**IP3 mediated global Ca2+ signals arise through two temporally and spatially distinct modes of Ca2+ release**

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**Abstract** The ‘building-block’ model of inositol trisphosphate (IP3)-mediated Ca2+ liberation posits that cell-wide cytosolic Ca2+ signals arise through coordinated activation of localized Ca2+ puffs generated by stationary clusters of IP3 receptors (IP3Rs). Here, we revise this hypothesis, applying fluctuation analysis to resolve Ca2+ signals otherwise obscured during large Ca2+ elevations. We find the rising phase of global Ca2+ signals is punctuated by a flurry of puffs, which terminate before the peak by a mechanism involving partial ER Ca2+ depletion. The continuing rise in Ca2+, and persistence of global signals even when puffs are absent, reveal a second mode of spatiotemporally diffuse Ca2+ signaling. Puffs make only small, transient contributions to global Ca2+ signals, which are sustained by diffuse release of Ca2+ through a functionally distinct process. These two modes of IP3-mediated Ca2+ liberation have important implications for downstream signaling, imparting spatial and kinetic specificity to Ca2+-dependent effector functions and Ca2+ transport.

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**Introduction**

Cytosolic Ca2+ signals generated by the liberation of Ca2+ ions sequestered in the endoplasmic reticulum (ER) through inositol trisphosphate receptor (IP3R) channels regulate ubiquitous cellular processes as diverse as gene transcription, secretion, mitochondrial energetics, electrical excitability and fertilization (Clapham, 2007; Berridge et al., 2000). Cells achieve such unique repertoires of Ca2+-dependent functions by generating a hierarchy of cytosolic Ca2+ signals with markedly different spatial scales and temporal durations, ranging from brief, localized Ca2+ transients called puffs (Parker and Yao, 1991; Yao et al., 1995) to larger and more prolonged Ca2+ elevations that engulf the cell. Global elevations in cytosolic Ca2+ typically last several seconds and may appear as waves that propagate throughout the cell (Woods et al., 1986). They can recur as oscillations with periods between a few seconds and a few minutes, and are thought to encode information in a ‘digital’ manner, whereby increasing stimulus strength results predominantly in an increase in frequency rather than amplitude (Parekh, 2011; Smedler and Uhlen, 2014). Puffs, on the other hand, are tightly localized elevations in cytosolic Ca2+ generated by stationary clusters containing small numbers of IP3Rs, which last only tens or a few hundreds of milliseconds and remain restricted within a few micrometers (Bootman et al., 1997a; Parker et al., 1996).

The patterning of cellular Ca2+ signals evoked by IP3 is largely determined by the functional properties of the IP3Rs and by their spatial arrangement in the ER membrane. Crucially, the opening of IP3R channels is regulated by cytosolic Ca2+ itself, in addition to IP3. Low concentrations of Ca2+ increase the open probability of the channel whereas high concentrations favor a closed state (Parker and Ivorra, 1990; Iino, 1990; Bezprozvanny et al., 1991). This biphasic modulation of IP3Rs by Ca2+ leads to the phenomenon of Ca2+-induced Ca2+ release (CICR). Ca2+ diffusing from one open channel may thus trigger the opening of adjacent channels, with self-reinforcing CICR...
countered by Ca\(^{2+}\) feedback inhibition. The clustered distribution of IP\(_3\)Rs further shapes the extent of this regenerative process. CICR may remain locally restricted to a single cluster, containing from a few to a few tens of functional IP\(_3\)Rs, to produce a puff; whereas it is proposed that a global response is generated by successive cycles of CICR and Ca\(^{2+}\) diffusion acting over longer spatial ranges to recruit successive puff sites (Bootman et al., 1997a; Parker et al., 1996; Berridge, 1997; Bootman and Berridge, 1996; Callamaras et al., 1998; Dawson et al., 1999; Marchant, 2001; Marchant et al., 1999). However, the transition between these modes remains an area of active investigation (Rückl and Rüdiger, 2016; Miyamoto and Mikoshiba, 2019; Sneyd et al., 2017a; Sneyd et al., 2017b; Rückl et al., 2015), and recent theoretical simulations have questioned whether Ca\(^{2+}\) released through puff activity is alone sufficient to propagate global cytosolic Ca\(^{2+}\) signals (Piegari et al., 2019).

Here, we examined the nature of Ca\(^{2+}\) liberation through IP\(_3\)Rs during global cellular Ca\(^{2+}\) signals, asking whether this accords with the widely-accepted ‘building block’ model (Bootman et al., 1997a; Parker et al., 1996; Berridge, 1997; Bootman and Berridge, 1996; Marchant, 2001; Marchant et al., 1999; Mataragka and Taylor, 2018) in which global signals are constructed by the summation of coordinated, pulsatile activation of Ca\(^{2+}\) release at puff sites; or whether global signals also involve an additional mode of Ca\(^{2+}\) liberation that is more homogeneous in space and time. Although Ca\(^{2+}\) puffs are often evident during the initial rising phase of global Ca\(^{2+}\) signals (Bootman et al., 1997a; Bootman and Berridge, 1996; Marchant, 2001; Marchant et al., 1999), a challenge in answering this question arises because puffs become obscured as the overall cytosolic Ca\(^{2+}\) level continues to increase. To reveal and monitor temporally rapid and spatially confined Ca\(^{2+}\) transients (puffs) during even large amplitude global Ca\(^{2+}\) elevations we developed image processing and analysis routines to analyze local fluctuations in Ca\(^{2+}\) fluorescence signals (Ellefsen et al., 2019). We applied these routines to Ca\(^{2+}\) recordings obtained both by total internal reflection fluorescence (TIRF) microscopy to resolve signals arising near the plasma membrane, and by lattice light-sheet (LLS) microscopy to acquire optical sections through the cell interior. We find that rapid flurries of Ca\(^{2+}\) puffs accompany the rising phase of global Ca\(^{2+}\) signals evoked by photoreleased IP\(_3\) and by agonist stimulation of the IP\(_3\) signaling pathway, but these rapidly terminate before the peak of the response through a mechanism regulated by ER Ca\(^{2+}\) store content. The punctate liberation of Ca\(^{2+}\) via transient, localized Ca\(^{2+}\) puffs contributes only a small fraction of the total Ca\(^{2+}\) liberated during global Ca\(^{2+}\) signals, which are instead sustained by diffuse Ca\(^{2+}\) liberation through a functionally distinct mode of release. These two modes of IP\(_3\)-mediated Ca\(^{2+}\) release will likely selectively activate different populations of effectors; those positioned close to the IP\(_3\)R clusters that mediate puffs and which respond to brief, repetitive transients of [Ca\(^{2+}\)], and others that respond to a more sustained, spatially diffuse elevation of bulk cytosolic [Ca\(^{2+}\)].

## Results

**Fluctuation processing of Ca\(^{2+}\) images highlights transient signals**

Our central question was whether IP\(_3\)-evoked Ca\(^{2+}\) liberation during cell-wide Ca\(^{2+}\) signals arises through coordinated activation of pulsatile, spatially localized events, analogous to the local Ca\(^{2+}\) puffs observed with weaker IP\(_3\) stimulation or after loading cells with EGTA to suppress global signals (Dargan and Parker, 2003; Smith et al., 2009). To better visualize and identify transient, localized Ca\(^{2+}\) events occurring during the course of larger, global elevations of Ca\(^{2+}\), we developed an image processing algorithm to highlight and quantify temporal fluctuations of the Ca\(^{2+}\) fluorescence signal. We previously described the use of pixel-by-pixel power spectrum analysis of temporal Ca\(^{2+}\) fluctuations for this purpose (Swaminathan et al., 2020), but this is computationally intensive and unfeasible for large data sets. Here, we adopted a faster approximation, by first temporally bandpass filtering image stacks and then calculating the standard deviation (SD) of the fluorescence fluctuations at each pixel over a running time window (Ellefsen et al., 2019).

The conceptual basis of the algorithm is illustrated in **Figure 1** (see also **Figure 1—video 1**). WT HEK293 cells were loaded with the fluorescent Ca\(^{2+}\) indicator Cal520 and imaged by TIRF microscopy during global cytosolic Ca\(^{2+}\) signals. The panels in **Figure 1A** show Cal520 fluorescence of individual image frames of a HEK cell captured before (i) and after (ii-v) photorelease of i-IP\(_3\), an active, metabolically stable analog of IP\(_3\). Photoreleased i-IP\(_3\) evoked a widespread increase in fluorescence...
Figure 1. Fluctuation analysis of Ca\(^{2+}\) signals. (A–D) Records from a single WT HEK cell loaded with Cal520 and stimulated by photorelease of i-IP\(_3\) to evoke a global Ca\(^{2+}\) elevation. (A) Panels show ‘raw’ TIRF fluorescence images of the cell before (i), during the rising phase (ii–iv) and at the peak (v) of the global Ca\(^{2+}\) signal. Images are Gaussian-blurred (sigma \(\sim 1\) \(\mu\)m) single frames (8 ms exposure time) captured at times as marked in C. Grey scale intensities depict fluorescence in arbitrary camera units, as indicated by the bar at the right. The yellow outline marks the TIRF footprint of the cell. (B) Figure 1 continued on next page
Panels show corresponding standard deviation (SD) images at the same times as in A, highlighting hot spots of local, transient Ca\(^{2+}\) release. Grey scale intensities (arbitrary units; A.U.) represent the shot noise-corrected standard deviation of fluorescence fluctuations within a 160 ms running time window. (C) Overlaid black traces show fluorescence monitored from 24 regions of interest (ROIs; marked by squares in panel A) placed on areas of local Ca\(^{2+}\) activity. The arrow indicates the time of the photolysis flash. (D) Power spectra of Ca\(^{2+}\) fluorescence fluctuations averaged from the 24 ROIs at baseline (blue trace) and during the rising phase of the global Ca\(^{2+}\) signal (red trace). Spectra were calculated from recordings during the respective times indicated by the colored bars in C, after low-pass (1 Hz) filtering of the fluorescence image stack to strip out the slow rise of the global signal. (E) Overlaid traces show shot noise-corrected SD signals from the 24 ROIs centered on hot spots of Ca\(^{2+}\) activity. The thicker yellow trace shows the mean SD signal monitored from a ROI encompassing the entire cell and is depicted after scaling up by a factor of 10 relative to the traces from small ROIs. (F–I) Corresponding images and plots from an HEK cell devoid of IP\(_3\)-mediated global Ca\(^{2+}\) signals. Fluorescence intensity at each pixel, calculated the standard deviation (SD) of the fluorescence signal at that pixel throughout the response. The SD signal was uniformly close to zero throughout the cell before drug release. Grey scale intensities (arbitrary units; A.U.) represent the shot noise-corrected standard deviation of fluorescence fluctuations within a 160 ms running time window. The online version of this article includes the following video and figure supplement(s) for figure 1:

**Figure supplement 1.** Optimization of space-time parameters used in SD fluctuation analysis algorithm.

**Figure supplement 2.** Correction of signal variance for photon shot noise.

**Figure supplement 3.** Spatial fluctuation analysis of IP\(_3\)-mediated global Ca\(^{2+}\) signals mirrors temporal fluctuations.

**Figure 1—video 1.** Fluctuation processing of Ca\(^{2+}\) image recordings.

https://elifesciences.org/articles/55008#fig1video1
stimulation (Figure 1B, panel i), while discrete, transient hot spots were clearly evident at several different sites during the rising phase of the global Ca\(^{2+}\) elevation (panels ii-iv), but ceased at the time of the peak response (panel v). This behavior is further illustrated by the black traces in Figure 1E, showing overlaid SD measurements from the 24 hot spots of activity. A flurry of transient events at these sites peaked during the rising phase of the global Ca\(^{2+}\) response to photoreleased i-IP\(_3\) but had largely subsided by the time of the maximal global Ca\(^{2+}\) elevation. Even though the global Ca\(^{2+}\) level then stayed elevated for many seconds the mean SD signals at these regions remained low. Measurement of the SD signal derived from a ROI encompassing the entire cell (yellow trace, Figure 1E) closely tracked the aggregate kinetics of the individual puff sites.

To further validate the fluctuation analysis algorithm, we examined a situation where cytosolic [Ca\(^{2+}\)] was expected to rise in a smoothly graded manner, without overt temporal fluctuations or spatial heterogeneities. For this, we imaged Cal520 fluorescence by TIRF microscopy in HEK293 3KO cells in which all IP\(_3\)R isoforms were knocked out (Alzayady et al., 2016). We pipetted an aliquot of ionomycin (10 µl of 10 µM) into the 2.5 ml volume of Ca\(^{2+}\)-free bathing solution at a distance from the cell chosen so that the diffusion of ionomycin evoked a slow liberation of Ca\(^{2+}\) from intracellular stores to give a fluorescence signal of similar amplitude (8.3 \(\Delta F/F_0\)) and kinetics to that evoked by photoreleased i-IP\(_3\) (6.9 \(\Delta F/F_0\) in Figure 1A-C). Figure 1F shows snapshots of ‘raw’ fluorescence captured before (i) and during (ii-v) application of ionomycin. The fluorescence rose uniformly throughout the cell without any evident hot spots of local transients in the SD images (Figure 1G and Figure 1—video 1). Measurements from 24 randomly located ROIs (squares in Figure 1F) showed only smooth rises in fluorescence (Figure 1H). Mean spectra from these regions (Figure 1I) displayed flat, substantially uniform distributions of power across all frequencies, consistent with photon shot noise in proportion to the mean fluorescence level. Notably, SD signals from local ROIs (Figure 1J, superimposed black traces) and from a ROI encompassing the entire cell (yellow trace) showed no increase in fluctuations beyond that expected for photon shot noise.

**Temporal fluctuations reflect spatially localized Ca\(^{2+}\) signals**

The SD image stacks generated by the temporal fluctuation algorithm showed transient hot spots of Ca\(^{2+}\) release associated with temporal fluctuations. However, the SD signal could also include temporal fluctuations in fluorescence that were spatially blurred or uniform across the cell. To determine whether these contribute appreciably, or whether the SD signal could be taken as a good reporter of localized puff activity, we developed a second algorithm to reveal spatial Ca\(^{2+}\) variations in Cal520 fluorescence image stacks (Figure 1—figure supplement 3).

Ca\(^{2+}\) image stacks were first temporally bandpass filtered as described above. The algorithm then calculated, frame by frame, the difference between strong and weak Gaussian blur functions (respective standard deviations of about 4 and 1 µm at the specimen), essentially acting as a spatial bandpass filter to attenuate high spatial frequencies caused by pixel-to-pixel shot noise variations and low-frequency variations resulting from the spread of Ca\(^{2+}\) waves across the cell, while retaining spatial frequencies corresponding to the spread of local Ca\(^{2+}\) puffs. The resulting spatial SD images were remarkably similar to images generated by the temporal fluctuation analysis routine (Figure 1—figure supplement 3A), and traces of mean cell-wide temporal and spatial SD signals during Ca\(^{2+}\) elevations matched closely (Figure 1—figure supplement 3B-E). We thus conclude that the temporal SD signals faithfully reflect transient, localized Ca\(^{2+}\) puff activity while minimizing confounding contributions from shot noise and slower changes in global fluorescence.

**Fluctuation analysis reveals a transient flurry of puffs during global Ca\(^{2+}\) signals**

In Figure 1, we show traces from discrete subcellular regions to illustrate how temporal SD images detect transient, local Ca\(^{2+}\) elevations while being insensitive to homogeneous global Ca\(^{2+}\) elevations. However, for all the following experiments in this paper we show SD signals derived from single ROIs that completely encompassed each cell, so as to obtain an aggregate measure of puff activity throughout the cell and obviate any subjective bias that might arise in selecting smaller, sub-cellular regions. Unless otherwise stated, all imaging was done by TIRF microscopy with cells bathed...
in a zero Ca\(^{2+}\) solution including 300 μM EGTA to avoid possible complication from entry of extracellular Ca\(^{2+}\) into the cytosol.

*Figure 2* and *Figure 2*—videos 1 and 2 present records from WT HEK cells loaded with Cal520 and caged i-IP\(_3\) showing how the SD signal reveals the patterns of puff activity underlying global Ca\(^{2+}\) signals. Under basal conditions, the shot noise-corrected cell-wide SD signals were almost flat, with a mean around zero (*Figure 2A*, *Figure 2*—video 1), indicating a negligible level of local Ca\(^{2+}\) activity at rest. Photorelease of small amounts of i-IP\(_3\) by brief (~100 ms) UV flashes evoked Ca\(^{2+}\) puffs - directly visible in the Cal520 fluorescence ratio movie in *Figure 2*—video 1, and more evident as sharp transients in the whole-cell SD trace - but without generating any appreciable global rise in basal Ca\(^{2+}\) (*Figure 2B*). Longer flashes (200–1000 ms) generated whole-cell elevations in cytosolic Ca\(^{2+}\) that rose and fell over several seconds, with fluorescence signals reaching peak amplitudes in rough proportion to the flash duration (smooth traces, *Figure 2C–E*; *Figure 2*—video 2). SD movies (*Figure 2*—video 2) and whole-cell SD traces (noisy traces, *Figure 2C–E*) revealed an underlying flurry of localized, transient Ca\(^{2+}\) events during the rising phase of the global Ca\(^{2+}\) responses. In instances where global Ca\(^{2+}\) signals were small and slowly rising, the SD traces showed Ca\(^{2+}\) transients persisting throughout the prolonged rising phase (*Figure 2C*). On the other hand, the SD traces from cells exhibiting intermediate (*Figure 2D*) and fast rising (*Figure 2E*) global signals revealed Ca\(^{2+}\) fluctuations that began almost immediately following photorelease of i-IP\(_3\), reached a maximum during the rising phase of the global signal, but then declined almost to baseline by the peak of the response.

The records in *Figure 2A–E* and *Figure 2*—videos 1 and 2 illustrate representative responses in individual cells. To pool data from multiple cells we grouped records into categories matching the examples in *Figure 2B–E*: that is responses showing puffs without an appreciable elevation of global Ca\(^{2+}\); and slow-, intermediate- and fast- rising global Ca\(^{2+}\) responses. *Figure 2G* shows overlaid traces depicting the mean Cal520 fluorescence ratios (ΔF/F\(_0\)) of the global Ca\(^{2+}\) responses from cells in these different categories, and *Figure 2H* shows the associated mean SD traces. Notably, in all three categories where global Ca\(^{2+}\) signals were evoked (*Figure 2C–E*) the mean SD signals were transient, indicating that puff activity was largely confined to the rising phase of the global Ca\(^{2+}\) elevation and largely ceased by the time the global signal reached a maximum. The durations of the puff flurries progressively shortened with increasing rates of rise in global Ca\(^{2+}\) and the magnitudes of the SD signal at the peak of the flurry activity increased.

