Immobile IP$_3$ Receptor Clusters: Building Blocks For IP$_3$-Evoked Ca$^{2+}$ Signals

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Co-regulation of IP$_3$ receptors (IP$_3$Rs) by IP$_3$ and cytosolic Ca$^{2+}$ allows them to mediate regenerative signals, amongst which are Ca$^{2+}$ puffs. These reflect the near-simultaneous opening of a few IP$_3$Rs within a small cluster. A long-standing conundrum is the observation that while most IP$_3$Rs appear to be mobile, Ca$^{2+}$ puffs repeatedly initiate from a limited number of fixed sites. Using gene-editing to attach GFP to endogenous IP$_3$Rs in HeLa cells has allowed the distribution of IP$_3$Rs and the Ca$^{2+}$ signals they evoke to be imaged simultaneously. This approach shows that most endogenous IP$_3$Rs are loosely assembled into small clusters, most of which are mobile. However, the Ca$^{2+}$ puffs evoked by histamine or photolysis of caged IP$_3$ invariably initiated at immobile IP$_3$R clusters adjacent to the plasma membrane (PM). Hence, only a small fraction of cellular IP$_3$Rs are ‘licensed’ to respond. The licensed IP$_3$R clusters sit alongside the sites where store-operated Ca$^{2+}$ entry (SOCE) occurs, suggesting that the IP$_3$Rs may allow local regulation of SOCE.

Keywords: Ca$^{2+}$ signal, Ca$^{2+}$-induced Ca$^{2+}$ release, endoplasmic reticulum, IP$_3$ receptor, spatial organization, store-operated Ca$^{2+}$ entry.

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INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) and their cousins, the ryanodine receptors (RyRs), are the largest of all ion channels. Each forms a large-conductance Ca$^{2+}$ channel with relatively weak selectivity for Ca$^{2+}$ over K$^+$ (Van Petegem 2014, Foskett et al. 2007). Both of these major families of intracellular Ca$^{2+}$ channels are expressed predominantly within the endoplasmic or sarcoplasmic reticulum (ER or SR), where the functional channel is assembled from four closely related subunits. For IP$_3$Rs, the channel can comprise four identical subunits or a mixture drawn from the products of the three IP$_3$R genes and their splice variants (Joseph et al. 2000). RyRs are invariably homomeric. The IP$_3$R subtypes differ in their expression between cell types, in their subcellular distributions, their association with accessory proteins and in their affinities for IP$_3$ and modulation by additional intracellular signals (Prole & Taylor 2016). The subtypes may also fulfil different biological roles. However, the similarities between IP$_3$R subtypes are more striking than the differences. The most important of the shared features is the co-regulation of all IP$_3$Rs by IP$_3$ and cytosolic Ca$^{2+}$: both ligands are required for the IP$_3$R channel to open. The simplest scheme envisages that binding of IP$_3$ primes IP$_3$Rs to bind Ca$^{2+}$, with Ca$^{2+}$ binding then triggering channel gating (Fig. 1A). This interplay is important because it endows IP$_3$Rs with a capacity to amplify, through Ca$^{2+}$-induced Ca$^{2+}$ release (CICR), the Ca$^{2+}$ signals evoked by IP$_3$Rs or other Ca$^{2+}$ channels. Higher concentrations of cytosolic Ca$^{2+}$ inhibit IP$_3$Rs. There is, therefore, a biphasic dependence of IP$_3$R gating on cytosolic Ca$^{2+}$ concentration: low Ca$^{2+}$ concentrations are stimulatory, while higher concentrations inhibit. RyRs are also biphasically regulated by cytosolic Ca$^{2+}$ (Van Petegem 2016). It is worth noting, since the observations are still cited, that it had been suggested that IP$_3$R2 and IP$_3$R3 were not biphasically regulated by cytosolic Ca$^{2+}$ (Hagar et al. 1998, Ramos-Franco et al. 1998). However, subsequent work established that all IP$_3$Rs are biphasically regulated by cytosolic Ca$^{2+}$ (Foskett et al. 2007, Taylor & Tovey 2012), although there are subtle differences between IP$_3$R subtypes in the interplay between IP$_3$ and Ca$^{2+}$.

