Analysis of Endoplasmic Reticulum–Endosome Association Using Live-Cell Imaging in Plant Cells

Chapter 3

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Abstract

The endoplasmic reticulum (ER) is one of the most abundant endomembrane compartments and is in close association with most of the other organelles. In mammalian and yeast cells, the physiological roles and the molecular machineries underlying such association have only recently begun to emerge. In plant cells, recent live-cell confocal imaging and electron microscopy studies have established that endosomes are associated with the ER [1]. Here, we describe confocal imaging methods and software to analyze ER–endosome association in plant cells.

Key words Endoplasmic reticulum, endosomes, contact sites, FRET, correlation analyses

1 Introduction

In recent years, mounting evidence has been accumulating that endomembrane compartments establish an exceptionally high number of contacts sites with each other and with ontogenetically distinct organelles [2–4]. Each of these contact sites has probably specific features with completely different physical and chemical properties. Particularly the ER, one of the most abundant endomembrane compartments of the cell, has been shown by electron microscopy [5] and live-cell imaging analyses [6, 7] to form numerous contact sites with the plasma membrane. The membrane associations occur at sites of close apposition of about 10–30 nm between the heterotypic membranes [6]. In mammalian cells, the ER forms contact sites with endosomes; this association increases as endosomes mature [4]. Furthermore, the association with the ER tubules seems critical to establish the site of the fission of the endosomes [4, 8].

In plant cells, it has been shown that endosomes associate with the ER and that a disruption of the spatial distribution of such association leads to an abnormal endocytic traffic [1].

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Here, we describe how the association between ER and endosomes can be analyzed by fluorescence resonance energy transfer (FRET) and colocalization analysis.

There are a number of suitable fluorescent markers to label ER and endosomes:

(a) *ER luminal markers*: the ER can be visualized in vivo using several luminal markers, such as ER-XFP or XFP-HDEL (Table 1) [9, 10], where X is a fluorescent protein (e.g., cyan, yellow, red). To our knowledge, these soluble ER markers do not affect the overall structure of the ER keeping the normal reticulated shape and arrangement of tubules and cisternae.

(b) *ER membrane markers*: several membrane markers are routinely used to mark the ER; it should be considered that in conditions of overexpression, some of these markers can modify the ER network architecture (*see* **Note 1**). Indeed, markers such as those based on fluorescent protein fusions to reticulons (e.g., RTNLB3) or calnexin protein domains have been shown to act as membrane modifiers when over-expressed, causing extensive remodeling of ER tubules and enlargement of ER cisternae [12, 13, 15] (Table 1).

(c) *Endosome markers*: in plant cells, the term endosomes defines populations of post-Golgi membrane compartments. The first endosomal compartments at the exocytosis and endocytic nexus are the early endosomes (EEs)[16]. Successively, multivesicular bodies (MVBs), late endosomes (LEs)/prevacuolar compartments, and late prevacuolar compartments originate from the EEs [17, 18]. Some of the most commonly used endosomes markers are listed in Table 1 (*see* **Notes 2** and **3**)

2 Materials

2.1 Plant Plasmids

1. Several plant binary vectors can be used for subcloning genes of ER or endosome proteins fused to fluorescent tags for imaging. The vectors can be used for transient transformation of *Nicotiana tabacum* or *N. benthamiana* or *Arabidopsis thaliana* seedlings or stable transformation of *A. thaliana*. Alternatively, binary vectors carrying the ER or endosome markers can be purchased through the ABRC (Arabidopsis Biological Resource Center) or NASC (Nottingham Arabidopsis Stock Centre).
2. Plasmids for FRET experiments; the following list of constructs are needed for the FRET experiments described in Subheading 3.4: (a) CHC-YFP; (b) VAP27-1-CFP; (c) cYFP (cytosolic-YFP).

