Experimental toolbox for quantitative evaluation of clathrin-mediated endocytosis in the plant model *Arabidopsis*

Running Title: Plant CME imaging toolbox

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Keywords; quantitative imaging, clathrin-mediated endocytosis, metal-replica electron microscopy, total internal reflection fluorescence microscopy, confocal microscopy, *Arabidopsis*

Summary Statement: We present detailed novel quantitative imaging protocols and review pharmacological and genetic manipulation to provide standardized experimental approaches for the investigation of plant clathrin-mediated endocytosis at multiple scales.

Abstract

Clathrin-mediated endocytosis (CME) is a crucial cellular process implicated in many aspects of plant growth, development, intra- and inter-cellular signaling, nutrient uptake and pathogen defense. Despite these significant roles, little is known about the precise molecular details of how it functions *in planta*. In order to facilitate the direct quantitative study of plant CME, here we review current routinely used methods and present refined, standardized quantitative imaging protocols which allow the detailed characterization of CME at multiple scales in plant tissues. These include: (i) an efficient electron microscopy protocol for the imaging of *Arabidopsis* CME vesicles *in situ*, thus providing a method for the detailed characterization of the ultra-structure of clathrin-coated vesicles; (ii) a detailed protocol and analysis for quantitative live-cell fluorescence microscopy to precisely examine the temporal interplay of endocytosis components during single CME events; (iii) a semi-automated analysis to allow the quantitative characterization of global internalization of cargos in whole plant tissues; and (iv) an overview and validation of useful genetic and pharmacological tools to interrogate the molecular mechanisms and function of CME in intact plant samples.

Introduction

Clathrin-mediated endocytosis (CME) is a major mechanism by which plasma membrane (PM) and extracellular cargo, which include cell surface receptors or extracellular materials, are internalized into cells (Bitsikas et al., 2014, McMahon and Boucrot, 2011). It is a dynamic and highly regulated multi-step process requiring the vesicle coat protein clathrin, and a large number of distinct endocytosis accessory proteins (EAPs). EAPs are dynamically recruited to CME events on the PM at different time points to regulate each unique step of the CME reaction; such as cargo recruitment, membrane invagination, scission/severing of the clathrin coated vesicle (CCV) from the PM and their uncoating (Kaksonen and Roux, 2018). CME plays an important role in many physiological processes in plants ranging from growth and development, cell polarity, intra- and inter-cellular signaling, nutrient uptake, stress response and pathogen defense (Barberon et al., 2011, Dhonukshe et al., 2007, Di Rubbo et al., 2013, Kitakura et al., 2011, Martins et al., 2015, Mbengue et al., 2016, Ortiz-Morea et al., 2016, Yoshinari et al., 2016, Zwiewka et al., 2015). Despite its physiological significance, little is known about the molecular mechanisms of how it functions in plants, especially when compared to mammalian and yeast model systems (Kaksonen and Roux, 2018, Lu et al., 2016).

The great advances in of CME studies in mammalian and yeast fields over the past 40 years are mainly thanks to key imaging technologies that have emerged as standard approaches allowing the direct quantitative characterization of CME at very high spatial and temporal resolutions (Kaksonen and Roux, 2018, Lu et al., 2016, Picco and Kaksonen, 2018, Robinson, 2015, Schmid, 2019, Sochacki and Taraska, 2019). For example, electron microscopy (EM) approaches have allowed the direct visualization of the clathrin coat itself (Fotin et al., 2004, Heuser, 1980) and live imaging of single events of CME on the cell surface have unraveled the complex temporal network of EAPs in live cells (Taylor et al., 2011). Importantly, the quantitative analysis systems combined with these imaging approaches are openly reported (Aguet et al., 2013, Loerke et al., 2009), thus providing standardized protocols making direct comparisons of experimental data from multiple groups possible. Despite the plant field lagging behind in characterization of this key process, in recent years there has been significant progress in the identification of evolutionarily conserved and plant-specific EAPs, and the understanding of their regulation, which have evolved to meet the unique requirements necessary for plant morphogenesis and growth (Adamowski et al., 2018, Barberon et al., 2011, Bashline et al., 2013, Beck et al., 2012,

Dhonukshe et al., 2007, Fan et al., 2013, Gadeyne et al., 2014, Gifford et al., 2005, Kim et al., 2013, Konopka et al., 2008, Martins et al., 2015, Mazur et al., 2020, Paciorek et al., 2005, Sharfman et al., 2011, Takano et al., 2005, Yoshinari et al., 2016, Zhou et al., 2018). While many of these studies have been driven by the application and optimization imaging protocols of endocytosis, there is a need to standardize and approaches to directly examining plant CME which will allow direct comparison of data from differing groups possible, thus improving our ability to work together to characterize a fundamental physiological process.

A major approach to characterize and identify *bona fide* plant EAPs, and thus unravel the mechanisms of plant CME, has been the use of biochemical methods such as pull-down assays coupled with mass spectrometry and *in vitro* binding studies. For example, using clathrin light chain 1 (CLC1) as bait, the potential uncoating factor Auxilin-like protein was identified (Adamowski et al., 2018). Also, the identification of two major plant EAP complexes, the AP2 and TPLATE complexes, were facilitated using similar approaches (Di Rubbo et al., 2013, Gadeyne et al., 2014, Yamaoka et al., 2013). While these approaches serve as a good starting point in the aid of plant CME characterization, their drawback is that they offer limited insight into the dynamics of the interactions, which are critical in characterizing a dynamic multi-step process like CME; where each of these steps requires a different subset of EAPs (Merrifield and Kaksonen, 2014). Further to this, pull-down and *in vitro* binding studies may not be sufficient to detect many of the key functional CME interactions, as many of them are reported to be transient (Smith et al., 2017). Therefore, in order to precisely characterize plant CME, it is critical to directly observe and quantitatively assess CME *in vivo*. Indeed, the optimization and application of imaging and quantitative analysis protocols to visualize CME structures and dynamics *in vivo* has been important for recent advances in our understanding of the molecular mechanisms of plant CME (Fujimoto et al., 2010, Gadeyne et al., 2014, Ito et al., 2012, Johnson and Vert, 2017, Konopka and Bednarek, 2008, Narasimhan et al., 2020, Stefano et al., 2018, Tinevez et al., 2017, Vizcay-Barrena et al., 2011, Wan et al., 2011, Wang et al., 2015, Yamaoka et al., 2013, Yoshinari et al., 2016).

To enhance and facilitate further detailed characterization of plant CME, here we briefly review currently available approaches and analytical tools for the characterization of the process of CME in plant cells. We also present detailed state-of-the-art microscopy-based methods and guidelines for the quantitative, direct and dynamic examination of CME at

multiple scales and pharmacological and genetic manipulations of CME in intact *Arabidopsis* seedlings.

Methodologies for the imaging and analysis of plant CME

While there are many different imaging modalities to study CME, there are two major categories: electron microscopy (EM) and light microscopy. Each modality offers different strengths and weakness based on how they physically function (Figure 1), which allows one to investigate different aspects of CME (Supplemental Table 1).

EM permits the imaging of subcellular structures, organelles and macromolecular complexes with high spatial resolution, as spatial resolution up to ~4 nm can readily be achieved (de Jonge et al., 2009). These high spatial resolutions are made possible as electrons are defined by their higher energy state than photons, which are optically diffraction limited to resolutions ~200 nm. While there are many EM methods, the two classical EM approaches in life sciences are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). They differ in that in TEM imaging electrons are detected once they have passed through the sample, whereas SEM detects electrons that are scattered off the sample. As both these methods subject the sample to high-energy electron stimulation under high-vacuum conditions, they are not generally suitable for hydrated organic material with limited electron dense contrast. Therefore, the sample must be fixed, dehydrated and contrasted using heavy metals, and either embedded into a resin for ultrathin sectioning in conventional TEM analysis, or replicated in SEM analysis. Thus, there is limited temporal information provided from a sample and one must be careful about the possibility of artifacts produced during sample preparation (Supplemental table 1)

There are two main types of light microscopy modalities routinely used to study CME, total internal reflection fluorescence microscopy (TIRF-M) and confocal microscopy. Both offer the possibility of conducting real time imaging of biological samples. They differ critically in how they illuminate the sample, TIRF-M makes use of a weak evanescent light wave, generated when the illumination beam hits an interface between two media with different refractive indexes at the critical angle, to illuminate just a small volume of the sample, ~100 nm in the Z dimension (Figure 1) (Mattheyses et al., 2010) (Axelrod, 2001). Whereas, confocal microscopy uses 'pinhole' in the optical pathway to physically excludes light from out of focus sample planes, allowing researchers to optically section a sample or focus on a single Z plane of interest (Figure 1). Typically, confocal microscopy refers to

confocal laser scanning microscopy (CLSM); which is where a single point scans multiple ‘lines’ across the sample to acquire the whole image. This results in CLSM having a relatively slow acquisition time. To overcome this, spinning disk (SD) confocal systems can be used. Instead of using a single confocal point to scan the imaging area, a series of pinholes and micro lenses are spun in the optical pathway resulting in multiple confocal ‘points’ in the imaging area (Figure 1), significantly increasing the speed of acquisition.

