Better to keep in touch: investigating inter-organelle cross-talk

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Abbreviations: BRET: Bioluminescence Resonance Energy Transfer; co-IP: co-immunoprecipitation; cryo-ET: cryo-electron tomography; ddFP: dimerization-dependent fluorescent protein; Dnm1: Dynamin-related protein DNM1; Drp1: Dynamin-related protein 1; EDF: Enhanced dark-field; EM: Electron microscopy; ER: Endoplasmic reticulum; ER-GFP: ER-targeted GFP; FLIM: Fluorescence lifetime imaging microscopy; FRET: Förster Resonance Energy Transfer; Grp75: Glucose regulated protein 75; IEM: Immuno-electron microscopy; LiMETER: Light-inducible membrane-tethered peripheral ER; MCS: membrane contact site; IMM: Inner mitochondrial membrane; IP3: Inositol 1,4,5-trisphosphate; IP3Rs: Inositol 1,4,5-trisphosphate receptors; MAM: Mitochondria-associated membranes; MCUC: Mitochondrial Ca^{2+} uniporter complex; MERLIN: Mitochondria-ER Length Indicator Nanosensor; MS: Mass spectrometry; mt-RFP: Mitochondria-targeted RFP; OMM: Outer mitochondrial membrane; ORAI1: Calcium release-activated calcium channel protein 1; PE: Phosphatidylethanolamine; 4Pi-SMS: 4Pi single-

molecule switching; PLA: Proximity Ligation Assay; PM: Plasma membrane; PS: Phosphatidylserine; RCA: Rolling-circle amplification; ROS: Reactive oxygen species; RyR-2: ryanodine receptor type 2; Sac1: Phosphoinositide phosphatase SAC1; SEM: Scanning electron microscopy; SERCA: Sarcoplasmic/ER Ca^{2+} ATPase; SIM: Structured illumination microscopy; SOCE: Store-operated Ca^{2+} entry; $SPLICS_L$: split-GFP-based contact site sensor (long); STIM1: Stromal interaction molecule 1; STED: Stimulated emission depletion; SXT: Soft x-ray tomography; TAP: Tandem affinity purification tagging; TEM: Transmission electron microscopy; TGN: trans-Golgi network; TIRFM: Total internal reflection fluorescence microscopy; TOM20: Translocase of the outer membrane 20; VAPs: VAMP (Vesicle-associated membrane protein)-associated proteins; VDACs: Voltage-dependent-anion-channels.

Abstract

The strategic importance for cellular organelles of being in contact with each other, exchanging messenger molecules, is nowadays well established. Different inter-organelle crosstalk pathways finely regulate multiple physiological cellular mechanisms and their dysregulation has been found to underlie different pathological conditions. In the last years, a great effort has been made to study such organelle interactions, to understand their functional roles within the cell and the molecules involved in their formation and/or modulation. In this contribution, some examples of organelle cross-talk and their contributions in regulating physiological processes are presented. Moreover, the pro and cons of the available methods for a proper, reliable investigation of membrane contact sites are described.

Introduction

Inter-organelle signals, regulating cellular homeostasis, are exchanged by vesicles or at inter-membrane contact sites (MCSs). Contacts may occur between identical organelles (homotypic MCSs), or between different ones (heterotypic MCSs); in some cases, triple interactions, involving more than two organelles, have been described [1]. MCSs can be defined as regions in which the membranes of different organelles are kept in close apposition by specialized proteins, often referred to as "tethers" (**Figure 1**). Importantly, tethers can exert their function by forming complexes with other proteins or by interacting with membrane lipids. Of note, at MCSs, the juxtaposed organelles never fuse their membranes, but exchange molecules or signals by mean of specific functional complexes (**Figure 1**). As to the distance between the opposing membranes, in most cases it ranges from 10 to 30 nm, though protein-mediated connections up to 300 nm have been described [2]. In general, however, the presence of both tethers and specific functionalities represents an essential condition that distinguishes MCSs from randomly occurring organelle proximity. The importance of organelle contacts in cell physiology has been recently well documented and further sustained by the fact that, in multiple pathological conditions, dysfunctional MCSs have been reported [1]. For instance, in neurodegenerative and age-related pathologies, such as Alzheimer's disease and Parkinson's disease, several disease-associated proteins are enriched at MCSs (in particular at endoplasmic reticulum (ER)-mitochondria contacts [3,4]), altering key functions (see below) and contributing to pathogenesis. Other pathologies in which MCS abnormalities have been reported include metabolic dysfunctions, like obesity [5], and cancer [6-8]. In this latter condition, for example, a reduced sensitivity to mitochondrial calcium (Ca^{2+}) overload, linked to altered ER-mitochondria Ca^{2+} crosstalk, underlies the resistance (induced by several oncogenes) of tumour cells to death. On the same line, the expression of tumour suppressor genes favours Ca^{2+} transfer from the ER to mitochondria, thus inducing pro-apoptotic effects on cancer cells [9].

Among MCSs, the most studied interactions are those between the ER and mitochondria [10] and between the ER and the plasma membrane (PM) [11]. In the last years, however, other important inter-organelle communications have been characterized, such as ER-endosomes, ER-Golgi apparatus, mitochondria-Golgi, mitochondria-lipid droplets, mitochondria-PM, mitochondria-vacuoles/lysosomes and mitochondria-peroxisomes [1,12,13]. Accordingly, different cell functions/processes have been linked to diverse MCSs and organelle interfaces. Although a wide range of activities is increasingly being reported to associate with specific MCS, below we briefly describe only those that have been better characterized (see also **Figure 2**).

