Calcium Release-Activated Channel (CRAC) assays on the FLIPR® Tetra System: Evaluation of a novel fluorescent calcium reagent

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Introduction

Calcium release-activated calcium (CRAC) channels play an important role in intracellular Ca^{2+} homeostasis. Like other store-operated calcium (SOC) channels, currents through the CRAC channel (I_{CRAC}) are activated by depletion of calcium in the endoplasmic reticulum (ER) and serve the purpose of slowly replenishing the ER with Ca^{2+}. The CRAC channel is endogenously expressed in a variety of non-excitable cells, including rat basophilic leukaemia cells (RBL) and Jurkat T cells. Both RBL and Jurkat cells have been widely used as model systems to study the properties and regulation of the CRAC channel.

CRAC channels are considered key for the activation of immune cells and abnormalities in I_{CRAC} have been associated with certain primary immunodeficiencies, acute pancreatitis and abnormal cell proliferation. Hence the development of a reliable and sensitive CRAC channel assay may lead to the detection of novel therapeutic agents aimed at treating these human disorders.

In this current study we compare ratiometric Fura2 AM, non-ratiometric Fluo4 NW and the recently introduced FLIPR® Calcium 6 and Calcium 6-QF Assay Kits in two CRAC channel assays, using both RBL and Jurkat cells. We also demonstrate how selection of the appropriate dye can enable detection of low affinity compounds under mock high-throughput screening conditions.

Materials and Methods

Dye Preparation

Calcium free loading buffer: 40 mM NaCl, 100 mM KCl, 17 mM $NaHCO_3$, 0.1 mM EGTA, 12 mM Glucose, 1 mM $MgCl_2$, 5 mM HEPES

Calcium 6

- Formulate one bottle of Calcium 6 kit dye (Component A) by adding 10 mL of calcium free buffer and vortexing until fully dissolved

Calcium 6-QF

- Formulate one bottle of Calcium 6 kit dye (Component A) by adding 10 mL of calcium free buffer and vortexing until fully dissolved, Component C is dissolved in 36 µL DMSO and added to Component A

Fluo4 NW

- Formulate one bottle of Fluo4 NW (Component A) by adding 10 mL of calcium free buffer and vortexing until fully dissolved

Fura2 AM

- Dissolve 50 µg of Fura2 AM in 50 µL DMSO. Add 30 µL to 10 mL calcium free buffer

Dye Loading

Jurkat E6.1 cells

- Fluo4 NW, Calcium 6 and Calcium 6QF:
  - Wash cells twice in calcium/magnesium free PBS then resuspend in loading dye buffer containing 2.5 mM probenecid
  - Plate cells at 40,000 cells/well in 30 µL/well and incubate for 2 hours at 37ºC
- Fura-2 dye:
  - Wash cells twice in calcium/magnesium free PBS then resuspend in loading dye buffer containing 2.5 mM probenecid
  - Dye load for 60 minutes at 37ºC in suspension then wash cells twice with calcium free buffer
  - Resuspend in calcium free buffer (with probenecid) and plate at 40,000 cells/well in 30 µL/well
  - Spin plate at 1000 rpm (with brake off) for 3 minutes, equilibrate at R.T for 15 minutes

RBL-2H3 cells

- Fluo4 NW, Calcium 6 and Calcium 6QF:
  - Cell plates were removed from the incubator, growth media was removed and replaced with 30 µL loading dye buffer containing 2.5 mM probenecid
  - Plates were incubated for two hours at 37ºC
- Fura-2 dye:
  - Cell plates were removed from the incubator, growth media was removed and replaced with 30 µL loading dye buffer containing 2.5 mM probenecid
  - Plates were incubated for 60 minutes at 37ºC then washed twice with calcium free buffer (with probenecid) leaving a final volume of 30 µL/well and left at R.T for 15 minutes

Assay

Prepare compound plates by diluting 100% DMSO stocks (inhibitor dose response curves and controls or randomly spiked inhibitor and controls for a mock screen) with calcium-free buffer containing thapsigargin (final assay concentration 1 µM thapsigargin).
Add 10 µL of test compound or control (+ thapsigargin) using the FLIPR Tetra System and measure the fluorescence signal for 180 seconds, then incubate the plate at R.T for 15 minutes.
To induce the Ca^{2+} signal, the FLIPR Tetra System was used to add 10 µL of calcium buffer (final assay concentration 2 mM) and the resultant signal was measured for up to 260 seconds.

