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Single-Particle Tracking for the Quantification of Membrane Protein Dynamics in Living Plant Cells

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ABSTRACT

The plasma membrane is a sophisticated, organized, and highly heterogeneous structure that compartmentalizes cellular processes. To decipher the biological processes involving membrane proteins, it is necessary to analyze their spatiotemporal dynamics. However, it is difficult to directly assess the dynamics and interactions of biomolecules in living cells using traditional biochemical methods. Single-particle tracking (SPT) methods for imaging and tracking single particles conjugated with fluorescent probes offer an ideal approach to acquire valuable and complementary information about dynamic intracellular processes. SPT can be used to quantitatively monitor the diverse motions of individual particles in living cells. SPT also provides super-spatiotemporal resolution that allows early-stage or rapid response information to be obtained for a better understanding of molecular basis of associated signal transduction processes. More importantly, SPT can be used to detect the motion paths of individual biomolecules *in vivo* and *in situ*, thus unveiling the dynamic behavior of the biomolecules that support developmental processes in living cells. In this review, we give an overview of SPT methods, from image acquisition to the detection of single particles, as well as tracking and data analysis. We also discuss recent applications of SPT methods in the field of plant biology to reveal the complex biological functions of membrane proteins.

Key words: single-particle tracking, dynamics, stoichiometry, dwell time, colocalization

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INTRODUCTION

Biological membranes are highly dynamic structures that are involved in very complex cellular processes. Membrane protein trafficking has been reported as an important mechanism underlying cell membrane organization (Alcor et al., 2009). To gain insight into the fundamental principles of membrane protein trafficking, it is necessary to examine and analyze the dynamics of protein molecules at the plasma membrane over time and space (Dehmelt and Bastiaens, 2010). The advent of fluorescence imaging techniques has provided the means to understand biomolecular dynamics in living cells. In particular, with the advances in fluorescence microscopy in combination with fluorescent protein labeling techniques, the spatial and temporal accuracy of living cell imaging technology has been greatly improved (Shaw and Ehrhardt, 2013). Single-particle microscopy techniques are new tools for the detection and manipulation of individual biological molecules that allow molecular characteristics and dynamics to be studied at the nanoscale level (Wang et al., 2018).

Various single-particle microscopy techniques have been developed, such as fluorescence correlation spectroscopy (FCS), single-molecule fluorescence recovery after photobleaching (smFRAP), and single-particle tracking (SPT) (Garcia-Saez and Schwille, 2008; Schuler and Eaton, 2008). The FRAP and FCS methods offer the advantage of collecting a large amount of data and allow immediate data analysis (Alcor et al., 2009; Rouger et al., 2012). SPT techniques allow researchers to quantitate the diffusion properties and motion paths of certain particles with high temporal resolution and a high signal-to-noise ratio (Kusumi et al., 2005). Here, the particles can be single protein molecules, multisubunit proteins, or protein or

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lipid complexes; the procedure for detecting and characterizing the dynamic behavior of single particles in a time-lapse image sequence is often called SPT (Chenouard et al., 2014). Tracking of the particle motion of interest is performed to understand the mechanisms underlying a wide variety of biological processes, from the basic mechanisms of molecular machines to protein–protein interactions (Yildiz, 2009). The application of SPT techniques has revolutionized the way we study biological systems. These techniques have been used to study various aspects of cell biology, including the dynamic behavior of membrane proteins and the elementary molecular processes responsible for this behavior (Hao et al., 2014; Wang et al., 2015a, 2015b).

In recent years, SPT techniques have been extensively applied for analyzing protein dynamics in plant cells and have led us to understand the functions of different plasma membrane proteins in living plant cells. In this review, we schematically illustrate the principles of SPT analysis. We also summarize the commonly used approaches, as well as the key parameters that should be considered in SPT and in data analysis. This may help researchers gain a more detailed view of the SPT technique. Furthermore, we describe current developments and applications of SPT in plant cells, including protein dynamics, stoichiometry, dwell time, and colocalization. We anticipate that this review will provide readers with a glimpse of the new world of quantitative information on molecular and subcellular processes obtainable by SPT and encourage researchers to become involved in research conducted at the level of single particles.

PRINCIPLE OF SINGLE-PARTICLE TRACKING

One of the key steps of SPT is obtaining series of time-lapse images of particles in a live cell. Tracking single particles requires identifying particles and determining their location in consecutive frames. While this is straightforward when single particle density is very sparse and SPT can be performed manually for few tracks, automation is necessary when tracking the hundreds or thousands of particles that can be acquired at once in a single cell. Moreover, extensive data analysis is required to describe the dynamic characteristics of particles. Accordingly, the whole process can be broken down into the following steps: image acquisition, background reduction, particle tracking, and data analysis.

Acquisition of Time-Lapse Image Sequences

The acquisition of images initially requires the specific labeling of proteins using a fluorescent probe (Langhans and Meckel, 2014). To date, the most commonly used probes include small-molecule fluorescent dyes, nanosized fluorescent particles, fluorescent proteins, and their variants (Wang et al., 2018). By comprehensively evaluating different probes, researchers may select the most suitable probes for certain investigations. Genetically encoded fluorescent proteins are the most popular fluorescent labels used for specifically labeling molecules inside live plant cells to improve the quality of single-particle images. The advantages of fluorescent proteins include stable inheritance, a 1-to-1 labeling ratio, low toxicity, and obviation of molecules passing through cell walls and the plasma membrane (Langhans and Meckel, 2014; Wang et al., 2018). Research studies have shown that fluorescent proteins, such as green fluorescent protein (GFP) and other variants, are advantageous for live cell experiments because of their reasonable photostability and quantum yields (Joo et al., 2008).

Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) is one of the key tools facilitating the tracking of fluorescent signals at the plasma membrane of plant cells (Wan et al., 2011). VA-TIRFM illumination offers the best combination of background reduction, wide-field data collection, and high imaging speed by using an evanescent field as the excitation source (Xiao, 2009) (Figure 1A). Using this technique, we were able to film single-particle movies in living plant cells. Here, as an example, we describe the detection of GFP-labeled plasma membrane aquaporin (GFP-PIP2;1) in living transgenic *Arabidopsis thaliana* plants. After being surface sterilized for 30 s in 85% EtOH/H_2O_2, the seeds were transferred to solid medium containing half-strength Murashige–Skoog salts with 0.7% plant agar. Subsequently, plates were transferred to a heated light chamber (22°C, 70% relative humidity with a 16-h-light/8-h-dark photoperiod). Seedlings that were grown for 4–6 days were used for further observation (Figure 1B). For observation, one seedling was transferred to a glass slide, immersed in Murashige-Skoog medium, and covered with a coverslip (Brand, Wertheim, Germany; borosilicate glass; thickness 0.13–0.17 mm; refractive index $n = 1.52 \pm 0.01$). The slide was then mounted onto a microscope equipped with a 100× oil-immersion objective (numerical aperture = 1.49, Olympus). GFP was excited by the laser (OBIS 488; Coherent, USA) and the emission fluorescence collected with an EMCCD camera (Evolve 512 Delta; Photometrics, USA). Time-lapse series images of GFP-PIP2 were acquired with a 100-ms exposure time and stored directly in a computer (Figure 1C). The recorded images were used to conduct further analyses (Figure 1D and 1E).