Membrane contact sites and cell functions

1. Ca^{2+} transfer and ROS signalling

Among the most studied inter-organelle functionalities, the channelling of the intracellular Ca^{2+} signal is the best characterized. ER-PM and ER-mitochondria MCSs are both strategically involved in different Ca^{2+} pathways. At the ER-PM interface a specific Ca^{2+} pathway, called store-operated Ca^{2+} entry (SOCE) [14] can be activated. In particular, when ER Ca^{2+} levels are sufficiently low, because of cell stimulations inducing ER Ca^{2+} release, a Ca^{2+} sensor, the stromal interaction molecule 1 (STIM1), located at ER membranes and sensing the cation concentration within its lumen, forms multimeric clusters. These structures connect and activate PM-located ORAIi1 Ca^{2+} channels, opening them and triggering Ca^{2+} entry from the extracellular space, thereby allowing ER Ca^{2+} refilling. For this key function, the close proximity between ER membranes and PM is needed, by the concerted action of different molecules [15-20].

Similarly, at the ER-mitochondria interface, Ca^{2+} exchange between the two organelles takes place at specific domains called mitochondria-associated membranes, MAM [21], thanks to specialized MCSs involving inositol 1,4,5-trisphosphate receptors (IP3Rs) at ER membranes, voltage-dependent-anion-channels (VDACs) at the outer mitochondrial membrane (OMM) and the cytosolic fraction of the protein Grp75 [22]. When Ca^{2+} is released from the ER, upon IP3-generating cell

stimulations, microdomains of high Ca^{2+} concentration are formed close to the mouth of the IP3R channel, at sites of close proximity with mitochondria, *i.e.*, at MAM [23-25]. These Ca^{2+} hot spots diffuse through VDACs from the OMM into the mitochondrial intermembrane space and here allows the inner mitochondrial membrane (IMM)-located mitochondrial Ca^{2+} uniporter complex (MCUC; [26]) to be activated and mediate mitochondrial Ca^{2+} uptake, a fundamental signal for several organelle metabolic functions [27]. Of note, ER-mitochondria Ca^{2+} crosstalk is of reciprocal interest for the two organelles. Indeed, the capacity of mitochondria to take up and buffer cytosolic Ca^{2+} can affect the local IP3R Ca^{2+}-dependent activation/inactivation [28], and modulate the activity of the Sarcoplasmic/ER Ca^{2+} ATPase (SERCA), by fuelling it with mitochondrial ATP [29], whose synthesis is Ca^{2+}-stimulated.

Moreover, mitochondrial production of reactive oxygen species (ROS), which in turn modulates Ca^{2+} signalling [30,31], can be influenced by the MAM-located ER-mitochondria Ca^{2+} exchange [32]. For instance, IP3Rs [33] and SERCA pumps [34,35] can be modulated by ROS, and high ROS production at ER-mitochondria contacts decreases mitochondrial motility [36], influencing ER-mitochondria MCS formation. In cardiac cells, ROS formed at MAM-located Ca^{2+} microdomains can oxidize the SR Ca^{2+}-releasing channel ryanodine receptor 2 (RyR-2), inducing a positive feedback further enhancing SR Ca^{2+} release [37,38]. Furthermore, in ER stress conditions, a potentiation of ER-mitochondria MCSs occurs; in turn, the associated higher Ca^{2+} exchanges at MAM can either stimulate mitochondrial oxidative phosphorylation [39] or eventually induce cell death [40].

2. Lipid exchange

Other important signalling molecules exchanged at MCSs are lipids [41]. Indeed, lipid synthesis takes place at the ER, their storage and transport occur mainly through vesicles, their oxidation is carried out in mitochondria and peroxisomes and they are ultimately hydrolyzed in lysosomes, making their exchange between several organelles necessary [42]. Moreover, lipid exchange

between ER and PM regulate phosphoinositide lipid signalling and local Ca^{2+} exchange, modulating several processes such as immunity response, muscle contraction, neuronal activity and hormone signalling [11].

Lipid channelling at MCSs could be driven by lipid consumption (for example, the transport of ceramide from the ER, where it is produced, to the Golgi, where it is converted to sphingomyelin [43]), or by their local production and thus enrichment, such as that of phosphatidylserine (PS), produced in the ER, at MAM, and then channelled to mitochondria for its conversion into phosphatidylethanolamine (PE) [44,45] (see also below). Additionally, metabolic lipid exchange at MCSs might be driven by the local enrichment of their specific transporters [46,47]. Of note, a MCS network can facilitate lipid movement, controlling their levels (and thus their function) at specific sites. For example, it has been reported recently that the phosphatase Sac1, an ER membrane protein, dephosphorylates phosphatidylinositol-4-phosphate on Golgi membranes, underlining the importance of ER-Golgi MCSs for this in-trans phosphatase activity [48].

3. Organelle dynamics

MCSs can also modulate organelle dynamics. In both yeast and mammalian cells, the ER wraps the mitochondrial network at precise sites, favouring mitochondrial fission [49]. ER and actin collaborate to generate constrictions on mitochondria, suitable for the local recruitment of dynamin-related proteins (Drp1 in mammals and Dnm1 in yeast), mediating mitochondrial fission through the mechano-chemical force generated by the assembly-stimulated GTP hydrolysis [50]. ER-mitochondria contacts, at nascent division sites, also activate *in situ* actin polymerization and guide the distribution of newly replicated mitochondrial DNA to new-generated daughter mitochondria [51-53]. In addition, mitochondria-lysosome contacts are also associated with mitochondrial division events [54]. Similarly, ER can modulate protein sorting, wrapping around endosomal budding regions [55]. Mitochondria might be anchored to PM and these type of MCSs are also reported to favour mitochondria repositioning during cell division [56,57]. Furthermore, the

anchoring of vesicles by MCSs to immobile organelles can physically stack them, avoiding their transport [58]. On the other hand, MCSs can also create organelle subpopulations with specific functional aspects. For instance, in brown adipose tissue, mitochondria forming MCSs with lipid droplets are metabolically different from those that are apart from them. Lipid droplet-contacting organelles, in fact, display increased ATP synthesis, reduced fatty acid β-oxidation and increased production of triacyl-glycerides [59].

