Optimization of a muscarinic M_3-receptor assay using frozen "Assay Ready" CHO cells on the FlexStation 3 System: The impact of calcium dye selection and cell culture conditions

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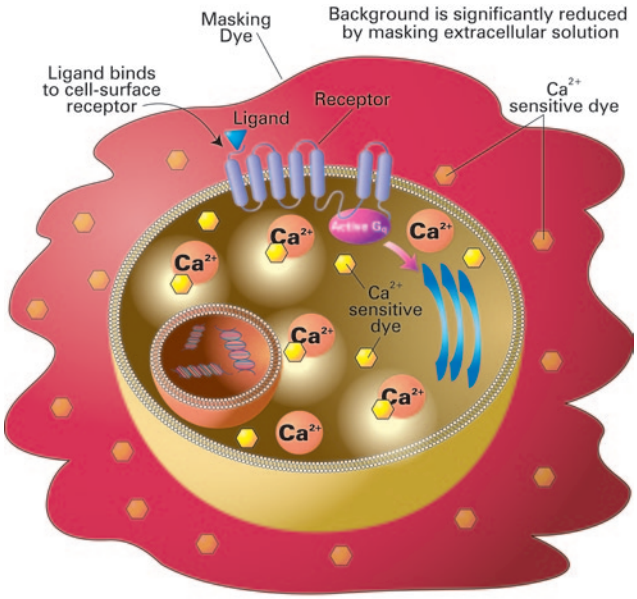
Introduction

Cell-based assays can often be challenging and time-consuming. To facilitate and streamline this complicated process, frozen cells which can be assayed without prior cultivation have become a suitable and frequently used alternative to cells in continuously growing culture. Frozen cells decouple cell culture preparation from functional testing, can help alleviate biological variability, improve plate-to-plate consistency and data fidelity, increase the flexibility of assay scheduling and significantly decrease cell culture costs.

The FLIPR® Calcium 5 Assay Kit (Figure 1), exhibits superior performance to other "no wash" calcium reagents, and has broad applicability across a varied selection of biological targets. Elimination of the usual wash steps has the key advantage of reduced plate handling and faster assay throughput.[1] In addition, under sub-optimal assay conditions the use of a homogenous assay has the potential for enhancements in data quality and reduced well-to-well variability.

FLIPR Calcium Assay principle (Figure 1)



FLIPR Calcium Assay Kits include a calcium sensitive dye that is taken into the cytoplasm of the cell during incubation. The kit's masking dye remains outside the cell and blocks background fluorescence. Upon ligand binding to the receptor, calcium is released into the cytoplasm of the cell. The dye binds to the intracellular calcium and becomes fluorescent.

The FlexStation® 3 Benchtop Multi-Mode Microplate Reader offers added flexibility over dispenser-based systems by directly transferring reagents from a source plate to the read plate, which reduces reagent and consumable consumption. The ability to define individual reagents and concentrations to be delivered to each column on the cell plate allows more assay conditions to be explored in a single experiment, and makes the system ideal for both agonist and antagonist studies. In this application note we utilize the FlexStation 3 Reader to compare different Ca^{2+} indicators (both single-wavelength and dual-wavelength) in a representative G_q-GPCR assay to highlight the potential benefits of using a homogeneous 'no wash' calcium reagent. In addition, we compare assay performance and data fidelity between cells in continual culture and those prepared from frozen reagent ("Assay Ready" cells available from the European Collection of Cell Cultures (ECACC)).

