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Calcium Imaging in T Lymphocytes: a Protocol for Use with Genetically Encoded or Chemical Ca^{2+} Indicators

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[Abstract] Elevations in cytosolic calcium (Ca^{2+}) drive a wide array of immune cell functions, including cytokine production, gene expression, and cell motility. Live-cell imaging of cells loaded with ratiometric chemical Ca^{2+} indicators remains the gold standard for visualization and quantification of intracellular Ca^{2+} signals; ratiometric imaging can be accomplished with dyes such as Fura-2, the combination of Fluo-4 and Fura-Red, or, alternatively, by expressing genetically-encoded Ca^{2+} indicators (GECI) such as GCaMPs. Here, we describe a detailed protocol for Ca^{2+} imaging of T cells *in vitro* using genetically encoded or chemical indicators that can also be applied to a wide variety of cell types. The protocol addresses the challenge of facilitating T cell attachment on various substrates prepared on glass-bottom dishes to enable T cell imaging on an inverted microscope. The protocol also emphasizes cell preparation steps that ensure optimal cell viability – an essential requirement for recording dynamic changes in cytosolic Ca^{2+} levels – and that ensure reproducibility between multiple samples. Finally, we describe a simple algorithm to analyze single-cell Ca^{2+} signals over time using Fiji (ImageJ) software.

Keywords: Calcium signaling, Single-cell imaging, T lymphocyte, T Cell Receptor (TCR) activation, Store-operated Ca^{2+} entry

[Background] Calcium indicators have revealed second-messenger cytosolic Ca^{2+} signals leading to cellular activation sequelae for nearly 40 years, since the pioneering work of Roger Tsien. Tsien and colleagues developed dyes based on the Ca^{2+} chelator EGTA, including quin-2 (Rink *et al*., 1982), and improved chemical indicators such as the dual-excitation ratiometric indicator fura-2 and the dual-emission ratiometric indicator indo-1 (Grynkiewicz *et al*., 1985); several other chemical indicators have been developed, including combinations of fura-red and fluo-4, which can be implemented for ratiometric imaging (McQuade *et al*., 2020). Chemical Ca^{2+} indicators are typically loaded into cells using a permeable ester-linked parent compound that is cleaved by intracellular esterases (Paredes *et al*., 2008). To overcome issues of dye loading and leakage, a variety of approaches have been taken to develop GECI based on mutated GFP-like structures, including FRET probes such as the Cameleons (Miyawaki *et al*., 1997), the GCaMP series (Tian *et al*., 2009; Akerboom *et al*., 2012; Chen *et al*., 2013), and tandem probes such as Salsa6f (Dong *et al*., 2017b). Salsa6f is a fusion protein comprising a GCaMP6f and tdTomato moiety linked by a V5 epitope tag. Being able to simultaneously monitor the GCaMP6f and tdTomato signal at every pixel permits the determination of a Green/Red or G/R Ratio, which is pseudo-ratiometric, independent of the GCaMP6f concentration at a given pixel, and a better reflection of

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cytosolic Ca^{2+}. Furthermore, the tdTomato moiety also allows for tracking of motile cells *in vitro* and *in vivo*, and a more accurate determination of Ca^{2+} in motile cells and in thin cell processes, which would not be possible through use of GCaMP6f alone (Dong *et al.*, 2017a and 2017b). Other advantages include excellent dynamic range with Ca^{2+} sensitivity in cytosol from 100-1,000 nM, stable expression restricted to cytosol in unperturbed mouse T cells, femtosecond excitation for two-photon microscopy, no effect on T cell development and activation, and ability to calibrate (Dong *et al.*, 2017a). Implemented in a transgenic mouse line for Cre-dependent expression, Salsa6f enables Ca^{2+} signaling to be monitored in a wide variety of cell types. Our group has used Salsa6f to examine Ca^{2+} signaling by confocal microscopy – in T cells from Cd4-Salsa6f mice (Dong *et al.*, 2017b; Jairaman *et al.*, 2021), macrophages from LSL-Salsa6f-Vav1$^{Cre/+}$ mice (Atcha *et al.*, 2021), and astrocytes from GFAP-Cre Salsa6f mice (Wakida *et al*., 2020) – and by two-photon imaging – in T cells in lymph nodes (Dong *et al*., 2017a) and pathogenic Th17 cells in the spinal cord (Othy *et al*., 2020).