Detection and Tracking

When tracking single particles in live cells, the main challenge is to enhance the signal-to-noise ratio. In plant cells there is generally autofluorescence from the cell wall components. Clearly, the higher the background noise the more difficult it is to identify a signal. Therefore, the background fluorescence should be subtracted prior to SPT analysis. In recent years, several methods have been developed to subtract background fluorescence, including the rolling-ball method (Zhang et al., 2009), median filtering (Wang et al., 2015a, 2015b), and spectral unmixing (Chen et al., 2009). The rolling-ball method is generally used for unevenly illuminated samples. However, for exclusion of random noise caused by scattered photons, dark current in the detectors, or electronic noise, median filtering of the measured data turns out to be a good method (Wang et al., 2015a, 2015b). In addition to rolling ball and median filtering, the use of spectral unmixing for correcting overlapping signals, such as the partially overlapping emission spectra of cyan and yellow fluorescent protein, should not be ignored (Chen et al., 2009). After background reduction, signals that exceed a threshold criterion are subsequently fitted to the Gaussian target image (Hinterdorfer and Van Oijen, 2009). Eventually, researchers can fit each of the identified signals to extract the information at each position.

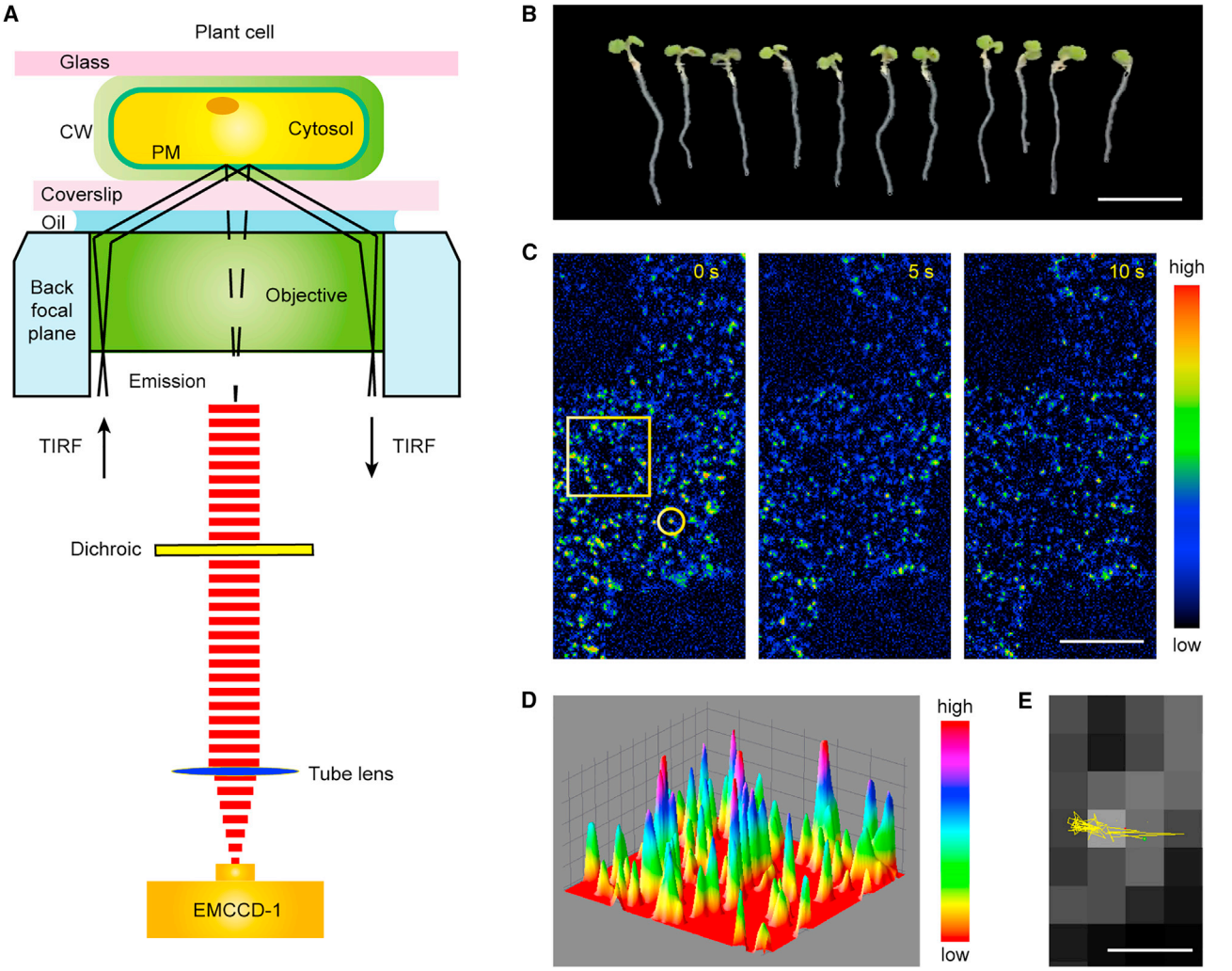


Figure 1. Detection of a GFP-Tagged Protein in *Arabidopsis thaliana*.

(A) Schematic drawing of the laser path and the total internal reflection fluorescence (TIRF).

(B) Plants grew on vertically oriented plates for 5 days before they were transferred to a slide for observation. Scale bar, 5 cm.

(C) VA-TIRFM image of epidermal cells in living plants. Scale bar, 5 μm.

(D) Three-dimensional luminance plots of GFP-PIP2 from the area indicated by the yellow box in **(C)** showing varied fluorescence intensity among different spots.

(E) The trajectory of the fluorophore-tagged molecules circled in **(C)**. Scale bar, 0.5 μm.

In fluorescence imaging, an arbitrary particle image appears as an Airy disk, which is a diffraction-limited spot with a radius of ~200 nm at a wavelength of 488 nm and a numerical aperture of 1.49 (Kues et al., 2001). The diffraction pattern of a point object or single particle is often referred to as a point spread function (PSF) (Kubitscheck et al., 2000). The width of a PSF can be mathematically represented by the Airy disk, which has a central lobe and concentric rings of decreasing intensity away from the center (Chen and Li, 2011). To obtain the position of a single fluorescent particle, we assume a Gaussian approximation for the fluorescence profile of the single particles. The fluorescent signals are analyzed by fitting a two-dimensional (2D) Gaussian function to the emission pattern of a single fluorescent probe to obtain the centroid of individual particles, yielding the position with an accuracy of ~50 nm. The equation of a 2D Gaussian curve is given by

$$I(x,y) = A_n \cdot \exp\left[-\frac{(x-x_n)^2+(y-y_n)^2}{2\sigma_n^2}\right], \qquad \text{(Equation 1)}$$

where A_n is the amplitude of the Gaussian (it is shown as a gray-scale value in the images). (x_n, y_n) are the (x, y) coordinates of the center of the curve, and the parameter σ_n is related to the full width at half maximum as follows: FWHM = $2\,(2\ln 2)^{1/2}\sigma_n$ (Hellriegel et al., 2005). The single-particle position data are plotted as Gaussians of the widths given by the respective confidence limits of the positional fit. Thus, directly fitting the equation 1 to the particle position data has become a common method for SPT. As for the centroid, independently fitted values of x_n and y_n are subtracted to find the displacement between any two images (Figure 2). Next, a tracking algorithm uses another set of criteria to track the detected particles and

