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

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

3 **Running Title:** Plant CME imaging toolbox

4  
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12 **Keywords;** quantitative imaging, clathrin-mediated endocytosis, metal-replica electron  
13 microscopy, total internal reflection fluorescence microscopy, confocal microscopy,  
14 *Arabidopsis*

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

18 **Abstract**

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

### 33 **Introduction**

34 Clathrin-mediated endocytosis (CME) is a major mechanism by which plasma membrane  
35 (PM) and extracellular cargo, which include cell surface receptors or extracellular materials,  
36 are internalized into cells (Bitsikas et al., 2014, McMahon and Boucrot, 2011). It is a  
37 dynamic and highly regulated multi-step process requiring the vesicle coat protein clathrin,  
38 and a large number of distinct endocytosis accessory proteins (EAPs). EAPs are dynamically  
39 recruited to CME events on the PM at different time points to regulate each unique step of the  
40 CME reaction; such as cargo recruitment, membrane invagination, scission/severing of the  
41 clathrin coated vesicle (CCV) from the PM and their uncoating (Kaksonen and Roux, 2018).  
42 CME plays an important role in many physiological processes in plants ranging from growth  
43 and development, cell polarity, intra- and inter-cellular signaling, nutrient uptake, stress  
44 response and pathogen defense (Barberon et al., 2011, Dhonukshe et al., 2007, Di Rubbo et  
45 al., 2013, Kitakura et al., 2011, Martins et al., 2015, Mbengue et al., 2016, Ortiz-Morea et al.,  
46 2016, Yoshinari et al., 2016, Zwiewka et al., 2015). Despite its physiological significance,  
47 little is known about the molecular mechanisms of how it functions in plants, especially when  
48 compared to mammalian and yeast model systems (Kaksonen and Roux, 2018, Lu et al.,  
49 2016).

50         The great advances in of CME studies in mammalian and yeast fields over the past 40  
51 years are mainly thanks to key imaging technologies that have emerged as standard  
52 approaches allowing the direct quantitative characterization of CME at very high spatial and  
53 temporal resolutions (Kaksonen and Roux, 2018, Lu et al., 2016, Picco and Kaksonen, 2018,  
54 Robinson, 2015, Schmid, 2019, Sochacki and Taraska, 2019). For example, electron  
55 microscopy (EM) approaches have allowed the direct visualization of the clathrin coat itself  
56 (Fotin et al., 2004, Heuser, 1980) and live imaging of single events of CME on the cell  
57 surface have unraveled the complex temporal network of EAPs in live cells (Taylor et al.,  
58 2011). Importantly, the quantitative analysis systems combined with these imaging  
59 approaches are openly reported (Aguet et al., 2013, Loerke et al., 2009), thus providing  
60 standardized protocols making direct comparisons of experimental data from multiple groups  
61 possible. Despite the plant field lagging behind in characterization of this key process, in  
62 recent years there has been significant progress in the identification of evolutionarily  
63 conserved and plant-specific EAPs, and the understanding of their regulation, which have  
64 evolved to meet the unique requirements necessary for plant morphogenesis and growth  
65 (Adamowski et al., 2018, Barberon et al., 2011, Bashline et al., 2013, Beck et al., 2012,

66 Dhonukshe et al., 2007, Fan et al., 2013, Gadeyne et al., 2014, Gifford et al., 2005, Kim et al.,  
67 2013, Konopka et al., 2008, Martins et al., 2015, Mazur et al., 2020, Paciorek et al., 2005,  
68 Sharfman et al., 2011, Takano et al., 2005, Yoshinari et al., 2016, Zhou et al., 2018). While  
69 many of these studies have been driven by the application and optimization imaging  
70 protocols of endocytosis, there is a need to standardize and approaches to directly examining  
71 plant CME which will allow direct comparison of data from differing groups possible, thus  
72 improving our ability to work together to characterize a fundamental physiological process.

