Supplemental Figure Legends

S1: Specificity of antisera. (A) E6.1 Jurkat cells were plated on anti-CD3 coverslips and immunostained with anti-STIM1 preimmune (left panel), antiserum (middle) or affinity purified antibodies (right) at 1:600 dilutions. Side views of 3D projections are shown to display caps. (B) Embryonic fibroblasts from either wild type (top panels) or STIM1 knockout mice were immunostained with anti-STIM1 antiserum (left panels) or affinity purified antibodies (right) (C) E6.1 Jurkat cells were plated on anti-CD3 coverslips and immunostained with non-immune serum (left panel), anti-Orai1 antiserum (middle) or affinity purified antibodies (right) at 1:200 dilutions. Side views of 3D projections are shown to show caps. (D) Immunohistochemistry of skin from either wild type (left panel) or Orai1 knockout mice (right). DAB anti-Orai1 reaction product appears brown and the sections are counterstained with hematoxylin. (E) Jurkat cells were transfected with GFP and Orai1 and then immunostained with affinity purified anti-Orai1 (left panel) or affinity purified anti-Orai1 pre-incubated with excess peptide. Anti-Orai1 staining is shown in red and marks both endogenous and transfected Orai1, while transfected cells also display GFP fluorescence (green). No immunostaining is seen after peptide blocking even in cells overexpressing Orai1 (green cells in right panel).

S2: In contrast to Orai1-YFP, YFP-Orai1 inhibits spreading of Jurkat T cells. All tagged constructs restore Ca^{+2} influx. (A) E6.1 Jurkat T cells expressing YFP-Orai1 were plated on anti-CD3 coated stimulatory coverslips and imaged with a spinning disk confocal microscope. Bar= 10μm (B and C) Ca^{+2} influx was analyzed in response to perfusion with 20 mM extracellular Ca^{+2} following store-depletion with 1μM thapsigargin. (B) Ca^{+2}

responses from Ca^{+2} influx-deficient fibroblasts from SCID patients stably transduced with vector alone, Orai1, N-terminally or C-terminally YFP-tagged Orai1. (C) Ca^{+2} responses from STIM1 knock-out MEFS stably transduced with vector alone, STIM1 or C-terminally CFP-tagged STIM1.

S3: STIM1-CFP puncta are induced by TCR activation in Jurkat T cells. E6.1 Jurkat T cells expressing STIM1-CFP were plated on anti-CD3-coated stimulatory coverslips and imaged with a laser scanning confocal microscope. Bar= 10μm, confocal section ~0.8 μm from coverslip.

S4: An intact cytoskeleton is needed for normal cap formation. (A) Cells treated with 100 μM colchicine to disrupt microtubules. Left panel: Live Jurkat T cells expressing STIM1-CFP (red) and Orai1-YFP (green) were plated onto stimulatory coverslips and imaged with a spinning disk confocal system. Z stacks were collected and 1 timepoint is displayed as a 3-D section view. Bar= 10μm. Right panel: Jurkat T cells were plated onto stimulatory coverslips, fixed after 15 min and stained for endogenous STIM1. (B) Cells treated with 300 nM latrunculin to disrupt actin filaments Left panel: Live Jurkat T cells expressing STIM1-CFP (red) and Orai1-YFP (green) were plated onto stimulatory coverslips and imaged with a spinning disk confocal system. Z stacks were collected and 1 timepoint is displayed as a 3-D section view. Bar= 10μm. Right panel: Jurkat T cells were plated onto stimulatory coverslips, fixed after 15 min and stained for endogenous STIM1. (C) Cells treated with 50 μM Blebbistatin to inhibit myosin II Left panel: Live Jurkat T cells expressing STIM1-CFP (red) and Orai1-YFP (green) were plated onto

stimulatory coverslips and imaged with a spinning disk confocal system. Z stacks were collected and 1 timepoint is displayed as a 3-D section view. Bar= 10μm. Right panel: Jurkat T cells were plated onto stimulatory coverslips, fixed after 15 min and stained for endogenous STIM1. (D) Time courses of changes in cytosolic Ca^{+2} levels in Jurkat T cells shown as the ratio of 405/510nm Indo-1 fluorescence emission vs. time for cells treated with colchicine, latrunculin and Blebbistatin.

S4: Inhibitor controls. (A) E6.1 Jurkat cells were transiently transfected with STIM1-CFP and Orai1-YFP, plated on anti-CD45 coverslips and treated with 1 μM thapsigargin. Puncta containing both STIM1-CFP and Orai1-YFP form on the surface in contact with the coverslip, but no cap forms. (B) Time courses of changes in cytosolic Ca^{+2} levels in Jurkat T cells shown as the ratio of 405/510nm Indo-1 fluorescence emission vs. time. Left panel: TCR vs non-TCR stimulation, anti-CD3 (OKT3), anti-CD45, thapsigargin or thapsigargin+ionomycin, Middle panel: Perturbations of Ca^{+2} flux, control, CCCP, EGTA, 2-APB or BAPTA+EGTA in OKT3 stimulated cells, Right panel: Perturbations of tyrosine phosphorylation, control, JCAM1-6, JCAM1-6 reconstituted with WT Lck, control cells treated with PP2, all stimulated with OKT3.