**Ca\(^{2+}\) signals evoked by agonist activation and photoreleased i-IP\(_3\) show similar patterns of puff activity**

UV photorelease of i-IP\(_3\) provides a convenient tool to activate IP\(_3\)Rs with precise timing and control of the amount released. However, this IP\(_3\) analog is slowly metabolized by the cell, remaining elevated for minutes following photo-uncaging (*Smith et al.*, 2009; *Dakin and Li*, 2007), and its uniform release throughout the cell differs from endogenous generation of IP\(_3\) at the cell membrane (*Keebler and Taylor*, 2017; *Lock et al.*, 2017). We thus compared responses evoked by photoreleased i-IP\(_3\) with those activated by the G-protein coupled muscarinic receptor agonist carbachol (CCH), locally applied through a picospritzer-driven micropipette (puffer pipet) positioned above WT HEK cells bathed in zero Ca\(^{2+}\) medium. A brief (5 s) pulse of CCH elicited a rapid, global rise in Ca\(^{2+}\) that was accompanied by an underlying burst of local Ca\(^{2+}\) signals (*Figure 2F*; *Figure 2*—video 3). As with responses evoked by photoreleased i-IP\(_3\), fluctuations arising from local Ca\(^{2+}\) signals occurred predominantly during the initial portion of the rising phase and then subsided to near basal levels before the peak of the global response. *Figure 2I* shows mean traces of whole-cell global Ca\(^{2+}\) signals (ΔF/F\(_0\)) and SD signals of CCH-evoked responses from 12 cells. Peak fluorescence amplitudes were similar to mean values for 11 cells stimulated by strong photorelease of i-IP\(_3\) (ΔF/F\(_0\)) of 8.89 ± 0.3 for CCH vs. 7.27 ± 0.4 for i-IP\(_3\); as were the rising phase kinetics of the global Ca\(^{2+}\) signal (rise from 20% to 80% of peak 0.70 s ± 0.05 s for CCH vs. 0.80 s ± 0.06 s for i-IP\(_3\)). However, global Ca\(^{2+}\) elevations evoked by CCH decayed more rapidly than those evoked by i-IP\(_3\) (fall from 80% to 20% of peak 6.33 s ± 0.3 s for CCH vs 20.05 s ± 3.2 s for i-IP\(_3\)) - likely because the slowly-degraded i-IP\(_3\) evoked a more sustained release of Ca\(^{2+}\).
Figure 2. Localized fluctuations in cytosolic [Ca\(^{2+}\)] occur predominantly during the rising phase of global Ca\(^{2+}\) elevations. Representative records show the Cal520 fluorescence ratio (ΔF/ΔF\(_0\); smooth traces) and the associated SD fluctuation measurements (noisy traces) from ROIs encompassing single WT HEK cells bathed in Ca\(^{2+}\)-free medium. (A) Record obtained under basal conditions without stimulation. (B–E) Responses evoked by progressively longer photolysis flashes to release increasing amounts of i-IP\(_3\) in cells loaded with caged i-IP\(_3\). The SD signals are presented in arbitrary units (A.U.) but are consistent throughout all panels. To better display responses to weaker stimuli, the y-axes are scaled differently between panels. (F) Responses evoked by application of carbachol (CCH; 10 μM) when indicated by the bar. (G, H) Pooled data plotting, respectively, means of the global Ca\(^{2+}\) fluorescence signals and SD signals of cells stimulated with progressively increasing photorelease of i-IP\(_3\) to evoke predominantly local Ca\(^{2+}\) signals (yellow traces; n = 7), and global elevations with slow (blue; n = 9), medium (green; n = 13), and fast rising Ca\(^{2+}\) signals (pink; n = 11). (I) Mean Cal520 Figure 2 continued on next page
Ca\(^{2+}\) puff activity terminates during the rising phase of global Ca\(^{2+}\) signals

Puff activity (SD signal) showed a characteristic rise and fall during the rising phase of global Ca\(^{2+}\) signals, and both parameters accelerated with increasing photorelease of i-IP\(_3\) (Figure 2). To investigate the relationship between the bulk Ca\(^{2+}\) level and puff activity in a time-independent manner, we took paired measurements of cell-wide SD signals and Ca\(^{2+}\) level (ΔF/ΔF\(_0\)) at intervals during the rising phase of IP\(_3\)-evoked Ca\(^{2+}\) elevations. Figure 3A shows a scatter plot of SD vs. ΔF/ΔF\(_0\) values for measurements from the cell in Figure 2D, and Figure 3B plots corresponding mean data pooled from groups of cells that gave IP\(_3\)-evoked global signals with fast (pink circles), intermediate (green triangles) and slow (blue squares) rising phases. Although the amplitudes of the SD signals were greater for the faster rising responses, all cells showed similar ‘inverted U’ shaped relationships. In all three groups, the SD signal was maximal when the Cal520 fluorescence ratio reached a ΔF/ΔF\(_0\) value of about two and then declined progressively as global Ca\(^{2+}\) rose higher. This is illustrated more clearly in Figure 3C, where the curves for the three groups of cells superimpose closely after normalization to the same peak SD level. A closely similar inverted U relationship was observed for Ca\(^{2+}\) elevations evoked by CCH (Figure 3D).

The decline in SD signal at higher Ca\(^{2+}\) levels during global signals cannot be attributed to a failure of our algorithm to detect local fluctuations because of saturation of the Cal520 indicator dye. Notably, maximal fluorescence responses evoked by addition of ionomycin in high (10 mM) Ca\(^{2+}\)-containing medium (ΔF/ΔF\(_0\) of 18.93 ± 1.5; n = 32 cells) considerably exceeded the peak fluorescence elevations evoked by even strong photorelease of i-IP\(_3\) (mean ΔF/ΔF\(_0\)=7.27 ± 0.4, n = 11 cells), and were greatly in excess of the fluorescence level (ΔF/ΔF\(_0\) ~2; Figure 3) at which the SD signal began to decline. Moreover, we observed instances of local Ca\(^{2+}\) signals even during large global Ca\(^{2+}\) elevations (ΔF/ΔF\(_0\)>8; Figure 3—figure supplement 1), and obtained SD signals using the lower affinity indicator fluo8L (Kd 1.86 μM vs. 320 nM for Cal520) confirming that puff activity was similarly suppressed prior to the peak of i-IP\(_3\) evoked Ca (Berridge et al., 2000) elevations (Figure 3—figure supplement 2).

Ca\(^{2+}\) puffs are independent of extracellular Ca\(^{2+}\)

We performed the experiments in Figures 1–3 using a bathing solution containing no added Ca\(^{2+}\) together with 300 μM EGTA to specifically monitor the release of Ca\(^{2+}\) from intracellular stores without possible confounding signals arising from entry of Ca\(^{2+}\) across the plasma membrane. To explore whether these results were representative of responses in more physiological conditions, we examined Ca\(^{2+}\) signals evoked by photoreleased i-IP\(_3\) in WT HEK cells bathed in solutions containing 2 mM Ca\(^{2+}\) (Figure 3—figure supplement 3). Cell-wide Ca\(^{2+}\) responses and flurries of local Ca\(^{2+}\) signals closely matched the patterns of activity in cells imaged in the absence of extracellular Ca\(^{2+}\) (Figure 3—figure supplement 3B–G), and scatter plots of SD signal vs. global Ca\(^{2+}\) fluorescence signal (Figure 3—figure supplement 3H,I) mirrored those in the absence of extracellular Ca\(^{2+}\) (Figure 3). Thus, the puff activity during IP\(_3\)-evoked global Ca\(^{2+}\) elevations appears independent of Ca\(^{2+}\) influx into the cell. However, global Ca\(^{2+}\) responses decayed more slowly when Ca\(^{2+}\) was included in the bath solution (fall\(_{80.20}\) for strong photoreleased i-IP\(_3\) of 35.87 s ± 3.9 s in 2 mM Ca\(^{2+}\) vs. 20.05 s ± 3.2 s in zero Ca\(^{2+}\); Fall\(_{80.20}\) for CCH of 13.06 s ± 0.5 s in 2 mM Ca\(^{2+}\) vs 6.33 s ± 0.3 in zero Ca\(^{2+}\)).
A likely explanation is that influx through slowly activating store-operated channels prolongs the response when extracellular Ca\(^{2+}\) is present.

**Patterns of Ca\(^{2+}\) release are largely unaffected by inhibition of mitochondrial and lysosomal Ca\(^{2+}\) uptake**

Mitochondria and lysosomes help shape intercellular Ca\(^{2+}\) dynamics by accumulating and releasing Ca\(^{2+}\) (Rizzuto et al., 2012; Mammucari et al., 2018; Morgan et al., 2011; Yang et al., 2019). To examine whether activity of these organelles influenced the spatial-temporal occurrence of puffs during IP\(_3\)-evoked global Ca\(^{2+}\) signals, we treated WT HEK cells for 10 min with FCCP to inhibit mitochondrial (Stout et al., 1998; Jensen and Rehder, 1991) and lysosomal (Churchill et al., 2002) Ca\(^{2+}\) uptake by dissipation of the proton gradient necessary for Ca\(^{2+}\) flux. (Figure 3—figure supplement 4A,B). Mean traces of whole-cell Ca\(^{2+}\) fluorescence (\(\Delta F/F_0\)) and associated SD fluctuations in FCCP-treated cells stimulated with CCH exhibited local and global Ca\(^{2+}\) signals similar to vehicle-treated controls, although with slightly smaller peak magnitudes (Figure 3—figure supplement 4C, D).
D). Scatter plots of SD signal vs. bulk Ca$^{2+}$ level during the rising phase of CCH-evoked Ca$^{2+}$ elevations were closely similar in control and following FCCP application (Figure 3—figure supplement 4E).

