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Review article

Spatial proteomics of vesicular trafficking: coupling mass spectrometry and imaging approaches in membrane biology

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Summary

In plants, membrane compartmentalization requires vesicle trafficking for communication among distinct organelles. Membrane proteins involved in vesicle trafficking are highly dynamic and can respond rapidly to changes in the environment and to cellular signals. Capturing their localization and dynamics is thus essential for understanding the mechanisms underlying vesicular trafficking pathways. Quantitative mass spectrometry and imaging approaches allow a system-wide dissection of the vesicular proteome, the characterization of ligand-receptor pairs and the determination of secretory, endocytic, recycling and vacuolar trafficking pathways. In this review, we highlight major proteomics and imaging methods employed to determine the location, distribution and abundance of proteins within given trafficking routes. We focus in particular on methodologies for the elucidation of vesicle protein dynamics and interactions and their connections to downstream signalling outputs. Finally, we assess their biological applications in exploring different cellular and subcellular processes.

Introduction

Vesicle trafficking pathways are essential cellular processes required for the precise transport and localization of proteins, polysaccharides and intermediates between cellular organelles, the plasma membrane (PM) and the extracellular space. Vesicles act as shuttles between membrane-bound cell compartments, transporting materials and also transducing signals. Vesicular trafficking requires a dynamic machinery comprising many key regulators, such as adaptor and coating proteins, as well as factors for vesicle targeting, scission, tethering and fusion (Waghmare *et al*., 2018; Zhang *et al*., 2021b). Compared with yeast (*Saccharomyces cerevisiae*) and humans, plants have evolved the expansion of gene families encoding key traffic regulators, such as soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) and RAB GTPases, suggesting the unique and complex vesicle trafficking pathways in plant cells. The identification, detection and functional characterization of these regulatory proteins and their cargos along the secretory and endosomal pathways are crucial for elucidating the complex interactions and behaviours of different molecules. In plants, endocytic and exocytic trafficking routes converge at the *trans*-Golgi network (TGN), a post-Golgi compartment that functions simultaneously as an early endosome and a cargo sorting station towards the PM and the vacuole (Zhang *et al*., 2019b) (Figure 1). Multivesicular bodies (also called prevacuolar compartments) serve as late endosomes to delivery cargo for vacuolar degradation (Cui *et al*., 2020; Liu *et al*., 2018).

Conventional biochemical methods based on subcellular fractionation have limitations in their capacity to purify subcompartment of organelles and to precisely define the location of proteins and cargos related to the endocytic and secretory pathways. Moreover, the endomembrane system includes specialized structures such as the endoplasmic reticulum (ER) exit site, the ER–Golgi intermediate compartment (ERGIC) and contact sites between membrane compartments that may remain elusive after standard purification procedures. Therefore, the proteomes of carriers targeted to specific membrane compartments are ill-defined since the vesicles and associated intermediates are transient in nature, low in abundance and hard to isolate. Moreover, the complexity of the overall system increases due to the dynamic relocation of proteins in response to external and internal signals.

Given the overlapping and dynamic nature of the vesicular proteome, its analysis has lagged behind that of other subcellular compartments in plants. Spatial proteomics aim to investigate the subcellular distribution and abundance of proteins. Recent advances in high-resolution mass spectrometry (MS)-based spatial

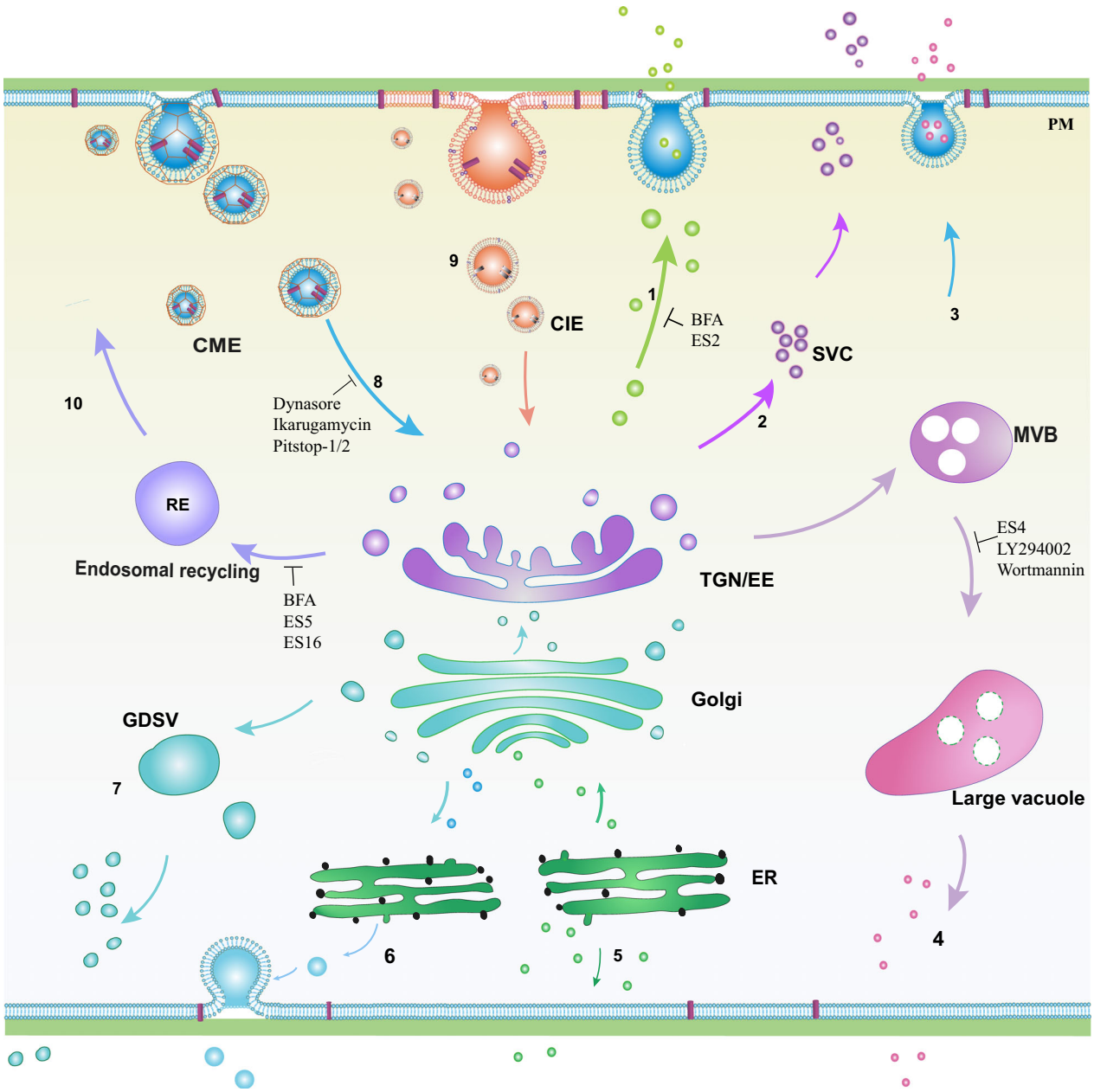


Figure 1 Hypothetical model of vesicle trafficking in plants. (1-7) Exocytic pathways. Newly synthesized cargo proteins are loaded into secretory vesicles and follow the conventional protein secretion (CPS) route from the endoplasmic reticulum (ER) to the Golgi apparatus for glycosylation, before being sorted to the *trans*-Golgi network (TGN) and sent to the plasma membrane (PM) or the extracellular space including the cell wall and intercellular spaces (outlined in route 1). In addition to the CPS, cargo proteins can be also secreted through unconventional protein secretion pathways for specific biological functions (routes 2–7), such as TGN-associated secretion through multivesicular bodies (MVBs) (route 3) or secretory vesicle clusters (SVCs) (route 2); Golgi bypass secretion (route 5); exocyst-positive organelle (EXPO)-mediated secretion (route 6); and Golgi-derived secretory vesicle-mediated secretion (route 7). (8-10) Endocytic pathways. PM proteins (including transporters, receptors and ion channels) undergo endocytic trafficking via clathrin-mediated endocytosis (CME) (route 8) or clathrin-independent endocytosis (CIE) (route 9) for different physiological processes. Some internalized cargo proteins are transported to the TGN/early endosome, which serves as a sorting platform, and are either trafficked back to the PM via recycling endosomes (REs) (route 10) or transported to the vacuole via MVBs. Chemical inhibitors affecting plant vesicle trafficking: During vesicle delivery, the inhibitors brefeldin A (BFA) and endosidin 2 (ES2) can be used to block secretory trafficking to the PM; ES4, ES17, LY294002 and wortmannin cause enlarged PVCs/MVBs and inhibit vacuolar transport; dynasore, ikarugamycin, Pitstop-1/2 and wortmannin inhibit CME; BFA, ES5 and ES16 block endocytic recycling from the TGN to the PM.

proteomics (Christopher *et al.*, 2021; Jorrin-Novo *et al.*, 2019) and advanced microscopy (Komis *et al.*, 2018), together with machine-learning data analysis, have accelerated the investigation of the protein contents of endosomes and endosomal protein interactions by providing proteome-wide spatial information and video-rate temporal resolution.

The breakthrough in efficient global analysis of vesicle trafficking comes with the development of suitable protein

detection and quantification methods (Box 1). Spatial proteomics profiling has been applied to mapping the localization of membrane proteins from diverse cell types, ranging from mammalian (Shin *et al.*, 2020) to plant cells (Heard *et al.*, 2015; Wilkop *et al.*, 2019). These studies represented essential different approaches to elucidate cellular signalling. The emerging consensus is that the complementary nature of MS and microscopy approaches (Box 2) promises exciting synergies for exploring the distribution and organization of vesicle components. Here, we use the term 'spatial proteomics' to describe the protein subcellular mapping, and we focus on both MS- and imaging-based methodologies to characterize the complex and dynamic secretory and endosomal vesicle networks in plants. We also provide examples of new methodologies used to determine spatial–temporal protein distributions essential for several key membrane signalling and trafficking processes in plant cells.

