

Multiplexing the production of two G protein-coupled receptor second messengers using spectrally resolved fluorescent dyes

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Understanding of the complex interactions of G protein-coupled receptor (GPCR) signalling pathways is needed

ION Biosciences has a variety of tools to study GPCR second messenger production

Detection of the spectrally resolved dyes in a multiplex fashion was assisted by the LVF monochromator™ on the CLARIOstar^{Plus}

INTRODUCTION

With about 1000 members, G protein-coupled receptors (GPCRs) represent the largest superfamily of proteins that serve as receptors localized to cell surface membranes. They share conserved elements and employ similar hetero-trimeric proteins to initiate production of second messengers to participate in a wide variety of physiological responses. Due to their physiological importance, GPCRs are the target of about 1/3 of approved drugs¹.

Despite all we have learned about GPCR's and their signalling there is still much to learn, for example, the complex interactions between receptors and the signalling cascades they initiate¹. To further our understanding of these important responses ION Biosciences has produced several tools to detect GPCR stimulated second messengers. They have used a variety of fluorophores that span the visible spectrum as a part of these tools thus enabling the detection of multiple signalling events simultaneously.

Here we show how the detection of calcium and thallium flux can be detected simultaneously. Using the CLARIOstar^{Plus} with LVF monochromator allowed easy adjustment of the detection wavelengths to optimize the multiplex detection.

ASSAY PRINCIPLE

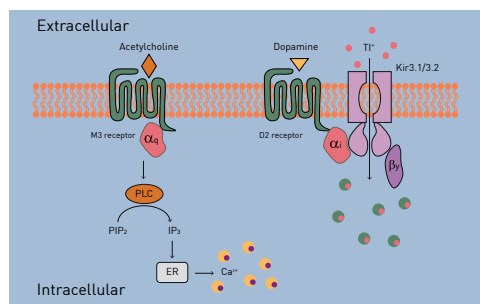


Fig. 1: Gq/Gi-GIRK Signal Multiplexing Assay Principle.

Cells expressing M3 receptor, D2 receptor and GIRK subunits (Kir3.1/3.2) are subsequently loaded with Thallos AM (green) and Fluo-Gold AM (yellow). Kinetic fluorescent signals of the Thallos AM and Fluo-Gold AM response can be measured simultaneously on a microplate reader.

Figure 1 shows two second messenger production events occurring after stimulation of the M3 and D2 receptors by acetylcholine and dopamine, respectively. Release of intracellular calcium

can be detected by Fluo-Gold because of Gq signalling following M3 activation. Simultaneously, stimulation of the D2 receptor initiates potassium flux through a G protein-coupled inwardly rectifying potassium channel (GIRK). This can be detected using surrogate thallium ions and Thallos.

MATERIALS & METHODS

- PDL coated 384-well plate, clear bottom, black (Corning® Falcon®, #CLS353219)
- Thallos AM and Fluo-Gold AM (ION Biosciences, #1381A, #1045E)
- CLARIOstar^{Plus} (BMG LABTECH)
- Cells, other chemicals and reagents were obtained from commercial sources

Experimental Procedure

HEK293 cells were transduced with D2 receptor, Kir3.1 and Kir3.2 for 24 hours and cultivated in a PDL coated microplate. Afterwards, cell culture media were removed, and cells were loaded with Thallos AM and Fluo-Gold AM dyes for 1 hour prior measurement. Cells were treated with 100 µM acetylcholine and/or 10 µM dopamine 20 seconds after the start of the measurement using the on-board reagent injectors on the CLARIOstar^{Plus}.