**Ca$^{2+}$ puffs do not terminate because of rising cytosolic Ca$^{2+}$ during cell-wide elevations**

In light of the resemblance between the inverted U relationship between puff activity and Ca$^{2+}$ level (Figure 3) and the well-known bell-shaped curve for biphasic modulation of IP$_3$R channel activation by Ca$^{2+}$ (Uyama, 1990; Bezprozvanny et al., 1991), we considered whether the suppression of puff activity during global elevations might result because IP$_3$Rs became inhibited by rising cytosolic Ca$^{2+}$ levels. To test this, we first examined the effect of elevating cytosolic Ca$^{2+}$ levels prior to evoking IP$_3$-mediated Ca$^{2+}$ signals. We loaded HEK WT cells with caged Ca$^{2+}$ (NP-EGTA) and delivered photolysis flashes of varying durations to cause jumps of cytosolic free Ca$^{2+}$ of different magnitudes before locally applying CCH from a puffer pipette (Figure 4A). Although the SD signals evoked by CCH declined progressively with increasing prior photorelease of Ca$^{2+}$, this reduction was matched by a similar diminution in peak amplitudes ($\Delta F/F_0$) of the global Ca$^{2+}$ signal. The open symbols in Figure 4B plot the ratio of puff activity (integral under SD traces) relative to the size of the CCH-evoked global Ca$^{2+}$ signal in each cell, and are presented after binning according to the magnitude of the preceding Ca$^{2+}$ jump evoked by photolysis of caged Ca$^{2+}$. Mean ratios (Figure 4B, filled symbols) remained almost constant for all Ca$^{2+}$ jumps; even at levels ($\Delta F/F_0 > 6$) corresponding to those where puff activity was strongly suppressed during the rising phase of global responses (Figure 3).

As a complementary approach, we then examined the effect of buffering the rise in cytosolic [Ca$^{2+}$] during global responses by strong cytosolic loading of EGTA.

Figure 4C shows representative SD and $\Delta F/F_0$ traces in response to photoreleased i-IP$_3$ from a WT HEK cell that was loaded with EGTA by incubation for 1 hr with 15 μM EGTA-AM. The cell showed a typical flurry of puff activity like that in non-EGTA-loaded cells. Puffs ceased before the peak of the global Ca$^{2+}$ signal, even though the amplitude of the signal (2.5 $\Delta F/F_0$) was strongly attenuated. Figure 4D summarizes mean data from multiple cells, plotting paired measurements of cell-wide SD signals and Ca$^{2+}$ level ($\Delta F/F_0$) at intervals during the rising phase of IP$_3$-evoked Ca$^{2+}$ elevations, as in Figure 3. The data again followed an inverted U relationship (solid circles), but in comparison to control, non EGTA-loaded cells (open circles) the relationship was shifted markedly to the left. Notably, the peak SD signal was attained at a fluorescence level of about 0.40 $\Delta F/F_0$ vs. about 2 $\Delta F/F_0$ for controls, and puffs were substantially suppressed at fluorescence levels ($\Delta F/F_0 < 2$) where the puff activity was near maximal in control cells.

Taken together, these results demonstrate that inhibition of IP$_3$Rs by elevated cytosolic [Ca$^{2+}$] is not the primary mechanism causing puff activity to terminate during whole-cell Ca$^{2+}$ responses. They further buttress other evidence that the decline in SD signal during the rising phase of the response does not arise because the indicator dye becomes saturated, but faithfully reflects a physiological termination of puff activity.

**Partial depletion of ER Ca$^{2+}$ selectively inhibits Ca$^{2+}$ puffs**

We next considered the possibility that puff activity may terminate during the rising phase of global Ca$^{2+}$ elevations because of falling luminal ER [Ca$^{2+}$], rather than rising cytosolic [Ca$^{2+}$]. We tested this idea by imaging i-IP$_3$ evoked global Ca$^{2+}$ signals after partially depleting ER Ca$^{2+}$ stores while minimizing changes in cytosolic free [Ca$^{2+}$].

In a first approach (Figure 5A), we transiently applied cyclopiazonic acid (CPA) to reversibly inhibit SERCA activity (Uyama et al., 1992), resulting in a net leak of Ca$^{2+}$ from the ER and a small elevation of cytosolic Ca$^{2+}$. Following wash-out of CPA, the cell was maintained in Ca$^{2+}$-free medium so that the cellular Ca$^{2+}$ content (including that of the ER) gradually depleted owing to passive and active extrusion across the plasma membrane. After about 4 min the resting cytosolic Ca$^{2+}$ level had returned close to the original baseline, and we delivered a photolysis flash to photorelease i-IP$_3$. This evoked a substantial elevation in global Ca$^{2+}$, yet the SD signal showed almost no transient puff activity during this response. Similar results were obtained in a further seven cells, as shown by the mean $\Delta F/F_0$ and SD traces in Figure 5B. To confirm that the suppression of puff activity resulted from cellular Ca$^{2+}$ depletion, we repeated this experiment, now making a paired comparison of
i-IP$_3$-evoked responses between cells that were bathed for 30 min after washing out CPA either in Ca$^{2+}$-containing medium to allow ER store refilling (Figure 5C; Figure 5—video 1), or in Ca$^{2+}$-free medium (Figure 5D; Figure 5—video 1). Cells in both groups showed substantial global Ca$^{2+}$ responses that were not appreciably different in peak amplitudes (Figure 5E); but whereas the SD signals showed that puff activity was strongly suppressed in cells maintained in zero Ca$^{2+}$ medium,
Figure 5. Ca\(^{2+}\) puffs are selectively depressed by reduced ER Ca\(^{2+}\) content. (A–F) Selective depression of puffs during i-IP\(_3\)-evoked global Ca\(^{2+}\) signals following depletion of ER Ca\(^{2+}\) content using transient application of cyclopiazonic acid (CPA; 50 μM) (A) The smooth trace shows fluorescence ratio (ΔF/F\(_0\)) from a WT HEK cell, and the noisy trace the corresponding SD signal (in arbitrary units). The cell was bathed throughout in solution containing no added Ca\(^{2+}\) and 300 μM EGTA, and CPA was locally applied from a puffer pipette during the time indicated by the bar. A UV flash was delivered when marked by the arrow to photorelease caged i-IP\(_3\) loaded into the cell. (B) Mean ΔF/F\(_0\) and SD signals from seven WT HEK cells in response to photoreleased i-IP\(_3\) following CPA treatment and wash in Ca\(^{2+}\)-free medium as in A. (C,D) Representative ΔF/F\(_0\) and SD responses to photoreleased i-IP\(_3\) in individual cells that were bathed, respectively, in Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free medium for 30 min following treatment with CPA as in A. (E) Peak amplitudes of global fluorescence signals evoked by photoreleased i-IP\(_3\) in experiments like those in C,D, for cells bathed in Ca\(^{2+}\)-containing (n = 8 cells; blue squares) or Ca\(^{2+}\)-free medium (n = 6; red circles). Open symbols denote measurements from individual cells; filled symbols are means. No significant difference between peak amplitudes (ΔF/F\(_0\)) of cells bathed in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium (Student T test; p=0.72). (F) Corresponding measurements of integral under SD traces (puff activity) during the time from the photolysis flash to the peak global fluorescence signal. SD integrals were significantly different between cells bathed in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium (Student T test; p=0.012). (G–I) Selective depression of puffs by depleting ER Ca\(^{2+}\) content by repeated applications of CCH in zero Ca\(^{2+}\) bathing solution. (G,H) Global Ca\(^{2+}\) signals (smooth traces; ΔF/F\(_0\)) and SD signals (noisy traces) evoked by successive, identical applications of CCH at 5 min intervals in two representative cells bathed, respectively, in medium containing 2 mM Ca\(^{2+}\) or 300 μM EGTA with no added Ca\(^{2+}\). Amplitudes of the SD signals are depicted after normalizing to the peak amplitude of the first response for each cell. (I) Data points show the ratio of puff activity (integral under the SD trace) vs. peak magnitude of Figure 5 continued on next page
Figure 5 continued

the global Ca\(^{2+}\) signal (\(\Delta F/F_0\)) for successive responses evoked by CCH application at 5 min intervals. Blue squares are data from cells bathed in medium containing 2 mM Ca\(^{2+}\) and red circles are from cells in Ca\(^{2+}\)-free medium; open symbols are ratios from individual cells and filled symbols are means. Data are plotted after normalizing to the mean SD integral and peak \(\Delta F/F_0\) evoked by the initial stimulus in each condition. Responses were significantly different between cells bathed in the presence and absence of external Ca\(^{2+}\) for times \(\geq 10\) min (Student T test, \(p=0.000008\)).

The online version of this article includes the following video for figure 5:
*Figure 5—video 1.* Partial depletion of ER Ca\(^{2+}\) selectively inhibits Ca\(^{2+}\) puff activity.
https://elifesciences.org/articles/55008#fig5video1

cells in Ca\(^{2+}\)-containing medium showed robust puff activity during the rising phase of the response (*Figure 5F*).

