Fluorescent Assay Technologies for G-protein Interactions

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Tamara Cooper

Abstract

Assay technologies for GPCRs and their associated G-proteins are in demand for drug screening and other biotechnology applications such as biosensors for diagnostic purposes or odorant/flavour assessment as well as for elucidating the remaining controversial mechanisms in G-protein mediated signalling. This study aims to make progress towards developing a TR-FRET assay for G-protein interactions that could be used as a generic assay platform for GPCR signalling that would be fluorescent, homogeneous and amenable to miniaturization. The first chapter of this study investigates the use of small molecule labels, CS124-DTPA-EMCH:Tb and Alexa546 in a TR-FRET assay. This TR-FRET pair had previously been applied to Gα, Gβγ and RGS4 proteins and during the characterization of this assay, the protein CrV2 was observed to interact with the G-protein. Using TR-FRET, it was demonstrated that a high affinity interaction appears to occur between $G\alpha_{i1}$ and CrV2 (Apparent *K*d 6.2 nM). CrV2 is encoded by a polydnavirus from endoparasitoid wasps, which is thought to mediate immune suppression, and the interaction with Gα could have important implications in the regulation of the immune system of invertebrates. Improvements to the labelling strategy used in this assay are then attempted through the creation of various G-protein subunit fusions with small, genetically encoded lanthanide binding tags (LBTs) or tetracysteine motifs (TCMs) for site-specific labelling with terbium or FlAsH, respectively. The consequence of the fusions on maintaining G-protein subunit integrity and on the affinity of the tags for their labels is characterized, and then the utility of these constructs as TR-FRET partners is demonstrated. TCM:FlAsH complexes could successfully be used as TR-FRET acceptors for CS124-DTPA-EMCH:Tb labelled binding partners. The interaction between $G\beta\gamma_2$-TCM:FlAsH and Gα:Tb could be measured using TR-FRET and generated an apparent *K*d of 3.6 nM. Likewise, LBT:Tb complexes could be used as TR-FRET donors to Alexa546 labelled binding partners which was demonstrated using the chimeric, promiscuous Gα subunit, LBT2:Tb-$G\alpha_{S25}$ and Gβγ:Alexa. Furthermore, the two site-specific

labelling strategies can be used together in TR-FRET studies and an interaction between LBT2:Tb-$G\alpha_{S25}$ and $G\beta\gamma_2$-TCM:FlAsH was shown to have an apparent *K*d of 2.3 nM. The TR-FRET assays were further validated using protease treatments and the addition of unlabelled binding partners reduced the TR-FRET signal. Finally, the feasibility of fusing lanthanide binding tags to GPCRs for alternate assay platforms or other applications was investigated. The β_2-adrenergic and M_2-muscarinic receptors were fused to LBTs and the integrity of the receptors determined using ligand binding and [^{35}S]GTPγS signalling assays. Terbium binding to the LBT was then demonstrated. The utility of these constructs in alternative TR-FRET platforms with G-proteins was then explored.

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Abbreviations

[^{35}S]GTPγS	^{35}S radiolabelled guanosine 5'-O-(3-thiotriphosphate)
a.u.	arbitrary units
ACP	acyl carrier protein
Alexa	Alexa fluor 546 C_5 maleimide
AlF_4^-	aluminium fluoride
AMP-PNP	adenosine 5'-(β,γ-imido)triphosphate
B2AR	β_2-adrenergic receptor
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine salt
BirA	*E. coli* biotin ligase
Bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
CNS	central nervous system
CSIRO	Commonwealth Scientific and Industrial Research Organization
Da	daltons
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTPA	diethylene triamine pentaacetic acid
DTT	dithiothreitol
EC_{50}	effective concentration
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FlAsH	4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein-(1,2-ethanedithiol)$_2$
FRET	Förster Resonance Energy Transfer
GDP	guanosine diphosphate
GFC	glass microfiber 1 µM filter papers
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
G-protein	heterotrimeric guanine nucleotide binding protein
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
Gα	Gα-subunit
Gβ	G-protein β subunit
Gγ	G-protein α subunit
hAGT	human O^6-alkylguanine-DNA alkyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-tag	6 histidine tag
hr	hours
IC_{50}	inhibitory concentration
IMAC	immobilized metal affinity chromatography
IMVS	Institute of Medical and Veterinary Science
IPTG	isopropyl-β-D-thiogalactopyranoside
Kd	dissociation constant
LBT	lanthanide binding tag

