Review Article

A Microscopic View of the Store-Operated Calcium Entry-Pathway

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Orai and STIM are the basic components of a highly complex and regulated mechanism for Ca^{2+} entry into the cell, known as store-operated calcium entry (SOCE). The activation of plasma membrane G-protein-coupled receptors associated with the phospholipase C cascade results in the rapid and massive production of inositol 1,4,5-triphosphate (IP_3). This second messenger triggers the massive efflux of Ca^{2+} from the endoplasmic reticulum and into the cytosol, resulting in the oligomerization of the stromal interacting molecule (STIM1), a sensor of ER Ca^{2+} . STIM1 oligomers (the so-called puncta) activate Orai channels at the plasma membrane, triggering the influx of Ca^{2+} into the cytosol. Several microscopy techniques have been implemented to study SOCE, resulting in stunning images of protein complexes assembling in real time. However, little attention has been paid to the findings about this complex mechanism from the imaging point of view, some of which appear to produce contradictory results. In the present review we gathered all the information about SOCE obtained with imaging techniques and contrast these findings with those obtained with alternative methods.

1. Introduction

Calcium (Ca^{2+}) is a key and ubiquitous second messenger, controlling a wide variety of cellular functions from cell proliferation to apoptosis [1]. Hence, Ca^{2+} signaling has to be highly regulated both in time and space [2]. To accomplish this task, cells rely on numerous pumps and channels, comprising the machinery to generate and regulate spatial-temporal Ca^{2+} signals [3].

There are two principal Ca^{2+} sources in the cell: (i) the extracellular medium and (ii) the intracellular stores, most notably the endoplasmic reticulum (ER), which plays a central role in Ca^{2+} homeostasis, not only as the major intracellular store, but also as the controller of the cytosolic entry of Ca^{2+} across the plasma membrane (PM). The modulation of calcium entry from the ER begins when phospholipase C (PLC) is activated by G-protein-coupled receptors on the cell surface, leading to the generation of the second messenger inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from the ER after binding the IP_3 receptor (IP_3R) located at the ER membrane [4, 5]. IP_3R activation results in the massive efflux

of Ca^{2+} from the ER and into the cytosol. This decrease of ER- Ca^{2+} content triggers the influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels in a process known as store-operated calcium entry (SOCE) [4].

The importance of SOCE in the immune system has been well established, where it plays a pivotal role in B- and T-cell activation, by triggering antigen recognition through activation of the transcription factor, NFAT [6, 7]. The molecular identities of the proteins involved in the SOCE were discovered recently in 2005, through a strategy of large-scale RNAi-based screening. dSTIM was initially identified in *Drosophila* as well as its human homologue STIM1 [8]. One year later Orai was identified almost simultaneously by a wide-genome screen strategy and by linkage analysis with single nucleotide polymorphism arrays in patients with a form of hereditary severe combined immune deficiency (SCID) [9, 10].

In the last seven years there have been major advances in our understanding of SOCE at the molecular level. The mechanism of gating and regulation of the Orai channel has been almost elucidated; inter- and intra-molecular dynamic

interactions between STIM1 and Orai achieve the opening of the channel leading to Ca^{2+} entry. Such important breakthroughs have been the product of a wide range of innovative and versatile tools derived from microscopy and imaging. In this review, we summarized what is known about the two most important proteins on SOCE (Orai and STIM) with emphasis in the different microscopy techniques that allowed the visualization of the molecular mechanisms of SOCE with such exquisite details.

2. Orai

The Orai protein is the pore-forming unit from CRAC channels [11, 12]. The calcium-release activated current (I_{crac}) was initially characterized in mast cells using patch clamp methods [13]. I_{crac} was identified as a calcium-selective current activation upon depletion of the ER [13, 14]. In mammals, the Orai channel family includes three homologs (Orai 1–3) [9]. All of them are highly selective for Ca^{2+} , just differing between them on their activation kinetics and fast and slow Ca^{2+} -dependent inactivation [15, 16].

There is evidence that Orail forms heteromeric channels [16, 17]. Nonetheless, stoichiometry of functional Orail channel is still controversial. Recently, the crystal structure of dOrai (Orai from *Drosophila*) was determined at 3.35 Å resolution [18]. By choosing among several orthologs of Orai, dOrai was selected by its biochemical properties and high homology with human Orail (73%). Following modification, expression, and purification of dOrai, an edited form of the channel was crystallized without loops between transmembrane segments and just covering amino acids 132–341 to produce well-ordered crystals. The final structure obtained was composed of a hexameric assembly of Orai subunits, with the ion pore at the center of the channel [18]. The open state of Orai crystal was engineered through the mutation in V174A (V102A in human Orail) which renders constitutively active channels in absence of STIM1 [19]. The question of whether engineered dOrai could reassemble functional cation channels was studied by Na^+ efflux using a fluorescence-based assay with purified channels reconstituted in liposomes [18].

Other approaches to explore the Orail conformation have been through cross-linking assays, where contradictory results have been obtained. Dimers in resting state, tetramers, hexamers, and even high molecular aggregates are common results in assays where concentration of the cross-linking agent is directly proportional to the grade of oligomerization [18, 20, 21].