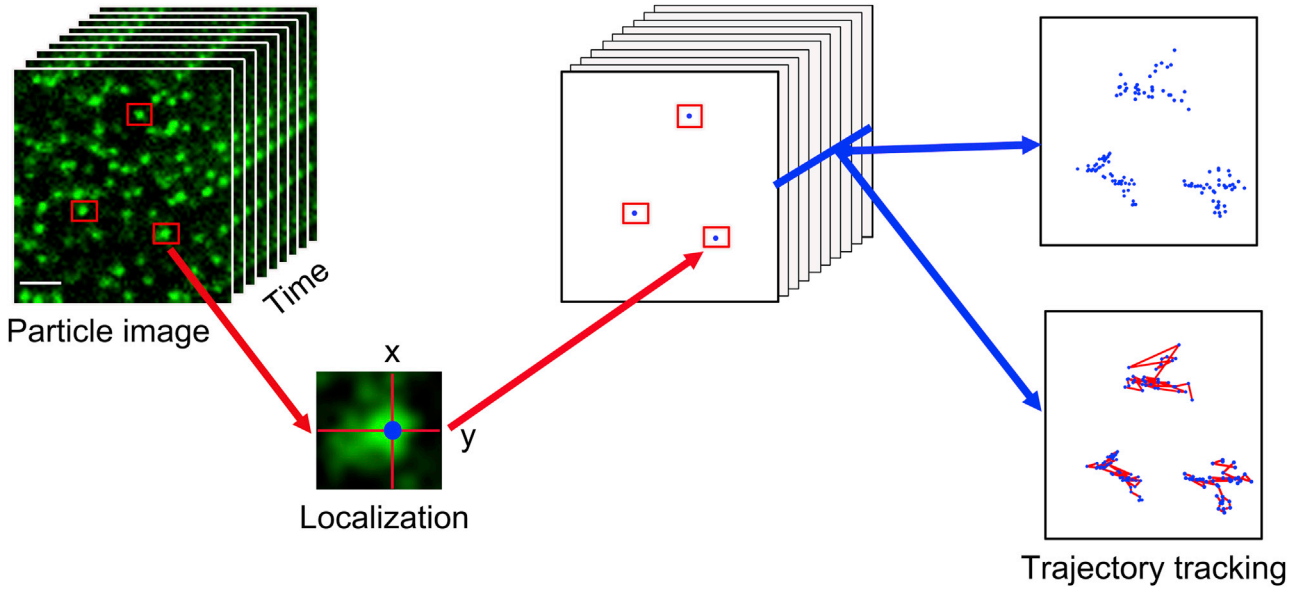


Figure 2. Model of Single-Particle Tracking.

Trajectory reconstruction consists of several processes. (1) Acquisition time series of the particle images. (2) Localization of individual particles at every given time. (3) Tracking the trajectories of individual particles. On the top right, the position of the particles is shown in blue. On the bottom right, the trajectory tracking of individual particles is shown in red. Scale bar, 2 μm.

quantify the dynamic changes in motion and temporary particle displacement from frame to frame. Different algorithms have been developed for the tracking of particle dynamics under different experimental conditions. The performance of the tracking algorithm defines the fundamental performance limit of the SPT method. Currently dozens of software tools are available for particle tracking, although no single method has performed best across all scenarios (Chenouard et al., 2014; Wang et al., 2015a, 2015b). We applied a multiple particle-tracking algorithm, described by Jaqaman et al. (2008), which accurately tracks particles in living plant cells, allowing us to address challenging and fundamental questions regarding the nature of molecular and cellular mechanisms.

Data Analysis

The software parameters are summarized in Box 1. The optimization of important parameters can improve the accuracy of tracking. The (*x*, *y*) position of a single particle in each frame of the time-lapse image series can be recorded by the tracking tool. These position data should then be further analyzed (Figure 3A and 3B). For each track, the mean square displacement (MSD) was calculated using the following equation:

$$\mathrm{MSD}(n\delta t) = \frac{1}{N-1-n}\sum_{i=1}^{N-n-1}\left\{[x(i\delta t + n\delta t) - x(i\delta t)]^2 + [y(i\delta t + n\delta t) - j(i\delta t)]^2, \right. \quad \text{(Equation 2)}$$

where $\Delta t = n\delta t$ and $(x(i\delta t + n\delta t), y(i\delta t + n\delta t))$ is the 2D position at a time interval Δt after starting at the position $(x(i\delta t), y(i\delta t))$. Also, n and i are positive integers, with n determining the time increment, and N is the total number of image frames before the molecule is bleached (Ma et al., 2007).

The pertinent parameters extracted from the trajectories are the diffusion coefficient and motion range. These elements reflect the biological properties of the plasma membrane and the molecular interactions. The diffusion coefficient for a particle was determined by fitting a line to MSD with n ranging from 1 to the largest integer $\leq L/4$ (where L is the length of the trajectory) (Saxton, 1997). The diffusion coefficients measured based on the trajectories of the particles were plotted as a histogram and

Box 1. Considerations for Tracking Parameters.

After the background was subtracted, the brightest pixel in each particle spot within the diffraction-limited size (3 × 3 pixels) was considered to be the central position of the particle, and the fluorescence signal position was calculated using MATLAB software. The accuracy of the tracking of the single particle depends on the tracking parameters used. The two best known tracking parameters are the search radius and the gap-closing time (Wang et al., 2015a, 2015b). The search radius is used to link the individual detections. Thus, the user can define the lower and upper limits of the search radius to avoid drifts in parameter estimation. In a single-particle experiment, the user can capture the motion of most particles in different plant cells and tissues by defining these limits. The gap-closing time plays an important role in capturing the trajectories resulting from temporary particle disappearance. In previous experiments, researchers found that using a time window of eight frames for the gap-closing duration was sufficient to capture most gaps, considering that longer gaps are less frequent than shorter gaps in experiments with plant cells (Wang et al., 2015a, 2015b). Therefore, optimizing these two parameters can help the user gain more precise information.