Materials

- FlexStation® 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices)
- AquaMax® 4000 Microplate Washer fitted with the 96-well cell wash head (Molecular Devices)
- ImageXpress® Micro Widefield High Content Screening System (Molecular Devices)
- FLIPR® Calcium 5 Assay Explorer Kit (Molecular Devices, Cat. #R8185)
- Fluo-4 AM (Invitrogen, Cat. #F-14201)
- Fura-2 AM (Invitrogen, Cat. #F-1221)
- DAPI Nucleic Acid Stain (Invitrogen, Cat. #D-1306)
- Cryopreserved "Assay Ready" CHO cells expressing the human muscarinic M_3 receptor (CHRM3, ECACC, Cat. #10031603)
- Water soluble probenecid (Invitrogen, Cat. #P-36400)
- Growth media for CHO cells: Hams F12 : DMEM with 10% FBS and 1% pen/strep (Invitrogen, Cat. #31331-093, 16140-071 and 15140-122)
- Hanks Balanced Salt Solution (HBSS) with calcium and magnesium with 20 mM HEPES (Invitrogen, Cat. #14025-050 and 15630-056)
- Cell plates (black-wall, clear-bottom 96-well CellBIND microplates, Corning, Cat. #3340)
- Acetylcholine, non-selective muscarinic receptor agonist (ACh, Sigma, Cat. #A6500)
- *p*-Fluorohexahydro-sila-difenidol hydrochloride, muscarinic receptor antagonist (*p*-F-HHSiD, Sigma, Cat. #H127)
- Methanol (Sigma, Cat. #179337)

Methods

Cell handling and plating methods

1. CHO cells expressing the muscarinic M_3 receptor (CHRM3) kept in continual culture were plated at 30,000 cells/well in 200 µL growth media, left to settle on the bench for 30 min. at room temperature then maintained overnight at 37°C, 95% humidity and 5% CO_2.

2. "Assay-Ready" frozen CHRM3 cells were thawed rapidly in a 37°C water-bath, pipetted gently into 10 mL warm growth media and centrifuged for 5 min. at 1000 rpm. Cells were resuspended in culture media and plated out at 50,000 cells/well in 200 µL media, plates were left on the bench for 30 min. at room temperature prior to being incubated at 37°C, 95% humidity and 5% CO_2 for 18 hours.

3. Cryopreserved CHRM3 cells were thawed rapidly in a 37°C water-bath, pipetted gently into 10 mL warm growth media and centrifuged for 5 min. at 1000 rpm. Cells were resuspended in 10 mL warm growth media before being returned to the CO_2 incubator for 60 min. Following centrifugation the cells were resuspended in FLIPR Calcium 5 Assay reagent and plated out at 75,000 cells/well in 100 µL of loading buffer. Finally, cell plates were centrifuged again at 1000 rpm (with no brake) before being returned to the CO_2 incubator for 45 min.

FLIPR Calcium 5 Assay Reagent loading

Dye loading buffer was prepared by dissolving the contents of one vial of dye completely with a final volume of 20 mL Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid adjusted to pH 7.4. Cell plates generated using cell preparation methods (1) and (2) were removed from the incubator, growth media was removed and 100 µL dye loading buffer was added to each well. Dye loaded plates were incubated for 45 min. at 37°C, 5% CO_2 and allowed to equilibrate to room temperature for 15 min. prior to assaying. Plates were not washed after dye loading and the initial assay volume was 100 µL per well.

Fluo-4 AM and Fura-2 AM dye loading

Cells prepared according to method (2) above were incubated with either 100 µL/well Fluo-4 AM or Fura-2 AM (2.5 µM with 2.5 mM probenecid) for 45 min. at 37°C following growth media aspiration. Cell plates were then washed with HBSS buffer plus 2.5 mM probenecid using an AquaMax 4000 Microplate Washer fitted with the 96-well cell wash head. Wash programs consisted of a series of programmed aspirate and dispense steps (Table 1).

Table 1. AquaMax 4000 Microplate Washer parameters for calcium flux assays using CHRM3 cells

Step	Action	Settings
1	Aspirate	Rate = 5, Descent speed = Fast, Dwell time = 2.0 sec. Probe height = 1.0, 2.0, or 3.0 mm
2	Dispense	Rate = 1 or 2, Volume = 300 µL
3	Aspirate	(Same as Step 1)
4	Repeat	1 time from Step 2
5	Dispense	Rate = 1 or 2, Volume = 300 µL
6	Aspirate	Rate = 5, Descent speed = Fast, Dwell time = 2.0 sec. Probe height = 4.5 mm

Cell number confirmation on the ImageXpress Micro System

To validate cell number and confluence for the dye comparison tests, plates prepared using cell method (2) above were stained with DAPI nucleic acid stain and imaged on the ImageXpress Micro Widefield High Content Screening System. Briefly, cells were prepared at varying densities and incubated at 37°C, 95% humidity and 5% CO_2 for 18 hours. Growth media was removed and replaced with 100 µL per well of ice cold methanol. After 5 minutes the methanol was removed and replaced with 100 µL per well DAPI solution (300 nM), this was incubated for 5 minutes before rinsing three times with HBSS. The cell monolayer was then imaged using a 4X objective to measure the entire well area before using the Count Nuclei Application Module for MetaXpress® Software to report total cell number per well.