Elucidating the crystal structure of Orai represents strong evidence about the hexameric conformation of the channel. However, there is vast microscopy evidence from several groups reporting a tetrameric configuration, both in functional and resting channels [20, 22–24], by using single molecule imaging technique, with total internal reflection fluorescence Microscopy (TIRFM) in HEK293 [22] and *Xenopus* oocytes [21]. Through the use of molecular constructs of Orail fused to the green fluorescent protein (GFP) and continued exposure to strong laser excitation, bleaching steps of the fluorescent protein were counted in order to estimate

the number of subunits in single molecule studies of the channel [21, 22, 25]. Such measurements provide strong evidence favoring the tetrameric conformation of the channel [21, 22]. However, single molecule photobleaching has been conducted in inactive channels, since STIM1 activation results in Orai clustering, preventing single molecule studies. Nonetheless, these limitations were resolved by using fixed cells that expressed low amounts of Orail and the constitutively active C-terminal of STIM1 to gate the channel. Ji et al. used the method originally reported by Ulbrich and Isacoff to determine subunits composition of membrane proteins [26]. By means of engineering several fluorescent Orail in tandem, which were fused to GFP, the correlation of bleaching steps with the tandem multimers was resolved. The results suggest a four-subunit arrangement of Orail as the active form of the channel. Furthermore, these photobleaching studies were confirmed by Förster resonance energy transfer (FRET) of the tandem constructs [22].

Counting discrete photobleaching steps of single fluorescent molecules requires reduced protein expression to lower the probability of having two assembled channels located in the same diffraction-limited spot and to eliminate the probability of fluorescent protein misfolding. Moreover, some limitations of this method are evident when the channel is conformed by 5 or more subunits, causing a similar distribution of bleaching steps for n and $n + 1$; in such case bleaching step size must be taken into account [26]. In the work of Ji et al. a hexameric Orail configuration was not explored. However, a bleaching step size undetected by a high order arrangement of Orail was abrogated by using concatenated OraIs fused to GFP, which prevented hidden photobleaching steps. These experiments were conducted using TIRFM, to reduce autofluorescence coming from the cytoplasm, which may interfere with single molecule fluorescence measurements [26].

Another relevant point still in discussion is the stoichiometry of Orail in the resting state; the results are contradictory even between groups that used single molecule photobleaching imaging. Demuro et al. reported a dimer conformation using *Xenopus* oocytes and HEK293 cells [21, 25]. On the other hand, Ji et al. reported a tetrameric state with the same methodology and using HEK293 as well [22]. The discrepancy between both results could be explained because Demuro et al. transfected Orail alone, while Ji et al. coexpressed Orail and STIM1 [25]. The later study suggests that if homodimers of Orail are forming in the resting state, STIM1 may be necessary to achieve the oligomerization in its active form. This last conclusion complicates the comparison of experiments like those using the V102A mutation in Orail, which renders constitutive active channels in absence of STIM1 [19] and even the crystal structure in which STIM1 was not employed [18].

In order to analyze the stoichiometry of Orail in the resting state with live cells and avoid possible artifacts of fixation, Madl et al. used a new fluorescent microscopy technique, which combines fluorescence recovery after photobleaching (FRAP), molecular tracking, and brightness analysis [24]. Through a highly specialized protocol of photobleaching in T24 cells expressing Orail-GFP, free diffusible channels were

obtained. This channel conformation is called Thinning Out Clusters while Conserving the Stoichiometry of Labeling (TOCCSL). Molecule tracking and brightness analysis of single fluorescent spots was conducted and the brightness values were fitted according to dimer, trimer, tetrameric, and pentameric Orai arrangements. A 4-Gaussian model was the best fit to the data, suggesting a tetrameric channel conformation in resting state [24]. Well-ordered TOCCSL must be obtained to ensure the appropriate fitting of the stoichiometric arrangement. To obtain such condition, photobleaching has to be performed in conventional epifluorescence. Furthermore, these results were confirmed by conducting FRET experiments between covalently linked Orai1 homodimers CFP/YFP-labeled, resulting in a significant interaction between homodimers, regardless of whether STIM1 is cotransfected or ER was depleted [24]. Maruyama et al. confirmed the same results through purified Orai1 FLAG tagged in reconstructed three-dimensional structures from negatively stained electron microscopy images with a resolution of 21 Å [20]. By using 3681 images from purified Orai1, they resolved a teardrop-shaped channel in its basal conformation, with 4-fold symmetry from its top view and with the largest portion of the protein facing the cytoplasm. A large cytoplasm portion of Orai1 was estimated of approximately 10 nm in length, supporting the interaction with the C-terminal of STIM1 previously reported by biochemical methods [20].

Because Orai1 may form functional homo- and heteromers, its stoichiometry may have important implications for the interaction with other partners (such as STIM1), reflecting modifications in its biophysical properties. Such is the case of the so-called ARC channels, which produce the arachidonic-regulated Ca^{2+} selective current, formed by the association between Orai1 and Orai3 in what appears to be a pentameric arrangement [27, 28]. The association between Orai1 and Orai3 has been demonstrated by FRET, where both Orai1 and Orai3 were tagged with CFP and YFP, respectively [17]. Their interaction resulted in a high value of FRET, even higher than that obtained when Orai1-CFP and Orai1-YFP were expressed to form homotetramers [17]. Nonetheless, the Orai1 interaction that has received more attention recently has been that with TRPC channels, which we will discuss in the following sections.