MS-based proteomic approaches to disentangle vesicular trafficking pathways

Inhibitor-based proteomics approaches

Proteomics analysis requires enrichment strategies to increase the yield of vesicles derived from specific compartments. Cell-permeable chemical inhibitors, which can be natural products or potent synthetic analogues, are routinely applied to investigate endomembrane transport (Rodriguez-Furlan *et al.*, 2017). The use of small molecules also overcomes limitations associated with genetic approaches, such as redundancy across protein families and isoforms, or time demands for the development of transgenic lines. These inhibitors can block specific vesicle trafficking pathways (Figure 1), leading to an enrichment of the proteins residing in the targeted endomembrane organelles, which results in the accumulation of intermediate compartments (Figure 2a) (Drakakaki *et al.*, 2012; Takac *et al.*, 2014). For instance, the

Box 1. Current quantitative MS approaches for organelle proteomics profiling.

The complexity and highly dynamic nature of vesicle trafficking present notorious challenges to precisely define the location of proteins and cargos associated with the secretory and endocytic pathways. Quantitative MS-based proteomics approaches have attracted increasing attention for organelle analysis, owing to their high coverage, sensitivity and resolution. Quantification broadly falls under either labelling or label-free based methods. Labelling strategies include isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tag (TMT) and SILAC. These methods, differing in the incorporation of stable isotope-enriched labels, are commonly used in organelle proteomics profiling. iTRAQ and TMT introduce labels onto proteins or peptides after cells have been lysed, whereas SILAC involves the incorporation of stable isotopes in plant medium. A general strategy for label-free quantitation (LFQ) is to compare the number or ion intensity of peptide spectra identified for a given protein across two or more biological samples (Chen and Hoehenwarter, 2019). LFQ allows the quantification of a virtually unlimited number of samples without any special chemical, metabolic or enzymatic modification. Due to the low cost and minimal sample processing steps, LFQ methods are increasingly employed to replace or complement labelling approaches in bulk proteomics analyses. However, LFQ is susceptible to high variability, with missing values between different MS runs. Despite various quantitative algorithms have been developed for data analysis, it remains challenging to overcome the random variability resulted from technical limitations, such as retention time shifts and poor ionization efficiency. Therefore, labelling approaches remain the gold standard for the quantification of organellar proteomics profiling due to their high accuracy and reproducibility. Recently, hyperplexing with a combination of SILAC and TMT or iTRAQ has been used to measure >15 samples/organelles simultaneously within a single MS run, resulting in increased multiplexing capability and robustness (Mertins *et al.*, 2018; Thompson *et al.*, 2019). Coupled with multiple-organelle proteomics, this method can lead to near-whole-cell coverage.

Box 2. Complementarity of MS and imaging methods for the dissection of vesicle trafficking.

In general, MS-based spatial proteomics is faster, as it does not require special reagents and can be used for large-scale quantitative analysis. Imaging methods require the visualization of proteins, usually with affinity-based reagents or by expressing a construct encoding a fluorescent fusion protein. Moreover, imaging methods are generally focused on a single protein, which makes them best suited to address targeted research questions. Recent innovations in high-content microscopy approaches can simultaneously analyse over 50 proteins, while these methods rely on various validated antibodies, hindering high-throughput proteomics analysis (Mattiazzi Usaj *et al.*, 2016). Although both MS and imaging methods allow for time-course analyses, MS techniques can only detect averaged information for heterogeneous proteins within samples, missing transient events that may take place in subpopulations. By contrast, imaging analysis using advanced microscopy offers easier characterization of proteins with dual localization and can provide nanoscale and nanosecond resolution. In particular, imaging methods can help in the discovery and characterization of the morphology of new structures or compartments; they can be quantitative but require manual processing. MS methods can be uniquely used to analyse protein isoforms and post-translational modifications. These two approaches provide complementary insights into the mechanisms of vesicle trafficking. However, any protein localization characterized by MS approaches should be validated in cells expressing genetically encoded tagged versions of each protein to confirm whether the observed localization is biologically relevant. The extent of agreement between the two methods in terms of protein localization is >75% (Zhang *et al.*, 2019c); integrated studies would enhance throughput and sensitivity. Overall, general limitations for both approaches stem from poorly resolved cellular compartments that can result in random and inconsistent organellar assignments.

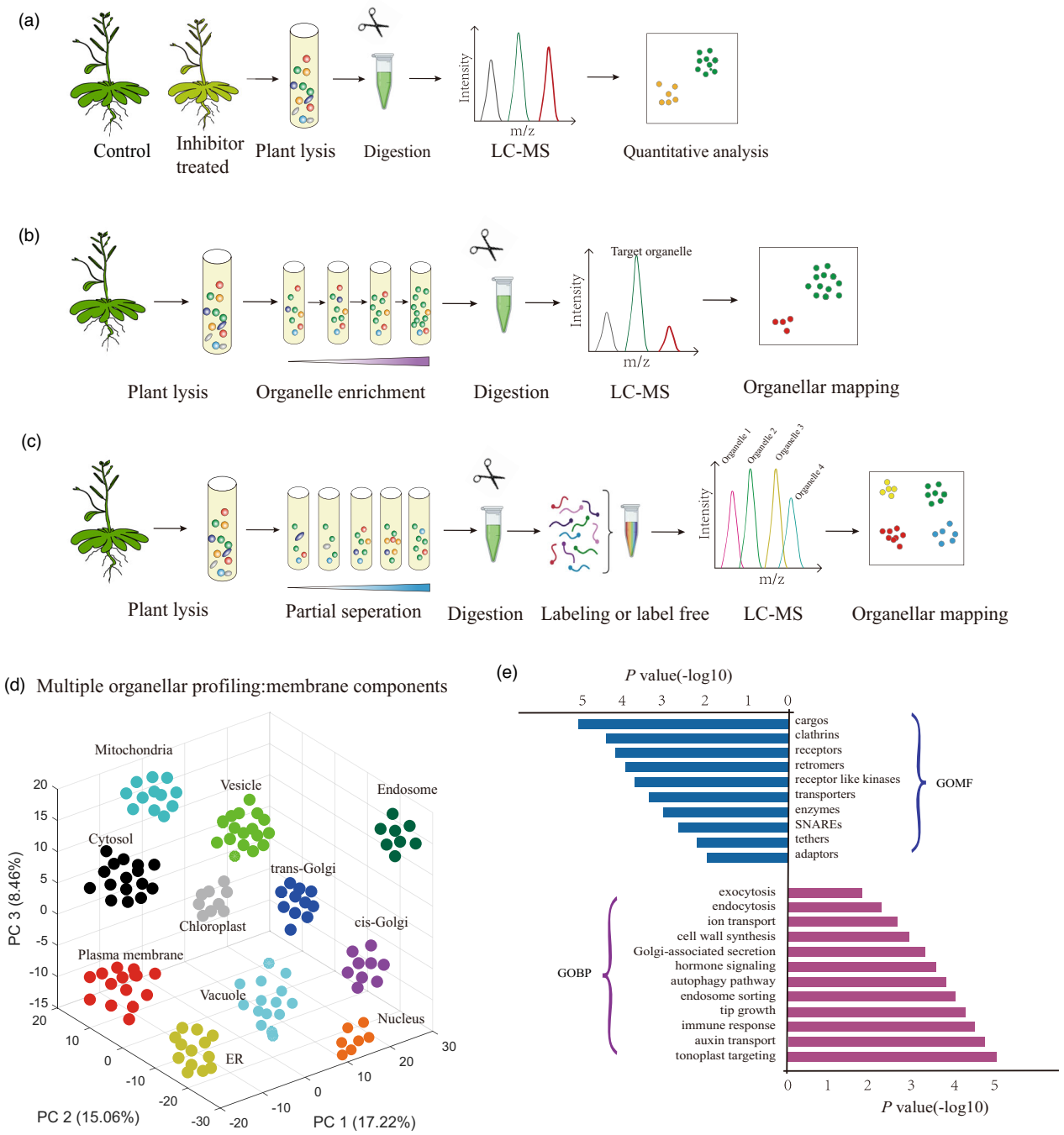


Figure 2 Dissection of vesicle trafficking with spatial proteomics. (a) Inhibitor-based proteomics workflow. Both control and inhibitor-treated plant materials are lysed, proteins are extracted and digested with trypsin, and the peptide mixtures are subjected to mass spectrometry (MS) identification and quantification. (b) Single organellar proteomics workflow. Plant materials are lysed, target organelles (e.g. Golgi stacks) are extracted, and a tailored subcellular fractionation method (e.g. gradient centrifugation or free flow electrophoresis [FFE]) is conducted to enrich the target organelle. The resulting enriched organellar protein fractions are immediately digested with trypsin and analysed by MS. Merged proteomics profile are presented as two-dimensional principal component analysis (PCA) projection. (c) Multi-organellar proteomics workflow. The organelles are partially separated in this workflow, and all fractions are subjected to label-free or labelling-based quantitative proteomics analysis (e.g. tandem mass tag [TMT] or isobaric tags for relative and absolute quantification [iTRAQ]). Each organelle has its own distribution profiling that is shared by all proteins predominantly associated with this organelle. (d) Multivariate analysis of spatial proteomic data. The whole MS data can be visualized in three dimensions using PCA to give a snapshot of organelle separation. The annotation of the PCA plot with well-known residents (markers) reveals protein localization. Each point represents one protein, and all the points are coloured according to their organelles of origin. The organelle map provides peptide-level resolution. Hierarchical clustering shows the vesicle and secretory (ER, *cis*-Golgi, medial-Golgi, endosome, vesicle, PM and vacuole) protein profiles. (e) GO enrichment of membrane proteins. GO terms for molecular function (MF) and biological process (BP) are statistically enriched.

application of brefeldin A promotes the accumulation of ERGICs to levels about 100-fold higher than in untreated samples (Breuza *et al*., 2004).