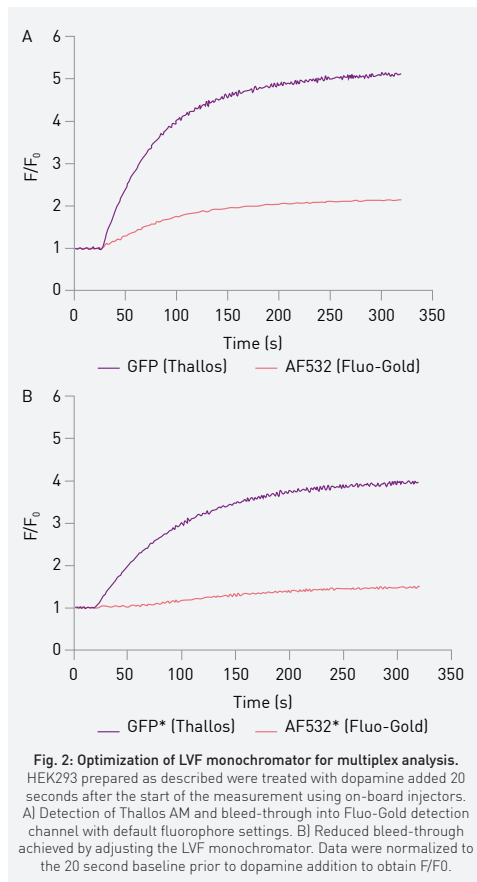
Instrument settings

Fluorescence Intensity [multichromatic], Bottom Reading, Kinetic (well mode)			
Optic Settings			
Thallos AM (= optimized)			
Preset	Excitation	Dichroic	Emission
GFP	470-15 [465-15]	491.2 [485.2]	515-20 [507-18]
Fluo-Gold AM (= optimized)			
Alexa Fluor 532	520-20 [525-17]	542.5 [546.2]	570-30 [574-30]
Gain and Focus		Adjusted	
Kinetic settings			
Number of intervals		320	
Interval time		1 second	
Flashes per chromatic		20	
Injector settings			
Volume		10 µl	
Pump speed		50 µl/second	
Injection start time		20 seconds	
General settings			
Target Temperature		37 °C	

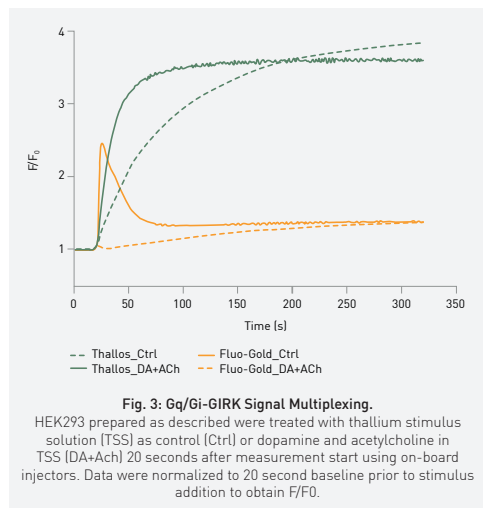


RESULTS & DISCUSSION

Initial studies showed that all dyes tested from ION Biosciences worked very well individually (data not shown), but we wanted to test multiplexing capacity. Figure 2 shows that the default settings available on the CLARIOstar *Plus* for the relevant fluorophores for Thallo AM and Fluo-Gold AM work well but exhibit some bleed-through (Fig. 2A). A small adjustment of the detection wavelengths, easily achieved because of the LVF monochromator, decreases the bleed-through (Fig. 2B).



Additional validation tests showed the specificity of detection of the Ca^{2+} and TL^+ signaling pathways (data not shown). Figure 3 shows the simultaneous detection of acetylcholine stimulated Ca^{2+} release via Fluo-Gold and dopamine-mediated activation of a GIRK channel via Thallos fluorescence. Both dyes were suitable for the multiplex approach.



CONCLUSION

We show the successful multiplexing of two fluorescent dyes to enable the simultaneous real time detection of activation of distinct GPCR signaling pathways. These results provide proof of principle for the application of these and similar dyes from ION Biosciences to further our understanding of complex GPCR signaling events.

REFERENCES

1. Zhang, M., et al. G protein-coupled receptors (GPCRs): advances in structure, mechanisms and drug discovery. *Sig Transduct Target Ther* (2024) **9**: 88