BRET[1]-assay using the FDSS/µCell imaging plate reader:

monitoring agonist-induced β-arrestin recruitment to a G protein-coupled receptor (GPCR)

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1. Introduction

The GPCRs represent the largest family of cell surface receptors and are the main target for drugs available on the pharmaceutical market. To prevent receptors from both acute and chronic overstimulation, GPCR activity is regulated by an intensively studied mechanism called desensitization or internalization (1).

Following ligand exposure, arrestins interact with phosphorylated GPCRs, uncoupling them from their cognate G protein, blocking further activation and promoting endocytosis (2). Interaction between receptors and arrestins is a measurable functional event in the GPCR-mediated signaling cascade.

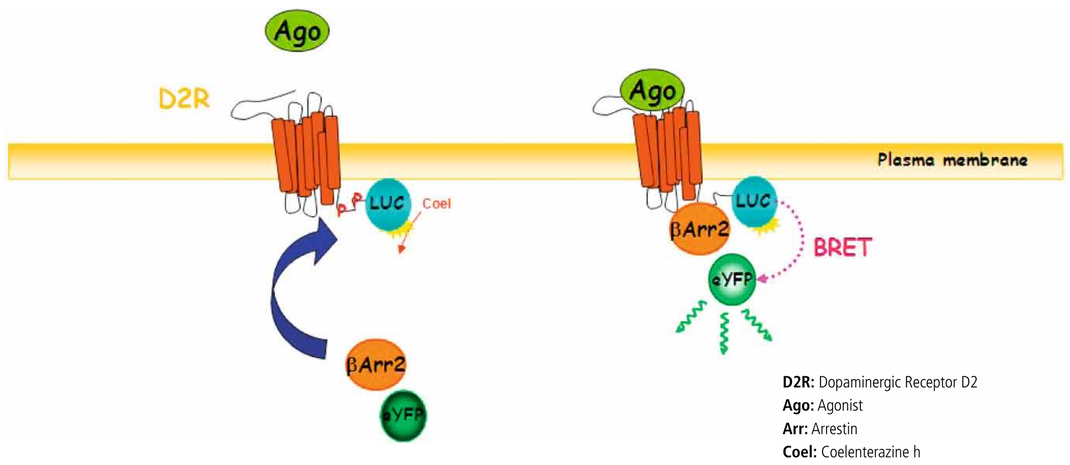
The biophysical technique named Bioluminescence Resonance Energy Transfer (BRET) has been widely used to monitor and quantify agonist-promoted β-arrestin recruitment (3), including in high throughput screenings (HTS) (4).

For the first time, we present this BRET[1] application on the FDSS µCell imaging plate reader (Hamamatsu Photonics) by monitoring the activity of the dopaminergic D2 receptor (short splice form), a prototypic and well characterized GPCR.

2. Principle

Renilla Luciferase (*R*luc) emits light at 480 nm in the presence of its substrate coelenterazine. When a *R*luc-labeled protein is brought into close proximity (<100Å) of an enhanced Yellow Fluorescent Protein (eYFP)-labeled protein, an energy transfer will occur between *R*luc and eYFP, and the latter will emit light at a higher wavelength (530 nm).

Receptor and β-arrestin2 constructs were transiently expressed in CHO cells. Upon receptor activation by the agonist, the eYFP-tagged β-arrestin2 approaches the phosphorylated *R*luc8 tagged-D2s receptor, which promotes the appearance of a BRET signal (cf. fig.1).



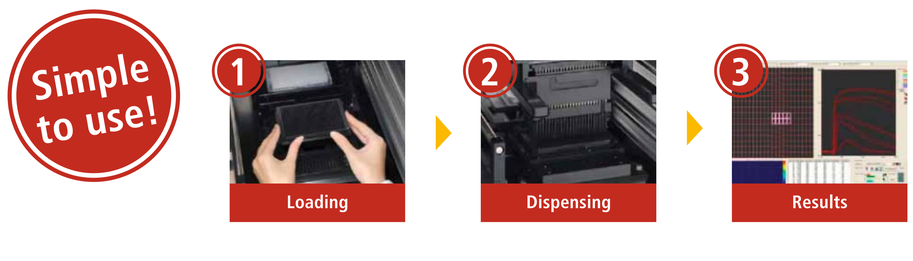
D2R: Dopaminergic Receptor D2
Ago: Agonist
Arr: Arrestin
Coel: Coelenterazine h
eYFP: enhanced Yellow Fluorescent Protein
P: phosphorylation Sites

