Jozef Šamaj *Editor*

Endocytosis in Plants

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Editor Biography

Jozef Šamaj received his Ph.D. degree in Plant Physiology from Comenius University in Bratislava, Slovakia. He completed three post-doctoral stays in France, Germany, and Austria, supported by Eurosilva, the Alexander von Humboldt Foundation, and the Marie Curie Programme. He co-edited four books and co-authored more than 115 research papers, reviews, and book chapters. He received his habilitation degree from the University of Bonn, Institute of Cellular and Molecular Botany in 2004, where he was a senior lecturer and group leader. Since 2010, he has been a full professor and department leader at the Palacký University Olomouc, Centre of the Region Haná for Biotechnological and Agricultural Research. His research is focused on the functional characterization of mitogen-activated protein kinases as well as cytoskeletal and vesicular trafficking proteins during plant development and stress responses. His laboratory is using integrated cell-biological, genetic, and functional proteomic approaches.

Preface

During the past 6 years plant endocytosis developed into a flourishing research field. The role of clathrin-mediated endocytosis in the internalization of some plasma membrane proteins was firmly established while alternative clathrin-independent endocytic routes such as fluid-phase and flotillin-dependent endocytosis were also described. Plant endosomes turned from enigmatic organelles to subcellular compartments with partially defined molecular topology and function. Chapter 1 of this book provides an overview of diverse methods recently introduced into plant endocytosis research. Up-to-date methodological approaches such as proteomics and advanced microscopy including light sheet microscopy or fluorescence recovery after photobleaching (FRAP) combined with super resolution microscopy start to be applied in plant endocytic research. Chapter 2 is devoted to chemical genomics, providing a new generation of more specific chemical inhibitors which in combination with automated quantitative microscopy (cellomics) provide another very powerful tool to study endocytosis in plants. Chapters 3 and 4 are focused on the crucial role of endocytosis in the establishment and maintenance of polarity in diverse types of plant cells. Chapter 5 describes fluid phase endocytosis in specialized storage plant cells while Chap. 6 provides a very useful overview of physical factors which have some impact on endocytosis. The next three chapters are focused on plasma membrane proteins such as receptors, auxin transporters and water channels, nicely demonstrating biologically relevant roles of endocytosis in the regulation of signalling proteins as well as auxin and water transport in plant cells and tissues. Chapters 10–14 deal with crucial molecular players regulating different steps of endocytosis, namely Rab GTPases, SNAREs, SCAMP, sorting nexins, retromer, and ESCRT proteins. The next two chapters summarize the importance of endocytosis in plant cell interaction with pathogens and symbiotic microbes. The final chapter provides an overview of the role of the cytoskeleton in the different types of endocytosis in plants and other organisms.

In total, the present book summarizes the latest advances in the field of plant endocytosis. Moreover, it also provides several excellent examples of biological relevance of endocytosis in physiological processes controlling cell polarity,

shape, water and nutrition uptake, or biotic interactions of plant cells with pathogens and symbionts. These surely belong to important fields of plant biology.

I would like to thank all authors for their excellent contributions to this book. I hope that reader will enjoy and appreciate it.

This book is dedicated to my family.

Olomouc, June 2012 Jozef Šamaj

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Update on Methods and Techniques to Study Endocytosis in Plants

Olga Šamajová, Tomáš Takáč, Daniel von Wangenheim, Ernst Stelzer and Jozef Šamaj

Abstract The growing interest in the investigation of endocytosis, vesicular transport routes, and corresponding regulatory mechanisms resulted in the exploitation of cell biological, genetic, biochemical, and proteomic approaches. Methods and techniques such as site-directed and T-DNA insertional mutagenesis, RNAi, classical inhibitor treatments, and recombinant GFP technology combined with confocal laser scanning microscopy (CLSM) and electron and immune-electron microscopy were routinely employed for investigation of endocytosis in plant cells. However, new approaches such as high-throughput confocal microscopy screens on mutants and proteomic analyses on isolated vesicular compartments and root cells treated with vesicular trafficking inhibitors (both focused on the identification of new endosomal proteins), together with chemical genomics and advanced microscopy approaches such as Förster resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), light sheet-based fluorescence microscopy, and super-resolution microscopy provided a significant amount of new data and these new methods appear as extremely promising tools in this field.

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1 Introduction

Endocytosis is a dynamic process of intracellular uptake of plasma membrane and extracellular cargos, which is controlled by a network of regulatory proteins. In addition, endocytosis of some plasma membrane proteins is modulated by their posttranslational modifications (PTMs) such as mono-ubiquitination and/or phosphorylation. Endocytosis is highly sensitive to the changes in external and internal physical (see chapter by Baluška and Wan in this volume) and chemical conditions (see chapter by Li et al. in this volume). Diverse molecules including proteins, lipids, and carbohydrates (Müller et al. 2007; Ovečka et al. 2010) are internalized by endocytosis. Spatial and temporal regulation and variability of endocytosis in diverse plant cell types, tissues, and organs emphasize the careful choice of methodological approaches used to study this highly dynamic biological process. This chapter provides an overview of methods and techniques, which were used to study plant endocytosis in recent years.

2 Chemical and Biochemical Methods

2.1 Using Conventional Chemical Inhibitors to Study Endocytosis

Well-established inhibitory compounds such as brefeldin A (BFA) and wortmannin are useful to study endocytosis in plant cells (Müller et al. 2007). The sensitivity of certain proteins to these inhibitors may indicate their subcellular localization and participation in specific steps of endocytic vesicular transport. During the last years, inhibitors were broadly employed for microscopic observations (Müller et al. 2007) and also in biochemical and proteomic studies (Luczak et al. 2008; Takáč et al. 2011a; Takáč et al. 2012).

BFA, a macrocyclic lactone, targets some BFA-sensitive adenosine diphosphate ribosylation factor-guanine nucleotide exchange factors (ARF GEFs), thus inhibiting secretory and recycling vesicular trafficking pathways in yeast, mammalian, and plant cells (Nebenführ et al. 2002; Geldner et al. 2003; Takáč et al. 2011a). A latest proteomic study revealed that profilin 2, an actin binding protein, is involved in the formation of BFA-induced compartments in Arabidopsis roots (Takáč et al. 2011a).

Concanamycin A, an inhibitor of V-ATPase was used to prove importance of V-ATPase activity for Golgi ultrastructure as well as to study *trans*-Golgi network (TGN) and multivesicular body (MVB) structural integrity (Dettmer et al. 2006; Viotti et al. 2010; Scheuring et al. 2011). Application of concanamycin A caused colocalization of TGN and MVB molecular markers, thus substantially contributing to the new finding that MVBs origin from TGN in Arabidopsis (Scheuring et al. 2011).

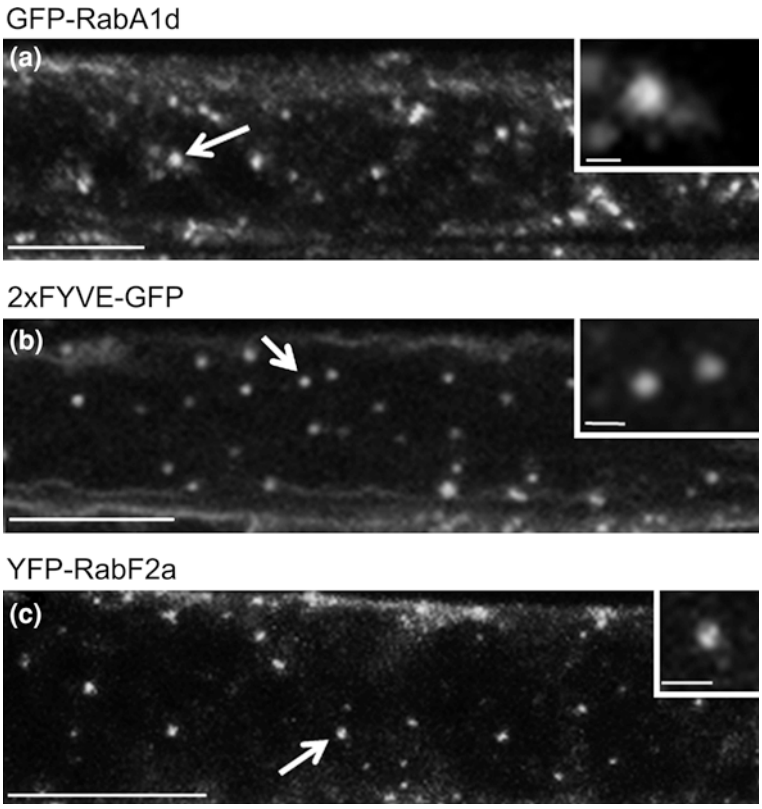


Fig. 1 Visualization of early endocytic compartment/TGN with GFP-tagged RabA1d and late endocytic compartments with 2xFYVE-GFP and YFP-tagged RabF2a using CLSM. Typical early and late endocytic compartments are indicated with Ï arrows. *Bars* represent 10 μm for panels **a**, **b**, and **c** and 1 μm for insets