Table 1
List of ER and endosome markers available as fluorescent protein fusions (*see* Note 2)

Organelle	Marker name	Reference
ER	ER-XFP (lumenal marker)	Nelson et al. [9]
ER	XFP-HDEL (lumenal marker)	Brandizzi et al. [10]
ER	Calnexin-XFP (ER-modifier)	Irons et al. [11]
ER	XFP-RTNLB3 (ER-modifier)	Stefano et al. [1];Sparkes et al. [12]; Tolley et al. [13]
Late endosome/prevacuole	XFP-RabF2a	Stefano et al. [1]; Geldner et al. [14]
Endosomal/recycling endosome	XFP-RabA1g	Stefano et al. [1]; Geldner et al. [14]
Late endosome/prevacuole	XFP-RabF2b	Stefano et al. [1]; Geldner et al. [14]

2.2 Agrobacterium Culture and Media

1. *Agrobacterium* strain GV3101::mp90 transformed with a binary vector (pBin20, pEarleyGate, etc.) containing the gene coding for an ER or endosome marker.
2. Appropriate antibiotics for selection of transformed *Agrobacterium* (e.g., gentamycin (#GTA202, Bioshop) 25 mg/mL, rifampicin (#R0079, TCI—Tokyo Chemical Industry) 50 mg/mL + antibiotic for binary vector resistance)
3. LB medium: 5.0 g/L yeast extract (#212750, BD Biosciences), 10.0 g/L tryptone (#211705, BD Biosciences), 10.0 g/L sodium chloride (#S3014, Sigma), pH 7.0.
4. Infiltration medium: 0.5% D-glucose (#1916-01, J.T. Baker Chem. Co.), 50 mM MES (#M2933, Sigma), 2 mM $Na_3PO_4{\cdot}12H_2O$ (#S7778, Sigma), 0.2 mM acetosyringone (#D134406, Sigma).
5. Cocultivation medium: LS (#LSP03-50LT, Caisson) Medium 0.25×, 100 μM acetosyringone, 0.005% Silwet L-77 (#NC0628903; Fisher Scientific).
6. Wash solution: 10 mM MgCl2 (#M8266, Sigma), 100 μM acetosyringone.

2.3 Plant Material

1. 3–4-week-old *N. tabacum or N. benthamiana* plants, or *A. thaliana* young seedlings (4–5 days after germination) can be used for transient transformation. For stable transformation *A. thaliana* plants at bolting stage can be used for floral dipping.

2. Stable *A. thaliana* seed lines carrying the ER or endosome markers can be purchased through the ABRC or NASC. Plants can alternatively be transformed with either marker through floral dipping (see below). Homozygous plants selected via antibiotic selection can be used for imaging. For dual imaging of both markers, a seed line carrying the ER marker should to be crossed with the seed line harboring the desired endosome marker. The F1 progeny can be already used for imaging at the confocal. Once the F2 progeny has been obtained, stable lines carrying both markers can be screened and selected. The selected plants can be transferred on soil. Their progeny can be used to grow plants that express both the markers for all the imaging analyses.

2.4 Seeds Sterilization

1. Sterile hood.
2. Sterile 1 mL pipette tips or sterile Pasteur pipettes.
3. 70% (v/v) ethanol.
4. 20% (v/v) bleach.
5. Sterile distilled water.
6. Seeds coexpressing ER and endosomal markers (*see* Table 1).

2.5 Plant Growth

1. Linsmaier and Skoog medium (LS) plates, with the pH buffered to 5.7 ± 0.2 containing macronutrients, micronutrients, and vitamins.
   (a) For *A. thaliana* stable lines use the following medium: For 1 L of medium (here named 1/2× LS) use 2.35 g of LS, 1% (w/v) sucrose (#S-0389, Sigma), and 0.8% (w/v) agar (#7558A, Acumedia), and then autoclave the media for 25 min.
   (b) For transient expression of reporters in *Arabidopsis* lines use the following: For 1 L of medium use 1.18 g of LS (here named 0.25× LS), 1% (w/v) sucrose, and 0.8% (w/v) agar, and then autoclave the media 25 min.
2. Growth chambers: For growing *A. thaliana plants* set the chamber at 16 h light (100 μmol m^{-2} s^{-1})/8 h dark, 22 °C and 55% humidity. For *N. tabacum* or *N. benthamiana* use a controlled chamber with a cycle of 23 °C for 18 h light (360 μmol m^{-2} s^{-1}) and 18 °C for 6 h dark, with humidity set to 90%.