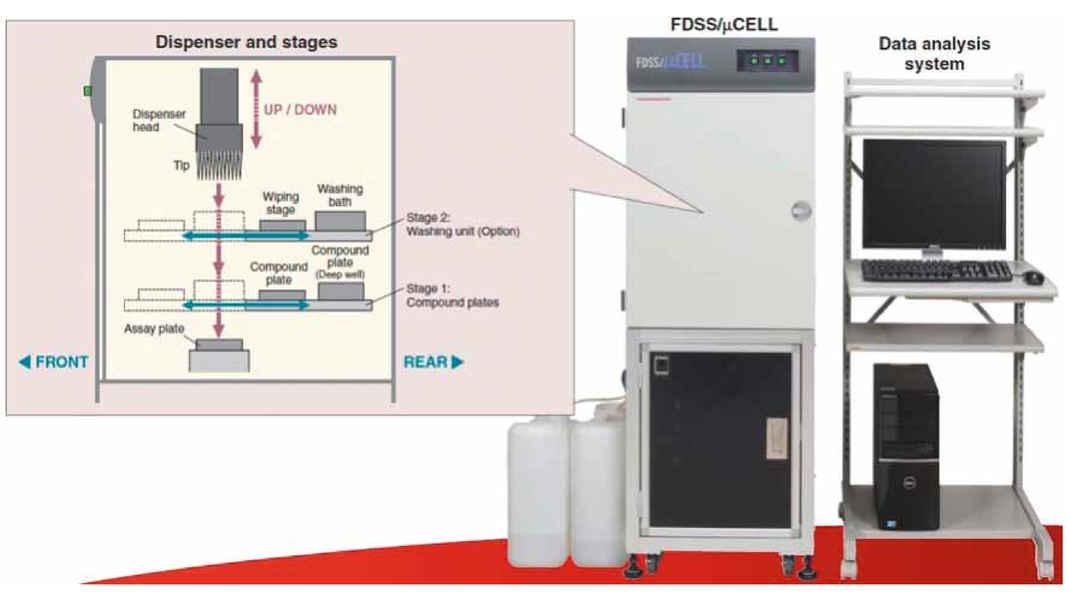
Fig. 1: principle of β-arrestin BRET[1]-assay

3. Experimental procedures

*R*luc8 tagged-D2s receptor (C-terminal) and eYFP-tagged β-arrestin2 (N-terminal) were transfected into CHO cells, and 36 hours after transfection, cells were starved overnight (16 hours) in serum-free medium. The next day, cells were washed twice with PBS plus glucose 1 g/l, detached with PBS plus 3 mM EDTA and resuspended in PBS plus glucose 1 g/l. A Dc protein measurement assay (Bio-Rad) was performed according to the manufacturer's protocol. For BRET monitoring, cells were then distributed (70 µg protein/well, about $4x10^5$ cells) in white (Costar, ref. #3903) or black (Costar, ref. #3603) flat and clear-bottomed 96-well microplates and incubated during 10 minutes in the presence or absence of various ligands before signal collection. BRET between *R*luc and eYFP was measured 9 minutes after the addition of the *R*luc substrate coelenterazine h (5 µM final) (Promega). The BRET[1] signals were calculated by the ratio of emission of eYFP (516-556 nm) to *R*luc8 (460-500 nm).

BRET signals were collected either by a photomultiplier (PMT) reader or by the FDSS/µCELL (Hamamatsu Photonics), a new imaging plate reader for kinetic cell-based assays. The latter can be equipped with one injection head (96, 384) and enables simultaneous injection and detection of all the wells.