This set of protocols describes the preparation of T lymphocytes for *in vitro* calcium imaging experiments. It is applicable to lymphocytes and to developing thymocytes that are resident in the thymus. Mature T and B lymphocytes migrate in the circulation throughout the body and home into lymphoid organs where they search for antigen. Lymphocytes can be easily isolated and purified from blood or lymphoid tissue and are maintained in suspension culture. The methods described below using either genetically encoded or chemical Ca^{2+} indicators can also be readily adapted to various suspension and adherent cell types.

Materials and Reagents

1. 6-well plates (Corning, catalog number: 3516)
2. 35 mm dish, with No. 1.5 Coverslip and 14 mm glass diameter (MatTek, catalog number: P35G-1.5-14-C)
3. Incubation Perfusion Lid for 35 mm dishes (Tokai Hit, catalog number: LV200-D35FME)
4. Anti-Mouse CD3e (Clone 145-2C11) (BioLegend, catalog number: 100359)
5. Anti-Mouse CD28 (Clone 37.51) (BioLegend, catalog number: 102121)
6. EasySep Mouse $CD4^+$ T Cell Isolation Kit StemCell (Technologies, catalog number: 19852)
7. RPMI 1640 Medium (Thermo Fisher Scientific, catalog number: 22400-089)
8. 0.5 M EDTA, pH 8.0 (Thermo Fisher Scientific, catalog number: 15575020)
9. Fetal calf serum (FCS) (Omega, catalog number: FB-12)
10. Bovine serum albumin (BSA) (ProSpec Bio, catalog number: PRO-422)
11. DMSO (Thermo Fisher Scientific, catalog number: D12345)
12. Penicillin-Streptomycin-Amphotericin (Sigma-Aldrich, catalog number: A5955)
13. Phosphate-Buffered Saline (PBS) (Corning, catalog number: 21-040-CV)
14. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
15. MEM Non-essential amino acids (Thermo Fisher Scientific, catalog number: 11140050)
16. Sodium pyruvate (Thermo Fisher Scientific, catalog number: 11360070)

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17. β-mercaptoethanol (Thermo Fisher Scientific, catalog number: 21985023)
18. Poly-L-Lysine Hydrobromide (Sigma-Aldrich, catalog number: 1399)
19. Pluronic acid F-127 (Thermo Fisher Scientific, catalog number: P3000MP)
20. Fluo-4 AM (Thermo Fisher Scientific, catalog number: F14201)
21. Fura-red AM (Thermo Fisher Scientific, catalog number: F3021)
22. EGTA (Sigma-Aldrich, catalog number: E8145)
23. HEPES (EMD Millipore, catalog number: 391338)
24. Recombinant Mouse IL-2 (Tonbo Biosciences, catalog number: 21-8021)
25. 1 mM Calcium Ringer's solution (see Recipes)
26. 0 mM or Calcium-free Ringer's solution (see Recipes)
27. Mouse T cell media (see Recipes)
28. RPMI complete (see Recipes)

Equipment

1. Confocal Laser Scanning Microscope (LSM) equipped with high-speed Resonant Scanner, IX3-ZDC2 Z-drift compensator, and 40× Silicone Oil Objective (Olympus FV3000)
2. Stage Top Incubation System (Tokai Hit, STXG)

Software

1. Fiji: ImageJ (https://imagej.net/Fiji)
2. GraphPad Prism (https://www.graphpad.com/scientific-software/prism/)
3. Olympus Viewer Plugin for ImageJ (https://imagej.net/OlympusImageJPlugin)

Procedure

A. The Day before T cell Isolation

1. Prepare antibody-coated 6-well plates

   Coat 6-well plate with antibodies against mouse CD3 (anti-CD3) and CD28 (anti-CD28). Dissolve the antibodies in PBS at final concentration of 2.5 µg/ml in a 15 ml Falcon tube. Use 2 ml of PBS/well (12 ml for all 6 wells). Mix the antibody solution by inverting the tube several times and let stand for a few minutes to ensure homogenous distribution before plating 2 ml/well. Seal the 6-well plate using parafilm to prevent evaporation of the antibody solution and incubate the 6-well plate at 4°C overnight. This substrate will be used to activate $CD4^+$ T cells isolated from mouse lymph nodes (LN) and spleen.