The structural basis of IP$_3$R activation has been most explored for IP$_3$R1, but the considerable amino acid sequence similarity between IP$_3$R subtypes suggests that the mechanisms of activation are likely to be similar for all subtypes. Indeed, the basic architecture of the activation mechanisms is probably similar for IP$_3$Rs and RyRs (Seo et al. 2012, Van Petegem 2016). For IP$_3$Rs, activation begins when IP$_3$ binds to the clam-like IP$_3$-binding core (IBC), which lies towards the N-terminal of the primary sequence of each subunit (Fig. 1B). The IBCs of all four subunits must bind IP$_3$ before the channel can open.
The 4- and 5-phosphate groups of IP₃, which are essential features of all IP₃R ligands, interact with basic residues lining opposite sides of the inner surface of the clam (Bosanac et al. 2002). This allows IP₃ binding to partially close the clam. Since one side of the clam, the α-domain, adheres tightly to the N-terminal domain of the IP₃R (the so-called suppressor domain, SD), clam closure causes movement of the SD. We suggest that this movement weakens interactions between IP₃R subunits, leading ultimately to channel gating (Seo et al. 2012). However, details of the structural links between the initial conformational changes around the IBC and SD, Ca²⁺ binding, and dilation of the pore that allows Ca²⁺ to pass from the ER into the cytosol, are not yet resolved. A major step towards revealing these details is the cryo-EM structure of IP₃R1 in a closed state (Fan et al. 2015). A notable feature of this structure, which may point the way towards a gating mechanism, is the rod-like α-helical C-terminal domain, which extends directly from TMD6, through a perpendicular linking domain, to make contact with the SD of an adjacent subunit (Fan et al. 2015).

The most pertinent points for subsequent discussion are the obligate co-regulation of IP₃Rs by IP₃ and cytosolic Ca²⁺; the large Ca²⁺ conductance of IP₃Rs (Foskett et al. 2007); the large size of an IP₃R (11,000 residues, ~1.2 MDa) with its mushroom-like cytosolic region extending some 20 nm from the ER membrane (Fan et al. 2015); and the observation that neither RyRs nor IP₃Rs are randomly distributed within intracellular membranes (Franzini-Armstrong 2018, Thillaiappan et al. 2017, Jayasinghe et al. 2018).

**RECRUITMENT OF CA²⁺ SIGNALS BY IP₃-REGULATED CICR**

High-resolution optical imaging, first with confocal microscopy and then with total internal reflection fluorescence microscopy (TIRFM), has revealed the subcellular organization of IP₃-evoked Ca²⁺ signals in cells loaded with a fluorescent Ca²⁺ indicator and EGTA to restrain the regenerative propagation of Ca²⁺ signals by CICR (Bootman et al. 1997, Wiltgen et al. 2010, Parker & Smith 2010). These ‘optical patch-clamp’ methods resolve the brief openings of individual IP₃Rs (as ‘Ca²⁺ blips’); the co-ordinated opening of several (typically fewer than 10) IP₃Rs within small clusters (‘Ca²⁺ puffs’); and the regenerative propagation of intracellular Ca²⁺ waves, which initiate more frequently as the IP₃ concentration increases (Smith & Parker 2009, Thurley et al. 2014). This hierarchy of Ca²⁺ release events is assumed to arise from CICR as higher concentrations of IP₃ progressively prime more IP₃Rs to respond to Ca²⁺ diffusing to them from nearby active IP₃Rs (Fig. 1C). The functional significance of the hierarchy comes from the changing nature of the Ca²⁺ signals as the stimulus intensity increases, and the opportunities that provides for encoding Ca²⁺ signals in
both spatial and temporal domains. Hence, the Ca\textsuperscript{2+} signal evolves from large focal increases in cytosolic [Ca\textsuperscript{2+}] delivered to targets adjacent to IP\textsubscript{3}Rs, to a global signal that can activate more remote targets, and these global signals can encode stimulus intensity in the frequency of the resulting Ca\textsuperscript{2+} spikes (Thurley et al. 2014). The extent to which IP\textsubscript{3}-evoked Ca\textsuperscript{2+} signals are amplified by this CICR mechanism, and so progress through the hierarchical pathway, depends on both the concentration of IP\textsubscript{3} and the separation of IP\textsubscript{3}Rs.

