

Image-Based Methods to Study Membrane Trafficking Events in Stomatal Lineage Cells

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Abstract

In eukaryotic cells, membrane components, including proteins and lipids, are spatiotemporally transported to their destination within the endomembrane system. This includes the secretory transport of newly synthesized proteins to the cell surface or the outside of the cell, the endocytic transport of extracellular cargoes or plasma membrane components into the cell, and the recycling or shuttling transport of cargoes between the subcellular organelles, etc. Membrane trafficking events are crucial to the development, growth, and environmental adaptation of all eukaryotic cells and, thus, are under stringent regulation. Cell-surface receptor kinases, which perceive ligand signals from the extracellular space, undergo both secretory and endocytic transport. Commonly used approaches to study the membrane trafficking events using a plasma membrane-localized leucine-rich-repeat receptor kinase, ERL1, are described here. The approaches include plant material preparation, pharmacological treatment, and confocal imaging setup. To monitor the spatiotemporal regulation of ERL1, this study describes the co-localization analysis between ERL1 and a multi-vesicular body marker protein, RFP-Ara7, the time series analysis of these two proteins, and the z-stack analysis of ERL1-YFP treated with the membrane trafficking inhibitors brefeldin A and wortmannin.

Introduction

Membrane traffic is a conserved cellular process that distributes membrane components (also known as cargoes), including proteins, lipids, and other biological products, between different organelles within a eukaryotic cell or across the plasma membrane to and from the extracellular space¹. This process is facilitated by a collection of membranes and organelles named the endomembrane system, which

consists of the nuclear membrane, the endoplasmic reticulum, the Golgi apparatus, the vacuole/lysosomes, the plasma membrane, and multiple endosomes¹. The endomembrane system enables the modification, packaging, and transport of membrane components using dynamic vesicles that shuttle between these organelles. Membrane trafficking events are crucial to cell development, growth,

and environmental adaptation and, thus, are under stringent and complex regulation². Currently, multiple approaches in molecular biology, chemical biology, microscopy, and mass spectrometry have been developed and applied to the field of membrane trafficking and have greatly advanced the understanding of the spatiotemporal regulation of the endomembrane system^{3,4}. Molecular biology is used for classical genetic manipulations of the putative players involved in membrane trafficking, such as altering the gene expression of the protein of interest or labeling the protein of interest with certain tags. Tools in chemical biology include the usage of molecules that specifically interfere with the traffic of certain routes^{4,5}. Mass spectrometry is powerful for identifying the components in an organelle that has been mechanically isolated by biochemical approaches^{3,4}. However, membrane traffic is a dynamic, diverse, and complex biological process¹. To visualize the membrane trafficking process in live cells under various conditions, light microscopy is an essential tool. Continuous progress has been made in advanced microscope techniques to overcome the challenges in measuring the efficiency, kinetics, and diversity of the events⁴. Here, this study focuses on the widely adopted methodologies in chemical/pharmacological biology, molecular biology, and microscopy to study membrane trafficking events in a naturally simplified and experimentally accessible system, the stomatal developmental process.

Stomata are micropores on plant aerial surfaces that open and close to facilitate gas exchange between the inner cells and the environment^{6,7,8}. Hence, stomata are essential for photosynthesis and transpiration, two events that are crucial for plant survival and growth. Stomatal development is dynamically adjusted by environmental cues to optimize the plant's adaptation to the surroundings⁹. Dating back to studies in 2002, the identification of the receptor protein

Too Many Mouths (TMM) opened the door to a new era of investigating the molecular mechanisms of stomatal development in the model plant *Arabidopsis thaliana*¹⁰. After just a few decades, a classical signaling pathway has been identified. From upstream to downstream, this pathway includes a group of secretory peptide ligands in the epidermal patterning factors (EPF) family, several cell-surface leucine-rich-repeat (LRR) receptor kinases in the ERECTA (ER) family, the LRR receptor protein TMM, a MAPK cascade, and several bHLH transcription factors including SPEECHLESS (SPCH), MUTE, FAMA, and SCREAM (SCRM)^{11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26}. Previous work indicates that one of the receptor kinases, ERLIKE 1 (ERL1), demonstrates active subcellular behaviors upon EPF perception²⁰. ERL2 also dynamically traffics between the plasma membrane and some intracellular organelles²⁷. Blocking the membrane trafficking steps causes abnormal stomatal patterning, resulting in stomatal clusters on the leaf surface²⁸. These results suggest that membrane traffic plays an essential role in stomatal development. This study describes a protocol to spatiotemporally investigate the ERL1 dynamics using protein-protein subcellular co-localization analysis combined with pharmacological treatment using some membrane trafficking inhibitors.