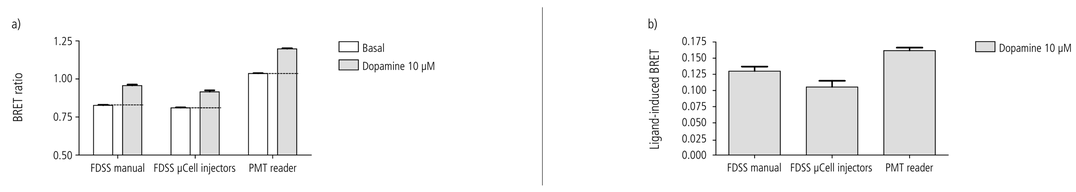




4. Results

Fig 2: agonist-induced β-arrestin recruitment to D2s-R. Real-time measurement of BRET signals in CHO cells. Results are expressed both as raw data ratio (a) and as the difference in BRET signal between cells exposed or not with the agonist (induced BRET) (b).

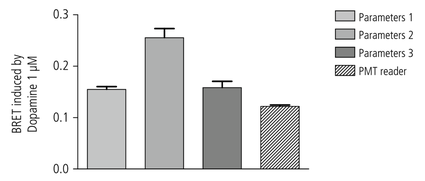
Comments: This experiment was performed by dispensing compounds and substrate manually or by using the dispensing head available on the FDSS µCell. Cells were distributed in **black MW96**.



5. Optimisation experiments

Fig 3: agonist-induced β-arrestin recruitment to D2s-R. Results are expressed as the difference in BRET signal in the presence or absence of the agonist. Data represent the mean ± s.e.m. of two independent experiments.

Comments: This experiment was performed by dispensing compounds and substrate manually. Cells were distributed this time in white MW96.
Different conditions were used for the readings. The best parameters, promoting the best BRET signal, are obtained with an exposure time of 100ms, a sensitivity of 4 and an interval of 1.1sec.

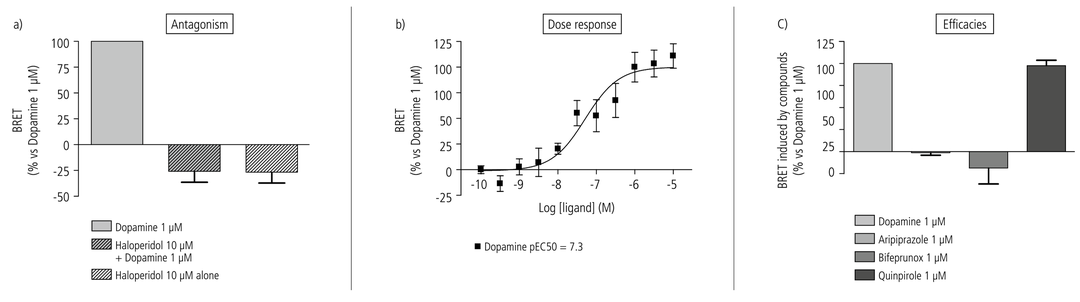


	Parameters 1	Parameters 2	Parameters 3
Exposure time	200 ms	100 ms	30 ms
Sensitivity	5	4	5
Interval	1.8 sec	1.1 sec	0.8 sec

6. Pharmacology

Fig 4: agonist-induced β-arrestin recruitment to D2s-R. Data represent the mean ± s.e.m. of at least 3 independent experiments.

Comments: This experiment was performed by using the best parameters. The antagonist Haloperidol fully reverses the BRET signal induced by dopamine (a). The stimulation by dopamine is dose-dependent (b). Dopamine efficacy can be discriminated from the activity of other compounds (c).



7. Conclusion

This work confirms the ability of the FDSS/µCell to monitor protein-protein interactions, using BRET[1] technology. The main advantages of the FDSS/µCell are its capability to dispense compounds and to read the light emitted simultaneously from the entire 96 well plate, thus potentially allowing HTS. Moreover, the switch time between the two filters for light emission readings is shorter than for the main other devices, which is a precious advantage for BRET measurements. The next step is to validate BRET[2] screening method, more sensitive and suitable for small distance changes.

Bibliography

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