Cell optimization assays

To compare the three different cell handling methods, Acetylcholine (ACh) agonist response curves and *p*-F-HHSiD antagonist inhibition curves were compared both qualitatively and quantitatively. After dye incubation and temperature equilibration the calcium mobilization assays were carried out at room temperature. A dilution series of Acetylcholine (ACh) was prepared at 3X final concentrations (assay concentrations 0.03 nM–300 nM) in HBSS buffer in 96-well polypropylene plates, 50 µL/well was added to each well of the cell plates to stimulate intracellular Ca^{2+} release.

For the antagonist studies, *p*-F-HHSiD was prepared at 3X concentrations and added offline 15 min. prior to addition of 50 µL of a 4X concentration of ACh (EC_{80}).

Fluorescence measurements were taken for 90 seconds before, during, and after compound addition.

Calcium indicator comparison assays

Calcium flux was measured with the FlexStation 3 Microplate Reader using the 'Flex' read mode. Cells loaded with FLIPR Calcium 5 Assay Kit, Fluo-4 AM or Fura-2 AM were challenged with varying concentrations of ACh (20 pM to 8 µM in 5-fold dilutions) using the FlexStation 3 Reader's integrated 8-channel pipettor. Fluorescence measurements were taken for 90 seconds before, during, and after compound addition using optimized parameters (Table 2.)

For the antagonist studies, *p*-F-HHSiD was added manually to appropriate wells and allowed to equilibrate for 15 minutes prior to placing the plates in the FlexStation 3 instrument. The instruments' on-board fluidic system was then used to dispense 50 µL/well of ACh (EC_{80} concentration) whilst changes in fluorescence were monitored in real time.

Table 2. Optimized SoftMax Pro Software settings for calcium dye comparison assays in CHRM3 cells on the FlexStation 3 Microplate Reader			
Parameter	**Settings**		
	Calcium 5	Fluo-4 AM	Fura-2 AM
Read Type	Flex		
Read Mode	**Fluorescence, Bottom Read**		
Ex Wavelength	485 nm	485 nm	340/380 nm
Em Wavelength	525 nm	525 nm	510 nm
Cut-off	515 nm	515 nm	None
Run Time	90 sec.		
Interval	1.6 sec.	1.6 sec.	3.5 sec.
	Compound Addition		
Initial Volume	100 µL		
Pipette Height	65 µL		
Volume	50 µL		
Rate	4 (64 µL/s)		
Time Point	20 sec.		

Data Analysis

Responses were measured as peak fluorescence intensity (FLIPR Calcium 5 Assay Kit or Fluo-4 AM) or the maximal 340/380 nm ratio for Fura-2 AM. To enable easy comparison, data were normalized as % response over baseline and expressed as mean ± s.e.m with n ≥ 4.

Individual sets of concentration-effect curve data were fitted to a four-parameter logistic of the form:

$$E = \beta + \frac{(\alpha - \beta)\,[A]^n}{[EC_{50}]^n + [A]^n}$$

where α, β, EC_{50} and n are the upper asymptote, lower asymptote, location (EC_{50} / IC_{50}) and slope parameters respectively. All curve-fitting routines were carried out using SoftMax Pro 6 Software.

