Advancing GPCR Drug Discovery: SMALP-Based FCS Screening for GPCR-RAMP receptors

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MRC DiMeN Doctoral Training Partnership

1- Introduction

G-protein coupled receptors (GPCRs) are the largest family of membrane receptors and can work in isolation or with accessory proteins, such as receptor activity modifying proteins (RAMPS).

GPCRs are targeted by ~1/3 of approved drugs[1] but their instability once extracted from the cell membrane in detergent still provides a challenge.

Styrene maleic acid lipid copolymer (SMALP) nanodiscs have been shown to preserve GPCR functionality when solubilized.[2]

Hit screening assays against solubilized GPCRs commonly require protein modifications and immobilization, reducing physiological relevance.

FCS provides an in-solution, high throughput hit screening assay for GPCR heterodimers in SMALPs.

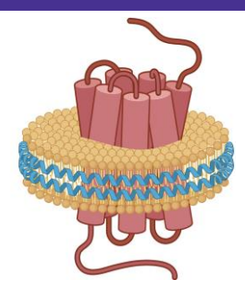

Figure 1- A diagram to show the cell membrane and GPCR encased in a nanodisc

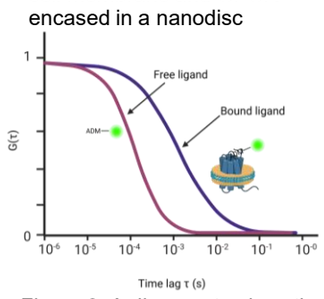

Figure 2- A diagram to show the difference between the autocorrelation curves of free and bound ligands in FCS

2- Methodology

Inducible Tet On HEK-293 3G cells were transfected with CLR and RAMP to produce stable cell lines, with receptor functionality tested through a cAMP secondary messenger assay

Detergent free extraction of membrane protein was followed by SMA isolation and Ni-NTA purification, with complex retention of the heterodimer quantified by FCCS

Saturation binding of GPCR-SMALP complexes was performed using TAMRA labelled adrenomedullin to determine kd values

Competition binding was performed with 1 uM of non-labelled ligand partner to determine non-specific binding

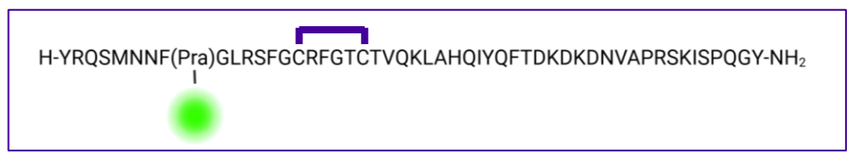

Figure 4- Structure of fluorescently labelled adrenomedullin, with TAMRA at propargylglycine [3]

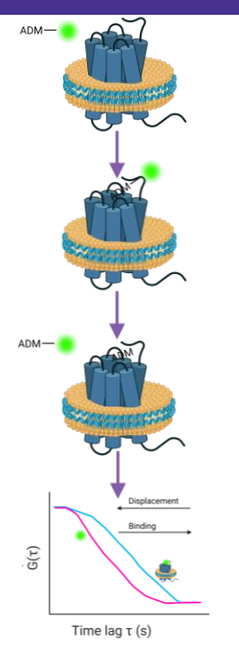

Figure 3- A diagram to show binding and competition assays for FCS using fluorescent peptide

3- Research question

Can Fluorescence correlation spectroscopy be used in high throughput screening as a SMALP based drug screening assay for GPCR-RAMP heterodimers?

4- Results

Secondary messenger assays confirm induction of functional receptor complexes in the cell, with SDS-PAGE showing successful method development for purified protein in SMA, and FCCS showing retention of receptor complexes in nanodiscs.