Protocol

1. Preparation of the solutions

1. Prepare seed sterilization solution by mixing 15 mL of bleach with 35 mL of distilled water and 50 μ L of Triton X-100.
2. Prepare brefeldin A (BFA) solution by dissolving the BFA powder in ethanol to a final concentration of 10 mM

(stock). Prepare wortmannin (Wm) solution by dissolving the Wm powder in DMSO to a final concentration of 10 mM (stock).

2. Sowing the seeds

1. Aliquot 10-50 seeds from each of the required transgenic plants into 1.5 mL microcentrifuge tubes. Add 1 mL of seed sterilization solution into each tube, and mix thoroughly by inverting the tube gently for 10 min on a shaker.
2. Discard the seed sterilization solution, and wash the seeds with 1 mL of autoclaved distilled water five times.
3. Add 300 μ L of autoclaved distilled water into each tube, and sow the seeds on half-strength MS media containing 1% (w/v) sucrose and 0.75% (w/v) agar. Supplement the medium with the corresponding antibiotics as needed.
4. Keep the plate upside-down at 4 °C in the dark for 2 days to synchronize the germination.
5. After 2 days, transfer the plate into a growth room with a 16 h light/8 h dark cycle ($80 \mu\text{mol}/\text{m}^2/\text{s}^1$) at 22 °C. This is considered the first day post germination (1 dpd).
6. Transplant the seedlings into soil at 10 dpd for further growth.

3. Preparation of dual-colored F1 transgenic plants

1. Grow homozygous transgenic plants bearing *ERL1-YFP* (plant A) and homozygous transgenic plants bearing an RFP-labeled marker protein *RFP-Ara7* (plant B)²⁰ side by side until flowering in a growth room with a 16 h light/8 h dark cycle ($80 \mu\text{mol}/\text{m}^2/\text{s}^1$) at 22 °C.

2. Choose a young inflorescence from plant A. Keep one flower that is just about to open on the inflorescence for genetic crosses. Remove all the older flowers and siliques. Additionally, carefully remove the younger flowers and floral meristems to avoid future confusion.
3. Gently dissect the unopened flower by removing the sepals, petals, and stamen using a pair of sharp tweezers. Only leave the pistil on the inflorescence.
4. Take a mature stamen from an opened flower on plant B, and deposit the pollen grains onto the stigma of the dissected pistil on plant A.
5. Label this manually crossed flower by indicating its father, mother, and the date of the genetic cross. Let the flower mature, and harvest the F1 seeds when the silique turns yellow/brown (~20 days after the cross). Check for a successful F1 plant that has both YFP and RFP signals.

4. Pharmacological treatment

1. For the BFA treatment, remove the cotyledons of 7-day-old seedlings, immerse the rest of the seedlings into either a mock solution (0.3% ethanol) or 30 μ M BFA solution, apply vacuum for 1 min, and keep the sample immersed for 30 min before imaging.
2. For the wortmannin treatment, remove the cotyledons of 7-day-old seedlings, immerse the rest of the seedlings into either 0.25% DMSO solution or 25 mM wortmannin, apply vacuum for 1 min, and keep the sample immersed for 30 min before imaging.
3. For the vacuum treatment of samples, in a 1.5 mL microcentrifuge tube, add 500 μ L of the corresponding drug solution, and immerse the dissected seedlings into the solution. Tightly attach a 10 mL syringe to the microcentrifuge tube, and apply vacuum for 1 min

(**Figure 1**). Remove the syringe, and keep the sample

in the solution for the required time. Gently take out the seedlings for imaging preparation.



Figure 1: Simple vacuum device. A 10 mL syringe is attached to a 1.5 mL microcentrifuge tube for the vacuum treatment.