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

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

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

## 100 **Methodologies for the imaging and analysis of plant CME**

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

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

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

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

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

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

#### 145 *Transmission electron microscopy*

146 Transmission electron microscopy (TEM) imaging approaches are widely used in CME  
147 studies in other model systems (Sochacki and Taraska, 2019). Whole cells, or tissues, are  
148 fixed and embedded into a resin for ultrathin slicing (40-70 nm). These sections can then be  
149 imaged to provide information on a single plane through the sample, or serial sections can be  
150 aligned and composed to produce a 3D ultra-structural view of the cell. While TEM has been  
151 used routinely in plants, and CCVs are visible and detectable in plant samples (Bonnett and  
152 Newcomb, 1966, Dejonghe et al., 2016, Dhonukshe et al., 2007, Lam et al., 2001, Li et al.,  
153 2012, Safavian and Goring, 2013), the preservation of CCVs is incredibly low regardless of  
154 fixing or embedding methods used. This has made it extremely difficult to visualize enough  
155 CME events to provide a robust quantitative analysis of plant CCVs.

#### 156 *Unroofing metal replica*

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

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

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

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

184

## 185 **Methods for directly imaging the cell surface; characterization of CME at the single** 186 **event level**

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

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

#### 205 *Single Channel EAP cell surface analysis*

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

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

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

#### 229 *Dual Channel EAP cell surface analysis*

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

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

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

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

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

265 As an alternative to TIRF-M and SD confocal microscopy, variable-angle  
266 epifluorescence microscopy (VAEM) (Chen et al., 2018, Higaki, 2015, Konopka and  
267 Bednarek, 2008, Wan et al., 2011), also known as highly inclined thin illumination (HILO)  
268 microscopy (Tokunaga et al., 2008), can be used. It overcomes some of the limitations of  
269 confocal systems (their lack of sensitivity for low levels of signal intensities) and TIRF-M  
270 (the shallow illumination volume). Instead of using TIRF illumination, the angle of incidence  
271 of the excitation beam is oblique such that it undergoes refraction instead of reflection  
272 towards the coverslip. In images where the excitation beam penetration is relatively shallow  
273 the signal to noise ratio of VAEM/HILO approaches that of TIRF-M (Wan et al., 2011).  
274 However, the depth of Z penetration is not uniform across the image (Supplemental Figure  
275 2B), which can introduce variability when one is trying to measure the dynamics of proteins  
276 in a single Z plane, such as the EAPs on the PM.

#### 277 *Considerations for the use of fluorescent protein tagged reporters*

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

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

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

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

304

### 305 **Methods for the quantitative analysis of cargo internalization; characterization of CME** 306 **at the whole tissue level**

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

#### 314 *FM Dyes*

315 FM dyes are a series of amphiphilic styryl dyes that have been used as tools across the model  
316 systems to measure net internalization of the PM (Bolte et al., 2004, Cheung and Cousin,  
317 2011, Jelinkova et al., 2010, Mueller et al., 2004). They contain a central region which is  
318 flanked by a hydrophobic tail and a polar head group (Figure 6A) (Betz et al., 1996). The

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

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

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

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

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

### 361 *Fluorescently labeled cargo uptake assays*

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

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

384 plant lines (Bandmann et al., 2012, Cao et al., 2020, Gadeyne et al., 2014, Leborgne-Castel et  
385 al., 2008, Mbengue et al., 2016). However, it is important to consider that to examine a  
386 protein of interest, it relies upon overexpression, and there appears to be differences in the  
387 vesicle trafficking machinery/processing between *Arabidopsis* and tobacco (Langhans et al.,  
388 2011).

#### 389 *Photo-convertible fluorescent tags of CME cargos*

390 Photo-convertible fluorescent tags such as EOS or Dendra can provide information about  
391 specific populations of tagged proteins. This is because after stimulation with a high intensity  
392 405 nm laser burst, its emission spectra irreversibly changes from green to red (Gurskaya et  
393 al., 2006), thereby enabling the discrimination between distinct protein populations that differ  
394 in terms of their lifetimes and/or subcellular distribution. In the case of CME cargo proteins  
395 that reside at the PM, the use of photo conversion permits the direct examination of the  
396 uptake of a specific cargo in any plant tissue and has been utilized in plants to address the  
397 internalization of PIN cargoes and their regulation by plant hormones (Jasik et al., 2016,  
398 Salanenka et al., 2018). As these assays are specifically looking at a defined cargo, they can  
399 provide greater insight into how CME is utilized by the cell in cellular processes. For  
400 example, in cases where PIN2-Dendra has been utilized to examine its internalization via  
401 CME, this direct approach has highlighted that the PIN2 dynamics reported from indirect  
402 measures of CME were not physiologically accurate (Jasik et al., 2016, Kleine-Vehn et al.,  
403 2008b).

