Monitoring Intracellular Calcium using fluorescent dyes in a mid-throughput assay

Thomas P. Keeley, Ron Jacob and Giovanni E. Mann
King's BHF Centre of Research Excellence, School of Cardiovascular Medicine & Sciences, King's College London, London SE1 9NH, UK

- **Ca^{2+} transients were measured in real time in human endothelial cells**
- **Mid/high-throupghput Ca^{2+} measurements require high dye retention – lowest leakage for Cal520-AM**
- **CLARIOstar® offers fast fluorescence detection and high temporal resolution for fast Ca^{2+} reactions**

Introduction

Changes in the intracellular concentration of Ca^{2+} ions is the basis for numerous cellular responses; from receptor signalling to mediating contractile function. Thus accurate techniques to monitor intracellular $[Ca^{2+}]$ are in high demand for both academic and industrial research. Traditionally, monitoring intracellular Ca^{2+} requires live-cell fluorescence imaging. Advances in microplate reader technology, including the ability to incubate at 37°C and 5% CO_2 and inject reagents automatically, have allowed the adaptation of the traditional fluorescence-based assays to a microplate format. This greatly increases the throughput and automation of such assays.

This application note compares the suitability of 3 commercially available fluorescent Ca^{2+} dyes; Fura-2AM, Fluo-8AM and Cal-520AM used to monitor histamine-stimulated Ca^{2+} mobilisation in human umbilical vein endothelial cells in the CLARIOstar® plate reader equipped with atmospheric control unit.

Assay Principle

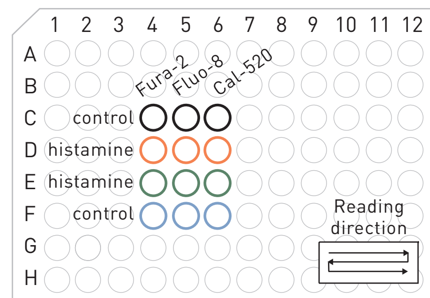

Fig. 1: Plate layout and assay principle. Cells were loaded with either Fura-2AM, Fluo-8AM or Cal-520AM and fluorescence recorded sequentially as indicated in well kinetic mode (2 min/well). Thus control replicate 1 and 2 are separated temporally by 20 minutes.

Materials & Methods

- 96-well microplate (black clear bottom, Greiner Bio-one)
- CLARIOstar® with atmospheric control unit (ACU), BMG LABTECH
- Fura-2AM and Fluo-8 (TefLabs, UK), Cal-520AM (Stratech, UK), histamine hydrochloride (Sigma-Aldrich, UK)

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords and cultured in black 96-well plates coated with gelatin (1 mg/ml) in M199 +20% FCS.

Calcium-dye loading and cell stimulation

Cells were loaded with 2 µM calcium dye (Fura-2AM, Fluo-8AM or Cal-520AM) in medium for 30 minutes at 37°C. Loading solution was washed off using Krebs buffer solution and then cells were incubated in 190 µL Krebs in the CLARIOstar® (set to 5% CO_2 and 37°C) for a further 10 min to allow complete deesterification. Histamine (20X working solution, 200 µM) was loaded into the on-board reagent injection system, and added to desired wells at 10 µL to achieve a final concentration of 10 µM. Background fluorescence was obtained in wells containing unloaded cells in Krebs buffer.

Data analysis

Fura-2AM: Background fluorescence at 340 and 380 nm (ex) was subtracted from raw fluorescent intensity at the corresponding wavelengths. Background corrected fluorescence at 340 nm (ex) was divided by that at 380 nm (ex) to obtain Ratio 340:380.

Fluo-8AM and Cal-520AM: Background fluorescence at 488 nm (ex) was subtracted from raw fluorescent intensity. The average baseline (pre-injection, F_0) fluorescence was taken and all data points expressed relative to this value (F/F_0).

