Light Microscopy of the Endoplasmic Reticulum-Membrane Contact Sites in Plants

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Summary

The existence of membrane contact sites (MCS) has been reported in different systems in the past decade, and their importance has been recognized by the cell biology community. Amongst all endomembrane structures, the endoplasmic reticulum (ER) plays vital roles in organizing the organelle interaction network with the plasma membrane (PM), Golgi bodies, mitochondria, plastids, endosomes and autophagosomes. A number of methods have been used to study the establishment and functions of these interactions, among them, light microscopy appears to be one of the most effective approaches. Here, we present an overview of the discovery of ER-PM contact sites, and highlight the latest developments in light microscopical-based techniques that can be used for their study.

Key words: Membrane Contact Sites, ER-PM contact sites, Endoplasmic Reticulum, Light Microscopy, Plants

Introduction

The endomembrane system is an elaborate network with various membrane-bound organelles that associate with each other through membrane contact sites[1-4]. Specific proteins and lipids usually accumulate at these sites, and the function or composition of each tethering organelles can be affected when the MCS is dysfunctional[1].

The endoplasmic reticulum (ER) is one of the most architecturally complicated membrane networks in eukaryotic cells (Figure 1A). It is able to directly connect with other organelles to regulate a variety of cellular activities, such as non-vesicular material transportation, cell signaling, organelle dynamics and autophagy[5-11] (Figure 1B-D). The ER-PM contact sites (EPCS) are typical MCSs and are conserved across eukaryotic cells[12-14]. In plants, these structures are organized by multiple EPCS resident proteins and cytoskeletal components[12,15] (Figure 1B), all of which contribute to the function and formation of EPCS in response to multiple biotic and abiotic stress responses[7-9,16-18].

The discovery of ER-PM contact sites in plants

In the early days, the close association between ER and other organelles was seen in muscle cells using transmission electron microscopy (TEM)[19], and further ultrastructural studies in plants have also revealed that the cortical ER was associated with the PM in different cell types[20-22]. Thanks to the application of fluorescent proteins (such as GFP) in endomembrane-related research, the clear observation of the ER network in living cells became possible[23,24]. It has been reported in numerous studies that the cortical ER network moves dramatically, but small areas of the ER remains persistent[25-27] (Figure 1A) and these areas were called 'ER-anchor points'. The molecular composition and biological function of these ER sub-domains remained unclear for a long time[28]. Only recently have proteins that localize to the EPCS been identified in several independent studies[7-10,17,18,29,30], and now the biological relevance of EPCS is becoming much clearer (Figure 1B-D).

The observation of ER-PM contact sites using light microscopy

A number of new systems and techniques have been developed for the investigation of MCSs. Among them, light microscopy is the most effective approach, especially for living cells. The ER polygonal structure changes dynamically within cells by altering its branching patterns of tubules, moving network junctions as well as transiting between cisternae and tubules[31,32] (Figure 1A). These events can be easily observed using laser scanning confocal microscopy in combination with ER targeted markers. Techniques, such as persistency mapping and "AnalyzER", have been developed in recent years to quantify the morphology and dynamics of the ER network[27,33-35]. In this way, changes in ER dynamics, morphology or putative EPCS in different conditions have been quantified[9,36].

However, the resolution of conventional confocal microscopy can hardly reach below 200nm due to the diffraction barrier. In the early days, high-resolution bright-field ultraviolet (UV) microscopy was used to obtain images with superior contrast and improved resolution (since the wavelength of UV light is shorter than that of visible light) in order to study the behavior of the plasma membrane and the cortical ER. Hechtian strands that originate from the plasma membrane and remain closely attached to the cell wall after plasmolysis have been studied in this way[37-39]. Interestingly, ER remnants are often found within the Hechtian reticulum, indicating that the cortical ER network is indirectly linked to the cell wall, and we now know these linkages are achieved through EPCS[36,40].

With the help of more advanced microscopical techniques, higher resolutions could be achieved. For example, using the super-resolution imaging technique Photoactivated Localization Microscopy (PALM), the individual ER-PM junctions in HeLa cells became more resolved and clearly visible[41]. The molecular localization obtained through PALM images could then be analyzed using an integrated software platform to quantify the morphology of the ER-PM junctions, as demonstrated for HeLa cells as well as for neurons and fibroblasts[41,42].