2.6 Microscopy Imaging

1. Razor blades.
2. Forceps.
3. Distilled water or half strength Linsmaier and Skoog (LS) liquid medium.
4. Glass slides.

5. Glass coverslips.
6. Confocal microscope with high resolution (e.g., 60× or 63× water or oil immersion objectives) and with double channel detection (*see* **Note 3**).
7. 25 μM Latrunculin B (Latrunculin B; #428020 Calbiochem) in distilled water.
8. 10 μM oryzalin (Oryzalin; 36182 Sigma) in distilled water.

2.7 Software for Imaging Analysis

Image J/Fiji or any advanced confocal suite software available for imaging analysis installed on any commercial confocal microscope can be used.

2.8 Other Materials Needed

1. Tips (1 mL, 200 μL, 20 μL)
2. Eppendorf tubes (1.5 mL)
3. Syringe (without needle) 0.5 mL
4. Permanent marker
5. Plant Pots (3-½ in.) and soil
6. Nalgene™ PPCO Centrifuge Bottle (500 mL) with Sealing Closure (#3141-0500PK Thermofisher)
7. Micropore 3M
8. Centrifuge

3 Methods

3.1 Plant Transformation

3.1.1 Transient Transformation in Tobacco

Tobacco transient transformation can be performed using a modified method based on a previous published protocol [19, 20]:

1. Inoculate 5 mL of LB medium with the *Agrobacterium* culture carrying the binary vector of interest (one culture with the bacteria carrying ER marker and the second one carrying the endosome marker) and add the appropriate antibiotics for selection;
2. Grow culture overnight at 28 °C and 200 rpm orbital shaking.
3. Transfer 1 mL of culture into a sterile 1.5 mL Eppendorf tube.
4. Pellet *Agrobacterium* cells at 2655 × *g*, 3 min at room temperature (20-25 °C).
5. Remove supernatant.
6. Add 1 mL of infiltration medium and gently resuspend the pellet.
7. Repeat **steps 4–6**.

8. Measure the absorbance at 600 nm of a 1 into 5 dilutions of cells, and calculate the OD of the culture by multiplying the OD value reads by 5.
9. Dilute the culture to 0.05–0.1 OD (*see* **Note 4**) for both *Agrobacterium* cultures and mix them in the same 1.5 mL Eppendorf tube.
10. Take a 3–4-week-old plant from the growth chamber and choose a well expanded leaf to be infiltrated.
11. Gently puncture a region of the leaf to be infiltrated using a 200 μL sterile tip.
12. Aspirate the resuspended solution containing the *Agrobacterium* cells with a 1 mL syringe (without the needle).
13. Using one hand, flip the leaf gently (to have the abaxial side facing upward) while placing one finger on the adaxial side of the region selected for infiltration.
14. Place the tip of the syringe on the abaxial side of the selected area and press the plunger down gently while supporting the adaxial side of the leaf with your finger, until the solution containing the *Agrobacterium* cells diffuses into the selected region
15. Mark the infiltrated area with a permanent marker.
16. Place the infiltrated plant back in the growth chamber for 48 up to 72 h.

3.1.2 Transient and Stable Transformation of Arabidopsis

The transient transformation of *Arabidopsis* seedlings often results in lower frequencies of transformed cells when compared to those achieved in tobacco. We tested several conditions to increase transformation efficiency in *Arabidopsis* and found that seedling age is one of the most critical factors. Based on our experience, it is best to use seedlings not older than 4–5 days after germination. Transient transformation of *Arabidopsis* seedlings can be performed using various protocols that have been previously published [21–24]. The protocol published by Li et al. (2009) has been the most successful for our experiments.