Electron microscopy methods; characterization of CME at the ultra-structural level

When EM approaches are combined with protocols for the enrichment of CCVs from plant tissues (for detailed methods see (Mosesso et al., 2018, Reynolds et al., 2014)), one can begin to define the molecular anatomy of the plant CCV. The draw back to examining CCVs isolated from plant tissues is that the preparation could be a mixture of PM- and TGN-derived CCV populations. Therefore, being able to examine CCVs *in situ* allows the direct examination of CCVs during the CME process and allows us to address key CME questions; such as, is the polymerization of clathrin coordinated with the bending of the PM (Bucher et al., 2018, Scott et al., 2018).

Transmission electron microscopy

Transmission electron microscopy (TEM) imaging approaches are widely used in CME studies in other model systems (Sochacki and Taraska, 2019). Whole cells, or tissues, are fixed and embedded into a resin for ultrathin slicing (40-70 nm). These sections can then be imaged to provide information on a single plane through the sample, or serial sections can be aligned and composed to produce a 3D ultra-structural view of the cell. While TEM has been used routinely in plants, and CCVs are visible and detectable in plant samples (Bonnett and Newcomb, 1966, Dejonghe et al., 2016, Dhonukshe et al., 2007, Lam et al., 2001, Li et al., 2012, Safavian and Goring, 2013), the preservation of CCVs is incredibly low regardless of fixing or embedding methods used. This has made it extremely difficult to visualize enough CME events to provide a robust quantitative analysis of plant CCVs.

Unroofing metal replica

An alternative EM approach to analyzing CME events is the utilization of scanning electron microscopy (SEM) on ‘unroofed’ plant cells, which allows the PM and its associated CCVs to be visualized. Recently, this approach has been successfully optimized for *Arabidopsis* protoplasts from suspension cultured root cells (Narasimhan et al., 2020) and is presented in

detail in Expanded Method 1. In this method, cells are fixed to adherent coverslips and membranes not in direct contact to the coverslip are ripped away, thereby exposing intact intra-cellular structures attached to the PM; like CCVs undergoing CME (Figure 2). Therefore, this permits the specific examination of budding CME CCVs, as they can be identified by their presence on the PM.

This approach produces images with unprecedented numbers of CCVs in a plant cell *in situ* situation, compared to previous plant EM approaches, and allows the direct characterization of the ultrastructural details of the CCV; such as shape, size and stage of CCV formation (Supplemental Figure 1). Therefore, this method is applicable to testing the effects of chemical and genetic manipulations on the clathrin coat itself and the molecular structure of the formation of CME vesicles.

The drawback of this approach is that at present it requires the generation of protoplasts which involves the enzymatic removal of the cell wall. Therefore, it needs to be considered that the CME-derived CCVs in these cells are formed under different physiological conditions from those that form in cells surrounded by cell walls (i.e., turgor pressures and membrane tensions). Further to this, digestion of the cell wall has been shown to result in the intracellular aggregation of certain EAPs (Kang et al., 2003). However, the average CCV size closely matches that of biochemically purified CCVs from plant tissues (Mosesso et al., 2018, Reynolds et al., 2014), suggesting that the protoplast CCVs are forming in a similar fashion as those in tissues. Using the unroofing method, one is further limited to examining just the top view of the CCV, which means hallmark features of CME, like the highly curved neck of CCVs, are obstructed by the vesicle itself. Additionally, as the samples are fixed, there is limited temporal information about the CCVs examined.

Methods for directly imaging the cell surface; characterization of CME at the single event level

The cell surface is a major site of CME, therefore high-resolution imaging of just the PM allows the direct visualization of single events of CME (Figure 3). By combining the imaging of single events, which can be marked using established EAP plant lines (Supplemental Table 2), with high-throughput analysis protocols, we can define the precise temporal characteristics of plant CME (Figures 3, 4 and 5); and is presented in 'Expanded Method 2'.

The methods presented in ‘Expanded Method 2’ uses an unbiased automated high-throughput analysis system to quantitatively analyze cell surface imaging data. Specifically, we make use of the detection and tracking components of the cmeAnalysis package (Aguet et al., 2013) and further processed the data with our own scripts (see ‘Expanded Method 2’, Note 7). This is because the processing step in the cmeAnalysis package, which is used to define *bona fide* CME events, is optimized for mammalian systems and fails to accurately identify the plant *bona fide* CME events (Johnson and Vert, 2017). This approach provides several significant advantages over manual methods for detection and quantitation of time lapse image sequences of CME events in plant cells. For example, the analysis is based on the parameters of the experimental setup and each detection is statistically tested removing subjective human input/bias (Aguet et al., 2013), the number of events analyzed by the described automated workflows are far larger than can be readily achieved by manual tracking thus giving them greater statistical significance and the reproducibility of results.

Single Channel EAP cell surface analysis

Live cell imaging of the PM in samples expressing fluorescent protein tagged EAPs, combined with automated unbiased detection, tracking and analysis provides key quantifiable physiological metrics to enable the analysis of the dynamics of plant CME. For example, live TIRF-M of clathrin light chain 2 (CLC2) and quantification using our automated single channel analysis (Figure 4A-F) (Expanded Method 2) gives the lifetimes (Figures 4G and H), density (Figure 4I) and fluorescent intensity profiles of CLC2 labeled clathrin coated pits (Figures 4F and J). The lifetimes of clathrin and other EAPs on the cell surface can provide information about the overall kinetics of CME. The density of EAPs is informative about the overall amount of CME occurring in a region of interest within the cell. The mean fluorescent profile of cell surface EAPs can provide clues of their functions; as CME is a reaction where CLC polymerize on a budding vesicle on the PM until freed from the PM, therefore one can expect its fluorescence profile to reflect this process (Figures 3B,C, 4F and J).

A major issue with single channel cell surface imaging data is that you are measuring the total population of your marker protein on, or near, the cell surface. Therefore, the results will include information about additional cellular processes that affect the lifetime and density of proteins at the PM, including its *de novo* synthesis, trafficking and recycling/degradation. If single channel images are used to assess the kinetics of CME, the results should be validated by population modeling (Loerke et al., 2009) and, or, by dual

channel imaging of *bona fide* CME marker proteins together with the protein of interest (Narasimhan et al., 2020). Once it has been established that a protein of interest functions in CME, single channel cell surface imaging can be utilized to test the effect of pharmacological and genetic manipulations on its recruitment and kinetics in CME (Supplemental Tables 3 and 4).

Dual Channel EAP cell surface analysis

To overcome the limitations of single channel cell surface imaging, dual channel imaging can be conducted with a second marker for CME. For example, examining the dynamics of an EAP which colocalizes with a second marker for CME, such as clathrin itself, aids the filtering of CME events to only consider *bona fide* events. Our analysis provides the same output metrics as the single channel analysis (lifetime, density and fluorescent intensity profile), but only for events where both markers are detected.

The greatest advantage of using dual channel cell surface imaging for the analysis of CME is that a departure assay can be conducted. This automated unbiased analysis allows the precise determination of when an EAP is recruited to CME events relative to a well characterized marker of CME; such as CLC2 (Johnson and Vert, 2017, Konopka et al., 2008, Mattheyses et al., 2011, Merrifield et al., 2002). In the departure assay, the fluorescence intensity profile of the candidate EAPs (in the secondary channel) is aligned to the end of CLC2 (in the primary channel); as this represents the moment a CME vesicle is scissioned from the PM and is able to leave the field of illumination (Figures 3B, C and 5). This gives a physiological reference on which to base the temporal dynamics of EAPs at single events of CME. This analysis of EAP dynamics therefore requires that the EAP of interest is co-expressed with a *bona fide* CME marker whose dynamics have been well characterized.

Comparison of cell surface imaging techniques for CME visualization on the PM

As the lifetimes of plant *bona fide* CME events are quite rapid (root cells, 42 s; hypocotyl, 33 s (Narasimhan et al., 2020)), it is necessary for dual channel imaging analysis to be conducted by either TIRF-M or spinning disk (SD) confocal microscopy which provide sufficient spatial and temporal resolution to capture CME events on the PM. However, TIRF-M provides a higher signal to noise ratio and sensitivity than SD confocal imaging and is thus better suited at detecting the early stages of CME; as many of the ‘early stage’ EAPs will be present in low numbers (Mettlen and Danuser, 2014). An additional benefit of the higher sensitivity of TIRF-M, is that less laser power is required for excitation, thus reducing phototoxicity effects

and increasing the duration it is possible to acquire images for. This is because TIRF-M limits the illumination volume of the sample and uses a low intensity evanescent wave, where all the emitted photons are collected. In contrast, SD microscopy uses pinholes to block out of focus photons and the whole sample is illuminated (Figure 1). The major disadvantage of TIRF-M is that because of its shallow illumination volume it can only be applied to imaging of cell surfaces that are in direct contact with the coverslip. For this reason, TIRF-M has been used almost exclusively to image epidermal cells of seedling roots and hypocotyls, whereas with SD confocal microscopy there is greater flexibility in terms of which tissues and cell surfaces that can be imaged.