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5. Sample preparation for imaging

1. Dissect the true leaf from the treated sample using a sharp razor blade. Gently put the true leaf in a drop of water on a glass slide, and keep the abaxial side up. Slowly cover the true leaf with a coverslip while avoiding trapping any bubbles.

6. Confocal imaging

NOTE: A Leica SP8 inverted scanning confocal microscope was used to image the fluorescence signal of the samples in this work.

1. Beam path setup

1. Select a 514 nm laser for YFP excitation. Use a high laser power to increase the signal intensity and, thus, obtain high image quality. However, laser powers above 5% run the risk of photobleaching, and the health of the sample may be affected. If the sample signal is not too dim, start at low laser intensity.

2. Turn on PMT/HyD for detection, and define the upper and lower emission band thresholds based on the spectrum for the YFP fluorophore. Set a detection window of 530-570 nm to collect the YFP signal.
3. Sequential imaging for the second color: Click on the Seq button, and add a new channel. By default, the previously designed YFP beam path will be Seq 1. In Seq 2, select a 561 nm laser for the RFP excitation, and set a detection window of 570-630 nm to collect the RFP signal.

2. Scan conditions setup (**Figure 2**)

1. To image the subcellular membrane traffic activity within stomatal precursor cells, choose a 63x/1.2 W Corr lens on the system for imaging.
2. The **format** refers to the image size in pixels. Start with 1,024 pixels x 1,024 pixels, and then optimize this based on the zoom factor and the objective settings for a good resolution of publication quality.

3. The **speed** refers to the speed of the scanhead as the laser passes over each pixel. Although slow scan speeds often result in a better signal-to-noise ratio, they may not efficiently capture the rapidly changing membrane trafficking events. Start with a speed of 400 Hz, and optimize this based on the specific situation of the samples.
4. The **zoom factor** is used to zoom in on a region of interest without changing the objective lens. Start with a zoom factor of 1, and optimize this based on the specific needs of the experiment.
5. The **line average** refers to the number of times each X-line will be scanned to obtain an average result. A greater line average reduces the noise in the resulting image, but it also increases the scanning time and exposure time to the laser light. To image membrane trafficking events, do not use a high line average. Start with a 2x line average, and optimize as needed.

3. Collecting a time series

NOTE: When studying membrane trafficking events, a time series is often needed to record the rapid movement of the subcellular endosomes.

1. In the **Acquisition Mode**, select the **xyt** scan mode to enable the time series utility.
2. Decide a waiting period between the time points under **Time Interval**. Alternatively, click on **Minimize** to take the images immediately one after another. A 7 s time interval was used in the following time series experiment.
3. Select **Duration**, and enter the total time for the experiment to run. Alternatively, define the number

of frames to be collected by selecting **Frames** (Figure 3).

4. To collect the three-dimensional information regarding the membrane trafficking event within the entire cell, use the Z-stack scan mode. The maximum intensity projection of the Z-stack images is often used for the analysis.

1. Select the **xyz** scan mode in the **Acquisition Mode**, and then the Z-Stack utility will become accessible.
2. Select the **Z-wide** option. Under the **Scan Mode**, define the top image and the bottom image of the Z-stack using the **Begin** and **End** buttons.
3. Define the thickness of the z-step by clicking on z-step size. Alternatively, define how many images to take in order to cover the entire range of the Z-stack. To ensure consistency between the samples, define the z-step size (Figure 4).

5. Image processing

1. The maximum intensity projection of the z-stack images is generated by the confocal software (<http://www.leica-microsystems.com>). In the primary **Process** tab, first choose the interested z-stack file under the **Open Projects** tab on the far-left side. Then, switch to the middle tab named **Process Tools**, select the **Projection** function, choose **Maximum** in the dropdown panel of **Method**, and click on the **Apply** button. A z-stack projection image will be generated under the Open Projects tab.
2. The video of the time series images is generated by Fiji (<https://imagej.net/Fiji>). In the **File** tab, use the **Open** function to open all the time series images in the correct order. In the **Image** tab, find the **Stack**

function, and choose **Images to stack** to generate a

video. Save the file in the .avi format with the desired frame rate (5 frames/s for **Video 1**).



Figure 2: The scan mode panel of the XY dimension. The scan mode panel is used to set up the image scan conditions.