S5: No cap structures form in mouse CD4+T cells in contact with B cells without peptide. Unpulsed CH12 B cells were plated on polylysine coated coverslips, then $CD4^{+}$ AND TCR cells were dropped onto the B cells and allowed to activate for 30 min at 37ºC, followed by fixation and immunostaing with anti-STIM1 (green) and anti-phosphotyrosine (red) antibodies. Bar= 4μm.

S6: Localization of distal pole and polarity markers. (A) 3-D section view of PBLs plated on stimulatory coverslips, fixed after incubation at 37°C for 30 min, and immunostained with anti-STIM1 (green) and anti-ezrin antibodies (red) Bar=4 μm. (B) 3-D section view of PBLs plated on stimulatory coverslips, fixed after incubation at 37°C for 30 min, and immunostained with anti-STIM1 (green) and anti-numb antibodies (red) Bar= 5 μm. (C) 3-D section view of PBLs plated on stimulatory coverslips, fixed after incubation at 37ºC for 30 min, and immunostained with anti-STIM1 (green) and anti-PKCζ antibodies (red) Bar=5μm. (D) 3-D section view of PBLs plated on stimulatory coverslips, fixed after incubation at 37ºC for 30 min, and immunostained with anti-STIM1 (green) and anti-scribble (red) antibodies Bar= 5μm.

S7: STIM1-CFP and Orai1-YFP are confined to the contact surface in some Jurkat T cells-B cells conjugates, but there is no redistribution in contacts with unpulsed B cells. (A) Jurkat T cell with STIM1-CFP and Orai1-YFP confined to the contact site between the two cells. The B cell (blue) has been visualized with CellTracker FarRed. Bar= 10μm. (B) Unpulsed Raji B cells were allowed to attach to polylysine coated coverslips. E6.1 Jurkat T cells expressing STIM1-CFP (red) and Orai1-YFP (green) were added to the chamber and imaged with a spinning disk confocal system. The 0 timepoint corresponds to the first image in the series.

Movie Legends

Movie 1 (Fig. 1A) Orai1-YFP localization in E6.1 Jurkat T cells. Transfected cells were plated on anti-CD3-coated coverslips and observed dynamically using a spinning disk confocal system. Z-stacks were collected, converted to maximum intensity projections and exported as an avi movie. Playback rates are 17.5x faster than real time.

Movie 2 (Fig. 2A) STIM1-CFP cap formation in E6.1 Jurkat T cells. Transfected cells were plated on anti-CD3-coated coverslips and observed dynamically using a spinning disk confocal system. Z-stacks were collected, converted to maximum intensity projections and exported as an avi movie. Playback rates are 29x faster than real time.

Movie 3 (Fig. 2B) Cap formation in E6.1 Jurkat T cells expressing both STIM1-CFP and Orai1-YFP. Transfected cells were plated on anti-CD3-coated coverslips and observed dynamically using a spinning disk confocal system. Z-stacks were collected, converted to maximum intensity projections and exported as an avi movie. Playback rates are 99x faster than real time.

Movie 4 (Fig. 2C) No cap forms in E6.1 Jurkat T cells expressing both STIM1-CFP and Orai1-YFP plated onto non-stimulatory coverslips. Transfected cells were plated on anti-CD45-coated coverslips and observed dynamically using a spinning disk confocal system.

Z-stacks were collected, displayed as section views and exported as an avi movie. Playback rates are 75x faster than real time.

Movie 5 (Fig. 2D) Rotation of 3-D projection of FRET efficiency between Orai1-YFP (green) and STIM1-CFP (red) in cells fixed 15min after plating on a stimulatory coverslip. Projection and rotation was performed on pseudo-color images using Imaris 5.0.

Movie 6 (Fig. 4A) Cap formation has little effect on an ER marker in E6.1 Jurkat T cells expressing both STIM1-CFP (red) and M1-YFP (green). Transfected cells were plated on anti-CD3-coated coverslips and observed dynamically using a spinning disk confocal system. Z-stacks were collected, displayed as a mosaic of maximum intensity projections images and exported as a quicktime movie. STIM1-CFP forms a cap (red in merged panel), while the distribution of MI-YFP shows little change (green in merged panel). Playback rates are 62.5x faster than real time.

Movie 7 (Fig. 7A) Cap in a Jurkat T cell activated by contact with superantigen pulsed Raji B cell. E6.1 Jurkat T cells expressing both STIM1-CFP (red) and Orai1-YFP (green) were plated in chambers containing superantigen pulsed B cells and observed dynamically using a spinning disk confocal system. B cells (blue) were stained with CellTrace Far Red. Z-stacks were collected, displayed as maximum intensity projections and exported as an avi movie. STIM1-CFP (red) and Orai1-YFP (green) formed a cap distal to the T cell-B cell contact surface. Playback rates are 105x faster than real time.

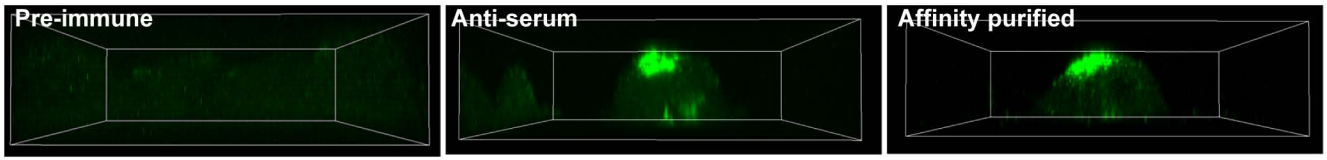
Movie 8 (Fig. 7C) STIM1-CFP and Orai1-YFP move from cap to contact surface in a Jurkat T cell activated by contact with superantigen pulsed Raji B cell. E6.1 Jurkat T cells expressing both STIM1-CFP (red) and Orai1-YFP (green) were plated in chambers containing superantigen pulsed B cells and observed dynamically using a spinning disk confocal system. Z-stacks were collected, displayed as maximum intensity projections and exported as an avi movie. STIM1-CFP (red) and Orai1-YFP (green) moved from a cap to the T cell-B cell contact surface. Playback rates are 65x faster than real time.