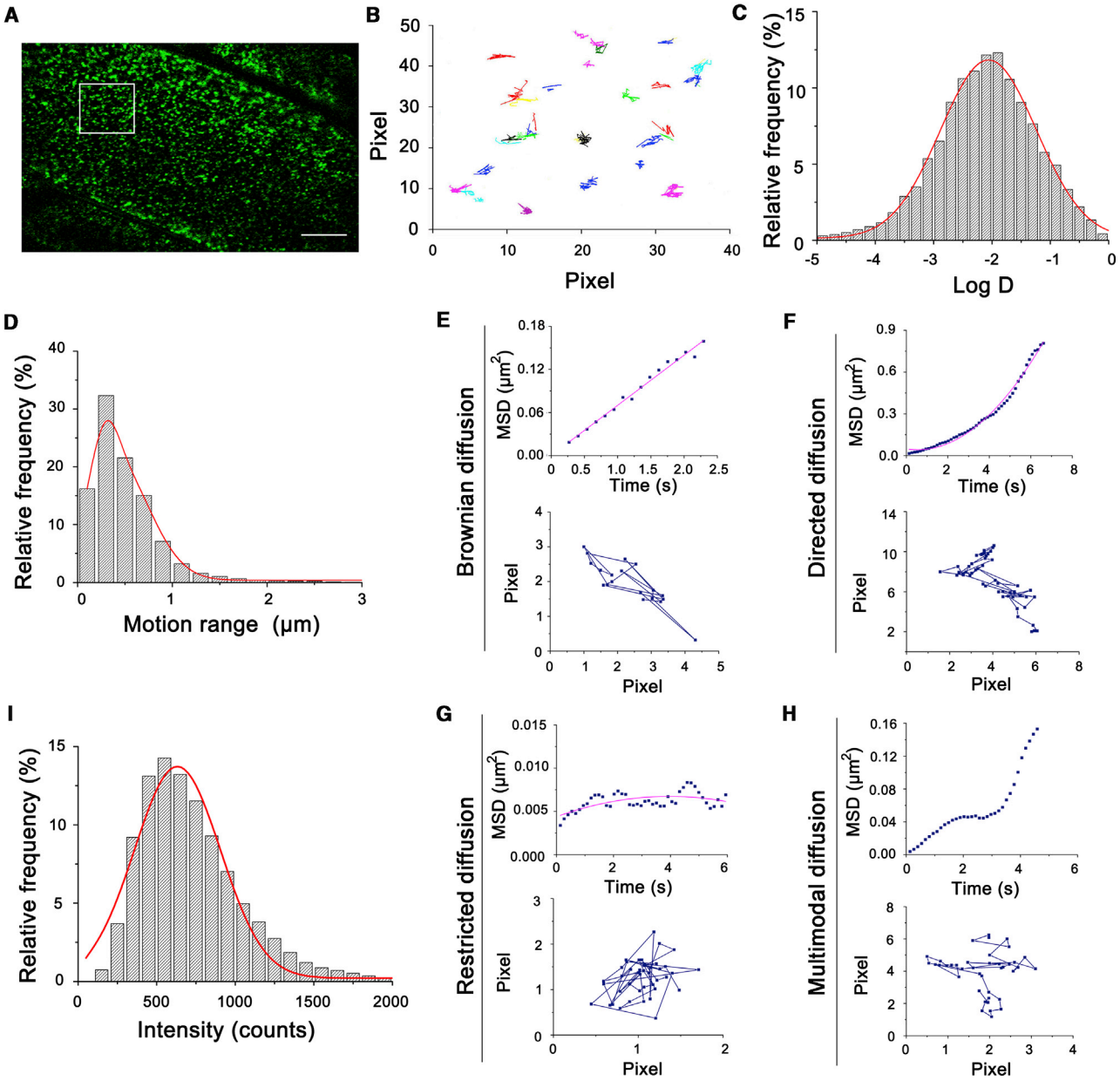


Figure 3. Illustration of *In Vivo* Protein Motion Analysis in *Arabidopsis*.

(A) TIRFM image of the plasma membrane proteins in living cells. Scale bar, 5 μm.

(B) Trajectories of the particles in the area indicated by the white box in **(A)**.

(C) Distribution of diffusion coefficients (n = 12 149 particles).

(D) Distribution of motion ranges (n = 13 877 particles).

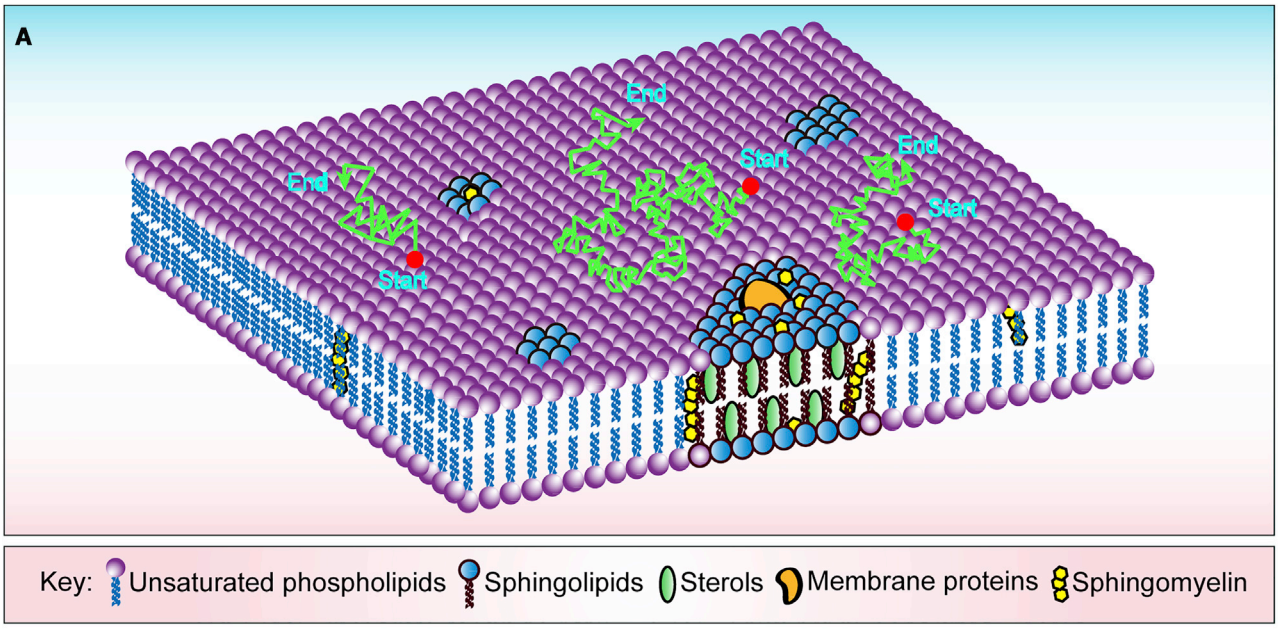
(E–H) MSD (mean square displacement) analysis of various protein trajectories and classification into different diffusion modes. The resulting MSD-t curves were fitted with pure Brownian **(E)**, directed **(F)**, restricted **(G)**, and multimodal diffusion models **(H)**.