3. STIM1

Historically, there were at least three hypotheses proposed to explain the mechanism of transmitting the signal from the depletion of intracellular stores to the PM: (i) a diffusible messenger, (ii) vesicle secretion, and (iii) a conformational coupling of proteins in the intracellular store membranes with PM channels [29]. The latter was confirmed with the discovery of new functions of STIM1 [8], a single pass transmembrane protein essential for SOCE [8, 30].

In mammals, STIM has two homologues (STIM1 and STIM2). STIM1 was first identified as a surface membrane protein [31] that confers binding capacity to pre-B lymphocytes [32, 33], although all the attention has been focused recently on its role in SOCE, where STIM1 serves as the sensor of the depletion of Ca^{2+} from the ER. Upon ER

depletion, Ca^{2+} unbinds from STIM1 EF hand, resulting in the oligomerization of this protein and its subsequent translocation to the so-called ER-PM junctions [33].

Under basal conditions, it was demonstrated that STIM1 colocalizes with ER markers [30] and by immunoelectron microscopy its presence was confirmed in the ER membrane [34]. In addition, STIM1 is associated with microtubule tracking protein EB1 [35, 36] and travels continuously through the ER under resting conditions (when the ER is filled with Ca^{2+}) [37]. Stathopoulos et al. published several studies reporting a monomeric conformation of luminal STIM1 in basal conditions and a dimer and high aggregate state produced after the depletion of Ca^{2+} from the ER [38, 39]. This depletion from the ER causes the loss of Ca^{2+} from the EF-Hand domain in STIM1, a motif located in the ER lumen, which binds Ca^{2+} with a dissociation constant in a range of 200–600 μM and a 1:1 stoichiometry [38]. Albeit, Stathopoulos et al. showed in great detail the molecular mechanisms underlying the binding to Ca^{2+} and the tertiary structure of the luminal region of STIM1; these *in vitro* assays differ from that reported by He et al. who showed direct interactions between STIMs in resting state [40], through the use of bimolecular fluorescence complementation (BiFC) technique, a simple and elegant method to study protein interactions in live cells which requires the generation of two recombinant STIM1, one expressing the amino terminus half of a fluorescent protein and the second one expressing the carboxyl terminus of the same fluorescent protein. BiFC takes place when both monomers of STIM1 associate, resulting in the generation of a functional fluorescent protein after STIM dimerization. The results obtained with this method were confirmed by FRET experiments between CFP-STIM1 and YFP-STIM1 [40].

The discrepancy between the two reports could lie in the fact that Stathopoulos's group used the luminal region of STIM1 and Jun He group employs the whole protein. It is relevant to point out that BiFC requires appropriate controls to avoid false-positive results [41]. First, expression of the fusion proteins had to be at concentrations approximating their endogenous counterparts, in order to minimize protein mislocalization and formation of nonnative complexes (check Table 1). Second, protein constructs in which the interaction interface has been mutated and fused to the fluorescent protein fragment are appropriate controls to avoid spontaneous complementation [42]. He et al. highlight the importance of C-terminal region of STIM1 to conduct the oligomerization in resting state, through the construct lacking the amino acids region 233–474 which is unable to interact and form oligomers. However, mutant constructs lacking the SAM domain, an important 5-helix luminal region of STIM1, that Stathopoulos et al. reported to be responsible for the oligomerization when the ER is depleted of calcium [38, 39] did not modify the fluorescence complementation of STIM1 in resting state [40].

The Ca^{2+} unbound condition of STIM1 destabilizes the EF-SAM domain [38, 39], which results in the oligomerization of STIM1 in a structure commonly referred to as a “puncta” which is an essential step to activate CRAC channels [30, 34, 37, 43, 44]. The first models of STIM1

TABLE 1: Fluorescence microscopy techniques.