Movie 9 (Fig. 7D) STIM1-CFP and Orai1-YFP form a single cap in a Jurkat T cell activated by contact with 2 superantigen pulsed Raji B cells. E6.1 Jurkat T cells expressing both STIM1-CFP (red) and Orai1-YFP (green) were plated in chambers containing superantigen pulsed B cells and observed dynamically using a spinning disk confocal system. B cells (blue) were stained with CellTrace Far Red. Z-stacks were collected, displayed as maximum intensity projections and exported as an avi movie. STIM1-CFP (red) and Orai1-YFP (green) moved from side to side eventually forming a cap distal to the second B cell. Playback rates are 150x faster than real time.

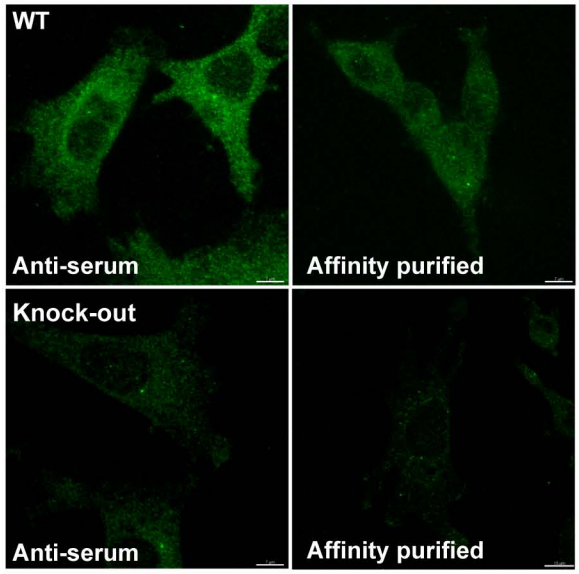
Movie 10 (Fig. 7F) STIM1-CFP and Orai1-YFP move from a cap to a new contact surface in a Jurkat T cell activated by contact with 2 superantigen pulsed Raji B cells. E6.1 Jurkat T cells expressing both STIM1-CFP (red) and Orai1-YFP (green) were plated in chambers containing superantigen pulsed B cells and observed dynamically using a spinning disk confocal system. B cells (blue) were stained with CellTrace Far Red. Z-

stacks were collected, displayed as maximum intensity projections and exported as an avi movie. STIM1-CFP (red) and Orai1-YFP (green) moved from a cap distal to the first B cell to the contact surface with the second B cell. Playback rates are 295x faster than real time.

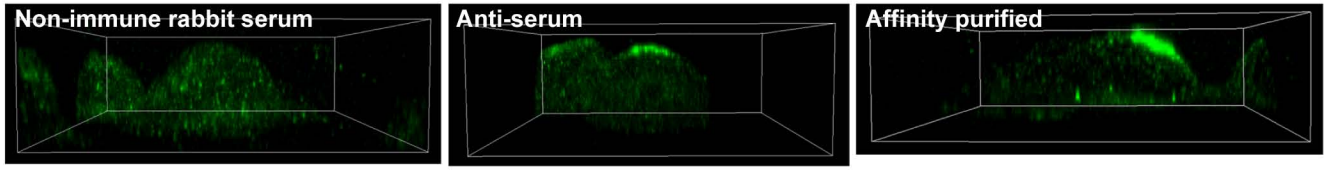
A Anti-STIM1 sera on activated Jurkat T cells



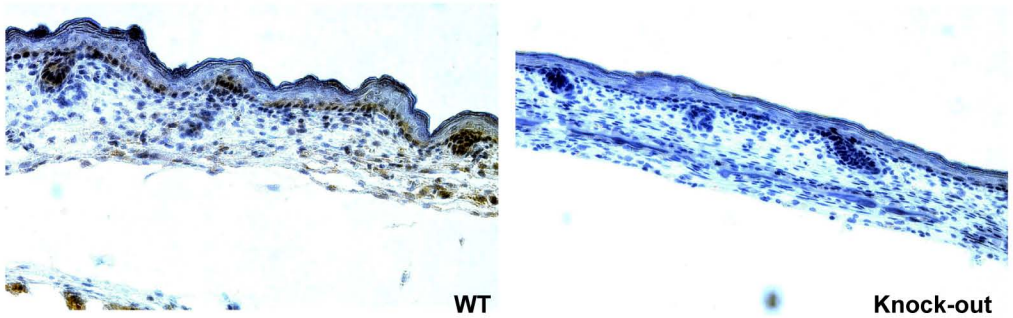
B Anti-STIM1 sera on WT and STIM KO mouse embryonic fibroblasts