Furthermore, there are several chemical compounds inhibiting clathrin-mediated endocytosis. Wortmannin inhibits phosphatidyl-inositol-3-kinase (PI3 K) and phosphatidyl-inositol-4-kinase (PI4 K) in a dose-dependent manner and also blocks clathrin-mediated endocytosis consequently to the aggregation and stabilization of clathrin-coated pits at the plasma membrane (Matsuoka et al. 1995; Ito et al. 2012; Figs. 1 and 2). The same effect was shown for LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) which is a synthetic compound selectively inhibiting PI3 K (Etxeberria et al. 2005; Baroja-Fernandez et al. 2006; Lee et al. 2008a). These two compounds also block vacuolar transport due to the fusion and swelling of prevacuolar compartments (PVCs), which are identical with MVBs (Matsuoka et al. 1995; Wang et al. 2009; Takáč et al. 2012). However, wortmannin also caused depletion of TGN compartments which probably fused with MVBs in wortmannin-treated Arabidopsis root cells (Takáč et al. 2012). Other inhibitors

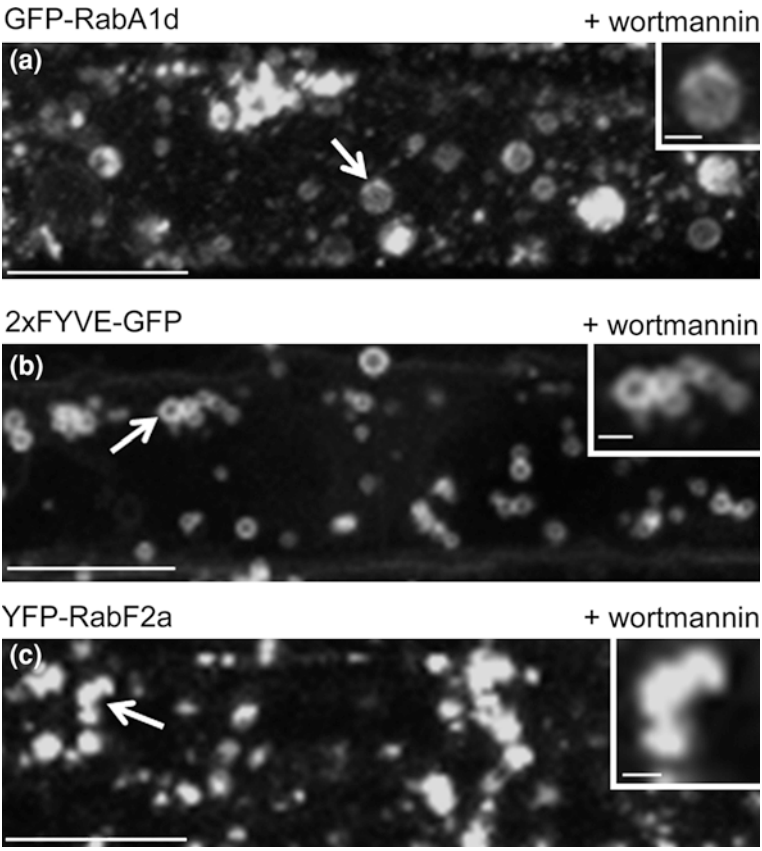


Fig. 2 Effect of wortmannin on early endocytic compartment/*TGN* (GFP-RabA1d) and late endocytic compartments (2xFYVE-GFP and YFP-RabF2a). The wortmannin-induced swelling and partial aggregation of *TGN* compartments as well as formation of larger clustered structures designated as wortmannin-induced compartments are indicated by *arrows*. Bars represent 10 μm for panels **a**, **b** and **c** and 1 μm for insets

such as tyrphostin A23 inhibited cargo sorting into clathrin-coated vesicles during clathrin-mediated endocytosis (Banbury et al. 2003) while ikarugamycin was used to inhibit clathrin-dependent endocytosis in tobacco protoplast culture (Onelli et al. 2008; Bandmann and Homann 2012; Nagawa et al. 2012).

Additionally, several routinely used auxin transport inhibitors such as 2,3, 5-triiodobenzoic acid (TIBA) and 2-(1-pyrenoyl) benzoic acid (PBA) inhibit vesicle trafficking in plant, yeast, and mammalian cells affecting actin dynamics (Dhonukshe et al. 2008) while nystatin and filipin inhibit lipid raft-mediated endocytosis (Kale et al. 2010; Ovečka et al. 2010; Luu et al. 2012).

2.2 Chemical Genomics Identifies New Inhibitors of Endocytosis and Vesicular Trafficking

More specific and targeted inhibitors are necessary to study complex vesicular trafficking pathways including the endocytosis. Significant effort has been devoted to the identification of new vesicular trafficking inhibitors through large-scale chemical genomics approaches (see chapter by Li et al. in this volume). In a chemical library screening study, altered pollen germination and pollen tube polar growth were used as selection criteria to find compounds altering vesicular trafficking (Robert et al. 2008). Using microscopic studies it was shown that endosidin 1 (ES1) interfered with endocytosis, causing the selective accumulation of PIN2, AUX1, and the brassinosteroid receptor BRI1 in distinct endomembrane compartments termed "endosidin bodies". ES1 also altered the circadian system of Arabidopsis, and it was proposed that the ability of ES1 to modify endosome trafficking is connected with an actin-stabilizing effect (Tóth et al. 2012). In a complementary study, a chemical library of 46418 compounds was screened for potential effects on vesicular transport by means of high-throughput CLSM (Drakakaki et al. 2011). For this purpose, distinct subcellular chemical-induced relocalizations of plasma membrane marker proteins such as PIN1-GFP, PIN2-GFP, and BRI1-GFP (all under the control of the respective native promoters) were tested in Arabidopsis root tips. A new compound named endosidin 3 was shown to target TGN but not Golgi, while it also altered the plasma membrane localization of small GTPase ROP6. Finally, another compound named endosidin 5 enhanced trafficking of plasma membrane proteins toward the vacuole.

2.3 Cell Fractionation and Isolation of Endosomes

The isolation of organelles involved in endocytosis and vesicular transport in combination with immunolocalization, biochemical, and proteomic analyses is one of the most effective ways to dissect the intracellular transport processes. Recently, vesicular TGN compartments containing SYP61 were isolated from Arabidopsis plants expressing *proSYP61*::*SYP61-CFP* using sucrose gradient fractionation, followed by immunopurification with antibodies against GFP (Drakakaki et al. 2012). SYP61 is a TGN-localized member of a Q-SNARE complex formed by SYP41/SYP61/VTI12 (Zouhar et al. 2009). Immunoprecipitation was performed using protein A agarose beads coupled to GFP antibody and rabbit IgG. Plant vesicle extracts were pretreated with unconjugated beads to remove non-specific binding proteins and then incubated with the antibody-coated beads to collect vesicles containing SYP61-CFP (Drakakaki et al. 2012). Sucrose density gradient fractionation was also used to isolate the MVB fraction and to prove the presence of vacuolar sorting receptors (VSR) in MVBs of germinating mung bean seeds.