4. Autophagy

Autophagy is a fundamental cell process modulated by MCSs. It is a lysosome-mediated process of controlled degradation of old/damaged or unnecessary cellular components, involved in molecule/organelle turnover and cell adaptation [60]. It requires membrane remodelling with the formation of an intracellular structure, called autophagosome, engulfing the material to be degraded. The formation of MCSs between a subdomain of the ER, termed omegasome, and a cup-shaped membrane, the phagophore, is the starting step towards autophagosome formation [61]. Indeed, lipid-synthesizing enzymes are enriched at this interface and lipids, transferred from the ER to the phagophore, seem to play an important role in autophagosome double-membrane growth. The existence of ER-autophagosome tethers has been reported also, such as vesicle-associated membrane protein-associated proteins (VAPs) [62]. In eukaryotic cells, autophagosome nucleation may occur also at ER-mitochondria [63] and ER–PM MCSs [64], with growing autophagosomes simultaneously contacting other organelles, including endosomes, Golgi and lysosomes [65]. As a proof of the importance of MCSs for the process, decreased formation of autophagy vesicles were found while artificially altering ER-mitochondria contacts [63,66]. Importantly, the regulation of autophagy by ER-mitochondria contacts seems to depend also on Ca^{2+} exchange between the two organelles [67].

5. Inflammation and proteostasis

Further processes involving organelle contacts are inflammation and protein folding, trafficking and turnover. As to the first, ER-mitochondria communication has been shown repeatedly to play a crucial role in multiple innate immune cell functions, from their lipid metabolism to their ability to engulf apoptotic cells, from their antiviral response to inflammasome activation [68]. On this latter aspect, in particular, during the inflammatory process the Nod-like receptor NLRP3 has been reported to redistribute from bulk ER membranes to MAM [69], where it becomes fully activated by Ca^{2+}-dependent, mitochondria-produced ROS [69]. Moreover, in cystic fibrosis (CF) experimental models, mitochondrial Ca^{2+} rises, sustained by ER-organelle juxtaposition, have been shown to be fundamental in both integrating pro-inflammatory signals induced by the pathogen and NLRP3 activation [70]. Along the same line, it was demonstrated very recently, that *Pseudomonas aeruginosa* infection in CF bronchial cells potentiates ER-mitochondria Ca^{2+} transfer by stabilizing the VAPB-PTPIP51 organelle tether, leading to autophagy dysregulation, mitochondrial unfolded protein response (mUPR) and NLRP3 inflammasome hyper-activation [71].

ER-mitochondria cross-talk also facilities stress responses evocated by cellular UPR [72] and key ER sensors have been shown to regulate ER homeostasis, by directly localizing at MAM and/or by regulating the expression of ER-mitochondria tethers/modulators [73-75]. Thus, activation of the UPR pathways may also regulate other cell processes by modulating MAM-resident proteins and, *vice versa*, ER-mitochondria MCSs might exert a control on UPR. For example, the UPR member inositol requiring enzyme 1 (IRE1) not only localizes at MAM during ER stress, but its dimerization-dependent activation is favoured by its interaction with the MAM-located ER chaperone sigma1 receptor [76], involved in ER-mitochondria Ca^{2+} cross-talk [77].

The UPR component PERK can also modulate, by F-actin network remodelling, ER-PM MCSs and SOCE, but its action seems to be independent from its UPR-linked activity [78]. Thus, key ER stress sensors modulate the connection between ER and other organelles/domains, such as mitochondria and PM, regulating different ER functionalities.

ER-endosome MCSs have been also implicated in protein turnover, regulating epidermal growth factor receptor (EGFR) sorting and degradation via a lysosomal-dependent pathway. Indeed, EGFR internalization requires its dephosphorylation on the cytosolic surface of the endosome by the ER protein Tyr phosphatase 1B (PTP1B), making these specific MCSs essential for the EGF signalling pathway [79].

Finally, in Drosophila, the homologue of the yeast ER-mitochondria tether EMC (ER membrane protein complex; [80]) has been implicated in the biogenesis and proper assembly of multi-pass transmembrane proteins, such as rhodopsin 1, with cells devoid of fly EMC components displaying disrupted ER-mitochondria tethering [80] and misfolded multipass membrane proteins [81].

Membrane contact site investigations

Generally, MCSs can be studied from a structural/morphological point of view (investigating also the molecular components needed for their formation) and/or from a functional point of view. Below, we briefly summarize the techniques that are commonly used to investigate these aspects, highlighting their advantages and drawbacks (see also **Table 1**).

1. Structural MCS analysis

1.1 Microscopy

Electron microscopy

Electron microscopy (EM) allows the direct visualization of MCSs and the calculation of the distance between organelles with a resolution of 0.2 nm [82]. It is therefore possible to evaluate whether the positioning of big functional complexes of interest, at specific MCSs, is compatible with the narrow gap at the inter-organelle interface. Different types of EM exist, allowing the generation of both 2D and 3D images. Transmission EM (TEM), for example, uses electron beams transmitted through thin sample slices, yielding 2D images [83]. A 3D MCS visualization can

instead be achieved by Scanning EM (SEM), based on topological information given by electrons scattered on the sample surface [84]. Moreover, 3D images can also be obtained by computer-assisted reconstructions of TEM 2D images. However, this process is time-consuming and hardly compatible with the analysis of a large number of interactions and cells.

