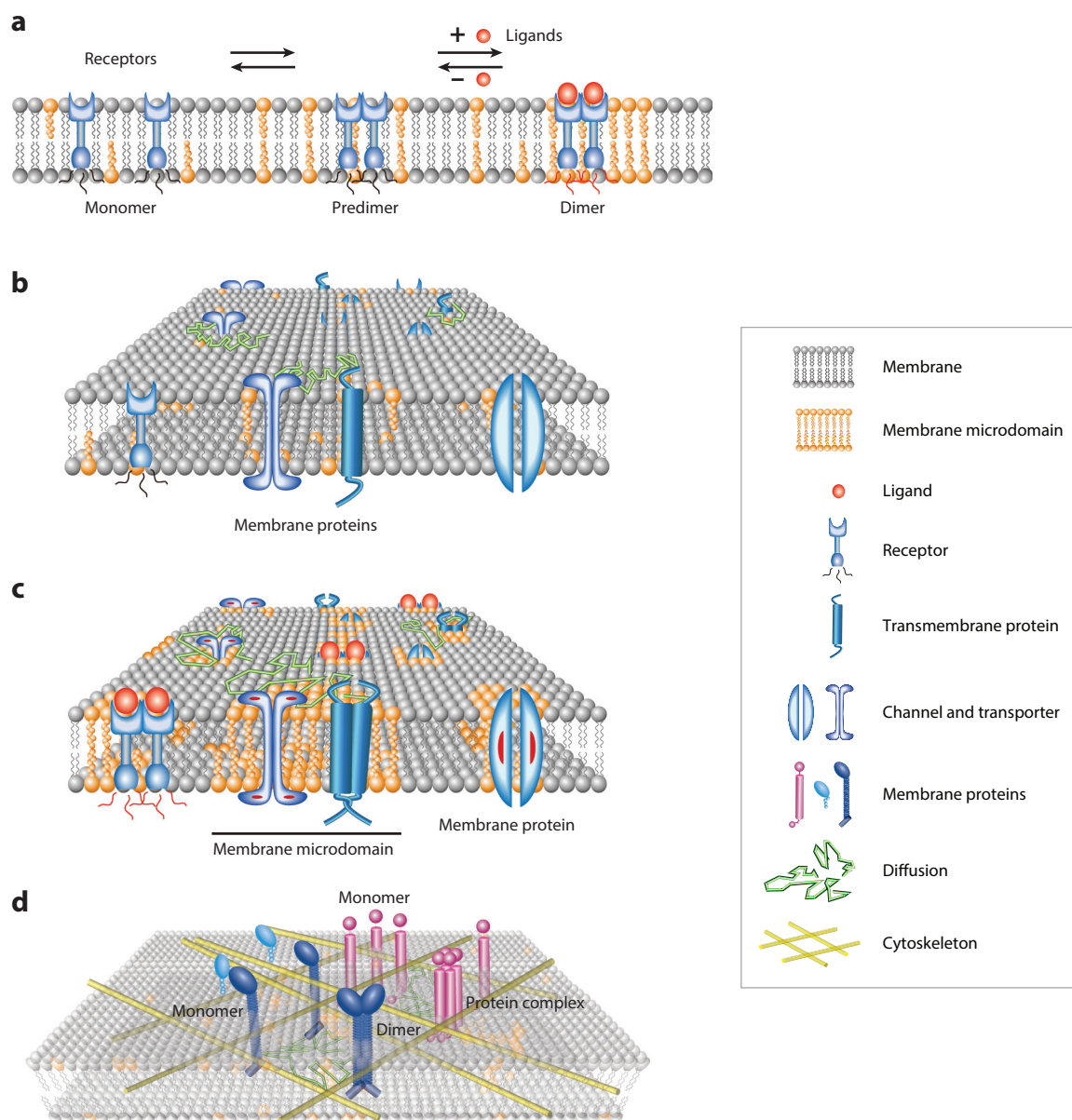


Figure 3

The techniques used to reveal biological significance in plants. (a) Membrane receptor assembly is involved in the regulation of monomer-predimer-dimer dynamic equilibrium. (b) Plasma membranes are highly heterogeneous lipid bilayers, comprising transmembrane proteins, receptors, ion channels and transporters, and membrane microdomains. (c) Stimuli can promote protein oligomerization and the recruitment of proteins into functional membrane microdomains to regulate signal transduction (protein icons with red markings represent activated proteins). (d) The cortical cytoskeleton can restrict the diffusion of membrane proteins, assist in their assembly, and potentially modulate signal transduction.

membrane proteins to regulate signaling activity. Lipid rafts or membrane microdomains might exist, in which clusters of membrane proteins and lipids form small but discrete platforms within the plane of the membrane (81, 126). These microdomains can facilitate dynamic cellular signaling by providing a niche for clustering previously isolated receptors and channels and for aggregating signaling components (72, 82).