As an alternative to TIRF-M and SD confocal microscopy, variable-angle epifluorescence microscopy (VAEM) (Chen et al., 2018, Higaki, 2015, Konopka and Bednarek, 2008, Wan et al., 2011), also known as highly inclined thin illumination (HILO) microscopy (Tokunaga et al., 2008), can be used. It overcomes some of the limitations of confocal systems (their lack of sensitivity for low levels of signal intensities) and TIRF-M (the shallow illumination volume). Instead of using TIRF illumination, the angle of incidence of the excitation beam is oblique such that it undergoes refraction instead of reflection towards the coverslip. In images where the excitation beam penetration is relatively shallow the signal to noise ratio of VAEM/HILO approaches that of TIRF-M (Wan et al., 2011). However, the depth of Z penetration is not uniform across the image (Supplemental Figure 2B), which can introduce variability when one is trying to measure the dynamics of proteins in a single Z plane, such as the EAPs on the PM.

Considerations for the use of fluorescent protein tagged reporters

The choice of fluorescent protein tags for imaging CME reporter proteins is an important consideration as they all have different properties; for example, excitation/emission spectra, brightness, photostability, maturation time, pH sensitivity and the ability to form dimers. These properties will make some better suited for different imaging modalities, so it is important to plan which microscopy and experimental approach will be used in order to optimize the fluorophore choice. An excellent resource for further information on the various available genetically encoded fluorescent protein tags and their parameters is ‘fpbase.org’ (Lambert, 2019).

A critical consideration when analyzing the localization and dynamics of fluorescent tagged proteins of interest is the expression level of that protein, as one must consider that

grossly overexpressing proteins involved in CME may affect the dynamics of the process. It is therefore good practice to use fluorescent fusion proteins that have been demonstrated to be functional (e.g. through their ability to rescue the phenotype of corresponding loss-of-function mutant lines (Supplemental Table 2)) and whose expression level is close to that of the endogenous protein of interest. However, from a practical standpoint the intensity of the candidate EAP fluorescence signal must be sufficiently above background in order for it to be detected by the instrumentation and detection software.

In recent years it has been considered best practice for studies of mammalian and yeast CME to utilize gene edited cells and systems for expression of fluorescent proteins of interest as it had been reported that the lifetime of certain EAPs was altered when transiently overexpressed (Doyon et al., 2011). However, it is important to note that the reported temporal difference between transient overexpression versus stable expression of CME reporters in gene edited cells was subsequently found to be due to the differences in sensitivity of the analysis software used. In particular in the recruitment and/or dynamics of fluorescent fusion protein tagged CLC2 were not affected by overexpression when analyzed using the robust and sensitive detection of the cmeAnalysis package (Aguet et al., 2013).

Methods for the quantitative analysis of cargo internalization; characterization of CME at the whole tissue level

The examination of the uptake of fluorescently labeled cargo, or dyes, from the PM provides another approach for assessing CME in plants (Supplemental Table 5). This is because after the CME event has occurred, cargo is trafficked to the TGN/early endosomes where it is either trafficked to the multi-vesicular bodies for delivery and degradation in the vacuole and/or be recycling back to the PM. Therefore, quantitative analysis of the intracellular levels of fluorescently labeled marker internalized from the PM, after a short time period, provides another approach for assessing the activity of the plant CME machinery and its regulation.

FM Dyes

FM dyes are a series of amphiphilic styryl dyes that have been used as tools across the model systems to measure net internalization of the PM (Bolte et al., 2004, Cheung and Cousin, 2011, Jelinkova et al., 2010, Mueller et al., 2004). They contain a central region which is flanked by a hydrophobic tail and a polar head group (Figure 6A) (Betz et al., 1996). The

central region determines the fluorescence properties of the dye, where differing lengths results in different colors of emission; for example two commonly used FM dyes in plant CME studies are FM4-64 and FM1-43, whose peak fluorescent emissions are in the red (Figure 6B) and yellow (Supplemental Figure 3A and B) spectrum, respectively. The hydrophobic tail can reversibly associate with the outer leaflet of membranes and upon this binding, there is a dramatic increase in their fluorescence quantum yield relative to their non-membrane bound state (Henkel et al., 1996). The polar head group prevents the FM dyes from being able to cross the PM, meaning that their entry into cells is solely dependent on endocytosis (Figure 6C-E).

To assess the level of PM endocytosis, cells are incubated with the FM dye and subsequently the level of internalized intracellular fluorescence signal is measured and compared to the signal of FM remaining on the PM. By using confocal based microscopy, these types of experiments provide the opportunity to analyze endocytosis at the cellular and whole tissue levels. As the only material requirement is the dye, they represent a rapid and convenient experimental approach to test the effects of pharmacological, or genetic manipulations of CME, with no need to generate genetic marker plants lines or crosses. In order to precisely quantify the total amount of FM dye internalization 3D imaging and analysis of the entire volume of a cell is required. This type of analysis is feasible when analyzing individual cells (Rosquete et al., 2019). However, a major challenge with the quantitation of the FM dye endocytosis in plant tissues is the non-uniform geometry of cells within the tissues, which when projected in 3D result in images with undefinable PM region, making the evaluation of the levels of PM FM signal relative to intracellular FM signal intensity prone to a subjective bias.

To date, the majority of FM dye uptake studies in plants have relied on the use of manual approaches to segment the PM and intracellular regions within single z-section image planes. Therefore, to facilitate the unbiased quantification of the total amount of FM dye labeling both the PM and intracellular compartments, we have developed a semi-automated analysis which is based on segmentation of the membrane and intracellular regions to provide a robust method for FM uptake quantification (Figure 6E-G; ‘Expanded Method 3’). Using this analysis, we found that the efficiency of internalization of FM4-64 and FM1-43 are similar (Supplemental Figure 3C and D). Due to the nature of the automated segmentation whole fields of view can be analyzed rapidly and only cells that are in a similar focal plane can be segmented, thereby removing manual selection and segmentation bias. Although this

analysis tool overcomes many of the previous difficulties in quantitating FM dye uptake, the analysis of the internalization of PM cargo in plant tissues and individual cells remains constrained to quantitating the level of FM dye signal within a single Z focal imaging plane.

A major consideration when using FM dyes to measure endocytosis is their internalization is mediated by both CME and clathrin independent endocytosis (CIE) internalization pathways. Additionally, it has been reported that FM dye labeling may perturb the localization of certain plant membrane proteins (Jelinkova et al., 2010). Nonetheless, despite these concerns, FM dye uptake studies have been and will continue to be an effective and informative tool for assessing global endocytosis in plant tissues.

Fluorescently labeled cargo uptake assays

To specifically examine the CME internalization pathway, one should make use of labeled known cargos of the CME internalization pathway. This requires a *bona fide* CME cargo protein. Recent studies have led to the identification of a number of plant cell surface proteins that undergo constitutive and/or ligand dependent CME; including PIN2, BOR1 and 4, FLS2, BRI1, IRT1, CEAS, PEPR1 and STRUBBELIG (Barberon et al., 2014, Bashline et al., 2013, Dhonukshe et al., 2007, Di Rubbo et al., 2013, Gao et al., 2019, Ortiz-Morea et al., 2016, Takano et al., 2005, Yoshinari et al., 2016). However, the expression of many of these proteins is often restricted to specific cell types, cellular domains and only under specific physiological conditions and thus are not well suited as general markers for the analysis of plant CME (Qi et al., 2018). Further work is needed to identify a ubiquitously expressed and constitutive recycling cargo protein to serve as a general marker for plant CME. A defined cargo satisfying these requirements in plants would provide researchers with a reference tool to directly and specifically examine the CME pathway in plants in all cells and tissues without requirements to activate the CME reaction.

One attempt to overcome these issues is using the canonical mammalian CME cargo, the human Transferrin receptor (hTfR). It has been demonstrated that *Arabidopsis* protoplasts are able to transiently express and recycle hTfR (Ortiz-Zapater et al., 2006), thus providing a rapid experimental system in which one can assess the rate of CME under different experimental conditions and treatments (Robert et al., 2010). However, this approach relies on a non-native plant cargo to examine the evolutionarily unique process of plant CME. Additionally, researchers have made use of transient expression of CME related proteins and cargos in tobacco leaf pavement cells as an rapid experimental alternative to generating novel

plant lines (Bandmann et al., 2012, Cao et al., 2020, Gadeyne et al., 2014, Leborgne-Castel et al., 2008, Mbengue et al., 2016). However, it is important to consider that to examine a protein of interest, it relies upon overexpression, and there appears to be differences in the vesicle trafficking machinery/processing between *Arabidopsis* and tobacco (Langhans et al., 2011).

