Alvarez-Curto, E., Pediani, J.D. and Milligan, G. (2010) *Applications of fluorescence and bioluminescence resonance energy transfer to drug discovery at G protein coupled receptors.* Analytical and Bioanalytical Chemistry, 398 (1). pp. 167-180. ISSN 1618-2642

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Deposited on: 13 August 2010

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spectroscopy detection techniques have given a great boost to the use of such ligands, as they enable resolution of receptor-ligand complexes to the level of a single cell or even single molecules. Using this particular inter-molecular FRET approach the kinetics of ligand binding and even receptor dimerization upon ligand binding may be analyzed in great detail, as shown in the work of Ilien and colleagues for the M_1 muscarinic acetylcholine receptor and the fluorescent antagonist BODIPY(558/568)-pirenzepine (Bo-PZ) (30). The authors showed binding of Bo-PZ in real time and in an assay that could potentially be miniaturized, therefore representing an interesting alternative to radio-labeled ligand binding and possibility of adaptation to high-throughput format.

Events involving G protein activation can also be monitored using inter-molecular FRET where it may be applied to analyze if activation is the result of conformational change or dissociation between the γ and $\beta\gamma$ subunits (Figure 1C). One of the first studies using FRET to explore G protein activation in live cells was performed using the social amoeba *Dictyostelium discoideum* (31). Changes in the heterotrimeric G protein FRET signal were monitored using CFP-$G_{\alpha 2}$ and YFP-G_{β} subunits. A loss of FRET upon addition of ligand, in this case cAMP, and stimulation of the cAMP specific receptor was interpreted as

subunits. These indicated that the β_{2A}-adrenoceptor is not pre-coupled to G proteins but rather interact upon agonist binding to the receptor (36).

Though some of these approaches contribute to answering important questions about mechanistic aspects of GPCR activation it is not difficult to envisage that they may prove hard to apply within high-throughput screening platforms (see next) and to translate to drug discovery; therefore, some alternative FRET approaches have been developed.

Emerging new FRET strategies: anisotropy FRET, photobleaching applications and FLIM

Given the innate sensitivity of FRET measurements to distance and orientation (12,37), changes in energy transfer are not easy to evaluate between FRET partners displaying significant spectral overlap because they contribute a substantial spectral bleed-through (SBT) background to the FRET signal. Using the sensitized emission intermolecular FRET technique, correct FRET data evaluation requires careful image processing from reference images acquired from control cells that only express donor or acceptor, respectively, to remove the SBT background. Furthermore, the various algorithms employed for ratiometric quantification vary in their accuracy and can introduce additional errors into the quantification of FRET (38). The transfer of this technique to high-throughput screening would require extensive control experiments, data processing and handling which could result in image storage issues and time consuming data analysis. Optimization of inter-molecular FRET, therefore, poses a number of technical challenges that are related to the spectral and physical properties of fluorescent proteins. A simpler solution to eliminate SBT background signal in the FRET channel and acceptor crosstalk excitation during donor excitation is the use of polarized light to detect FRET, i.e. anisotropy FRET (AnFRET) (39). The basis of this technique is that in the absence of FRET, emitted fluorescence from the directly excited donor fluorescent protein is highly polarized and can be easily resolved from intermolecular FRET fluorescence, which is highly depolarized (Figure 2). Rizzo and Piston (39) have demonstrated that AnFRET can unambiguously resolve cyan-yellow FRET with 10-fold greater contrast than other FRET approaches such as time or frequency domain fluorescence lifetime imaging microscopy (FLIM). FLIM is a very robust method for detecting protein-protein interactions as read out measurements are independent of fluorophore concentrations and no SBT subtraction is required. Time-gated or frequency-domain fluorescence lifetime measurements using HTS microplate readers equipped with pulsed lasers has only recently become possible. Microplate readers with the capability to measur