The use of inhibitors may induce changes in gene expression or in protein turnover that regulate the targeted trafficking route and organelle and thus affect the respective proteomes (Takac *et al*., 2012). Therefore, the combination of proteomics and chemical biology approaches may reveal previously inaccessible findings, including the identification of new proteins regulating or participating in vesicle trafficking pathways. Such integration can also uncover new biochemical or physiological events connected to the blocked pathway or the inhibitor itself (Takac *et al*., 2011, 2012). However, this approach depends on drug specificity and strict discrimination between primary and secondary effects. The implementation of rigorous imaging and bioinformatics methods, such as mapping of subcellular targeting motifs or secretory signals, can substantially improve the experimental outcome.

Single-organelle proteomics mapping

Single-organelle proteomics involves the isolation of cellular compartments by subcellular fractionation, which is based on unique physical properties of the respective organelle. In principle, endomembrane-derived microsomes and vesicles can be further fractionated based on differences in their buoyant density (e.g. ultracentrifugation in inert solute gradients), the surface charge of their membranes (e.g. free flow electrophoresis [FFE]) (Guo *et al*., 2021; Islinger *et al*., 2018; Parsons *et al*., 2014) or after fluorescent labelling and subsequent sorting (e.g. flow cytometry) (Wrasman *et al*., 2018) (Figure 2b). For example, the separation of microsomal fractions through sucrose density gradients has resolved membrane microdomains (Chu *et al*., 2021) and vesicle proteomes (Pertl-Obermeyer *et al*., 2016) in Arabidopsis (*Arabidopsis thaliana*). Similarly, the enrichment of autophagosomes by differential centrifugation allows the identification of specific cargos and receptors capable of recognizing and trafficking each given cargo to lytic compartments (Suzuki *et al*., 2014). Coupled with a cell-free coat protein complex II (COPII) vesicle reconstitution assay, a recent proteomic study revealed novel mechanisms underlying ABA-induced stress responses via the giant COPII vesicles (Li *et al*., 2021). Typically, FFE is effective for the isolation of dynamic subdomains of the Golgi apparatus based on the small differences in surface charge carried by the membranes (Guo *et al*., 2022; Parsons *et al*., 2014). These methods have been extensively used to purify specific vesicle populations to near homogeneity and to characterize their resident proteins in plants.

In addition, immunoaffinity purification has been widely used to analyse membrane-bound organelles. As this method relies on the presence of epitopes, it is amenable to the isolation of functionally distinct subcellular organelles with common immunoreactivity properties. Recombinant marker proteins and imaging analysis are needed for such strategies to visualize and confirm their subcellular localization, as revealed in the identification of novel trafficking components and cargos of vesicles in Arabidopsis (Drakakaki *et al*., 2012). Using multiple fluorescent-tagged endosomal markers, this workflow can be applied to the large-scale mapping of cargo proteins and regulators involved in endosomal and secretory pathways (Heard *et al*., 2015). Overall, these studies have provided a long list of proteins proposed to participate in vesicle trafficking routes.

Despite the progress in endosomal protein determination, these approaches can suffer from low purity of isolated organelles or limitations of endomembrane protein markers. In addition, membrane proteins are dynamic and not distinguished using single-organelle isolation or affinity purification. To overcome these limitations, quantitative MS methodologies can be implemented in the organelle proteomics studies, allowing accurate determination of vesicle protein localization in a high-resolution and quantitative manner (Box 1). A major breakthrough in the study of vesicle proteome was recently achieved by stable isotope labelling (SILAC)-MS-based quantitative proteomics, whereby >1000 true resident proteins were determined in enriched exosomes from multiple human cell lines (Kugeratski *et al*., 2021). The combination of FFE and quantitative Golgi proteomics has revealed the transient localization patterns of proteins in the *cis*-Golgi, medial-Golgi, *trans*-Golgi and specific vesicle populations in Arabidopsis seedlings (Parsons *et al*., 2019). Similar approaches identified >700 novel PM-localized proteins (de Michele *et al*., 2016) and numerous Golgi membrane proteins (Okekeogbu *et al*., 2019).

Additionally, organellar proteomics profiling can be combined with the ablation of an entire compartment or of highly abundant proteins. Following this strategy, proteomics analysis of vesicle-enriched fractions has revealed protein contents of various types of clathrin-coated vesicles (Borner *et al*., 2012). These methodologies are sensitive and specific in analysing membrane proteins and are well suited to address organelle-targeting questions.

Multiple-organelle proteomics mapping

To avoid the need for isolating pure endomembrane organelles, spatial proteomics approaches have been developed to analyse multiple cellular compartments simultaneously (Figure 2c). One powerful method is localization of organelle proteins by isotope tagging (LOPIT). This strategy does not rely on the isolation of specific subcellular organelles. Rather, LOPIT involves the partial separation of organellar membranes by density gradient centrifugation in self-forming iodixanol gradients (Chen and Heazlewood, 2021; Dunkley *et al*., 2004). The membrane compartments and large protein complexes can be simultaneously separated based on their physical differences. Finally, isotope tagging-based quantitative proteomics and multivariate statistical methods like principal component analysis (PCA) are exploited to correlate proteins with similar characteristics relative to well-curated organelle markers to assign their subcellular localizations (Dunkley *et al*., 2004) (Figure 2d).

In combination with quantitative MS (Box 1), LOPIT can determine large protein complexes and proteins residing in multiple compartments. Though originally developed in mammalian cells, LOPIT-MS has been successfully applied to plant proteomics analyses and has offered high-resolution mapping of organelle proteins (Groen *et al*., 2014; Nikolovski *et al*., 2012; Shin *et al*., 2020). This method can give a comprehensive distribution profiling and allow subcellular assignments to multiple organelles per experiment, making it possible to retrieve data about non-secretory organelles. The removal of cytosolic contamination, or employment of non-photosynthetic tissues and etiolated materials, can enhance the vesicle proteome coverage. LOPIT revealed the spatial organization of numerous novel Golgi-resident proteins, including those involved in sugar nucleotide metabolism and protein sorting (Nikolovski *et al*., 2012). Combining LOPIT-MS with immunoisolation, high-confidence proteomics datasets have been generated for the Arabidopsis TGN, ER and other endomembrane compartments (Groen *et al*., 2014; Nikolovski *et al*., 2014).

Moreover, the updated workflow hyperLOPIT can simultaneously analyse up to 11 organelles in a single experiment, allowing the spatial dissection of the proteome at sub-organelle resolution in large protein complexes (Mulvey *et al*., 2017). Variations of LOPIT have been recently implemented by integrating novel methods for sample preparation and MS data analysis, and shown to be more efficient for high-confidence data assignment (Geladaki *et al*., 2019). Employing vesicle relocalization and Bayesian analysis, LOPIT-MS determined the protein contents of different endosomal vesicles destined for the TGN, revealing the protein trafficking mechanisms from the endosome to the Golgi along retrograde transport pathways (Shin *et al*., 2020). LOPIT allows simultaneous quantification of numerous purified fractions in a single experiment, minimizing the technical variability and leading to an enhanced quantitative reproducibility and efficiency. The combination of LOPIT and FFE offers a means to distinguish the protein localization patterns of residents and trafficking proteins, or proteins that are shared by other organelles, as well as to determine the locations of proteins that reside at multiple sites (Parsons *et al*., 2019) (Figure 2).

Other proteomics methods such as dynamic organellar maps and SubCellBarCodes are reproducible and comprehensive for the global mapping of protein translocation events and static organellar compositions (Itzhak *et al*., 2016; Orre *et al*., 2019; Qian *et al*., 2021). Another strategy similar to LOPIT but using label-free quantification approaches is protein correlation profiling (PCP) (Mann, 2020). In general, these methods are based on similar basic principles, with differences in sample separation, MS data acquisition and quantification approaches.

In recent years, these methodologies have achieved a high level of sub-organellar resolution, proteome coverage and classification accuracy. Thus, these new strategies have enabled a deeper characterization of the spatial organization underlying the subcellular proteome. Overall, multi-organelle proteomics profiling analysis provides novel perspective on vesicle trafficking and can be used for cell biology models in a systems biology approach.

Imaging-based techniques: visualizing the spatiotemporal organization and dynamics of proteins involved in vesicular trafficking

Fluorescent labelling methods for probing vesicular trafficking

Vesicle-mediated protein trafficking within plant endomembrane compartments is a tightly regulated process that involves multiple membrane-bound organelles. The live imaging of plant cells with specific probes or reporters greatly contributes to visualizing and validating membrane protein localization and dynamics at multiple scales (Colin *et al*., 2022). In the past decade, the popular approach of labelling proteins by genetic fusion to fluorescent markers has extended our understanding of protein function. To determine the subcellular localization of proteins of interest, transgenic plant lines stably expressing fluorescent fusion proteins targeted to specific compartments have been established and are essential for colocalization experiments. For Arabidopsis, the Wave line collection provides multiple markers tagged with several colours for each endomembrane compartment, thereby facilitating the localization of a target protein. The widely used membrane marker proteins include aquaporin PIP1;4 for PM; SNARE proteins such as SYP32 for Golgi; VTI12 for TGN; VAMP711 for tonoplast; Rab GTPases such as Rab F2b for prevacuolar compartment (PVC); and Rab A5d/Rab A1e for early and recycling endosomes (Geldner *et al*., 2009). As PM-residing proteins generally undergo constitutive or ligand-inducible endocytosis, fluorescent protein fusions to several well-documented PM proteins, such as the auxin transporters PIN1 and PIN2, the brassinosteroid receptor BRI1, the pattern recognition receptor FLS2 and the boron transporter BOR1, are widely used to investigate the regulatory mechanisms of plant endocytosis (Aniento *et al*., 2022; Bai *et al*., 2020). In addition, novel fluorescent proteins offer new opportunities to dissect plant vesicle trafficking. For instance, the exocytic and endocytic trafficking processes during pollen tube tip growth can be measured simultaneously based on corrected fluorescence recovery after photobleaching analysis using a PM-localized cargo protein tagged with a photoconvertible fluorescent protein (Luo *et al*., 2016). Recently, the delivery route of the endo-β-1,4-glucanase KOR1 from PM to vacuole was traced using tandem fluorescent timer (tdFT) technology, which enabled simultaneous monitoring of the location and age of KOR1 (Nagashima *et al*., 2020). Indeed, fluorescent reporter proteins have the advantage that they can be expressed and tracked in organelles, cells or even intact plant tissues. However, care must be taken to assure that the fluorescent protein is fused in-frame with the N-/C-terminus or interior of the target protein without disturbing its *in vivo* functions. Ideally, constructs containing the native promoter and the fused coding sequences of the target protein and the fluorescent marker should be introduced into corresponding knockout mutants to test for functional complementation (Fujimoto *et al*., 2020).