The interactions between organelles and the cytoskeleton are highly dynamic and high-resolution microscopy which requires long acquisition times (e.g. PALM, STORM) is not easily used to study EPCS or any MCS in real time. Grazing Incidence Structured

Illumination Microscopy (GI-SIM) has been developed to overcome the limitations in resolution, speed and z-depth of conventional light microscopy[43]. By employing multi-colour GI-SIM, the dynamic events of microtubules and ER network can be visualized and measured precisely. New ER remodeling mechanisms were also identified by studying the interactions between ER, microtubules and other organelles in different animal cell types using this technology[43].

Fluorescence density mapping in combination with common total internal reflection microscopy (TIRFM) is another approach for studying the morphology and dynamics of EPCS structures. High-resolution can be achieved by exciting the fluorophore within only a 200 nm depth below the PM, where cortical ER and EPCS are normally located. Information on the size and shape of contact sites can be calculated by constructing an intensity map[44]. Taken together, although the resolution of any light microscopical technique to date cannot achieve the size of the MCS interface (normally 10-30nm), the current technologies do provide the quickest and most effective way to study membrane and cytoskeleton dynamic at the EPCS. Although some of these novel techniques (e.g. GI-SIM) are of significant interest to plant community, they have only been tested on mammalian cells. Further modifications to imaging techniques are required for plant cell imaging.

Studying protein dynamics at the ER-PM contact site

The dynamics of EPCS-resident proteins can be measured by using the technique of Fluorescence Recovery After Photobleaching (FRAP). The recovery of EPCS resident proteins is much slower than that of proteins that localize to the ER membrane[40]. A few studies have also indicated that monitoring EPCS protein dynamics may provide useful information on long-term plant adaptive responses to long-term exposure to ionic stress[9]. In order to study the nanoscale dynamics of EPCS-resident proteins, super-resolution techniques combined with single-particle tracking can be used. Single molecule microscopy methods are extremely powerful tools for investigating concealed properties of a complex system. By using this technique, the diffusion and trapping of STIM1 and Orai1 at EPCS of mammalian cells has been analyzed[45-47]. Similar techniques are also well established in plants[48-50], and their application to EPCS studies

in plants would be an interesting direction to progress.

Artificially designed markers and tethers used for labelling EPCS

A set of genetically encoded markers and tethers have been designed to investigate the function and dynamic regulation of ER-PM junctions. MAPPER (membrane-attached peripheral ER) is a commonly used marker for labelling EPCS in both animal and plants. MAPPER is comprised of a genetically engineered STIM1 domain fused to GFP at the ER lumen, and a FKBP12-rapamycin binding (FRB) domain fused with a polybasic motif at the cytosol; these two parts are connected by a transmembrane (TM) domain[51]. More recently, an *Arabidopsis* version of MAPPER, called MAPPER-GFP, has also been created[9]. The localization of MAPPER-GFP strongly resembles the plant EPCS marker SYT1 (synaptotagmin 1) and differentiates between luminal ER and ER-membrane markers[9]. Meanwhile, artificial ER-PM tethers have also been generated for studying the cortical ER architecture in yeast cells, namely TM-mCherry-CSS_{Ist2} and TM-mCherry-PH_{Osh3}. In these constructs, two alternative lipid binding motifs including a cortical sorting signal (CSS) from *Saccharomyces cerevisiae,* Ist2, and a pleskstrin homology (PH) domain from the *Schizosaccharomyces pombe* homolog of Osh3 were fused to the C-terminus of the TM-mCherry backbone. These were shown to successfully tether ER to PM[52].

Proximity-dependent methods using fluorescent probes provide a useful toolbox for EPCS research

Benefitting from the general application of confocal microscopy, a number of fluorescent protein (FP)-based tools have been developed for targeting and identifying putative MCS. Examples include Bimolecular Fluorescence Complementation (BiFC)[53], Proximity Ligation Assay (PLA)[54] and dimerization-dependent Fluorescent Proteins assay (ddFP)[55]. All of these techniques have been designed on the basis that signals are generated as a consequence of the close distance between target proteins and they have been used in detecting protein-protein interactions at MCS.