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

407

#### 408 **Probing the mechanisms of CME through pharmacological and genetic manipulation**

409 A classical way to determine the mechanisms underlying biological processes is to disrupt it  
410 using pharmacological agents and/or through genetic manipulation. In this manner, testable  
411 hypotheses regarding the function of proteins of interest can be formulated and  
412 experimentally evaluated. Pharmacological and genetic manipulations that interfere directly  
413 with the process of CME (Supplemental Tables 3 and 4) are a very useful tools for the  
414 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 (Ruscinova 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).

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

#### 449 *Specific CME pharmacological agents*

450 Pharmacological manipulations of EAPs and CME are routinely used in CME investigation,  
451 where most of the drugs have been developed in other model systems (Dutta and Donaldson,  
452 2012, von Kleist and Haucke, 2012). Recent advances in our understanding of plant CME  
453 have highlighted that plant CME functions in many evolutionary unique ways (Adamowski et  
454 al., 2018, Barberon et al., 2011, Bashline et al., 2013, Beck et al., 2012, Dhonukshe et al.,  
455 2007, Fan et al., 2013, Gadeyne et al., 2014, Gifford et al., 2005, Kim et al., 2013, Konopka  
456 et al., 2008, Martins et al., 2015, Paciorek et al., 2005, Sharfman et al., 2011, Takano et al.,  
457 2005, Yoshinari et al., 2016, Zhou et al., 2018), thus when using inhibitors from non-plant  
458 systems it is important to validate their activity and examine potential off target effects. For  
459 example, the commonly used CME inhibitor, tyrphostinA23 (TyrA23) (Banbury et al., 2003),  
460 was found to operate through distinct mechanisms. In animal cells, TyrA23 targets the EAP  
461 AP-2 complex but in plant cells it appears to predominantly act by disrupting intracellular pH  
462 gradients (Dejonghe et al., 2016). Additionally, the small molecule inhibitor of mammalian  
463 CME Pitstop 2, while reducing FM uptake in a concentration dependent manner, it was  
464 reported that the internalization of CME cargos were not inhibited in *Arabidopsis* seedlings  
465 (Dejonghe et al., 2019).

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

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

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

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

#### 499 *Genetic manipulations of EAPs to investigate plant CME*

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

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

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

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

### 537 *Quantitative Analysis of CME inhibition*

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

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

561

## 562 **Discussion and Conclusions**

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

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

576 Another important area for the standardization of plant CME investigations is the  
577 highlighting of robust EAP antibodies. To date, many antibodies have led to key insights in to  
578 the interactions and localization of EAPs in both pull-down assays and immunohistochemical  
579 staining of plant tissues (Dejonghe et al., 2019, Dhonukshe et al., 2007, Di Rubbo et al.,  
580 2013, Gadeyne et al., 2014, Gao et al., 2019). Therefore, to further broaden the  
581 standardization of plant CME tools to cover antibodies, researchers should be encouraged to  
582 submit their routinely used antibodies to online databases, such as Antibodypedia (Bjorling  
583 and Uhlen, 2008).

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

595

## 596 **Expanded Methods and Materials**

597 Matlab code with detailed additional detailed instructions can be downloaded at  
598 10.5281/zenodo.3888519 and will be maintained/updated at  
599 [www.github.com/ajohnsoncode/plantCmeMethods](http://www.github.com/ajohnsoncode/plantCmeMethods). Example data can be found at  
600 10.5281/zenodo.3888531.

### 601 **Expanded Method 1: SEM visualization of *Arabidopsis* protoplast CCVs using metal** 602 **replica of unroofed cells**