A direct visualization of specific proteins at MCSs could be achieved by using Immuno-EM (IEM), in which colloidal gold particles attached to antibodies are used to mark the location of specific proteins [85]. Importantly, the use of antibodies conjugated to gold particles of different size allows the simultaneous detection of two or more proteins. To enhance the quality and the sensitivity of this detection, a strategy to control the binding orientation of the antibody to nanoparticles, while preserving the capacity of the antibody to bind its target, has been developed [86]. Since complex MCS structures could not be easily interpreted using 2D EM images, SEM has been combined with an additional, FRET (Förster Resonance Energy Transfer; see below) -based technique, to screen for candidate MCS tethering molecules [87]. Additional information on the molecular nature of structures involved in MCS formation can be obtained by correlating cryogenic fluorescence microscopy and soft X-ray tomography (SXT). Because soft X-rays penetrate biological materials more deeply than electrons, SXT can image intact, fully hydrated cells up to 15 μm thick [88]. This approach was used to define the 3D structure of ER–mitochondria MCSs [89].

In addition to the distance between juxtaposed organelles, the length of MCSs can be also calculated from EM images. These data are usually normalized to the number/size of the organelle under investigation, as previously reported [90,91]. Importantly, EM can show changes in organelle morphology, induced by specific experimental conditions, that should be taken into account to precisely quantify MCSs.

The main EM limitation is the need to chemically treat the sample (fixation, dehydration and staining), possibly altering delicate tissues/structures. However, cryo-electron tomography (cryo-ET), in which samples are quickly frozen instead of chemically treated, has been shown to limit

artefacts [92]. For example, organelle contacts have been found more numerous in chemically fixed samples (where swelling or shrinkage of some organelles was observed) than in cryo-treated ones [93]. Cryo-ET was successfully used to visualize the ultrastructure of ER-PM contacts in 3D [94].

It is important, however, to underline that neither EM nor cryo-ET are compatible with live imaging. Considering that MCSs are dynamic [1], this represents a major limitation of all the EM-based techniques.

Fluorescence microscopy

Fluorescence microscopy can give an estimation of MCSs (and of their dynamics) by co-localization signal analyses. The use of light microscopy, however, offers a limited resolution (in the range of hundreds of nm), which is theoretically insufficient to accurately detect organelle contacts, typically formed with a range of distances between 10 and 100 nm [90]. Moreover, it needs the expression of fluorescent proteins targeted to specific organelles, or the use of specific antibodies (and of immunofluorescence protocols) against organelle-located proteins, to mark the intracellular structures of interest. In both cases, MCSs result as zones of co-localized signals (coming from different organelles; **Figure 3A**) [95].

The use of multispectral imaging and computational analysis has helped in distinguishing signals from multiple fluorophores, thus permitting, for instance, the simultaneous dynamic visualization of contacts among six different organelles [12]. This approach, using confocal and lattice light sheet spectral imaging, allowed to obtain information on organelle number and volume, their position and dynamicity in terms of inter-organelle contacts and organelle speed [12]. Moreover, the use of a confocal microscope, with a spectral detector and a linear unmixed algorithm, yielded the number of contacts between different pairs of organelles and calculated the fraction of each organelle contacting the others [96].

Fluorescence microscopy allows live-imaging studies but needs sample transfection/infection of exogenous fluorescent proteins, possibly altering the expression of endogenous proteins or organelle morphology [95]. Moreover, in the case of immunofluorescence, fixation is needed and live-imaging is not possible, with the final outcome strongly relying on antibody goodness and specificity [95].

Several super-resolution methods have been developed to improve the resolution of fluorescence microscopy [97]. Examples are structured illumination microscopy (SIM) [98], stimulated emission depletion (STED) [99] and total internal reflection fluorescence microscopy (TIRFM). A combination of SIM and TIRFM has been used to visualize cell structures [100]. Using TIRFM, ER-PM MCSs have been visualized in living cells [101] but this technique suffers from a small range of illumination. Moreover, all these approaches require quite expensive equipment, are not easy to perform and have multiple drawbacks, such as photo-bleaching and fluorescent dye photo-quenching. Interestingly, in a recent work, a fluorescence-free, 3D super-resolution method, by enhanced dark-field (EDF) microscopy combined with plasmon nanoparticles, was used to visualize ER-mitochondria contacts at nanoscale resolution in living cells, reporting a distance of ~45 nm between the two organelles at MCSs [102]. Finally, by combining interferometric 4Pi-SMS imaging with ratiometric colour assignment, 3D multicolor imaging of mammalian cells with a localization precision of 5-10 nm has been reported, highlighting the structure of different intracellular organelles and of ER-PM MCSs [103].

FRET and BRET

Two particular techniques that allow to increase the resolution of standard fluorescence microscopy, limiting the need for expensive equipment, are FRET and BRET (Bioluminescence Resonance Energy Transfer). FRET occurs when a donor fluorophore, in its excited state, transfers energy to an acceptor chromophore [104]. Importantly, FRET can occur only when the two fluorophores are closer than 10 nm and in an appropriate orientation [95]. Moreover, FRET efficiency is inversely

proportional to the distance between fluorophores so that, fusing each of the two with specific proteins located in two target membranes, it is possible to dynamically measure their proximity [25,104].