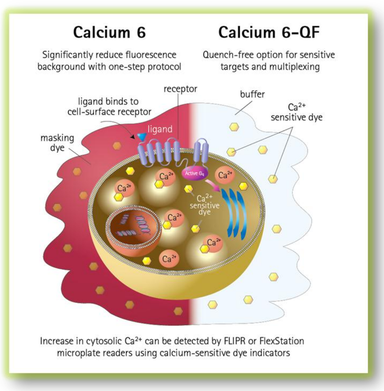

Figure 1. Principle of the new FLIPR® Calcium 6 Assay Kits

Calcium 6 & Fluo4 NW		Fura2 AM	
Parameter	Setting	Parameter	Setting
Read Mode 1		Read Mode 1	
Excitation	470 - 495 nm	Excitation	335-345 nm
Emission	515 - 575 nm	Emission	475-535 nm
% LED	30	% LED	100
Exposure	0.3	Exposure	0.5 sec
Gate	6%	Gate	23%
Gain	2000 (fixed)	Gain	2000 (fixed)
Read Mode 2		Read Mode 2	
Excitation	N/A	Excitation	380-390 nm
Emission	N/A	Emission	475-535 nm
% LED	N/A	% LED	80
Exposure	N/A	Exposure	0.2 sec
Gate	N/A	Gate	6%
Gain	N/A	Gain	2000 (fixed)
Read time interval	1.0 sec.	Read time interval	1.15 sec.

Table 1. FLIPR® Tetra optics setup parameters

- Background fluorescence is reduced by masking technology
- New dye formulation delivers larger signal window due to enhanced retention of dye within the cell
- Anion exchange protein inhibitor sensitive targets can be run with less or no probenecid
- Calcium 6-QF formulation is a new flexible option for quench sensitive targets or multiplexing applications

Results

Initial experiments were done to ascertain the pharmacological profile of the CRAC channels in both cell lines, and IC_{50} values were obtained with three known inhibitors of the channel: YM 58483, Econazole and 2-Aminoethoxydiphenylborane (2-APB).
The resultant inhibition curves are shown in **Figures 2** and **3**, and the mean –log IC_{50} estimates (±sem) are displayed in **Table 2** along with the average assay Z' factor.

A second series of experiments were run as a mock HTS screen using IC_{15}, IC_{30} and IC_{75} concentrations of YM 58483 and IC_{15} concentration of 2-APB 'spiked' in the compound addition plate. **Figure 4** shows the typical kinetic traces obtained on the FLIPR Tetra system with the four calcium dyes, and in **Table 3** the resulting statistical analyses are shown.

	YM 58483	Econazole	2-APB	Assay Z'
RBL-2H3				
Calcium 6	6.96 ± 0.02	4.81 ± 0.02	5.47 ± 0.02	0.73
Calcium 6QF	6.82 ± 0.04	4.80 ± 0.07	5.45 ± 0.03	0.84
Fluo4 NW	6.76 ± 0.08	4.73 ± 0.07	5.46 ± 0.09	0.76
Fura2 AM	6.19 ± 0.08	4.74 ± 0.06	5.37 ± 0.13	0.38
Jurkat				
Calcium 6	7.10 ± 0.05	5.22 ± 0.04	4.98 ± 0.03	0.75
Calcium 6QF	6.91 ± 0.11	5.19 ± 0.08	5.01 ± 0.13	0.73
Fluo4 NW	6.79 ± 0.12	4.97 ± 0.05	4.95 ± 0.05	0.78
Fura2 AM	6.77 ± 0.11	5.17 ± 0.09	4.66 ± 0.17	0.68