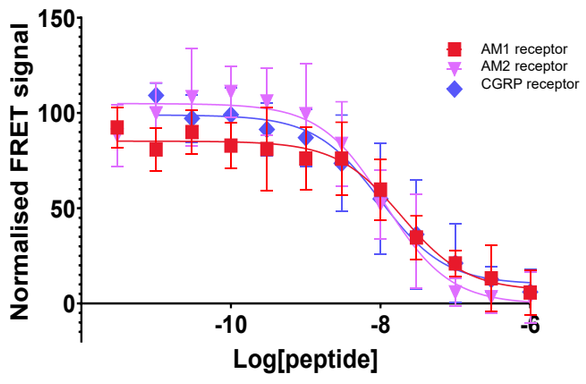

Figure 5- cAMP assays with the CGRP, AM1 and AM2 receptor overexpressing cells stimulated with CGRP (CGRP receptor (n=4)) or ADM (AM1/AM2 receptors (n=3)), demonstrating a production of functional receptor upon induction with 25 ng/mL doxycycline

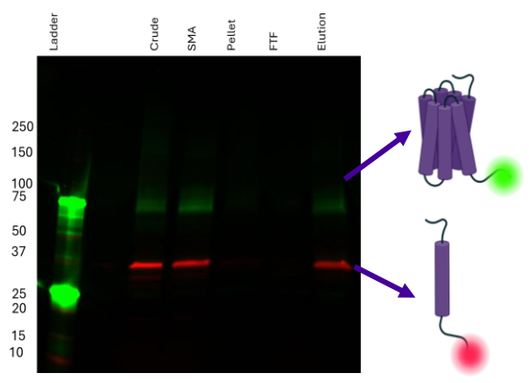

Figure 6- In-gel florescence SDS-PAGE gel for the CGRP receptor, where RAMP1=red and CLR= green to show the process of SMALP isolation and purification

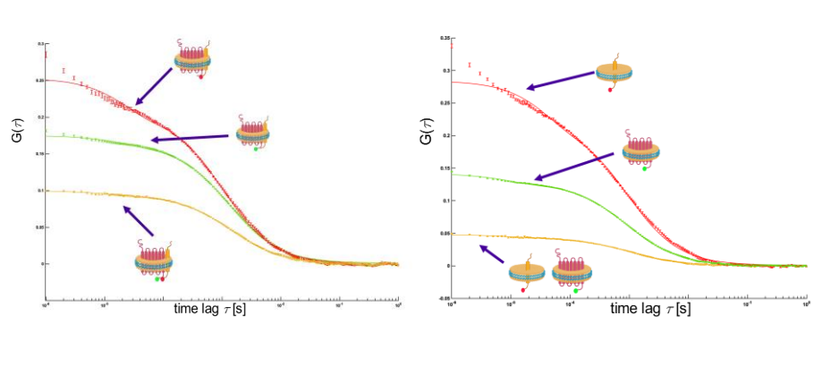

Figure 7- Characterization of Protein isolated in SMA200 through FCCS, showing autocorrelation curves (left) and quantified complex retention in the SMALP (right) normalized to a 0% control (n=3)

FCS can discriminate between small molecules, peptides and proteins, showing good dynamic range to allow a titration of the isolated protein complex to a labelled binding partner (Adrenomedullin), resulting in the determination of kd values,

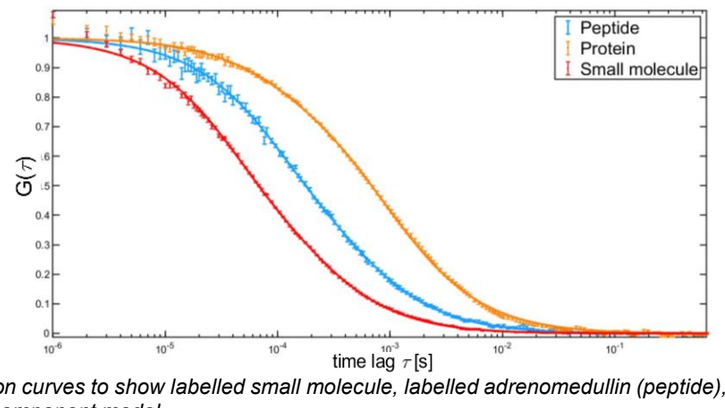

Figure 8-Autocorrelation curves to show labelled small molecule, labelled adrenomedullin (peptide), and labelled protein (AM2r) fitted with a 1 component model.