In the BiFC system, FP is divided into FP-N and FP-C and fused to the N- and C- termini of target proteins, respectively. A bright fluorescence will be generated only when the

target proteins interact with each other and get close enough. For example, Tao et al. (2019) employed BiFC to produce artificial ER-PM tethers in plants, and they proposed that integral membrane proteins and phosphoinositide-binding proteins contributed to ER-PM tethering. Similarly, ddFP technology involves the reversible binding of two nonfluorescent FP monomers to form a fluorescent dimer, which relies on the increased proximity or effective concentration of monomers caused by FP-FP interaction. ddFP has been successfully applied to confirm ER-mitochondria juxta-positioning in living mouse embryonic fibroblasts by targeting one half of GFP to the ER surface and the other half to the outer mitochondrial membrane (OMM). In this way, it reduces operator bias in the tethering measurements based on EM or confocal image analysis [56]. Although both BiFC and ddFP are highly sensitive, care should be taken when using these techniques. Both may raise the risk of altering cellular structures and membrane organization when they are being used to study membrane-membrane interactions[57], especially when the constructs are highly over-expressed.

To overcome this potential problem of over-expression artifacts, PLA has been applied in the study of MCS localized proteins at the endogenous level and with high specificity and sensitivity in human carcinoma cell lines[58]. To do this, target proteins are probed with antibodies conjugated to complementary oligonucleotide extensions. The close proximity results in a rolling-circle amplification of the signal, which can then be detected by hybridized fluorophore-labeled oligonucleotide probes[58]. Recently, PLA has been used to visualize and quantify endogenous ER-mitochondria interactions between the outer mitochondrial membrane protein VDAC1 (voltage-dependent anion channel) and the ER membrane protein IP3R1 (inositol 1,4,5-triphosphate receptor) in hepatocarcinoma cell lines[59]. A similar method could be applied in EPCS studies although PLA can be only used in fixed cells which is one of its main limitations.

Photo-inducible and photo-convertible markers for MCS research

It is worth mentioning that photo-inducible/convertible technologies could also contribute to MCS research. EosFP encodes a fluorescent protein that originally comes from the stony coral *Lobophyllia hemprichii*[59].It displays a green-to-red fluorescence conversion when exposed to near-UV irradiation ($\approx$390 nm)[60]. EosFP has been used

successfully in plants to identify putative MCS between plastids, which have been labelled with EosFP fusion proteins. Live-cell imaging has demonstrated that plastids are interlinked by tubular membranes. However, these direct connections do not appear to be transferring macromolecules as the EosFP signal could not be transferred between the interlinked plastids[61].

Another optogenetic tool called LiMETER (Light-inducible Membrane-Tethered peripheral ER) has been designed to specifically label cortical ER. It contains an ER-lumen-localized fragment fused to GFP, followed by a light-sensitive LOV2 domain (Light, Oxygen or Voltage-sensing domain), and a PM-targeting polybasic tail at the C-terminus[62]; upon blue light stimulation, the polybasic domain becomes exposed, and the protein is able to bind to plasma membranes in a close proximity[62]. The application of these photo-sensitive markers could be extended to plant cells in order to visualize the formation and dynamic changes of EPCS in a controlled manner.

Future Perspective

Professor Chris Hawes was one of the pioneers using modern light microscopy techniques for plant endomembrane research. Before the term of membrane contact site was known by the plant cell biology community, he has proposed the existence of direct interactions between ER and Golgi[63-64] and identified ER-PM anchor sites in plants[26-27]. His contribution will be remembered by the plant cell biology society around the world. No doubt, the identification of MCS is revolutionary in the cell biology field. Significant progress has been made in characterizing the molecular composition of MCS in plants, as well as identifying new MCS between different structures. Although electron microcopy-related techniques (e.g TEM immunogold labelling) provide the most direct evidence to confirm the protein composition of MCS, successful application of light microscopical techniques can certainly promote our understandings in this field.