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Figure 3: The time series utility under the xyt scan mode. The time series utility is used to set up the imaging conditions to consecutively collect a series of images. [Please click here to view a larger version of this figure.](#)



Figure 4: The z-stack utility under the xyz scan mode. The z-stack utility is used to set up the imaging conditions to collect a series of images on the z-axis. [Please click here to view a larger version of this figure.](#)

Representative Results

A previous study indicated that ERL1 is an active receptor kinase that undergoes dynamic membrane trafficking events²⁰. ERL1 is a transmembrane LRR-receptor kinase on the plasma membrane. Newly synthesized ERL1 in the endoplasmic reticulum is processed in the Golgi bodies and further transported to the plasma membrane. The ERL1 molecules on the plasma membrane can perceive EPF ligands using their extracellular LRR domain¹⁸. Upon activation by the inhibitory EPFs, including EPF1, ERL1 is internalized into endosomes, transported to the multi-

vesicular bodies (MVB), and then transported into the vacuoles for degradation to terminate the signal²⁰. In contrast, the antagonistic EPFL9 ligand, which promotes stomatal formation¹⁴, retains ERL1 in the endoplasmic reticulum. Alternatively, the plasma membrane-localized ERL1 can recycle between endosomes and the plasma membrane²⁰.

Here, transgenic plants bearing *ERL1 promoter::ERL1-YFP* were grown for 7 days following the protocol mentioned above. When just emerging, the true leaf was dissected for imaging, as *ERL1* is only expressed in stomatal lineage cells in young leaves²⁰. As expected, scattered cells were

highlighted by the YFP signal, where ERL1-YFP labeled the plasma membrane (**Figure 5**). In addition, multiple punctate signals were also observed, suggesting that ERL1 was also localized to the endosomes.

Several organelles in plant cells present as punctate fluorescent signals when observed under a light microscope, such as the trans-Golgi network and the MVBs. When the MVB marker line bearing *RFP-Ara7* was genetically crossed with the *ERL1-YFP* transgenic line, both marker proteins were observed using the sequential imaging approach as described above. As shown in **Figure 6**, RFP-Ara7 highlighted multiple punctate signals in almost all the cells, indicating the MVBs in these cells (**Figure 6B**). When merging with the ERL1-YFP signal, most of the ERL1-YFP-positive punctate overlapped with the RFP-Ara7-labeled punctate. This result suggests that some of the ERL1-YFP molecules are transported to the MVBs.

To monitor the dynamics of these fast-moving endosomes, time series images of the 7-day-old true leaves bearing *ERL1-YFP* and *RFP-Ara7* were collected (**Figure 7**). Time intervals of 7 s were applied for 203 s, and the collected 30 images were used to generate a video in Image J (**Video 1**). The

video showed that ERL1-YFP-labeled endosomes moved together with RFP-Ara7 within the stomatal lineage cells, thus confirming that ERL1-YFP was localized to the MVBs.

To examine the ERL1 trafficking routes using a pharmacological approach, the effect of two commonly used membrane trafficking drugs, brefeldin A (BFA) and wortmannin (Wm), were analyzed. BFA, by inhibiting the ADP-ribosylation factor guanine-nucleotide exchange factors, including GNOM, can block the endosomal recycling and endocytosis of membrane proteins²⁹. After 30 min of exposure to the BFA solution, ERL1-YFP was detected in some large compartments known as BFA bodies (**Figure 8B, C**). This indicates that ERL1 trafficking can be blocked by BFA, suggesting that ERL1 undergoes recycling or endocytosis. Wm can inhibit phosphatidylinositol-3 and phosphatidylinositol-4 kinases and, thus, causes the fusion of MVBs⁵. When treated with Wm for 30 min, some ring-like structures highlighted by ERL1-YFP were observed, which are typically called Wm bodies (**Figure 8C**). This result indicates that ERL1-YFP is transported to MVBs, thus suggesting that ERL1 follows the route to vacuoles for degradation.