1. Sterilize around 200 seeds of *A. thaliana* in a 1.5 mL eppendorf tube, as follows:
   (a) Add 1 mL of ethanol 70%, mix, centrifuge at low speed (around 100 rpm) and discard the solution by pipette.
   (b) Add 1 mL of 20% bleach solution, mix, centrifuge as before and discard the liquid by pipette.
   (c) Wash at least two times with sterile water then stratify the seeds on plate (LS 0.25× medium) and vernalize at 4 °C for at least 48 h.
2. Incubate the plates in the growth chamber.

3. Prepare the cocultivation media.
4. Three days after the seeds have germinated, inoculate the *Agrobacterium* (GV3101) carrying the binary vector with the fluorescent marker of interest into LB medium with appropriate antibiotics, incubate at 28 °C with shaking at 200 rpm, until the optical density (OD) read at 600 nm (OD_{600}) reach a value which is greater than 1.5.
5. Centrifuge the *Agrobacterium* culture at low speed 2655 × *g* for 3 min then discard the supernatant.
6. Resuspend the *Agrobacterium* in 10 mL of wash solution by vortex. Repeat the last two steps one more time.
7. Resuspend the *Agrobacterium* pellet in 1 mL of wash solution.
8. Add *Agrobacterium* to 10 mL of cocultivation media to reach a final OD_{600} of 0.5, and then pour the solution in the plate containing the seedlings.
9. Incubate the plates in the dark for about 24 h.
10. Open the plates remove the media, and wash at least twice with 10 mL of co cultivation media.
11. Seal the plate with micropore 3M and incubate in standard growth conditions.
12. Perform confocal microscopy on cotyledons or root tissue 2–3 days after transformation.

Stable *Arabidopsis* transformation can be performed with the most used method described earlier [25], which is based on floral dipping. To have a high percentage of independent transformants using this method, the health and age of the plants used for the transformation are extremely important factors to consider. Based on our experience, we found that healthy plants that are 4–5 weeks old work best for a successful transformation.

1. Grow four *Arabidopsis* plants for each 3-½ in. pot (one plant in each corner of the pot) until flowering.
2. Grow *Agrobacterium* (carrying fluorescent marker of interest) in 250 mL of LB media with antibiotics and incubate at 28 °C with shaking at 200 rpm, until OD_{600} ranging between 0.8 and 1.0 is reached.
3. Using a 500 mL Nalgene centrifuge bottle spin down *Agrobacterium* at 4225 × *g* for 10 min then discard supernatant.
4. Add 250 mL of LB media supplemented with 5% sucrose (dipping solution) and resuspend.
5. For each 3-½ in. pot to be dipped use 250 mL of dipping solution.
6. Before dipping, add Silwet L-77 into dipping solution to a concentration of 0.05% (125 μL/250 mL) and mix.

7. Dip flowering stems of plants in *Agrobacterium* solution for 10–30 s, with gentle agitation.
8. Place *Agrobacterium*-treated plants under a dome in a dark O/N to maintain high humidity (lay pots on their side).
9. Grow plants in normal growth chamber conditions.
10. Harvest dry seeds.
11. Select for transformants using antibiotic or herbicide selectable marker.
12. Transplant transformants to soil.

3.2 Imaging Leaf Material

For confocal microscopy, the use of oil or water immersion lenses of the highest possible numerical aperture is recommended (e.g., 60×, 63× objectives).

1. Within the *Agrobacterium*-infiltrated zone, cut a square section (approximately 0.5 cm^2 area) from tobacco leaf or *Arabidopsis* cotyledon tissue using a razor blade.
2. Place the excised tissue in a drop of water on a glass slide with the abaxial side oriented toward the objective lens, and cover it with a glass coverslip.
3. Image the sample using a confocal microscope.