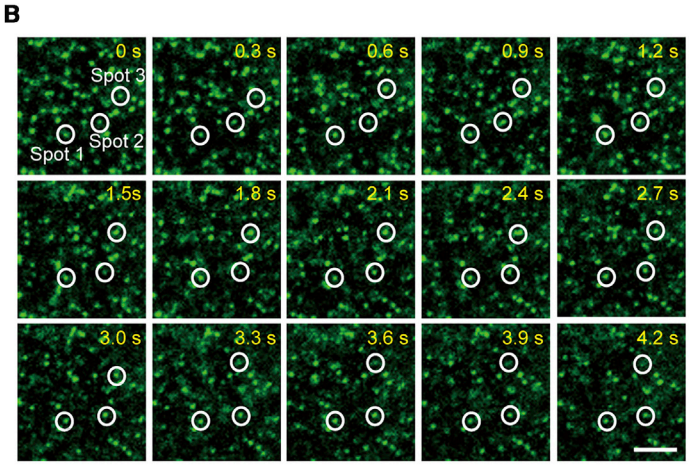
(I) Distribution of fluorescence intensities (n = 11 723 particles).

fitted by a Gaussian function to characterize the mobility. The Gaussian peaks (denoted as $\hat{G}$) were defined as the characteristic values (Figure 3C). The motion range was computed as the largest displacement during the lifetime (Figure 3D). The MSD for a given time lag in the MSD versus time plot describes the diffusion properties of a particle. The time dependence of the MSD reflects one four categories of motion, namely the Brownian, restricted, directed diffusion, and multimodal diffusion models (Figure 3E–3H) (Saxton and Jacobson, 1997). The confinement area was calculated by fitting the MSD data plotted against time (t), using the equation

$$\mathrm{MSD} = \frac{L^2}{3}\left[1 - \exp\left(\frac{-12D_t}{L^2}\right)\right], \qquad \text{(Equation 3)}$$

where L^2 is the confinement area (in μm^2) as described previously (Xiao et al., 2008; Mercer et al., 2012). D_t denotes the diffusion coefficient.





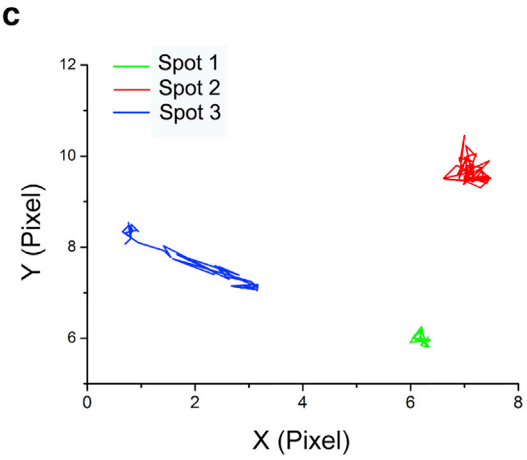


Figure 4. SPT Probing of Membrane Protein Lateral Diffusion.

(A) Schematic diagram showing the membrane organization during diffusion and the trajectories of individual particles.

(B) The lateral diffusion of foci imaged by VA-TIRFM at the plasma membrane: relatively static motion (spot 1), confinement to a specific region (spot 2), and free diffusion (spot 3). Scale bar, 2 μm.

(C) Trajectories of the three spots shown in **(B)**.

In addition to the coordinate of a pixel on the *x* and *y* axes, the fluorescence intensity of a single particle at a given time can also be analyzed (Figure 3I). Under the same conditions, the fluorescence intensity of an individual particle is proportional to the number of photobleaching steps and, by association, the subunit stoichiometry.

BIOLOGICAL APPLICATIONS AT THE SINGLE-PARTICLE LEVEL IN LIVING PLANT CELLS

Quantification of Protein Dynamics

Biological membranes are highly organized structures that exhibit complex dynamics. Early evidence shows that membrane dynamics play crucial roles in cellular motility, adhesion, trafficking, and signaling (Groves et al., 2008). Using SPT approaches, we can directly "observe" how particles diffuse in living cells in front of our eyes. As shown in Figure 4, some particles are confined to a small area (spot 2) and have a very small motion range, some particles are in relatively static motion in a limited area (spot 1), and other particles show visible mobility (spot 3), indicating that the motion states of fluorescently labeled membrane proteins are heterogeneous.

Some studies have established that the lateral diffusion of many membrane proteins is related to their activated/inactivated status (Low-Nam et al., 2011). For example, Ma et al., (2007) showed that the mobility of the transforming growth factor β type I (TGF-β1) receptor (TβRI) was significantly reduced upon

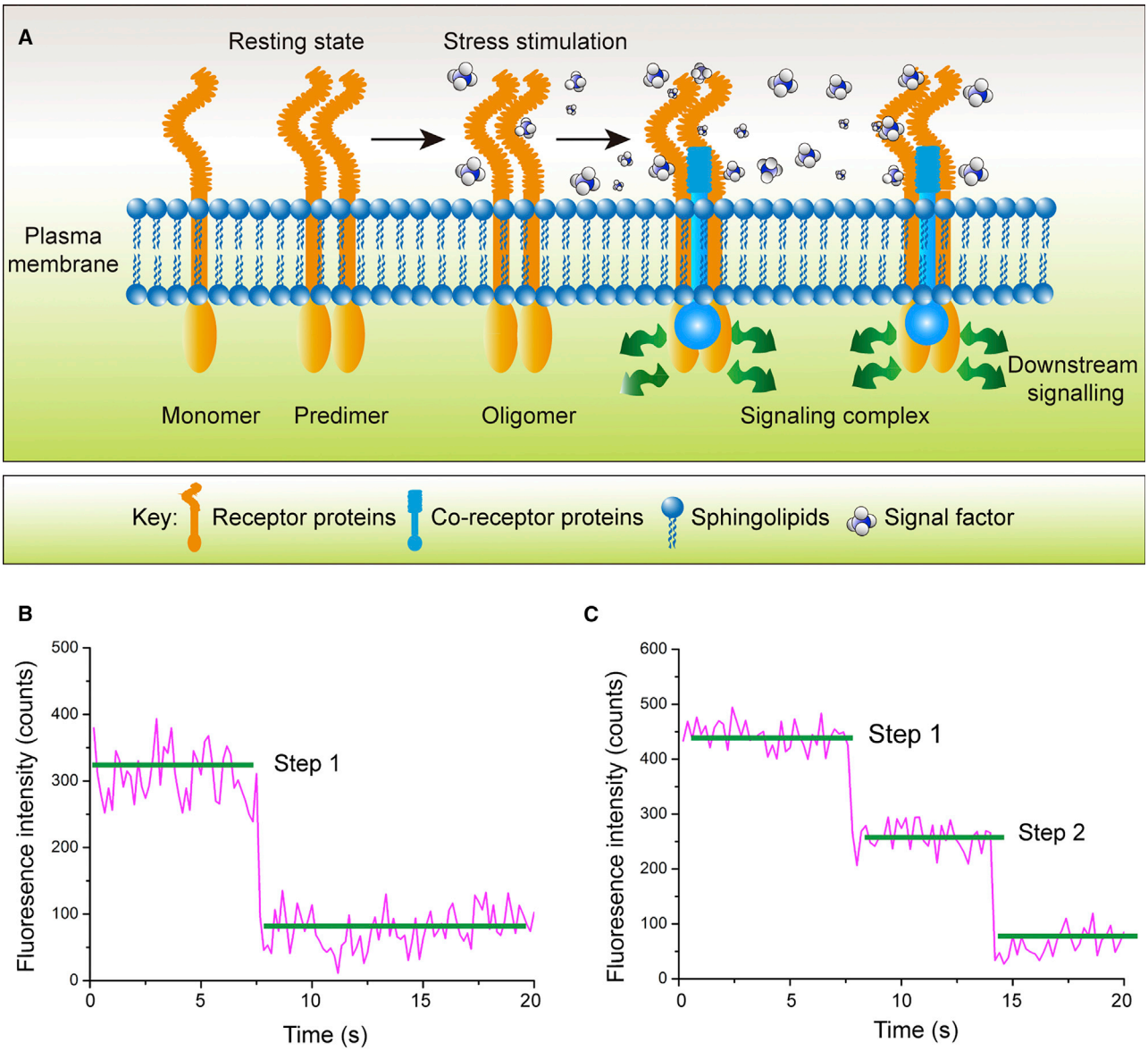


Figure 5. SPT Approach Used in the Study of Protein Oligomerization.
(A) Schematic showing the dynamic equilibrium between plasma membrane protein monomers and oligomers.
(B–C) Time course of fluorescence signal from a single fluorophore, showing one-step bleaching (B), two-step bleaching (C).