Recently, stable or transient expression of recombinant proteins with SNAP- or Halo-tags in plant cells or intact plants incubated with synthetic chemical dyes has provided another tool to study protein localization and dynamics (Iwatate *et al*., 2020; Minner-Meinen *et al*., 2021). The efficient labelling and visualization of mitotic microtubule arrays were achieved in BY-2 cells and Arabidopsis seedlings stably transformed with SNAP-tag α-tubulin (Iwatate *et al*., 2020). In combination with fluorescent protein, SNAP-tag PIN2 with DRBG-488 dye labelling was used to observe the endocytic route of PIN2 as well as confirm that newly synthesized PIN2 is preferentially moved to the forming cell plate during mitosis (Iwatate *et al*., 2020).

Using chemical dyes to detect covalently tagged proteins has several advantages over using fluorescent protein fusions, including the wide range of fluorescent dyes of various colours available from commercial resources; the smaller size of chemical dyes and their higher quantum yield, photostability and ultra-resistance to photobleaching, which makes them potentially more suitable for noninvasively monitoring protein activities and trafficking over longer time periods (Iwatate *et al*., 2020). It is recommended that the permeability, low background and pH sensitivity of dyes should be determined if their tag forms have never been tested. Because dyes for specific targeting to endomembrane compartments and other organelles such as mitochondria, chloroplasts and peroxisomes have not yet been evaluated or developed for plants, covalent target labelling currently has limited applications in plant biology.

Similar to genetically tagged fluorescent reporter proteins, fluorescent dyes can be used to label specific compartments in the plant endomembrane network. In Arabidopsis, the lipophilic styryl dye FM4-64 has been widely used as a promising tool to chart the dynamics of endocytic processes by timing the flow of FM4-64 uptake from the PM to the tonoplast within 1 to 2 h passing through the TGN/early endosomes and the PVC/late

endosomes (Jelinkova *et al.*, 2019; Sommer *et al.*, 2021; Zhang *et al.*, 2021a). The Golgi dye BODIPY TR ceramide and the vacuole lumen dye 2′,7′-Bis-[2-Carboxyethyl]-5-[and-6]-Carboxyfluorescein (BCECF) have been employed for colocalization and analysis of vacuole formation (Takemoto *et al.*, 2018; Wang *et al.*, 2015a). Although fluorescent dyes are convenient for plant cell labelling without time-consuming genetic manipulations, the current dye resources are too limited to track the complex endomembrane compartments in plants.

Current state of advanced microscopy in imaging vesicular trafficking

A mechanistic understanding of membrane protein function requires the combination of interdisciplinary proteomics, biochemical and cell biology methods. Recent developments in advanced microscopy platforms have enabled unprecedented live imaging of subcellular membrane structures in high spatiotemporal resolution (Table 2). Laser scanning confocal microscopy (CLSM) was initially regularly employed to study the subcellular localization and dynamic trafficking of membrane proteins (Zhang *et al.*, 2019a, 2021a) (Figure 3a–c). However, imaging with CLSM is not always satisfactory in terms of its resolution and rate of acquisition when attempting to detect rapid trafficking and complex compositions of endomembrane vesicles. Excitingly, the super-resolution confocal live imaging microscopy (SCLIM) appears to be suitable to study fast cellular protein trafficking in plants at high spatiotemporal resolution (Shimizu *et al.*, 2021; Uemura *et al.*, 2019). Using SCLIM and three-coloured 4D imaging, a recent study revealed that two distinct membrane domains for cargo sorting zones existing in plant TGN are responsible for exocytosis and vacuolar trafficking (Shimizu *et al.*, 2021).

To analyse the membrane dynamics, variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) is currently a gold-standard technique for studying events that occur at the PM of plant cells located near the coverslip (Cui *et al.*, 2018). VA-TIRFM does not collect any fluorescence from out-of-focus planes, allowing to image membrane behaviours at single-molecular level, including membrane dwell time, diffusion coefficient, motion range and oligomerization status of PM-resident proteins (Xue *et al.*, 2018). For example, the analysis with VA-TIRFM demonstrated that the light receptor phototropin 1 remains inactive state as monomers predominately, while blue light treatment contributes to phototropin 1 dimerization and relocation into sterol-rich membrane microdomians for signalling activation (Xue *et al.*, 2018). Owing to the high resolution at z-axis, VA-TIRFM can be used to qualitatively and quantitatively characterize events of vesicular fusion and excision during exocytosis and endocytosis or PM protein dynamics in plant cells (Cui *et al.*, 2018).

As vesicles and membrane domains are very tiny; moreover, membrane trafficking is extremely fast, making it challenging to study endomembrane system with optical resolution of light microscopy (~200 nm). Several types of super-resolution microscopes have been developed to overcome the diffraction barrier of classical microscopy (Table 1). The application of these techniques has made it possible to characterize the structural and dynamic heterogeneity of membrane proteins with an accuracy of ~20–80 nm, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), stimulated emission depletion (STED) microscopy and structured illumination microscopy (SIM) (Ovecka *et al.*, 2022). They also allow the quantification of interactions via multicolour colocalization and the recording of protein dynamics at nanosecond and nanometre scales (for review see (Schubert, 2017)).

In comparison with other super-resolution methods, the superiority of SIM is that most experimental protocols and labelling fluorophores used for wide-field fluorescence microscopy are applicable without any modifications, thus allowing large-scale studies (Komis *et al.*, 2018). In this case, appropriate endomembrane markers such as fluorescently tagged HDEL signal peptide for ER-resident proteins, or the fluorescently tagged FYVE lipid-binding domain and small GTPase RabF2A, can be used to visualize the ER network (Figure 3f,g) and decipher the morphology of late endosomes (Figure 3h,j) respectively, which remains unresolved by standard epifluorescence visualization (Figure 3i,k). SIM can visualize various plant subcellular structures, ranging from endosomes to membrane nanodomains (von Wangenheim *et al.*, 2015; Yu *et al.*, 2017) (Figure 3l–n). For long-term monitoring organelles and subcellular compartments in intact developing plants, light-sheet fluorescence microscopy (LSFM) allows fast and live cell imaging at a wide range of spatiotemporal resolution (Ovecka *et al.*, 2022). LSFM is suitable for capturing developmental processes deep inside the root using fluorescent proteins targeted to subcellular compartments including endosomes and the PM (Ovecka *et al.*, 2015; Vyplelova *et al.*, 2017), as illustrated by the visualization of late endosomes in root tissues (Figure 3d,e). In addition, endocytic clathrin-coated pits can be imaged across the entire PM surface by LSFM (Aguet *et al.*, 2016).

These examples illustrate the potential of super-resolution microscopy platforms to tackle some key biological questions depending on the organization of the plant cell membranes. However, trade-offs with different imaging capabilities are inevitable. While super-resolution microscopy can uncover intricate protein localization patterns at the sub-organelle scale, it can be limited by the speed of image acquisition. A combination of super-resolution methods with LSFM can provide high acquisition frame rates and lower incidence of photodamage and photobleaching in living cells.

In addition, recently developed electron microscopy (EM) techniques can overcome the relatively limited resolution (20–100 nm) of fluorescence-based super-resolution microscopy, enabling to resolve 3-D ultrastructure of fixed cells at nanometre spatial resolution level (Liu *et al.*, 2021). Compared with conventional EM, electron tomography (ET) offers higher resolution for analysing 3-D membrane morphology of plant organelles (Otegui and Pennington, 2019). After capturing stack of 2-D images under multiple viewing directions, the 3-D ultrastructure can be made following back projecting these 2-D images with suitable weighting (Otegui and Pennington, 2019). ET methodologies have been successfully adapted to study various endomembrane organelles in plant cells, including TGN, autophagosome and extracellular vesicles (Roth *et al.*, 2019; Wang *et al.*, 2017; Zhuang *et al.*, 2017). To construct 3-D models of large-size structures or organelles, ET can be applied to whole-cell morphological analysis (Cui *et al.*, 2019a). Moreover, the serial block-face scanning electron microscopy (SBF-SEM) allows *in situ* automated volume imaging and can be efficiently used to resolve cellular structures and organelles in 3-D mode (Titze and Genoud, 2016). Recently, these EM techniques have been used to investigate protein storage vacuole (PSV) and lytic vacuole (LV) biogenesis in plants (Feeney *et al.*, 2018). To bridge fluorescence microscopy and EM, the correlative light and electron microscopy (CLEM) combines the strengths of these two modalities to analyse