   Note: As a general rule, plating antibodies at 4°C overnight leads to better coating. If imaging chambers need to be prepared in a hurry on the same day as imaging, 2-3 h plating at 37°C can also lead to acceptable antibody coating.

2. Prepare antibody-coated imaging chambers

   If planning to image TCR-activated Ca^{2+} signals in freshly isolated T cells on plate-bound anti-CD3/anti-CD28 the next day, prepare imaging chambers by coating 35 mm MatTek glass-bottom dishes with anti-CD3/anti-CD28. Use 2.5 µg/ml of each antibody in PBS and add 250 µl to the center of the 35 mm dish. Incubate at 4°C overnight. Seal the chamber with parafilm to prevent evaporation of liquid. Confirm that the chamber is on a flat surface to ensure uniform concentration of the coated antibody throughout.

   Note: The concentration of coating antibodies can be varied depending on the research question being asked. Lower concentrations of antibodies may be used to study Ca^{2+} signals in response to a smaller TCR activation stimulus. Cross-linking of CD3 is the primary stimulus for TCR induced Ca^{2+} signaling. Cross-linking of CD28 provides a co-stimulatory signal, which may not be critical for short-term Ca^{2+} signaling but helps the T cells to attach to the substrate better and may be important to sustain Ca^{2+} signaling after several hours of plating.

3. Prepare Poly-L-Lysine (PLL) coated chambers

   Add 250 µl of 100% PLL (1 mg/ml in molecular grade water) to the center of 35 mm dishes at room temperature (RT). Let it sit for 1 hour. Wash off the PLL 2-3 times with PBS and let the chambers dry. They are now ready to be used anytime within 2-3 weeks. Store at room temperature (RT) away from light.

   Note: PLL-coated chambers provide positively charged but non-activating substrates to attach T cells and are useful to study changes in cytosolic Ca^{2+} in response to receptor activators and inhibitors, Ca^{2+} channel agonists and antagonists, etc. These are usually added by perfusion/solution exchange to the 35 mm dishes containing the cells.

4. Prepare mouse T cell media containing RPMI with 10% FCS, 2 mM L-glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate, 55 µM β-mercaptoethanol, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of Amphotericin B.

B. On the Day of T cell Isolation

1. Dissect LN and spleen from mouse. Place in 3 ml of RPMI complete or T cell media. Use the bottom of a 3 ml syringe to mash and pass cells through a 70 µm filter into a 50 ml Falcon tube. Use the EasySep™ mouse $CD4^+$ T cell isolation kit to isolate $CD4^+$ T cells following kit instructions.
2. After isolation is complete, wash cells with excess media to remove all traces of EDTA. Resuspend cells in 4-5 ml of fresh warmed T cell media and count the cells. Each mouse yields approximately 10 million $CD4^+$ T cells. Meanwhile, bring the 6-well plate to RT in the tissue culture (TC) hood and wash the antibody-coated wells 2-3 times with PBS to remove the uncoated antibody. Add approximately 2 million cells into each 6 well to activate T cells. Cells should be suspended in T cell media + 30 U/ml of recombinant mouse IL-2. Add 4 ml of media per well.

Note: Cells activated for 2 days are ideal for imaging, although cells can be used freshly after isolation (resting T cells) or after 1, 2, or 3 days of activation. Note that some cells may start losing their viability after 48 h due to excessive activation. Spinning the cells at a low speed may help to get rid of some dead cells, but not all.

C. Prepare Cells for Imaging

Note: The plating protocol under this section applies to T cells that express genetically encoded Ca^{2+} probes and that do not require loading with chemical Ca^{2+} dyes. When using dyes, additional loading steps need to be followed (see Section D on Dye loading).

1. Re-suspend cells in media at an optimal concentration just before they are plated on the imaging dishes. When working with freshly isolated T cells (which are small), re-suspend at 4-5 million/ml. This gives a 60-70% confluency when plated on anti-CD3/anti-CD28 coated or PLL coated 35 mm dishes. When working with 2-day activated T cells, which are larger in size, re-suspend at 1 million/ml. The cell suspension should be kept at 37°C (either in 1.5 ml Eppendorf tube or 15 ml Falcon tube, depending on the volume, with the caps loose to ensure CO_2 exchange) in the TC incubator on the day of imaging. For imaging, plate approximately 50-100 µl of cell suspension into the center of the 35 mm dish and place the dish (and remaining cells) back in the incubator. Incubation time for T cells plated on imaging dish: 15 min for PLL and approximately 1 h for anti-CD3/anti-CD28.