As an alternative approach to partially deplete ER Ca\(^{2+}\) without pharmacological intervention, we evoked Ca\(^{2+}\) signals by repeated applications of CCH at 5 min intervals, and compared responses in cells bathed in Ca\(^{2+}\)-containing (*Figure 5G*) and Ca\(^{2+}\)-free solutions (*Figure 5H*). In both cases, the amplitudes of the global Ca\(^{2+}\) signals progressively declined, likely a result of inhibition of IP\(_3\)Rs. However, whereas the amplitude of puff activity reported by SD signals in cells bathed in Ca\(^{2+}\)-containing medium fell roughly in proportion to the amplitude of the global fluorescence signal, puff activity in Ca\(^{2+}\)-free medium declined abruptly. In the example depicted in *Figure 5H*, no activity was evident in the SD signal after the fifth stimulus at 20 min even though an appreciable global Ca\(^{2+}\) elevation remained. To quantify these data, we determined puff activity as the integral under the SD trace, and plotted the normalized ratio of puff activity vs. peak global Ca\(^{2+}\) amplitude (*Figure 5I*). For cells in Ca\(^{2+}\)-containing medium, the mean ratio remained constant across successive stimuli (blue squares, *Figure 5I*), whereas it declined almost to zero for cells in Ca\(^{2+}\)-free medium (red circles, *Figure 5I*).

We conclude from these results that Ca\(^{2+}\) puff activity is modulated by ER Ca\(^{2+}\) store content, and that when stores are partially depleted IP\(_3\) can still evoke Ca\(^{2+}\) release by a process that is independent of puff activity, and occurs without detectable temporal fluctuations. We term this mode of Ca\(^{2+}\) liberation as ‘diffuse’ release and refer to Ca\(^{2+}\) puffs as a ‘punctate’ mode of Ca\(^{2+}\) liberation.

**All three IP\(_3\)R isoforms mediate punctate and diffuse modes of Ca\(^{2+}\) liberation**

In common with many other cell types, WT HEK and HeLa cells express all three major IP\(_3\)R isoforms – types 1, 2, and 3 – that are encoded by separate genes and translated into structurally and functionally distinct proteins that co-translationally oligomerize to form heterotetrameric channels. We (Lock et al., 2018) and others (Mataragka and Taylor, 2018) recently demonstrated that all three isoforms can individually mediate Ca\(^{2+}\) puffs. We now utilized HEK cells genetically engineered to express single IP\(_3\)R isoforms to evaluate the respective roles of each isoform in liberating Ca\(^{2+}\) via punctate, localized transients versus sustained, diffuse release.

We evoked Ca\(^{2+}\) liberation in WT HEK cells and cells exclusively expressing type 1, 2, or 3 IP\(_3\)Rs by local application of CCH (*Figure 6*). All three single-isoform-expressing cell lines exhibited patterns of responses qualitatively similar to WT cells. The SD traces showed flurries of puffs during the foot and rising phase of global Ca\(^{2+}\) signals that ceased before the time of the peak global Ca\(^{2+}\) elevation (*Figure 6A–H*). Nevertheless, notable differences were apparent between the isoforms. Cells expressing IP\(_3\)R1 generated whole-cell Ca\(^{2+}\) signals having much smaller amplitudes and slower rising phases than WT and R2- and R3-expressing cells, and localized fluctuations persisted longer (*Figure 6C,D,I*). In contrast, IP\(_3\)R2-expressing cells displayed fast rising, large amplitude Ca\(^{2+}\) signals, with a transient flurry of Ca\(^{2+}\) fluctuations concentrated during the initial portion of the rising phase (*Figure 6E,F*). Ca\(^{2+}\) signals in cells expressing IP\(_3\)R3 (*Figure 6G,H*) were similar in amplitude to WT and IP\(_3\)R2-expressing cells, but with slower rates of rise and more prolonged flurries of puffs. Scatter plots of puff activity (SD signal) as a function of the global Ca\(^{2+}\) level (\(\Delta F/F_0\)) during the rising phase of the global response further highlighted these differences (*Figure 6J,K*). Ca\(^{2+}\) fluctuations were maximal when Cal520 fluorescence (\(\Delta F/F_0\)) rose to roughly 1.5, 6, and 3 for types 1, 2, and 3 IP\(_3\)Rs, respectively; and similarly large differences were evident in the global Ca\(^{2+}\) level attained when puff activity terminated.
HeLa and HEK cells exhibit similar patterns of Ca\(^{2+}\) signals

We utilized HEK cells for most experiments because of the availability of cell lines expressing individual IP\(_3\)R isoforms (Alzayady et al., 2016). The patterning of local, transient Ca\(^{2+}\) signals during IP\(_3\)-mediated whole-cell Ca\(^{2+}\) elevations was not unique to this cell type. Stimulation of HeLa cells with histamine also evoked global Ca\(^{2+}\) signals accompanied by flurries of local Ca\(^{2+}\) activity during the rising phase, which subsided as Ca\(^{2+}\) levels continued to rise (Figure 6—figure supplement 1).
Diffuse Ca\textsuperscript{2+} signals in TIRF do not reflect punctate release in the cell interior

The data in Figures 1–6 derive from TIRF imaging of Ca\textsuperscript{2+} signals in close proximity to the plasma membrane, where a majority (~80%) of puff sites in WT HEK cells are located (Lock et al., 2018). However, TIRF microscopy provides no direct information from the interior of the cell, leaving open the question as to whether slow diffusion of Ca\textsuperscript{2+} ions from puffs at internal sites may contribute to the diffuse component of the Ca\textsuperscript{2+} signal visualized in TIRF images after the puff flurry has ceased. To address this issue, we applied fluctuation analysis to images obtained using lattice light-sheet (LLS) microscopy to record Ca\textsuperscript{2+} signals within diagonal optical ‘slices’ through the cell volume (Ellefsen and Parker, 2018).

Figure 7A,B illustrate LLS Ca\textsuperscript{2+} fluorescence ratio images and corresponding SD images recorded before and after photorelease of i-IP\textsubscript{3} to evoke a global Ca\textsuperscript{2+} response. Similar to observations with TIRF imaging, the SD images revealed local Ca\textsuperscript{2+} transients that began soon after photorelease, and before any appreciable rise in the global Ca\textsuperscript{2+} signal (Figure 7A, panel ii). Discrete events then continued during much of the rising phase of the global signal (panels iii-v) but had largely ceased at the time of the peak global signal (panel vi). In this cell Ca\textsuperscript{2+} puffs were primarily restricted to the cell periphery, whereas Figure 7B shows an example from another cell where local activity was observed both around the periphery and in the cell interior.

![Light sheet imaging of global Ca\textsuperscript{2+} elevations evoked in HEK cells by photoreleased i-IP\textsubscript{3}.](image-url)
Figure 7C,D shows respective measurements from these two cells, plotting fluorescence ratio changes (ΔF/F₀) and SD signals from ROIs encompassing peripheral (red traces) and central (blue traces) regions of the cells, as indicated in the leftmost lower panels of Figure 7A,B. In both cells, the local Ca⁡²⁺ activity monitored by SD fluctuations started within a few hundred ms of the photolysis flash and was maximal during the early portion of the rising phase. The SD signal then declined, returning close to baseline as the global Ca⁡²⁺ signal approached a peak. For the cell illustrated in Figure 7A, the SD signal within the peripheral region was much greater than in the central region, even though the rise in global Ca⁡²⁺ was slightly smaller. In contrast, the cell illustrated in Figure 7B showed a SD signal in the interior that was similar in size to the periphery (Figure 7D). On average, however, mean SD signals from the cell interior were about one quarter of that at the periphery, and fluctuations arising from interior sites followed a similar relation with bulk Ca⁡²⁺ level as peripheral sites (Figure 7E).

Given the relatively low average level of puff activity in the cell interior, and the similar termination of internal and peripheral puff flurries during the rising of global Ca⁡²⁺ signals, we conclude that the diffuse component of the Ca⁡²⁺ rise observed by TIRF microscopy cannot be accounted for by Ca⁡²⁺ spreading from punctate release at internal sites and becoming blurred by diffusion in space and time.

Puff activity contributes only a fraction of the total Ca⁡²⁺ liberated during global signals

To assess the relative contributions of punctate versus diffuse modes of Ca⁡²⁺ release during global Ca⁡²⁺ signals, we derived the kinetics of Ca⁡²⁺ flux into the cytosol through IP₃Rs on the basis that the cell-wide fluorescence signal reflects a balance between Ca⁡²⁺ release into the cytoplasm and its subsequent removal. To obtain a rate constant for removal of cytosolic Ca⁡²⁺ in WT HEK cells, we recorded the decline of fluorescence Ca⁡²⁺ signals following transient photorelease of Ca⁡²⁺ from caged Ca⁡²⁺ loaded into the cytosol (Figure 8—figure supplement 1A), and during the final ‘tail’ of CCH-evoked Ca⁡²⁺ signals when Ca⁡²⁺ liberation would have almost ceased (Figure 8—figure supplement 1B). Both fitted well to single exponential decay functions, consistent with a dominantly first order removal process, with respective mean rate constants of 0.22 and 0.32 s⁻¹.

