SUPPLEMENTAL DATA

for:

Single-molecule analysis of diffusion and trapping of STIM1 and Orai1 at ER-plasma membrane junctions

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Supplemental Figure 1. GFP-STIM1 & Orai1-GFP particles in resting HEK cells have a narrow range of fluorescence intensities and show single-step photobleaching. (A-B) The amplitude distributions of GFP-STIM1 and Orai1-GFP particles detected and tracked by u-track, calculated from Gaussian fits of the normalized fluorescence intensity for each particle (Jaqaman et al., 2008). Histograms are of 1744 STIM1 and 4994 Orai1 particles. (C-D) Fluorescence intensity vs. time graphs for representative GFP-STIM1 (C) and Orai1-GFP (D) particles. The background-subtracted mean intensity over a 6x6 pixel ROI centered on a single GFP-STIM1 or Orai1-GFP particle (tracked manually) was calculated for each frame of a burst movie. Raw movie images were background-subtracted, but not filtered or normalized. The majority of particles showed single-step photobleaching, but in a few cases, particles bleached in more than one step (far right).

The narrow range of intensities and the fact that the majority of tracked particles bleached in a single step indicate that under our experimental conditions Orai1-GFP was expressed at near or below endogenous levels, and most channels were likely made of one to at most a few Orai1-GFPs combining with endogenous Orai1 subunits. We conclude that these particles represent Orai1 channels in the plasma membrane rather than intracellular compartments, based on observations that new fluorescent Orai1-GFPs entered from the edges of the cell and that treatment with an antibody to the extracellular domain of Orai1 (to cluster channels) significantly slowed Orai1 diffusion (data not shown). Similarly for GFP-STIM1, the narrow distribution of measured amplitudes and photobleach steps indicate GFP-STIM1 was expressed at levels similar to endogenous STIM1 in HEK cells, and that tracked particles contained 1-2 GFP-STIM1 molecules.
Supplemental Figure 2. Experimentally determined trajectory lengths contribute to uncertainty in estimating diffusion coefficients for single STIM1 and Orai1 particles. Because of the stochastic nature of diffusion, finite track lengths introduce variation in the estimation of the diffusion coefficient of individual particles (Qian et al., 1991; Saxton, 1997). In our SPT experiments, GFP bleaching limited our trajectory lengths (mean length of 2.7 s for Orai1-GFP; 1.8 s for GFP-STIM1). To estimate the spread in apparent diffusion coefficients due to finite track length, we simulated free diffusion of 1744 STIM1 and 4994 Orai1 particles (corresponding to our experimental data sample sizes). Each particle was assigned an experimentally measured track length and a fixed diffusion coefficient corresponding to the most frequently measured D value for STIM1 or Orai1 (0.081 or 0.072 µm²/s, respectively). D values for each simulated particle were then calculated from the MSD vs. Δt curve of each simulated trajectory. (A) Freely diffusing particles assigned a fixed D of 0.081 µm²/s display a range of D values estimated from their short trajectories (blue), but this range is significantly smaller than that of our experimentally-measured STIM1 D values (black, reproduced from Figure 1C). Thus, the heterogeneity in D values we measured for STIM1 in resting cells is not merely due to statistical variation. (B) Histogram of Orai1 D values calculated from simulated trajectories of free diffusion (blue) superimposed on the distribution of Orai1 D values from experimental data (red, from Figure 1C). As for STIM1, the spread in measured D values for Orai1 in resting cells was wider than predicted from statistical variation alone.
Supplemental Figure 3. Defining immobility based on experimental estimation of diffusion coefficients of fixed Orai1-GFP. The distribution of D values for immobile particles was estimated by tracking Orai1-GFP particles in HEK cells after 20 min fixation with 4% paraformaldehyde (n=5 cells, 165 tracks). (A) The histogram of measured D values was best fit by a Gaussian function with a standard deviation of 0.0035 μm²/s. We set the threshold D for “immobile” particles to be 2 x s.d. of the Gaussian fit. Particles with D < 0.007 μm²/s were considered immobile. (B) Comparison of the D values for Orai1 particles in live cells in 2 Ca Ringer’s (gray) vs. fixed cells (black). Based on the D < 0.007 μm²/s threshold, the fraction of immobile Orai1 particles in resting cells was ~4%.
Supplemental Figure 4. Using Monte Carlo diffusion simulations to test whether movement of STIM1 and Orai1 particles is Brownian or subdiffusive. Freely diffusing particles should produce linear MSD vs. Δt plots, but Brownian diffusion can appear sublinear if the diffusion coefficient is correlated with track length. To test whether STIM1 and Orai1 MSD plots are consistent with pure Brownian diffusion, we simulated Brownian diffusion of particles that were assigned the same set of diffusion coefficients and track lengths we measured experimentally for STIM1 and Orai1 in resting cells. (A) Comparison of MSD vs. Δt derived from experimental STIM1 data (black, n=1744 tracks, from Figure 1E) with MSD curves calculated from 10 simulations of free diffusion for the same 1744 particles (gray). The green curve shows the average of the 10 simulations. For STIM1, the experimental MSD vs. Δt plot falls within the range of variation predicted from simulations, indicating that STIM1 diffusion is probably Brownian in resting cells. (B) Comparison of MSD vs. Δt from experimental Orai1 data (red, n=4994 tracks, from Figure 1E) with MSD curves from 10 simulations of free diffusion for the same 4994 particles (gray, average in green). For Orai1, the experimental MSD vs. Δt plot falls outside the range expected from
freely diffusing particles, suggesting that Orai1 diffusion is slightly restricted due to environmental factors. (C) MSD vs. Δt from experimental STIM1-ΔK SPT data from store-depleted cells (blue, n=3305 tracks, from Figure 4C) and MSD curves from 10 simulations of free diffusion for the same 3305 particles (gray, average in green). The sublinearity of the MSD vs. Δt curve for STIM1-ΔK in depleted cells exceeded the variation predicted for free diffusion, suggesting that STIM1-ΔK subdiffusion in depleted cells is real. Therefore, although STIM1-ΔK does not slow down significantly after store depletion (Figure 4A-B), its movement does become slightly restricted.