Microscopy technique	Brief concept	Advantages and uses	Disadvantages	Ref.
Colocalization	This technique measures the coexistence of signal at the same pixel location when at least two different fluorochromes are visualized by multichannel fluorescence microscopy [93]. This method is helpful in answering the question of whether two or more fluorochromes are located in the same physical structure, within the diffraction limit.	(i) Examine multichannel signals. (ii) Experiments can be conducted with live and fixed cells. (iii) Detection with antigens and fluorescent proteins. (iv) Dynamic experiments with subcellular location. (v) Simple evaluation. (vi) Studies conducted at tissue and cellular levels.	(i) Not tested protein interactions. (ii) Does not provide direct proof of functional relationship.	[93, 94]
Föster resonance energy transfer (FRET)	FRET is a process by which energy is transferred from one fluorophore (donor) to another nearby molecule (acceptor) through a nonradioactive pathway. For FRET to occur 3 conditions must be met: (1) less than 10 nm of distance between donor and acceptor; (2) the emission spectrum of the donor must overlap the acceptor molecule excitation spectrum; (3) the emission dipole of the donor and excitation dipole of the acceptor must not be oriented perpendicular to each other [80, 95].	(i) Examine protein interactions and dynamics. (ii) Molecular ruler. (iii) Development of biomolecules sensors by FRET. (iv) Intramolecular transition studies. (v) Proteolysis studies. (vi) Live-cell molecular interactions. (vii) Several FRET microscopy techniques available. (viii) FRET goes beyond the resolution limit of light microscopy (diffraction limit).	(i) Lack of FRET not necessarily indicates lack of interaction, due to incorrect orientation (dipolar moment) of fluorophores. (ii) Limited number of interactions can be explored at once.	[80, 95]
Fluorescence recover after photobleaching (FRAP)	FRAP is used to assess the molecular mobility in living cells. Proteins of interest tagged with a fluorophore or fused to a fluorescent protein are subjected to irreversible bleaching in a region of interest (ROI) using a high-power laser illumination [96–98].	(i) Live-cell molecular dynamics. (ii) Studies of protein dynamics in a lipid raft context. (iii) Studies of lipids dynamics.	(i) Photodamage of the cells. (ii) Density of detectable molecules limited.	[96–98]
Bimolecular fluorescence complementation (BiFC)	A technique to visualize protein interactions. It is based on structural complementation between two nonfluorescent fragments of a fluorescent protein [41].	(i) Irreversible BiFC complexes are useful to analyze weak or transient interactions. (ii) High sensitivity. (iii) Simplicity of analysis. (iv) BiFC does not require specialized equipment and postacquisition image processing.	(i) Irreversibility of BiFC complexes makes measurement of dynamic interaction impossible to assess. (ii) Overexpression of the protein of interest might force complementation, resulting in false-positives.	[40–42]
Superresolution microscopy (nanoscopy)	This microscopy offers spatial resolution beyond the diffraction limit of conventional microscopes by exploiting nonlinear phenomena. There are at least four approaches to reach superresolution: (1) stimulated emission depletion (STED), (2) structured illumination microscopy (SIM), (3) photoactivation localization microscopy (PALM), (4) stochastic optical reconstruction microscopy (STORM) [98, 99].	(i) XY-resolution in a range of 130–20 nm. (ii) Z-resolution in a range of 100–350 nm. (iii) Good for smaller or filamentous objects. (iv) Most techniques are not suitable for dynamic studies, because image acquisition is very slow (minutes).	(i) Dyes require special characteristics (photostability). (ii) Temporal resolution in minutes. (iii) Not suitable for live-cell imaging studies. (iv) Postprocessing imaging required (except for STED). (v) The performance critically depends on the labeling density and which biological structure is studied. Best suited for filamentous structures.	[98, 99]

TABLE 1: Continued.

Microscopy technique	Brief concept	Advantages and uses	Disadvantages	Ref.
		(i) Compatible with different kinds of microscopy: (1) confocal microscopy (2) superresolution microscopy (3) electron-microscopy (4) two-photon microscopy (5) TIRFM (6) epifluorescence (7) HILO (8) SPIM (ii) Live- and fixed-cells experiments. (iii) High spatial resolution for location and diffusion studies. (iv) Dynamic information with single particle tracking. (v) Conformational changes, vesicle movement, and oligomerization studies. (vi) Compatible with FRET, photobleaching, and photoactivation techniques. (vii) provide information about molecular kinetics.	(i) Very high signal-to-noise ratio. (ii) Dyes require photostability. (iii) High density of label and autofluorescence entails complications. (iv) It is limited by illumination depth and quenching of the signal.	[100, 101]
Single-molecule imaging	A powerful tool to examine a wide range of properties of individual molecules.			
		(i) Increases the signal-to-background ratio. (ii) Illumination beam always passes through the center of the focal plane, which confers a powerful tool for three-dimensional imaging. (iii) Reduced photobleaching. (iv) Allows single molecule studies.	(i) Restricted visual plane. (ii) Limited penetration depth. (iii) Not suitable for molecular tracking.	[101–103]
Highly inclined and laminated optical sheet microscopy (HILO)	HILO uses a laser beam slightly below the critical angle to illuminate the sample in a laminated and inclined thin optical sheet, also called shallow angle fluorescence microscopy (SAFM).	(i) Increases the signal-to-background ratio. (ii) Reduced photobleaching. (iii) Good penetration. (iv) Allows single molecule studies. (v) Fast acquisition. (vi) Phototoxicity limited.	(i) Requires time-consuming calibration of the system. (ii) Scattering light in the sample due to absorption by the specimen. (iii) Optical artifacts, like shadows and stripes, are common by the angle of illumination.	[103]
Selected plane illumination microscopy (SPIM)	SPIM illuminates the sample from one side in a focal plane where detection is done perpendicularly.		(i) Limited studies in structures other than plasma membrane. (ii) Requires time-consuming calibration to find the critical angle of illumination. This procedure must be repeated for every excitation wavelength. (iii) Multichannel (multicolor) experiments are difficult to produce unless expensive equipment is acquired to automate the changing of the critical illumination angle.	[100, 101, 104]
Total internal reflection fluorescence microscopy (TIRFM)	TIRFM is a surface selective imaging technique which illuminates a coverslip with a laser beam at an incident angle greater than critical angle, producing a nonpropagating electromagnetic field known as evanescent wave. The energy of evanescent wave decays exponentially with the distance, exciting fluorophores at distances not greater than 100 nm from the coverslip.	(i) Excellent tool to minimize background fluorescence. (ii) Dynamics of plasma membrane, like vesicle fusion, adhesion, and cell motion. (iii) Z-resolution in a range of 60–200 nm. (iv) Allows single molecule studies. (v) Live- and fixed-cells experiments.		