Accumulating evidence suggests that many proteins, which were first identified in detergent-resistant membranes, reside in membrane microdomains. Most of these proteins are involved in signaling and transport, suggesting that distinct plant plasma membrane microdomains represent association platforms for signaling and transport processes (92). Specifically, stomatin/prohibitin/flotillin/HflK/C-domain-containing proteins, such as flotillins, are commonly accepted markers for membrane microdomains (66, 77). In addition, plant-specific remorin proteins may act as molecular scaffolds that recruit plasma membrane-associated and cytoplasmic proteins into microdomains to preassemble signaling complexes (60). Single-molecule analysis showed that flotillins and remorin proteins label coexisting and distinct membrane domains in living plant cells; at least two major classes of membrane domains have been identified (29). Nanodomains (10–100 nm) can coalesce into larger microdomains (>100 nm) via interactions between their individual components. Finally, different types of microdomains contribute to the functioning of the associated proteins (59).

Numerous studies support the notion that membrane microdomains are involved in regulating the assembly of membrane proteins and influencing their function in plant cells. For example, an experiment using STED showed that the ABA signaling phosphatase ABSCISIC ACID INSENSITIVE1 and the calcium-dependent protein kinase 21 localize to membrane microdomains, thereby preventing anion channel activation (29). Other membrane proteins, including the potassium channel KAT1 (133) and the NADPH oxidase RbohD, also localize to membrane microdomains (75). Notably, some membrane proteins can move into and out of membrane microdomains, and some stimuli may promote their oligomerization and recruitment into functional membrane microdomains to regulate signal transduction (**Figures 3b,c**) (79, 141), indicating that membrane microdomains help alter the activity of some membrane proteins by positively or negatively affecting their clustering and signal transduction. Furthermore, using FRET-FLIM, Bhat et al. (13) determined that powdery mildew fungus might induce membrane microdomains to form at entry sites. Further analysis showed that oligomeric remorin proteins attach to the host plasma membrane and control plant–bacteria interactions (74). Quantitative image analysis also showed that after inoculation with the symbiotic bacterium *Sinorhizobium meliloti*, dynamic LYK3:GFP puncta became relatively static and were recruited into laterally immobile microdomains labeled by flotillin 4 (45). Although membrane microdomains undoubtedly act as an organizing platform and populations of membrane microdomains in plant cells are diverse and heterogeneous, precisely how the proper functioning of membrane microdomains is linked to long-range signaling remains unknown. Single-molecule methods are unique for their ability to investigate the spatiotemporal dynamics of protein segregation into membrane microdomains and to specify protein function.

Dynamics Associated with the Cytoskeleton

The cytoskeleton is a ubiquitous and dynamic network of filamentous structures found throughout the cell. The cortical cytoskeleton is involved in modulating the mobility of proteins and lipids in the membrane and influencing their clustering (50, 57). Single-molecule imaging and tracking analysis suggested that the cytoskeleton and membrane proteins constitute different membrane compartments: All transmembrane proteins generally undergo short-term confined

diffusion within a compartment but must undergo long-term hopping movements between compartments (67). The cortical cytoskeleton can restrict the diffusion of membrane proteins, assist in their assembly, and potentially modulate signal transduction (**Figure 3d**) (57).

Actin filaments and microtubules are organized into networks that enable the intracellular trafficking and delivery of cell wall material to the extracellular environment. Both structures are linked to organizational events associated with the plasma membrane and cell wall. For example, the localization of KORRIGAN1 and COBRA, two proteins that function in cell wall biosynthesis (118, 119), as well as GPI-anchored proteins, which are associated with membrane microdomains (122), is dependent on microtubules in the plasma membrane. Microtubules are also thought to influence tropisms by positioning regulatory proteins or complexes in the plasma membrane. Spinning disk microscopy and kymograph analysis showed that cortical microtubules can maintain a stable lateral position to direct the movement of cellulose synthase (CesA) complexes along the microtubule (84, 110). In contrast to microtubules, the actin cytoskeleton has a fundamental role primarily in intracellular transport and motility (52). Moreover, actin is associated with the plasma membrane (94). Thus, the plant cortical cytoskeleton is associated with the cell wall and plasma membrane and may help regulate protein assembly.