   Notes:

   a. *Ensure sufficient CO_2 exchange by keeping the cap loose or open in the incubator. Gently mix cells in the Falcon or Eppendorf tubes every 30 min to prevent formation of cell pellets. Cells will be fine at RT for short periods of time, but it is important to have them at 37°C for imaging, especially with anti-CD3/anti-CD28 coated dishes. It is highly recommended to plate and incubate cells for imaging in a TC Hood close to the microscope. Move cells very gently to the microscope. Moving imaging chambers that contain T cells plated on anti-CD3/anti-CD28 over longer distances can easily cause detachment of the cells. Cells plated on PLL tend to attach better.*

   b. *In our experience, having a low volume of cell suspension (50-100 µl) in the imaging dish for up to an hour does not adversely affect cell viability. Volume can be increased up to 250 µl if cell viability is a concern. Additional media (2 ml) should be gently added to the sides of the chamber if cells are to be kept in the incubator for a longer time.*

   c. *Plating times for cells on PLL: Do not exceed 15 min. Cell viability will be adversely affected after 30 min on PLL. This gives ~15-20 min of imaging time. Less than 10 min of plating may not lead to good cell attachment.*

   d. *Plating times for cells on anti-CD3/anti-CD28: While 1 h plating is more than sufficient to ensure proper cell attachment and TCR signaling activation, shorter and longer plating times are also possible. Cells can potentially attach within 5-10 min, which might be useful to study the early Ca^{2+} events of TCR signaling. One may also consider adding the cells to the*

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imaging chamber when on the microscope to get the very early events of T cell signaling. For this, cells would need to be added close to the imaging field right where the objective is, at a very high density and low volume (say 500,000 cells in 10 µl or so). Cells can be imaged up to 24-48 h after plating if the cells are healthy and bathed in sufficient fresh media. This applies more to freshly isolated cells as 2 day-activated cells are more likely to start dying due to the additional period of TCR stimuli.

2. At the end of the plating time, wash attached cells very gently with 2 ml of the final imaging solution (1 mM Ca^{2+} Ringer's solution) twice to remove unattached cells. Gently add fresh imaging buffer to attached cells and mount on the microscope for imaging.
3. Synchronize the timing for plating and imaging multiple samples: When imaging multiple dishes in a single session, ensure the same plating times between different samples for reproducibility. **Figure 1** shows an example protocol for imaging four imaging dishes (T cells plated on PLL) in a single session. The protocol may be modified depending on the imaging time for each sample.

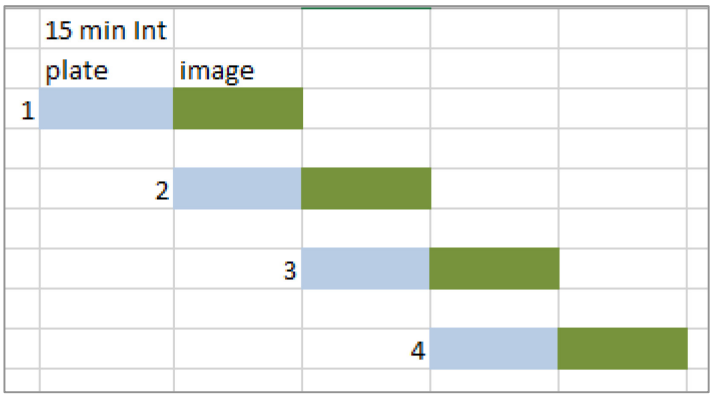

Figure 1. Sequential preparation and imaging of T cell samples expressing Salsa6f GECI. Each box represents a 15 min time interval. Blue box: Plate T cells onto imaging chamber and incubate at 37°C for 15 min. Green box: Wash attached cells, mount on microscope, and image. The numbers on the left refer to sample or chamber. The protocol can be modified to accommodate longer or shorter imaging times.