FRET pairs in which the interaction between donor and acceptor fluorophore can be induced and maximized by rapamycin have been also used. Importantly, on one hand, rapamycin cross-linking increases the generated signal. On the other hand, however, a progressive forced proximity of the two membranes is observed [25,105]. Moreover, the addition of rapamycin to live samples could affect their physiology, since the drug is a potent autophagy inducer. Recently, by combining specific FRET couples with fluorescence lifetime imaging microscopy (FLIM), ER-trans-Golgi MCSs have been visualized in living cells, allowing the identification of molecules involved in the formation of these organelle contacts [87].

BRET uses a bioluminophore (luciferin/coelenterazine), oxidized by luciferase, which is able to transfer its energy to a fluorescent acceptor by resonance [106]. Compared to FRET, it reduces photo-toxicity, because it does not require sample illumination and does not depend on the donor/acceptor orientation, thereby increasing the signal-to-noise ratio [106]. However, the need for specific experimental equipment, the low intensity of the generated signal, the need to supply cells with luciferin/coelenterazine to fuel luciferase, as well as the extremely high sensitivity of luciferase activity to endogenous ATP levels and pH changes, severely limited the versatility of this technique in the study of MCSs. A recent work reported a BRET-based reversible system (MERLIN) for dynamically mapping ER-mitochondria contacts without, however, providing information about individual MCSs [107].

Bimolecular fluorescence complementation

Another technique used to measure MCSs is the dimerization-dependent fluorescent protein (ddFP) technology. It involves the reversible binding of two dark fluorophore monomers that become fluorescent when close enough to form the dimer [108]. This technique, however, is endowed with

limited brightness, low contrast and very low signal, features that are incompatible with a satisfactory visualization of MCSs.

A similar approach relies on split-GFP, a GFP divided into two non-fluorescent polypeptides (each fused with one target protein) which, when close enough, reconstitute the entire, fluorescent GFP [109]. The addition of spacers of specific length between each GFP fragment and the relative fusion protein can help to map and discriminate between different types of MCSs, *i.e.*, between contacts in which organelle distance is below a threshold of choice (**Figure 3B**) [110]. Indeed, different functionalities have been associated with different gaps between organelle membranes [111].

When using these multicomponent probes, however, an accurate stoichiometry between the two parts of the probe is really important and systems allowing their equal expression (*e.g.*, bicistronic vectors) are strongly recommended. Moreover, this system presents all the limitations of genetically-encoded probes described above; in addition, the two GFP portions bind irreversibly, limiting the possibility of recording MCSs dynamically and possibly leading to the formation of non-physiological MCSs (see for example [112]). To avoid the forced tethering induced by split-GFP, other systems have been developed. Among these, the light-inducible membrane-tethered peripheral ER (LiMETER) allowed an inducible, reversible, specific labelling of ER-PM contacts by GFP [113].

Proximity Ligation Assay

Another technique based on reciprocal positioning of specific targets *in situ* (at distances < 40 nm) is the Proximity Ligation Assay (PLA) [114]. It employs specific antibodies, recognizing proteins located in the two target membranes, conjugated with short DNA oligonucleotides. When a connector DNA strand is added, it ligates the two DNA strands and form a single DNA molecule. One of the PLA probes can then work as a primer in presence of DNA polymerase, inducing rolling-circle amplification (RCA). Thanks to the addition of fluorescent complementary oligonucleotides, the newly synthetized DNA is visualized by fluorescence [115]. By tagging

abundant resident proteins on membranes of two organelles, this assay offers the possibility of detecting MCSs [116]. The steric hindrance of the PLA system, however, might be a critical point, because its proper folding could be incompatible with the very narrow spaces in which MCSs take place. Moreover, before drawing conclusions, the expression levels of target proteins must be checked carefully. Indeed, an increased signal may arise from a higher abundance of the proteins recognized by the antibodies, rather than from an increased organelle apposition. Thus, this approach relies strongly on target protein expression and antibody specificity and, in addition, needs sample fixation.

1.2 Biochemical analysis

The majority of biochemical analyses aim at identifying protein localization or protein-protein interactions involved in MCS formation, as well as their functional involvement. They start from collecting cellular extracts, thus indirectly estimating organelle interactions on entire organs or tissues. Although these approaches do not need to express exogenous proteins, without interfering with MCS physiology, they could destroy weak interactions or artificially bring together non-contacting structures. Bioinformatic tools, predicting protein cellular localization by machine learning-based determination, can further validate biochemical results or help the design of new experiments [117,118].

Co-immunoprecipitation

Several biochemical purification techniques have been used to purify protein complexes present at MCSs, like affinity chromatography, often using tags, tandem affinity purification (TAP) tagging [119] and co-immunoprecipitation (co-IP). By this latter technique, a MCS-involved target protein is purified with the help of specific bead-linked antibodies, starting from a non-denaturing protein extract, containing the protein of interest. Consequently, any protein that is tightly associated with the target will be also co-precipitated, allowing the collection of many proteins, possibly contributing to the formation of a MCS multi-protein complex [120,121].

Mass spectrometry (MS) helps the analysis of co-IP partners. MS is extremely sensitive and requires low amount of material. However, the accuracy of MS results relies on sample purity, which requires several specific steps and appropriate controls during sample preparation. MS could also be involved in the analysis of co-IP partners after the use of cross-linkers, molecules able to stabilize transient inter-protein interactions. Recent discoveries in the MS field further improved its applications in the study of MCSs, like the analysis of post-translational protein modifications. For example, the phosphorylation state of a protein might suggest its cellular re-localization or enzymatic modification at MCSs [122].