LBT1	lanthanide binding tag of the amino acid sequence: Tyr Ile Asp Thr Asn Asn Asp Gly Trp Tyr Glu Gly Asp Glu Leu Leu Ala
LBT2	Lanthanide binding tag of the amino acid sequence: Ala Cys Val Asp Trp Asn Asn Asp Gly Trp Tyr Glu Gly Asp Glu Cys Ala
M2R	M_2-muscarinic receptor
min	minutes
MOI	multiplicity of infection
MW	molecular weight
NBT	nitro-blue tetrazolium chloride
Ni-NTA	nickel-nitriloacetic acid
OD	optical density
PBS	phosphate buffered saline
PCP	peptide carrier protein
PCR	polymerase Chain Reaction
PMSF	phenylmethanesulphonylfluoride
PPtase	phosphopantetheinyl transferase
RGS	regulator of G-protein signalling
*R*luc	*Renilla* luciferase
SARDI	South Australian Research and Development Institute
s	seconds
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
TCEP	tris(2-carboxyethyl)phosphine
TCM	tetracysteine motif
TR-FRET	Time resolved - Förster Resonance Energy Transfer
UK	UK 14304, synthetic adrenalin analogue
UV	ultra violet
YFP	yellow fluorescent protein

Academic Prizes and Awards

2008	Lorne Protein Conference Committee travel grant $100
2005-2008	School of Molecular Biosciences travel awards 4 x $500
2007	Doreen McCarthy bursary: The Australian Federation of University Women – South Australian Inc. Trust fund $3000
2007	Informa Life Sciences: 5th Annual Congress: GPCRs in Drug Discovery Poster Prize. "Time-resolved fluorescent technologies for GPCRs, G-protein and Regulator of G-protein signalling interactions".
2006	ARC/NHMRC Research Network: Fluorescence Applications in Biotechnology and Life Sciences (FABLS) $400 to attend Fluoro2006
2005	The Biochemical Journal Poster Prize for biochemistry and molecular biology (Presented at ComBio2005, Adelaide)
2005-2008	Australian Postgraduate Award ~ $19 000 p.a.
2005-2008	CSIRO Postgraduate Studentship $7000 p.a. + $6000 travel
2004	Commonwealth Accommodation Scholarship $4000
2004	Chancellors Letter of Commendation in recognition of outstanding results towards B.Biotechnology (Hons)
2002	Admission into the Advanced Entry Program in 2002 allowing completion of the Bachelor of Biotechnology (Hons) in 3 years rather than 4.

Publications arising from this thesis

2008 **Tamara Cooper** and Wayne R. Leifert (accepted for publication date 2009). [^{35}S]GTPγS binding in G-protein coupled receptor assays. *In Methods in Molecular Biology: G-protein Coupled Receptors in Drug Discovery*. Editor, Wayne R. Leifert. Humana Press, Totowa, New Jersey.

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2007 Glatz, R. V., Leifert, W. R., **Cooper, T. H.,** Bailey, K., Barton, C. S., Martin, A. S., Aloia, A., Bucco, O., Waniganayake, L., Wei, G., Raguse, B., Weiczorek, L. & McMurchie, E. J. (2007). Cell-free assaying of G-protein Coupled Receptors and G-proteins. *Aust. J. Chem*. Research Front "Bionanochemistry". 60, pp.309–313. (Invited Rapid Communication)

2006 Wayne R. Leifert, Kelly Bailey, **Tamara H. Cooper**, Amanda L. Aloia, Richard V. Glatz , Edward J. McMurchie (2006). Measurement of heterotrimeric G-protein and regulators of G-protein signalling interactions by time-resolved fluorescence resonance energy transfer. *Analytical Biochemistry,* 355, pp.201–212.

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and Experimental Pharmacologists and Toxicologists (ASCEPT). **Invited talk – student oral prize session**

2006 **T. Cooper**, R. Glatz, W. Leifert, J. Wallace, E. McMurchie (2006). Development of site-specific fluorescent labelling of G-protein subunits using a lanthanide (Tb^{3+}) binding tag and a FlAsH binding tetracysteine motif. *Proceedings of the Australian Society for Medical Research Scientific Meeting*.

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