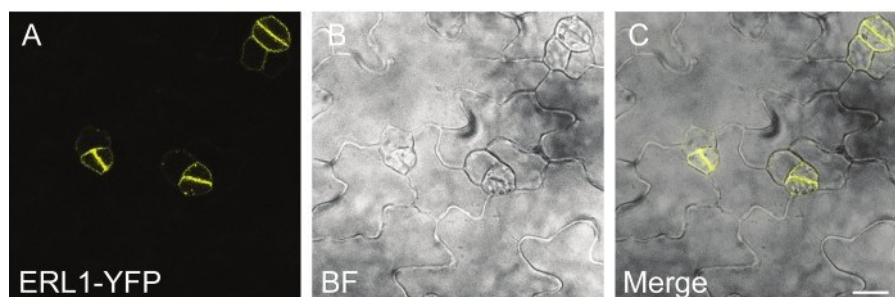


Figure 5: Single plane image of ERL1-YFP. (A) The fluorescence image, (B) the bright-field image, and (C) the merged image show that ERL1-YFP labels the plasma membrane and some highly mobile punctate within stomatal lineage cells.

Scale bar: 10 μ m. [Please click here to view a larger version of this figure.](#)

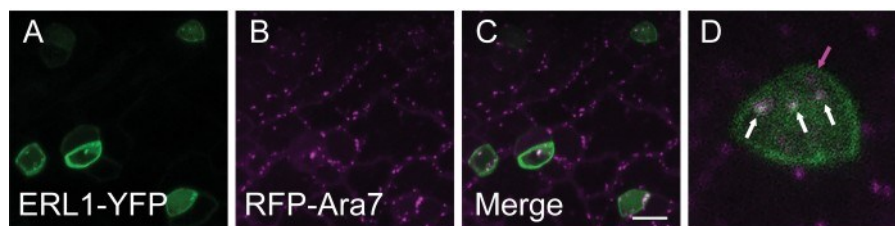


Figure 6: Sequential images of ERL1-YFP and RFP-Ara7. (A) ERL1-YFP and (B) RFP-Ara7 both label punctate signals within the cell. The white punctate in (C) the merged image and (D) the inset image indicates that the two proteins co-localize on the MVBs within stomatal lineage cells. The white arrows indicate endosomes labeled by both ERL1-YFP and RFP-Ara7.

The red arrows indicate endosomes labeled by RFP-Ara7. Scale bar: 10 μ m. [Please click here to view a larger version of this figure.](#)