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

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

### 609 *Cell Preparation*

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

634

### 635 *Unroofing of cells*

- 636 8. The excess protoplasts are removed gently from the coverslips using a Pasteur pipette,  
637 and samples are washed briefly with PBS (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ). Then they are  
638 equilibrated to room temperature in the same dish.
- 639 9. Extraction solution (2  $\mu\text{M}$  phalloidin, 1% Triton X-100 (w/v) and 1% polyethylene glycol  
640 (PEG; MW 20000; w/v) in PEM buffer (100 mM PIPES free acid, 1 mM  $\text{MgCl}_2$ , 1mM  
641 EGTA; pH 6.9 adjusted with KOH)) was equilibrated to room temperature and applied  
642 for 4 min at RT with gentle agitation.
- 643 10. Samples are then washed in PEM buffer plus 1% PEG 3 times 1 min each at RT.
- 644 11. They are then fixed with 2% glutaraldehyde (v/v) in 0.1 M PB, pH 7.4, for 20 min at RT  
645 and then washed in ddH<sub>2</sub>O 3 x 5 min each at RT.
- 646 12. Then the sample are further incubated with 0.1% tannic acid in water (w/v) for 20 min at  
647 RT and then washed twice with excess of ddH<sub>2</sub>O 5 min each.
- 648 13. The samples are treated with 0.2% uranyl acetate (w/v) in water for 20 min at RT and  
649 then washed twice with ddH<sub>2</sub>O.

650

#### 651 *Dehydration*

- 652 14. Lens tissue with loosely arranged fibers (Kimberly-Clark®) are cut into squares, a little  
653 larger than the diameter of sample holders for critical point drying (CPD; round wire  
654 baskets, Leica Microsystems).
- 655 15. The CPD sample holders are placed in a glass beaker filled with distilled water and a  
656 piece of lens tissue is put at the bottom of each holder. A coverslip with cells facing up is  
657 then placed on the lens tissue, covered with another piece of lens tissue. This loading  
658 procedure, alternating coverslips and lens tissue, is continued until the holders are filled.  
659 Samples are always kept in water.
- 660 16. Sample holders are then placed on a homemade standing device for quick transfer from  
661 glass beaker to glass beaker with increasing concentrations of ethanol in water. These are  
662 10%, 20%, 40%, 60%, 80%, 96% and 3 times 100%. Incubation times are 5 min each at  
663 RT. The standing device is constructed in a way that a magnetic rod fit under the  
664 mounting plate for sample holders and the solution can be kept under constant agitation  
665 on a magnetic stirrer.

666

#### 667 *Critical point drying and coating*

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

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

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

675

#### 676 *Imaging*

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

679

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

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

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

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

688

#### 689 Notes and considerations;

690 1 – The specific cells used here are Col-0 *Arabidopsis* root-derived suspension cultures, and  
691 were gifted to us by Eva Kondorosi (Gif-sur-Yvette, France). They were maintained and  
692 grown in growth media (GM) (4.25 g/l of MS salts, 30 g/l of sucrose, 0.250 mg of 2,4D,  
693 0.015 mg of kinetin and 2 ml Vitamin B5 stock (100 ml stock contains 0.1 g nicotinic acid,  
694 0.1 g pyridoxine HCl, 1 g thiamine HCl. 10 g myo-inositol) at pH 5.7).

695 2 - The enzyme solution and the GM buffer are filter sterilized and stored at 4°C.

696 3 – Centrifugations from step 3 are done at RT with no breaks to avoid damaging of digested  
697 cells.

698

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

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

710 *Tissue Preparation;*

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

718 *Coverslip preparation;*

- 719 4. During this incubation period, coverslips 24 mm x 50 mm, thickness 1.5 (VWR #631-  
720 0147) are prepared and cleaned as follows (Note 2);
  - 721 a. Coverslips are placed in a coverslip holder (Sigma, Wash-N-Dry) and washed in  
722 ‘cleaning’ solution (Decon 90 0.01% (w/v), NaOH 100 mM) in a 250 ml beaker  
723 (Supplement Figure 7, Photo 2) and incubated for 15 mins.
  - 724 b. Coverslips are then cleaned using lens tissue (GE Healthcare, Whatman) and placed  
725 back into the coverslip holder.
  - 726 c. The coverslips are washed again in ‘cleaning’ solution for a further 15 mins.
  - 727 d. The coverslips are then washed at least 5 times in ddH<sub>2</sub>O, 5 mins each, until there are  
728 no more detergent bubbles in the solution.

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

736 *Sample preparation;*

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

749 *TIRF-M Imaging;*

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

787 5 – The location of imaging is critical for producing good TIRF-M images and for  
788 reproducible results (Figure 3A). This is because it has been reported that the kinetics of  
789 CME change with the different stages of develop in the roots (Konopka et al., 2008). Also,  
790 cells in the elongation zone are used for imaging as they are normally lie flat on the coverslip,  
791 which allows distortion free TIRF imaging of the roots. It is important to pick a cell which  
792 presents a uniform field of illumination across the sample, and it free from large auto-  
793 fluorescent structures (Supplemental Figure 2). It is also essential to image cells located at the  
794 same distance from the root tip so comparison between genotypes/conditions. The end of the  
795 lateral root cap can be used as a reference (Figure 3).