First, the VSR-enriched fraction in sucrose gradients was detected by using VSR-1 antibody, and subsequently immunogold negative staining of isolated MVBs was performed with VSR-1 and BP-80 CT antibodies (Wang et al. 2007).

Sucrose gradient fractionation combined with immunoblots also represents a widely used approach to localize proteins related to endosomal transport. For example, the retromer-like protein complex is associated with PVC and with high-density sedimenting membranes. Immunogold negative staining identified these membranes as coated microvesicles with 90 nm diameter (Oliviusson et al. 2006). GRV2/RME-8 protein was identified as a protein controlling the transport between MVB and vacuole. The sucrose gradient fractionation showed that this protein may be distributed along an array of diverse but interconnected endomembrane compartments (Silady et al. 2007). Membrane fractions separated in sucrose gradients were either probed against RabA4b, or spotted onto nitrocellulose membranes and immunoblotted with monoclonal antibodies recognizing cell wall polysaccharides (Kang et al. 2011). The pattern of terminally fucosylated xyloglucan distribution nearly followed the pattern of RabA4b distribution, suggesting TGN localization. To simplify the detection of the protein of interest, microsomal membranes of BY-2 cells overexpressing SCAMP2-YFP were separated by sucrose density gradient fractionation and subsequently separated by SDS-PAGE. SCAMP2-YFP was found in plasma membrane and TGN fractions suggesting that it is involved in the transport between these two subcellular compartments (Toyooka et al. 2009). Recently, signal transducing mitogen-activated protein kinase MPK6 was localized to the plasma membrane and TGN using subcellular fractionation combined with immunoblotting, and these results were supported by diverse microscopic methods (Müller et al. 2010).

2.4 Isolation of Plasma Membrane Lipid Rafts

Lipid rafts are sterol/sphingolipid-enriched liquid crystalline-ordered membrane microdomains important for protein and lipid subcellular trafficking and for intracellular signalling (Simon-Plas et al. 2011). They are usually isolated from the plasma membrane fraction prepared by using an aqueous two-phase polyethylene glycol (PEG)-dextran partitioning system. Detergent-resistant membrane (DRM) proteins are isolated by the solubilization of plasma membrane by the addition of Triton X-114, followed by the addition of sucrose in certain, tissue-dependent final concentration, overlaid with successive concentration steps of sucrose in TBS buffer, and then centrifuged at 200,000 g for 16 h. DRMs could be recovered above the 30–35 % interface as an opaque band (Borner et al. 2005; Morel et al. 2006; Lefebvre et al. 2007). Alternatively, an Optiprep step gradient can be used (40/30/0 % OptiPrep). In this case, DRMs are isolated on the top of the gradient simplifying the procedure (Carmona-Salazar et al. 2011).

In the search of lipid raft origins, DRMs were isolated also from endoplasmic reticulum (ER) and Golgi fractions again on the basis of Triton X-100 insolubility (Laloi et al. 2007). The lipid composition of Golgi DRMs showed a marked similarity to plasma membrane-derived DRMs suggesting that plasma membrane lipid rafts originate in Golgi apparatus (Laloi et al. 2007).

2.5 Proteomic Approaches to Study Endocytosis

Proteomics is a valuable tool for the investigation of various aspects of plant development (Takáč et al. 2011b; Weckwerth 2011) and also represents a challenging strategy to investigate endocytosis. With the help of proteomic techniques it is possible to identify a spectrum of proteins in certain tissues at certain time. However, the capability of proteomics to investigate endocytosis is limited due to the dynamic character and spatial variability of endocytotic proteins.

A significant advancement of the knowledge on organelle proteomes was achieved during the last few years (Dunkley et al. 2006; Lilley and Dupree 2007; Agrawal et al. 2010).

The organelle proteomic analysis, like all other biochemical subcellular localization studies, requires absolute purity of the respective organelle fractions. One of the most powerful approaches to overcome this problem is membrane density gradient fractionation combined with isotope tags for relative and absolute quantitation (iTRAQ) (Dunkley et al. 2006). In brief, proteins of membrane fractions are solubilized, digested, and the resulting peptides are labeled by different iTRAQ reagents. Such peptides are subjected to 2D LC MS/MS analysis in conjunction with 2D liquid chromatography, which enables the quantification of protein distributions within a gradient. Proteins with similar density gradient distributions and therefore, localizations, are clustered (Lilley and Dupree 2007).

Very recently, the SYP61 compartment was isolated using two-step immunopurification combined with sucrose gradient fractionation (Drakakaki et al. 2012). Isolated proteins were identified by nano-liquid chromatography coupled to tandem mass spectrometry and two-dimensional nano-ultra-performance liquid chromatography (UPLC). Proteins detected in the SYP61 sample but not in IgG control were considered specific to TGN compartment possessing SYP61. About 60 % of the identified proteins were known or predicted to be associated with the endomembrane system and 32 % were predicted as a putative cargo transported by vesicular transport. Microscopic observations were employed to validate the localization of individual proteins. This study detected all members of SYP61 SNARE complex in the proteome of SYP61 compartment while SYP43 was identified as a putative SYP61 interactor. In addition, the proteome of SYP61 compartment encompasses important regulators of membrane trafficking such as RabD2b and YIP1, as well as the VSR3, VSR4, and VSR7 (Drakakaki et al. 2012).

Since endocytosis and vesicular trafficking are dynamic processes affected by chemical compounds in certain defined steps, differential proteomics using such

compounds can significantly contribute to our better understanding of these processes. For example, the differential proteomic analysis of Arabidopsis roots treated by BFA was recently published (Takáč et al. 2011a). Using comprehensive, gel-free, and gel-based proteomic approaches it was shown that proteins known to accumulate in BFA compartments are upregulated in the whole cell protein fraction. A combination of proteomic approaches with microscopic observations revealed an important role of cytoskeletal protein profilin 2 in the interplay of vesicular transport and the cytoskeleton. Additionally, the BFA-induced accumulation of ER resident proteins revealed by proteomics was confirmed by independent CLSM and electron microscopy approaches (Takáč et al. 2011a). In a similar way, the changes in the Arabidopsis root proteome were analyzed after wortmannin treatment. This study revealed that TGN-localized RabA1d, a small Rab GTPase is downregulated by wortmannin (Takáč et al. 2012). Wortmannin also affected post-Golgi compartments leading to depletion of TGN and fusion/swelling of MVBs, indicating the possible consumption of TGN by MVBs, as revealed by CLSM and electron microscopy analyses (Wang et al. 2009; Takáč et al. 2012).

Several proteomic studies employing Triton X-100-based isolation procedures aimed to analyze lipid rafts in Arabidopsis (Borner et al. 2005), BY-2 tobacco cells (Morel et al. 2006), *Medicago truncatula* (Lefebvre et al. 2007) and the two monocotyledonous species oat and rye (Takahashi et al. 2012). The solubilization of DRMs using buffers containing high concentrations of detergents may be incompatible with reverse phase separation and substantially decrease the efficiency of trypsin to digest proteins. Therefore, the separation of proteins either by 1D SDS-PAGE followed by excision of protein lanes (Morel et al. 2006; Lefebvre et al. 2007) or 2D electrophoresis (Borner et al. 2005) overcomes these problems. Proteins involved in signalling and response to biotic and abiotic stresses, cellular trafficking, and cell wall metabolism are more abundant in DRMs compared to the rest of the plasma membrane (Morel et al. 2006; Takahashi et al. 2012).