Z′ factors were calculated using the formula:

$$Z' = 1 - \frac{(3\sigma c^+ + 3\sigma c^-)}{|\,\mu c^+ - \mu c^-\,|}$$

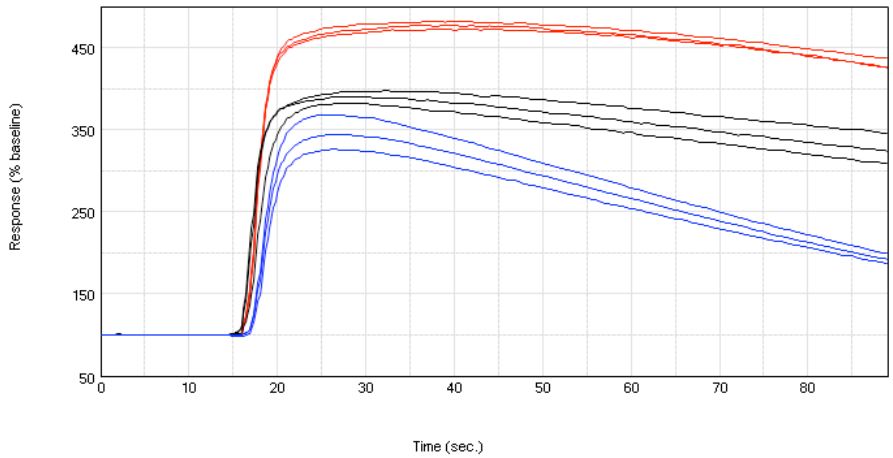
where σc^+ denotes standard deviation of the positive control and σc^- represents standard deviation of the negative control. The term $|\,\mu c^+ - \mu c^-\,|$ denotes the absolute value of the difference between the mean of the positive control signal and the mean of the negative control signal. A Z' factor > 0.5 indicates a large separation band between the negative and positive controls, and therefore a robust assay.[2]

Results

Cell optimization assays

CHRM3 cells[3] loaded with the FLIPR Calcium 5 Assay kit showed robust fluorescence increases in response to ACh application (Figure 2) under all three cell preparation methods.

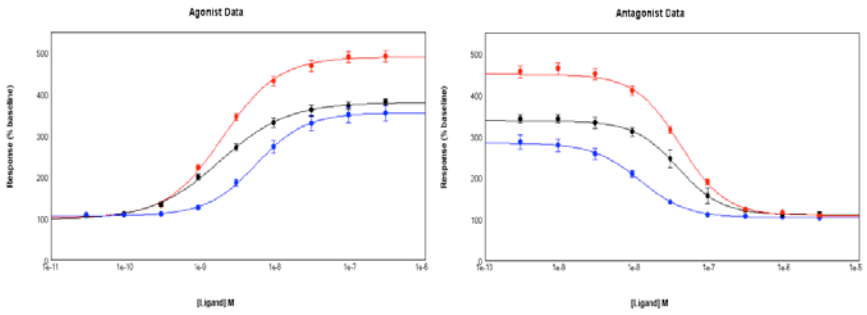
Kinetic traces (Figure 2)



Representative kinetic traces exported from SoftMax Pro 6 Software for ACh (300 nM) in FLIPR Calcium 5 Assay Kit loaded CHRM3 cells on the FlexStation 3 System. Cell conditions shown were (•) cells in continual culture, (•) frozen cells used 18 hours after thawing or (•) cells used from frozen. Plot shows % response over baseline on the *y*-axis against time in sec. on the *x*-axis.

The calculated EC_{50} values for ACh were within 0.5 log units of each other (Table 3), however there was a significant ($P < 0.05$) dextral shift in the EC_{50} values when using cells directly from frozen (Figure 3). Likewise, IC_{50} estimates for *p*-F-HHSiD were similar between the three cell preparation methods. Z' estimates were greater for cells in continual culture and frozen cells used 18 hours than those obtained when using frozen cells directly.

Activation/inhibition curves (Figure 3)



Activation/Inhibition curves for ACh ± *p*-F-HHSiD in FLIPR® Calcium 5 Assay Kit loaded CHRM3 cells. Cell conditions tested were (•) cells in continual culture, (•) frozen cells used 18 hours after thawing or (•) cells used from frozen.