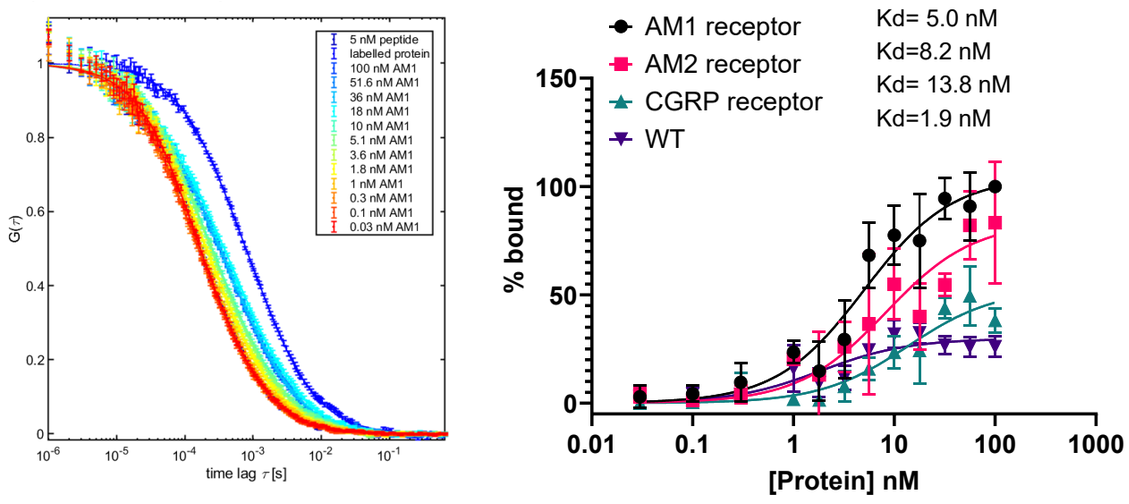

Figure 9-Autocorrelation curves (Left) for AM1 receptor titration with TAMRA-ADM peptide using a 2-component fit constrained with a 100% and 0% control, allowing for the calculation of a kd from saturation binding curves (right), for AM1 (n=3), AM2 (n=3) and CGRP (n=4) receptors, compared to wildtype protein (n=3).

Addition of a saturating concentration of unlabelled ligand (Adrenomedullin), competes off the labelled peptide, recovering its binding to <10% showing minimal non-specific binding of the labelled peptide to the receptors.

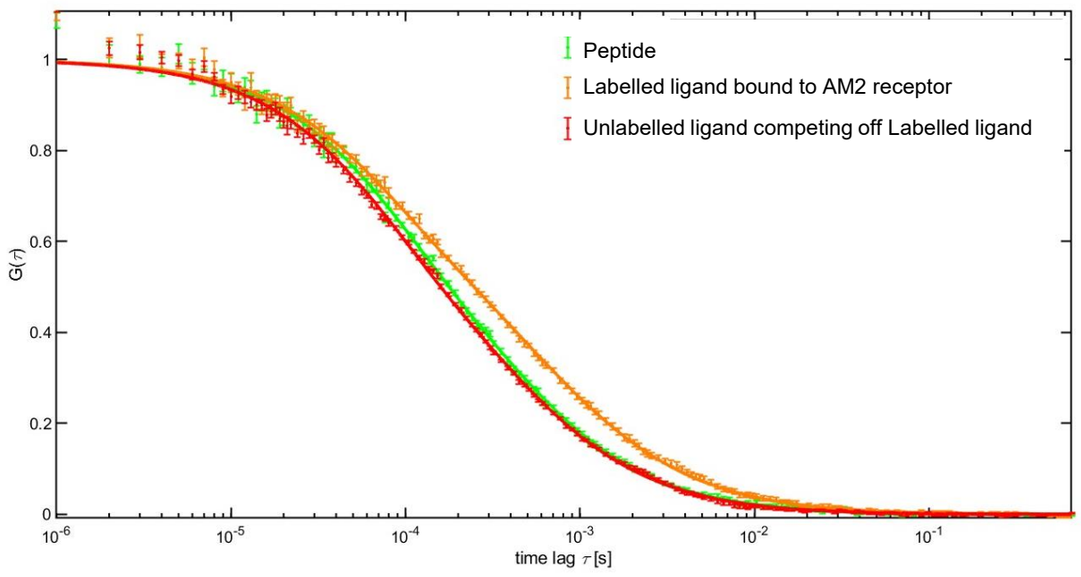

Figure 10- Autocorrelation curves to show changes in binding when adding saturated unlabeled peptide to AM2 receptor, using a 2-component fit constrained with a 100% and 0% control.