3 Molecular Biological Methods

3.1 Cloning and Fluorescent Tagging of Endocytic Proteins for Visualization and Colocalization Studies

Recently, the most common method of subcellular visualization of endocytic proteins is based on their cloning and tagging with fluorescent proteins such as GFP and its structural analogs such as YFP and CFP. Occasionally, other fluorescent proteins such as mCherry and mRFP were used. The number of fluorescently tagged endocytic proteins for cell biological studies is continuously increasing. Here, we provide a brief overview and highlight the most important examples of these proteins.

In most cases, these proteins were cloned under the control of the strong constitutive cauliflower mosaic virus promoter 35S. In order to minimize problems with silencing, variable expression, and expression bias, the ubiquitin promoter UBQ10 was also used for the constitutive expression of some endocytic marker proteins (Geldner et al. 2009; Herberth et al. 2012). Nevertheless, there are also examples of gene cloning under their own native promoters as it was in the case of *GNOM* (Geldner et al. 2003; Miyazawa et al. 2009), *GNL1* and *GNL2* (Richter et al. 2011), *VAN3* (Naramoto et al. 2010), *BRI1* (Wang et al. 2005), *BAK1* (Chinchilla et al. 2007), *FLS2* (Robatzek et al. 2006), *SNARE* (Ebine et al. 2008; Zhang et al. 2011), *AP-3* δ and *AP-3* β (Zwiewka et al. 2011), *PIN1* (Benková et al. 2003), *PIN2* (Xu and Scheres 2005), *PIN3* (Friml et al. 2002a), *PIN7* (Blilou et al. 2005), *AUX1* (Swarup et al. 2004), *BOR1* (Takano et al. 2010), *DRP1A* (Konopka and Bednarek 2008a) and *SNX1* (Jaillais et al. 2006) genes. Importantly, some work focused on the preparation of molecular markers which proved to be very useful for visualization of endocytic compartments including GFP-tagged VHA1, RabA1d, RabA1f, RabA2, RabA3 for early endosomes/TGN and RabF2a, RabF2b, ESCRT for late endosomes/MVBs (Ueda et al. 2001, 2004; Voigt et al. 2005; Chow et al. 2008; Spitzer et al. 2009; Ovečka et al. 2010). Some of above-mentioned molecular markers for endocytic compartments were tagged also with YFP (YFP-RabA1e, YFP-RabF2a, YFP-RabD1 and YFP-RabD2) or with red fluorescent proteins as in the case of DsRed-FYVE, VHA1-RFP, ARA6-mRFP, mRFP-Ara7, VAN3-mRFP, DRP1A-mRFP1, mRFP-Rha1, and mCherry-RabF2a/Rha1 (Voigt et al. 2005; Dettmer et al. 2006; Miao et al. 2008; Geldner et al. 2009; Naramoto et al. 2009; Takano et al. 2010; Mravec et al. 2011) and were subsequently used for successful colocalization studies with other endocytic proteins tagged with GFP (e.g. Ueda et al. 2004; Voigt et al. 2005; Miao et al. 2008; Pinheiro et al. 2009).

In the last years, several plasma membrane proteins have been identified to be internalized to plant cells by endocytic pathways including clathrin-dependent and lipid raft-dependent endocytosis (Lam et al. 2007; Mayor and Pagano 2007; Müller et al. 2007; Doherty and McMahon 2009; Bassil et al. 2011; Chen et al. 2011; Kitakura et al. 2011; Reyes et al. 2011; Li et al. 2012; see also chapters by Li et al. and Bassil and Blumwald in this volume). Some of these proteins are crucial for the control of plant development, water, and ion homeostasis and for interactions of plants with pathogens.

Polar auxin transport is a fundamental process shaping the plant body and it is controlled primarily by plasma membrane localized auxin transporters such as PIN-FORMED1 (PINs) and AUXIN-RESISTANT1 (AUX1). These transporters are polarly organized in the plasma membranes of opposite cellular poles. Their turnover is regulated via endocytosis as revealed by dynamic studies using GFP/YFP-tagged PIN1, PIN2, PIN3, and AUX1 in diverse cell types (Swarup et al. 2004; Dhonukshe et al. 2007, 2008; Kleine-Vehn et al. 2008; Jelínková et al. 2010; Kitakura et al. 2011; see also chapter by Nodzinski et al. in this volume). PIN proteins and their polar subcellular localization at the plasma membrane determine the direction and rate of cellular export and intercellular transport of auxin

(Petrášek et al. 2006; Wisniewska et al. 2006). PIN proteins are constitutively recycled between the plasma membrane and endosomes (Geldner et al. 2003; Dhonukshe et al. 2007), aiming at polarity establishment and rapid polarity alterations during plant development and organogenesis (Kleine-Vehn et al. 2010). Endocytosis of PIN proteins is clathrin-dependent (Dhonukshe et al. 2007) while their vesicular recycling depends on an ARF-GEF protein called GNOM (Geldner et al. 2003). Moreover, the clathrin-dependent endocytosis of PINs is inhibited by auxin (Paciorek et al. 2005; Robert et al. 2010). AUX1-YFP polar localization and subcellular trafficking, unlike PIN1 dynamics, is independent of GNOM but it is sensitive to sterol disruption. Thus, AUX1 and PINs seem to use different trafficking pathways in plants (Kleine-Vehn et al. 2006). Further, it was shown that PIN transcytosis occurs by endocytic recycling and alternative recruitment of the same cargo molecules by apical and basal sides of polarized cells (Kleine-Vehn et al. 2008).

Fluorescently tagged (with GFP) plasma membrane aquaporin PIP2.1 can be internalized to plant cells by two alternative pathways, including a classical clathrin-dependent pathway, or a lipid raft-associated pathway employed during salt stress, as recently shown by GFP-tagged PIP2.1 in combination with FRAP technology (Mongrand et al. 2004; Morel et al. 2006; Li et al. 2011; Luu et al. 2012). Thus, PIP2.1 might be involved in the multiple modes of regulating water permeability through the dynamic heterogeneous distribution in the plasma membrane and recycling pathways (Li et al. 2011).

Plant brassinosteroids (BR) are recognized at the plasma membrane by the receptor called BRASSINOSTEROID INSENSITIVE 1 (BRI1) belonging to transmembrane serine/threonine protein kinases. Interestingly, GFP-tagged BRI1 localizes to the plasma membrane and to the endosomes of Arabidopsis root cells independently of brassinosteroid treatment (Geldner et al. 2007; Irani et al. 2012). Another plasma membrane localized receptor kinase involved in perception and signalling of bacterial flagellin during plant–pathogen interactions is FLAGELLIN SENSITIVE2 (FLS2) (Robatzek et al. 2006). Ligand-activated GFP-FLS2 moves to endosomes and it is further sorted to the vacuole for lytic degradation, depending on ubiquitination and phosphorylation of this receptor (Robatzek et al. 2006). It is not clear yet, whether signalling of flg22-activated FLS2 is associated with plasma membrane or rather with endosomes.

Other molecular components essential for internalization of plasma membrane proteins, such as clathrin and dynamin, were studied in plant cells using GFP recombinant technology. The large GTPase dynamin is required for scission of CCVs. Dynamics of clathrin foci as revealed by fluorescently tagged clathrin light chain CLC-GFP at the plasma membrane was studied by using variable-angle epifluorescence microscopy (VAEM) (Konopka and Bednarek, 2008b). Furthermore, it was correlated with the dynamics of two dynamins, DRP1A and DRP1C, by dual color labeling and live cell imaging. DRP1C-GFP colocalized with a clathrin light chain fluorescent fusion protein (CLC-FFP) in dynamic foci which depend on functional clathrin-mediated endocytosis, cytoplasmic streaming and cytoskeleton (Konopka et al. 2008). Recently, it was shown that clathrin plays a

fundamental role in plant cell polarity, growth, patterning, and organogenesis (Kitakura et al. 2011).

