Ratiometric calcium flux measurements using Fura-2 Ca^{2+} indicator on the FLIPR® Tetra system

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Introduction

Receptor-ligand mediated change in the concentration of intracellular calcium is one of the most common signal transduction events measured in cell-based assays. Calcium mobilization assays monitoring Gq-coupled GPCR activity using single wavelength dyes such as Fluo-4, can on occasion miss responses due to interference from a number of factors; autofluorescent compounds excited at the same wavelength as the dye, large intracellular calcium concentration changes, the use of GFP tagged receptors or greater than desired well to well variability. During intracellular calcium mobilization, dual-wavelength calcium indicators such as Fura-2 undergo a spectral shift proportional to intracellular calcium concentration, its peak absorbance shifts from 340 nm in the Ca^{2+} bound state to 380 nm in the Ca^{2+} free state. The primary advantage of Fura-2 is that it is a dual excitation dye and is typically not sensitive to the issues seen with most single wavelength dyes.

Molecular Devices has recently developed excitation LED light sources with 340 nm and 380 nm wavelengths, and a complementary 505 nm emission filter, to enable ratiometric Fura-2 measurements on the FLIPR® Tetra system. In this poster, we demonstrate the change in 340nm/380nm ratio with change in calcium concentration driven by receptor ligand interaction. Ligand EC_{50} and IC_{50} values are compared to typical values. Using the flexibility of the FLIPR® Tetra system, Fura-2 based assays can now be scaled up from compound-confirmation to HTS screening by simply changing the plate format from 96-well to 384-well or even 1536-well format.

Materials & Methods

Fura-2 Assay

- WT3 M1 CHO and HEK-293 Cells (ATCC) cultured overnight at 8,000 cells/well in 25 µL volume, in black wall clear bottom 384-well plates (Corning Cat # 3172) @ 37ºC and 5% CO_2.
- Fura-2 AM Ultrapure grade dye (AAT Bioquest Cat # 21021) stock solution (10 mM in DMSO)
- Wash Buffer containing 20 mM HEPES (Invitrogen Cat # 15630080) in HBSS (Invitrogen Cat # 14025-134) and 2.5 mM probenecid (Sigma Cat # P8761), an anion reuptake inhibitor to prevent dye extrusion from the cells
  - Note: Probenecid was used only for CHO cell assays, not required for HEK-293 cells
- Dye Loading Buffer for 1 plate (2x concentration), 4 µM Fura-2 AM concentration final in assay
  - 10 µL Fura-2 AM from stock
  - 40 µL 10% Pluronic Acid F-127 (AAT Bioquest Cat # 20052)
  - 11 mL Wash Buffer
- Compound Plates (5x concentration) in Wash Buffer
  - Muscarinic M1 and M3 receptor agonist carbachol (Sigma Cat # C4382)
  - Muscarinic receptor antagonist atropine (Sigma Cat # A1032)
  - P2Y2 receptor agonist ATP (Sigma Cat # A28383)
- Assay steps
  - Remove cell plate from the incubator and add 25 µL Dye Loading Buffer directly to the cells
  - Incubate for 1 hour @ 37ºC and 5% CO_2
  - Using the Aqua*Max 4000 Microplate Washer equipped with the 384–well Cell Wash Head from Molecular Devices, LLC, and Wash Buffer, wash the cells leaving a volume of 50 µL Wash Buffer in the wells
  - Prepare the FLIPR Tetra system protocol according to the steps in Table 1.
  - On the Data Corrections tab in the Analysis Section, click on Ratiometric Options (Read_Mode_1/Read_Mode_2) to display the data in ratiometric mode.
  - Add 5x compound and read. Data is easily analyzed on the system or exported to other programs for further analysis.