Table 2. Mean –log IC_{50} estimates obtained with YM 58483, Econazole and 2-APB in RBL-2H3 and Jurkat cell lines loaded with various calcium sensitive dyes. Results are mean ±sem, n ≥ 4

Comparison of results between the FLIPR® Calcium 6 Assay Kits, Fluo4 NW and Fura2 AM in two CRAC channel inhibitor assays

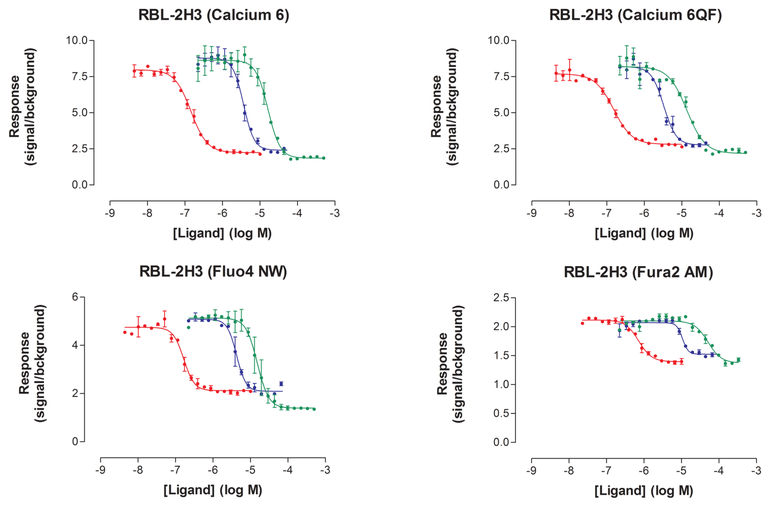

Figure 2. CRAC channel inhibitor assay in adherent RBL-2H3 cells. Dyes were loaded according to protocols outlined in the methods section and inhibition curves obtained with YM 58483 (●), Econazole (●) and 2-APB (●). The rank order of inhibitor potency in this assay was YM 58483 » 2-APB > Econazole. –log IC_{50} and Z' estimates are shown in **Table 2**.

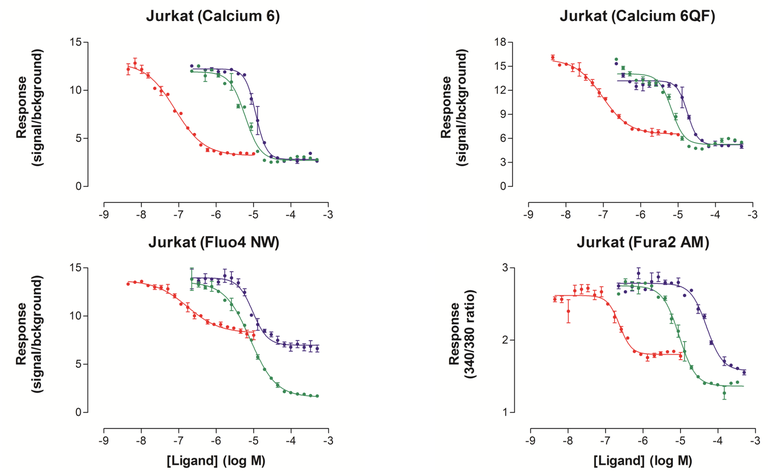

Figure 3. CRAC channel inhibitor assay in suspension Jurkat cells. Dyes were loaded according to protocols outlined in the methods section and inhibition curves obtained with YM 58483 (●), Econazole (●) and 2-APB (●). The rank order of inhibitor potency in this assay was YM 58483 » Econazole ≥ 2-APB. -log IC_{50} and Z' estimates are shown in **Table 2**.