TABLE 1: Continued.

Microscopy technique	Brief concept	Advantages and uses	Disadvantages	Ref.
Light-guide-based TIRFM (LG-TIRFM)	This novel method for TIRFM uses an excitation light propagation via the coverslip to produce the evanescence wave, and uses the coverslip as a light guide.	In comparison with other systems of TIRFM, like objective or prism-based systems, LG-TIRFM confers the following advantages: (i) it can be used with dry, water, and oil immersion objectives, and a broad range of illuminators (not limited to laser excitation); (ii) excellent for multicolor experiments, and simple to setup; (iii) evanescence wave propagates in a large, evenly illuminated area; (iv) the excitation light does not enter the emission channel and does not interfere with detection, thus providing better signal/background ratios; (v) LG-TIRFM is compatible with open perfusion chambers, allowing simultaneous patch clamp or microinjections experiments; (vi) calibration of the system is much less demanding; (vii) low photobleaching in continuous laser excitation.	(i) Limited studies in structures other than plasma membrane. (ii) Expensive sapphire coverslip is required for oil-immersion objectives.	[104]
Surface plasmon resonance (SPR)	SPR is an optical technique to characterize interactions between different molecules. Through a monochromatic polarized light directed on a gold surface under total internal reflection, this produces an evanescence wave, which interacts with a sensor surface with a specific refractive index. When a molecule is covalently bound to the gold surface, it is possible to characterize in real-time the modifications in the refractive index similar to a signal measured in resonance units.	(i) Sensitive tool for identification and characterization of protein-protein interactions. (ii) Characterize antibody affinities. (iii) Protein-DNA interactions. (iv) Protein-lipids interactions. (v) Competitions and binding assays.	(i) Only for <i>in vitro</i> experiments. (ii) Not suitable for small analytes. (iii) High concentration of the analyte is required.	[86, 105, 106]

puncta formation argued that STIM1 translocates to the PM to activate CRAC channels [34]. However, through flow cytometry using a FICT-anti-FLAG antibody in permeabilized and nonpermeabilized cells, puncta structures were localized underneath the PM [37]. The same result was obtained by acid-induced quenching of GFP-STIM1 in real-time measurements by TIRFM imaging, taking advantage of GFP fluorescence pH sensitivity. The experiment consisted in acidifying the extracellular medium in a range from pH 7.3 to 5.2 to quench the fluorescence of GFP when present in the extracellular face of the plasma membrane but having no quenching effect if GFP was facing the cell cytosol [45]. The lack of any quenching effect argued in favor of STIM1 not being translocated to the PM.

Nevertheless, resolution of light microscopy imposes limits to attributing a spatial subcellular location to puncta. Studies using immunoelectron microscopy were conducted to attain a higher spatial resolution with adequate preservation of membrane structures [46]. Richard Lewis's group reported that STIM1 redistribution is a critical early step in Orai channel activation and measured the distance from STIM1 puncta to the PM in Jurkat cells. The calculated distance was 10 to 25 nm, which is sufficient for direct interactions between STIM1 (embedded in the ER membrane) and Orai at the PM [45, 47]. Using colocalization analysis and a system of chemically inducible (variable length) bridge formation between PM and ER membrane in COS-7 cells, a distance between 11 to 14 nm was suggested [47]. By means of a similar strategy, using a chemically inducible bridge, Luik et al. demonstrated that STIM1 oligomerization is redistributing very close to the PM, using a recombinant STIM1 in which the complete luminal region was replaced with 2 rapamycin-binding proteins, causing oligomerization when bound to rapamycin analogue, rapalogue [44].

STIM1 and its puncta formation are considered like a new paradigm in signal transduction, in which the ER-PM junctions play a critical role to create a restricted Ca^{2+} signal and facilitate Ca^{2+} gradients. The sequential translocation of STIM1 was described by Liou et al. by means of FRET and FRAP in live cell measurements [43]. STIM1 interactions were first measured by FRET showing that the initial interactions between STIM monomers occur without signs of puncta formation. Then, FRAP measurements were conducted in YFP-STIM1 to monitor the mobility in basal and activated state, resulting in a larger time for fluorescence recovery when Ca^{2+} store depletion occurs, which suggest a large immobile fraction of YFP-STIM1 anchored to the channels and lipids at the PM. They concluded that STIM1 ER-PM signaling is comprised of 4 sequential steps: (i) dissociation of Ca^{2+} from the EF-Hand domain, (ii) rapid oligomerization of STIM1-SAM motif, (iii) translocation of STIM1 to ER-PM junctions (puncta formation), and (iv) activation of CRAC channels [43].