In contrast to membrane proteins in animal systems, some plant membrane proteins are rather immobile, such as PIN2, KAT1, KNOLLE, BOR1, and NIP5;1, which display low lateral mobility in the plasma membrane (62, 96, 135). However, other membrane proteins display dynamic behavior in the plasma membrane. Using single-molecule techniques combined with SPT, researchers have quantified the diffusion coefficients and displacement of membrane proteins such as BRI1 and FLAGELLIN SENSITIVE2 (FLS2) (17), PIP2;1 (79), and RbohD (47). Interestingly, the diffusion coefficient and modes of these proteins can change under different environmental conditions, indicating that diffusion has a role in regulating plasma membrane function. Recently, dual-color VA-TIRFM was used to analyze the dynamics of fluorescent protein–labeled *Arabidopsis* hypersensitive-induced reaction and microtubules (TUA5-mCherry) in living *Arabidopsis* cells. The investigators revealed that the microtubule-based membrane skeleton can create relatively stable membrane compartments and restrict lateral diffusion of AtHIR1 in the plasma membrane (90). Other studies have shown that the cytoskeleton can limit the lateral diffusion of membrane proteins such as CesAs and POM2 (14, 121).

However, two recent studies showed that the cytoskeleton and membrane microdomain organization have little effect on the diffusion of some membrane proteins. For example, using FRAP, Martinière et al. (95) found that the cell wall can limit the lateral diffusion of membrane proteins, including GPI and MAP. Similarly, live-cell SPT with PALM analysis demonstrated that membrane microdomains and microtubules do not confine the mobility of AtPIP2;1-mEOS2. However, actin is partially implicated in its confinement, and the cell wall can immobilize membrane proteins, indicating that cell walls have a major role in controlling the dynamics of plasma membrane proteins (54). Although it is difficult to reconcile these results, various proteins connect the cell wall to the cytoskeleton and this implies that the cell wall, plasma membrane, and cytoskeleton should not be regarded as independently acting components.

Responses to Environmental Stimuli

Cells sense the environment and communicate with each other through regulating the spatial organization of proteins at the cell surface, representing a critical step in signal transduction and endocytic membrane trafficking (129). When cells perceive the extracellular environment, the dynamics of membrane proteins change, and these proteins are internalized and sorted into various endosomal compartments (109). Endocytosis regulates cell signaling by controlling the density

and dwell time of active proteins on the plasma membrane; cell signaling and endocytosis are intimately and bi-directionally linked (7).

Plants also possess a homeostatic mechanism in which endocytosis regulates the internal concentrations of nutrients in response to changes in nutritional status. Typical examples include the endocytic regulation of the boron transporter BOR1, the phosphate transporter PHT1;1, and the ammonium transporter AMT1;3. BOR1 rapidly undergoes endocytosis and degradation under high-boron supply to prevent toxic levels of boron from accumulating (135). Similarly, PHT1;1 is controlled by regulatory mechanisms including oligomerization and endocytosis to prevent cellular toxicity associated with high internal concentrations of phosphate (8, 38). Single-molecule analysis showed that AMT1;3 forms clusters that are rapidly internalized in the cytoplasm under external high-ammonium stress (143). These findings demonstrate that clustering and endocytosis effectively help plant cells avoid overaccumulation of nutrients by removing active ion channels and transporters from the plasma membrane.

Another striking example of these mechanisms is the response of plants to abiotic stimuli. Low temperature inhibits the internalization of some membrane proteins, light regulates membrane protein localization, and hyperosmotic stress induces changes in membrane dynamics and lipid composition to maintain cellular integrity (reviewed in 73). After salt treatment, the rate of cycling by water transport proteins PIP1;2 and PIP2;1 is significantly enhanced (88). VA-TIRFM imaging combined with SPT revealed that the number of GFP-PIP2;1 subunits in a complex ranges from one to four, and under salt treatment, the diffusion coefficients and percentage of restricted diffusion increase. These findings indicate that PIP2;1 exists in various multimeric forms and that PIP2;1 internalization is enhanced in response to salt stress (79). Importantly, environmental stimuli function as specific regulators of the endocytic pathways of membrane proteins. FCS and FCCS analysis confirmed that both clathrin-dependent and membrane microdomain-associated endocytic pathways can cooperatively regulate endocytosis of membrane proteins and that membrane microdomain-associated pathways can be stimulated under salt stress (47, 79). These findings suggest that enhanced internalization and endocytic pathways represent specific responses of membrane proteins to hypertonic stress. Such responses alter the mobility and amount of the active forms of these proteins on the plasma membrane, acting as a regulatory mechanism to protect plant cells against stress conditions.