Table 1. Fura-2 Assay Optics Set-up Parameters

ICCD Camera Settings		EMCCD Camera Settings	
Parameter	Setting	Parameter	Setting
Read Mode 1		Read Mode 1	
Excitation	335-345 nm	Excitation	335-345 nm
Emission	475-535 nm	Emission	475-535 nm
% LED	100	% LED	100
Exposure	0.2	Exposure	0.5 sec
Gate	80%	Gate	NA
Gain	2000(fixed)	Gain	150
Read Mode 2		Read Mode 2	
Excitation	380-390 nm	Excitation	380-390 nm
Emission	475-535 nm	Emission	475-535 nm
% LED	0.5	% LED	50
Exposure	0.1sec	Exposure	0.08 sec
Gate	25%	Gate	NA
Gain	2000(fixed)	Gain	150
Read time interval	1.0 sec	Read time interval	1.05 sec

The FLIPR Tetra System

Standard FLIPR Calcium Kit Assay

- The same cells and compounds were used in both assays.
- FLIPR® Calcium 5 Assay Explorer Kit (Molecular Devices Cat # R8185)
- Wash Buffer as listed above with or without probenecid
- Assay steps
  - Formulate 1 bottle of Calcium 5 kit dye according to instructions in the package insert
  - Add 25 µL dye to the cells
  - Incubate for 1 hour @ 37C and 5% CO_2
  - Do NOT wash the cell plates
  - Prepare the FLIPR Tetra system protocol according to the settings for standard calcium kit assays found in the Calcium 5 Kit package insert
  - Add 5x compound and read. Data is easily analyzed on the system or exported to other programs for further analysis.

Results

Calcium mobilization assay results in WT3 CHO M1 cells: comparing results from a traditional Fluo-4 wash assay with the Fura-2 wash assay

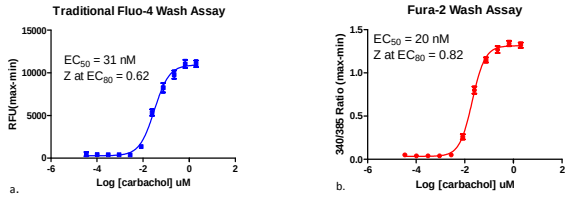

Figure 1. Comparison of calcium flux assay results in WT3 M1 CHO cells stimulated by carbachol. a) Response in Fluo-4 assay. b) Response in Fura-2 assay. EC_{50} values are very similar. The Z factor at EC_{80} in the Fura-2 wash assay was higher.

Stimulation of endogenous muscarinic M3 receptor in HEK 293 cells by carbachol

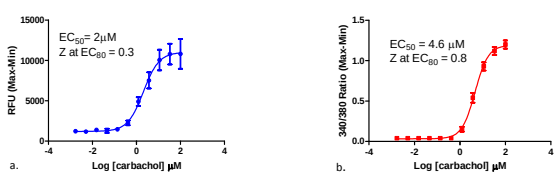

Figure 2. Comparison of calcium flux assay in HEK 293 cells stimulated by carbachol. Muscarinic M3 is endogenously expressed and as a result, the assay can be variable in standard calcium flux assays (Figure a). The ratiometric nature of the Fura-2 dye assay helps to correct for variable results with a resulting increase in Z factor @ EC_{80} as seen in figure b.

Comparison of Fura-2 Wash and FLIPR Calcium 5 Kit Assays in a Muscarinic M1 receptor antagonist assay

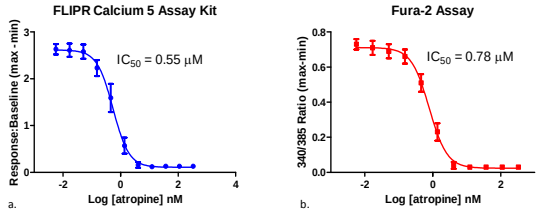

Figure 3. Fura-2 assay comparison in WT3 M1 CHO cells. An EC_{80} concentration of challenge agonist, carbachol, was added 15 minutes after a dose response of atropine, a Muscarinic M1 blocker. Compounds were added and assays were read on the FLIPR Tetra System according to the optics settings for each optics protocol. IC_{50} values are very similar.