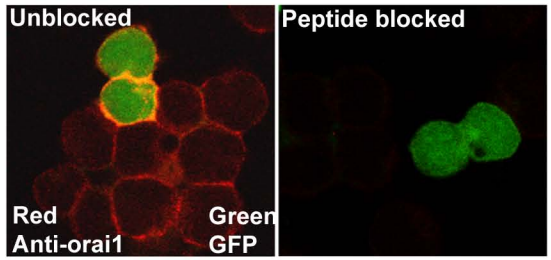
C Anti-Orai1 sera on activated Jurkat T cells



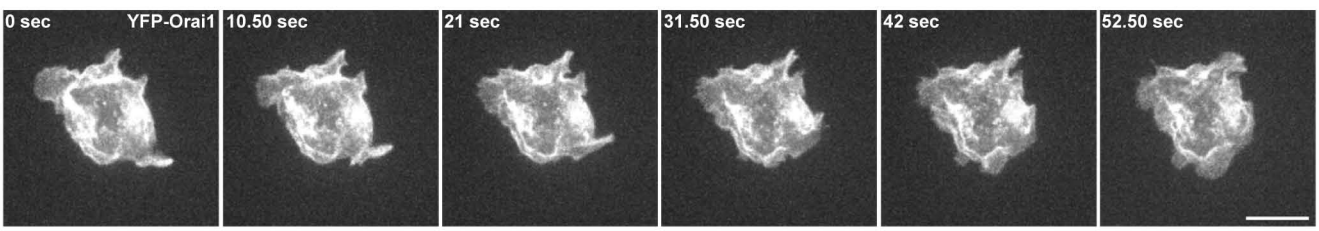
D Affinity purified anti-Orai1 serum on WT and Orai KO mouse skin



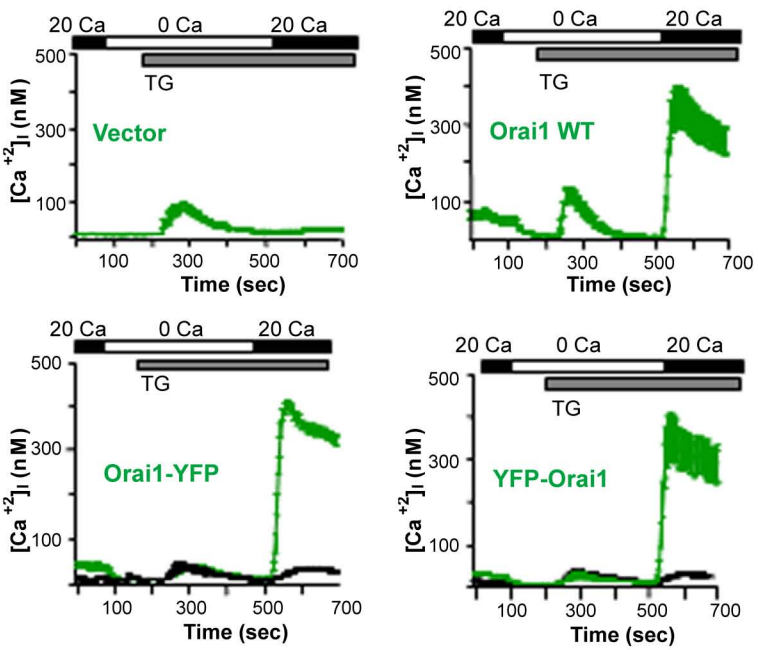
E Pretreatment with peptide blocks staining with affinity purified anti-Orai1 serum



