

**Fluorescent Assay Technologies**  
**for**  
**G-protein Interactions**

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## Declaration

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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  $G\alpha$ ,  $G\beta\gamma$  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  $G\alpha$  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 FIAsH, 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:FIAsH 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:FIAsH and  $G\alpha$ :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  $G\alpha$  subunit, LBT2:Tb- $G\alpha_{S25}$  and  $G\beta\gamma$ :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:FIAsH 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  $\beta_2$ -adrenergic and M $_2$ -muscarinic receptors were fused to LBTs and the integrity of the receptors determined using ligand binding and [ $^{35}$ S]GTP $\gamma$ S 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

[ <sup>35</sup> S]GTPγS	<sup>35</sup> S radiolabelled guanosine 5'-O-(3-thiotriphosphate)
a.u.	arbitrary units
ACP	acyl carrier protein
Alexa	Alexa fluor 546 C <sub>5</sub> maleimide
AlF <sub>4</sub> <sup>-</sup>	aluminium fluoride
AMP-PNP	adenosine 5'-(β,γ-imido)triphosphate
B2AR	β <sub>2</sub> -adrenergic receptor
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine salt
BirA	<i>E. coli</i> 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 <sub>50</sub>	effective concentration
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FIAsH	4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein-(1,2-ethanedithiol) <sub>2</sub>
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 <sup>6</sup> -alkylguanine-DNA alkyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-tag	6 histidine tag
hr	hours
IC <sub>50</sub>	inhibitory concentration
IMAC	immobilized metal affinity chromatography
IMVS	Institute of Medical and Veterinary Science
IPTG	isopropyl-β-D-thiogalactopyranoside
K <sub>d</sub>	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 <sub>2</sub> -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
Rluc	<i>Renilla</i> 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: 5<sup>th</sup> 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). [<sup>35</sup>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.
- Tamara Cooper**, Wayne R. Leifert, Richard V. Glatz and Edward J. McMurchie. (2008). Expression and characterisation of functional lanthanide-binding tags fused to a Gα-protein and muscarinic (M2) receptor. *J. Bionanoscience*. (In press).
- Wayne Leifert, **Tamara Cooper** and Kelly Bailey. (2008). G-protein Coupled Receptors: Progress in Surface Display and Biosensor Technology. *Springer Handbook of Nanotechnology, 3<sup>rd</sup> Edition*. (In press)
- Wayne Leifert, **Tamara Cooper**, Kelly Bailey, Richard Glatz, Marta Bally, Brigitte Stadler, Eric Reimhult and Joe Shapter. (2008). Biosensors and Biochips. *Annual Reviews in Nanotechnology*. (In preparation)
- 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.

## Abstracts arising from this thesis

- 2008**      **T. Cooper**, R. Glatz, W. Leifert, E. McMurchie (2008). The use of Lanthanide Binding Tags (LBTs) in the development of TR-FRET assay technologies for G-protein coupled receptors (GPCRs). *Lorne Protein Conference*, Lorne, Victoria.
- 2007**      **T. Cooper**, K. Bailey, R. Glatz, W. Leifert, J. Wallace, E. McMurchie (2007). Time-resolved fluorescent technologies for GPCRs, G-protein and Regulator of G-protein signalling interactions. Informa Life Sciences - 5<sup>th</sup> annual congress: GPCRs in Drug Discovery, Lisbon, Portugal. **Winner of the Poster Prize – Oral presentation**
- T. Cooper**, K. Bailey, R. Glatz, A. Aloia, W. Leifert, J. Wallace, E. McMurchie (2007). Time-resolved FRET assay development for GPCRs, G-protein and Regulator of G-protein signalling interactions. *Molecular Pharmacology of G-Protein-Coupled Receptors. Proceedings of The Australasian Society of Clinical*

*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<sup>3+</sup>) binding tag and a FIAsh binding tetracysteine motif. *Proceedings of the Australian Society for Medical Research Scientific Meeting*.

Richard V. Glatz, Wayne R. Leifert, Kelly Bailey, **Tamara H. Cooper**, Chris S. Barton, A. Scott Martin, Amanda Aloia, Olgatina Bucco, Lakshmi Waniganayake, Gang Wei, Burkhard Raguse, Lech Wieczorek, and Edward J. McMurchie (2006). Cell-free receptor-based biosensors. *International Conference of Nanoscience and Nanotechnology Proceedings*.

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**2005**

**Tamara Cooper**, Wayne R. Leifert, Kelly Bailey, Richard V. Glatz and Edward J. McMurchie (2005). Time Resolved Fluorescence Resonance Energy Transfer assay for studying RGS4 interactions with G-protein G $\alpha$ -subunits in varying states of activation. *Proceedings of the Australian Society for Biochemistry and Molecular Biology*. **Biochemical Journal Poster Prize for Biochemistry and Molecular Biology**

R. Glatz, **T. Cooper**, W. Leifert, C. Barton, L. Wieczorek, E. McMurchie (2005). Engineering of G-proteins for production of cell-free ligand biosensors. *Proceedings of the Australian Society for Biochemistry and Molecular Biology*.

Wayne R. Leifert, Kelly Bailey, **Tamara Cooper**, Amanda Aloia, Richard V. Glatz, Edward J. McMurchie (2005). Measurement of heterotrimeric G-protein and RGS interactions by a novel homogeneous TR-LRET assay. Drug Discovery: From Targets to Candidates. *Proceedings of the Society for Biomolecular Screening*. P04049. Geneva PalExpo, Switzerland, Sept 11-15.

**T. Cooper**, W. R. Leifert, K. Bailey, R. V. Glatz, E. J. McMurchie (2005). Analysis of RGS4 and G $\alpha_{i1}$  interactions in different activation states. *Proceedings of the Australian Society for Medical Research Scientific Meeting*.

**Tamara Cooper**, Wayne R. Leifert, Kelly Bailey, Richard V. Glatz, John Wallace and Edward J. McMurchie (2005). Measurement of RGS4 interactions with G-protein subunits in varying states of activation using time-resolved fluorescence resonance energy transfer. *Molecular Pharmacology of G-Protein-Coupled Receptors*. *Proceedings of The Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT)*.