796 6 - The resultant lifetime is highly dependent upon the frame rate used to image. This is due  
797 to the high level of short transient visits of EAPs to the cell surface in plants, therefore a rapid  
798 acquisition rate (<1 Hz) will include many more of these events compared to a slow rate  
799 (Johnson and Vert, 2017).

800 7 – Particle detection and tracking of the EAPs is conducted using just the detection and  
801 tracking programs of the cmeAnalysis package (Aguet et al., 2013), as the further filtering in  
802 this package is highly optimized for mammalian CME events and not suitable for plant CME  
803 analysis. Although there are other automated detection systems available (see (Loerke et al.,  
804 2009, Tinevez et al., 2017) for alternatives), the detection method presented here outperforms  
805 manual and other programs in robustness and sensitivity (Mettlen and Danuser, 2014). The  
806 tracks are filtered for analysis to only include; tracks absent from the first and last 10 frames  
807 of the movie, not within 10 pixels of the edge of the movie and which persist long than 5  
808 frames. The lifetime is then calculated as the mean of all the combined tracks. The density is  
809 generated by imposing a 100 x 100 pixel region of interest (ROI) in the center of the movie,  
810 and then mean number of valid tracks is calculated over 100 frames (where the time range of  
811 frames used is 50 before the middle time lapse image to 50 after the middle time lapse  
812 image). This means the image of the cell must be larger than this 100 x 100 pixel ROI and the  
813 duration must be 101 or more frames. To test for significance, data from multiple  
814 experiments were combined and subjected to an unpaired, parametric two tailed T-Test. The  
815 mean profile is generated by combining and normalizing all the intensity profiles of tracks  
816 which have a lifetime within the range of mean lifetime +/- 3 frames and aligned to their  
817 termination.

818 8 – Dual channel analysis was conducted using a primary channel/secondary channel  
819 approach. The tracks in the primary channel are detected, tracked and filtered as in the single  
820 channel analysis (Note 5) A further filtering is conducted where the master track must have a  
821 significant signal in the secondary channel which persists for more than 5 frames. The filtered  
822 tracks are then used to calculate the mean lifetime and density as in note 5. The mean profile  
823 is generated in the same way as ‘Note 5’, but additionally the slave profiles normalized and  
824 both the primary and secondary profiles are plotted.

825 9 – These analysis systems have been tested and found to work on spinning disc PM data, but  
826 strong levels of fluorophore expression are required (Wang et al., 2020).

827

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

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

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

#### 844 *Tissue Preparation;*

- 845 1. 10-20 sterilised Col-0 seeds were plated on to AM+ and 1% sucrose agar plates, with  
846 ample space for the roots to grow vertically.
- 847 2. The plates were incubated for 2 days at 4 °C in darkness.

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

#### 850 *Treatment and Imaging*

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

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

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

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

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

#### 871 *Analysis*

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

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

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

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

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

882 Notes and considerations;

883 1 – IKA (Abcam; ab143408) was used at 30  $\mu$ M for 15 minutes.

884 2 - FM4-64 (ThermoFisher; T3166) is a ‘red’ dye, so can be used to with a GFP marker to  
885 determine if a labelled cargo can enter the FM labelled early endosomes. There are other dyes  
886 available, for example FM1-43 (ThermoFisher; T3163), emitting in the yellow range which  
887 function in the same way (Supplemental Figure 3). FM4-64 and FM1-43 were used at 2  $\mu$ M  
888 and incubated for 15 mins.

889 3 – Prolonged FM treatment has been reported to be toxic to plant tissues (Meckel et al.,  
890 2004), so long term treatments are not advised.

891 3 – The location of cells, and region of the plant, chosen for analysis is important, as different  
892 developmental stages of the root have been reported to have different kinetic rates of  
893 endocytosis (Konopka et al., 2008).

894 4 – The value entered effects the strength of the image segmentation (Supplemental Figure  
895 6). First, a Gaussian filter is applied to the image and then the enter threshold value  
896 determines the Luminance threshold to binarize the image, which is used to define the PM  
897 and intracellular regions.