Where Ca\textsuperscript{2+} blips have been resolved, they arise from sites that appear either immobile or to move with diffusion coefficients (D = 0.003 µm\textsuperscript{2} s\textsuperscript{-1}) (Wiltgen et al. 2010) at least ten-times slower than those of IP\textsubscript{3}Rs determined from fluorescence recovery after photobleaching (FRAP) or single-particle tracking of tagged IP\textsubscript{3}Rs (Smith et al. 2014, Thillaiappan et al. 2017). It is not yet clear whether Ca\textsuperscript{2+} blips arise from lone IP\textsubscript{3}Rs or from IP\textsubscript{3}Rs within clusters that fail to ignite the activity of their neighbours. Ca\textsuperscript{2+} puffs also initiate at sites that remain immobile for many minutes (Wiltgen et al. 2010, Bootman et al. 1997, Keebler & Taylor 2017, Thillaiappan et al. 2017). The pioneering studies of IP\textsubscript{3}R puffs suggested there were no more than a handful of initiation sites per cell (Bootman et al. 1997, Smith et al. 2014, see Smith & Parker 2009), but recent work suggests they may be more abundant with perhaps a hundred sites per cell (Keebler & Taylor 2017). Nevertheless, it is clear that Ca\textsuperscript{2+} puffs repeatedly initiate at sites that remain immobile for many minutes, and which include only a small fraction of the total complement of IP\textsubscript{3}Rs. There is, therefore, a conundrum in that most IP\textsubscript{3}Rs (typically ~70%) appear to be mobile, yet IP\textsubscript{3}-evoked Ca\textsuperscript{2+} signals initiate at fixed sites, leading Parker and his colleagues to speculate that anchoring of immobile IP\textsubscript{3}Rs into clusters may prime them to respond to IP\textsubscript{3} (Parker & Smith 2010, Smith et al. 2014). To gain further insight into this problem, we used gene-editing to attach enhanced green fluorescent protein (GFP) to the endogenous IP\textsubscript{3}R1 of HeLa cells (Thillaiappan et al. 2017). We demonstrated that all IP\textsubscript{3}R1s, the major subtype in HeLa cells, were tagged with GFP, the tagged IP\textsubscript{3}R1s were functional and assembled with other IP\textsubscript{3}R subtypes, and they were expressed in clusters within ER membranes. Subsequent sections of this short review focus on our analyses of these tagged endogenous IP\textsubscript{3}Rs insofar as they illuminate our understanding of the relationship between the geography of IP\textsubscript{3}Rs and the Ca\textsuperscript{2+} signals they elicit.

**WHAT IS AN IP\textsubscript{3} RECEPTOR CLUSTER?**

Functional analyses of Ca\textsuperscript{2+} puffs (Dickinson et al. 2012), single-particle tracking of over-expressed mEos2-IP\textsubscript{3}R1 (Smith et al. 2014) and patch-clamp recordings from the outer
nuclear envelope (Rahman et al. 2009, Vais et al. 2011, Rahman et al. 2011) suggest that typical puff sites are likely to include 2-9 functional IP$_3$Rs. There is, however, considerable heterogeneity in the number of active IP$_3$Rs, both between sites and between successive Ca$^{2+}$ puffs at the same site (Smith & Parker 2009). This suggests that not all IP$_3$Rs within a cluster are recruited during each Ca$^{2+}$ puff.