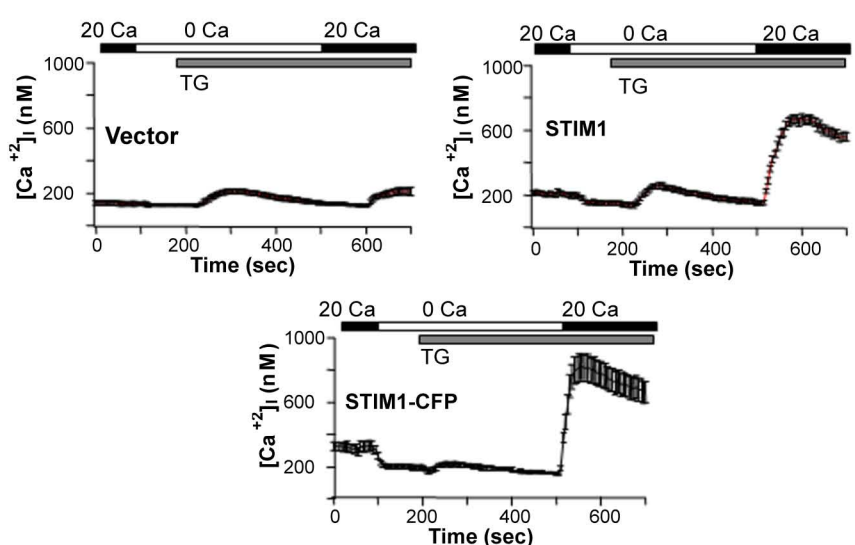
A Plated on CD3

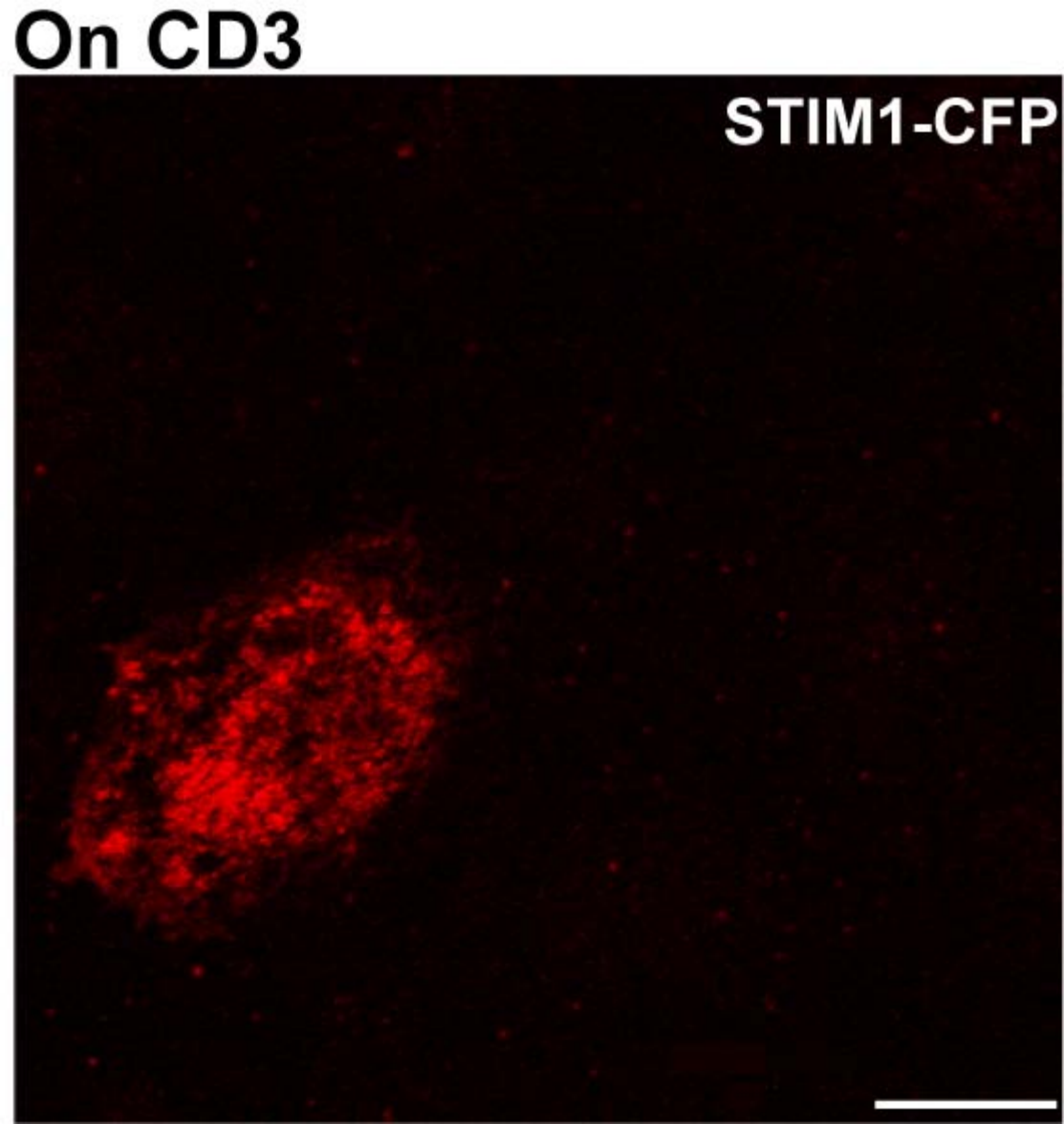