Several proteins such as epsin, auxilin, synaptojanin, synaptotagmin and annexin are positioned at the interface between clathrin coats and the cytoskeleton (Šamaj et al. 2004) during clathrin mediated endocytosis in yeast, animals and likely also in plants. So far, epsin-homology domain proteins EHD1 and EHD2 were identified in plants. They were visualized as GFP-tags and implicated in endocytosis (Bar et al. 2008).

Boron and iron transporters as well as Na^+/H^+ antiporters were also tagged with GFP and their internalization via endocytosis was visualized using CLSM (Takano et al. 2010; Bassil et al. 2011; Yoshinari et al. 2012).

Except for the aforementioned plasma membrane localized and associated proteins, also proteins regulating vesicular trafficking, associated with early and late endosomes represented by TGN and MVB/PVC compartments, were extensively studied in plant cells using recombinant GFP technology. Among these proteins several small Rab GTPases tagged with GFP, YFP, or mRFP such as RabA4b, RabA1d, RabA1e, RabA1f, RabA2 were localized to TGN (Preuss et al. 2004, 2006; Chow et al. 2008; Ovečka et al. 2010) while others such as RabF2a and RabF2b were rather localized to MVBs (Haas et al. 2007).

Further, secretory carrier membrane proteins (SCAMPs) tagged with GFP, YFP, or RFP are localized to the plasma membrane and TGN/early endosome, whereas VSRs tagged with GFP mediate the sorting of soluble vacuolar cargo molecules, and they are localized to the MVBs and vacuole of pollen tubes (Lam et al. 2007; Wang et al. 2010).

The retromer, a multiprotein complex, is involved in the recycling of transmembrane VSRs from late endosomes/MVBs to the TGN. VSRs mediate the transport of vacuolar/lysosomal hydrolases from TGN to the lytic compartments (lysosome/vacuole). It was shown that GFP-tagged VSRs are localized preferentially to MVBs in tobacco BY-2 cells and in Arabidopsis suspension culture cells (Miao et al. 2006, 2008; Oliviusson et al. 2006). In addition to retromer and VSRs, sorting nexins have also been studied in plant cells with fluorescent-tagging technology (Jaillais et al. 2006, 2008).

Another protein complex essential for the formation of internal vesicles in MVBs is the endosomal sorting complex required for transport (ESCRT). Using GFP technology ESCRT components ELC and VPS23 were shown to colocalize with MVB markers (Spitzer et al. 2006, 2009; Richardson et al. 2011; see also chapter by Otegui et al. in this volume).

An alternative method to visualize endosomes is to use GFP/RFP-tagged specific peptide domains such as FYVE which binds to phosphoinositol 3-phosphate enriched in the endosomal membranes (Gaullier et al. 1998; Voigt et al. 2005; Veermer et al. 2006). It is likely that this molecular marker binds to two populations of endosomes, likely representing early endosomes/TGN and late endosomes/MVBs in different cell types (Bar et al. 2008; Salomon et al. 2010).

3.2 Molecular Interactions Between Endocytic Proteins

Endocytosis is a complex process depending on highly regulated interactions between diverse types of molecules, e.g. ligand-receptor, protein–protein, and protein-lipid interactions. Increased internalization of brassinosteroid receptor BRI1 was observed in protoplasts as a consequence of coexpression of BRI1 together with its co-receptor BAK1. It was proposed that BAK1 regulates BRI1 endocytosis and trafficking (Russinova et al. 2004). In vivo, BAK1 can form a complex with FLS2 in a ligand-dependent manner (Chinchilla et al. 2007). It was shown in Arabidopsis protoplasts that epsin1, a homolog of epsin, binds and interacts with clathrin, AP-1, vacuolar sorting receptor1 (VSR1) and VTI11, and it is involved in the vacuolar trafficking of soluble proteins at the TGN (Song et al. 2006). Further, EHD2 is essential for endocytosis of the plasma membrane receptor LeEix2 tagged with GFP because binding of the coiled-coil region of EHD2 to the cytoplasmic domain of the LeEix2 receptor is required for inhibition of receptor internalization and signalling (Bar and Avni 2009; Bar et al. 2009). PIN polarity and transcytosis are regulated by Ser/Thr protein kinase PINOID and protein phosphatase 2A in the pathway which is independent of GNOM (Kleine-Vehn et al. 2009).

Clathrin and adaptor proteins (AP) form complexes, which are associated with clathrin-coated pits (CCPs) at the plasma membrane and with clathrin-coated vesicles (CCVs) during their budding. APs are recruited to CCPs and CCVs from the cytosol and they are necessary for further recruitment of clathrin and cargos to CCPs and CCVs. AP2 complex has a role in trafficking from the plasma membrane to TGN while AP1 complex takes part on clathrin-dependent endosomal sorting at the level of TGN/early endosome (Drake et al. 2000). Recently, it was proposed that AP3 complex likely functions as a clathrin adaptor complex and plays a role in protein sorting at the TGN and/or endosomes in plants (Zwiewka et al. 2011).

One major class of proteins associated with vesicular trafficking are SNARE proteins. Recently, RabF1/Ara6 was found to interact with SNARE and to regulate complex formation at the plasma membrane (Ebine et al. 2011).

4 Genetic Methods

Several genetic approaches such as forward genetics on ethyl methane sulfonate (EMS)-induced mutants, reverse genetics on knock-out T-DNA insertional mutants as well as site-directed mutagenesis of functional sites in proteins undergoing or regulating endocytosis was used for functional studies on these proteins. Here, we selected some examples of these genetic approaches.

Most functional analyses on these proteins were likely performed on knockout T-DNA insertional mutants. For example, single, double, and triple knock-out *pin1*, *pin2*, *pin3*, *pin4*, *pin7* mutants as well as single *aux1* mutant in auxin

transporters were used to study their function and complex regulation of auxin distribution in the Arabidopsis root (Friml et al. 2002a, b; Benková et al. 2003; Friml et al. 2003; Swarup et al. 2004; Blilou et al. 2005). Knockout mutants *ara6, ara7, rha1*, (Haas et al. 2007; Ebine et al. 2011) were used to study biological functions of small Rab GTPases and together with *skd1* mutant also to study biogenesis of MVBs (Haas et al. 2007). Other mutants such as *vamp72* and *syp22* were used to study biological functions of plant SNARE proteins in endocytosis, seed development as well as their functional link with Rab GTPase Ara6 (Ebine et al. 2008, 2011). Next, *bak1* (Chinchilla et al. 2007), *epsin1* (Song et al. 2006), *snx2b* (Phan et al. 2008), *van3* (Naramoto et al. 2010), and *trs120* (Thelmann et al. 2010) carrying T-DNA insertions disrupting functions of corresponding genes helped to determine roles of these key endocytic protein players. The double *chmp1a chmp1b* mutant in ESCRT-related Charged MVB Proteins show MVBs with less lumenal vesicles, mislocalization of auxin transporters, and defects in embryo polarity establishment (Spitzer et al. 2009). Recently, the *chc1*, *chc2* knockout mutants together with dominant-negative CHC1 (HUB) transgenic lines, all defective in clathrin heavy chain, showed aberrant endocytosis of PINs leading to auxin transport-related phenotypes (Kitakura et al. 2011).

Another set of mutants were point mutants obtained by classical EMS-induced mutagenesis. Among these mutants were $gnom^{R5}$ (Geldner et al. 2003), *aux1* (Swarup et al. 2004), and *bor1-1* (boron transporter, Noguchi et al. 1997), again revealing the crucial role of mutated proteins in the plant endocytosis. Additionally, fluorescence imaging-based screen of an EMS-mutated plant population identified the *ben1* mutant, shown by forward genetics to be an ARF-GEF protein called BIG, which is involved in early endocytosis of plasma membrane proteins (Tanaka et al. 2009). A similar approach led to the identification of *pat2* and *pat4* mutants, defective in AP necessary for biogenesis of vacuoles (Feraru et al. 2010; Zwiewka et al. 2011).