D. Working with chemical Calcium indicator dyes (Fluo-4 AM and Fura-Red AM, or Fura-2 AM for Ratiometric Imaging)
   1. Prepare stocks of the Ca^{2+} dyes at 2 mM in DMSO. Stocks are stored at -20°C. Bring stock to RT before imaging. All dyes are used at 3 µM final concentration. Dye is mixed with an equal volume of pluronic acid to facilitate loading into cells. Pluronic acid is stored at RT and needs to be warmed up to 40°C for 20-30 min before use (it is a gel at RT, which will make pipetting small volumes impossible).
   2. 'Intermediate working' concentration of dye solution: Prepare dye solution in T cell media that is twice (6 µM) the final concentration (3 µM). Each imaging dish requires around 100 µl of cell suspension (50 µl of dye solution at 6 µM + 50 µl of cell suspension at 2× concentration). In a

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1.5 ml Eppendorf tube, add the required volume of the dye from the 2 mM stock, then add an equal volume of pre-warmed pluronic acid and mix vigorously by vortexing several times. Finally, add the required volume of T cell media and vortex again. This is the 'intermediate' 2× working solution of the dye.

For experiments on PLL: For each imaging chamber, take 50 µl of 2× dye solution and mix with 50 µl of 2× cell suspension in a 1.5 ml tube. Incubate for 15 min at 37°C in the TC incubator, then add to the center of a PLL coated dish. Plate for 15 min at 37°C in the TC incubator. Wash 2-3 times with imaging buffer and image on the scope. **Figure 2** depicts an example protocol for performing multiple experiments in a single imaging session using Ca^{2+} dyes.

Note: Cell suspension also needs to be at 2× concentration (10 million/ml for resting or freshly isolated T cells and 2 million/ml for activated T cells). Mixing with the 2× dye solution at a 1:1 ratio will result in the optimal concentration of dye and cells. The 2× dye solution and 2× cell suspension need to be stored in the TC incubator for further experiments. Ensure sufficient CO_2 exchange.

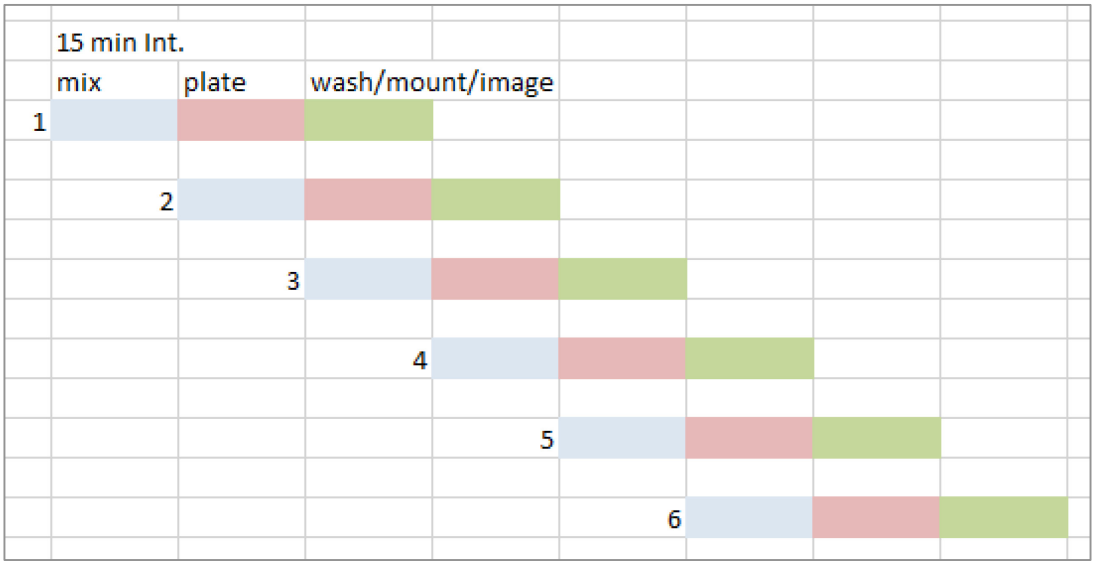

Figure 2. Sequential preparation and imaging of T cells using chemical Ca^{2+} indicators. Each box represents a 15 min time interval. Blue box: Mix 2× dye solution (50 µl) with 2× cell suspension (50 µl) in a 1.5 ml Eppendorf tube and incubate for 15 min in TC Incubator. Red box: Plate 100 µl of dye-loaded T cell suspension onto the imaging chamber and further incubate for 15 min in TC Incubator. Green box: Wash unattached cells, mount imaging chamber on microscope and image. The numbers on the left refer to the samples. The protocol can be modified to accommodate longer or shorter imaging times.