TGF-β1 stimulation, if TβRI was co-expressed with TGF-β type II receptor (TβRII). In addition, the diffusion rate of TβRI was not changed further even with TGF-β1 treatment when cholesterol was depleted, suggesting that the association of TβRI and TβRII for cell signaling requires membrane rafts. Moreover, the phosphorylation state of many membrane-associated tyrosine kinase receptors can lead to the formation of dimers or higher-order aggregates, which further alters the mobility of receptor molecules (Lemmon and Schlessinger, 2010). Therefore, spatiotemporal information about protein motion can contribute to an understanding of transient interactions or intermediates along reaction pathways.

In recent years, SPT has been extensively applied to study the initial responses to environmental stimulation in plant cells, and has greatly contributed to the understanding of response mechanisms. For example, Hao et al. (2014) used SPT to monitor the lateral mobility of *Arabidopsis thaliana* respiratory burst oxidase homolog D (AtRbohD) at the plasma membrane. After treatment with NaCl, the diffusion coefficient of AtRbohD increased compared with the control, indicating that salt stress induces a change in AtRbohD dynamics. Moreover, examination of Brassinosteroid-Insensitive 1 (BRI1) motion using SPT further supported the idea that brassinosteroids (BRs) may activate BRI1, the BR receptor, leading to dramatically faster diffusion, which may be necessary for further related signal transduction (Wang et al., 2015a, 2015b). In addition, Bucherl et al. (2017) showed that in response to ligand binding, Flagellin Sensitive 2 (FLS2) exhibited a decrease in lateral displacement within the plasma membrane. Additionally, the diffusion coefficients of

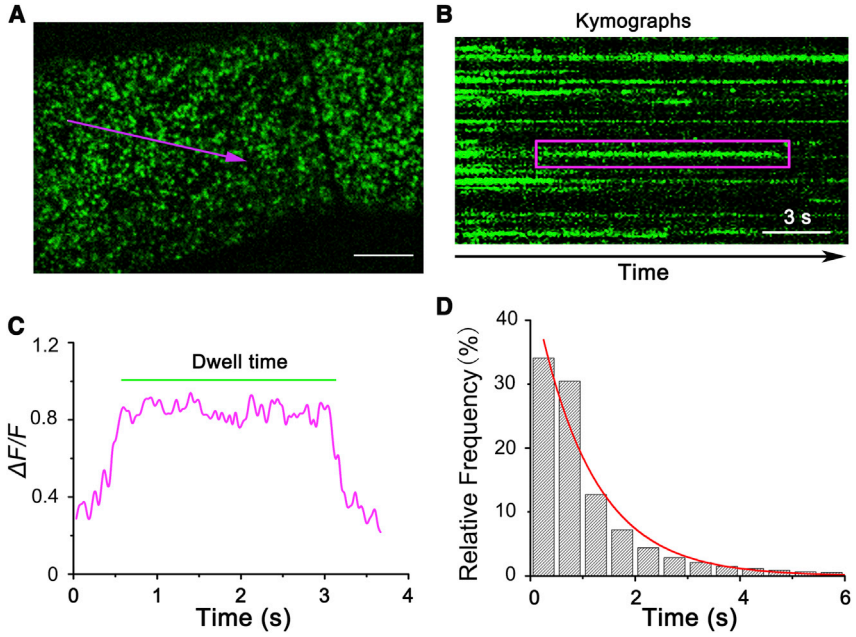


Figure 6. Illustration of Fluorescence Intensity and Dwell Time in *Arabidopsis*.
(A) TIRFM images of GFP. Scale bar, 5 μm.
(B) Representative kymograph showing the dwell times of individual fluorescent proteins.
(C) Representative normalized fluorescence intensity scanned from an individual fluorescent protein.
(D) Distribution of the dwell times of different single particles.

aquaporin PIP2;1, an intrinsic plasma membrane protein, significantly increased in response to salt stress (Li et al., 2011). Taken together, these studies show that SPT is a powerful approach to directly monitor protein dynamics in plant systems.

Determination of Stoichiometry

For the study of oligomerization of plasma membrane proteins, different experimental approaches have been exploited, such as chemical crosslinking and detection by SDS–PAGE analysis, coimmunoprecipitation, and fluorescence complementation (de Heus et al., 2013). However, it is very difficult to directly detect the oligomeric state of molecules within living cells at a nanometer scale with a time resolution in the millisecond range using traditional techniques (Garcia-Saez and Schwille, 2008).

Emerging studies have shown that plant plasma membrane proteins can form multimers and potentiate downstream signaling (Figure 5A) (Wang et al., 2013; Xue et al., 2018). Therefore, determining protein complex stoichiometry is critical for the analysis of structural and functional changes. Using SPT technology the subunit stoichiometry of membrane proteins in living cells can be determined by counting the number of fluorescence signal bleaching steps (Figure 5B and 5C) (Ulbrich and Isacoff, 2007). The fluorescence intensity of a complex containing proteins with one or several fluorescence tags drops in a stepwise fashion, and the step number matches the number of subunits per protein assembly (Reiner et al., 2012). It is worth noting that it is essential to use a standard monomer fluorescent protein as the control to rule out the effects of inherent dimer or even oligomer formation between fluorescence tags (Song et al., 2017). To statistically quantify the fractions of various oligomers, the ''Progressive Idealization and Filtering'' program is applied; this program automatically identifies the subunit stoichiometry of any fluorescently tagged protein (McGuire et al., 2012). This approach enabled us to study how the composition of certain complexes is fine-tuned in response to the external environment.