898 5 – Cells selected should be not contain areas where there are clear dark shadows visible  
899 inside the cell, as these are most likely organelles deep in the cell which physical exclude FM  
900 containing structures from the cytoplasmic space (Supplemental Figure 6A).

901 6 – Selected cells should be separated from the surrounding cells and outlines of badly  
902 segmented cells (Supplemental Figure 6B). This step can be checked immediately after the  
903 cell selection.

904 7 - No sample size calculation was conducted. Data from multiple experiments were  
905 combined and tested for significance using an unpaired, parametric two tailed T-Test in  
906 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 1<sup>st</sup> 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.

935 3 - To test for significance, data from multiple experiments were combined and subjected to  
936 an unpaired, parametric two tailed T-Test. No sample size calculation was conducted.

937 4 – Inhibitor experiments used IKA (Abcam; ab143408) and Dyngo-4a (Abcam; ab120689)  
938 at 30  $\mu$ M and with a 15 minute incubation.

939

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#### 949 **Competing Interests**

950 All authors declare there are no conflicts of interests.

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#### 956 **Data availability**

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

#### 958 **Author Contributions**

959 A.J. and J.F. conceived the project and designed the experiments. Data collection was  
960 conducted by A.J., N.G., W.A.K. and M.N. Analysis was completed by A.J. and N.G, where  
961 the analysis protocols presented were developed by A.J. The article was written by A.J.,  
962 S.Y.B and J.F, with contributions of intellectual content from W.A.K., M.N, G.V. and  
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## 1341 **Figure Legends**

### 1342 **Figure 1 – Schematic of the principles of the major CME light microscopy methods**

1343 TIRF-M uses a weak uniform evanescent wave that penetrates ~100-200 nm into the sample,  
 1344 thereby illuminating cell surfaces in contact with the coverslip. The evanescent illumination  
 1345 wave is generated when the illumination beam hits a refractive index mismatch between the  
 1346 coverslip and sample/media at the critical angle ( $\theta$ ). The energy of the evanescent wave is  
 1347 directly proportional to the distance away from the point of generation, meaning that  
 1348 fluorophores closer to the PM are stimulated more than those deeper in the cell (green-grey  
 1349 GFP spots). CLSM makes use of a single pinhole, which blocks out-of-focus emitted light  
 1350 from reaching the detector (grey lines). In CLSM, the illumination beam passes directly into

1351 the sample. SD confocal microscopy makes use of disks with many pinholes and micro-lens  
1352 which rapidly spins, thus creating many simultaneous confocal points.

### 1353 **Figure 2 – Schematic of unroofing protoplasts and SEM**

1354 A) *Arabidopsis* protoplasts cells are plated onto poly-L-lysine coated coverslips. B) The cells  
1355 are washed with detergent to ‘unroof’ the cells. C) The unroofed cells are then covered with a  
1356 thin coat of platinum. D) Samples are imaged using SEM. Main image shows a high  
1357 magnification view of a replica in which CCVs associated with the PM are identifiable  
1358 (yellow arrows). Scale bar = 200 nm. Insert in panel D) represents low magnification view of  
1359 a whole cell replica, scale bar 2  $\mu$ m.

### 1360 **Figure 3 – Schematic of cell surface TIRF-M imaging**

1361 A) To facilitate TIRF-M, the sample needs to be flat and in direct contact of the coverslip.  
1362 The location of imaging within the root is critical in obtain reproducible results. The end of  
1363 the lateral root cap (LRC) is used as a developmental marker, and cells 5-8 up the root are  
1364 used for imaging. B) Lifetimes of proteins on the PM are only when the protein is within the  
1365 illumination volume. C) Time and intensity fluorescent profiles of proteins can provide hints  
1366 about their physiological function. For example, as clathrin triskelia containing fluorescently  
1367 labeled clathrin subunits (blue) polymerize on the PM to form invaginating clathrin coated  
1368 pits, there is an increase in the level of fluorescence signal. Upon scission of the labeled  
1369 CCVs from the PM they rapidly depart from the illumination field resulting in a sharp  
1370 decrease in fluorescence signal. Dual channel imaging of proteins of interest with a  
1371 fluorescently tagged clathrin marker permits the quantitative comparison of its temporal  
1372 dynamics relative to clathrin coated pit initiation, maturation and CCV departure from the  
1373 PM (green and red examples as depicted in B).