Table 3. Comparative results for FLIPR Calcium 5 Assay Kit loaded CHRM3 cells under the three different cell handling conditions*									
	Cells in culture			Frozen cells after 18 hr.			Cells from frozen		
	EC_{50}/IC_{50}	% R/B	Z'	EC_{50}/IC_{50}	% R/B	Z'	EC_{50}/IC_{50}	% R/B	Z'
ACh	1.9 x 10^{-9} M	378	0.61	2.5 x 10^{-9} M	501	0.78	6.3 x 10^{-9} M	352	0.41
p-F-HHSiD	3.6 x 10^{-8} M	341	0.57	3.7 x 10^{-8} M	450	0.81	4.9 x 10^{-8} M	286	0.24

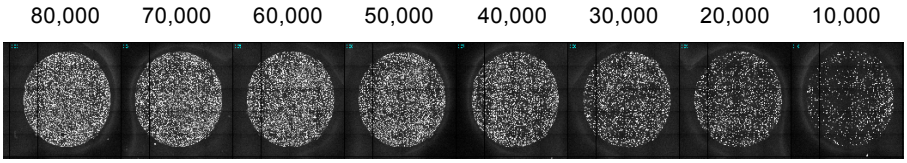
* *Note, the Z' estimate for the agonist data was calculated using the EC_{80} response. The % response over baseline (% R/B) parameter was obtained by calculating the peak response above baseline, with the baseline set as the 100% response.*

On the basis of the EC_{50} estimates, greater signal magnitude and improved Z' values from these initial studies, the easier cell preparation and lower consumable costs, all subsequent experiments were carried out with frozen cells used 18 hours after plating (method 2).

Cell number confirmation on the ImageXpress Micro System

To empirically verify cell number and confluence two representative cell plates were prepared according to cell preparation method (2) and the following day were stained with DAPI nucleic acid stain. The plates were then imaged on the ImageXpress Micro Widefield High Content Screening System (Figure 4.)

Cell density (Figure 4)



Representative cell density images taken on the ImageXpress Micro Widefield High Content Screening System. The original cell plating densities are indicated numerically above each representative well.

Table 4. Cell density results obtained with DAPI stained CHRM3 cells, data was analyzed using the Count Nuclei Application Module for MetaXpress Software	
Plating Density (cells per well)	Cell Number after 18 hours (average cells per well)
80,000	71,935
70,000	64,496
60,000	58,209
50,000	53,374
40,000	44,002
30,000	34,348
20,000	24,515
10,000	14,482

The optimal cell density giving a consistent monolayer was determined to be 50,000 cells/well in this series of experiments (Table 4). This cell density was then used for all subsequent experiments.

Calcium indicator comparison assays

ACh produced a concentration-dependent increase in intracellular Ca^{2+} in CHRM3 cells for cells loaded with the FLIPR Calcium 5 Assay Kit, Fura-2 AM and Fluo-4 AM respectively (Figure 5.).

One-way Analysis of Variance (ANOVA) indicated that the EC_{50} estimates for the Fluo-4 AM and Fura-2 AM data sets were not significantly different from one another (P value = 0.92).

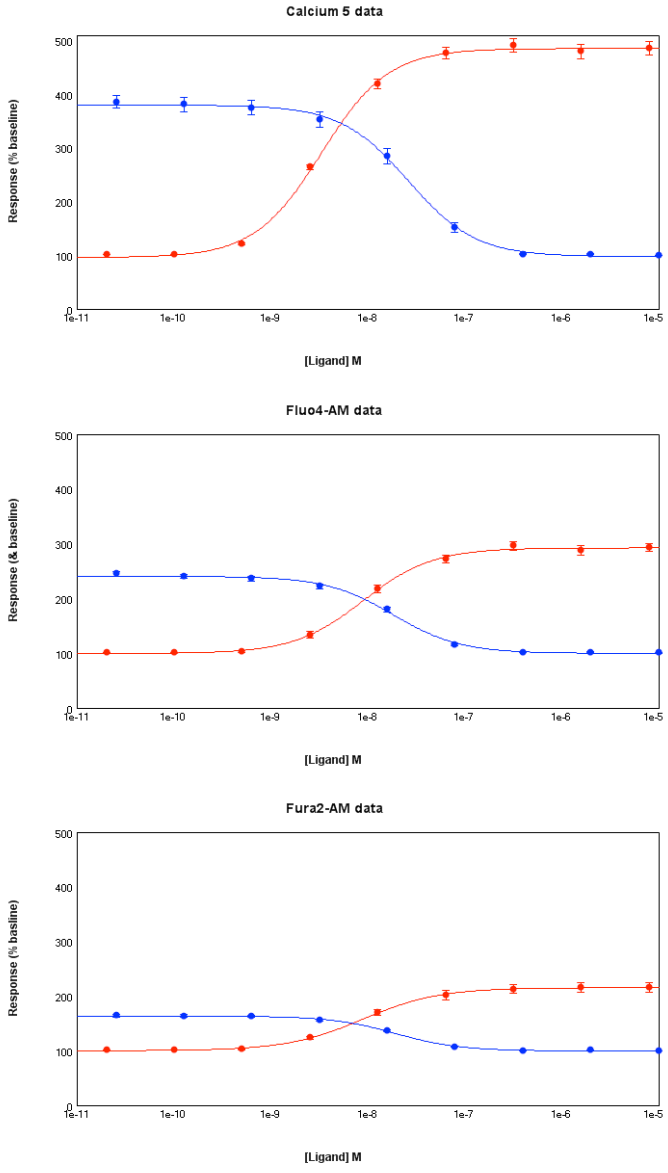
However, with the FLIPR Calcium 5 Assay Kit there was a significant ($P < 0.05$) leftward shift of the EC_{50} values, higher Z' values and greater response/baseline values (Table 5) suggesting this 'no-wash' approach is more optimal.