Plants specifically regulate endocytic trafficking in response to pathogen attack. Some plasma membrane-resident pattern recognition receptors (PRRs), such as FLS2, the PRR kinases EF-TU RECEPTOR (EFR) and CHITIN ELICITOR RECEPTOR KINASE (CERK), and receptors for damage-associated endogenous peptides PEP RECEPTOR1 (PEPR1) and PEPR2, can sense potential pathogens, and their internalization is triggered upon ligand perception (11, 27, 102). One of the best-studied examples is the perception of bacterial flagellin. Imaging analysis of the spatiotemporal dynamics of FLS2 revealed that flg22 enhances FLS2 internalization and induces trafficking of FLS2 into the late endosomal pathway (9, 127). Furthermore, trade-offs between endogenous hormone pathways and immunity may affect plant growth (3). In a recent study, SPT and quantitative colocalization analysis showed that FLS2 and BRI1 cluster within distinct remorin-labeled plasma membrane nanodomains, which may contribute to signaling specificity between immune and growth signaling (17). Plants also secrete defense molecules via exocytosis to repel invading pathogens (69). For example, during plant–fungus interactions, the endomembrane-compartment-associated R-SNAREs VAMP721 and VAMP722 participate in secretory pathways to deliver pathogen-inducible cargos (68). However, the mobility, docking, and priming process of single secretory vesicles containing pathogen-defense-response components must be precisely investigated in real time by means of single-molecule imaging and tracking methods to shed light on the mechanism underlying plant innate immunity.

CONCLUSIONS AND FUTURE ISSUES

Many cellular functions take place in membranes through the intimate clustering or oligomerization of proteins, but how these proteins are spatiotemporally organized and the depth of their biological significance in plants remain enigmatic. Clarifying these issues will require sophisticated imaging technology, as well as data analysis, to reveal the location and dynamics of proteins in the membrane at high spatiotemporal resolution. The emergence of single-molecule methodology has led to great advances in our understanding of the functions of different plasma membrane proteins in plant cells.

Despite substantial progress, the use single-molecule techniques to analyze the dynamics of protein oligomers and protein–protein interactions remains challenging. In the future, it will be necessary to develop standard experimental conditions and smart automated tracking algorithms to perform high-throughput analysis of the accumulating data. Investigations involving single-molecule 3D tracking with multifocal plane imaging techniques and super-resolution imaging with spectroscopic measurements are needed to obtain more reliable information to better understand the molecular basis of signal transduction processes. Single-molecule experiments and bulk experiments such as biochemical analysis should also be combined to develop quantitative models that incorporate data about dynamic regulation with data describing proteins and biological functions. The use of combined techniques supported by ever-progressing single-molecule methodology will help us monitor the transduction pathways of individual signaling molecules and detect their chemical fingerprints during signal transduction.

SUMMARY POINTS

1. The plant plasma membrane is highly dynamic, and deciphering spatiotemporal changes in proteins in the membrane provides insights into protein function and the organization of the cell membrane.
2. Single-molecule techniques offer an unprecedented means to address the assembly and interactions of single biomolecules in their native but complex environment, where molecular events take place in a stochastic manner in both time and space.
3. The labeling of membrane proteins is a crucial step in single-molecule analysis because fluorescent signals from single molecules or labels in living cells are extremely weak.
4. New developments in optical microscopy and super-resolution microscopy, with increased sensitivity and signal-to-noise ratios, open exciting new avenues for exploring the organization, dynamics, and functions of membrane proteins at the nanosecond and nanometer scales.
5. Several single-molecule techniques (based on fluorescence brightness, photobleaching, fluorescence fluctuation, FRET, and colocalization and interactions) have great potential for quantifying the assembly and dynamics of membrane proteins.
6. These techniques have been used to help decode complex signaling messages at multiple levels, and their potential for use in biological function studies will eventually be realized.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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