3. For experiments on anti-CD3/anti-CD28: For each imaging chamber, take 50 µl of 2× dye solution and mix with 50 µl of 2× cell suspension in a 1.5 ml tube. Plate immediately to the center of an anti-CD3/28 coated dish. Plate for 30-60 min at 37°C in the TC incubator. Wash 2-3 times with imaging buffer and image on the microscope.

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Notes:

a. *Note that on both PLL and anti-CD3/28, the total dye incubation time with cells is 30 min.*
b. *For anti-CD3/28 coated chamber, longer dye incubation times may be used if cells do not attach to the substrate in 30 min. Consider reducing dye concentration in that case. Ensure the loading conditions are consistent between all samples. This is critical for proper comparison, especially for Fluo-4 and Fura-Red imaging.*
c. *For cells with genetically encoded Calcium indicators from transgenic mice, steps in section D do not apply.*

Data acquisition

Images were acquired at 37°C on the Olympus FV3000 confocal laser scanning microscope equipped with high-speed Resonant Scanner, IX3- ZDC2 Z-drift compensator, 40× Silicone Oil Objective, high sensitivity GaAsP detectors, and Tokai Hit Stage Top Incubation System (**Figure 3**). Exposure time using the resonant scanner is 0.034 msec/ single line scan, which was averaged six times for each frame. Images are acquired on a single plane (no Z stack). For Salsa6f imaging, GCaMP6f (green channel) and tdTomato (red Channel) are excited sequentially using 488 nm (0.3% laser power, 450V PMT) and 561 nm (0.05% laser power, 450V PMT) Diode lasers respectively. The detectors are set at 494-544nm and 580-680nm for the green and red channels, respectively. For ratiometric imaging using Fluo-4 and Fura-Red, both dyes are excited at 488 nm at 0.07% laser power and detection settings are similar to Salsa6f. Time-lapse images are recorded at 2.5 s/ frame on the Olympus fluoview software and saved as .OIR files.

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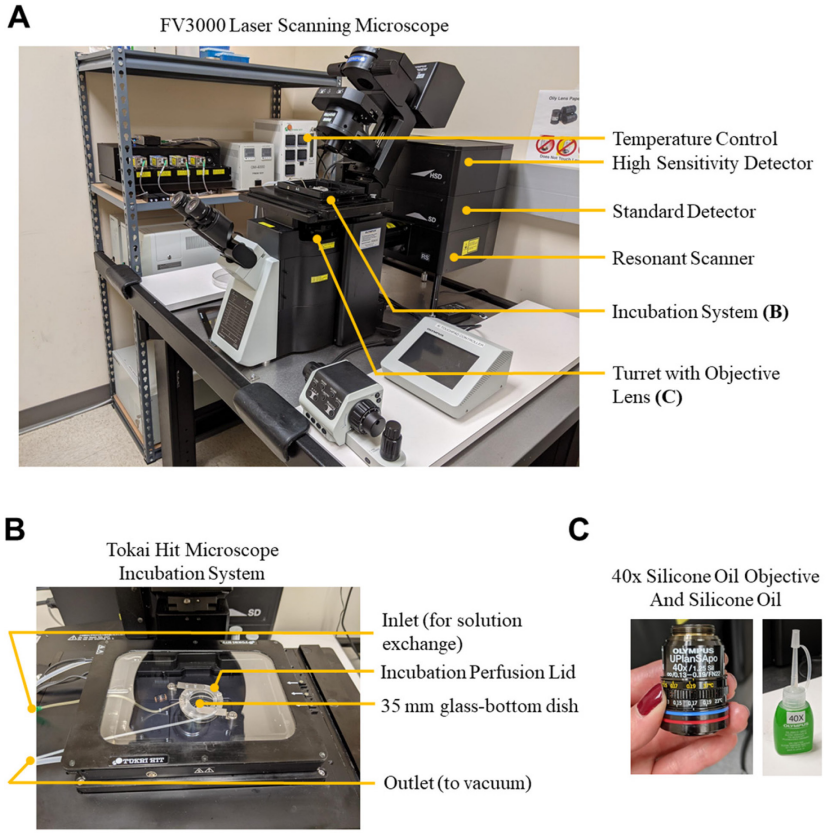

Figure 3. Olympus FV3000 Confocal Imaging System. (**A**) Image of the FV3000 Laser Scanning Microscope at SCRC Core at UCI. The resonant scanner permits image acquisition at faster rates, but a standard Galvanometer scanner may be used for live-cell imaging if faster imaging rates are not critical. (**B**) Tokai hit stage top incubation system for imaging live cells at 37°C. The imaging dish is placed on the stage using a 35mm dish adaptor. Solution exchange is performed using an incubation perfusion lid with an inlet and outlet. (**C**) Image of the 40× Silicone Oil Objective and Silicone Oil.