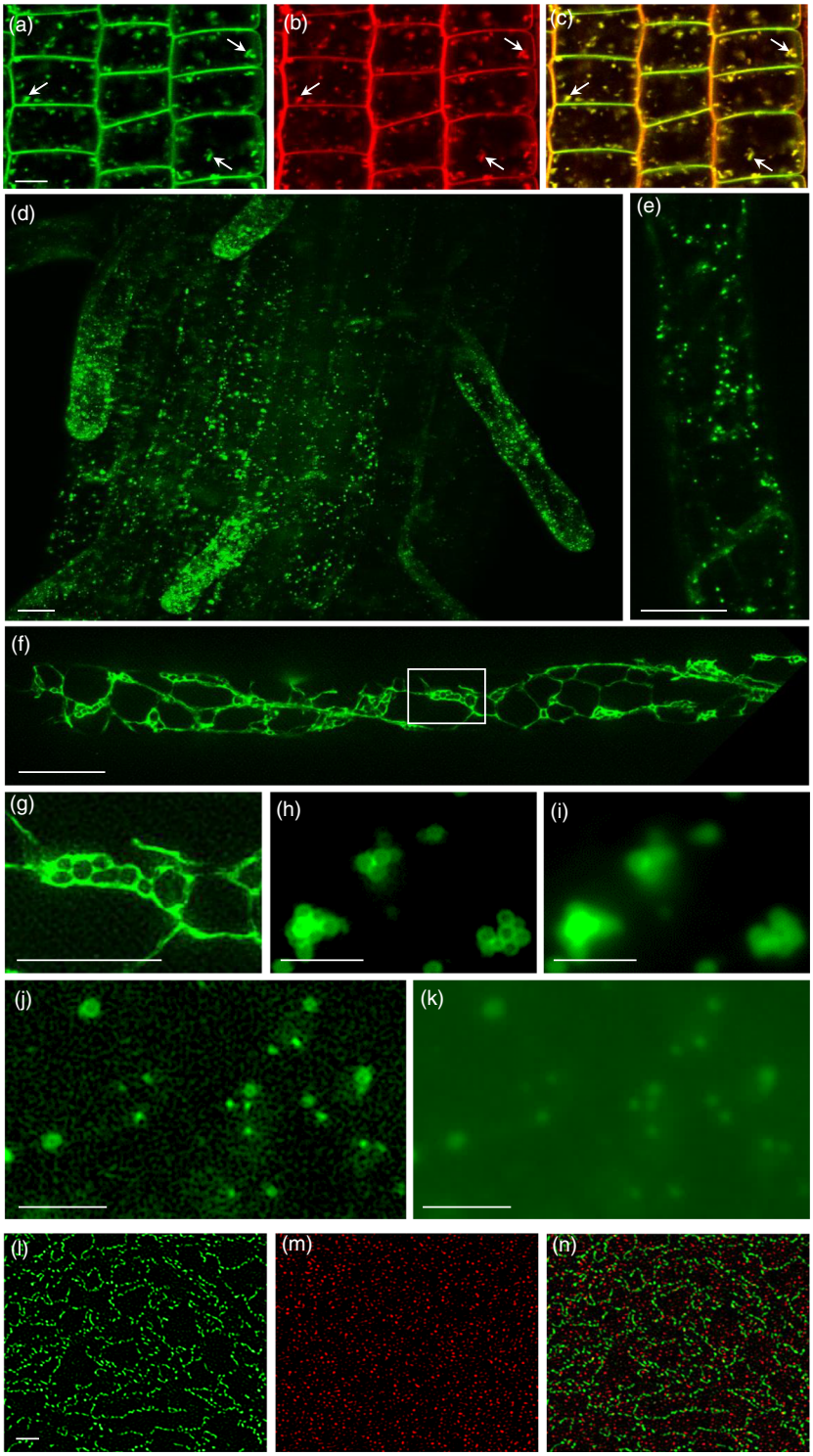


Figure 3 Survey of membrane-bound organelles in vesicle trafficking pathways by laser scanning confocal microscopy (CLSM), light-sheet fluorescence microscopy (LSFM), structured illumination microscopy (SIM) and wide-field epifluorescence microscopy. (a) PM and TGN/early endosomes labelled with GFP-VAMP721 in Arabidopsis root cells, as visualized by CLSM. (b) FM4-64-labelled early endosomes and PM in root cells after a short incubation with the dye. (c) Colocalization between GFP-VAMP721- and FM4-64-labelled endosomal compartments, as indicated by arrowheads. (d) Late endosomes labelled with YFP-RabF2a, as visualized by LSFM in Arabidopsis roots and root hairs. (e) Resolution of individual late endosomes and their subcellular distribution pattern by LSFM in root epidermal cells. (f) SIM overview of GFP-HDEL-labelled ER in an Arabidopsis root hair. (g) Magnified view of the boxed area in (f). (h,i) Clusters of late endosomes labelled with GFP-FYVE in an Arabidopsis hypocotyl cell, as visualized by SIM (h) or wide-field epifluorescence microscopy (i). (j, k) Individual late endosomes of variable size labelled with YFP-RabF2a in an Arabidopsis hypocotyl cell, as visualized with SIM (j) and wide-field epifluorescence microscopy (k). In both cases, SIM reveals the hollow structure of vesicles (h, j) which was not resolved by wide-field epifluorescence microscopy (i, k). Markers tagged with YFP are presented in artificial green colour. (l–n) The analysis of colocalization between a GFP-tagged lipid-binding protein and a mCherry-tagged membrane raft protein in Arabidopsis cotyledon epidermal cells, as visualized by VA-TIRF-SIM. Scale bars: 5 μm (a–c), 10 μm (d–f), 5 μm (g), 2 μm (h–k) and 10 μm (l–n).

rare cellular events in the same section (de Boer *et al.*, 2015). When applied to Arabidopsis root cells, a recent study revealed that homotypic fusion and protein sorting (HOPS) complex subunit VPS41 localized to membrane-less condensates and likely have regulatory roles in vacuolar transport (Jiang *et al.*, 2022). Unfortunately, EM and ET techniques based on fixed probes do not allow live cell imaging.

Overall, advanced microscopy methods allow the observation of both the spatial distribution of vesicles and their trafficking over time. Though still in their infancy in plant science, these methods show great promise for studying the dynamics of vesicular trafficking and structures at the cellular to macromolecular nanoscales.

Characterizing protein–protein interactions across vesicular trafficking pathways

MS-based techniques

MS has been extensively employed to analyse protein interactions using antibody-based immunoprecipitation followed by MS

Table 1 Comparison of microscopy techniques to explore vesicular trafficking pathways

Method	Microscope type	Lateral resolution	Axial resolution	Speed of imaging	Photo bleaching	Disadvantages	Applications in plant cells	References
PALM and STORM	Wide-field	10–30 nm	10–75 nm	Very slow (several minutes)	Low	• Very slow imaging rate; • Relies on labelling density of specific fluorescent probes.	• Protein dynamics and nanodomains at PM; • Protein colocalization; • Ultrastructure of cell wall polysaccharides.	Martiniere *et al*. (2019), Platre *et al*. (2019), Peaucelle *et al*. (2020)
STED	Laser scanning	20–90 nm	~500 nm	Fast (ms–s)	Moderate to high	• Phototoxicity with high-power illumination; • Small field of view; • Not suitable for 3D imaging.	• Architecture of cell wall; • PIN clustering distribution.	Coto Hernandez *et al*. (2016), Paes *et al*. (2018)
SIM	Wide-field	100–130 nm	~300–400 nm	Fast to moderate (ms–s)	Moderate to high	• Artefacts during image reconstruction; • Limited frame rate for fast time-lapse imaging.	• Endosomal movement, • 3D imaging of cortical microtubules; • Architecture of plasmodesmata.	Knox *et al*. (2015), Komis *et al*. (2018), Vavrdova *et al*. (2019)
SCLIM	Laser scanning	~300 nm	~700 nm	Very fast (up to 100 frames per second)	Low	• Lack of a scan zoom function; • Inability to adjust pinhole size.	• ER–Golgi transport; • TGN subdomains for cargo sorting.	Ito *et al*. (2018), Shimizu *et al*. (2021), Uemura *et al*. (2019)
LSFM	Laser scanning	~300–700 nm	~700 nm	Very fast (up to 100 frames per second)	Low	• Relative low resolution for subcellular imaging.	• Endosomal movement, cell division; • Calcium oscillations, root development.	Baesso *et al*. (2018), Ovecka *et al*. (2018)
VA-TIRFM	Wide-field	~300 nm	~100 nm	Very fast (10 ms per frame)	Low	• Imaging restricted to the cell surface; • no Z-sectioning.	• Endocytic pathway, PM microdomain; • smPPI analysis.	Cui *et al*. (2018), Wang *et al*. (2015b)
CLSM	Laser scanning	~300 nm	~700 nm	Slow (4 to 30 frames per second)	High	• Slow frame rate; prone to photobleaching / toxicity; not suitable for long-term developmental imaging.	• Detection of protein localization, membrane trafficking, protein interaction.	Ovecka *et al*. (2018), Xing *et al*. (2019), Xue *et al*. (2018)
ET	Electron microscope	<1 nm	2–7 nm	Slow (h)	Low	• Limited sample section thickness; • Complicated 3-D reconstruction.	• Analysis of membrane structures, such as TGN, vacuole and autophagosome.	Cui *et al*. (2019a), Otegui and Pennington (2019), Zhuang *et al*. (2017)

(Continues)