The co-IP/MS approach is, however, poorly quantitative and, to get the final protein pool from cell homogenates, several chemicals have to be used, possibly altering protein structures and affecting protein-protein interactions [119]. To further validate the results obtained by co-IP, additional techniques, such as the two-hybrid system, can be used [123].

Purification of organelle interface fractions

Another widely used biochemical method to investigate MCSs, at least some of them, is the isolation of organelle interface fractions. Originally, this was the first method utilized to define MAM [21]. It is based on sequential density gradient ultracentrifuge of tissue/cell homogenates and allows the separation of nuclear, membrane, organelle and cytosolic fractions. By this protocol, organelle membrane interface fractions can be isolated and analyzed further by Western blotting, giving information about the presence of particular proteins. Moreover, MS can discover additional unknown molecular components involved in the formation/modulation of the specific organelle interface.

In particular, the original protocol to isolate MAM fraction has been recently optimized [124], yet this technique remains poorly quantitative. Moreover, some experimental conditions can affect sample purification yield. For this reason, rigorous normalization with subcellular markers is always

necessary. All the obtained fractions are indeed never pure but only enriched in specific target components [95].

2. Functional MCS analysis

The discovery of a MCS is both a starting point and a proof of concept of its possible functional role. Multiple analyses, however, must be performed in order to understand the specific function of a MCS within the cell. Generally, the physiological meaning of a MCS could be defined by analyzing changes in a particular function predicted to be affected by the specific organelle interface, such as Ca^{2+} signalling and bioenergetics, cell proliferation and death, autophagy, lipid metabolism, unfolded protein response or inflammation [1,10].

For instance, the presence of certain lipids (labelled by radioactive ^{3}H precursors) in specific isolated cell fractions may be informative on the pathways involved in lipid synthesis/metabolism at MCSs [21]. Following radioactivity at different times, it has been demonstrated that PS is synthesized at ER membranes and then efficiently translocated from ER to mitochondria, where it is converted into PE before translocating back to the ER [44,45,125]. More recently, radiolabelling has been combined with a fluorescent-conjugated form of cinnamycin, an antibiotic that binds to PE, allowing its visualization [126]. On the same line, the subcellular distribution of specific lipids, such as PS, can be investigated by using the PS-binding domain of lactadherin and evectin proteins, as distinct genetically-encoded probes. This approach allowed to discover that the lipid composition of the trans-Golgi network (TGN), and in particular its PS content, is of crucial importance for the stability of ER–TGN contact sites [87].

In addition, measurements of Ca^{2+}fluxes at MCSs can be used as a functional assay of organelle coupling. In particular, PM-ER and ER-mitochondria Ca^{2+} cross-talk have been extensively investigated. The quantification of ER to mitochondria Ca^{2+} transfer can be assessed by ER- and mitochondria-targeted genetically-encoded Ca^{2+} indicators. Generally, the higher the net amount of Ca^{2+} transferred from ER to mitochondria, the closer the juxtaposition between the two organelles

(**Figure 3C**). However, appropriate controls are necessary to genuinely evaluate the impact of organelle contacts on the overall process. Indeed, mitochondrial Ca^{2+} uptake not only relies on the vicinity to ER, but also on the intrinsic capacity of mitochondria to take up Ca^{2+} and on the overall concentration of the cation ($[Ca^{2+}]$) to which these organelles are exposed [95,127]. The first parameter can be checked by evaluating the expression levels of the different MCUC components, as well as of the mitochondrial Ca^{2+} efflux proteins. Furthermore, mitochondria exposure to different fixed, known $[Ca^{2+}]$, after cell permeabilization, can be informative on the intrinsic properties of mitochondrial Ca^{2+} handling machineries [91]. On the other hand, the overall cytosolic Ca^{2+} rise induced by the specific stimulation, evaluated by cytosolic probes (*i.e.*, fluorescent chemical molecules, such as fura-2 or genetically-encoded indicators [128]), need to be considered. Finally, a more direct evaluation of the number/extension of Ca^{2+} hot-spots generated at the OMM can be evaluated by specific, OMM-targeted genetically-encoded Ca^{2+} indicators [24,25]. However, so far, the need for both complex pixel-by-pixel analysis and high spatial/temporal resolution of the detection apparatus has limited their use.

Conclusion

In the last years, MCSs have emerged as key determinants in the physio-pathological control of cellular metabolism and homeostasis. Therefore, the deep analysis of such inter-organelle contacts is of utmost importance for the understanding of several cellular processes. Moreover, the elucidation of their functional roles and molecular determinants could favor the design of new therapeutic treatments whenever their alterations underlie particular pathological conditions. To this aim, multiple approaches have been recently developed to investigate MCSs, each one, however, with specific strengths and drawbacks. Therefore, the best approach to investigate MCSs is to look at them in parallel by multiple techniques, making a puzzle with different collected morphological and functional data, with the final goal of clarifying this fundamental field in cell biology.

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Conflict of interest

The authors declare no conflict of interest.

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MR, PP and RF wrote the manuscript.

Figure legends

Figure 1. Organization of a possible MCS. Structural components, such as tethering proteins, keep organelles in close apposition, and may form a platform for the assembly of protein complexes exerting specific functionalities. Among these, the exchange of lipids, soluble metabolites and the transmission of signals (for instance, Ca^{2+}) have been frequently reported.

Figure 2. Organelle contacts within the cells. The cartoon represents different inter-organelle contacts and their major functionalities. Frequently, molecular signals, such as Ca^{2+}, are locally exchanged at specific MCSs (often involving the ER). Lipid transfer is another common functionality associated with different MCSs, as well as the assembly of protein complexes regulating organelle morphology/dynamics or specific signalling pathways, such as inflammasome activation. Finally, the formation of certain organelles (for instance, autophagosomes), takes advantage of MCSs, likely because of a favored protein/lipid exchange.