Photo-convertible fluorescent tags of CME cargos

Photo-convertible fluorescent tags such as EOS or Dendra can provide information about specific populations of tagged proteins. This is because after stimulation with a high intensity 405 nm laser burst, its emission spectra irreversibly changes from green to red (Gurskaya et al., 2006), thereby enabling the discrimination between distinct protein populations that differ in terms of their lifetimes and/or subcellular distribution. In the case of CME cargo proteins that reside at the PM, the use of photo conversion permits the direct examination of the uptake of a specific cargo in any plant tissue and has been utilized in plants to address the internalization of PIN cargoes and their regulation by plant hormones (Jasik et al., 2016, Salanenka et al., 2018). As these assays are specifically looking at a defined cargo, they can provide greater insight into how CME is utilized by the cell in cellular processes. For example, in cases where PIN2-Dendra has been utilized to examine its internalization via CME, this direct approach has highlighted that the PIN2 dynamics reported from indirect measures of CME were not physiologically accurate (Jasik et al., 2016, Kleine-Vehn et al., 2008b).

Critically, as one is looking specifically at a certain cargo, the internalization pathway of this cargo should be well defined, for example in terms of clathrin-dependent and -independent internalization.

Probing the mechanisms of CME through pharmacological and genetic manipulation

A classical way to determine the mechanisms underlying biological processes is to disrupt it using pharmacological agents and/or through genetic manipulation. In this manner, testable hypotheses regarding the function of proteins of interest can be formulated and experimentally evaluated. Pharmacological and genetic manipulations that interfere directly with the process of CME (Supplemental Tables 3 and 4) are a very useful tools for the general studies of plant endocytic trafficking at multiple scales. Pharmacological agents are

415 advantageous due to their rapid action, application and reversibility upon removal. In
416 contrast, the use of genetic manipulation allows the customized targeting of specific proteins,
417 domains and interactions. Both approaches can be used in conjunction with the ‘Expanded
418 Methods’ presented in this paper.

419 *Brefeldin A*

420 An example of internalization assays of labeled CME cargos combined with pharmacological
421 disruption of the trafficking pathway to study CME is the use of Brefeldin A (BFA). BFA is a
422 fungal metabolite which interferes with interactions of certain ARF GTPases and GEFs
423 (Helms and Rothman, 1992), resulting in the reversible agglomeration of misshaped
424 endosomal and Golgi compartments (Geldner et al., 2001, Grebe et al., 2003), which are
425 often referred to as ‘BFA bodies’. Within these BFA bodies, endocytic cargo and newly
426 synthesized secretory proteins become entrapped and are prevented from entering
427 downstream trafficking pathways to be degraded or recycled. The levels of PM proteins in
428 BFA bodies can thus be used as a proxy to estimate CME cargo internalized. Indeed, it has
429 been shown that many cell surface cargoes co-localize with BFA bodies (Beck et al., 2012,
430 Gifford et al., 2005, Karlova et al., 2006, Kwaaitaal et al., 2005), thus the rate of
431 internalization of PM associated proteins can be determined by measuring the signal intensity
432 of fluorescent endocytic reporters upon entrapment in BFA bodies. However, to discriminate
433 between the accumulation of endocytic cargo versus newly synthesized proteins in BFA
434 bodies, the sample needs to be treated with both the protein synthesis inhibitor cycloheximide
435 and BFA.

436 Although BFA treatment provides often a quick and easy estimate of the rate of
437 internalization of different cargoes, it is a problematic approach for multiple reasons. First
438 consideration is that cells of different tissues and developmental stages shows different
439 sensitivities to BFA. Another issue is that the ‘BFA body pathway’ is not a common pathway
440 for all potential CME cargos (Russinova et al., 2004), thus the cargo used in such
441 experiments must be well characterized. Further concerns are that BFA has been reported to
442 partially inhibit the endocytosis at least of some cargoes (Naramoto et al., 2010) and after
443 prolonged incubations some endocytic cargoes (such as PINs) gradually disappear from the
444 BFA bodies (Kitakura et al., 2011, Kleine-Vehn et al., 2008a). The most problematic issue is
445 that BFA does not lead only to intracellular accumulation of endocytic cargoes but also to the
446 aggregation of endosomes, TGN and Golgi at the periphery (Naramoto et al., 2014).

Therefore, the cargo accumulation in the BFA bodies is a net result of all these processes and thus the outcome needs to be interpreted with caution.

Specific CME pharmacological agents

Pharmacological manipulations of EAPs and CME are routinely used in CME investigation, where most of the drugs have been developed in other model systems (Dutta and Donaldson, 2012, von Kleist and Haucke, 2012). Recent advances in our understanding of plant CME have highlighted that plant CME functions in many evolutionary unique ways (Adamowski et al., 2018, Barberon et al., 2011, Bashline et al., 2013, Beck et al., 2012, Dhonukshe et al., 2007, Fan et al., 2013, Gadeyne et al., 2014, Gifford et al., 2005, Kim et al., 2013, Konopka et al., 2008, Martins et al., 2015, Paciorek et al., 2005, Sharfman et al., 2011, Takano et al., 2005, Yoshinari et al., 2016, Zhou et al., 2018), thus when using inhibitors from non-plant systems it is important to validate their activity and examine potential off target effects. For example, the commonly used CME inhibitor, tyrphostinA23 (TyrA23) (Banbury et al., 2003), was found to operate through distinct mechanisms. In animal cells, TyrA23 targets the EAP AP-2 complex but in plant cells it appears to predominantly act by disrupting intracellular pH gradients (Dejonghe et al., 2016). Additionally, the small molecule inhibitor of mammalian CME Pitstop 2, while reducing FM uptake in a concentration dependent manner, it was reported that the internalization of CME cargos were not inhibited in *Arabidopsis* seedlings (Dejonghe et al., 2019).

To circumvent these issues, the novel ES9-17 compound was designed in order to produce a specific block of CME in plant tissues (Dejonghe et al., 2019). It targets CHC but without the off target protonophore effects of its predecessor endosidin 9 (ES9). The impact of ES9-17 on trafficking was analyzed using a variety of assays including FM4-64, cargo uptake, as well as measuring the lifetimes of EAPs on the cell surface (Supplemental Table 3). It produced a very strong block of FM uptake and a prolonged lifetime of the EAPs, which suggests that it reliably blocks plant CME (Dejonghe et al., 2019). However, it is important to note that the effects of ES9-17 may not be restricted to CME as CHC is also involved in post-Golgi clathrin dependent trafficking, rather than just CME at the PM.

In addition to CME inhibitors that target AP2 and clathrin, several compounds that interfere with other components essential EAPs have been tested in plants samples. For example, the mammalian dynamin GTPase inhibitor Dynasore (Macia et al., 2006) has been used in *Nicotiana benthamiana* and been shown to be an effective blocker of endocytosis

(Sharfman et al., 2011), presumably by interfering with the plant DRP homologues of dynamin. A more potent analogue of Dynasore, Dyngo 4a, which also inhibits mammalian dynamin GTPase activity (McCluskey et al., 2013), has also been used in plant tissues (Hunter et al., 2019, Zhang et al., 2017). Here we demonstrate using TIRF-M that Dyngo 4a also prolongs the lifetime of CLC2 in intact *Arabidopsis* root tissues (Supplemental Figure 4). This prolongation, rather than the stalling of CLC2 on the PM, suggests that Dyngo 4a might not have such a strong affinity for the plant DRPs as for the mammalian Dynamins. It is important to note that because Dyngo 4a absorbs light in the range of ~500 – ~700 nm it is unsuitable for use in studies with FM dyes 4-64 and 1-43 (peak emissions at 725 nm and 580 nm respectively) (Supplemental Figure 5 and unpublished observations from multiple labs).

Ikarugamycin (IKA), a naturally occurring compound, has also been used to inhibit CME in plant protoplasts, pollen tubes and mammalian systems (Bandmann et al., 2012, Elkin et al., 2016, Moscatelli et al., 2007). While its mechanism of action is not known, IKA is reportedly specific for the CME pathway in mammalian systems (Elkin et al., 2016). We therefore tested its effect on plant CME in intact *Arabidopsis* root samples. We show that treatment of *Arabidopsis* roots with (30 µM) IKA for 15 min resulted in a significant increase of persistent cell surface foci of the CLC2 lifetime on the PM and that it reduces but not completely blocks FM4-64 uptake, indeed suggesting IKA may specifically inhibit CME mediated FM uptake, but not CIE (Figure 7A and B). However, caution is required until its precise mechanism of function is uncovered.

Genetic manipulations of EAPs to investigate plant CME

Analysis of mutants that disrupt the expression of proteins involved in CME has been a powerful approach validating the function of key CME EAPs, including evolutionarily conserved and plant specific EAPs (Supplemental Table 6). However for plant CME, there appears to be an extensive amount of functional redundancy; for example, mutations in the individual genes encoding the two *Arabidopsis* CHC, CHC1 and CHC2, isoforms display no, or only weak, CME defects (Kitakura et al., 2011). Another complication with the genetic analysis of proteins involved in CME in plants is that mutations that result in the complete loss of expression of essential proteins are homozygous lethal. Homozygous double *chc1chc2* mutants cannot be isolated likely because at least one isoform is required for gametogenesis and/or zygote development (Kitakura et al 2011). Likewise, loss-of-function mutants in

genes encoding subunits of the TPLATE complex (TPC), a key plant-specific EAP, result in pollen lethality (Gadeyne et al., 2014).

