Functional characterisation of recombinant muscarinic M_3 receptors in Assay-Ready frozen cells using a novel Calcium Assay Kit on the FLIPR$^{TETRA®}$ instrument

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Introduction

Cell-based assays have become an indispensible method for screening and compound profiling in the early drug discovery process, and Ca^{2+} flux assays using the FLIPR® instrument are the assay of choice for Gq-coupled GPCR targets.

Use of cryopreserved cells as a 'reagent', instead of harvesting from a growing culture, uncouples cell culture from drug-screening activities decreasing time and consumable requirements. This technique increases the flexibility and reliability of a screening campaign by decreasing variability, removing unwanted passage effects and improving assay consistency.

In this study we describe our success in transitioning a recently established muscarinic M_3 receptor assay from one using cells in culture, to a more simple and robust FLIPR$^{TETRA®}$ assay using ECACC Assay-Ready frozen cells and the new FLIPR® Calcium 5 Assay Kit.

Success was confirmed by the demonstration of consistent muscarinic M_3 receptor pharmacology between growing and frozen cells, good Z' factors and robust assay performance.

Methods

Establishment of a SCREENflex cell line:

The SCREENflex technique from InSCREENeX is an accelerated methodology for producing recombinant cell lines. The basis of this method is the pre-establishment of an engineered master cell line containing a reporter gene and a 'silent' genetic marker. A vector encoding the gene of interest is introduced into the master cell line whereby site-directed recombination allows for the replacement of the reporter gene with a gene of interest and the subsequent expression of the genetic marker. Cells are selected for the absence of reporter gene expression and the restoration of the selection marker. For this study, a vector encoding the human muscarinic M_3 receptor was used. The resultant CHO cell line was designated CHRM3 and was transferred to ECACC for banking, characterisation and the generation of growing and Assay-Ready frozen cells.

Establishment of a qualified cell bank at ECACC:

The frozen vial of CHRM3 cells was resuscitated and expanded to a 50 vial Master Cell Bank (MCB) following full QC testing for mycoplasma and DNA fingerprinting. Analysis of cell growth and morphology using the IncuCyte™ ensured that cells were sub-cultured in log phase growth.

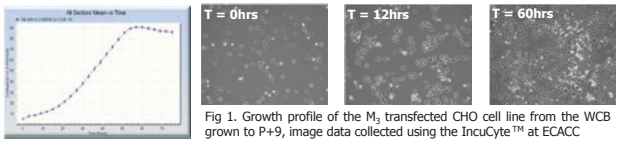


Fig 1. Growth profile of the M_3 transfected CHO cell line from the WCB grown to P+9, image data collected using the IncuCyte™ at ECACC

A vial from the frozen MCB was resuscitated and expanded to a 100 vial ($3x10^6$ cells/vial) Working Cell Bank (WCB) using optimised cell culture scale-up processes, subculture and seeding density routines and cryopreservation methods. Finally, a vial from the frozen WCB was resuscitated and expanded to create the 50 vial frozen Assay-Ready Cell Bank ($1.3x10^7$ cells/vial) used in the FLIPR® studies.

FLIPR® Calcium 5 Assay Kit:

The FLIPR® Calcium 5 Assay Kit contains a new, superior performance calcium sensitive indicator that uses the same proven quench technology as the market leading FLIPR® Calcium 4 Assay Kit. This masking dye technology is licensed to MDS Analytical Technologies from Bayer AG (patent no. US 6,420,183, EP 0906572). The masking technology combined with the novel indicator significantly lowers background fluorescence and improves signal-to-noise without the requirement for media removal or washing the cells.

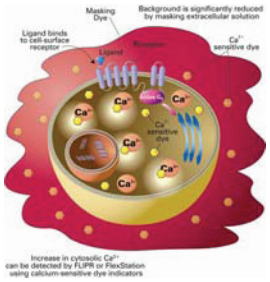


Fig 2. FLIPR® Calcium 5 Assay Kit response to intracellular calcium mobilisation.
During cellular response to GPCR signaling, the fluorescent signal increases as released calcium molecules bind to the novel indicator. Background signal is decreased by the extracellular masking technology.

Cell handling and plating methods:

Before dye loading, cells were dispensed into Corning® CellBIND® black/clear base 384-well plates.

(1) Cells in continual culture were plated at 10K cells/well in 50 L Hams F-12, left on the bench for 60 min. at room temperature then grown overnight at 37ºC, 95% humidity and 5% CO_2.

(2) Assay-Ready frozen cells were thawed rapidly in a 37ºC water-bath, pipetted gently into 10 ml warm growth media and centrifuged for 5 min. at 1000 rpm. Cells were resuspended in Hams F-12 and plated out at ~13K cells/well, left on the bench for 60 min. at room temperature prior to being incubated at 37ºC, 95% humidity and 5% CO_2 for 18 hours.

(3) Assay-Ready frozen cells were thawed rapidly in a 37ºC 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 Hams F-12 before being returned to the CO_2 incubator for 60 min. Following centrifugation the cells were resuspended in FLIPR® Calcium 5 Assay loading buffer and plated out at ~26K cells/well, plates were finally centrifuged before being returned to the CO_2 incubator.