The apparent sublinearity of our STIM1 SPT data likely results from an experimental bias in which slower particles tended to yield longer tracks, while faster moving particles had shorter tracks. In resting cells, STIM1 particles with short trajectories (1-2 s) had a mean D = 0.120 μm²/s (n=1309 particles), while particles with long tracks (>5 s) moved >2 times slower, with mean D=0.051 μm²/s (n=28). For Orai1, particles with short and long trajectories moved with similar mobilities: for short tracks (1-2s), mean D = 0.091 μm²/s (n=2657); for tracks >5 s, mean D = 0.086 μm²/s (n=549). We hypothesize that faster moving STIM1 particles had shorter tracks because they were more likely to move out of the evanescent field, given the 3-dimensional architecture of the ER.

MSD vs. Δt curves are expressed as weighted means of all the square displacements, weighted by particle track length. Error bars for experimentally measured MSD vs. Δt curves are weighted standard deviation (weighted by particle track length). Error bars for average simulation curves show the 99% confidence interval about the mean.
Supplemental Figure 5. MSD vs. Δt for the fastest STIM1 particles does not indicate directed or active transport. We analyzed only the fastest STIM1 particles to check whether a subpopulation undergoing directed movement was masked by the ensemble MSD. Particles moving directionally produce supralinear MSD vs. Δt curves, but the MSD vs. Δt curve for STIM1 particles with D ≥ 0.2 μm²/s (green, n=258) was sublinear, showing no indication that STIM1 moves by directed or active transport. The linear least squares fit to MSD points 2-5 (Δt = 50-200 ms) is shown by the green dashed line.
Supplemental Figure 6. Orai1-GFP and mCherry-STIM1 are concentrated ~10-fold inside puncta. HEK cells coexpressing moderate to high levels of Orai1-GFP and mCh-STIM1 were store depleted with 1 μM TG in 0 Ca Ringer’s for >5 min and imaged in TIRF mode. The degree of accumulation of Orai1-GFP and mCh-STIM1 in puncta was measured by analyzing fluorescence intensity line scans (after background subtraction) through puncta. TIRF images of Orai1-GFP (A) and mCh-STIM1 (B) in store-depleted HEK cells. Colored lines show the line scans measured for these cells. (C) Fluorescence intensity profile for the green-colored line scan (shown in A) through an Orai1-GFP punctum. Arrows indicate an 8.8-fold accumulation of Orai1-GFP in this punctum. (D) Intensity profile for the red-colored line scan (shown in B) through a mCh-STIM1 punctum. Arrows indicate a 10.7-fold accumulation of mCh-STIM1 in this punctum. (E) Average accumulation of mCh-STIM1 and Orai1-GFP calculated from 21 and 17 puncta, respectively; bars = standard deviation.
**Supplemental Figure 7.** STIM1-ΔK oligomerizes after store depletion as measured by FRET. FRET efficiency (FRET-E) measured in HEK cells expressing either CFP-STIM1 and YFP-STIM1 (black) or CFP-STIM1-ΔK and YFP-STIM1-ΔK (red) before and after store depletion with 1 μM ionomycin in 0 Ca Ringer’s. Both dimers showed similar FRET increases after store depletion, indicating that STIM1-ΔK undergoes a similar store-dependent oligomerization as STIM1. Mean FRET-E ± sem shown for STIM1-ΔK (10 cells) and STIM1 (11 cells).
Supplemental Figure 8. The probability of escape of STIM1 or Orai1 from puncta in SPT experiments is less than 1%. (A) Average MSD vs. Δt of STIM1 particles starting in puncta (black, from Figure 2E; n=1225 STIM1 particles) compared to the average MSD vs. Δt from simulated Brownian diffusion with a 1% escape probability (blue). The blue curve shows the average (and 99% confidence interval) of 10 simulations; in each simulation, 1225 particles were assigned D values and track lengths corresponding to the experimentally-measured STIM1 particles in depleted cells and were placed randomly within a circular corral of diameter 0.76 μm. Upon leaving the corral, particles were assigned a D of 0.116 μm²/s (mean D measured for STIM1 in resting cells). Unlike