Extensive mutagenesis analysis has been directed to explore the role of several amino acids in the ability of STIM1 to generate puncta. For example, the function of the EF-hand domain was determined from single-cell Ca^{2+} imaging experiments with the EF-hand mutant (D76A), which resulted

in a constitutive Ca^{2+} entry and puncta formation [30, 34]. However, puncta structures have been explored by means of recombinant expression of STIM1 often fused to fluorescent proteins. Just a few groups have demonstrated endogenous STIM1 clusters formation [34, 48, 49].

Both, confocal and TIRFM are far below to the z-resolution needed to resolve ER-PM junctions, because of the fact that Zhang et al. showed by immune-electron microscopy, with single molecule resolution, that STIM1 appears in clusters after depletion of the ER [34]. Nonetheless, Golovina used high spatial resolution imaging in astrocytes with the membrane-associated Ca^{2+} indicator, FFP-18. With this method a subplasma membrane Ca^{2+} concentration was visualized showing that SOCE starts at PM-regions overlying the ER [48]. Recently, our group showed reorganization of endogenous STIM1 in primary cultures of cortical astrocytes stimulated with thrombin [49]. In this study we showed for the first time that agonists (and not only depletion of the ER with drugs) induced puncta formation of endogenous STIM1 proteins.

The stoichiometry of STIM1 to gate Orai is reported to be of eight molecules of STIM1 [19, 50, 51]. In several studies STIM tandems have been used to activate CRAC channels, indicating that maximal CRAC current is reached when four Orai subunits and eight STIM1 are expressed [19, 50]. However, Icrac activation is not an "all-or-none" process and can be gradual, based on the amount of STIM1 oligomerization [51]. Activation of Orai by STIM1 requires interactions by coiled-coil domains, but this will be tackled in detail in the next section.

STIM1 has a polybasic domain in its C-terminus which functions like an electrostatic phosphoinositide lipid-binding domain [52, 53] and plays a crucial role in STIM1 clustering at the ER-PM junctions [54, 55]. Deletion of the STIM1 polybasic domain does not affect its oligomerization but prevents its recruitment to ER-PM junctions, showing that oligomerization and translocation are separated steps in the signal transduction [43]. Phosphoinositides are not essential for the accumulation of STIM1 in ER-PM junctions but can enhance the interaction between STIM1 and Orai [52, 56]. We have recently identified at the carboxyl terminus of STIM1 an APC-binding domain [57]. This domain overlaps with the phosphoinositide lipid-binding domain mentioned above. Adenomatous Polyposis Coli (APC) is a cortical cytoskeleton protein that interacts with microtubules via EB1 [58]. In the aforementioned study, we showed that a form of relay mechanism between EB1 and APC facilitates the positioning of STIM1 near ER-PM junctions after ER depletion [57].

4. STIM-Orai Coupling

STIM and Orai interaction is necessary to achieve calcium influx [35, 59–63]. The group of Murali Prakriya showed through live-cell FRET microscopy an enhancement of STIM1-Orai interaction when store depletion is induced [63]. The increment of FRET occurs in parallel with STIM1 and Orai redistribution to clusters [63]. In the same way Muik et al. analyzed the dynamic of interactions between STIM1 and Orai and explored the role of the C-terminal from

Orai1. Their results positioned the coiled-coil domain from STIM1 as relevant to maintain Orai-STIM1 coupling. A point mutation on the central amino acid leucine at position 273 of Orai1 destabilizes the coiled-coil structure, which results in loss of interaction with STIM1, inhibition of Icrac and suppression of Orai1-STIM1 clusters [64].

Nonetheless, STIM1 clustering is not sufficient to activate Orai1; this was demonstrated by Yuan et al. who mapped the minimal STIM1 domain necessary to activate Orai1 channels. By using a colocalization analysis between mCherry-Orai1 and the minimum fragment of STIM1 tagged with eGFP, they identified a segment that covers amino acids 344 to 442, named STIM1 Orai-activating region (SOAR) which can fully activate Orai1 channels. However, mutations within the SOAR domain from STIM1 prevent activation of Icrac but not puncta formation [60]. Almost simultaneously, Park et al. identified the same region of 110 amino acids referred to in their study as CRAC activation domain (CAD), comprising amino acids 342–448 [65, 66]. STIM1 possesses three putative coiled-coil domains within its cytosolic portion; the role of these domains was studied by Romanin's group using a combined approach of confocal FRET microscopy and electrophysiology [67].

Using a novel STIM1-FRET sensor, consisting in an Orai-activating small fragment (OASF, covering 233–474 amino acids from STIM1), double-labeled with CFP and YFP in N and C terminal, determined the intramolecular transition to achieve the interaction with Orai1 [68]. YFP-OASF-CFP (the sensor), when Orai1 is not overexpressed, showed strong FRET in basal state, being indicative of a compact conformation and close proximity between both fluorescent proteins. If Orai1 is overexpressed, YFP-OASF-CFP decreases its FRET value. Mutations in OASF, which disrupt the coiled-coil domains by changing their hydrophobic interactions, also reduced FRET. These results suggest that STIM1 C-terminal undergoes an intramolecular transition to an extended conformation to interact with Orai1 [68]. This work supports the previous results of Balla's group, who reported an acid patch in the first coiled-coil domain in STIM1 required for Orai activation [69]. Neutralization of the first coiled-coil domain resulted in a constitutive active STIM1; otherwise, neutralization of basic patch within SOAR resulted in inactive STIM1, concluding that in resting state, SOAR is occluded by the first acid patch inside the first coiled-coil domain. When STIM1 interacts with Orai1, SOAR is released and extended to interact with the channel [69].