Our single-step photobleaching analyses of endogenous GFP-IP$_3$R1 in HeLa cells suggest that most IP$_3$Rs form clusters, with up to ~40 IP$_3$Rs in each, and a mean of ~8 IP$_3$Rs per cluster (Thillaiappan et al., 2017) (Fig. 2A). The dimensions of a cluster, typically several 100 nm across, are similar to the dimensions estimated from a single-particle tracking analysis (~400 nm) (Smith et al. 2014). These small IP$_3$R clusters, which we suggest may be the elementary structural units of IP$_3$R signalling, are expressed throughout the cell. A surprising observation is the apparent independence of each cluster. There is no evident mixing of IP$_3$Rs between mobile and immobile clusters, and we observe clusters apparently moving past each other without losing their identities. Hence, once IP$_3$Rs are assembled into a cluster, it seems to be a long-lasting relationship. Super-resolution analyses of the distribution of IP$_3$Rs within clusters suggests that many of the component IP$_3$Rs are too far apart to interact with each other directly (Fig. 2A). We suggest, therefore, that IP$_3$R clusters are loose confederations held together by scaffolding complexes that might involve cytosolic or ER proteins, lipid microdomains, or contacts between ER and other organelles (Fig. 2A). The notion that IP$_3$Rs might be relatively loosely distributed within their stable clusters sits comfortably with several features of Ca$^{2+}$ puffs. Firstly, although the rising phase of Ca$^{2+}$ puffs is usually very brisk, consistent with rapid recruitment of closely spaced IP$_3$Rs, it is sometimes possible to discern steps in the rising phase, suggestive of a looser coupling (Smith & Parker 2009). Secondly, and notwithstanding the blurring of signals as Ca$^{2+}$ diffuses away from IP$_3$Rs, the dimensions of Ca$^{2+}$ puffs (~500 nm) are much larger than needed to accommodate ten or fewer IP$_3$Rs (each ~20 nm across). Thirdly, although puff sites are immobile, the peak of the Ca$^{2+}$ signal wanders by up to 300 nm within the site during a puff (Wiltgen et al. 2010), suggesting that active IP$_3$Rs may be as much as 300 nm apart. Finally, even the most mobile of Ca$^{2+}$ blips (D = 0.003 µm$^2$s$^{-1}$) (Wiltgen et al. 2010), which may represent IP$_3$Rs within clusters that fail to ignite a Ca$^{2+}$ puff, would be expected to move only about 35 nm during the typical duration of a Ca$^{2+}$ puff (i.e. well within the confines of the loose cluster). Hence, we suggest that most IP$_3$Rs, whether mobile or immobile, are corralled into loose confederations by scaffolding complexes that typically hold ~8 IP$_3$Rs in a long-lasting relationship.
We concluded from our previous patch-clamp analyses of IP$_3$Rs in the outer nuclear envelope, which is continuous with the ER, that low concentrations of IP$_3$ cause IP$_3$Rs to assemble into small clusters (Rahman et al. 2009, Rahman et al. 2011). Others have challenged this conclusion by suggesting that IP$_3$R clusters assemble without the need to increase the intracellular IP$_3$ concentration (Vais et al. 2011, Smith et al. 2009b), and our results from HeLa cells also show that stable IP$_3$R clusters are present in unstimulated cells (Thillaiappan et al. 2017). How might these observations be reconciled? It may be that the nuclear envelope is not an appropriate model for the ER, or basal levels of IP$_3$ may be sufficient to ensure assembly of IP$_3$R clusters in unstimulated cells (Smith et al. 2009b). However, a more attractive possibility, which we have yet to address experimentally, is that most IP$_3$Rs are already assembled into loose corrals in unstimulated cells, and IP$_3$ then promotes tighter clustering within the corral. Although preliminary analyses failed to provide support for this scheme (Smith et al. 2014), it deserves closer attention since such local ‘huddling’ would not increase the size of each pre-formed cluster, but it would be expected to improve the CICR-mediated recruitment of IP$_3$Rs within a cluster. Hence, IP$_3$ might initiate Ca$^{2+}$ puffs by stimulating gating of IP$_3$Rs and by enhancing CICR by causing loosely pre-clustered IP$_3$Rs to huddle.

**CA$^{2+}$ PUFFS OCCUR AT IMMOBILE IP$_3$ RECEPTOR CLUSTERS NEAR THE PLASMA MEMBRANE**

Both direct measurements, using FRAP (Fukatsu et al. 2004, Pantazaka & Taylor 2011, Ferreri-Jacobia et al. 2005) or single-particle tracking (Smith et al. 2014, Thillaiappan et al. 2017), and evidence that stimuli can regulate IP$_3$R clustering (Wilson et al. 1998, Tateishi et al. 2005, Iwai et al. 2005, Chalmers et al. 2006, Tojyo et al. 2008, Rahman et al. 2009, Geyer et al. 2015) attest to the mobility of IP$_3$Rs within ER membranes. Typically, these studies suggest that most IP$_3$Rs are mobile (mobile fractions, M$_f$, typically ~70%) and that most movement is by diffusion. A caveat remains that most such studies have observed over-expressed IP$_3$Rs, with the attendant risk that they may not faithfully report behaviours of endogenous IP$_3$Rs. Using single-particle tracking of endogenous GFP-IP$_3$Rs in HeLa cells to record the movement of IP$_3$R clusters, we observed that most clusters (~70%) were mobile, while the remainder remained immobile for periods of many minutes. Within the population of mobile clusters, most clusters moved by diffusion (D ~0.03 µm$^2$.s$^{-1}$), but a small fraction of the mobile clusters (~10%) moved directionally along microtubules, driven by kinesin and dynein motors (Thillaiappan et al. 2017). Our results are similar to those obtained using
single-particle tracking of over-expressed mEos2-IP$_3$R1, in suggesting that ~70% of IP$_3$Rs are mobile, but they differ in that only we observed directional movement along microtubules. The difference may be due to the different cell types used or to over-expression of mEos2-IP$_3$R1 masking the small number of actively moving IP$_3$R clusters. The key points are that most native IP$_3$Rs are clustered, and most of these clusters are mobile.