Data analysis

1. Time-lapse videos acquired on the Olympus FV 3000 LSM are saved as .OIR files. Open Fiji (ImageJ) and Convert .OIR files into .TIF files using the "Olympus Viewer" Plugin (needs to be installed, link included in Software section): *Plugin* → *Olympus Viewer* → *DragDrop*. If a Z-stack time-lapse was acquired, convert to a single-plane time-lapse using Maximum Intensity Projection option, either on the Olympus Fluoview Image Acquisition software or using ImageJ.
2. Save the Green and Red channel .TIF image stack separately after background subtraction (**Figures 4A and 4B**). The stack represents images acquired on a single imaging plane over time. Note that for both Salsa6f and Fluo-4/Fura-Red Ratiometric Imaging, Green and Red channel image stacks will be acquired for each run (in addition to Bright Field Image stack).

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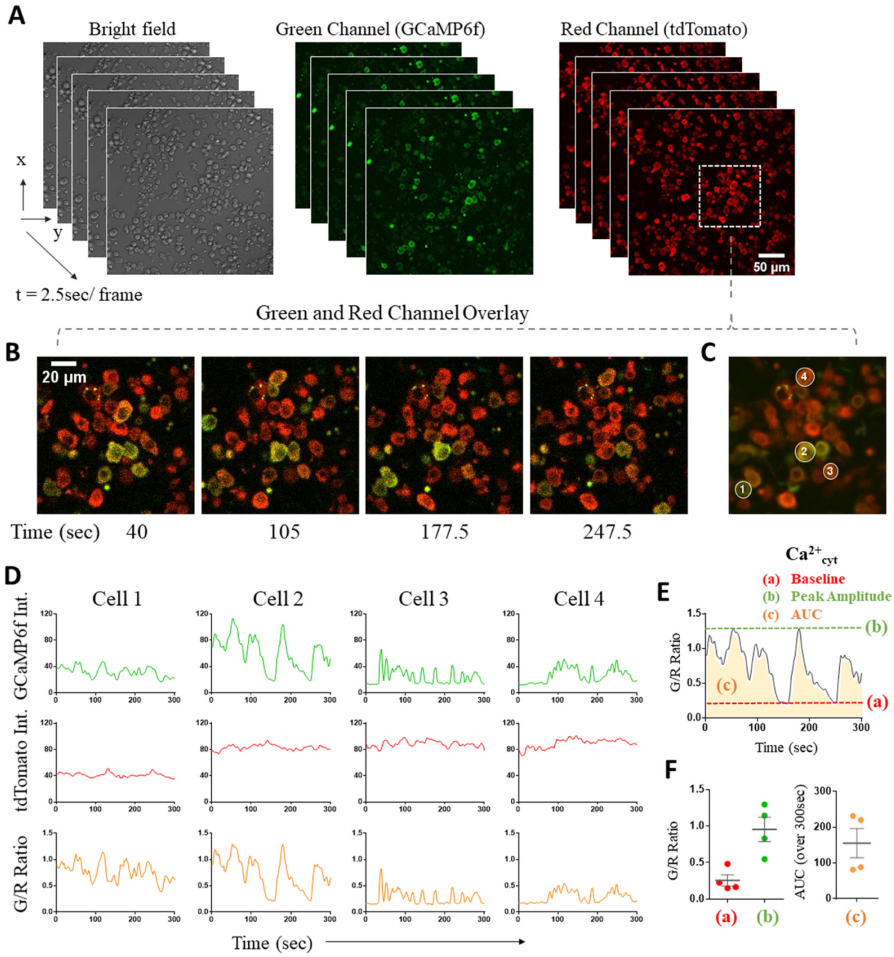