We then calculated the instantaneous Ca⁡²⁺ release flux at intervals throughout the time course of a global Ca⁡²⁺ response by differentiating the whole-cell fluorescence Ca⁡²⁺ signal and adding to this the estimated rate of Ca⁡²⁺ removal; for i-IP₃ signals we used a rate constant of 0.22 s⁻¹; for CCH evoked responses we applied rate constants (0.3 s⁻¹ to 0.6 s⁻¹) that were determined from the tail-end of the global Ca⁡²⁺ decay for that particular cell.

We used data from the experiment of Figure 5C,D to compare the kinetics of Ca⁡²⁺ liberation during Ca⁡²⁺ signals under normal conditions, and when puff activity had been inhibited by partial depletion of ER Ca⁡²⁺ store content. Figure 8A,B show records from two representative cells that gave global Ca⁡²⁺ responses of comparable peak amplitudes (black traces). However, whereas the SD signals (grey traces) exhibited the normal flurry of puff activity in the control cell (Figure 8A) this activity was almost completely suppressed in the cell pretreated with CPA (Figure 8B). The red traces show the respective rates of Ca⁡²⁺ release into the cytosol, revealing a larger initial transient of Ca⁡²⁺ liberation in the control cell during the flurry of puff activity. Figure 8C shows overlaid mean traces of Ca⁡²⁺ release from control (n = 5) and CPA-treated cells (n = 6). Colored areas indicate the relative cumulative amounts of Ca⁡²⁺ entering the cytosol (integral under the release trace) in CPA-treated cells where puff activity was substantially abolished (blue shading), and the additional Ca⁡²⁺ flux (pink shading) in control cells showing flurries of puffs. From these respective areas, we estimate that, in normal conditions, the punctate liberation of Ca⁡²⁺ through puff activity contributes about 41% of the total Ca⁡²⁺ release responsible for the initial rise of Ca⁡²⁺ toward its peak. Figure 8D,E further illustrate representative records of SD signals (grey traces), global Ca⁡²⁺ (black), rate of Ca⁡²⁺ release into the cytosol (red), and cumulative amount of Ca⁡²⁺ released (blue) during the entire time course of global Ca⁡²⁺ signals evoked by photoreleased i-IP₃ (Figure 8D) and by CCH (Figure 8E). Because much of the cumulative Ca⁡²⁺ release through IP₃Rs arises from a sustained, low level flux that continues after the peak, Ca⁡²⁺ puffs on average contribute only about 13% of the total Ca⁡²⁺ liberation during global i-IP₃-evoked signals, and about 17% during shorter-lasting responses evoked by CCH (Figure 8F).
Discussion

Ca\textsuperscript{2+} puffs are transient, localized elevations in cytosolic Ca\textsuperscript{2+} that arise from concerted opening of small numbers of IP\textsubscript{3}Rs clustered at fixed intracellular sites (Parker and Yao, 1991; Thillaiappan et al., 2017). Puffs are apparent as discrete events superimposed on a steady basal Ca\textsuperscript{2+} level when cytosolic IP\textsubscript{3} concentrations are modestly elevated (Parker and Yao, 1991; Yao et al., 1995; Parker et al., 1996), whereas higher concentrations of IP\textsubscript{3} evoke global, cell-wide Ca\textsuperscript{2+} signals on which puffs are evident on the rising phase (Bootman et al., 1997a; Marchant et al., 1999; Mataragka and Taylor, 2018); acting as local signals in their own right at low [IP\textsubscript{3}] and mediating global Ca\textsuperscript{2+} signals at higher [IP\textsubscript{3}] by a fire-diffuse-fire mechanism whereby Ca\textsuperscript{2+} released by a puff site diffuses to activate CICR at neighboring sites (Bootman et al., 1997a; Parker et al., 1996; Berridge, 1997; Dawson et al., 1999). However,
it has been difficult to definitively test this ‘building block’ model because puffs become obscured by the large global Ca\(^{2+}\) elevations; and recent theoretical simulations have questioned whether the summation of Ca\(^{2+}\) released through coordinated puff activity at multiple sites is alone sufficient to propagate global cytosolic Ca\(^{2+}\) signals (Piegari et al., 2019). Here, we addressed this topic by analyzing temporal and spatial fluctuations in Ca\(^{2+}\) image data to resolve local Ca\(^{2+}\) transients during global signals (Swaminathan et al., 2020). Our main conclusion is that global Ca\(^{2+}\) signals involve two modes of Ca\(^{2+}\) liberation through IP\(_3\)R: ‘punctate’ release as a flurry of transient, local events, and a more sustained, ‘diffuse’ release mode.

As with any new approach, we first needed to validate the ability of our algorithm to faithfully report local Ca\(^{2+}\) transients during even large global Ca\(^{2+}\) elevations, when resolution may be impaired by factors including the dynamic range of the indicator dye and by increased photon shot noise at high fluorescence levels. A particular concern was whether the indicator (Cal520) we used for most experiments may have approached saturation, thus ‘clipping’ the signals to artifactually suppress the temporal SD signal and giving a false impression that puff activity terminates as the Ca\(^{2+}\) level and fluorescence rise during global signals. Several lines of evidence convincingly argue that this is not the case. Notably: (i) maximal, saturating signals evoked by ionomycin (~19 ΔF/Φ) were much higher than mean peak IP\(_3\)-evoked fluorescence signals (~7 ΔF/Φ), and puff activity began to decline as fluorescence rose above ~2 ΔF/Φ; (ii) we observed patterns of puff activity using the low affinity indicator fluo-8L (K\(_{d}\) 1.86 μM) that closely matched those obtained with Cal520 (K\(_{d}\) 320 nM) (Figure 3—figure supplement 2); (iii) we were able to resolve instances of local puff activity even at the peak of IP\(_3\)-evoked global fluorescence elevations (Figure 3—figure supplement 1); (iv) the kinetics of puff activity were closely similar in cell lines individually expressing single IP\(_3\)R isoforms, despite large differences in the amplitudes of the global Ca\(^{2+}\) signals (Figure 6); (v) the onset and termination of puff activity during the rise of IP\(_3\)-mediated global Ca\(^{2+}\) signals were little altered when the initial basal Ca\(^{2+}\) level was elevated (Figure 4A,B) or, conversely, when the global Ca\(^{2+}\) rise was attenuated by buffering with cytosolic EGTA (Figure 4C,D). Finally, the suppression of punctate Ca\(^{2+}\) liberation throughout all phases of IP\(_3\)-evoked Ca\(^{2+}\) responses when ER Ca\(^{2+}\) stores were partially depleted (Figure 5) strongly supports our proposal that Ca\(^{2+}\) liberation can arise in a diffuse manner, independent of local puff events.

### Puff activity during global Ca\(^{2+}\) signals

In agreement with previous findings (Bootman et al., 1997a; Marchant et al., 1999) our fluctuation analyses reveal flurries of puffs during the initial rise of IP\(_3\)-mediated global Ca\(^{2+}\) signals. However, although puff activity was evident during the initial foot of the response and peaked early during the rising phase, it then subsided during the later portion of the rising phase, with few or no transient, local Ca\(^{2+}\) signals evident by the time of the peak. Notably, overall Ca\(^{2+}\) levels continued to rise after puffs had largely ceased, and cytosolic Ca\(^{2+}\) remained elevated for several seconds in the face of rapid removal from the cytosol, during which time Ca\(^{2+}\) fluctuations were largely suppressed.

This ‘noise-free’ component of the fluorescence signal cannot be attributed to slow diffusion of Ca\(^{2+}\) liberated as puffs to fill in spaces between release sites. Diffusion would be rapid (e.g. mean time of ~300 ms to diffuse 5 μm assuming an effective diffusion coefficient of 20 μm\(^2\) s\(^{-1}\)). In any case, the average fluorescence signal would not be expected to increase appreciably if the total amount of Ca\(^{2+}\) in the imaging volume remained constant. Utilizing lightsheet imaging we further excluded the possibility that the continuing rise in near-plasmalemmal Ca\(^{2+}\) observed by TIRF imaging might arise through diffusion of Ca\(^{2+}\) over longer distances after liberation at sites in the cell interior. Finally, as noted previously, the observation of large global Ca\(^{2+}\) signals in the absence of detectable fluorescence fluctuations (Figure 5) definitively points to a mode of Ca\(^{2+}\) liberation that is independent of puff activity.

By deriving the time course of cumulative Ca\(^{2+}\) liberation during global responses we estimated that puffs contribute only ~41% of the initial Ca\(^{2+}\) flux that drives the peak of the Ca\(^{2+}\) response, and an even smaller proportion (~15%) of the cumulative flux during its entire time course. Thus, a second component of continuous, spatially diffuse release of intracellular Ca\(^{2+}\) is responsible for generating and sustaining a large part of whole-cell Ca\(^{2+}\) signals. These two components are further discriminated by procedures that selectively promoted either puff activity (Dargan and Parker, 2003; Smith et al., 2009) (by cytosolic loading of slow Ca\(^{2+}\) buffers), or global Ca\(^{2+}\) elevations in the absence of localized Ca\(^{2+}\) transients (by partial depletion of ER Ca\(^{2+}\) store content). We term these...
Two modes of IP₃-mediated Ca²⁺ release as ‘punctate’ (discontinuous in time and space) and ‘diffuse’ (smoothly varying in time and space).