Table 1 Continued

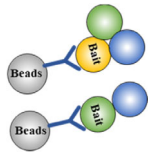
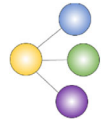
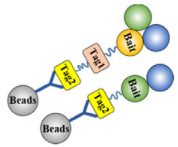
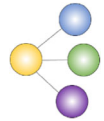
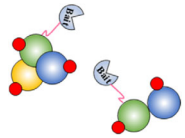
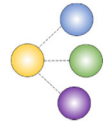
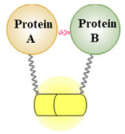
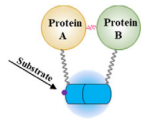
Method	Microscope type	Lateral resolution	Axial resolution	Speed of imaging	Photo bleaching	Disadvantages	Applications in plant cells	References
SBF-SEM	Scanning EM	~10 nm	~20–100 nm	Slow (h)	Low	• Restricted Z-axis resolution; • Relative low contrast for imaging; • Image quality is not consistent	• Analysis of the 3-D architecture of PSV and vacuole.	Feeney *et al.* (2018), Scheuring *et al.* (2016); Titze and Genoud (2016)
cryo-ET	Electron microscope	<1 nm	2–7 nm	Slow (h)	Low	• Limited sample section thickness; • Challenges of sample preparation and image analysis.	• Analysis of organelle structures, including ribosomes, ER, vacuole and lipid droplets in pollen tube.	Liu *et al.* (2021), Otegui and Pennington (2019)
CLEM	Combined light microscopy and EM/ET	Related to microscope type	Related to microscope type	Slow (h)	Low to high	• Diffuse precipitates induced by DAB.	• Analysis of HOPS subunit VPS41 localization at membrane-less condensates.	de Boer *et al.* (2015), Jiang *et al.* (2022)

identification (IP-MS), an affordable method that requires minimal equipment and can be carried out in most laboratories (Table 2). However, this method can suffer from lack of selectivity and low signal relative to background noise (Wendrich *et al.*, 2017). By contrast, tandem affinity purification (TAP) relies on the expression of a construct encoding a bait protein with specific affinity tags in cells (Van Leene *et al.*, 2015). The resulting bait protein can be used as an affinity probe for purifying interactions; thus, this method requires no specific antibodies against proteins of interest. When combined with MS, TAP empowers the identification of protein–protein interactions (PPIs) under near-physiological conditions and can be used for high-throughput studies (Figure 4), as illustrated by the isolation of seven putative interactors of the plant-specific adaptor protein (AP) complex TPLATE (Gadeyne *et al.*, 2014). TAP-MS has been widely applied to identify partners of membrane proteins in various plant species, showing a high level of intra- and inter-laboratory reproducibility (Adamowski *et al.*, 2018; Chen and Weckwerth, 2020). While this approach is extremely powerful, it often suffers cross-contamination with other organelles. In addition, the organelles or protein complexes under study must be efficiently purified under native condition and must maintain their integrity throughout the whole experimental process.

A recently developed method named proximity labelling overcomes some limitations of TAP-MS and can detect weak and transient PPIs in their physiological state (Branon *et al.*, 2018; Qin *et al.*, 2021). The basic strategy exploits engineered enzymes, which generate reactive and short-lived reagents that covalently tag proteins with biotin when in the vicinity (within nanometres) of the enzyme. Proximity labelling-based MS method has become a complementary and alternative approach to TAP-MS. This strategy can be extended to organelle-focused proteomics by anchoring the engineered enzyme to specific subcellular compartments using genetically encoded organelle localization signals (Lobingier *et al.*, 2017). Because the biotin 'tag' is covalently attached, it permits the use of very stringent buffers for disruption and efficient solubilization of membranes and protein interactions. The biotin-tagged proteins are then purified using streptavidin beads and followed by MS identification. Since the proteins need not be isolated in their native state, this method allows thorough washing to minimize non-specific background, thus capturing interactions with improved specificity and efficiency.

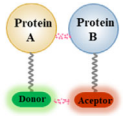
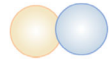
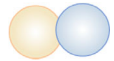
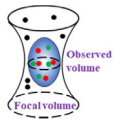
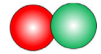
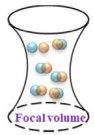
To date, the most commonly used enzymes for proximity-dependent biotinylation are biotin ligases (e.g. BioID and TurboID) and ascorbate peroxidases (e.g. APEX1 and APEX2) (Table 2). Conceptually, the interacting partners of a given protein define a local spatial proteome. The analysis of a range of distinct bait proteins from the same system generates an interaction map in the form of an interconnected network informing about the subcellular localization of its constituent proteins (Figure 4). One limitation here may stem from the extensive movement of vesicles across trafficking pathways, resulting in very different labelling dynamics compared with more stable organelles. To circumvent these caveats, the smaller labelling radius (less than 10 nm) of BioID favours protein interactors that are in close proximity to the bait, thus allowing to investigate both direct and indirect interactions (Kim *et al.*, 2014). The major advantage of such labelling is that it is applicable for detecting the immediate intracellular interacting partners of a bait protein. For instance, if a protein localizes throughout the entire Golgi apparatus, essentially all proximal associations can be labelled. As labelling

Table 2 Comparison of strategies used for the analysis of protein–protein interactions

Method		Principle	Requirements	Type of interactions	Throughput	Information from single experiment	Limitations	References
MS-based	IP-MS		Antibody	Very stable interactions	Small scale		• Not suitable for transient, weak or hydrophobic interactions; • Susceptible to organelle cross-contaminations; • Low accuracy and low coverage.	Bludau and Aebersold (2020)
	TAP-MS		Genetic tagging	Very stable interactions	Large scale		• Not suitable for transient, weak or hydrophobic interactions; • Susceptible to organelle cross-contamination	(Van Leene *et al.* (2015)
	Proximity-labelling MS		Genetic tagging with enzyme	Transient or stable interactions	Large scale		• Not suitable for detecting interactions in oxidative or acidic organelles or upon cold stress; • Depends on surface-exposed negatively charged amino acids.	Yang *et al.* (2021)
Imaging-based	BiFC		Reporter-fragment tagging	Stable interactions	Two proteins		• Low accuracy; • Not suitable for transient and dynamic interactions.	Fujii *et al.* (2018, Kodama and Hu (2012)
	Split luciferase system		Reporter-fragment tagging	Stable interactions	Two proteins		• Not suitable for detecting localization of interactions.	Azad *et al.* (2014)

(Continues)

Table 2 Continued

Method	Principle	Requirements	Type of interactions	Throughput	Information from single experiment	Limitations	References
FRET		Fluorophore tagging	Stable interactions	Two proteins		• Not suitable for low-abundance proteins; • Photobleaching, spectral bleed-through.	Shrestha *et al*. (2015)
FRET-FLIM		Fluorophore tagging	Stable interactions	Two proteins		• Difficult to quantify the fluorescence lifetimes in multi-exponential decays.	Bucherl *et al*. (2014)
FCCS		Fluorophore tagging	Stable or transient interactions	Two proteins		• Large axial excitation; • Depends on correlation curves.	Li *et al*. (2016)
Colocalization		Fluorophore tagging	Stable or transient interactions	Two proteins		• Medium accuracy; • Depends on high spatiotemporal resolution.	Cui *et al*. (2019b)

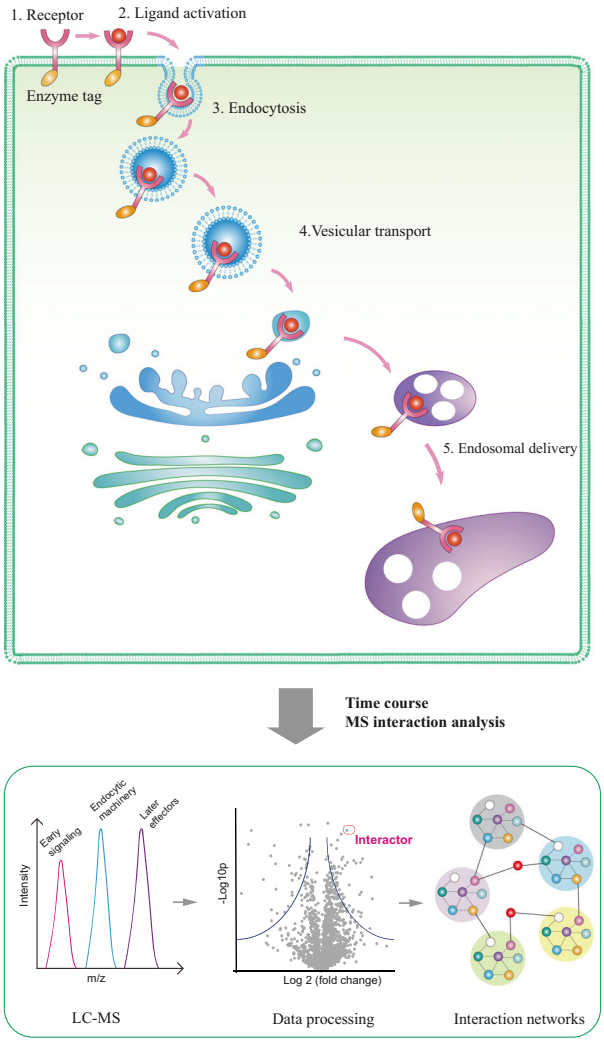


Figure 4 Spatiotemporal proteomics mapping of vesicular trafficking through interaction networks. Quantitative MS-based interactomics methodologies can be uniquely applied for the study of transient interactions, including signalling activation (upon ligand binding), endocytic uptake and vesicle delivery processes. The dynamic abundance of groups of protein interactors screened at different time points is shown in the lower panel, and the protein interaction networks can be established with bioinformatics tools.

duration increases, dynamic vesicle structures and components can be labelled over time, depending on the moving speed and half-lives of the bait protein and its biotinylated interactors.

The application of proximity labelling has been limited in plants mainly to systems with transient and/or high expression. In addition, the background activity of endogenous plant peroxidases and biotin contents of plant tissues have been the major hindrances in proximity labelling. To overcome these challenges, two active variants of biotin ligase (TurboID and miniTurboID) have been optimized by adding a small chemical tag to proteins that are close to them (Mair *et al.*, 2019). Such improvements have been utilized to generate different organellar proteomes in plants and have allowed the detection of proteins in subcompartments of plant cells, as shown with the identification of interactors of the endocytic adaptor TPLATE complex (Arora *et al.*, 2020).

Besides biotin ligases, APEXs oxidize phenol derivatives to biotin–phenoxyl radicals that covalently react with electron-rich amino acids, thus labelling proteins with biotin in minutes (Martell *et al.*, 2012). The rapid labelling capabilities of APEXs provide speeds comparable to those of many cellular processes, making it suitable to analyse transient or dynamic PPIs. APEX-based proximity labelling can provide spatially specific sub-organelle mapping and identify interactions, as shown for the endosome, the ER lumen and membrane (Hung *et al.*, 2017; Lobingier *et al.*, 2017), to reveal PPIs that necessitate higher spatial resolution.

Taken together, these technologies bridge the gap between classical protein interaction studies and cell biology, resulting in more accurate cellular localization of proteins as well as more clearly defined protein interactomes.