reference images acquired from control cells (Figure 3). One of the disadvantages of photoacceptor bleaching, however, is that the sample is irreversibly destroyed and measurement of dynamic interactions between proteins is not possible. Several photobleaching approaches have been used in the study of GPCR oligomerization from the characterization of 5-HT_{2C} receptor homo-dimers at the cell surface to experiments proving evidence for homo-dimerization and oligomerization of this same receptor at the level of endoplasmic reticulum and Golgi (11,40). Recently, Demarco et al. (41) working with a UV photo-activatable (PA) FRET acceptor, (i.e. PA-GFP), have developed a novel live cell FRET imaging method which can be used to simultaneously measure the mobility of a protein and its dynamic interaction with cyan donor partner proteins. This technique, known as photo-quenching FRET (PQ-FRET), is based on PA-GFP which can be switched from a dark to bright fluorescent state on excitation by a brief pulse of intense 400 nm laser light (42). If the cyan donor partner fusion protein is in close proximity and the correct orientation to the PA-GFP fusion protein then upon photo-activation excitation energy is transferred from the donor to the acceptor and the donor fluorescence becomes quenched. One of the benefits of PQ-FRET is that unlike FRET measurement of sensitized emission from the acceptor, the detection of donor quenching does not require SBT background correction. Another advantage is that in contrast to the photo-destructive technique apFRET, photo-activation of the acceptor in a defined region within the cell allows real time quantification of dynamic donor de-quenching. The main potential of PQ-FRET, however, is that it can be transferred to high-throughput screening as this technique can be used to quantify donor quenching from a substantial number of cells and, therefore, it should be simple to apply PQ-FRET to various multi-well plate formats. Furthermore, some of the commercially available live cell imaging high-throughput screening systems are now equipped with an integrating structured illumination device, (e.g. Arrayscan VTI reader plus Apotome module) to generate confocality images. The structured illumination process detects strong fluorescence wherever focus is sharp and weak fluorescence (blur) where focus is soft. An algorithme focus 7fgenefCaz. F ulcorionpr

Applications of FRET to GPCR quaternary structure

GPCR dimerization, both homo- and hetero-dimerization, has been widely reported as a mode of regulating receptor function (5,44) and FRET-based techniques have been extensively used to study this (45-48). Although FRET analysis of receptor dimerization has contributed to the understanding of receptor internalization and the effects of ligands it is unlikely that, due to the technical issues discussed before, it will prove a generic ligand screening strategy. However, there are relevant examples of fine advances in this technology to study oligomeric receptor complexes in live cells (49,50). An illustration of this can be found in the work of Lopez-Gimenez and colleagues. GPCR multimers are extremely difficult to determine using biochemical and two-chromophore resonance energy transfer methods (51); however, the authors showed an elegant application of three-chromophore FRET (52) to the study of complexes of the α_{1b}-adrenoceptor in living cells (38). Using sequential CFP to YFP to DsRed2, and direct, CFP to DsRed2 FRET, the authors demonstrated that the α_{1b}-adrenoceptor forms oligomeric structures and that disruption of these complexes has severe consequences for cell surface delivery and function of the GPCR. Time-resolved (TR-FRET) and homogeneous time-resolved FRET (HTRF) are good methods to monitor GPCR complexes at the cell surface in living cells. TR-FRET is based on the use of two different labeled antibodies against an N-terminal tag in the receptor. One antibody is normally labeled with a lanthanide, such as europium crussed betha goabe-4(itnd-esc1Hows6ŠŠ5Ô2Ïaní3À3¾*ÊÍu3-B

SNAP tag is a 20 kDa enzyme, based on a O6-alkylguanine-DNA-alkyltransferase (56) that can be fused to the N-terminal end of a GPCR of interest. SNAP reacts specifically with benzylguanine substrates which can be in turn labeled with a FRET partner, e.g. a terbium cryptate donor or d2 acceptor. This complex can be linked to a peptide or small molecule of interest for use in ligand binding assays. The CLIP tag is a modified version of SNAP that reacts specifically with benzylcytosine substrates. The combination of SNAP/CLIP tags and HTRF technologies has been described recently as a means to detect and quantify receptor homo and hetero-dimerization at the cell surface (57). The cell-based and homogeneous nature of this type of assay has opened the gates to new drug development opportunities. Data obtained using Tag-lite® HTRF may shed

strategy that allows visualization of protein-protein interactions within a small living animal. This technology, developed by De and colleagues, combines a mutant red fluorescent protein (mOrange) and a mutant *Renilla reniformis*

receptor B, however, it potentially may elicit a unique hetero-dimer effect specific for that receptor pairing and drug. This set up does not use RET technologies but a similar strategy could be applied to the development of a BRET-based β-arrestin recruitment dependent system, in which one of the two protomers of the GPCR dimer is tagged with a BRET acceptor (e.g. YFP), whilst the second remains untagged. These two proteins are then expressed in cells in the presence of β-arrestin-Rluc and treated with agonists specific for only one of the receptor pairs. The ligand-dependent nature of the system will clearly allow separating specific BRET signal from that obtained from controls. The advantages of this method are quite obvious, one of them being that the system is G protein-independent and, therefore, it may be applied to pairings that couple to different G proteins. Other advantages of this method is that only GPCRs that are at the cell surface will be activated, given the agonist is not cell permeable, therefore avoiding background signals coming from proteins in the endoplasmic reticulum that might have been misfolded.