Figure 4. Analysis of Calcium signals in Salsa6f expressing T cells. (**A**) Images from a time-lapse experiment of Salsa6f expressing T cells on plate-bound anti-CD3/anti-CD28 (Channels: Bright Field, left; Green, middle; Red, right). Images (512 × 512 pixels, 320 µm × 320 µm) were acquired at 2.5 s per frame (120 frames total, only 1st five frames are shown) on the FV3000 confocal microscope using a resonant scanner in a single Z-plane. (**B**) Green-Red Channel Overlay images (Zoomed in) depicting four time points from the above time-lapse run. (**C**) Average Intensity Projection Image over time showing ROIs drawn around four T cells that were analyzed. (**D**) Changes in GCaMP6f intensity (top row), tdTomato intensity (middle row), and GCamP6f/tdTomato intensity ratio (G/R Ratio) of the four T cells analyzed. G/R Ratio reflects changes in cytosolic Ca^{2+} over time. (**E**) Quantification of various parameters that capture the dynamics of Ca^{2+} changes at the single-cell level. Single-cell G/R Ratio traces were used as the basis to calculate (a) Baseline Ca^{2+} (average of lowest 10 ratio values over time), (b) Peak Ca^{2+} Amplitude (highest Ratio value over time), and (c) Area under the Curve (AUC) using the Area under the curve function in GraphPad prism. All parameters were calculated over the 5 min time period of the traces. (**F**) Summary of baseline, peak Ca^{2+} amplitude, and AUC in the four T cells analyzed.

3. Use the freehand or Oval tool to draw ROIs around single cells (**Figure 4C**). Save each ROI in

the ROI Manager (Analyze → Tools → ROI Manager). Ensure that the ROI closely envelops the cell through all the frames of the time-lapse run before saving. Pressing the key 't' after each drawn ROI saves it temporarily in the ROI manager. To save all ROIs permanently for a given experiment as a .zip file, select all ROIs in the manager, then hit *More* → *Save*).

4. Create a separate image window from either the Green or Red channel image stack using *Image* → *Stack* → *Plot Z axis profile* → *Average.* Note that this image is a single frame and not a Z stack.
5. Overlay all the saved ROIs on this new image window using the ROI Manager. Hit *More* → *Draw.*
6. Now select the green channel image stack, overlay all saved ROIs on this stack by clicking the *Show All* option on the bottom right of the ROI Manager, then hit *More* → *Multi Measure* option in the ROI manager. Copy the Green channel intensities over time and paste them on Excel.
7. Now select the Red Channel Image stack and repeat the procedure as outlined in #6 for the Green Channel. The Ratio reflecting changes in Ca^{2+} over time can be calculated on the Excel sheet by dividing the Green and Red channel intensities at each time point. Look at each trace carefully to ensure that the changes in Ca^{2+} are expected and valid. Outliers may be eliminated at this stage (for instance, cells with elevated unchanging baseline could be non-viable cells, cells that moved out of the ROI leading to spurious changes in ratio, *etc*.).
8. Other parameters like average baseline, peak amplitude, and cumulative change in Ca^{2+} over time can all be calculated from the single cell ratio changes over time (**Figure 4E**).
9. Plot the final data using GraphPad Prism (**Figures 4D and 4F**). The average baseline, average peak amplitude can be plotted as scatter bar graph. If comparing 2 groups, use Mann-Whitney U Test to determine statistical significance. Use One-Way ANOVA with Multiple Comparison if there are more than 2 test groups to compare.

Recipes

1. 1 mM Calcium Ringer's solution
   155 mM NaCl
   4.5 mM KCl
   1 mM $CaCl_2$
   0.5 mM $MgCl_2$
   10 mM glucose
   10 mM HEPES (pH adjusted to 7.4 with NaOH)
2. 0 mM or Calcium-free Ringer's solution
   155 mM NaCl
   4.5 mM KCl
   1.5 mM $MgCl_2$
   10 mM glucose
   10 mM HEPES

1 mM EGTA (pH adjusted to 7.4 with NaOH)

3. Mouse T cell media
   RPMI 1640 with 10% FCS (fetal calf serum)
   2 mM L-glutamine
   1× non-essential amino acids
   1 mM sodium pyruvate
   55 µM β-mercaptoethanol
   100 units/ml of penicillin
   100 µg/ml of streptomycin
   0.25 µg/ml of Amphotericin B
4. RPMI complete
   RPMI 1640 with 10% FCS (fetal calf serum) and antibiotics as above

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Competing interests

There are no conflicts of interest or competing interests.

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