Imaging-based techniques

Protein–protein interaction studies based on imaging rely on the visualization of proteins in their cellular states without tissue disruption or organelle isolation (Table 2). Classic techniques for detecting protein interactions employ protein-fragment complementation assays, including bimolecular fluorescence complementation (BiFC) and the split luciferase system (also referred to as luciferase complementation imaging assays), which are the simplest and most popular methods to assess protein interactions in living plant cells (Fujii *et al.*, 2018). However, these methods suffer from low accuracy and are prone to false positive and false negative interactions.

Fluorescence resonance energy transfer (FRET) harnesses the changes in fluorescence intensity of physically interacting fluorophore-tagged donor and acceptor proteins due to proximity-dependent energy transfer and is widely used to investigate protein interactions (Hayes *et al.*, 2016). The combination of FRET and fluorescence lifetime imaging microscopy (FLIM) can reveal the fluorescence lifetime differences in fluorescent molecules independently of fluorophore concentration or excitation intensity (Bucherl *et al.*, 2014). FRET–FLIM helps to overcome some of the drawbacks of intensity-based FRET, such as autofluorescence induction, photobleaching of the donor and spectral bleed-through. It is efficient in detecting membrane PPIs in their native cellular state at nanometre and nanosecond resolution (Cui *et al.*, 2019b), as shown in the interaction studies between phototropin 1 and Arabidopsis REMORIN 1.3 (Rem1.3) (Xue *et al.*, 2018). In addition to FRET–FLIM, single-particle microscopy techniques like fluorescence correlation spectroscopy (FCS) can also be used to monitor PPIs. The principle of FCS is based on fluctuations in fluorescence intensity of fluorescent molecules that pass through the tiny detection volume (10^{-15} L) of a laser beam (Li *et al.*, 2016). Fluorescence intensity, molecule concentration, mobility and diffusion coefficient in the observation volume can be analysed with single-molecule sensitivity (Hao *et al.*, 2014; Iwai *et al.*, 2013). Dual-colour fluorescence cross-correlation spectroscopy (FCCS) is a powerful extension of FCS that can quantitatively measure a wide range of molecular interactions (Table 2). The fluctuations in fluorescence produced from two spectrally distinct fluorophores are recorded and analysed through their cross-correlation function, thereby providing information about the extent of interaction or colocalization (Krieger *et al.*, 2015). Both FCS and FCCS are limited by the classical optical diffraction limit (~200 nm). However, the inclusion of these techniques with modern microscopy approaches such as TIRFM or STED will facilitate a better characterization of

protein dynamics and interactions (Li *et al*., 2016). In comparison with bulk protein analysis by BiFC and FRET, FRET–FLIM and FCCS facilitate accurate quantification of spatial PPIs at single-molecule resolution.

Colocalization describes the spatial proximity between different fluorophore-labelled proteins of interest. With the help of advanced microscopy techniques, this approach can precisely map and reveal protein interaction networks. The degree of interaction can be quantitatively determined by calculating a protein proximity index, making it suitable for membrane protein studies. For instance, VA-TIRFM revealed that the AP2 subunit colocalizes with clathrin light chains, demonstrating a role for AP2 in regulating the formation, assembly and maturation of clathrin-coated vesicles (Fan *et al*., 2013). Later studies shed new light on other vesicle-mediated endocytic processes (Baral *et al*., 2015). Single-molecule protein proximity index (SMPPI) was recently developed based on single-molecule imaging techniques captured by VA-TIRFM (Cui *et al*., 2018), which can be calculated to quantitatively determine the colocalization of membrane proteins with improved accuracy. Success in these colocalization studies depends on the resolution of microscopy, proper preparation of analysed images to extract coefficients and correct interpretation of the results. For instance, SIM and STORM can map the coupling and analyse nanometre-scale assemblies of vesicle proteins (Lagache *et al*., 2018). Super-resolution microscopy coupled with powerful imaging analysis tools, such as 3D dual-particle tracking or the ImageJ tool DiAna, which overcome the limitations of incomplete spatial state descriptions and inaccurate evaluation in two-dimensional dual-colour images, can enhance the estimation of correlation coefficients and provide highly accurate quantification of colocalization, thus enabling more precise investigation of *in vivo* PPIs (Bon *et al*., 2018).

The imaging-based techniques described above constitute a versatile toolbox for assessing the spatial aspects of protein dynamics and interactions, which greatly facilitate the molecular characterization of vesicles in cellular processes in their native environment.

Data analysis of spatial proteomics

Spatial proteomics data processing

MS-based spatial proteomics analysis produces thousands of peptide dataset, making it hard to represent in a simple diagram. To reduce the complexity and improve visualization, proteins with similar characteristics can be correlated in the data map, allowing rapid assignment of resolved multi-organelles (Parsons *et al*., 2019). Methods to reduce data dimensions such as PCA and t-distributed stochastic neighbour embedding can resolve subcellular compartment proteomes, as proteins associated with the same organelles should form defined clusters (Figure 2d). These methods are often adopted to reveal the overall underlying data structure, as well as to compare variable features across conditions and replicates. Multi-organelle maps produced by such visualization methods only reflect the resolution and underlying principles of the selected fractionation approach (Parsons *et al*., 2019). As membrane proteins are highly dynamic and dually targeted in multiple cellular organelles, validation of known reference proteins of a target organelle within different biological contexts is critical.

Employed with established organelle marker proteins, supervised machine-learning algorithms can be used to convert spatial proteomics data into lists of organellar proteins (Crook *et al*., 2020a,b). Due to the lack of universally accepted organellar markers, the choice of marker proteins can be challenging for supervised workflows. As vesicle proteins are shuttled along endomembrane systems, membrane marker proteins should be confidently associated with a single organelle (e.g. calnexin as ER marker) in order to capture an accurate snapshot of protein distribution by spatial proteomics (Rosquete *et al*., 2019). Quantification of protein abundance in the cytosolic *versus* organellar fractions that are independent from their assigned subcellular compartments offers the possibility to make predictions for dual-localized proteins. Furthermore, organelle profiling obtained from larger gradients with more fractions (e.g. >20 fractions) can enhance assignment confidence and resolution. To precisely deconvolve proteomics data into multiple constituent organelles, the pure proteomics profiles of individual organelles are suggested to be normalized with marker protein averages. In this regard, spatial proteomics coupled with imaging-based quantification of a range of marker proteins can accurately deconvolve the underlying profiles of 'pure' compartments.

Unsupervised clustering algorithms do not require marker proteins, as they can define clusters from the provided profiling data, followed by assigning a compartment to each cluster based on available external subcellular localization information of constituent proteins from each cluster (Orre *et al*., 2019). These methods are tailored to the type of data generated within experiments and can be employed for the studies when marker proteins are unavailable. Choosing a machine-learning algorithm will depend on how much computing power can be accessed and which hypothesis might be appropriate about the data.

Processing interactomics data follow a reductionist approach: Its potential is to identify a small set of *bona fide* binding proteins from thousands of background proteins. Because quantitative interaction experiments are intrinsically noisy, stringent false discovery rate cut-offs and appropriate controls must be used to identify genuine PPIs. When analysing compartments that are not membrane-bound, constituents from adjacent organelles can be used as markers to identify these compartments (Hesketh *et al*., 2020). In addition, the use of multiple organellar baits is an efficient means for describing the organization of vesicle proteins and contact sites between organelles (Christopher *et al*., 2021) (Figure 4).

Image data acquisition and processing

The effective implementation and application of super-resolution microscopy to biological specimens largely depend on developing platforms and special software for image data acquisition and analysis and keeping them up to date with imaging platforms. For commercial imaging platforms, their modes of operation for data acquisition are described and maintained up to date by the manufacturer (Bayle *et al*., 2021; Komis *et al*., 2015). The microscope and imaging parameters, such as illumination pattern, objective, scanning mode, frame rates, time interval and z-stacks, can thus be set up and adjusted based on sample brightness according to the manufacturer's recommendations (Schubert, 2017). Moreover, image processing and editing are critical to produce a final dataset that can be viewed and assessed for the biological question at hand. Since advanced microscopy technologies are fully digital, they require new approaches for the exploration of reconstruction algorithms for the raw images. Compared with other super-resolution microscopy techniques, various pre-processing steps are required for the reconstruction of 3D data from long-term developmental imaging and multi-view

imaging by LSFM. Although stack projections, such as maximum or sum intensity, provide a visualization of the acquired data, several additional processing steps including virtual clipping, slicing and rotation are required to avoid hiding the region of interest in 3D view (Ovecka *et al*., 2018). For the four-dimensional representation of physiological or developmental tracking by LSFM, multiple and large 3D image stacks require the registration of individual angles, fusion of raw views into a single output image, and repeated processing of fused images for hundreds or thousands of programmed time points (Wolff *et al*., 2018). Recently, the development of algorithmic solutions implemented in open-source platforms like MATLAB and ImageJ/Fiji have substantially eased the reconstruction of raw data into super-resolved images. For example, the FairSIM plugin integrated into ImageJ/Fiji provides a ready-to-use, easy-to-operate and free solution for SIM reconstructions (Muller *et al*., 2016). Other open-source solutions, such as rapid STORM, sptPALM_viewer, MaMuT for light-sheet imaging, are available with different features for data analysis (Bayle *et al*., 2021; Wolff *et al*., 2018). More recently, a deconvolution algorithm that incorporates both sparsity and continuity has been developed to overcome resolution limits of current conventional and super-resolution microscopy (Zhao *et al*., 2022). In terms of multi-view or thousands of tracks, individual experiments tend to produce very large datasets, which makes smooth deployment and proper handling challenging for biologists lacking advanced computational skills. Therefore, packaging various algorithms into open-source platforms will greatly help researchers to evaluate the progress and quality of the image reconstruction quickly and efficiently.