Conclusions and outlook

There are a number of FRET-based biosensors used to study activation of GPCRs and downstream signaling such as intra-molecular sensors for protein kinase activity (88,89), as well as in monitoring the regulation of cAMP levels using Epac-based FRET biosensors (90) or intracellular calcium levels (37). These are certainly useful for high content and high throughput screening. In terms of GPCR pharmacology, the biophysical techniques outlined here have led to enhanced understanding of many aspects of GPCR activation and regulation mechanisms, and some of the approaches described, such as the use of FlAsH-FRET experiments to substitute the much larger YFP tag have proven very useful in dissecting the range of conformational changes that occur during receptor activation. [illegible]

Figure legends

Figure 1 Optimal requirements for FRET

A. The emission spectrum of the donor (blue) must overlap the absorption spectrum of the acceptor (yellow) for FRET to occur.

B. The dipole moments of both fluorescent proteins (blue and yellow cylinders), represented here as red and black arrows, must be oriented at an angle that is near 0°. Efficient resonance energy transfer can only take place if the distance between the two FRET partners is less than 100 Å.

C. Inter-molecular FRET applied to study receptor dimerization where each GPCR is tagged at the C-terminal tail with a donor or acceptor protein, or in experiments in which a G-protein is tagged with one of the FRET partners and the GPCR as the second one. Intra-molecular FRET using internal YFP and CFP tagging (and also FlAsH) tags within the receptor structure is illustrated on the right panel.

Figure 2 Polarization anisotropy FRET using fluorescent proteins

A schematic diagram illustrates the processes involved in polarisation anisotropy FRET. (A). fluorescent proteins are excited with linearly polarized light (cyan wave), and only those fluorescent proteins whose absorption dipole vector is oriented parallel to the incoming polarized excitation light will be optimally excited. The resultant emitted signal is detected using a vertical analyser that is positioned parallel and perpendicular to the polarization axis (green wave). A decrease in polarization anisotropy FRET is observed if the fluorescent protein rotates during the timescale of the experiment, (B), or if FRET occurs between two interacting proteins which have different orientations, (C). Since fluorescent proteins are of substantial size, resonance energy transfer will occur more rapidly than molecular rotation so depolarization due to FRET can be readily distinguished from the loss of anisotropy that would just occur during rotation. (Adapted from www.microscopyu.com).

Figure 3 Acceptor photobleaching of a mCerulean-mCitrine fluorescent protein tandem

Images presented in the top row show the acceptor, (i), and donor pre-bleach images, (ii and iii),

YFP (BRET[1]), GFP[2] (BRET[2]), or the red-shifted mOrange GPF variant (BRET[3]). Note that donor-acceptor overlap is minimal in the case of BRET[3] whereas in BRET[1] there is considerable donor overlap with the YFP spectrum. mOrange acceptor signal has a highly reduced donor bleed-through.

B. Schematic representation of β-arrestin recruitment assay. In this type of assay the GPCR is tagged with Rluc (blue) and co-expressed with β-arrestin-YFP. Upon ligand (red dot) activation of the receptor β-arrestin-YFP gets recruited to the membrane where it interacts with the tagged receptor, bringing the Rluc and YFP molecules into close proximity and resulting into measurable BRET signal.

C. Example of a BRET-based ligand induced β-arrestin recruitment assay using β$_2$-adrenoceptor Rluc and β-arrestin-YFP in the presence of coelenterazine H. The graph shows a dose-response curve to the agonist isoprenaline after 5 minutes of stimulation performed in a 96-multiwell format.

Figure 5 Illustrations of BRET applications to GPCR dimerisation and of GPCR-G protein interactions

A. Homo- or heterodimer specific BRET. Two receptor tagged with e0 Tw 38ons127.62 535sig3(ons)l Tw 21.

B. Example of a BRET-based, ligand-induced β-arrestin recruitment assay using β2-adrenoceptor-Rluc and β-arrestin-YFP in the presence of coelenterazine h. The graph shows a concentration response curve to the agonist isoprenaline after 5 minutes of stimulation performed in 96 multiwell format.

Acknowledgments

These studies were supported by the Biotechnology and Biosciences Research Council (grant BB/E006302/1) and the Medical Research Council (Grant G0900050).

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FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1

FRET		BRET	
Advantages	Limitations	Advantages	Limitations
Single cell studies	Complex and specific instruments required	Cell based assays	Not as amenable for high-content assays
Positive signals indication of proximity between partners	Potential issues with photobleaching and bleed-through	Highly amenable for high-throughput formats	Some reagents are expensive
Possible to use small molecule tags	Absence of FRET difficult to interpret	No external excitation light source required, no photobleaching or photodamage to cells	Luminescence signal can be too weak to quantify accurately
Possible to use time-resolve formats	GPCRs tagging with fluorescent proteins required	Reduced “signal to noise” ratio permits detection of protein interactions44ghtall	