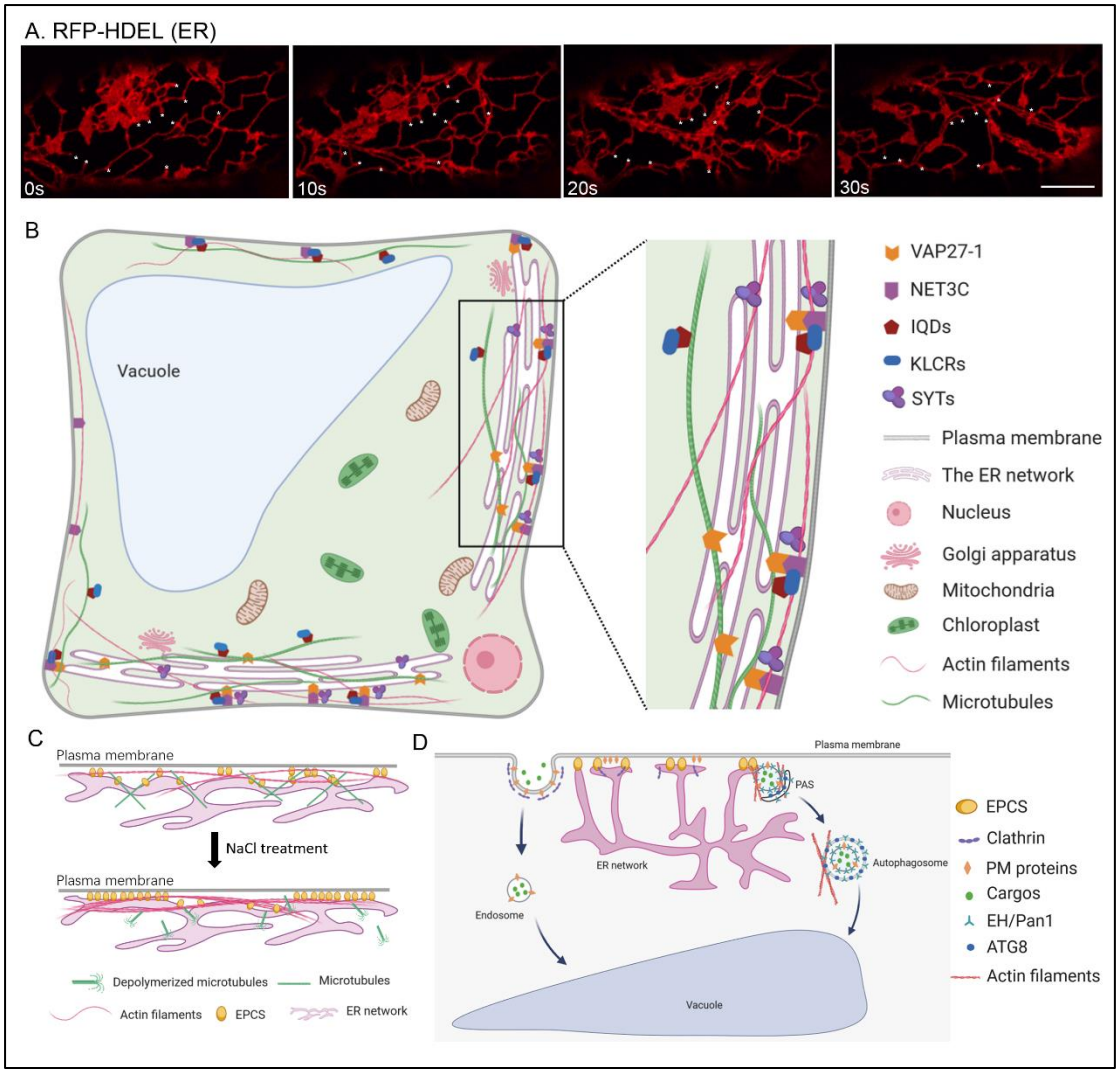

Figure 1. ER network and ER-PM contact sites in plants. A. The endoplasmic reticulum (labeled by RFP-HDEL) moves dramatically in plants cells, with persistent sites that connect to the plasma membrane. **B.** Diagram illustrations of EPCS-associated proteins that have been identified to date. These include ER-integral membrane proteins of the VAP27 and SYT family; an actin cytoskeleton-associated protein, Networked 3C (NET3C) and microtubule-associated proteins of the IQ67 domain (IQD) and kinesin light chain-related protein (KLCR) family[18,29]. **C.** The attachment between ER and PM is enhanced when plants are under ionic stresses, a process that is regulated by protein-phospholipids interactions at the EPCS[9]. **D.** Known functions of EPCS in plants, such as regulation of endocytosis and autophagosome biogenesis[11,17] (scale bar = 10μm).

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