An alternative approach for the analysis of proteins involved in CME that are encoded by genes essential plant gametogenesis and/or development, is the use of conditional mutants. This allows one to regulate the timing of the downregulation of expression of essential CME proteins of interest. It also facilitates the characterization of their function to stages of plant development and/or tissues more tractable for live-cell imaging and cargo uptake studies. Use of conditional mutants is also advantageous as it limits the possibility for the plant to develop compensatory mechanisms. For example, to overcome the pollen lethality associated with loss-of-function mutation in genes encoding TPLATE and other subunits of the TPC, inducible artificial micro RNAs (amiR) have been used to silence the expression of TPLATE in seedlings thereby permitting the analysis of the function of the TPC in CME (Gadeyne et al., 2014, Wang et al., 2016). Similar conditional amiR knockdown strategies have been successfully used to study the role of other key plant EAPs (Supplemental Table 4).

A complementary approach for the analysis of essential and/or functionally redundant proteins involved in CME is to utilize inducible overexpression of wild type, or dominant negative versions of proteins of interest to regulate the process of CME (Supplemental table 4). For example, the inducible overexpression of the Arabidopsis auxilin-like proteins, homologues of the mammalian auxilin protein involved in CCV uncoating, abolished the formation of CME foci at the PM (Adamowski et al., 2018). Overexpression of the C-terminal of CHC1 (termed CHC HUB), which binds and prevents CLCs from forming the CCV, has also been shown to effectively disrupt CME (Dhonukshe et al., 2007). Similarly, the overexpression of a dominant negative GTPase defective DRP1a resulted in the significant prolonging of the lifetime of CLC2 on the PM (Yoshinari et al., 2016). However, it is important to consider potential off target effects of the use of dominant negative constructs in other cellular processes. For example, overexpression of dominant mutant DRP1a resulted in thylakoid biogenesis defects (Park et al., 1998).

Quantitative Analysis of CME inhibition

The inhibitory effects of both pharmacological and genetic mutants on CME can be assessed at the ultrastructural and global levels using assays like the FM4-64 uptake method detailed in ‘Expanded Methods’ 1 and 3. However, to determine the effects at single events on the PM, additional analysis is required as cell surface lifetime analysis alone is not sufficient to

reliably quantify the effects of inhibitors and/or genetic manipulation of the dynamics of individual CME events. A major reason for this, is that the quantification of the lifetime of clathrin and EAPs at the PM involves measuring the duration (i.e. time between the initiation and disappearance) of CME marker proteins recruited to CME events. Inhibition of CME manifests in non-productive CME events (i.e., stalled or delayed) in which clathrin and/or EAP marker proteins are present before and after the image acquisition window, thus preventing/reducing the visualization of their appearance and disappearance; making it almost impossible to accurately determine their lifetimes. Therefore, we developed an alternative robust method to quantitate the level of inhibition of CME dynamics following pharmacological and/or genetic manipulation termed the ‘spot persistence assay’ (Figure 7C). It measures the duration of the EAP foci on the PM to determine a ratio between the number of dynamic and persistent foci of EAPs within the first 100 seconds of a cell surface imaging experiment (see Expanded Method 4). The 100 second window is used as it is over twice the duration of the mean lifetime of bona fide CME events (42 s in roots, 35 s in hypocotyl (Narasimhan et al., 2020)). As the output is a relative metric, i.e. the percentage of persistent tracks of the total tracks within the first 100 seconds, the effectiveness of different inhibitors on the process of CME in wild-type plants can be compared. For example, we found that the CME inhibitory effects of IKA are stronger than those of Dyngo 4a (Figure 7C-E and Supplemental Figure 4).

Discussion and Conclusions

Plant CME is vital to many key physiological processes, but it remains largely uncharacterized at multiple scales compared to other model systems. Significant progress has been made in the last decade to enhance our understanding of CME, which has been enabled through the identification of evolutionarily conserved and plant specific factors involved in CME. Further in depth analysis of the temporal and spatial dynamics of the protein-protein and protein-membrane interactions necessary for the initiation maturation and release of CCV during CME will require a comprehensive set of tools optimized for the direct, quantitative and unbiased examination of plant CME at multiple scales. Here we present refined imaging and analysis protocols which allow the quantifiable characterization of plant CME at the ultra-structural, single CME event and tissue levels. The aim is to provide the plant community working on endocytosis in many different physiological and developmental

contexts with a standardized set of tools enabling quantitative and directly comparative studies.

Another important area for the standardization of plant CME investigations is the highlighting of robust EAP antibodies. To date, many antibodies have led to key insights in to the interactions and localization of EAPs in both pull-down assays and immunohistochemical staining of plant tissues (Dejonghe et al., 2019, Dhonukshe et al., 2007, Di Rubbo et al., 2013, Gadeyne et al., 2014, Gao et al., 2019). Therefore, to further broaden the standardization of plant CME tools to cover antibodies, researchers should be encouraged to submit their routinely used antibodies to online databases, such as Antibodypedia (Bjorling and Uhlen, 2008).

While further work is required to characterize and develop more specific pharmacological and genetic tools to interrogate each individual step of plant CME, combining the presented imaging methods with pharmacological and genetic manipulation tools will enable and accelerate our understanding of plant CME. Further to this, the further development of super-resolution imaging for plants combined with computational analysis tools will further our ability to improve the precise characterization of plant CME. For example, establishment of correlative light electron microscopy (CLEM), and live approaches such as structure illumination microscopy (SIM), stochastic optical reconstruction microscopy (STORM), Stimulated Emission Depletion (STED) and light sheet microscopy techniques will allow us to further characterize the precise structures, interplays of EAPs and tissue effects of CME in plants.

Expanded Methods and Materials

Matlab code with detailed additional detailed instructions can be downloaded at 10.5281/zenodo.3888519 and will be maintained/updated at www.github.com/ajohnsoncode/plantCmeMethods. Example data can be found at 10.5281/zenodo.3888531.

Expanded Method 1: SEM visualization of *Arabidopsis* protoplast CCVs using metal replica of unroofed cells

Here we present a method which allows the visualization of CCVs in *Arabidopsis* protoplast cells (adapted from (Doczi et al., 2011, Svitkina, 2007)). The cells are unroofed and coated

with metal to produce a replica of the cell interior, where PM associated elements can be observed (Figure 2). By analyzing these samples with SEM, one can directly count the number of CME vesicles and quantify their size, structural arrangement and localization within the cell.

Cell Preparation

1. Protoplasts are isolated from 3-day-old *Arabidopsis* suspension culture cells derived from roots (Note 1). 25 ml of suspension culture is initially centrifuged at 1500 rpm for 5 min at room temperature (RT).
2. After removing the supernatant, the pelleted cells are resuspended in 25 ml of enzyme solution (GM buffer [2.2 g MS with vitamins (Duchefa Biochemie, # M0222, 15.25 g glucose, 15.25 g mannitol, H₂O to 500 ml, pH to 5.5, with KOH] supplemented with 1% cellulose (Yakult), 0.2% Macerozyme (Yakult) (Note 2)). The whole resuspension is then incubated for 4 hours in the dark with gentle agitation to digest the cell wall.
3. Once the majority of the cells are round, the cells are washed twice and spun at 1200 rpm and 1000 rpm for 5 min each with GM buffer (Note 3).
4. After removing the supernatant, the cells are resuspended in sucrose buffer (4.4 g/l of MS powder and 0.28 M sucrose; pH 5.5 with KOH) and centrifuged at 800 rpm for 7 min. The protoplasts which are now suspended on top of the sucrose gradient are carefully removed with a Pasteur pipette and stored overnight at 4°C.
5. Coverslips for plating the cells are prepared as follows;
 - a. 12 mm diameter glass coverslips are washed in absolute ethanol and air dried
 - b. Coverslips are carbon coated to a thickness of 10 nm using the ACE600 high-vacuum coating device (Leica Microsystems).
 - c. The coverslips are then coated with poly-L-lysine (Sigma) at 4°C overnight.
 - d. The coverslips are washed with ddH₂O to remove any excess poly-L-lysine
6. The protoplasts prepared are plated on the coated coverslips and incubated at RT for 4 hours, to allow the cells to adhere to the coverslips.
7. Plated protoplasts together with the coverslips are spun at 800 rpm for 5 min to further aid cell adhesion.