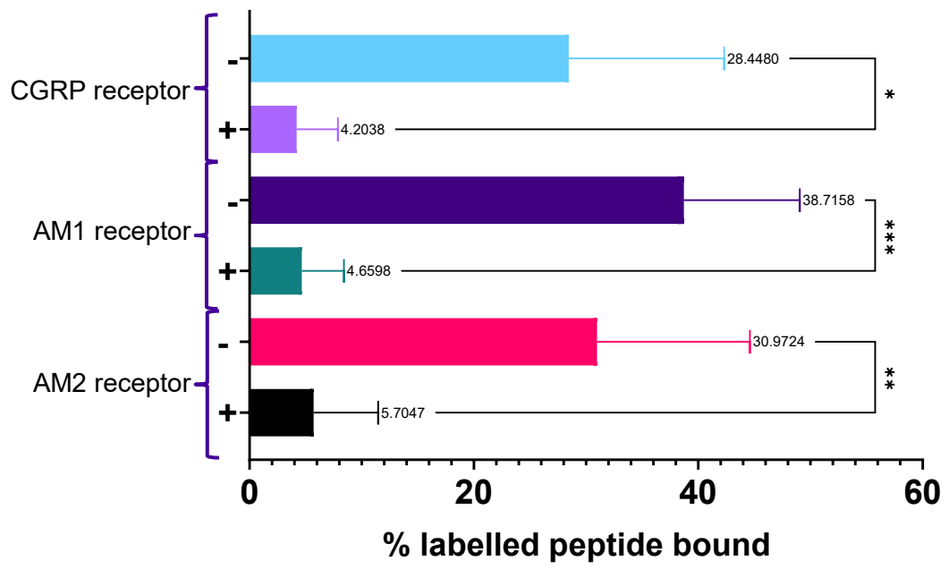

Figure 11- % fluorescent ligand bound before (-) and after (+) a saturating concentration (1 uM) of unlabeled peptide was added to 0.75x the calculated kd of each (CGRPr n=3, AM1/2r n=4)

5- Conclusion

- CLR-RAMP receptor complexes can be functionally overexpressed in HEK-293 cells using an inducible promoter
- SMA200 Allows for isolation and purification of the CLR-RAMP receptor complexes using His tags
- FCCS has enabled us to probe the receptor complex formation following isolation using fluorescent protein tags
- FCS can show the detection of binding using fluorescent probes, with Kd determined and can also detect competition binding

6- Future work:

- The competition assay shows potential to be scaled up into a high throughput drug screening assay using GPCR compound libraries
- There is a scope for wider application across a range of GPCRs and heterodimeric complexes
- Newly developed alternative native nanodisc systems can also be trialled to improve the complex retention

Figure 12- A diagram to show the process of screening SMA solubilized protein from Tet-on HEK293 3G cells

[1] Sriram, K.; Insel, P. A. G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs? *Mol. Pharmacol.* **2018**, *93* (4), 251–258. https://doi.org/10.1124/mol.117.111062.
[2] Jamshad, M.; Charlton, J.; Lin, Y.-P.; Routledge, S. J.; Bawa, Z.; Knowles, T. J.; Overduin, M.; Dekker, N.; Dafforn, T. R.; Bill, R. M.; Poyner, D. R.; Wheatley, M. G-Protein Coupled Receptor Solubilization and Purification for Biophysical Analysis and Functional Studies, in the Total Absence of Detergent. *Biosci. Rep.* **2015**, *35* (2), e00188. https://doi.org/10.1042/BSR20140171.
[3] Schönauer, R.; Kaiser, A.; Holze, C.; Babilon, S.; Köbberling, J.; Riedl, B.; Beck-Sickinger, A. G. Fluorescently Labeled Adrenomedullin Allows Real-Time Monitoring of Adrenomedullin Receptor Trafficking in Living Cells. J Pept Sci 2015, 21 (12), 905–912. https://doi.org/10.1002/psc.2833.
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