Small elevations of [Ca²⁺]ᵢ promote opening of IP₃R channels (Iino, 1990; Bezprozvanny et al., 1991) and increase puff frequency (Yamasaki-Mann et al., 2013). The accelerated puff activity during the foot and initial upstroke of the global Ca²⁺ signal is thus likely a consequence of a rising basal cytosolic Ca²⁺ level, resulting both from the puffs themselves and from diffuse Ca²⁺ liberation. This positive feedback of cytosolic Ca²⁺ to promote opening of IP₃Rs is inherently regenerative, so it is imperative that mechanisms exist to ‘put out the fire’. Our results illuminate at least two mechanisms, acting across different time scales, to terminate punctate Ca²⁺ liberation through IP₃Rs. Individual puffs terminate rapidly as IP₃R channels close within tens of ms (Parker et al., 1996; Bootman et al., 1997b) via stochastic inhibition by high local Ca²⁺ levels (Shuai et al., 2008) and potential allosteric interactions between clustered IP₃Rs at a puff site (Wiltgen et al., 2014). However, during the rising phase of a global Ca²⁺ signal, each IP₃R cluster may generate a flurry of repeated puffs - indicating that although the fast-inhibitory processes that terminates individual puffs recovers quickly, a slower process terminates the flurry. This does not appear to result from IP₃Rs becoming inactivated by the rise of bulk cytosolic Ca²⁺, because we found puff flurries were not suppressed by prior Ca²⁺ elevations, and still terminated normally during global responses when cytosolic Ca²⁺ levels were attenuated by buffering with EGTA (Figure 4C,D). Instead, our observations that partial depletion of ER Ca²⁺ stores suppressed puff activity during global Ca²⁺ responses (Figure 5) implicate the depletion of luminal Ca²⁺ as a dominant mechanism responsible for terminating the local signals; analogous to the central role of luminal Ca²⁺ depletion in terminating the Ca²⁺ sparks mediated by ryanodine receptors (Stern et al., 2013). Although individual puffs appear not to affect luminal ER [Ca²⁺] (Lock et al., 2018), it is plausible that ER depletion may occur during the rapid flurries of puffs evoked at multiple sites during the rising phase of global signals. A related question is whether the Ca²⁺ depletion is locally confined to the ER around puff sites and arises through Ca²⁺ released by the puffs themselves, or whether diffuse Ca²⁺ liberation causes global ER Ca²⁺ depletion throughout a luminally continuous ER network (Okubo et al., 2015; Park et al., 2000).

Two functionally distinct modes of IP₃R-mediated Ca²⁺ liberation

The two modes of Ca²⁺ liberation we describe – punctate and diffuse – might, in principle, arise from two functionally and physically distinct populations of IP₃Rs, or through functional regulation of the clustered IP₃Rs underlying puffs such that they switch from a pulsatile to continuous mode of release. At present, we cannot discriminate between these mechanisms, but favor the former for congruence with studies revealing two distinct populations of IP₃Rs in terms of their spatial distributions and motilities. A small (~30%) fraction of the IP₃Rs in a cell are stationary (Thillaiappan et al., 2017; Smith et al., 2014), grouped in small clusters that are anchored at fixed sites predominantly near the plasma membrane (Smith et al., 2009; Lock et al., 2018; Thillaiappan et al., 2017), whereas the majority of IP₃Rs are distributed throughout the bulk of the cytoplasm and are motile within the ER membrane (Thillaiappan et al., 2017; Smith et al., 2014; Tateishi et al., 2005; Gibson and Ehrlich, 2008; Ferreri-Jacobia et al., 2005; Fukatsu et al., 2004). Puffs are proposed to originate from IP₃Rs within the immotile clusters that are endowed with the ability to preferentially respond to low concentrations of IP₃ (Smith et al., 2009; Thillaiappan et al., 2017). In contrast, the widely distributed, motile IP₃Rs remain apparently silent under conditions when puffs are selectively activated by low concentrations of IP₃R or in the presence of slow cytosolic Ca²⁺ buffers to inhibit global Ca²⁺ elevations (Dargan and Parker, 2003; Dargan et al., 2004). The motile, distributed IP₃Rs would be an attractive candidate for the diffusive mode of Ca²⁺ liberation.

Functional differences between putative populations of IP₃Rs mediating punctate and diffuse Ca²⁺ release cannot be attributed to their being constituted of different isoforms of IP₃R, because all three isoforms mediate Ca²⁺ puffs (Mataragka and Taylor, 2018; Lock et al., 2018), and we show here that cells exclusively expressing individual isoforms generate cell-wide Ca²⁺ elevations involving both punctate and diffuse modes of Ca²⁺ liberation. Instead, the functional properties of the IP₃Rs may be affected by factors including their location in the cell, their mutual proximity to enable interactions by CICR, and by their association with modulatory and anchoring proteins (Prole and Taylor, 2016; Lock et al., 2019; Konieczny et al., 2012).
**Consequences of bimodal Ca\(^{2+}\) signals for downstream signaling**

Stimulation of the IP\(_3\)/Ca\(^{2+}\) signaling pathway by activation of cell-surface receptors evokes repetitive oscillations in cytosolic Ca\(^{2+}\) in numerous cell types (Thomas et al., 1996). Signaling information is encoded in the amplitude and frequency of these Ca\(^{2+}\) oscillations activate distinct targets, including the selective activation of NFkB in Jurkat T cells by low frequencies (Dolmetsch et al., 1998), and the frequency-dependent control of gene expression in RBL mast cells (Li et al., 1998). However, signaling information is not restricted to frequency and amplitude components of bulk cytosolic Ca\(^{2+}\) elevations, and numerous findings implicate a component of spatial Ca\(^{2+}\) profiling (Smedler and Uhlén, 2014; Dupont et al., 2011; Berridge et al., 2003; Thul et al., 2009). Because of the restricted diffusion of free Ca\(^{2+}\) ions in the cytosol (Allbritton et al., 1992; Schwaller, 2010), the specificity of downstream signaling by Ca\(^{2+}\) liberated through IP\(_3\)Rs will be strongly influenced by the proximity of target proteins, as well as by their Ca\(^{2+}\) binding kinetics (Samanta and Parekh, 2017; Atakpa et al., 2018; Csordás et al., 2010). The two modes of IP\(_3\)-evoked Ca\(^{2+}\) liberation we describe are, therefore, likely to selectively activate different populations of effectors; those positioned close to the IP\(_3\)Rs at puff sites that experience brief, repetitive transients of high local [Ca\(^{2+}\)], and others that respond to a more sustained, spatially diffuse elevation of bulk cytosolic [Ca\(^{2+}\)].

Based on a close juxtaposition of stationary IP\(_3\)R clusters to ER-plasma membrane junctions where STIM and Orai interact to induce store-operated Ca\(^{2+}\) entry (SOCE) (Thillaiappan et al., 2017; Thillaiappan et al., 2019), it has been proposed that local depletion of ER Ca\(^{2+}\) content at puff sites might rapidly and selectively activate SOCE, without requiring substantial overall loss of the ER Ca\(^{2+}\) that is necessary to sustain numerous ER functions (Thillaiappan et al., 2017; Thillaiappan et al., 2019; Taylor and Machaca, 2019). On the other hand, we previously reported puffs to be unaffected by removal of extracellular Ca\(^{2+}\) (Lock et al., 2018), and we show that patterning of local puff activity during IP\(_3\)-evoked global Ca\(^{2+}\) signals is unaffected by the presence or absence of Ca\(^{2+}\) in the bath solution. Thus, influx of extracellular Ca\(^{2+}\) does not appear to contribute acutely to the initial flurry of puffs or to the rapid rise in global Ca\(^{2+}\), although the more prolonged decay phase of the Ca\(^{2+}\) signal in Ca\(^{2+}\)-containing medium points to a slower activation of SOCE. The relative contributions of local puffs vs. diffuse Ca\(^{2+}\) liberation in activating SOCE remain to be determined. Another example of proximate Ca\(^{2+}\) signaling is the close tethering between ER and mitochondria (Giorgi et al., 2009) that underlies a rapid shuttling of Ca\(^{2+}\) released through IP\(_3\)Rs to the mitochondrial matrix (Csordás et al., 2010; Filadi and Pozzan, 2015), such that Ca\(^{2+}\) transients within a signaling microdomain, rather than the bulk cytosolic Ca\(^{2+}\) signal, regulate mitochondrial bioenergetics and induction of autophagy (Cárdenas et al., 2010). A similar close coupling has recently been described between IP\(_3\)Rs and lysosomes (Atakpa et al., 2018). Our description of two modes of IP\(_3\)-mediated Ca\(^{2+}\) liberation thus raises questions regarding their respective roles in downstream signaling. Is organellar Ca\(^{2+}\) signaling via structurally defined nanodomains restricted to the predominantly peripheral ER contact sites where puffs originate, or might a separate population of IP\(_3\)Rs that mediate diffuse Ca\(^{2+}\) liberation also transmit their signals via restricted domains?

**Materials and methods**

**Cell culture and loading**

HEK293 wild-type (WT) cells were kindly provided by Dr. David Yule (University of Rochester). An IP\(_3\)R null cell line (3KO; #EUR030) and cell lines natively expressing exclusively type 1 (IP\(_3\)R1; #EUR031), type 2 (IP\(_3\)R2; #EUR032) and type 3 (IP\(_3\)R3; #EUR033) isoforms generated from that parental WT HEK293 cell line by CRISPR/Cas9 genetic engineering in the Yule lab were purchased from Kerafast (Boston, MA). HEK cell lines were characterized as described in Alzayady et al., 2016 and were certified mycoplasma free prior to distribution. HeLa cells (#CCL-2) purchased from ATCC (Manassas, VA) were characterized by STR profiling and certified free of mycoplasma prior to distribution. HEK WT, 3KO, and single IP\(_3\)R isoform-expressing cell lines were cultured in Eagle’s Minimum Essential Medium (EMEM; ATCC #30–2003), and HeLa cells were cultured in Dulbecco’s modified Eagle Medium (DMEM; #11965092) from Thermo Fisher Scientific (Waltham, MA). Both EMEM and DMEM were supplemented with 10% fetal bovine serum (#FB-11) from Omega Scientific.