Unroofing of cells

8. The excess protoplasts are removed gently from the coverslips using a Pasteur pipette, and samples are washed briefly with PBS (without Ca^{2+} and Mg^{2+}). Then they are equilibrated to room temperature in the same dish.
9. Extraction solution (2 μM phalloidin, 1% Triton X-100 (w/v) and 1% polyethylene glycol (PEG; MW 20000; w/v) in PEM buffer (100 mM PIPES free acid, 1 mM MgCl_2 , 1mM EGTA; pH 6.9 adjusted with KOH)) was equilibrated to room temperature and applied for 4 min at RT with gentle agitation.
10. Samples are then washed in PEM buffer plus 1% PEG 3 times 1 min each at RT.
11. They are then fixed with 2% glutaraldehyde (v/v) in 0.1 M PB, pH 7.4, for 20 min at RT and then washed in ddH₂O 3 x 5 min each at RT.
12. Then the sample are further incubated with 0.1% tannic acid in water (w/v) for 20 min at RT and then washed twice with excess of ddH₂O 5 min each.
13. The samples are treated with 0.2% uranyl acetate (w/v) in water for 20 min at RT and then washed twice with ddH₂O.

Dehydration

14. Lens tissue with loosely arranged fibers (Kimberly-Clark®) are cut into squares, a little larger than the diameter of sample holders for critical point drying (CPD; round wire baskets, Leica Microsystems).
15. The CPD sample holders are placed in a glass beaker filled with distilled water and a piece of lens tissue is put at the bottom of each holder. A coverslip with cells facing up is then placed on the lens tissue, covered with another piece of lens tissue. This loading procedure, alternating coverslips and lens tissue, is continued until the holders are filled. Samples are always kept in water.
16. Sample holders are then placed on a homemade standing device for quick transfer from glass beaker to glass beaker with increasing concentrations of ethanol in water. These are 10%, 20%, 40%, 60%, 80%, 96% and 3 times 100%. Incubation times are 5 min each at RT. The standing device is constructed in a way that a magnetic rod fit under the mounting plate for sample holders and the solution can be kept under constant agitation on a magnetic stirrer.

Critical point drying and coating

17. The chamber of the critical point drying device EM CPD 30 (Leica Microsystems) is filled with absolute ethanol, just sufficient to cover the CPD holder. The holder is then

placed in the ethanol bath and the CPD operated according to the manufacturer's instructions.

18. The samples are then fixed onto SEM specimen mounts using carbon conductive adhesive tabs (Ø 12 mm) and coated with gold- or platinum to a thickness of 5 nm by rotary shadowing at a 45° angle using an ACE600 coating device.

Imaging

19. Samples are examined in a FE-SEM Merlin Compact VP (Zeiss) and imaged with an In-lens Duo detector (SE and BSE imaging) at an accelerating voltage of 0.5 to 5 kV.

Analysis (this is an example analysis to determine the size of the CCVs)

20. The image is opened in Fiji/ImageJ (NIH).

21. Set the scale of the image to the correct calibration (analysis menu > 'set scale').

22. Regions of interest (ROIs) are created on the structures/features you want to measure using the line tool. Press 't' to add them to the ROI manager. When all the ROIs are made, it is important to save them, and the ROIs can be measured by pressing the 'measure' button. Results are copied and pasted into excel where further analysis can be conducted (Supplemental Figure 1).

Notes and considerations;

1 – The specific cells used here are Col-0 *Arabidopsis* root-derived suspension cultures, and were gifted to us by Eva Kondorosi (Gif-sur-Yvette, France). They were maintained and grown in growth media (GM) (4.25 g/l of MS salts, 30 g/l of sucrose, 0.250 mg of 2,4D, 0.015 mg of kinetin and 2 ml Vitamin B5 stock (100 ml stock contains 0.1 g nicotinic acid, 0.1 g pyridoxine HCl, 1 g thiamine HCl, 10 g myo-inositol) at pH 5.7).

2 - The enzyme solution and the GM buffer are filter sterilized and stored at 4°C.

3 – Centrifugations from step 3 are done at RT with no breaks to avoid damaging of digested cells.

Expanded Method 2: Cell surface TIRF-M of *Arabidopsis* root epidermal cells

Here we present a method which allows the automated direct examination of EAPs and single events of CME on the PM. Briefly, plants expressing fluorescently labelled EAPs are imaged over time using a microscopy approach which directly examines the PM (Figure 3). Automated analysis detects and tracks the proteins over the duration of the movie. The detections are unbiased and based upon parameters of the experimental setup used and quantifiable outputs include; the lifetimes (mean and distribution), density and mean fluorescent profiles for both single (Figure 4) and dual channel experiments (Figure 5). Using dual channel data, a departure assay is conducted, which produces a recruitment profile of a protein of interest for single events of CME. This provides an imaging and analysis method to precisely determine the physiological temporal dynamics of proteins in plant CME.

Tissue Preparation;

1. Seeds expressing a suitable CME marker (Supplemental Table 2) are sterilised and sown in a row at the top of AM+ 1% sucrose agar plates, spaced at least 1 cm apart.
2. The plates are then incubated for 2 days at 4 °C in darkness.
3. The plates are then transferred to growth rooms; 21° C, 16- and 8-hour light and dark cycles and grown for 5-7 days (Note 1). It is critical that the plates are slight tilted backwards; if the plates are too vertical then the seedlings will grow too many hairs to enable a flat contact with the coverslip (Supplement Figure 7, Photo 1).

Coverslip preparation;

4. During this incubation period, coverslips 24 mm x 50 mm, thickness 1.5 (VWR #631-0147) are prepared and cleaned as follows (Note 2);
 - a. Coverslips are placed in a coverslip holder (Sigma, Wash-N-Dry) and washed in 'cleaning' solution (Decon 90 0.01% (w/v), NaOH 100 mM) in a 250 ml beaker (Supplement Figure 7, Photo 2) and incubated for 15 mins.
 - b. Coverslips are then cleaned using lens tissue (GE Healthcare, Whatman) and placed back into the coverslip holder.
 - c. The coverslips are washed again in 'cleaning' solution for a further 15 mins.
 - d. The coverslips are then washed at least 5 times in ddH₂O, 5 mins each, until there are no more detergent bubbles in the solution.

- e. The coverslips are then washed twice with 100% ethanol for 5 mins.
- f. The coverslips are removed from the washing beaker and allowed to air dry (this is normally done in a flow hood to speed up the drying and ensure the coverslips remain sterile). Ensure the coverslips are separated to allow each one is able to dry (Supplement Figure 7, Photo 3).
- g. The coverslips are washed in acetone, for at least 5 mins. And then air dried and stored in a sterile manner. Coverslips should not be stored longer than 3 weeks.

Sample preparation;

5. The root of interest is cut about 1 cm from the tip of the root and gently laid flat on a microscopy slide (76 mm x 26 mm; Carl Roth #H869) (Note 3).
6. The root is covered in an excess of experimental media (~60 μ l) and a pre-cleaned coverslip is slowly placed on top of the root, ensuring there are no bubbles created and that the root stays in the middle of the coverslip (Supplemental Figure 7, photos 4-5).
7. The excess media is aspirated away (by tissue or pipette), resulting in the coverslip providing a small amount of pressure to ensure that the root is in direct contact with the coverslip (Supplement Figure 7, photo 6). It is important not to apply any additional force, as it will damage the tissue structure.
8. The coverslip is sealed on to the slide by applying nail polish, to prevent the media evaporating away. Start with the corners and sides, then seal the whole coverslip allow to dry (Supplement Figure 7, photos 7-9).

TIRF-M Imaging;

9. The slide is then mounted onto the TIRF microscope and the appropriate settings for the system and fluorophores are used. Typically, an 100X 1.49 NA oil immersion objective (Note 4) and 488 nm and 561 nm lasers for green or red fluorescent proteins.
10. Cells in the elongation zone (determined to be 6-10 cells away from the end of the lateral root cap (Figure 3A)) are imaged. This is critical to obtain reproducible results (Note 5).
11. Time lapse movies are acquired typically at 1 frame per second, for either 5- or 10- minutes total duration (301 or 601 frames in total) (Note 6). Dual channel images are captured sequentially.

758 *Analysis;*

759 12. Movies are opened in ImageJ (NIH) and cropped to include only the area of interest.

760 13. For single channel analysis (*Arabidopsis thaliana* Col-0, plants expressing
761 pCLC2::CLC2-GFP (AT2G40060) (Konopka et al., 2008) were used in this manuscript);

762 a. singChan_cellSurfaceAnalysis is run in Matlab (Note 7) (for further detail on how to
763 use this program, see the cell surface analysis instruction PDF).

764 b. To combine data from multiple experiments, combineSingChanData is run in Matlab
765 (for further detail on how to use this program, see the cell surface analysis instruction
766 PDF).

767 14. For the dual channel departure analysis (*Arabidopsis thaliana tplate*, plants expressing
768 pLAT52::TPLATE-GFP (AT3G01780) x pRPS5::CLC2-RFP (Gadeyne et al., 2014) were
769 used in this manuscript);

770 a. dualChan_cellSurfaceAnalysis is run in Matlab (Note 8) (for further detail on how to
771 use this program, see the analysis instruction PDF).

772 b. To combine data from multiple experiments, combineDualChanData is run in Matlab
773 (for further detail on how to use this program, see the cell surface analysis instruction
774 PDF).