### 1374 **Figure 4 –Single channel TIRF-M cell surface analyses**

1375 A) Example TIRF-M image from a root epidermal cell expressing CLC2-GFP. B) An  
1376 example kymograph generated of the yellow line in A. C) Zoomed in image of the region of  
1377 the yellow square in A. D) The results of the automated detection and tracking analysis. E)  
1378 Time series of an example single CLC event on the PM and F) quantitation of its lifetime  
1379 based on the fluorescent intensity profile, which is significantly above the intensity of  
1380 surrounding pixels. Tracking data from multiple independent tracks and experiments are  
1381 combined to generate G) a mean lifetime, H) the lifetime distribution, I) a mean density of

1382 spots and J) a mean fluorescent profile. Plots are mean +/- SEM. N = 4 cells from  
1383 independent roots, 20098 tracks. Scale bar, A = 5  $\mu$ m.

#### 1384 **Figure 5 – Dual Channel TIRF-M cell surface analyses**

1385 A) TIRF-M images of a root epidermal cell expressing fluorescently tagged CLC2-tagRFP  
1386 and TPLATE-GFP. Scale bar = 5  $\mu$ m. B) Representative kymograph of CLC and TPLATE  
1387 lifetimes on the PM. C) Example of time lapse image sequence from the single endocytic  
1388 event positive for both CLC2 and TPLATE. Black arrows mark the appearance and  
1389 disappearance of the fluorescence signals on the plasma membrane. Each frame represents 1  
1390 second. D) Fluorescence intensity quantification of the CLC2 (magenta) and TPLATE  
1391 (green) spots in the example image sequence of the single CME event shown in C. Data from  
1392 five independent experiments were combined to generate D) a mean lifetime, E) the lifetime  
1393 distribution, F) a mean density of CME events and G) a mean recruitment profile of TPLATE  
1394 to the site of endocytosis. Plots are mean +/- SEM. N = 5 cells from independent roots, 11361  
1395 tracks.

#### 1396 **Figure 6 – FM4-64 uptake assay**

1397 A) The chemical structure and B) fluorescence excitation/emission spectra of FM4-64  
1398 (adapted from ThermoFisher bioscience Fluorescence SpectraViewer). C) Schematic diagram  
1399 of FM dye uptake assay; at  $T_0$  the plant is incubated with the dye and at  $T_{end}$ , after washing  
1400 the non-internalized dye away, FM dye is internalized via endocytosis from the PM. D) A  
1401 basic FM experimental plan. E) An example CLSM image of the epidermal layer of the  
1402 *Arabidopsis* root after FM incubation. F) (upper) A zoom of the yellow region of interest,  
1403 (middle) example of the automated membrane and bottom (intra-cellular) thresholding used  
1404 for quantification. G) An example of a single cell and its PM and intra-cellular segmentations  
1405 (yellow asterisks in F), which is used to calculate a ratio between the PM and intra-cellular  
1406 signals.

#### 1407 **Figure 7 – Inhibition of endocytosis by Ikarugamycin**

1408 A) Representative confocal images of epidermal root cells incubated with FM4-64 in the  
1409 presences of mock (DMSO) treatment and IKA (30 $\mu$ M) treatment. Scale bar = 20  $\mu$ m. B)  
1410 Quantification of the membrane uptake. Plots are mean +/- SEM. N, DMSO = 6 independent  
1411 seedlings, 170 cells; IKA = 6 independent seedlings, 144 cells. T-test,  $p$ -value = 0.0009. C)  
1412 Example TIRF-M images and kymographs corresponding to mock- (DMSO) or IKA- (30  $\mu$ M

1413 15 mins) treated root cells expressing CLC2-GFP. The detections panel highlights tracks  
1414 which are present within the first 100 frames of the movie; D) green and red denote tracks  
1415 that do or do not persist over 100 frames (100 s), respectively relative to the beginning frame  
1416 (start of sequence). E) Data from multiple experiments are combined and plotted as the  
1417 percentage of tracks within the first 100 frames which persist for this duration. Plots are mean  
1418 +/- SEM. N = DMSO, 5 cells from independent roots, 3861 tracks; IKA, 3 cells from  
1419 independent roots, 3614 tracks. T-test,  $p$ -value = 0.0005.

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