Figure 3. Structural and functional techniques to measure ER-mitochondria coupling. A) Representative confocal image of a HeLa cell, expressing ER-targeted GFP (ER-GFP) and mitochondria-targeted RFP (mt-RFP). Yellow regions of signal co-localization represent sites of organelle proximity. Bar: 10 μm. B) Representative confocal image of a HeLa cells, expressing the $SPLICS_L$ probe (highlighting ER-mitochondria contacts (< 50 nm), see text for details) and immuno-stained with a specific, anti-TOM20 antibody (to mark the entire mitochondrial network). Bar: 10 μm. C) The cartoon shows typical traces of Ca^{2+} transients recorded, by specific probes, in the bulk cytosol, the mitochondrial matrix and at ER-mitochondria interface upon an IP3-linked cell stimulation.

Table 1. Pro and cons of the main techniques used to investigate MCS structure.

Imaging Techniques for structural MCS analysis	Advantages	Disadvantages
Electron microscopy (EM)	• High resolution (0.2 nm) [82] • Both 2D and 3D MCS images [83,84] • Direct visualization of MCSs and specific MCS-located proteins (by Immuno-EM (IEM)) [85] • Imaging of intact, fully hydrated cells up to 15 μm thick by x-ray tomography (SXT) [88]	• Alteration of delicate tissues by sample fixation, dehydration and staining (avoided in cryo-electron tomography (cryo-ET)) [92,93] • Incompatibility with live imaging, failing to map MCS dynamicity • Time consuming
Fluorescence microscopy	• Simultaneous dynamic live imaging of MCSs among different organelles [12] • Imaging of specific protein dynamics • Relatively accessible equipment	• Low resolution of ~ 250 nm (overcome by super-resolution methods [96-101]) • Need to express organelle targeted fluorescent proteins, or use specific antibodies • Alteration of endogenous protein expression or organelle morphology by sample transfection/infection of exogenous fluorescent proteins [95] • With immunofluorescence, need of sample fixation, impossibility to perform live imaging and final outcome dependent on antibody specificity [95] • With most super-resolution methods, small range of illumination, photo-bleaching and fluorescent dye photo-quenching, fixation is required
FÖrster Resonance Energy Transfer (FRET)	• Higher resolution (~ 10 nm) than classical fluorescence microscopy [95] • Live imaging • High temporal resolution	• Photo-toxicity • Dependence on donor/acceptor orientation [95] • Specialized equipment required • Specific couples of fluorescent proteins necessary • Need for appropriate controls (evaluation of the dynamic range)
Bioluminescence Resonance Energy Transfer (BRET)	• Higher resolution than classical fluorescence	• Low signal intensity [106] • Need for specific

	microscopy [106] • Live imaging • Limited photo-toxicity [106]	experimental equipment and luciferin/coelenterazine • Luciferase sensibility to endogenous ATP levels and pH changes
Bimolecular fluorescence complementation	• Live imaging [110] • Increased specificity by genetic engineering • Resolution can be modified by genetic engineering of the probes (fusion with spacers of specific length) [110] • Accessible equipment required • Relatively simple quantification	• Limited brightness in the reversible systems [108] • Need of accurate stoichiometry in the expression of multicomponent probes • Alteration of endogenous protein expression or organelle morphology by sample transfection/infection of exogenous fluorescent proteins [95] • Irreversible systems can affect MCS dynamics • Reversibility only in few systems [108]
Proximity Ligation Assay	• Higher resolution than classical fluorescence microscopy (< 40 nm) [114] • Relatively simple quantification	• Antibody steric hindrance • Outcome dependent on the expression level of target proteins and antibody specificity [115] • Need for sample fixation
Co-immunoprecipitation + Mass Spectrometry (MS)	• Estimation of organelle interactions on entire organs or tissues • Possible detection of post-translational protein modifications [122] • Discovery of unknown MCS players • Detection of protein physical interaction at MCS [120,121]	• Reliability of MS results depends on sample purity • Poorly quantitative • Possible alterations of protein structure and protein-protein interactions by sample chemical treatment [119] • Need of other techniques to validate the obtained results
Purification of organelle interface fractions + Mass Spectrometry (MS)	• Discovery of unknown MCS players [21,124] • Estimation of organelle interactions on entire organs or tissues	• Poorly quantitative • Need of rigorous normalization with known fraction markers to evaluate sample purification yield [124] • Contamination by other fractions [95]

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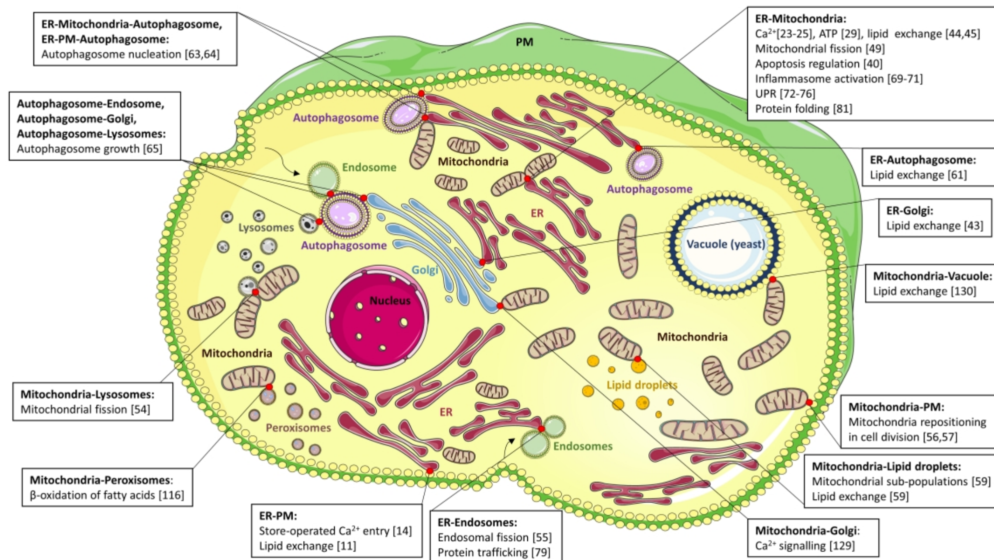