In HeLa cells, almost all Ca$^{2+}$ puffs initiate close to the plasma membrane (PM) (Thillaiappan et al. 2017), consistent with similar observations in SH-SY5Y cells (Smith et al. 2009a). Furthermore, the pattern was similar whether the Ca$^{2+}$ signals were evoked by histamine to stimulate endogenous signalling pathways, or by photolysis of caged-IP$_3$ to allow uniform release of IP$_3$ throughout the cytosol. Indeed, both we (Keebler & Taylor 2017) and others (Lock et al. 2017) have shown that endogenous signalling pathways and photo-released IP$_3$ activate the same Ca$^{2+}$ puff sites. By simultaneously recording the Ca$^{2+}$ puffs evoked by IP$_3$ and the underlying distribution of endogenous GFP-IP$_3$Rs in HeLa cells, we showed that Ca$^{2+}$ puffs initiate only at immobile clusters of IP$_3$Rs (Thillaiappan et al. 2017). The observation is important, because IP$_3$R clusters are expressed throughout the cell, not just near the PM, and most IP$_3$R clusters are mobile. Hence, only a small subset of the few thousand IP$_3$Rs in a cell, namely those within immobile clusters adjacent to the PM, is ‘licensed’ to respond to IP$_3$ (Fig. 2B). There must, therefore, be an additional level of regulation of IP$_3$Rs that endows them with the competence to respond to IP$_3$. The nature of that regulation has yet to be resolved.

In addition to causing an increase in cytosolic [Ca$^{2+}$], activation of IP$_3$Rs also causes a decrease in [Ca$^{2+}$] within the ER, and that leads to activation of store-operated Ca$^{2+}$ entry (SOCE) (Hogan 2015). The core features of SOCE are now clear: they involve direct interactions between the ER Ca$^{2+}$ sensor, stromal interaction molecule 1 (STIM1), and the PM Ca$^{2+}$ channel, Orai1. Loss of Ca$^{2+}$ from the luminal EF-hands of STIM1 causes STIM1 to cluster and expose cytosolic domains that bind to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and Orai1. Binding of STIM1 to Orai opens its channel, allowing Ca$^{2+}$ to flow into the cell. Hence, loss of ER Ca$^{2+}$ causes STIM1 to be captured, through its interaction with PIP$_2$ and Orai, within narrow junctions where the ER and PM are no more than ~20 nm apart (Hogan 2015, Prakriya & Lewis 2015). Our results from HeLa cells show that the sites to which STIM1 translocates after loss of Ca$^{2+}$ from the ER are immediately adjacent to (but not perfectly coincident with) the immobile near-PM IP$_3$R clusters that we know to be the sites where Ca$^{2+}$ puffs occur (Thillaiappan et al. 2017). Because IP$_3$Rs project about 20 nm from the ER membrane (Fan et al. 2015), they may be too large to enter the narrow ER-PM
junctions wherein STIM and Orai interact. This physical exclusion may account for the lack of perfect colocalization of STIM1 with immobile IP₃R clusters, but it leaves unexplained our observation that immobile near-PM IP₃R clusters are preferentially juxtaposed to the ER-PM SOCE junctions (Thillaiappan et al. 2017). Future work will need to identify the tether that positions the licensed IP₃R clusters adjacent to SOCE junctions. We can, however, speculate on the possible physiological significance of the juxtaposition.