Lock and Parker. eLife 2020;9:e55008. DOI: https://doi.org/10.7554/eLife.55008
(Tarzana, CA). All cell lines were cultured in plastic 75 cm flasks and maintained at 37°C in a humidified incubator gassed with 95% air and 5% CO₂. For imaging, cells were collected using 0.25% Trypsin-EDTA (Thermo Fisher Scientific #25200–056) and grown on poly-D-lysine (Millipore Sigma #P0899; St. Louis, MO) coated (1 mg/ml) 35 mm glass bottom imaging dishes (#P35-1.5–14 C) from MatTek (Ashland, MA) or 12 mm glass coverslips for 2–3 days.

Immediately before imaging, cells were incubated with the membrane-permeant fluorescent Ca²⁺ indicator Cal520/AM (5 μM; AAT Bioquest #21130; Sunnyvale, CA) for 1 hr at room temperature (RT) in a Ca²⁺-containing HEPEs buffered salt solution (Ca²⁺-HBSS). Where indicated cells were additionally loaded with membrane permeant esters of either the caged IP₃ analogue ci-IP₃ (1 μM; SiChem #cag-iso-2-145-10; Bremen, Germany) or the caged Ca²⁺ buffer NP-EGTA [o-nitrophenyl EGTA/AM] (200–500 nM; Thermo Fisher Scientific #N6803) in conjunction with Cal520. For the experiments in Figure 4C,D cells were additionally loaded with EGTA/AM (15 μM; Thermo Fisher Scientific #E1219) for 1 hr at RT in Ca²⁺-HBSS to attenuate global Ca²⁺ elevations. To address possible saturation of Cal520 at high Ca²⁺ levels, cells were alternatively loaded with the lower affinity Ca²⁺ indicator fluo8L/AM (5 μM, AAT Bioquest #21096) together with 1 μM ci-IP₃/PM for 1 hr at RT in Ca²⁺-HBSS. Following incubation with AM esters, cells were incubated for 30 min at room temperature in Ca²⁺-HBSS. Cal520/AM, ci-IP₃/PM, and NP-EGTA/AM, EGTA/AM, and fluo8L/AM were all solubilized with DMSO/20% pluronic F127 (Thermo Fisher Scientific #P3000MP).

FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) and CPA (cyclopiazonic acid), were purchased from Millipore Sigma (#C2920 and #C1530, respectively), and solubilized in 100% ethanol. Carbachol (#C4832) and histamine (#H7125), also from Millipore Sigma, were reconstituted in deionized H₂O. Ca²⁺-HBSS contained (in mM) 135 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH = 7.4). Nominal Ca²⁺-free HBSS consisted of the same formulation as Ca²⁺-HBSS except that CaCl₂ was omitted; for zero Ca²⁺-HBSS, 300 μM EGTA was added to nominal Ca²⁺-free HBSS. For lattice light-sheet imaging, the plasma membrane was stained by adding Cell Mask Deep Red (Thermo Fisher Scientific #C10046) to the bathing solution in the imaging chamber at 1/50,000 dilution.

Ca²⁺ imaging
Total internal reflection fluorescence (TIRF) imaging of Ca²⁺ signals was accomplished using a home-built system, based around an Olympus (Center Valley, PA) IX50 microscope equipped with an Olympus 60X oil immersion TIRF objective (NA 1.45). Fluorescence images were acquired with an Evolve EMCCD camera (Photometrics; Tucson, AZ) with a bit depth of 16 bits, using 2 × 2 binning for a final field at the specimen of 128 × 128 binned pixels (one binned pixel = 0.53 μm) at a rate of ~125 frames s⁻¹. Image data were streamed to computer memory using Metamorph v7.7 (Molecular Devices; San Jose, CA) and stored on hard disk for offline analysis.

Lattice light-sheet imaging was performed using a home-built system as previously described (Ellefsen and Parker, 2018). Images were acquired with an Andor Zyla 4.2 sCMOS camera (Oxford Instruments; Abingdon, England) from a single, diagonal light-sheet slice (512 × 256 pixels, one pixel = 0.11 μm) at 100 frames s⁻¹ and 16 bit depth. Ca²⁺ signals were imaged in Cal520-loaded cells for several seconds following photorelease of i-IP₃ by a flash from a 405 nm laser diode, utilizing 473 nm laser fluorescence excitation and a 510–560 nm bandpass emission filter. A 562 nm laser and 590 nm long-pass filter were used to image the plasma membrane stained with Cell Mask Deep Red. Images were streamed to disk using MicroManager (Vale Lab UCSF; San Francisco, CA).

Photo-uncaging and local application of agonist
Photorelease of i-IP₃ was evoked by UV light from a xenon arc lamp filtered through a 350–400 nm bandpass filter and introduced by a UV-reflecting dichroic in the light path to uniformly illuminate the field of view. The amount of i-IP₃ released was controlled by varying the flash duration, set by an electronically controlled shutter (Uniblitz; Rochester, NY). The same system was used for photolysis of NP-EGTA (i.e. caged Ca²⁺). For the local delivery of solutions to cells during imaging, glass micropipettes were prepared from borosilicate glass capillary filaments (1.5 mm x 0.86 mm, O.D. x I.D.) using a micropipette puller (Sutter Instruments; Novato, CA) to produce tip diameters of ~1–2 μm.
Micropipettes were positioned above the cell understudy with local delivery controlled using a pneumatic picospritzer. The delivery of micropipette contents and the duration and intensity of the UV-flash were empirically adjusted to evoke rapid rises in whole-cell cytosolic Ca\(^{2+}\) levels.

All imaging was performed while cells were bathed in HBSS containing 2 mM Ca\(^{2+}\) or zero Ca\(^{2+}\)-HBSS containing 0.3 mM EGTA and no added Ca\(^{2+}\).

**Image analysis**

Image data imported in 16 bit integer MetaMorph stk or multi-plane TIF format were processed using a script written in Flika (http://flika-org.github.io), a freely available open-source image processing and analysis software in the Python programming language (Ellefsen et al., 2019; Ellefsen et al., 2014). All internal processing and data output were performed using 64 bit floating-point arithmetic.

To analyze and derive movies representing the pixel-by-pixel standard deviation (SD) of temporal fluctuations in fluorescence of the Ca\(^{2+}\) indicator dye we used a custom Flika script as described previously (Ellefsen et al., 2019). In brief, following subtraction of camera black offset level, the raw image stack from the camera was first spatially filtered by a Gaussian blur function with a standard deviation (sigma) of 2 pixels (~1 \(\mu\)m at the specimen). The python package (skimage) used to perform this function applies a two-dimensional Gaussian blur with a specified standard deviation (sigma) out to a range of 4 sigma. To attenuate high-frequency photon shot noise and slow changes in baseline fluorescence, it was then temporally filtered with a bandpass Butterworth filter with low and high cutoff frequencies of 3 and 20 Hz. A running variance of this temporally filtered movie was calculated, pixel by pixel, by subtracting the square of the mean from the mean of the square of a moving 20 frame (160 ms) boxcar window of the movie. The running standard deviation was calculated by taking the square root of the variance image stack to create a standard deviation (SD) stack. Finally, to remove the mean predicted photon shot noise which increases in linear proportion to the mean fluorescence intensity, the SD stack was corrected, pixel-by-pixel, by subtracting the square root of a running mean of the spatially filtered fluorescence movie multiplied by a scalar constant; derived as illustrated in Figure 1—figure supplement 2.

We also applied a second FLIKA script to analyze spatial fluctuations in Ca\(^{2+}\) image stacks. For this, black-level subtracted image stacks were first temporally band-pass filtered as before, and then new image stacks were derived by taking the difference of weak (sigma two pixel) and stronger (sigma eight pixel) Gaussian blur functions. Essentially, this functioned as a spatial bandpass filter, attenuating high frequencies caused by pixel-to-pixel shot noise variations and low frequency variations resulting from spread of Ca\(^{2+}\) waves across the cell, while retaining spatial frequencies corresponding to the spread of local Ca\(^{2+}\) puffs. Next, for each frame, we calculated the spatial variance among pixels within an imaging field, took the square root to obtain a measure of the spatial SD signal and subtracted the predicted increase in spatial shot noise with increasing fluorescence. Finally, we derived traces showing the average spatial fluctuations across a region encompassing the entire cell within a moving boxcar window of 160 ms (Figure 1—figure supplement 3).

The scripts used to perform temporal and spatial fluctuation analysis are presented as Supplementary file 1.

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Jeffrey T Lock, Data curation, Formal analysis, Writing - original draft, Writing - review and editing; Ian Parker, Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing - original draft, Writing - review and editing

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**Additional files**
**Supplementary files**
- Supplementary file 1. Ca\textsuperscript{2+} Image Processing Routines.
- Transparent reporting form

**Data availability**
Algorithms used to generate SD fluctuation images from Ca2+ image recordings are provided in the Supplementary file 1.

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