Xue et al. (2018) demonstrated that inactive phot1 mostly exists as monomers, and that the dimerization rate significantly increases with increased exposure to blue light (BL), indicating that BL can act as a physical ligand-like factor that induces phot1 dimerization at the plasma membrane. In another study, activation of the BRI1 kinase upon ligand binding was found to induce the formation of BRI1 and BAK1 hetero-oligomers, enabling downstream signaling (Bücherl et al., 2013). Similar conclusions have also been reached in early studies using SPT techniques in animal cells; for example, it was reported that activation of a membrane-located receptor kinase generally leads to homo-/heterodimerization or hetero-oligomerization, which ultimately initiates cellular responses (Katritch et al., 2013). Also, by measuring the maximum bleaching steps of *Arabidopsis* ammonium transporter 1;3 (AMT1;3), Wang et al. revealed that AMT1;3 exists as a trimer at the plasma membrane under normal conditions, while under external high-ammonium stress it assembles into clusters and is then internalized into the cytoplasm (Wang et al., 2013). More recently, single-particle analysis revealed that under salt stress RbohD assembles into clusters and is then internalized into the cytoplasm (Hao et al., 2014). These findings indicate that activated dimers or multimeric forms can further aggregate into clusters and that clustering functions as an important regulator of signal transduction. Therefore, by applying SPT techniques we can not only detect the oligomerization state of membrane proteins but, more importantly, monitor the transformation between the multimeric states of specific molecules in real time before and after stimulation.

Analysis of the Dwell Time on the Membrane

Ligand-activated receptors promote receptor endocytosis via the clathrin-mediated pathway or another other endocytic pathway and subsequent delivery to the endosomes (Tsvetanova and von Zastrow, 2014). In recent years the dwell time, defined as the time during which receptors are clustered into endocytic pits before endocytosis, has received more and more attention by researchers (Xue et al., 2018). Dwell time is calculated as the time between the first frame in which the spot tracker detects an event above the background fluorescence level and the last frame in which the particle disappears (Romanvendrell et al., 2014). By applying automated detection and a tracking assay, the dwell time can be determined (Figure 6A). In kymographs, the horizontal lines illustrate endocytosis of the labeled protein (Figure 6B–6D). More recently, techniques combining SPT with

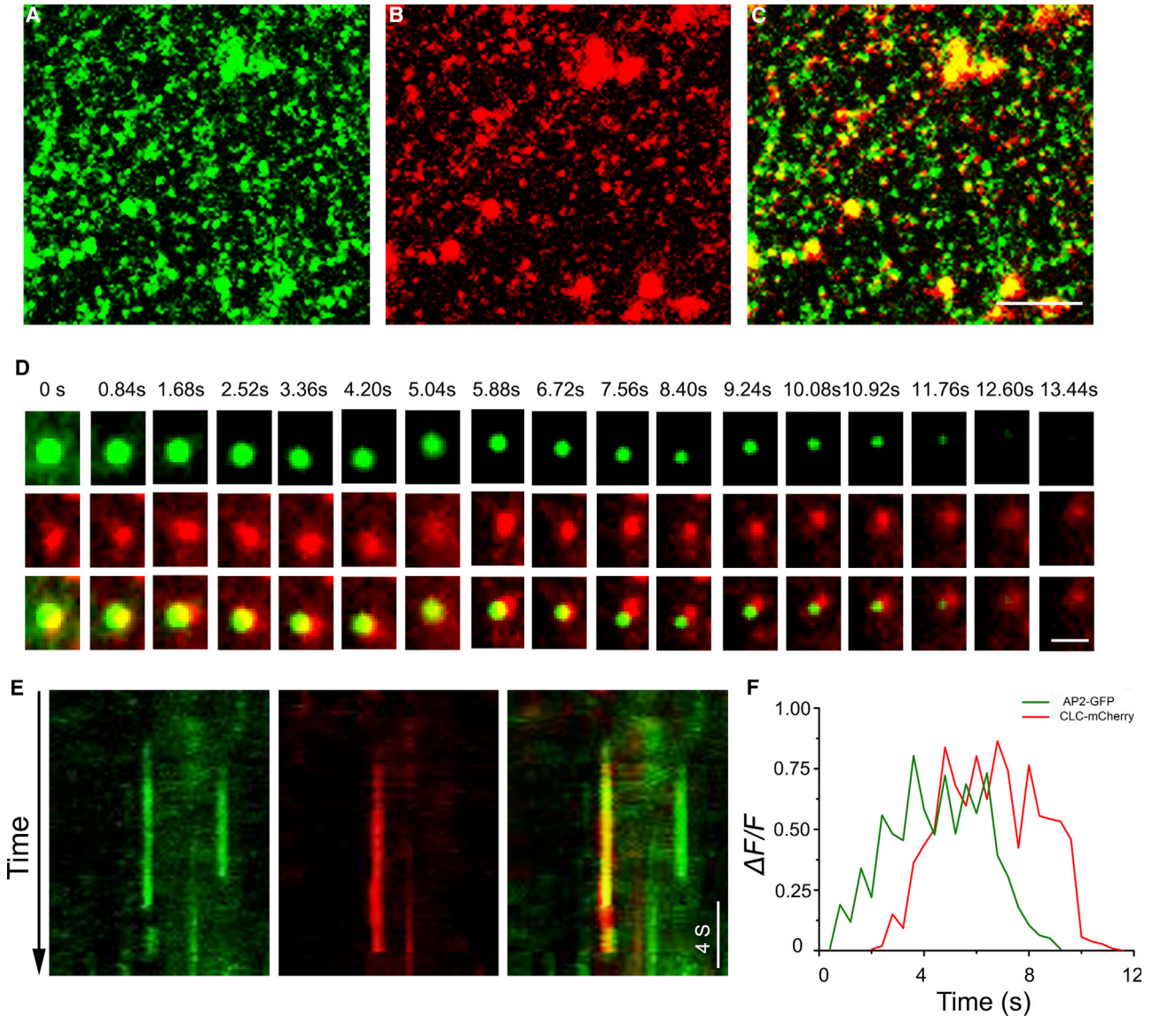


Figure 7. Dual-Color Analysis of Two-Protein Colocalization at the Plasma Membrane.

(A) VA-TIRFM image of AP2-GFP clusters proximal to the plasma membrane.

(B) VA-TIRFM image of CLC-mCherry clusters proximal to the plasma membrane.

(C) Merged image of **(A)** and **(B)**; the yellow dots indicate colocalization of the two signals. Scale bar, 5 μm (applies also to **A** and **B**).

(D) Representative images of a spot containing AP2 σ-GFP (top) and CLC-mCherry (middle) over a time course (time shown in seconds). The yellow signal in the merged images (bottom) indicates co-localization. Scale bar, 1 μm.

(E) Kymograph representation of the time course of green and red fluorescence. Left, middle, and right panels show AP2 σ-GFP, CLC-mCherry, and merged signal, respectively.

(F) Representative normalized fluorescence intensity scanned from AP2 σ-GFP and CLC-mCherry.

kymography were developed in order to further dissect the time-normalized intensity profiles and dwell times of different proteins from individual endocytic events (Flores-Otero et al., 2014). Such techniques have great potential for analyzing the key parameters of endocytosis in relation to signal transduction. For example, Flores-Otero et al. (2014) reported that ligand-specific endocytic dwell time controlled the functional selectivity of the cannabinoid receptor 1 (CB1R). They found that the time during which CB1R was clustered with β-arrestin into endocytic pits before endocytosis controlled β-arrestin-biased signaling and that agonists produced specific endocytic dwell times. In addition, extending the CB1R ligand-specific endocytic dwell time substantially increased β-arrestin signaling (Flores-Otero et al., 2014).