775 Notes and considerations;

776 1 – Different durations of growth periods will affect the location of a good ‘TIRF region’ in
777 the root. See Note 5.

778 2 – It is essential to clean the coverslips to remove any possibility of disruptions in the optical
779 pathway.

780 3 - Roots are imaged one at a time to maximise the chances of having a perfectly flat cell for
781 imaging.

782 4 – TIRF-M was conducted on an Olympus IX83 inverted microscope equipped with a
783 Cell[^]TIRF module using an OLYMPUS Uapo N 100x/1.49 Oil TIRF objective. Wavelengths
784 of 488 and 561 nm were to stimulate GFP and RFP signals, a quad line beam splitter
785 emission filter (Chroma) was used in combination with an EM-CCD camera (Hamamatsu) to
786 acquire the data.

5 – The location of imaging is critical for producing good TIRF-M images and for reproducible results (Figure 3A). This is because it has been reported that the kinetics of CME change with the different stages of develop in the roots (Konopka et al., 2008). Also, cells in the elongation zone are used for imaging as they are normally lie flat on the coverslip, which allows distortion free TIRF imaging of the roots. It is important to pick a cell which presents a uniform field of illumination across the sample, and it free from large auto-fluorescent structures (Supplemental Figure 2). It is also essential to image cells located at the same distance from the root tip so comparison between genotypes/conditions. The end of the lateral root cap can be used as a reference (Figure 3).

6 - The resultant lifetime is highly dependent upon the frame rate used to image. This is due to the high level of short transient visits of EAPs to the cell surface in plants, therefore a rapid acquisition rate (<1 Hz) will include many more of these events compared to a slow rate (Johnson and Vert, 2017).

7 – Particle detection and tracking of the EAPs is conducted using just the detection and tracking programs of the cmeAnalysis package (Aguet et al., 2013), as the further filtering in this package is highly optimized for mammalian CME events and not suitable for plant CME analysis. Although there are other automated detection systems available (see (Loerke et al., 2009, Tinevez et al., 2017) for alternatives), the detection method presented here outperforms manual and other programs in robustness and sensitivity (Mettlen and Danuser, 2014). The tracks are filtered for analysis to only include; tracks absent from the first and last 10 frames of the movie, not within 10 pixels of the edge of the movie and which persist long than 5 frames. The lifetime is then calculated as the mean of all the combined tracks. The density is generated by imposing a 100 x 100 pixel region of interest (ROI) in the center of the movie, and then mean number of valid tracks is calculated over 100 frames (where the time range of frames used is 50 before the middle time lapse image to 50 after the middle time lapse image). This means the image of the cell must be larger than this 100 x 100 pixel ROI and the duration must be 101 or more frames. To test for significance, data from multiple experiments were combined and subjected to an unpaired, parametric two tailed T-Test. The mean profile is generated by combining and normalizing all the intensity profiles of tracks which have a lifetime within the range of mean lifetime ± 3 frames and aligned to their termination.

8 – Dual channel analysis was conducted using a primary channel/secondary channel approach. The tracks in the primary channel are detected, tracked and filtered as in the single channel analysis (Note 5) A further filtering is conducted where the master track must have a significant signal in the secondary channel which persists for more than 5 frames. The filtered tracks are then used to calculate the mean lifetime and density as in note 5. The mean profile is generated in the same way as ‘Note 5’, but additionally the slave profiles normalized and both the primary and secondary profiles are plotted.

9 – These analysis systems have been tested and found to work on spinning disc PM data, but strong levels of fluorophore expression are required (Wang et al., 2020).

Expanded Method 3: FM 4-64 Uptake Assay in *Arabidopsis* root epidermal cells

Here we present a method which allows the rapid assessment of the overall efficiency of internalization of the PM. Briefly, by determining the amount of FM dye has been internalized into the cell over a certain time period, one can assess the overall efficiency of endocytosis in a whole seedling (Figure 6). By combining this approach with pharmacological agents (Supplement Table 3) or genetically-altered plants (Supplement Table 4), one can directly assess the effect of such an experimental manipulation.

Intact *Arabidopsis* seedlings are pre-incubated with either control or treatment solutions. Then incubated with the FM4-64 in either control or treatment conditions. Once the samples are mounted on to a microscopy slide, images are then obtained on a confocal microscope (Figure 6D). Once samples images are acquired (Figure 6E), the experiments can be quantified using our semi-automated Matlab analysis system. Cells are segmented using a user entered threshold value, and then a ratio between the PM and intra-cellular signal is determined (Figure 6F and G). Thereby proving the direct assessment of PM internalisation (using a novel semi-automated analysis system), thus indicated potential effects of CME disruption.

Tissue Preparation;

1. 10-20 sterilised Col-0 seeds were plated on to AM+ and 1% sucrose agar plates, with ample space for the roots to grow vertically.
2. The plates were incubated for 2 days at 4 °C in darkness.

3. The plates were incubated in growth rooms for 5-7 days; 21° C, 16- and 8-hour light and dark cycles.

Treatment and Imaging

4. The seedlings are incubated in 2 ml AM+ broth supplemented with 1% sucrose with either mock or a chemical treatment for 15 mins in a 6 well cell plate (Note 1). Several seedlings can be processed and imaged together. To avoid disruption of the samples due to the static interaction of the roots with the plate it is recommended to incubate not more than two seedlings in the same time.

5. The seedlings are then incubated in 2 ml AM+ broth supplemented with 1% sucrose with mock or a chemical treatment, and 2 µM FM (Note 2), for 15 mins (Note 3) in a 6 well plate.

6. The seedlings are then washed gently twice by dipping then in a clean well containing 2 ml of the required experimental media (without FM4-64 to reduce background signal from weakly bound FM).

7. The seedlings are then transferred on to a microscope slide (76 mm x 26 mm; Carl Roth #H869), covered in experimental media and then carefully covered with a coverslip (24 mm x 50 mm, thickness 1.5; VWR #631-0147); ensuring that there are no bubbles and the root remains undamaged (Supplemental Figure 7, Photos 10-12).

8. The seedlings are then imaged with a confocal microscope. Here we made use of an inverted 800 or 880 Zeiss confocal microscope, equipped with a 40x 1.2 NA water emersion objective. The elongation zone (Note 3) of the root epidermis are imaged, and a small Z-stack which captures both signal from cell wall and the inner part of the sample was taken.

Analysis

9. The z-plane which shows clean signal from the lateral sides of the FM stained plasma membranes, and no clear sign of organelles blocking the cytoplasm region in which FM could be present in, are chosen for analysis (Supplemental Figure 6A).

10. The single plane should then be saved as a TIF file using ImageJ (NIH).

11. In Matlab, the fmUptakeAnalysis should be initiated (for further detail on how to use this program, see the fmAnalysis PDF manual).

12. When prompted, enter a threshold value (Note 4) to attempt to generate a good segmentation of the individual cells. It is critical to set the threshold which produces the most filled cells (Supplement Figure 6B).

13. The individual segmented cells are selected for analysis by user selection (Note 5 and 6).

Notes and considerations;

1 – IKA (Abcam; ab143408) was used at 30 μ M for 15 minutes.

2 - FM4-64 (ThermoFisher; T3166) is a ‘red’ dye, so can be used to with a GFP marker to determine if a labelled cargo can enter the FM labelled early endosomes. There are other dyes available, for example FM1-43 (ThermoFisher; T3163), emitting in the yellow range which function in the same way (Supplemental Figure 3). FM4-64 and FM1-43 were used at 2 μ M and incubated for 15 mins.

3 – Prolonged FM treatment has been reported to be toxic to plant tissues (Meckel et al., 2004), so long term treatments are not advised.

3 – The location of cells, and region of the plant, chosen for analysis is important, as different developmental stages of the root have been reported to have different kinetic rates of endocytosis (Konopka et al., 2008).

4 – The value entered effects the strength of the image segmentation (Supplemental Figure 6). First, a Gaussian filter is applied to the image and then the enter threshold value determines the Luminance threshold to binarize the image, which is used to define the PM and intracellular regions.

5 – Cells selected should be not contain areas where there are clear dark shadows visible inside the cell, as these are most likely organelles deep in the cell which physical exclude FM containing structures from the cytoplasmic space (Supplemental Figure 6A).

6 – Selected cells should be separated from the surrounding cells and outlines of badly segmented cells (Supplemental Figure 6B). This step can be checked immediately after the cell selection.

7 - No sample size calculation was conducted. Data from multiple experiments were combined and tested for significance using an unpaired, parametric two tailed T-Test in GraphPad Prism 6.0.

907

908 **Expanded Method 4: Spot persistence assay**

909 Here we present a method which allows the determination as to whether a chemical or
910 genetic manipulation of CME significantly disrupt EAPs on the PM. TIRF-M imaging is
911 conducted as detailed in expanded method 2, but with a different analysis system. This is
912 critical and overcomes the shortcomings of using a standard lifetime measurement to assess
913 the effects of CME inhibitors. The percentage of spots which is persist from the total amount
914 of spots detected within the 1st 100 frames of the time lapse is calculated (Figure 7C).