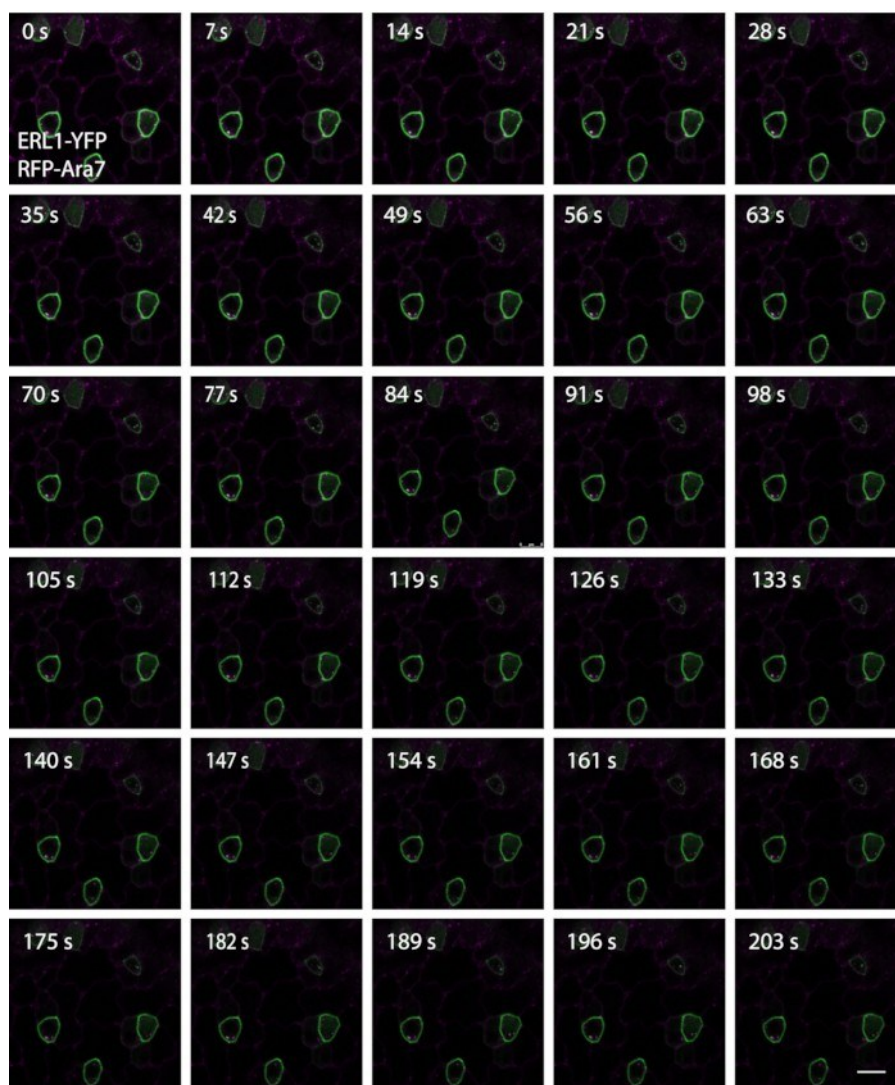


Figure 7: Time series images of ERL1-YFP and RFP-Ara7. The images were taken with a 7 s time interval. The time point of each image is indicated in the top-left corner. Scale bar: 10 μ m. [Please click here to view a larger version of this figure.](#)

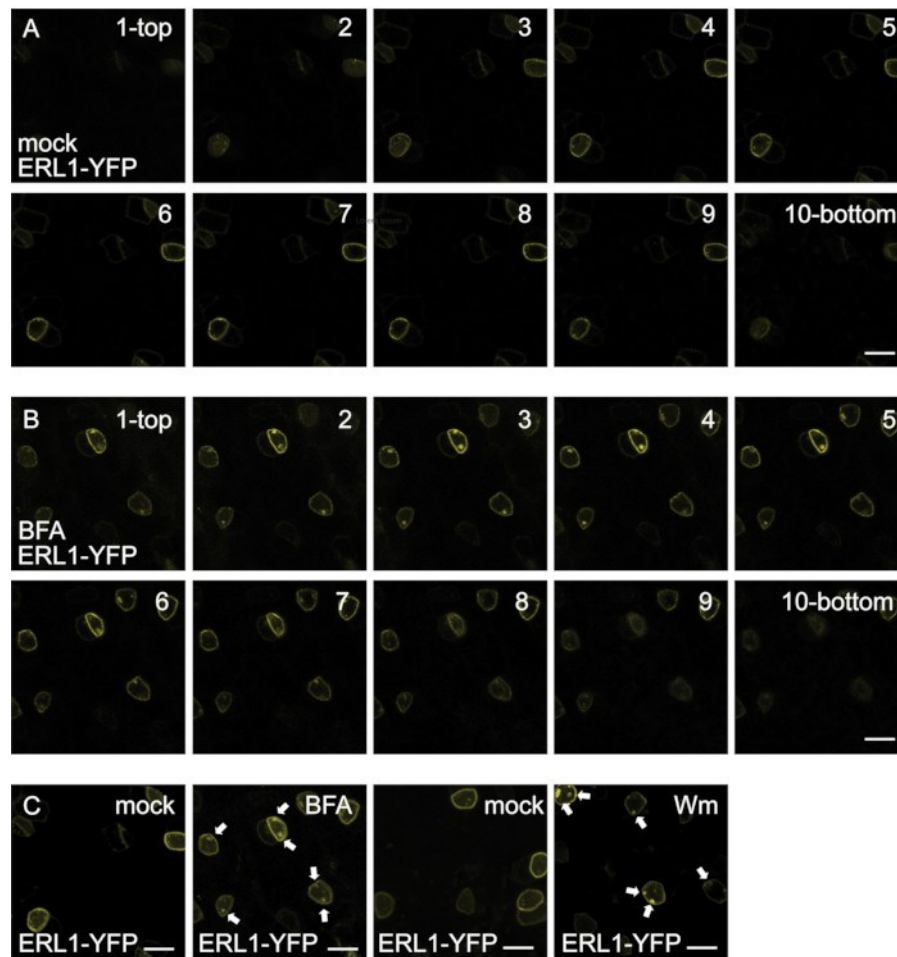


Figure 8: Z-stack images of ERL1-YFP. (A, B) Z-stack images of the ERL1-YFP plants treated with (A) mock or (B) BFA. The numbers in the top-right corner indicate the position of the image in the z-stack from top to bottom. Scale bar: 10 μ m. (C) Projection of the z-stack images of ERL1-YFP treated with mock or BFA solution and mock or Wm solution, as indicated in the top-right corner. The arrows indicate the aggregation bodies of ERL1-YFP caused by the drugs. Scale bar: 10 μ m. [Please click here to view a larger version of this figure.](#)