FLIPR® Calcium 5 Assay Kit loading:

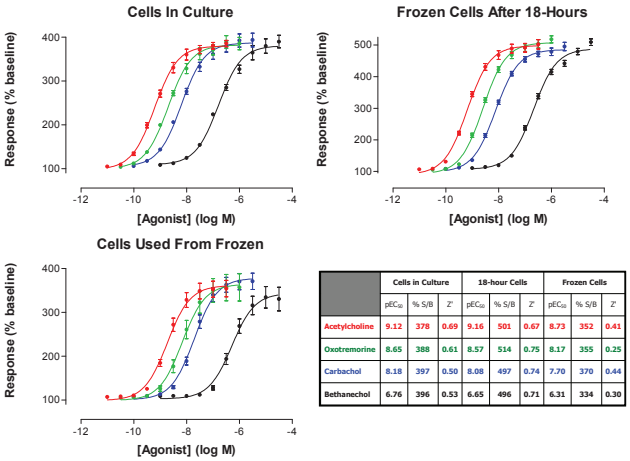
Dye loading buffer was prepared by dissolving the contents of one vial of dye completely with a final volume of 20 mL Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid adjusted to pH 7.4. Cell plates for cell conditions (1) and (2) were removed from the incubator, growth media removed and 50 L dye loading buffer was added to each well. Dye loaded plates were incubated for 45 min. at 37ºC, 5% CO_2 and allowed to cool to room temperature for 15 min. prior to reading on the FLIPR$^{TETRA®}$ system. Plates were not washed after dye loading.

Calcium mobilisation assay on FLIPR$^{TETRA®}$ Instrument:

A 5X volume of agonist was prepared in HBSS buffer in 384-well polypropylene plates. Agonist (12.5 L) was added during detection on the FLIPR$^{TETRA®}$ instrument at optimised parameters. Antagonist was prepared at 5X concentration and added 15 min. prior to addition of a 6X volume of EC_{80} concentration of challenge agonist.

Results

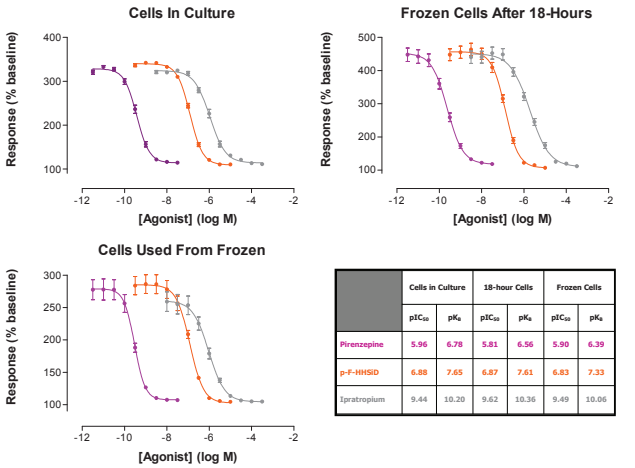
FLIPR® Calcium 5 Assay Kit loaded cells were challenged with multiple concentrations of the following cholinergic agonists;
(●) Acetylcholine, (●) Oxotremorine M, (●) Carbachol or (●) Bethanechol



	Cells in Culture			18-hour Cells			Frozen Cells		
	pEC_{50}	% S/B	Z'	pEC_{50}	% S/B	Z'	pEC_{50}	% S/B	Z'
Acetylcholine	9.12	378	0.69	9.16	501	0.67	8.73	352	0.41
Oxotremorine	8.65	388	0.61	8.57	514	0.75	8.17	355	0.25
Carbachol	8.18	397	0.50	8.08	497	0.74	7.70	370	0.44
Bethanechol	6.76	396	0.53	6.65	496	0.71	6.31	334	0.30

Fig 3. Agonist E/[A] curves in FLIPR® Calcium 5 Assay Kit loaded CHRM3 cells. Data were exported from ScreenWorks® software as % maximum response above baseline and analysed using GraphPad Prism®.

FLIPR® Calcium 5 Kit loaded cells were incubated with multiple concentrations of the following cholinergic antagonists before being challenged with an EC_{80} concentration of acetylcholine;
(●) Ipratropium , (●) p-F-HHSiD or (●) Pirenzepine



	Cells in Culture		18-hour Cells		Frozen Cells	
	pIC_{50}	pK_B	pIC_{50}	pK_B	pIC_{50}	pK_B
Pirenzepine	5.96	6.78	5.81	6.56	5.90	6.39
p-F-HHSiD	6.88	7.65	6.87	7.61	6.83	7.33
Ipratropium	9.44	10.20	9.62	10.36	9.49	10.06

Fig 4. Antagonist IC_{50} curves in FLIPR® Calcium 5 Kit loaded CHRM3 cells. Data were exported from ScreenWorks® as % maximum response over baseline and analysed using GraphPad Prism®.

Summary

We have shown that FLIPR® Calcium 5 Assay Kit loaded CHRM3 cells, transfected using the novel SCREENflex method, show pharmacology consistent with literature reports;

Agonists pEC_{50}: Acetylcholine > Oxotremorine M > Carbachol » Bethanechol
Antagonist pK_B: Ipratropium » p-F-HHSiD > Pirenzepine

Assay-Ready frozen CHRM3 cells supplied by ECACC generally gave similar results on the FLIPR$^{TETRA®}$ to CHRM3 cells in continual culture, with the advantage of significant savings in both time and resources, and potentially more consistent day-to-day results.

The signal window and Z' values obtained using Assay-Ready frozen cells plated out 18-hours before use were consistently better than those from cells in continual culture, or frozen cells used on the day of the FLIPR® assay.

The signal window and Z' values from frozen cells used on the day of the assay tended to be lower, additionally there was a small but significant rightward shift in agonist pEC_{50} estimates.

In our hands, the ECACC Assay-Ready frozen cells, in combination with the high performance FLIPR® Calcium 5 Assay Kit, are a suitable reagent for screening and compound profiling on the FLIPR$^{TETRA®}$ and allow researchers to routinely perform robust, reliable assays 5 days a week.

Acknowledgements

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