Site-directed mutagenesis of functional amino acid residues in endocytic proteins was also used to study function of these proteins in plant endocytosis. Point mutations generating the amino acid substitutions Ser-26 to Asn (S26N), Gln-71 to Leu (Q71L), and Asn-125 to Ile (N125I) were created to study biological functions of RabA2 and RabA3 localizing to TGN during cell plate formation (Chow et al. 2008). Similar artificial locking of Rab and Arf GTPases in either GTP (constitutive active membrane-bound form), or GDP (inactive/dominant-negative cytoplasmic form) state represents a powerful and widely used tool to study biological function of these proteins (Xu and Scheres 2005; Dhonukshe et al. 2006; Chow et al. 2008; Nielsen et al. 2008; Böhlenius et al. 2010; Ebine et al. 2011). Site-directed mutagenesis was also used for elucidation of the ubiquitination of plasma membrane proteins for internalization and vacuolar trafficking (Herberth et al. 2012) as well as to study functional relevance of some domains in auxin influx carrier AUX1 (Yang et al. 2006), brassinosteroid receptor BRI1 (Wang et al. 2005), inositol transporters INT1 and INT4 (Wolfenstetter et al. 2012), and epsin homology domain protein EHD2 (Bar et al. 2009).

5 Electrophysiological Methods

Electrophysiological approaches to study exo- and endocytosis were mostly based on patch-clamp capacitance measurements. Capacitance measurements take advantage of the fact that exo- and endocytosis are associated with changes in plasma membrane area leading to proportional changes in the electrical membrane capacitance (Bandmann et al. 2011). As the membrane capacitance is proportional to the membrane area, the surface area and thus the diameter of the vesicle can be determined from the vesicle capacitance. The observed different kinetics of single-vesicle membrane capacitance could be grouped into four different categories, representing a variable behavior with respect to transient or permanent fusion and fission of vesicles. Using capacitance measurements, it is also possible to estimate the diameter of the vesicles. Real-time patch-clamp recordings were undertaken to monitor single-vesicle fusion and fission in order to resolve the kinetic properties of the release and incorporation of the secretory cargo to the plasma membrane (Thiel et al. 2009). The data show that single vesicles can, in a rhythmic fashion, make and break contact with the plasma membrane of plant protoplasts. Such oscillations are only possible if the two processes are linked by a distinct feedback system.

Recently, patch-clamp capacitance measurements were applied for the investigation of glucose uptake in BY-2 cells (Bandmann and Homann 2012). In the presence of glucose a strong decrease in the number of fusion events and transient fission events was recorded, while the frequency of permanent endocytic events increased fourfold (Bandmann and Homann 2012). The inhibition of clathrin coat formation by ikarugamycin did not prevent the stimulatory effect of glucose on endocytosis indicating the clathrin-independent endocytosis of glucose in BY-2 cells.

6 Vital Fluorescent Markers for Endocytosis

6.1 Fluorescently Labeled Endocytic Cargo

The existence of distinct fluid phase endocytosis (FPE) in plant was proved using Na-dependent fluorescent marker Coro-Na (membrane-impermeable form) and membrane marker FM 4-64 (Etxeberria et al. 2009). Recently it was shown that FPE is clathrin-independent (Onelli et al. 2008; Bandmann and Homann 2012). Using inhibitors of CME, uptake of fluorescent-labeled glucose derivative 2-NBDG [2(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] was not affected (Bandmann and Homann 2012). Further evidence for FPE independence on CME is the demonstration that GFP-Flot1 does not colocalize with CLC-mOrange and also TyrA23 (tyrphostin A23, an inhibitor of endocytotic cargo recruitment in clathrin-mediated endocytosis) did not affect GFP-Flot1 movement (Li et al. 2012). Very

recently, the fluorescently labeled endocytotic marker FITC-BSA was used to discover and describe the endocytic route of nutrient uptake in diverse carnivorous plants (Adlassnig et al. 2012).

6.2 Vital FM Styryl Dyes

Fluorescent FM dyes, water-soluble lipophilic styryl compounds, are virtually nonfluorescent in aqueous media but turn to be fluorescent following incorporation to the outer leaflet of the plasma membrane. The plasma membrane incorporation of FM dyes and their subsequent endocytic internalization and distribution among endomembranes appointed them a crucial role in endocytic trafficking visualization in living plant cells. The most widely used FM dyes in plant cell biology are FM 4-64 and FM 1-43 which were successfully used as live endocytic tracers not only in different fungal and plant cells including fungal hyphae, *Chara* internodal cells, pollen tubes, root epidermal cells and root hairs, stomata, leaf epidermal cells, but also in protoplasts and tobacco BY-2 suspension cells (Ovečka et al. 2005; Voigt et al. 2005; Xu and Scheres 2005; Sousa et al. 2008; Zonia and Munnik 2008; Scheuring et al. 2011). Studies based on FM4-64 uptake experiments combined with colocalization of this dye with TGN markers revealed that TGN acts as an early endosome, receiving first endocytosed material from the plasma membrane (Dettmer et al. 2006; Lam et al. 2007, 2009; Jaillais et al. 2008). FM 4-64 was also microinjected directly into the cytosol of BY-2 cells and *Tradescantia virginiana* stamen hair cells (van Gisbergen et al. 2008). Additionally, advanced CLSM technique based on FRET measurements comparing membrane-bound FM 4-64 with cytoplasmic GFP also showed that the dye is taken up into cells preferentially by endocytosis (Griffing 2008). Recently, it was shown that FM 4-64 and FM 5-95 in tobacco BY-2 and Arabidopsis cell suspensions initialize transient re-localization of auxin carriers, and FM 1-43 affects their activity. Re-localization was not blocked by inhibitors of endocytosis or cytoskeletal drugs. However, no changes in localization of auxin carriers were observed in Arabidopsis root epidermis and cortex cells labeled with FM dyes (Jelínková et al. 2010).

6.3 Filipin and di-4-ANEPPDHQ

The antibiotic filipin is a polyene fluorochrome that binds to structural sterols. Since filipin has fluorescent properties it can serve as a vital probe for in vivo visualization of structural sterols in the plasma membrane and endosomes (Grebe et al. 2003; Kleine-Vehn et al. 2006; Liu et al. 2009; Ovečka et al. 2010; Boutté et al. 2011). Additionally, sterols complexed by filipin can be visualized at the ultrastructural level (Ovečka et al. 2010). Filipin was used to label structural

sterols in the plasma membrane and to study their internalization and endosomal trafficking in epidermal cells of intact Arabidopsis roots (Grebe et al. 2003) and in root hairs (Ovečka et al. 2010). Early endocytic trafficking of structural sterols seems to be actin dependent and BFA-sensitive, and might involve endosomes enriched with RabF1/Ara6 (Grebe et al. 2003). Another fluorescent dye visualizing structural sterols is di-4-ANEPPDHQ (Liu et al. 2009). Recently, it was shown that phosphatidyl-inositol-3-phosphate (PI3P) is abundant on the outer surface of plant cell plasma membranes and it mediated pathogen effector proteins entry involving lipid raft-mediated endocytosis (Kale et al. 2010). The inhibition of the accumulation of effector proteins was achieved using wortmannin (inhibitor of PI3P biosynthesis and clathrin-mediated endocytosis) and filipin and nystatin (inhibitors of lipid raft-mediated endocytosis). This observation suggested that the disruption of lipid rafts impaired the distribution of PI3P on the outer surface of plasma membrane (Kale et al. 2010).

6.4 Labeled Ligands for Signalling Receptors

Very recently, a fluorescently labelled brassinosteroid analog (castasterone coupled to Alexa Fluor 647) was used as a ligand for GFP-tagged BRI1 (Irani et al. 2012). This study, in contrary to a previous report regarding BRI signalling from endosomes (Geldner et al. 2007), revealed that BRI1 signalling is actually restricted to the plasma membrane, while BRI1 internalization to endosomes leads to the attenuation of castasterone-induced signalling (see also chapter by Di Rubbo and Russinova in this volume).