3.3 FRET (Förster Resonance Energy Transfer) Imaging for ER–Endosome Interactions

FRET measurements can be used to characterize the association between the ER-plasma membrane contact sites and endosomes [7]. Here, as example, we describe the FRET imaging of a clathrin heavy chain fused to YFP (CHC-YFP) that labels early endosomes and VAP27-1-CFP, which localizes to the ER and ER-plasma membrane contact sites. Controls for this type of experiments should include combinations of VAP27-1-CFP with cytosolic free YFP and a CHC-YFP coexpressed with a CFP-based ER marker.

1. Incubate samples (*Arabidopsis* cotyledons or a 0.5 cm^2 square section of a tobacco leaf) for 30 min in 25 μM Latrunculin B to depolymerize actin filaments and 10 μM oryzalin to depolymerize microtubules. This will stop any kind of cytoskeleton-dependent organelle movement [7].
2. Mount samples on microscope slides and cover them with coverslips using the solution containing Latrunculin B and oryzalin.
3. To set up the confocal for FRET imaging, scan the sample, find and select the region of interest (ROI) using nonphotobleaching and nonsaturating imaging settings. Ensure that the microscope settings prevent signal cross talk between the YFP and CFP channels.
4. Using the line-switching mode of the microscope, capture at least 10 prebleach images using the CFP and YFP channels.

5. Bleach YFP (acceptor) only for 10 s using high laser power intensity (use laser line 514 nm, power 100%).
6. Collect at least 1 postbleach image of the CFP and YFP channels (postbleaching acquisition).
7. Perform at least 10 FRET imaging acquisitions as detailed above and repeat the entire image acquisition scheme three times for each combination of fluorescent protein fusions, including controls.
8. Calculate the energy transfer efficiency (EFRET) between the paired proteins based on the change in fluorescence intensity of the donor before (last prebleach image) and after photobleaching (first postbleach image) using the formula $EFRET = (CFP_{after} - CFP_{before})/CFP_{after} \times 100$ [26]. The CFP_{after} image is the first one obtained after the photobleaching step.

3.4 ER-Endosome Colocalization

Generally, to evaluate the colocalization between two fluorescent markers the Pearson correlation coefficient (PCC), Manders's overlap coefficient (MOC), and Manders's colocalization coefficient (MCC) are used [27].

We found that to quantify the degree of colocalization between ER and endosome signals MOC or MCC are the most appropriate correlation coefficient measurements. MOC reaches value equal to 0 only when the two probes are completely reciprocally exclusive. The MCC measures specifically the fraction of one fluorophore that colocalizes with another fluorophore.

These coefficients are provided in almost any image analysis software, such as Nikon AR elements, Imaris, Volocity, and ImageJ via the JACoP plugin.

MOC or MCC can be calculated using ImageJ and JACoP plugin [28, 29] as follows:

1. Having acquired the colocalization images with two fluorochromes (X_1FP and-X_2FP) in nonsaturating conditions, select an ROI in the merged image.
2. Open the X_1FP channel image and X_2FP channel independently in ImageJ via JACoP plugin.
3. Select automatically the thresholds for X_1FP and-X_2FP to remove background and signal noise, then calculate the colocalization levels [28, 29] (*see* **Note 5**).

4 Notes

1. Use low OD_{600} if transient transformation is performed (e.g., 0.005-0.01 OD_{600}).
2. For additional markers of the ER and other secretory organelles, *see* refs. 9, 14.

3. Choose fluorescent protein combinations that do not favor fluorescence signal cross talk.
4. In case of very low transformation efficiency, we recommend infiltrating *Agrobacterium* at different OD_{600} starting from 0.05 to 0.5.
5. It is possible to measure colocalization over time by acquiring time-lapse images with a narrow temporal interval between frames. The colocalization analysis for ER-endosomes should be performed manually on the ROI of interest over the desired time frame.

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