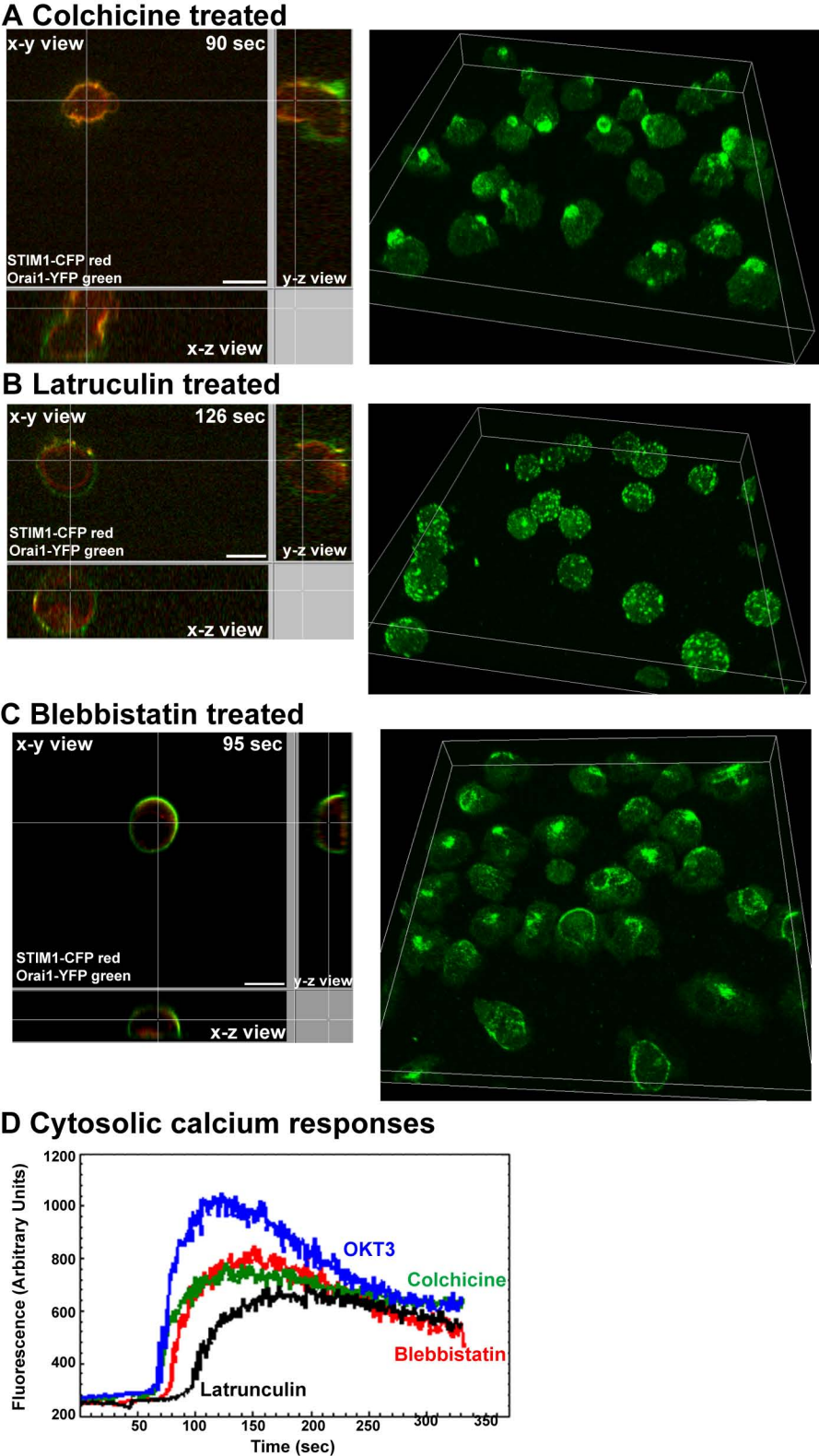
B Complementation of SCID fibroblasts with YFP tagged Orai 1