Instrument Settings

	Fluorescence intensity, well mode kinetic			
Optic settings	Monochromator settings			
		Excita-tion	dich-roic	Emission
	Fura-2M	335-10 380-10	420.5	520-30
	Fluo-8AM	480-10		
	Cal-520AM			
	Gain	2000		
	Focus height	4.3 mm		
Optic	Bottom optic			
General settings	Number of flashes			50 per interval
	Settling time			0.5 s
Kinetic settings	Number of intervals			120
	Interval time			1 s
Injections	10 µl in interval 11			
Incubation	37°C			
Atmospheric control	5% CO_2			

Rev. 03/2019
Keywords: Calcium, Injectors, ACU – atmospheric control unit

Results & Discussion

To investigate which Ca^{2+} dye performs best in a 96-well plate format assay, cells were loaded with either Fura-2AM, Fluo-8AM or Cal-520AM and treated with control (ddH_2O) or histamine (10µM) and intracellular Ca^{2+} levels monitored as outlined in Fig 1. Figure 2 illustrates the results from duplicate wells, expressed as background corrected fluorescence intensity at 520nm. The wellmode kinetic function of the CLARIOstar enables rapid cycle times, and the recording of rapid calcium release after stimulation. Independent of dye used, stimulation with histamine results in a peak/plateau pattern reminiscent of similar measurements using epifluorecent imaging. Cal-520 demonstrated the highest F/F_0 upon histamine stimulation, which was comparable to the R340/380 obtained with Fura-2.

When using the wellmode option, each well is read in turn, and there is a time difference between measuring the first and last well. Therefore, a factor to consider when choosing a suitable dye is the extent of leakage from the cytosol of the dye, particularly when monitoring intracellular Ca^{2+} levels, which are ~10^5X lower than extracellular concentrations. Dye leakage would be detected as an increase in baseline fluorescence over time, as shown in Figure 3. Fluorescence was only monitored periodically to avoid photo-bleaching effects. While Fluo-8 showed the strongest baseline shift over time, with Fura-2 also showing a gradual increase, no substantial baseline increase was detected with Cal-520 over the time course investigated.

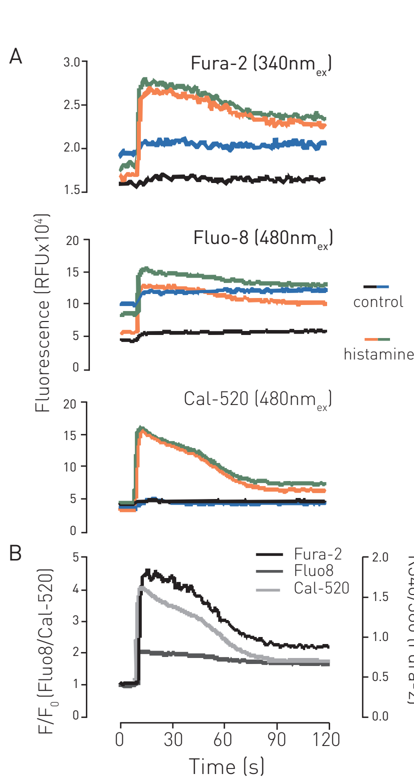

Fig. 3: Intracellular Ca^{2+} measurements in a 96-well plate using various calcium dyes. Cells were loaded with 2µmol/L dye and stimulated with either ddH_2O (control) or histamine (10µmol/L). Duplicate traces are shown in A, and mean data is shown in B as either baseline-corrected (F/F_0) or as the ratio of fluorescence intensity at 340/380nm_{ex}.

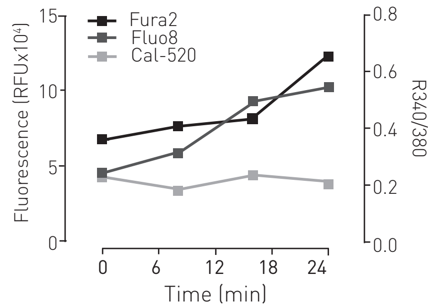

Fig. 3: Increases in baseline fluorescence or R340/380 over time in well kinetic mode.

Conclusion

Fluorescent dyes have long been used to monitor intracellular Ca^{2+} levels in living cells by fluorescent imaging. Here we have adapted such protocols for use in a 96-well plate format in the CLARIOstar® plate reader. Dye leakage can contribute significantly to the accuracy of such measurements, and here we have shown that Cal-520AM appears as good as Fura-2 at reporting histamine-stimulated Ca^{2+} mobilisation in endothelial cells in a 96-well plate format, with minimal leakage over a 30 minute time frame. Such attributes make this dye useful for mid/high-throughput analysis of intracellular Ca^{2+} levels in living cells.