Compared with the extensive research on the dwell time of membrane proteins in mammalian systems, there are very limited studies on dwell time and its potential roles in cellular signaling and endocytosis processes in plant cells. Previous studies have shown that the dwell time of FLS2 and BRI1 showed a similar distribution in the absence of ligand, but was different in the presence of ligand, indicating that the ligand can change the dwell

Plant species	Proteins	Parameters	References
Arabidopsis thaliana	Plasma membrane intrinsic protein 2; 1 (PIP2; PIP1) clathrin light chain (CLC)	Oligomerization state Diffusion coefficient Colocalization	Li et al., 2011
A. thaliana	Flotillin1	Diffusion coefficient Colocalization	Li et al., 2012
A. thaliana, *Nicotiana benthamiana*	Remorin	Lateral stability	Jarsch et al., 2014
A. thaliana	Ammonium transporter 1; 3 (AMT1; 3)	Oligomerization state Lifetime Colocalization	Wang et al., 2013
A. thaliana	Chloroplast Division Protein FtsZ	Diffusion coefficient	Johnson et al., 2013
Arabidopsis thaliana	Adaptor protein 2 (AP2) proteins Phot1	Colocalization	Fan et al., 2013; Xue et al., 2018
A. thaliana	Respiratory burst oxidase homolog D (RbohD)	Oligomerization state Diffusion coefficient Colocalization	Hao et al., 2014
A. thaliana	Brassinosteroid insensitive 1 (BRI1)	Oligomerization state Diffusion coefficient Colocalization	Wang et al., 2015a, 2015b
A. thaliana	Hypersensitive induced reaction (AtHIR)	Oligomerization state Colocalization	Lv et al., 2017
A. thaliana	Cellulose synthase (CC) proteins	Colocalization	Endler et al., 2015
A. thaliana, *N. benthamiana*	Flagellin-Sensing 2 (FLS2) Brassinosteroid-Insensitive 1 (BRI1)	Colocalization	Bucherl et al., 2017
A. thaliana	The plant K^+ channel KAT1	Diffusion coefficient Colocalization	Reuff et al., 2010

Table 1. Applications of SPT Techniques in Plants.

time of the receptor (Bucherl et al., 2017). Wang et al. (2013) also measured the dwell time of fluorescently labeled *Arabidopsis* AMT1;3 under various ammonium conditions Remarkably, they found that under low ammonium concentrations, 44.5% of AMT1;3-EGFP spots in the plant cell membrane were short-lived while 55.5% of them were long-lived, but that under N-deprived conditions the proportion of long-lived proteins significantly increased. These results provided new insights into the regulation of AMT1;3 activity under different ammonium conditions. In addition, Xue et al. (2018) found that the dwell time of phot1 at the plasma membrane progressively decreased as the BL intensity increased, suggesting that BL induced the dissociation of phot1-GFP from the plasma membrane. Therefore, SPT-based research on the dwell time of plant membrane proteins will help us examine the endocytosis of membrane proteins at the plasma membrane in plant cells and its role in signal transduction.

Detection of Colocalization of Plasma Membrane Proteins

Fluorescence imaging allows the study of the spatiotemporal dynamics of fluorescently labeled proteins in living cells and organisms. Single-particle co-tracking, in which different particle species labeled with spectrally separated fluorophores are simultaneously tracked and imaged, has also been used to determine particle trajectories and further analyze the interactions taking place in a spatiotemporally organized way (Figure 7). This approach can be used to determine whether two proteins diffuse as a single complex and can thus provide valuable information about cotracking and colocalization between proteins. For instance, Flores-Otero et al. (2014) used SPT to establish that CB1R colocalizes with clathrin, but not with caveolin-1, thus revealing that clathrin-mediated endocytosis is the major mechanism for ligand-induced endocytosis of CB1R.

It was also reported that the appearance and internalization of *Arabidopsis* GPI-anchored proteins occur in two ways. Specifically, mCherry-GPI was found to gradually diffuse together with, as well as independently of, clathrin light chain (CLC) spots, indicating that the endocytosis of GPI-anchored proteins occurs through both clathrin-dependent and clathrin-independent pathways (Baral et al., 2015). Moreover, using an SPT assay, the AP2 σ subunit, which is an adaptor protein involved in endocytosis, was found to display dynamic behavior similar to that of CLC and to accumulate in the endocytic clathrin-coated pits. Time-lapse imaging and kymograph analysis further demonstrated that the AP2 σ-mCherry fluorescence spots appeared and disappeared before the CLC-GFP spots, suggesting that AP2 plays an important role in clathrin-coated vesicle formation, assembly, and maturation, but is not required for the separation of endocytic vesicles from the plasma membrane in *Arabidopsis* (Fan et al., 2013). More recently, Bucherl et al. (2017) demonstrated that the FLS2 and BRI1 receptors are indeed located within nanodomains of the plasma membrane in leaf epidermal cells.

Additionally, the differential colocalization of the FLS2 and BRI1 receptors with four different remorin (REM) protein nanodomain markers clearly showed that FLS2 and BRI1 reside within different nanodomains (Bucherl et al., 2017). Taken together, these research findings indicate that the SPT technique is a strong method for defining the relationships among plasma membrane proteins.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last decade, fluorescence microscopy has provided an essential and extremely powerful tool for visualizing biological processes in living cells. However, measurement of the spatial resolution of molecules and molecular complexes with complex dynamics at high spatial resolution is a difficult task using traditional confocal microscopy. Single-particle microscopy succeeds in delivering the most precise and complete quantitative information by combining high-precision visualization of single particles with smart automated tracking algorithms (Lv et al., 2017; Xue et al., 2018), as has recently been shown for several plasma membrane proteins (Table 1). At the single-particle level, fluorescence microscopy combined with data analysis can reveal the location and dynamic behavior of proteins at the plasma membrane at high spatiotemporal resolution (Bucherl et al., 2017; Xue et al., 2018). Therefore, SPT has high potential as a method to address future challenges in plant biology by enabling quantitative analysis in living cells.

Although SPT has already been developed and implemented, the use of this technique to analyze the dynamics of protein and protein–protein colocalization remains challenging. Due to the heterogeneous background and particle movements, temporal resolution can deteriorate and limit the density or concentration in living cells. Recently, emerging research has led to the constant development of new SPT modalities. For example, the combination of photoactivation localization microscopy and SPT overcame the problems of poor fluorescence signal and rapid photobleaching of fluorescent proteins (Manley et al., 2008; Wang et al., 2018). Furthermore, combined with fluorescence resonance energy transfer, SPT can detect the activation of single molecules and help us monitor the transduction pathways of individual signaling molecules (Coban et al., 2015; Sustarsic and Kapanidis, 2015; Sasmal et al., 2016). Accordingly, it is anticipated that SPT will be further improved and develop into a robust and reliable tool for the investigation of proteins in plant cells.

Single-particle experiments and bulk experiments provide information at complementary time and spatial scales. Single-particle systems can also be combined with other techniques to obtain a more complete picture. It is anticipated that in the near future these approaches will fundamentally change our views of the molecular processes actually occurring in living cells.

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AUTHOR CONTRIBUTIONS

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