Assay of Intracellular Free Calcium in RAW 264.7 Cells Loaded with Fluo-3 (with FLEXstation)

AfCS Procedure Protocol PP00000210
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This protocol describes a method to assess concentrations of free cytoplasmic calcium, $[Ca^{2+}]_i$, in cultured adherent RAW 264.7 cells, using a 96-well plate format. This objective is accomplished by using the Ca^{2+}-sensitive fluorescent dye, fluo-3, which permeates cell membranes as an ester and is hydrolyzed in the cell to its Ca^{2+}-sensitive acidic form. Fluorescence for the adherent cells is measured over time by using a bottom read of a 96-well plate, with cells that have been washed free of extracellular dye. Free $[Ca^{2+}]_i$ is estimated as described below (see *Calculations*). A FLEXstation benchtop scanning fluorometer and integrated fluid transfer workstation, which works with 96-well assay plates, are used to measure fluorescence. Sets of eight wells are read in a single column simultaneously. (The wells in each column are read sequentially, but with only a fraction of a second between wells, and with multiple scans of the column.) The machine monitors all the wells in a column over a time course, during which up to three compounds can be added robotically. Multiple columns are read sequentially for additional run times. Data are transferred from the FLEXstation software (Softmax Pro) for calculation of free $[Ca^{2+}]_i$, as described below.

Loading Cells with Intracellular Free Calcium Indicator Fluo-3

1. Suspend RAW 264.7 cells at 3 x 10^5 cells/ml in RAW 264.7 growth medium 1 (RAWGM1), and distribute 200 μl of the cell suspension in each well of a 96-well clear bottom black-walled plate, avoiding the outside wells (rows A and H and columns 1 and 12), due to potential variability in water loss with overnight culture. Plate up to 6 columns of 6 wells each per plate. (It may be possible to use all 10 columns available, but we have only tested the stability of the cells for 6 columns.) Fill empty wells with sterile, distilled/deionized water.
2. Incubate plated cells at 37 °C in a mixture of air and 5% CO_2 for 18 to 24 hr.
3. Mix equal volumes (20 μl) of 1 mM fluo-3 acetoxymethyl (AM) and 20% pluronic F-127.
4. To make the fluo-3 loading solution, add the entire fluo-3 mixture (from step 3) to 5 ml of Hanks' balanced salt solution-bovine serum albumin (pH 7.2) containing 2.5 mM probenecid, pH 7.45 (HBSS-BSA-probenecid, pH 7.45). The final concentration of fluo-3 is 4 μM.
5. Remove the assay plate of cells from the incubator. Using a multichannel pipette, remove 180 μl of the growth medium from the wells, leaving approximately 20 μl. Add 180 μl of fluo-3 loading solution to the 36 wells (6 x 6 format) of the black-walled plate containing cells.
6. Incubate the assay plate in the dark at room temperature for 30 min.
7. Using a multichannel pipette, remove 180 μl of the dye loading solution, taking care not to contact the bottom of the well with any tips used (leaves

~20 μl in the wells). Wash the wells twice (by adding, then removing, 180 μl HBSS-BSA-probenecid, twice), using prewarmed HBSS-BSA-probenecid buffer. Finally, pipette off 180 μl of the last wash (leaves ~20 μl in the wells), and add 55 μl prewarmed HBSS-BSA-probenecid to give 75 μl final volume of per well.

8. Immediately place the assay plate into a 37 °C incubator (no CO_2 required, because the HBSS-BSA-probenecid is not bicarbonate buffered), and incubate for 30 min in the dark.
9. While the cells are incubating, turn on the FLEXstation, and set the machine to warm up to 37 °C. Check the FLEXstation setup, and program (or recall on the instrument) the correct settings for the assay.
   a) Set up the assay plate template.
   b) Set the excitation/emission wavelengths (using fluo-3, set fluorometer optics for excitation at 485 nm and emission at 525 nm) reading sensitivity level at 8 (normal/high), with the PMT at high.
   c) Set the first robotic addition time T1 at 20 sec (allows a 20-sec baseline to be taken prior to the addition). Monitor emissions for 170 sec after ligand addition at T1.
   d) Program the robotic addition times for two subsequent additions during the same run: T2 = 190 sec for addition of the Fmin solution and T3 = 320 sec for addition of the Fmax solution.
   e) Set the total run time (used for every column, for each set of 6 wells, which run together) at 320 sec.
   f) The FLEXstation uses tips set up in a tip rack. Specify the robot's use of tips from the tip rack for the transfer of compounds from the compound plate to the assay plate (i.e., which column of tips for each column in the compound plate).

Preparation of the Compound Plate

10. Prepare a 96-well U-bottomed compound plate with the correct layout, concentrations, and volumes of solutions to be added during the assay run at the set time points (T1, T2, and T3) to the assay plate (see below). Compounds can be prepared in advance (up to 30 min, subject to stability) and kept on ice. Ten min prior to beginning the assay, warm compounds to 37 °C in a water bath for 5 min, and then pipette into a prewarmed compound plate (set in a plate warmer at 37 °C).
11. Make ligands and negative and positive control compounds (up to 6 different ligands or compounds), to be added at T1 during the assay runs, at 4X the final desired concentration. Dilute ligands in HBSS-BSA-probenecid. Make solutions for addition of 25 μl of ligands (4X) to wells in the assay plate (final volume/well is 100 μl). Add a specific compound to be used for a row of replicate wells in an assay plate to a single well in the compound plate. Line up the ligands (for the specific row replicates) in the first column of the compound plate. Allow 25 μl per well, plus 25 μl excess volume (175 μl for 6 replicate ligand wells).

12. Add 5X Fmin solution (25 μl added at T2 during the assay) to the next column of the compound plate; use 175 μl for additions to 6 wells in each row.
13. Add 6X Fmax solution (25 μl added at T3 during the assay) to the next column of the compound plate; use 175 μl for additions to 6 wells in each row.
14. Transfer the loaded compound plate from the plate warmer into the 37 °C FLEXstation compound plate chamber.

Intracellular Calcium Flux Assay

15. Remove the assay plate from the incubator, and place it in the warmed FLEXstation. Allow approximately 2 min for temperature to settle.
16. Start the assay using the FLEXstation software.

Data Collection and Processing

17. Relative fluorescent unit (RFU) data are exported from the FLEXstation and ultimately converted into estimates of intracellular free Ca^{2+} concentration using the formula below.

Calculations:

$$[\text{free } Ca^{2+}] = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where the K_d for fluo-3 is assumed = 390 nM

Reagents and Materials

RAW 264.7 growth medium 1 (RAWGM1): AfCS Solution Protocol ID PS00000510

Clear bottom black-walled plate, 96 well: Greiner Bio-One; catalog no. 655090

Fluo-3, acetoxymethyl (AM), 1 mM solution in dimethyl sulfoxide (DMSO): Molecular Probes; catalog no. F-14218

Pluronic F-127, 20% solution in dimethyl sulfoxide (DMSO): Molecular Probes; catalog no. P-3000

Hanks' balanced salt solution-bovine serum albumin (pH 7.2) with 2.5 mM probenecid, pH 7.45 (HBSS-BSA-probenecid, pH 7.45): AfCS Solution Protocol ID PS00000574

FLEXstation microplate fluorometer with Softmax Pro software: Molecular Devices; catalog no. 0200-4000

Fmin solution, 5X: AfCS Solution Protocol ID PS00000607

Fmax solution, 6X: AfCS Solution Protocol ID PS00000608

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