915 *Sample preparation and imaging;*

916 Sample preparation and imaging is conducted as described in expanded method three (Note
917 1).

918 *Analysis;*

919 1 – Run singChan_cellSurfaceAnalysis as detailed in expanded method 3, point 13.

920 2 – Then run the persisTest program (for further detail on how to use this program, see the
921 cell surface analysis instruction PDF) (Note 2)

922 Notes and considerations;

923 1 – The time lapse of the experiment must be over 100 frames, as this is this number of
924 frames used to determine persistence. Typically, the frame rate of these experiments should
925 be 1 Hz, therefore 100 frames represented a time point which is over twice the duration of a
926 *bone fide* CME event in the root (Narasimhan et al., 2020). If the frame rate is changed, it
927 should cover a duration greater than this. However, if the frame rate is too quick, then the
928 persistent spots will be subjected to more bleaching effects.

929 2 – The detection and tracking of spots in the movies are made in the same way as expanded
930 method 3. However, tracks present in the first and last frames are still retained for analysis,
931 and the duration of tracks measures is greater than 24 frames (as this is the mean lifetime of
932 single channel CLC2 and thus filters additional noise from use of an inhibitor). Then all the
933 frames which are present at the start of the movie are counted, and then a percentage of these
934 tracks which persist longer than 100 frames is calculated.

3 - To test for significance, data from multiple experiments were combined and subjected to an unpaired, parametric two tailed T-Test. No sample size calculation was conducted.

4 – Inhibitor experiments used IKA (Abcam; ab143408) and Dyngo-4a (Abcam; ab120689) at 30 μ M and with a 15 minute incubation.

Acknowledgments

This paper is dedicated to the memory of Christien Merrifield. He pioneered quantitative imaging approaches in mammalian CME and his mentorship inspired the development of all the analysis methods presented here. His joy in research, pure scientific curiosity and microscopy excellence remain a constant inspiration. We thank Daniel Van Damme for gifting us the CLC2-GFP x TPLATE-TagRFP plants used in this manuscript. We further thank the Scientific Service Units at IST Austria; specifically, the Electron Microscopy Facility for technical assistance (in particular Vanessa Zheden) and the BioImaging Facility BioImaging Facility for access to equipment.

Competing Interests

All authors declare there are no conflicts of interests.

Funding

A.J. is supported by funding from the Austrian Science Fund (FWF): I3630B25 to J.F. N.G. is supported by European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Grant Agreement No. 665385. This work was partially supported by an award from the National Science Foundation to S.Y.B.; Award No. 1614915.

Data availability

Raw data presented in this study is be available at [10.5281/zenodo.3888544](https://doi.org/10.5281/zenodo.3888544).

Author Contributions

A.J. and J.F. conceived the project and designed the experiments. Data collection was conducted by A.J., N.G., W.A.K. and M.N. Analysis was completed by A.J. and N.G, where the analysis protocols presented were developed by A.J. The article was written by A.J., S.Y.B and J.F, with contributions of intellectual content from W.A.K., M.N, G.V. and especially N.G. All authors edited the final version.

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Figure Legends

Figure 1 – Schematic of the principles of the major CME light microscopy methods

TIRF-M uses a weak uniform evanescent wave that penetrates ~100-200 nm into the sample,
 thereby illuminating cell surfaces in contact with the coverslip. The evanescent illumination
 wave is generated when the illumination beam hits a refractive index mismatch between the
 coverslip and sample/media at the critical angle (θ). The energy of the evanescent wave is
 directly proportional to the distance away from the point of generation, meaning that
 fluorophores closer to the PM are stimulated more than those deeper in the cell (green-grey
 GFP spots). CLSM makes use of a single pinhole, which blocks out-of-focus emitted light
 from reaching the detector (grey lines). In CLSM, the illumination beam passes directly into

the sample. SD confocal microscopy makes use of disks with many pinholes and micro-lens which rapidly spins, thus creating many simultaneous confocal points.

Figure 2 – Schematic of unroofing protoplasts and SEM

A) *Arabidopsis* protoplasts cells are plated onto poly-L-lysine coated coverslips. B) The cells are washed with detergent to ‘unroof’ the cells. C) The unroofed cells are then covered with a thin coat of platinum. D) Samples are imaged using SEM. Main image shows a high magnification view of a replica in which CCVs associated with the PM are identifiable (yellow arrows). Scale bar = 200 nm. Insert in panel D) represents low magnification view of a whole cell replica, scale bar 2 μ m.

Figure 3 – Schematic of cell surface TIRF-M imaging

A) To facilitate TIRF-M, the sample needs to be flat and in direct contact of the coverslip. The location of imaging within the root is critical in obtain reproducible results. The end of the lateral root cap (LRC) is used as a developmental marker, and cells 5-8 up the root are used for imaging. B) Lifetimes of proteins on the PM are only when the protein is within the illumination volume. C) Time and intensity fluorescent profiles of proteins can provide hints about their physiological function. For example, as clathrin triskelia containing fluorescently labeled clathrin subunits (blue) polymerize on the PM to form invaginating clathrin coated pits, there is an increase in the level of fluorescence signal. Upon scission of the labeled CCVs from the PM they rapidly depart from the illumination field resulting in a sharp decrease in fluorescence signal. Dual channel imaging of proteins of interest with a fluorescently tagged clathrin marker permits the quantitative comparison of its temporal dynamics relative to clathrin coated pit initiation, maturation and CCV departure from the PM (green and red examples as depicted in B).

Figure 4 –Single channel TIRF-M cell surface analyses

A) Example TIRF-M image from a root epidermal cell expressing CLC2-GFP. B) An example kymograph generated of the yellow line in A. C) Zoomed in image of the region of the yellow square in A. D) The results of the automated detection and tracking analysis. E) Time series of an example single CLC event on the PM and F) quantitation of its lifetime based on the fluorescent intensity profile, which is significantly above the intensity of surrounding pixels. Tracking data from multiple independent tracks and experiments are combined to generate G) a mean lifetime, H) the lifetime distribution, I) a mean density of

spots and J) a mean fluorescent profile. Plots are mean $\pm$ SEM. N = 4 cells from independent roots, 20098 tracks. Scale bar, A = 5 μ m.

Figure 5 – Dual Channel TIRF-M cell surface analyses

A) TIRF-M images of a root epidermal cell expressing fluorescently tagged CLC2-tagRFP and TPLATE-GFP. Scale bar = 5 μ m. B) Representative kymograph of CLC and TPLATE lifetimes on the PM. C) Example of time lapse image sequence from the single endocytic event positive for both CLC2 and TPLATE. Black arrows mark the appearance and disappearance of the fluorescence signals on the plasma membrane. Each frame represents 1 second. D) Fluorescence intensity quantification of the CLC2 (magenta) and TPLATE (green) spots in the example image sequence of the single CME event shown in C. Data from five independent experiments were combined to generate D) a mean lifetime, E) the lifetime distribution, F) a mean density of CME events and G) a mean recruitment profile of TPLATE to the site of endocytosis. Plots are mean $\pm$ SEM. N = 5 cells from independent roots, 11361 tracks.

Figure 6 – FM4-64 uptake assay

A) The chemical structure and B) fluorescence excitation/emission spectra of FM4-64 (adapted from ThermoFisher bioscience Fluorescence SpectraViewer). C) Schematic diagram of FM dye uptake assay; at T_0 the plant is incubated with the dye and at T_{end} , after washing the non-internalized dye away, FM dye is internalized via endocytosis from the PM. D) A basic FM experimental plan. E) An example CLSM image of the epidermal layer of the *Arabidopsis* root after FM incubation. F) (upper) A zoom of the yellow region of interest, (middle) example of the automated membrane and bottom (intra-cellular) thresholding used for quantification. G) An example of a single cell and its PM and intra-cellular segmentations (yellow asterisks in F), which is used to calculate a ratio between the PM and intra-cellular signals.

Figure 7 – Inhibition of endocytosis by Ikarugamycin

A) Representative confocal images of epidermal root cells incubated with FM4-64 in the presences of mock (DMSO) treatment and IKA (30 μ M) treatment. Scale bar = 20 μ m. B) Quantification of the membrane uptake. Plots are mean $\pm$ SEM. N, DMSO = 6 independent seedlings, 170 cells; IKA = 6 independent seedlings, 144 cells. T-test, p -value = 0.0009. C) Example TIRF-M images and kymographs corresponding to mock- (DMSO) or IKA- (30 μ M

15 mins) treated root cells expressing CLC2-GFP. The detections panel highlights tracks which are present within the first 100 frames of the movie; D) green and red denote tracks that do or do not persist over 100 frames (100 s), respectively relative to the beginning frame (start of sequence). E) Data from multiple experiments are combined and plotted as the percentage of tracks within the first 100 frames which persist for this duration. Plots are mean $\pm$ SEM. N = DMSO, 5 cells from independent roots, 3861 tracks; IKA, 3 cells from independent roots, 3614 tracks. T-test, p -value = 0.0005.