7 Immunolocalization Methods for In Situ Localization of Endosomal Proteins

Immunolocalization with specific antibodies raised against endocytic proteins is a very reliable technique which can considerably support and strengthen results obtained by fluorescent GFP/YFP/RFP-tagging of these proteins. For more robust species such as maize, a technique based on embedding in Steedman wax and subsequent immunolocalization on dewaxed semithin sections is a common and useful method of choice (Vitha et al. 2000a, b; Baluška et al. 2004). In Arabidopsis seedlings and suspension BY-2 cells, the whole mount immunolocalization was the technique mostly used so far (Hejátko et al. 2006; Szechyńska-Hebda et al. 2006). The main advantage of immunolocalization techniques is that immunofluorescence data obtained on lower resolution can be correlated with high-resolution localization using immunogold electron microscopy (EM). The latter technique allows the unambiguous identification of proteins associated with

endocytotic uptake as well as with early and late endosomes. Thus, clathrin was localized at the plasma membrane (Dhonukshe et al. 2007; Müller et al. 2010), VHA and Rab GTPases were localized to TGN (Nielsen et al. 2008) while RabF2a and RabF2b were localized to MVBs (Haase et al. 2007) by immunogold EM.

Specific antibodies against PIN1 (Gälweiler et al. 1998; Benková et al. 2003), PIN2 (Müller et al. 1998), Ara6 and Ara7 (Ueda et al. 2001) were used in Arabidopsis and anti-VSR (Miao et al. 2006, 2008) in BY-2 cells for subcellular localization of corresponding proteins by using immunolocalization methods. Moreover, immunolocalization with specific antibodies against SCAMP1 and VSR revealed that SCAMP is localized to apical endocytic vesicles, while VSRs are localized to the MVB and vacuole in lily pollen tubes (Wang et al. 2010).

Very recently, immunogold EM helped to identify fusions of the PVC/MVB (including internal MVB vesicles) with vacuole. Thus, cargo ubiquitination-independent and PVC-mediated degradation of plasma membrane proteins in the vacuole was proposed for plant cells (Cai et al. 2012).

8 Microscopic Methods

The progress in microscopic imaging in the past few years, involving for example high-throughput analyses, or sophisticated new instrumentation, such as light sheet and super-resolution microscopy, brought novel analytic tools, also for the investigation of proteins undergoing or regulating endocytosis.

8.1 Identification of New Endocytic Proteins by High-Throughput Microscopy Screen

A high-throughput fluorescence imaging uses epifluorescence or confocal microscopy to screen tissues or cells for specific phenotype. The exact targeting (visualization) of specific vesicular transport processes requires fluorescence-tagged proteins, which selectively localize to certain organelles, or take part in specific processes. A fluorescence imaging-based forward genetic screen, was performed on EMS-mutagenized *PIN1pro::PIN1-GFP* Arabidopsis transgenic plants treated with BFA by using epifluorescence microscopy (Tanaka et al. 2009). Arabidopsis mutants that do not efficiently internalize and/or accumulate PIN1-GFP in the BFA compartments were identified in this search. Using this approach, three mutant loci, *BFA-visualized endocytic trafficking defective1* (*ben1*), *ben2* and *ben3* were identified. Fine mapping revealed that *BEN1* encodes an ARF-GEF vesicle trafficking regulator belonging to the BIG class. Further detailed study suggested that this ARF-GEF is involved in endocytosis of plasma membrane proteins and localizes to early endocytic compartments distinct from GNOM-positive recycling endosomes.

In a similar study, EMS mutagenized *PIN1pro::PIN1-GFP* Arabidopsis transgenic plants were screened for aberrant PIN1-GFP distribution resulting in the detection of several *protein-affected trafficking* (*pat*) mutants (Feraru et al. 2010). It was found that *pat2* is specifically defective in the biogenesis, identity, and function of lytic vacuoles but shows normal sorting of proteins to storage vacuoles. *PAT2* encodes a putative b-subunit of adaptor protein complex 3 (AP-3) (Feraru et al. 2010). High-throughput confocal microscopy was carried out also to analyze the quantitative differences between distinct endomembrane vesicles in leaf epidermal tissue and to find differences in the quantity of GFP-2xFYVE in endosomal compartments upon exposure of Arabidopsis plants to biotic or abiotic stresses (Salomon et al. 2010). Toward this goal, a spinning-disk microscope enabling the observation of samples in multi-well plates was employed and marked differences in the quantity of endomembrane compartments were found in leaf cells. Moreover, substantial increase in the number of GFP-2xFYVE compartments after biotic and cold stress was observed, whereas dark caused decreased numbers of these endosomes.

8.2 Confocal Laser Scanning Microscopy

CLSM became the most common and valuable method in endocytic investigations being a platform for visualization of fluorescently labeled endocytotic markers or proteins fused with fluorescent tags. For example it was widely used for defining the colocalization of endocytic proteins with the membrane tracker FM4-64 (Ito et al. 2012) and for colocalizations of clathrin, SNXs, VSR2, AAA ATPase Vps4p/SKD1, AtVSR reporters, and storage vacuolar cargo with endocytic markers (Haas et al. 2007; Miao et al. 2008; Foresti et al. 2010; Niemes et al. 2010; Pourcher et al. 2010; Ito et al. 2012). CLSM was also used for the detection of the sensitivity of PIN1, GNOM, SCAMP1, SNX, selected Rab GTPases, and other proteins to vesicular trafficking drugs such as BFA, concanavalin A and wortmannin (Geldner et al. 2003; Dettmer et al. 2006; Lam et al. 2007; Jaillais et al. 2008; Takáč et al. 2011a; Takáč et al. 2012; Fig. 2). The dynamics of vesicles and visualization of zones of exocytosis and endocytosis in tobacco pollen tubes was performed by pulse-chase observations of endocytic tracker FM4-64 and FM1-43 using CLSM (Zonia and Munnik 2008). For the observations of highly dynamic endosomal systems, a spinning-disk confocal time-lapse microscopy proved to be a promising tool (Nakano 2002). It is capable for high-speed sequential acquisition and significantly minimizes sample bleaching. For example, EYFP-RabA4d in growing pollen tubes was monitored by this imaging system (Szumlanski and Nielsen 2009). It also helped to identify TGN/EE as a dynamic and independent compartment which only temporarily moves together with Golgi (Viotti et al. 2010). It was also used for high-throughput CLSM screen using FYVE-labeled endosomes (Salomon et al. 2010).

In conventional CLSM, the laser beam is focused through the tissue whereas in total internal reflection fluorescence microscopy (TIRFM) the laser beam is reflected off the surface at a critical angle of surface plasmon generation (Sparkes et al. 2011).