We suggest that immobile near-PM IP₃R clusters sit alongside SOCE junctions and face the PM (Fig. 2B). Since PIP₂ recruits STIM to ER-PM junctions, we suggest that the licensed IP₃R clusters are located immediately alongside the substrate (PIP₂) from which endogenous signalling pathways will generate IP₃. Activation of SOCE requires substantial loss of Ca²⁺ from the ER (Brandman et al. 2007, Prakriya & Lewis 2015), yet while regulating SOCE the ER must also fulfil numerous additional functions, many of which require luminal Ca²⁺ (Berridge 2002). We speculate that the positioning of licensed IP₃R clusters alongside SOCE junctions might allow IP₃R activation to locally deplete the ER and activate SOCE without trespassing into the other Ca²⁺-requiring functions of the ER. Finally, SOCE is acutely regulated by Ca²⁺ passing through the low-conductance Orai1 channel (Prakriya & Lewis 2015). If IP₃Rs, with their very large Ca²⁺ conductance, were too close to SOCE junctions, they might disrupt this local feedback regulation. Hence, having the licensed IP₃Rs that will respond to IP₃ alongside, rather than within, SOCE junctions may provide the best compromise between local regulation of SOCE by local depletion of ER Ca²⁺ stores, while retaining acute auto-regulation of SOCE by Ca²⁺ passing through Orai channels (Fig. 2B).

CONCLUSIONS
IP₃Rs are co-regulated by IP₃ and cytosolic Ca²⁺. This allows them to mediate regenerative intracellular Ca²⁺ signals as IP₃ primes IP₃Rs to respond to Ca²⁺ (Fig. 1C). Ca²⁺ puffs, which report the near-simultaneous opening of a small number of IP₃Rs within a cluster, are the smallest units of these regenerative Ca²⁺ signals. Each cluster is a loose, but stable, confederation of a small number of IP₃Rs that must be held together by an as yet unidentified scaffold. Most IP₃R clusters are mobile, but only immobile clusters immediately adjacent to the PM initiate Ca²⁺ puffs. The additional signal that provides these licensed IP₃Rs with competence to respond to IP₃ is unknown. Nor is it resolved whether the large population of mobile IP₃R clusters contributes to Ca²⁺ signals evoked by more intense stimulation. Since the licensed IP₃R clusters sit alongside the ER-PM junctions where SOCE occurs, we speculate that they may contribute to local regulation of SOCE.
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**Figure 1.** Hierarchical recruitment of IP$_3$-evoked Ca$^{2+}$ release. (A) Many receptors in the plasma membrane (PM) stimulate phospholipase C (PLC), with concomitant formation of IP$_3$. Binding of IP$_3$ to each of the four subunits of a tetrameric IP$_3$R primes the IP$_3$R to bind Ca$^{2+}$, and that Ca$^{2+}$ binding then triggers channel gating. Co-regulation of IP$_3$Rs by IP$_3$ and Ca$^{2+}$ endows them with the capacity to mediate IP$_3$-regulated Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). (B) IP$_3$ binds within the clam-like cleft formed by the $\alpha$- and $\beta$-domains of the IP$_3$-binding core (IBC), causing partial closure of the clam. This clam closure causes re-orientation of the suppressor domain (SD) and thereby initiates channel gating. The pore of the channel is formed by transmembrane domains (TMD) towards the C-terminal of the primary sequence. Structure from Seo et al. (2012). (C) By tuning the gain on CICR by IP$_3$Rs, IP$_3$ allows hierarchical recruitment of intracellular Ca$^{2+}$ release events.

**Figure 2.** Immobile IP$_3$R clusters at the PM are licensed to respond to IP$_3$. (A) TIRFM image shows a portion of a HeLa cell in which endogenous IP$_3$R1s are tagged with GFP; the ER is shown in red. STORM image of a single mobile IP$_3$R cluster shows the localization of GFP-IP$_3$R1 in red, and the underlying TIRFM image in green. The yellow box beneath the scale bar shows the approximate dimensions of a single IP$_3$R tetramer. The image shows that IP$_3$Rs are often relatively loosely associated within clusters, even though individual clusters retain their individuality for prolonged periods. Single-step photobleaching analyses suggest that IP$_3$R clusters contain variable numbers of tetrameric IP$_3$Rs, but with a mean of ~8 IP$_3$Rs/cluster. Results from Thillaiappan et al. (2017). (B) We suggest that most IP$_3$R clusters are mobile and inactive (red), but licensed IP$_3$R clusters (green) are immobilized alongside the ER-PM junctions where SOCE occurs. This, we suggest, may allow local regulation of SOCE through local depletion of the ER by licensed IP$_3$Rs, while retaining acute feedback regulation of SOCE activity by Ca$^{2+}$ passing through Orai channels. See text for further details.