Hydrophobic interactions between the first coiled-coil of STIM1 and SOAR domain unmask SOAR and keep it inactive; when the first coiled-coil domain interacts with Orai1 C-terminal, SOAR is released and free to interact with Orai1 [70]. There is some evidence suggesting that Orai1 N-terminal interacts through a weaker extent with STIM1 [65], and truncation of the entire N-terminus of Orai1 completely abolishes SOCE [64].

5. Other Players in SOCE

STIM1 and Orai1 are the basic components of SOCE. Experiments using heterologous expression in yeast showed that

STIM1 and Orai1 are sufficient to reconstitute SOCE [71]. However, a multitude of molecules, including lipids and proteins, have been identified to play a role in modulating SOCE [72]. We referred to these multiprotein interactions as the store-operated calcium influx complex (SOCIC) [1].

Before the discovery of Orai channels, all the attention was directed to some members from the TRP family, in particular TRPC1. The first study reported was conducted by our group, showing that *Drosophila*'s TRP channel was activated after store depletion with thapsigargin (TG), a drug commonly used to deplete ER stores from calcium [73]. There is solid evidence indicating that TRPC1 interacts with Orai1 and STIM1 [1, 74–77]. Electrophysiology studies demonstrated that STIM1 gates TRPC1 by electrostatic interaction with a polybasic domain of STIM1 and two conserved negatively charged aspartates in TRPC1 [78]. Moreover, interactions between TRPC1 and STIM1/Orai1 occur in specialized membrane domains composed of cholesterol and sphingolipids, such domains are called lipid rafts lipids rafts [59, 74, 75]. Sampieri et al. used a high resolution form of multicolor TIRFM-FRET method to show the dynamic association between STIM1 and TRPC1; such interaction is produced when the ER is depleted and takes place in subcellular regions rich in cholesterol and colocalizing with caveolin, an important protein in caveolae, a member of lipid raft family. STIM1-TRPC1 interaction was abolished when reducing the PM cholesterol content with methyl- β -cyclodextrin. Interestingly, reducing PM cholesterol content did not inactivate TRPC1 channels and cholesterol reduction transformed the channel from a store-operated to a receptor-operated channel [74]. This study suggests that cholesterol depletion converts TRPC1 channels from store operated to receptor operated by altering their activation mechanism.

Other reports have shown that STIM1 interacts with other channels besides Orai and TRPCs. Wang et al. showed that the SOAR domain within STIM1 strongly suppresses voltage-operated calcium channels (Cav1.2) and reciprocally activates Orai channels [79].

The list of proteins that interact and regulate SOCE keeps increasing, in spite of the diffraction limit imposed on fluorescence microscopy, which makes the study of molecular interactions at high resolution difficult; several studies have shown STIM1-EB1 interactions forming comet-like structures in resting state [35, 36]. To detect protein interactions by FRET is better accomplished in a TIRFM or HILO system, which possesses better resolution in the Z-axis (Figure 1). Because the ER is found at greater depths in the cell and is not completely visualized with TIRFM, Sampieri et al. developed a real-time shallow angle fluorescent microscopy (equivalent to HILO. in Table 1 and Figure 1) combined with FRET to follow STIM1-EB1 interactions [35]. Nonetheless, FRET not always depicts protein interactions because absence of FRET not necessarily reflects absence of interactions [80]. Such is the case of SERCA2A which is reported to interact with STIM1 puncta in coimmunoprecipitation assays, but FRET interactions were not detected, probably due to an incorrect dipole orientation of both (donor and acceptor) fluorescent proteins. In spite of the lack of FRET signal, SERCA2A forms

FIGURE 1: Optical principles of the fluorescence microscopy techniques and their contribution to SOCE. Numbers in red depict the z-resolution.

a ring decorating STIM1 puncta, as visualized by novel high-resolution form of TIRFM microscopy (LG-TIRFM) [1, 35].

There are several ways for conducting FRET, with various compatible microscopy techniques. Wide-field microscopy is the simplest and most widely used technique [81]. However, the use of a specific microscopy technique depends on the scope of the investigation [81]. It can be complicated to resolve FRET with small structures, where TIRFM and HILO are excellent tools to measure FRET. On the other hand, when it is required to detect FRET sensors on the cytoplasm for time-lapse experiments or interactions between proteins resident into large structures, confocal and even wide-field microscopy are substitutable and provide better results.