Integrative membrane biology: combining data from MS and imaging analysis

Measurement of signalling dynamics associated with the PM

Since PM serves as a signalling platform controlling solute transport, characterization of PM proteins provides crucial information to elucidate *in vivo* cellular regulation. Phosphoproteomics profiling of single organelles defined early phytohormone signalling cascades, which revealed that the phosphorylation of several critical regulators of vesicle trafficking was regulated in response to phytohormone cues (Chen *et al*., 2010). Shotgun proteomics conclusively shows qualitative and quantitative changes in post-translational modification (PTM) of PM proteins; imaging data provide direct visualization of PTM-mediated interactions or translocations. For instance, the combination of phosphoproteomics and imaging analysis have functionally assessed nutrient limitation-induced and phosphorylation-dependent regulation of membrane transporters or kinases (Wu *et al*., 2019). Previous vesicle proteomics quantitatively determined the phosphorylation dynamics of aquaporin AtPIP2;1, subsequent site-directed mutagenesis analysis and microscopy validation demonstrated the requirement of phosphorylation in the C-terminus of aquaporin AtPIP2;1 for its trafficking to the PM (Prak *et al*., 2008). Although MS approaches are unable to directly provide spatiotemporal localization information, their quantitative capabilities are a powerful tool for investigating protein interactions in whole-cell lysates. Employed with IP-MS, large-scale interactomics screens have been performed across a wide range of Qa-SNARE-depleted plant materials, followed by imaging analyses that validated the essential roles of Qa-SNARE proteins in vesicle transport and lignin biosynthesis (Fujiwara *et al*., 2014). The most important impact that advanced microscopy likely have on MS data is to provide insight into the trafficking pathways of membrane proteins. Combined with chemical labelling, recent vesicular proteomic studies indicate the potential of imaging-based approaches to supplement MS data to determine previously uncharacterized clathrin-coated vesicle components and cargos (e.g. AP-4 and vacuolar sorting receptors), resulting in valuable biological insights (Dahhan *et al*., 2022).

The PM undergoes compartmentalization into numerous interactive and specialized microdomains. Quantitative membrane proteomic studies indicate that rapid and discrete modulation of microdomain proteins involved in vesicle trafficking and secretory pathways take place upon perception of pathogen elicitors (Keinath *et al*., 2010; Stanislas *et al*., 2009). These data indicate a new link between environmental signalling events and the function of specialized sub-membrane structures. The advantage of MS-based proteomics is their ability to identify the mixture of all proteoforms present in a given sample; therefore, validation of subcellular localization derived from MS data is the most straightforward integration of advanced microscopy. Compared with MS methods, imaging analyses preserve fine microdomain structures to enable the detection of dynamic localization and behaviour of their constituent proteins across membranes. Single-molecule imaging analysis using VA-TIRFM revealed that the endocytic pathways mediated by clathrin and microdomain cooperatively regulate redox signalling pathways at the PM (Hao *et al*., 2014). Another advantage of imaging methods is that protein purification is not required, making it the only available approach for some receptor targets. For instance, previous colocalization analysis revealed that microdomains are crucial for endocytic trafficking of the pattern recognition receptor FLAGELLIN-SENSING 2 (FLS2) in response to the effector flg22 (Cui *et al*., 2018). These examples revealed the pivotal roles of membrane microdomains in regulating cargo protein sorting.

Examining the trafficking and deposition of cell wall components

In addition to mediating membrane trafficking, plant Golgi apparatus and TGN have specialized functions in the biosynthesis and delivery of cell wall components. A proteomics screen of Arabidopsis cargo proteins demonstrated that cell wall proteins are completely depleted from purified protein membrane fractions of *ap-3β* and *ap-4β* knockout plants, indicating the important functions of adaptor protein complexes in the delivery of cell wall components (Pertl-Obermeyer *et al*., 2016). High-resolution MS is capable of measuring hydrophobic proteins in the dynamic subdomains of the Golgi apparatus; using immuno-isolated Golgi fractions and selected endomembrane marker proteins, previous proteomics analysis precisely determined the localization of cell wall biosynthetic enzymes into discrete Golgi sub-compartments (Heard *et al*., 2015). Using a combination of vesicle glycomics and imaging validation, a large-scale cell wall glycan profiling was performed for the SYP61-TGN compartments; mutant analysis indicated varied non-cellulose-based glycans as the cargos of these vesicles (Wilkop *et al*., 2019). Taken together, integration of MS and imaging methods enable to resolve the critical roles of vesicle proteins in the delivery and deposition of detail structural polysaccharides and glycoproteins. Although membrane compartments associated with a specific marker protein exist as heterogeneous populations that are pooled by affinity purification, scaling up proteomics of various vesicles using distinct marker proteins of endo-exocytic pathways

provides insight into how these pathways regulate polysaccharide delivery and how dynamic tethering proteins influence TGN/early endosome organization (Rosquete *et al*., 2019). Because membrane vesicle types vary with cell type, tissue and species, which adds another layer of complexity to the analysis of cell wall component delivery, developing more subcellular markers will facilitate the characterization of cell wall synthesis and assembly.

A major breakthrough came with the characterization of the Golgi proteome, where the combination of FFE/LOPIT-MS and super-resolution microscopy enabled the establishment of a high-resolution proteome map across distinct Golgi compartments and determination of the spatial organization of transient cargo proteins, tethering and docking factors related to secretory systems (Parsons *et al*., 2019). In addition to visualizing proteins, super-resolution microscopy has become a powerful tool for exploring the nano-architecture of cell walls. STED-deconvolved techniques can accurately measure the architecture of the secondary cell wall and the middle lamella in the wood section when focusing on lignin fluorescence (Paes *et al*., 2018). Direct STORM (3D-dSTORM) revealed the organization of homogalacturonan polysaccharides into nanofilaments in pavement anticlinal cell walls (Haas *et al*., 2020). Ultimately, super-resolution microscopy allows the description of the dynamic localization, intricate ultrastructure and mutual spatial arrangement of major cell wall precursors with nanometre resolution.

These reports demonstrate the power of MS and super-resolution microscopy but also the necessity to combine proteomics mapping with targeted imaging to characterize the proteins detected and the corresponding structural changes of subcellular compartments as a function of vesicle trafficking.

Characterization of plant immune responses

Plants specifically activate membrane receptors and regulate vesicle trafficking to fight off pathogen attacks (Gu *et al*., 2017; Yun and Kwon, 2017). In this context, the combination of quantitative MS and imaging methods has been widely adopted to investigate TGN-mediated secretion. For example, a comparison of intracellular proteins between wild-type plants and mutants lacking various cargo proteins determined that the SYP4-VAMP721 secretory pathway mediates the constitutive secretion of cell wall-modifying enzymes to enhance pathogen-inducible extracellular defences (Uemura *et al*., 2019). The investigation of membrane-localized interactors has provided novel insights into the molecular mechanisms of plant–pathogen interactions. Several MS-based interaction studies have identified immunity-related proteins, such as SYP122, phot1, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and REM1.3; these proteins are involved in processes targeted by bacterial type-III secretion systems to promote pathogenesis (Bozkurt *et al*., 2014; Khan *et al*., 2018; Vijayapalani *et al*., 2012).

Combination of MS and imaging-based methods can capture transient and weak interactions, enabling previously unresolved parts of the interactomes to be characterized. An elegant application of TurboID-MS was recently reported in Arabidopsis, where the interactomic studies uncovered new regulators of nucleotide-binding leucine-rich repeat (NLR) immune receptor-mediated immunity by E3 ubiquitin ligases and other proteins (Zhang *et al*., 2019c). Further validations using BiFC showed that 85% of the tested candidates interact with the NLR (Zhang *et al*., 2019c). The coupling of IP-MS and colocalization analysis was recently applied to globally map the complement of interactions between host proteins and pathogen effectors. In this study, the vesicle interactomic screen revealed that the PexRD12/31 family of effectors targets the vesicle trafficking machinery during pathogen infection in *N. benthamiana* (Petre *et al*., 2021). Compared with BiFC, live cell imaging analysis captures spatiotemporal snapshots of effector accumulation at higher resolution; this confirmed that PexRD12/31 effectors accumulate at plant-derived extrahaustorial membranes and alter vesicle trafficking by increasing endosome numbers in pathogen-infected *N. benthamiana* cells (Petre *et al*., 2021). These studies bridge the gap between MS and imaging methods of the endocytic and secretory trafficking pathways. The advantage of imaging approaches is that membrane proteins are characterized within their native lipid context, allowing for more complete interaction profiling in membrane regions. However, MS and imaging approaches target different aspects of the interactions, suggesting that both methods should be applied simultaneously or integrated into a single pipeline for a more complete characterization of biological systems.

To conclude, MS and imaging techniques are complementary approaches for the characterization of immune responses, allowing a system-wide discovery of cellular signalling networks.

Concluding remarks

Dynamic vesicle transport systems mediate intra- and extracellular cargo transport and thus regulate plant growth, development and stress responses. The deconvolution of individual vesicle trafficking networks between endomembrane organelles is becoming a major goal of plant cell biology. As discussed in this review, current quantitative MS can discriminate sub-stoichiometric interactions. When combined with advanced microscopy imaging capabilities, these approaches enable multiplexed mapping of membrane proteins to very high resolution and accuracy. The use of new chemical modulators of vesicle transport has also proven beneficial. Although vesicles are one of the most technically challenging organelles to study, the technologies described above have determined the spatial organization of >1000 proteins and key regulators involved in diverse vesicle trafficking pathways. Nevertheless, several studies have shown the strong synergy resulting from the combination of MS-based spatial proteomics and advanced imaging.

Continue development of new technologies may address more challenging questions, such as determination of organelle-specific turnover rates and organellar interfaces. In the future, we expect that both MS and microscopy will continue to improve in resolution, sensitivity and scanning speed. As these technologies are becoming more accessible to cell biologists, they will be increasingly important among the arsenal of techniques that offer us ways to understand the cell in a systems biology manner.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Author contributions

Zhang L wrote the manuscript and contributed to CLSM images. Liang X provided drawing image files. Takáč T, Komis G, M Ovečka and Šamaj J revised the manuscript and provided microscopy images. Li X contributed to the project discussion. Zhang Y provided VA-TIRF-SIM images. Chen Y designed the project and wrote the manuscript. All the authors read and approved the manuscript.

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