Calculation of average cytosolic free [Ca^{2+}] released during a calcium flux assay

Average intracellular or cytosolic free [Ca2+] can be calculated using the Calcium Calibration Buffer Kit (Invitrogen Cat# C-3008MP) and Fura-2 salt form indicator (Invitrogen Cat # F1200) with the same optical settings used for Fura-2 AM cell based assays on the FLIPR Tetra System. Unlike calculations performed for calcium released in an individual cell, this calculation reflects the average free calcium released per cell in an entire well. In Figure 4a., the fluorescence spectrum of the dye is shown at increasing concentrations of free Ca^{2+}. (figure reprinted from Molecular Probes Handbook Fluorescent Probes and Research Chemicals) In figure 4b., using varying calcium concentrations mixed with Fura-2 dye as described in the product information sheet that comes with the kit, a standard curve is generated using the buffer kit and taking fluorescence readings at each concentration. The relationship is linear. The next step is to run the Fura-2 assay as described earlier in the poster. The temperature and settings of the FLIPR Tetra System matched those run during generation of the Calcium concentration standard curve. Concentration response curve is shown in Figure 5a. Finally, the 340/385 ratio at each carbachol concentration on the CRC is entered into the equation for the standard curve to generate the free [Ca^{2+}] for each value. The linear relationship is show in Figure 5b.

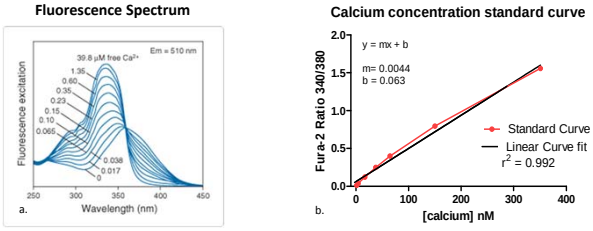

Figure 4a. Fluorescence excitation spectra of Fura-2 in solutions containing zero to 39.8 µM free Ca^{2+}. Reprinted from Molecular Probes Handbook of Fluorescent Probes and Research Chemicals. 4b. Calcium concentration standard curve. The LLD of calcium calculated in this assay is 3.0 nM (data not shown). Concentration of calcium in the well plotted against the Fura-2 Ratio 340/385 nm excitation and 510 nm peak emission. Linear fit r^2 = 0.992

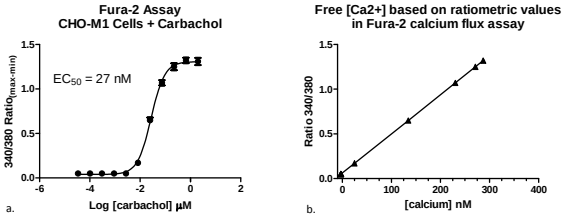

Figure 5a. Concentration response curve values generated with Fura-2 dye on the FLIPR Tetra with the same settings and at the same temperature used to generate the standard curve in Figure 4b. Figure 5b. Using the y= mx + b equation generated in the standard curve (figure 4b) with the ratio values generated in the CRC (figure 5a), the cytosolic free [Ca^{2+}] can be calculated at each concentration of carbachol added.

Summary

The FLIPR Tetra System now offers 335-345 and 380-390 nm excitation LEDs and 475-535 nm emission filters necessary for Fura-2 assays. The benefits of the Fura-2 Assay for measurement of calcium mobilization are:

- Ratiometrics provides an internal control that normalizes for cell and dye loading , low receptor expression and small responses to ligand.
- By using the UV wavelengths, it is possible to avoid interference from GFP-tagged cells or compounds that autofluorescence at 488 nm, the wavelength for standard calcium dyes.
- It is possible to calculate the average cytosolic free [Ca2+] concentration released during an assay.
- Leveraging the power of the FLIPR Tetra System, Fura-2 assays can be scaled to meet the needs of both smaller labs as well as high throughput screening.

References

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