Video 1: Video of the time series images of ERL1-YFP and RFP-Ara7. The time series images of ERL1-YFP and RFP-Ara7 are stacked into a video using Fiji at a speed of 5 frames/s. [Please click here to download this Video.](#)

Discussion

The endomembrane system separates the cytoplasm of a eukaryotic cell into different compartments, which enables the specialized biological function of these organelles. To deliver cargo proteins and macromolecules to their final destination at the right time, numerous vesicles are guided to shuttle between these organelles. Highly regulated membrane

trafficking events play fundamental roles in the viability, development, and growth of cells. The mechanism regulating this crucial and complicated process is still poorly understood.

Several approaches have been described here for observing the subcellular localization of membrane proteins, analyzing the co-localization between two membrane proteins tagged with different fluorescent proteins, monitoring the dynamic movement of a membrane protein using time-series images, and collecting 3D information about a membrane protein by performing z-stack imaging covering the whole cell. Besides the membrane trafficking events described here, these approaches can be easily applied to other biological processes. For instance, the time-series imaging can be conducted over a long-term period (several days) to monitor a developmental process³⁰. The z-stack imaging can be performed to quantify the abundance of a protein within a cell³⁰. In addition, these imaging approaches can perfectly combine with pharmacological applications, including various drugs, hormones, and even environmental cues, indicating that these imaging approaches are easily applicable to live biological samples. Besides the flexibility in the sample treatment, the methods described here require only economically affordable devices, except the confocal microscope, and only light training of the personnel in the technical skills, thus meaning these methods can be widely adopted.

Caution is needed in several critical steps of the protocol. First, the optimal concentrations of the membrane trafficking drugs vary depending upon the material and the treatment. A previous study suggests that 30 μM BFA is a low concentration that can trigger characteristic BFA body formation without causing the collapse of the Golgi apparatus in meristemoids²⁰. Likewise, 25 μM Wm is a low but effective

concentration that can confer Wm body formation without severely damaging the meristemoids. When a new material (different plant species, different parts of the plant, etc.) is treated, the concentrations of the drugs need to be optimized accordingly. Second, to maintain the physiological condition of the samples to a maximal extent during the treatment, the seedlings are kept partially intact (only removing the cotyledons) until the imaging step. True leaves are dissected for slide preparation, and the sample is imaged afterward. Each sample needs to be treated individually, and the slide needs to be made freshly for imaging. Lastly, both z-stack imaging and time-series imaging require repeated laser scanning of the sample. To minimize the risk of photobleaching and phototoxicity on the samples, a low laser power is strongly recommended.

The high efficiency of membrane trafficking events is ensured by the diversity of the trafficking routes and their kinetics in response to the specific developmental and environmental conditions of the cell¹. The described methods have limited spatiotemporal resolution, so more sophisticated light microscopy techniques are emerging to gain detailed knowledge of membrane traffic within a eukaryotic cell. For instance, super-resolution microscopy, including stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM), greatly increase the spatial resolution of a fluorescence-tagged protein^{31, 32}, though only fixed samples can be analyzed; spinning-disk microscopy substantially accelerates the scanning speed and reduces the photobleaching of the samples³³, though it is challenging to record the dynamics of proteins with low abundance using this method; light-sheet microscopy provides fast and gentle 3D imaging^{34, 35}; and two-photon microscopy enables the detection of fluorescence signals from deeper tissues^{35, 36}, though at the cost of

slightly lower resolution, etc. In addition, chemical biology has identified more specific drugs interfering with certain trafficking routes. Despite the limitations, these techniques together with other emerging advanced techniques will greatly improve our knowledge and shed light on the mechanisms of the cell membrane trafficking system.

Disclosures

The authors declare no conflicts of interest.

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