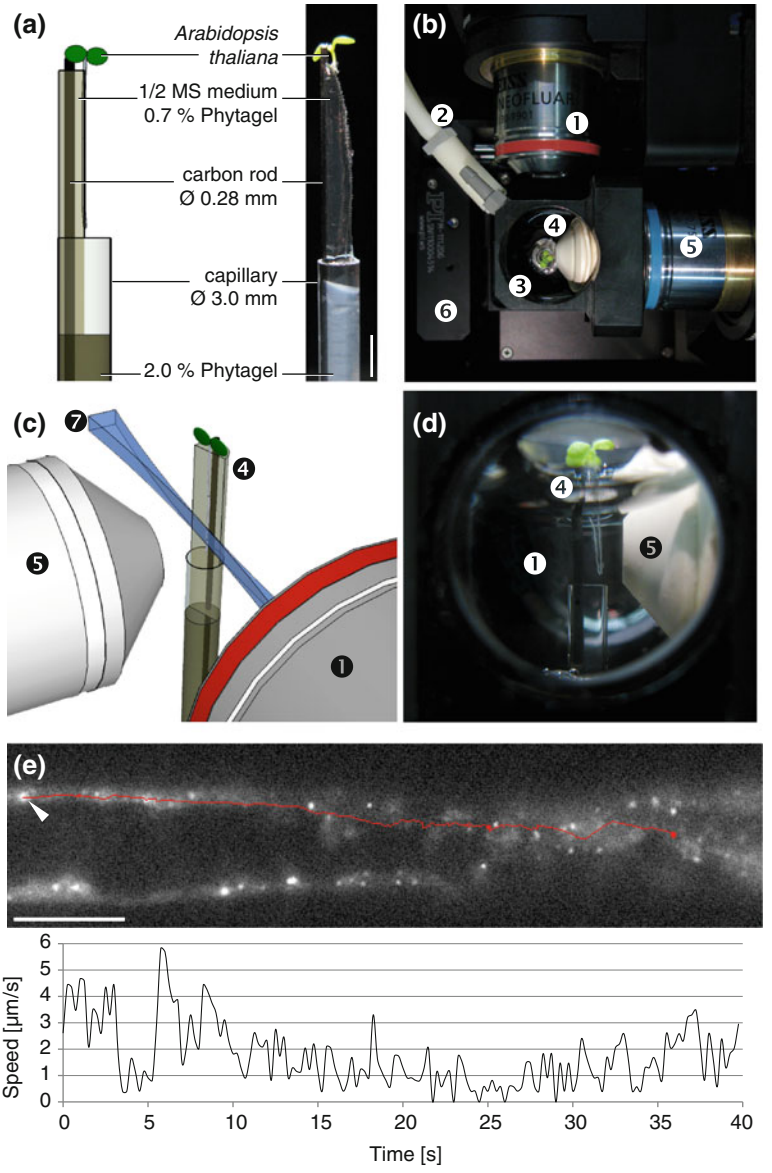
It is valuable mostly for the visualization of processes in the close proximity of plasma membrane as it was shown to monitor the behavior of FM-labeled vesicles in growing pollen tubes (Wang et al. 2009), GFP-tagged clathrin, dynamin, and flotillin at the plasma membrane (Konopka and Bednarek 2008a; Li et al. 2012) or GFP and GDP locked versions of small RabGTPase Ara6 (Ebine et al. 2011).

8.3 Light Sheet-Based Fluorescence Microscopy

Live microscopic imaging is one of the major tools in experiments targeted at understanding the development of organisms. The primary constraint in all such experiments is to maintain the specimen at near-physiological conditions. This requires minimally invasive imaging methods with sufficient spatial resolution and the temporal capability to capture the biological processes on site. Processes such as ion dynamics, cytoskeletal reorganization, cell division, cytokinesis, and intracellular trafficking are completed within seconds to minutes, and, therefore, require short imaging intervals but a high spatial resolution. Capturing and tracking endosomes that move at a speed of several μm/sec is one of the most challenging efforts. Since plants scatter and absorb light well, they have to be exposed to relatively strong light fluencies to ensure a proper illumination and the detection of signals across their entire volume. This potentially results in heating and photo-toxicity problems, which can induce the malfunction and finally the death of the plant. Therefore, if the experimental measurements are to represent a normal development, the specimen must be maintained at an appropriate physiological condition, and must remain accessible for microscopic observation at high image acquisition rates.

Conventional microscopy and CLSM usually employ the same objective lens for both fluorescence excitation and detection. Confocal theta fluorescence microscopy (Stelzer and Lindek 1994) introduced the systematic use of at least two separate lenses for illumination and detection. In such a system the optical axes are arranged orthogonally, improving the axial resolution and resulting in an almost spherical point spread function. The wide-field implementation of the theta principle, illuminates the specimen throughout an entire plane with a light sheet and collects the emitted light at a perpendicular axis. The use of light sheets for imaging purposes has been known for more than one hundred years and their use has been suggested for macroscopic imaging (e.g. Voie et al. 1993), but the applicability for high-resolution light microscopy was not realized until a few years ago (Huisken et al. 2004). Almost all theoretical aspects (single/multiple photons, circular and annular apertures) have been covered in a series of papers published by Stelzer and Lindek (Lindek and Stelzer 1996; Lindek et al. 1996a, b) and have been picked up and further developed by many other authors (e.g., Sätzler and Eils 1997).

However, in light sheet-based fluorescence microscopy (LSFM), optical sectioning arises from the overlap between the focal plane of the detection system and the central plane of a light sheet. The thickness of the light sheet is similar to, and in many instances even thinner than the depth of field of the detection system,



which means that only fluorophores close to the focal plane of the detection system are excited and contribute to the image. It also means that illumination causes no photo damage either in front or behind the focal plane. Therefore, LSFM exposes a specimen to 200 times less energy than a conventional and up to 5,000 times less energy than a confocal fluorescence microscope (Keller and Stelzer 2008). Importantly, LSFM takes advantage of state-of-the-art scientific cameras (e.g.,

◀ **Fig. 3** *Arabidopsis thaliana* in the monolithic digital scanned laser light sheet-based fluorescence microscope (mDSLM). The plant grows in an upright position in the specimen chamber. While the leaves remain in the air, the root system is perfused with liquid half-strength MS medium. Only the fluorophores in a thin planar volume that overlaps with the focal plane of the detection lens are excited. Thus, fluorophores outside this volume do not blur the image and are not subject to photo bleaching. Four motors below the chamber move the plant along x/y/z and rotate it around the vertical axis, which is orthogonal to the detection axis. **a** Sample holder for microscopy of upright plants. *A. thaliana* seedlings grow in a capillary on the surface of a vertically positioned ½ MS medium containing 0.7 % Phytagel. For the image acquisition process, the phytagel cylinder is extruded from the capillary, which is rigidified by an embedded carbon rod. **b** Top view of the specimen chamber. **c** 3D model of the sample in front of the objective lens. **d** Side-view into the chamber with the root in front of the detection lens. Legend: *1* illumination objective lens, *2* perfusion system, *3* specimen chamber, *4 A.thaliana* in capillary, *5* detection objective lens, *6* microscope stage, *7* laser light sheet. **e** The initial image of a single plane time-lapse recording of an *A. thaliana* root hair. The 6-day-old plant expresses the late endosome marker YFP-RabF2a (Geldner et al. 2009). The *red line* indicates the track of a single endosome during a 40-second-long time frame. **d** The endosome speed over time. Images were recorded at a rate of 4 fps for a 40 s stretch with the pair EC Plan-Neofluar 5×/0.16 and N-Apochromat 63×/1.0 W. Scale bar: **a** 3 mm, **e** 10 μm

CCD, EM-CCD, sCMOS, time-of-flight sensitive cameras) and easily records 100 frames per second (fps) with a dynamic range of 12–14 bits and image sizes exceeding 2,000 pixels by 2,000 lines. It typically achieves an isotropic resolution between 250 and 300 nm.

The first results of live imaging of growing multicellular plant structures with LSFM were published recently (Maizel et al. 2011; Sena et al. 2011; Fig. 3). Dynamics of the movement of subcellular organelles is a typical and fast event. We recorded the movements of endosomal compartments labeled by an YFP-RabF2a reporter (Geldner et al. 2009) and successfully tracked single endosomes.

The plant is placed vertically in a medium-filled chamber close to the common focal point of the two objective lenses. The root grows on the surface of a phytagel cylinder cast in a glass capillary. For the image acquisition process, the phytagel cylinder is extruded from the capillary, which is rigidified by an embedded carbon rod. A perfusion system ensures that the entire medium in the plant chamber is exchanged every 15 min. The plant is inserted from above but it is held from below. Thus, the opening remains accessible for diurnal illumination provided by a standard lamp and can be spectrally adjusted (Fig. 3).

8.4 Fluorescence Recovery After Photobleaching and Super-Resolution Microscopy

FRAP has many important applications, mainly for the investigation of the dynamics of plasma membrane located proteins and highly motile compartments such as endosome. It was also used to monitor vesicle fusion to the plasma membrane during exocytosis in growing pollen tubes (Lee et al. 2008b). The apical region of tobacco