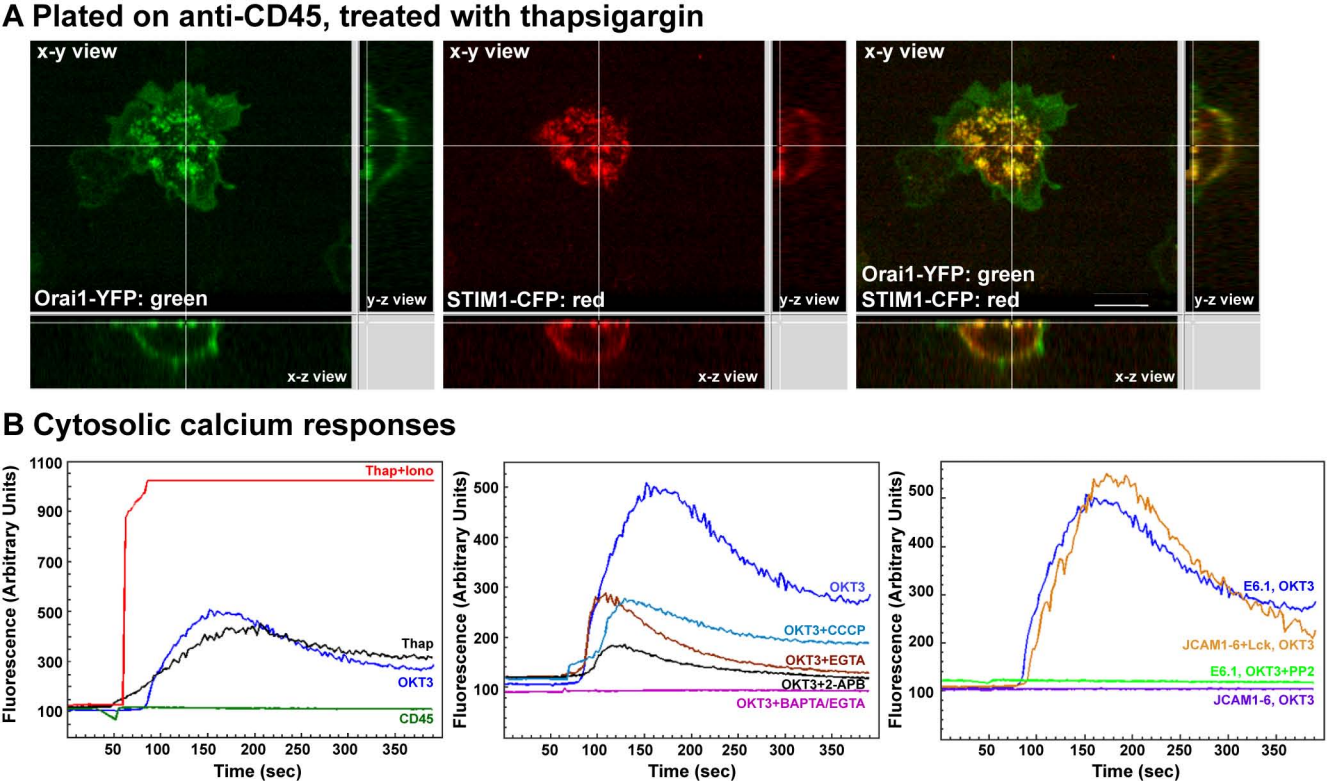


C Complementation of STIM1 knock-out MEFS with STIM1-CFP

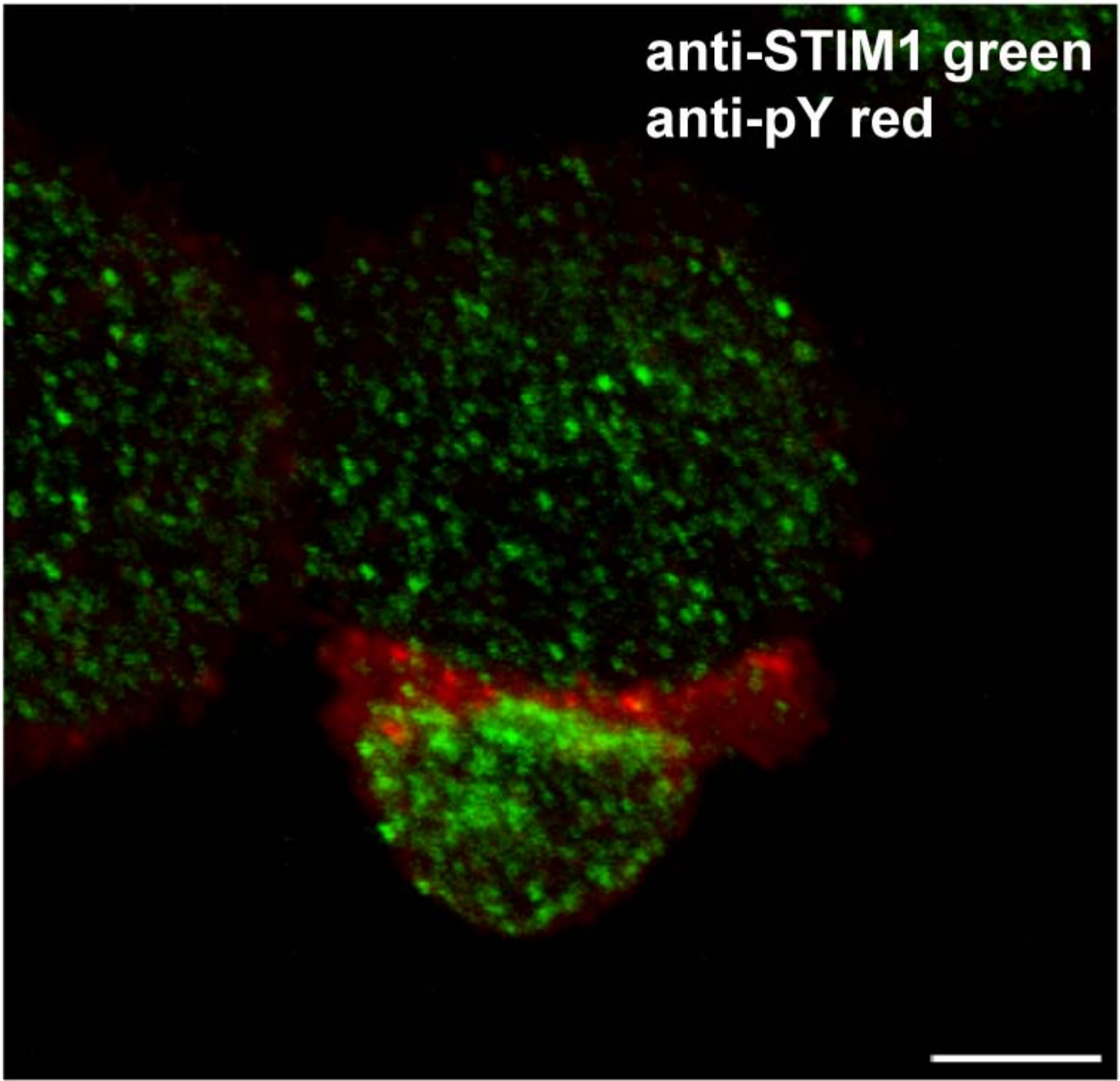


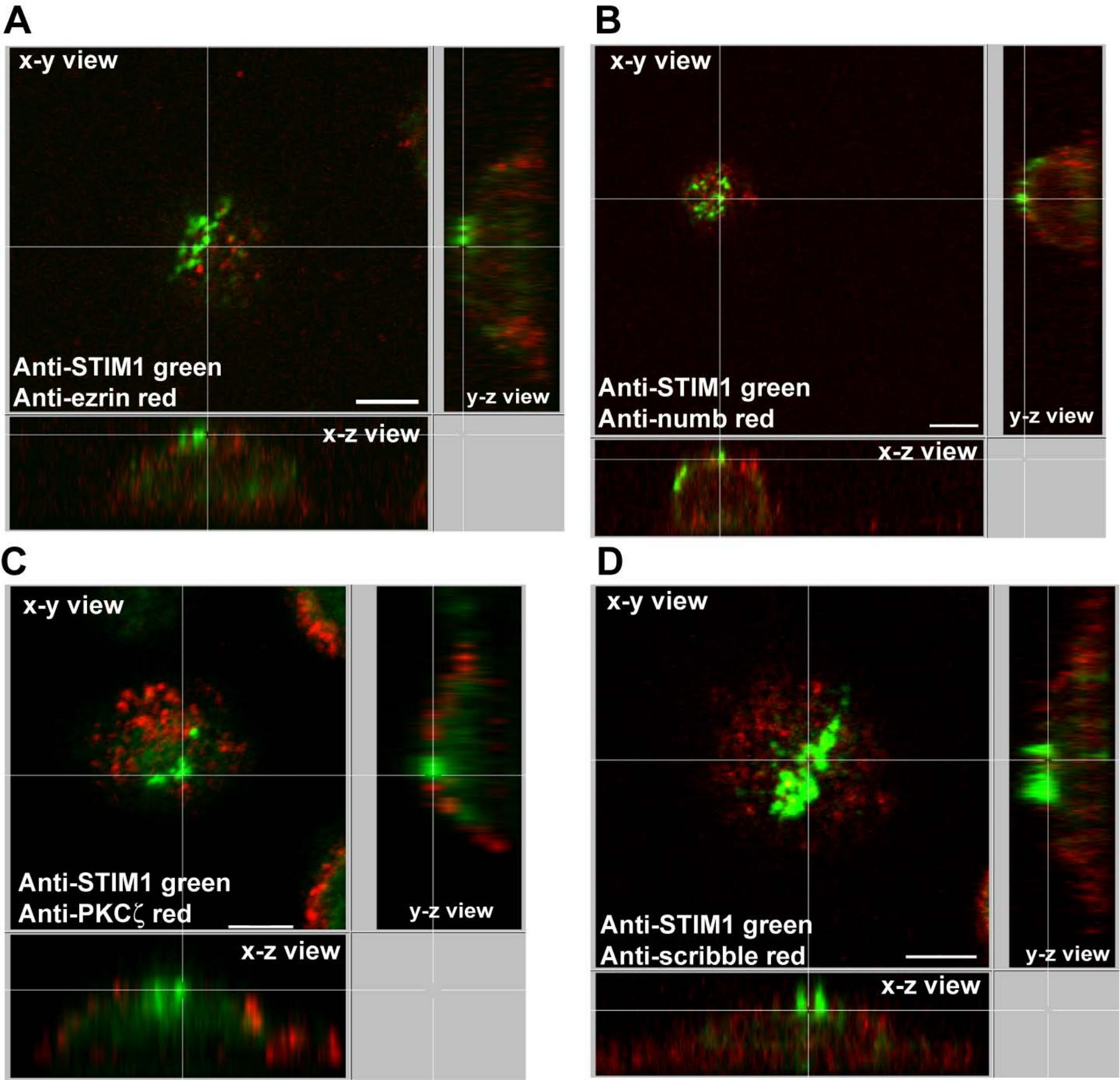

On CD3
STIM1-CFP


A Colchicine treated
x-y view
90 sec
STIM1-CFP red
Orai1-YFP green
y-z view
x-z view
B Latruculin treated
x-y view
126 sec
STIM1-CFP red
Orai1-YFP green
y-z view
x-z view
C Blebbistatin treated
x-y view
95 sec
STIM1-CFP red
Orai1-YFP green
y-z view
x-z view
D Cytosolic calcium responses
Fluorescence (Arbitrary Units)
Time (sec)
OKT3
Colchicine
Blebbistatin
Latrunculin
1200
1000
800
600
400
200
50
100
150
200
250
300
350

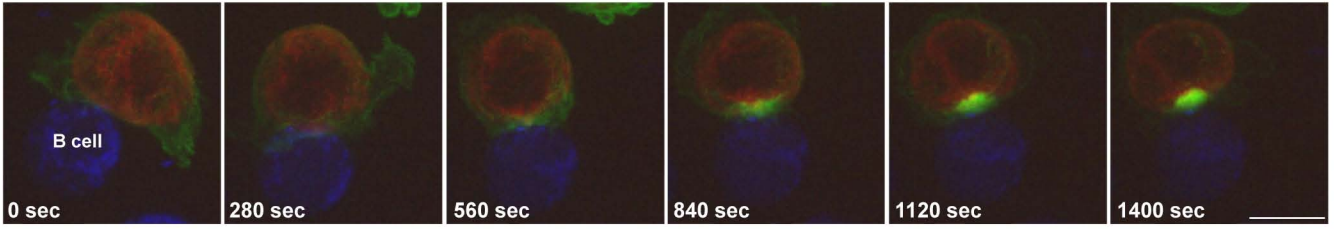

A Plated on anti-CD45, treated with thapsigargin
x-y view
Orai1-YFP: green
y-z view
x-z view
x-y view
STIM1-CFP: red
y-z view
x-z view
x-y view
Orai1-YFP: green
STIM1-CFP: red
y-z view
x-z view
B Cytosolic calcium responses
Fluorescence (Arbitrary Units)
Time (sec)
Thap+Iono
Thap
OKT3
CD45
OKT3
OKT3+CCCP
OKT3+EGTA
OKT3+2-APB
OKT3+BAPTA/EGTA
E6.1, OKT3
JCAM1-6+Lck, OKT3
E6.1, OKT3+PP2
JCAM1-6, OKT3

T cell from AND TCR mouse, no peptide




A
x-y view
Anti-STIM1 green
Anti-ezrin red
y-z view
x-z view
B
x-y view
Anti-STIM1 green
Anti-numb red
y-z view
x-z view
C
x-y view
Anti-STIM1 green
Anti-PKCζ red
y-z view
x-z view
D
x-y view
Anti-STIM1 green
Anti-scribble red
y-z view
x-z view

A STIM1 and Orai at the IS after contact with superantigen pulsed Raji B cell



B No redistribution of STIM1 or Orai1 after contact with Raji B cell without superantigen