the experimental MSD plot, the MSD vs. Δt curve for the simulations does not plateau, indicating that under our SPT conditions, the STIM1 escape probability is <1%. (B) Average MSD vs. Δt of Orai1 particles starting in puncta (red, from Figure 2E; n=439 Orai1 particles) compared to the average MSD vs. Δt from simulated Brownian diffusion with a 1% escape probability (blue). The blue curve shows the average (and 99% confidence interval) of 10 simulations; in each simulation, 439 particles with D values and track lengths corresponding to the experimentally-measured Orai1 particles in depleted cells were placed randomly within a circular corral of diameter 0.68 μm. Upon leaving the corral, particles were assigned a D of 0.090 μm²/s (mean D measured for Orai1 in resting cells). Unlike the experimental MSD plot, the MSD vs. Δt curve for the simulations does not plateau, indicating that under our SPT conditions, the Orai1 escape probability is <1%.
Supplemental Figure 9. The effect of junctional corral size on estimates of diffusion coefficients. We simulated diffusion of 1744 particles placed randomly within a 0.76 µm-diameter corral with impenetrable barrier (0% escape probability). Each particle was assigned an experimentally-measured D (from STIM1 in resting cells) and a track length of 5 s (to ensure that particles would encounter the corral boundary). D values were calculated from the MSD vs. Δt curves of each simulated trajectory. (A) Histograms of D values from the simulated particles trapped in a corral (blue) and D values from experimental STIM1 data from resting and depleted cells (gray & black, from Figure 2C). The mean D of simulated particles trapped in a corral was 0.059 ± 0.001 µm²/s (mean ± sem, n=1744, blue); mean D from experimental STIM1 SPT data from resting cells = 0.116 ± 0.002 µm²/s (n=1744, gray), and depleted cells, 0.031 ± 0.001 µm²/s (n=1288; black). Limiting mobility to within a corral reduces the apparent D values, but not to the extent measured in depleted cells; in particular it does not account for the increase in immobile particles observed after store depletion. Therefore, collisions of particles with the junctional boundary cannot account for the shift in D values we measured after store depletion. (B-C) Trajectories of single Orai1 particles (yellow) overlaid on the corresponding Ch-STIM1 TIRF image (red) from store-depleted cells. Some particles diffuse throughout the punctum (B, reproduced from Figure 3B), while diffusion of other particles is tightly restricted (C).
**Supplemental Figure 10.** Identifying ER-PM junctions by the Bernsen local thresholding method. All cells were store-depleted with 1 μM TG in 0 Ca$^{2+}$ Ringer’s. (A) Summed image of the mCherry emission channel of a 1000-frame burst movie (20 Hz, in TIRF mode) of a HEK cell expressing mCh-STIM1 and Orai1-GFP. The summed image shows the location of ER-PM junctions. (B) The image in A after application of a Bernsen local threshold using Fiji (Image -> Adjust -> Auto Local Threshold -> Bernsen method, radius 5). (C) Merged image of A and B, with summed mCh-STIM1 image in green and the Bernsen-thresholded image in red. (D) MSD vs. Δt for complete STIM1 tracks starting in puncta (black, from Figure 2E) compared with the MSD calculated from the junctional sojourns only (blue) (for definitions, see Methods). (E) MSD vs. Δt for complete Orai1 tracks starting in puncta (red, from Figure 2E) compared with the MSD from the junctional sojourns only (blue). When calculated from junctional sojourns, the MSD vs. Δt curves are artificially limited by the dimensions of the Bernsen-thresholded puncta. In contrast, analysis of the complete tracks shows the true spatial extent of the corral. For both STIM1 and Orai1, the MSD calculated from junctional sojourns plateaus at a slightly lower value than the MSD from complete tracks, suggesting that the thresholded image slightly underestimates the true extent of the junctions.
SUPPLEMENTAL VIDEO LEGENDS