Organelle contacts within the cells. The cartoon represents different inter-organelle contacts and their major functionalities. Frequently, molecular signals, such as Ca2+, are locally exchanged at specific MCSs (often involving the ER). Lipid transfer is another common functionality associated with different MCSs, as well as the assembly of protein complexes regulating organelle morphology/dynamics or specific signalling pathways, such as inflammasome activation. Finally, the formation of certain organelles (for instance, autophagosomes), takes advantage of MCSs, likely because of a favored protein/lipid exchange.

338x190mm (300 x 300 DPI)

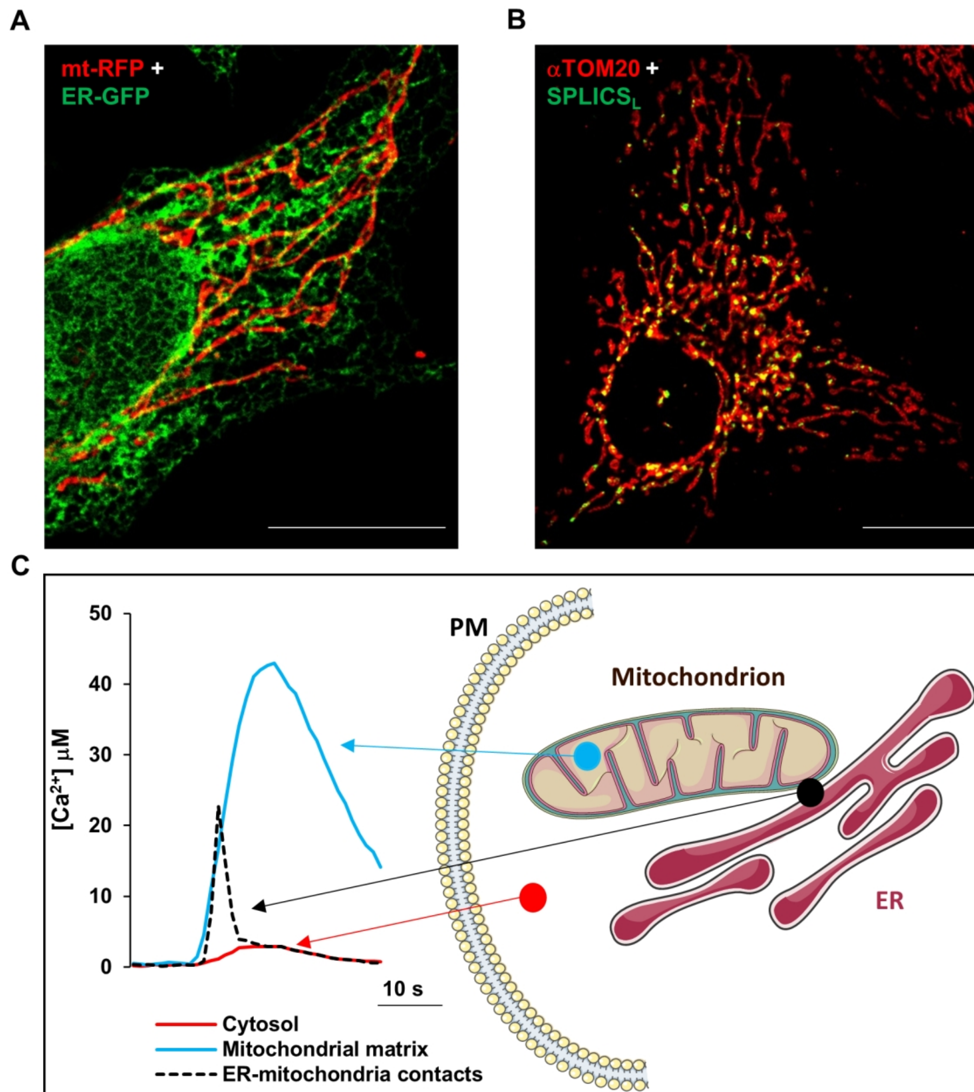

Figure 3. Structural and functional techniques to measure ER-mitochondria coupling. A) Representative confocal image of a HeLa cell, expressing ER-targeted GFP (ER-GFP) and mitochondria-targeted RFP (mt-RFP). Yellow regions of signal co-localization represent sites of organelle proximity. Bar: 10μm. B) Representative confocal image of a HeLa cells, expressing the SPLICSL probe (highlighting ER-mitochondria contacts (< 50 nm), see text for details) and immuno-stained with a specific, anti-TOM20 antibody (to mark the entire mitochondrial network). Bar: 10 μm. C) The cartoon shows typical traces of Ca2+ transients recorded, by specific probes, in the bulk cytosol, the mitochondrial matrix and at ER-mitochondria interface upon an IP3-linked cell stimulation.