In comparison, the *p*-F-HHSiD data suggest that there was negligible difference between the calculated pIC_{50} values, suggesting that antagonist binding is not significantly affected by the different assay preparation methods (Table 5.)

Table 5. FlexStation 3 Microplate Reader results for CHRM3 cells loaded with the three different calcium indicators (n ≥ 6)									
	FLIPR Calcium 5 Kit			Fluo-4 AM			Fura-2 AM		
	EC_{50}/IC_{50}	% R/B	Z'	EC_{50}/IC_{50}	% R/B	Z'	EC_{50}/IC_{50}	% R/B	Z'
Acetylcholine	3.1 x 10^{-9} M	485	0.81	7.9 x 10^{-9} M	294	0.58	8.7 x 10^{-9} M	215	0.63
p-F-HHSiD	2.3 x 10^{-8} M	377	0.87	2.0 x 10^{-8} M	240	0.72	2.1 x 10^{-8} M	174	0.78

The screening coefficient window (Z' factor), which reflects the dynamic range of the signal and the data variation for the assay, was calculated using the buffer addition (negative control) and the EC_{80} concentration of ACh (agonist positive control). Z′ factors obtained with all three dyes were > 0.5 (Table 5). This shows a large separation band between the negative and positive controls and reproducible, high quality assays. However, the FLIPR Calcium 5 Assay Kit data consistently yielded higher Z' values than the other 2 calcium indicators, and significantly larger responses (% R/B).

Calcium 5 Assay Kit data (Figure 5)



SoftMax 6 Pro Software plots showing ACh (•) or *p*-F-HHSiD (•) concentration (*x*-axis) against the % change in signal (*y*-axis) for cells loaded with FLIPR Calcium 5 Assay Kit, Fluo4-AM and Fura2-AM. Data is mean ± s.e.m.

Conclusions

The FlexStation 3 Benchtop Multi-Mode Microplate Reader in combination with the FLIPR Calcium 5 Assay Kit has been shown to optimally measure changes in intracellular Ca^{2+} in "Assay Ready" frozen CHO cells expressing the muscarinic M_3 receptor. The dual monochromators facilitate optimal excitation and emission wavelengths selection, and allow both single-wavelength and dual-wavelength ratiometric indicators to be used.

The emergence of frozen cells recently as viable assay reagents has further streamlined the assay development and screening processes, and we have successfully demonstrated the data quality benefits of frozen cells versus cells in continual culture. Additional benefits of using frozen cells include significant savings in consumables, growth media and FTE resources.

Comparisons to similar experiments[4] carried out previously on the previous generation of FlexStation Systems, where the cells were washed manually, suggests that the use of the AquaMax 4000 Cell Washer can contribute to enhancements in assay quality.

Furthermore, we have shown simple assay optimization on the FlexStation 3 System and the improvements in data fidelity facilitated with a "no wash" reagent, such as the FLIPR Calcium 5 Assay Kit.

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