Supplemental Video 1: Single GFP-STIM1 particles diffusing in the ER of a resting HEK cell.
A 50 s movie (acquired and displayed at 20 frames per s) of a resting HEK cell (in 2 mM Ca Ringer’s) expressing low levels of GFP-STIM1, imaged with 488-nm laser illumination in TIRF mode. This movie was acquired from the cell shown in Figure 1A. Bar, 5 µm.

Supplemental Video 2: Single Orai1-GFP particles diffusing in the PM of a resting HEK cell.
A 50 s movie (acquired and displayed at 20 frames per s) of a resting HEK cell (in 2 mM Ca Ringer’s) expressing low levels of Orai1-GFP, imaged with 488-nm laser illumination in TIRF mode. This movie was acquired from the cell shown in Figure 1B. Bar, 5 µm.

Supplemental Video 3: Diffusion and trapping of Orai1-GFP in a store-depleted HEK cell.
A 50 s movie (acquired and displayed at 20 frames per s) of a HEK cell expressing low levels of Orai1-GFP and moderate/high levels of mCherry-STIM1 imaged with 488-nm laser illumination in TIRF mode (488-nm strongly excites GFP and weakly excites mCherry). The cell was treated with 1 µM TG in 0 Ca Ringer’s for 6 min before imaging to deplete Ca^{2+} stores. The white box highlights a freely diffusing Orai1-GFP particle becoming trapped by a mCh-STIM1 punctum and then escaping (the same particle is shown in Figure 3A and Supp. Video 4). Bar, 5 µm.

Supplemental Video 4: Real-time trajectory of a tracked Orai1-GFP particle in a store-depleted HEK cell.
The 23 s trajectory of the same Orai1-GFP particle highlighted in Supp. Video 3 is depicted as a yellow dot moving in real time (20 frames per s). The trajectory is overlaid on the mCh-STIM1 image (summed from the mCherry channel from the same 1000 frame TIRF movie). Particle detection and tracking was done using u-track software (see Methods). The entire particle trajectory is shown in Figure 3A. Bar, 1 µm.

Supplemental Video 5: Real-time trajectory of a tracked Orai1-GFP particle in a store-depleted HEK cell.
A 16 s trajectory of an Orai1-GFP particle depicted as a yellow dot moving in real time (20 frames per s). The trajectory is overlaid on the corresponding summed mCh-STIM1 TIRF image (summed from the mCherry channel from the same 1000 frame movie). This HEK cell was expressing low levels of Orai1-GFP and high/moderate levels of mCh-STIM1 and treated with 1 µM TG in 0 Ca Ringer's for 5 min before imaging. Particle detection and tracking was done using u-track software (see Methods). The entire particle trajectory is shown in Figure 3B. Bar, 1 µm.

Supplemental Video 6: Real-time trajectory of a tracked GFP-STIM1 particle in a store-depleted HEK cell.
A 14 s trajectory of a GFP-STIM1 particle depicted as a yellow dot moving in real time (20
frames per s). The trajectory is overlaid on the corresponding summed mCh-myc-Orai1 TIRF image (summed from the mCherry channel from the same 1000 frame movie). This HEK cell was expressing low levels of GFP-STIM1 and moderate levels of mCh-myc-Orai1 and treated with 1 µM TG in 0 Ca Ringer’s for 3 min before imaging. Particle detection and tracking was done using u-track software (see Methods). The entire particle trajectory is shown in Figure 3C. Bar, 1 µm.

**Supplemental Video 7: Real-time trajectory of a tracked Orai1-L273D-GFP particle in a store-depleted HEK cell.**
A 12 s trajectory of an Orai1-L273D-GFP particle depicted as a yellow dot moving in real time (20 frames per s). The trajectory is overlaid on the corresponding summed mCh-STIM1 TIRF image (summed from the mCherry channel from the same 1000 frame movie). This HEK cell was expressing low levels of Orai1-L273D-GFP and high/moderate levels of mCh-STIM1 and treated with 1 µM TG in 0 Ca Ringer’s for 5 min before imaging. Particle detection and tracking was done using u-track software (see Methods). The entire particle trajectory is shown in Figure 3D. Bar, 1 µm.
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