Comparison of results between the FLIPR® Calcium 6 Assay Kits, Fluo4 NW and Fura2 AM under mock CRAC channel screening conditions using RBL-2H3 cells

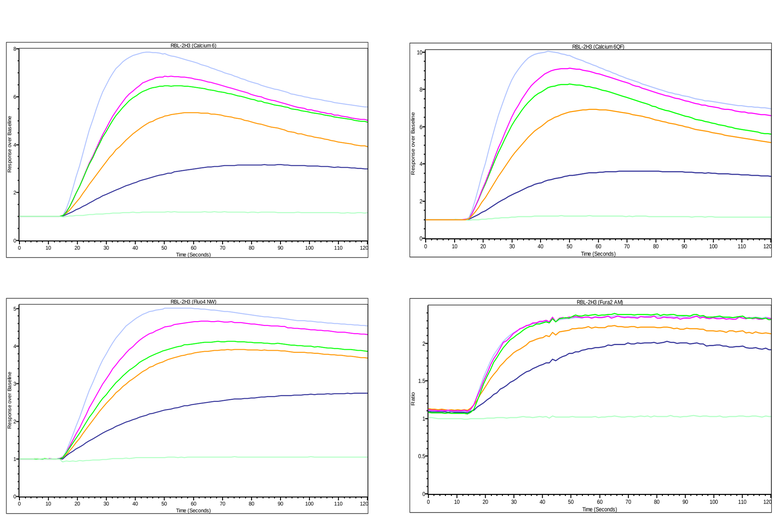

Figure 4. Representative kinetic traces from RBL-2H3 cells loaded with FLIPR® Calcium 6 Assay Kit (top left), FLIPR® Calcium 6QF Assay Kit (top right), Fluo4 NW (bottom left) or Fura2 AM (bottom right). Kinetic traces shown are positive control (---, 2mM Ca^{2+}), YM 58483 (---, IC_{15}), YM 58483 (---, IC_{30}), YM 58483 (---, IC_{75}), 2-APB (---, IC_{15}) and negative control (---, HBSS)

	YM 58483 IC_{15}	YM 58483 IC_{30}	YM 58483 IC_{75}	2-APB
RBL-2H3				
Calcium 6	** P < 0.01	*** P < 0.001	*** P < 0.001	*** P < 0.001
Calcium 6QF	* P < 0.05	*** P < 0.001	*** P < 0.001	*** P < 0.001
Fluo4 NW	** P < 0.01	*** P < 0.001	*** P < 0.001	*** P < 0.001
Fura2 AM	ns	** P < 0.01	*** P < 0.001	ns

Table 3. Statistical analyses from the 'mock screen' experiments in RBL-2H3 cells shown in **Figure 4**, results show the effect of the dye selection and cell handling protocol, especially with lower inhibitor concentrations

Summary

The FLIPR® Tetra System now offers 335-345 nm and 380-390 nm excitation LEDs and a 475-535 nm emission filter necessary for Fura2 assays. We have compared Fura2 AM and Fluo4 NW to the recently released FLIPR® Calcium 6 and FLIPR® Calcium 6QF Assay Kits in calcium release-activated calcium (CRAC) channel assays using two different cell lines;

- We have developed robust Ca^{2+} flux assays to pharmacologically profile the CRAC channels in adherent RBL-2H3 and non-adherent Jurkat cells on the FLIPR® Tetra System
- The rank order of inhibitor potency in RBL-2H3 cells was YM 58483 » 2-APB > Econazole, in Jurkat cells the profile was YM 58483 » Econazole ≥ 2-APB
- Despite the ratiometric benefits of Fura2 AM, the three no wash reagents used consistently gave lower IC_{50} estimates and higher Z' values in the profiling assays. In addition to this, the signal window tended to be smaller with Fura2 AM
- When testing low concentrations of YM 58483 under mock HTS screening conditions to simulate weakly active compounds, the choice of dye had a significant effect on detection rates. With Fura2 we were consistently unable to see an inhibitory effect with an IC_{15} concentration of YM 58483
- A no wash formulation of Fura2 may provide better data fidelity as well as offering the benefits of a ratiometric dye such as an internal control that normalizes for cell and dye loading variability, low receptor expression and small responses to ligands. Molecular Devices has just released the Fura-2 QBT™ Calcium Kit (http://www.moleculardevices.com/Products/Assay-Kits/GPCRs/Fura-2-Calcium.html) that employs a proprietary masking technology with the industry-standard Fura-2 dye

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