Other proteins like Calmodulin plays an important role mediating the Ca^{2+} -dependent inactivation of Orail by direct interaction with its N-terminus [61]. CRACR2 and SARAF are proteins recently identified. CRACR2 interacts with both Orail and STIM1 to form a ternary complex which associate in lower Ca^{2+} concentrations with the cytoplasm and is important to achieve the clustering of Orail and STIM1 [82]. SARAF, on the other hand, is a negative regulator of SOCE, which interacts with STIM1, as demonstrated by a competitive decrease of FRET in which SARAF-GFP and STIM1-mCherry were used like a pair-FRET and untagged STIM1 was used to corroborate the interaction in a competitive manner (by displacing STIM1-mCherry and abolishing FRET). The same results were obtained by fluorescence lifetime imaging microscopy (FLIM) [83], one of the most direct

methods for measuring FRET, consisting in monitoring donor fluorescent life-time in the presence or absence of the acceptor [80].

Other proteins from the ER have been reported to be part of SOCE complex. For example, Juctate, an ubiquitous component of PM-ER junctions, is recently reported to interact with both Orail and STIM1 when the complex is formed [84]. Furthermore, mutation of an EF-hand domain in Juctate induces STIM1 clustering independently of store depletion [84]. Another protein that has been shown to interact with STIM1 inside the ER lumen is Erp57, an oxidoreductase identified by screening ER resident proteins that binds to the luminal domain of STIM1 [85]. Stahelin showed this interaction by surface plasmon resonance, a nonmicroscopic technique that uses the same optic principles of TIRFM [86]. This technique measures the refractive index changes produced by covalently binding of interacting proteins with the target (STIM1) bound to a gold chip. This *in vitro* methodology allows performing massive screening tests. The Erp57-STIM1 interaction was corroborated *in vivo* by FRET [85].

SOCE can initiate diverse cellular processes, the best studied of which are activation of NFAT and the triggering antigen recognition in B and T cells [6]. However, SOCE can initiate cell division, gene expression, cell migration, cell differentiation, and so forth. Many stimuli can induce increments in intracellular calcium; thus an open question is how the cell differentiates this widely used signal to respond selectively.

Recently, Willoughby et al. exposed the link between the two more important second messengers, Ca^{2+} and cyclic adenosine monophosphate (cAMP), in one particular signaling route. By using FRET measurements showed direct interactions between the N-terminal of the Ca^{2+} -stimulated Adenylyl cyclase 8 (AC8) and Orail [87]. By using sensitive emission to measure FRET it was possible to map the site of interaction between both proteins [87]. Sensitive emission is one of the most popular methods for measuring FRET, which consists in obtaining FRET signal from images acquired using three different fluorescence excitation and emission parameters: (1) donor excitation and emission (donor channel), (2) donor excitation and acceptor emission (FRET channel), and (3) acceptor excitation and emission (acceptor channel). Emission of one fluorophore being detected on the second fluorophore channel (Spectral bleed through) is a contaminant signal; for that, subtracted spectral bleed-through is required to obtain a clean FRET signal [88, 89]. The main advantage of sensitive emission is that it can be carried out on simple wide-field microscopes [80].

Finally, other studies used AC8 constructs tagged with the genetics sensors GCaMP3 and Epac2-camps, to measure in real-time Ca^{2+} and cAMP, respectively [90–92]. With these tools it was possible to resolve distinct behaviors in mutated forms of the AC8. First, with the AC8 tethered to GCaMP3, it was shown that AC8 was exclusively activated by SOCE. Deletion of the N-terminal from AC8, a sequence required to interact with Orail, shifts the sensitivity of this enzyme, which now is activated by calcium coming from the ER. On the other hand, the AC8 construct fused to Epac2-camps detects the elevation of cAMP after activating AC8 from Ca^{2+} influx produced by depletion of the ER. Interestingly, when Orail is silenced with siRNA, AC8-Epac2-camps detect the production of cAMP mostly from the release of the ER. These results demonstrate that Orail conveys an organized signaling complex where Ca^{2+} and cAMP respond in a coordinated manner. These results positioned both Ca^{2+} and cAMP microdomains as key elements to differentiate the nature of the stimuli and the type of cellular response to be conveyed [87].

6. Concluding Remarks

Since Anton Van Leeuwenhoek in the 17th century developed the first microscope and Ernest Abbe and Lord Rayleigh developed the equations describing the basic principles of the diffraction of light and the resolution limit in the 19th century, advanced microscopy equipment and techniques have been developed in combination with molecular tools. The palette of fluorescent proteins keeps increasing and the detection systems are increasingly sensitive, facilitating measurements far beyond the diffraction limit.

In the present review we discussed the main findings about SOCE and its regulatory mechanisms from the light (and only briefly electron) microscopy point of view. The interest lies in understanding in greater detail how SOCE and SOCIC function has driven many groups to develop novel microscopy systems specifically designed for this task.

From this microscopy view a complex and sophisticated mechanism emerged, which involves the dynamic assembly of macromolecular complexes, the opposing of distinct cell membranes, and the intriguing control of Ca^{2+} , cAMP, and (most likely) other messenger microdomains. Our understanding of the fine-tuning of this spatial-temporally controlled symphony is now slowly emerging.

The future advances in superresolution and the generation of novel bioluminescence and fluorescence methods in combination with electrophysiology and molecular biology may provide real-time details about inter- and intramolecular interactions of the different components involved in modulating SOCE.

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