

## Adenosine A<sub>2A</sub> Receptor Ligand Binding Experiments by Using Real-time Single-cell FRET

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**[Abstract]** We designed a fluorescence resonance energy transfer (FRET)-based approach to study the ligand binding constants of the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R). Our assay is based in the interaction of a fluorescent A<sub>2A</sub>R agonist ligand (MRS5424) with an A<sub>2A</sub>R tagged with the cyan fluorescent protein (CFP) at the N-terminus (*i.e.* A<sub>2A</sub>R<sup>CFP</sup>) and expressed in living cells. Thus, upon fast superfusion of the A<sub>2A</sub>R<sup>CFP</sup> expressing cells with MRS5424, the ligand-receptor interaction is determined by single-cell FRET in a real-time mode. Accordingly, our approach allowed immediate 'real-time' readout of the ligand-receptor interaction, thus allowing kinetic binding experiments, a feature impossible to achieve using conventional radioisotope-labelled ligands. In addition, since our assay permitted the visual confirmation of receptor localization it also allowed localized saturation binding experiments.

### **Materials and Reagents**

1. Cell line (*i.e.* HEK-293 cells)
2. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich)
3. Sodium pyruvate
4. L-glutamine
5. Antibiotics: streptomycin and penicillin
6. Fetal bovine serum
7. TransFectin™ Lipid Reagent (Bio-Rad Laboratories)
8. Hank's balanced salt solution (HBSS) (see Recipes)
9. Cell culture medium (see Recipes)

### **Equipment**

1. 18 mm diameter glass coverslips
2. Attofluor holder
3. Inverted Axio Observer microscope (ZEISS) equipped with a 63x oil immersion objective

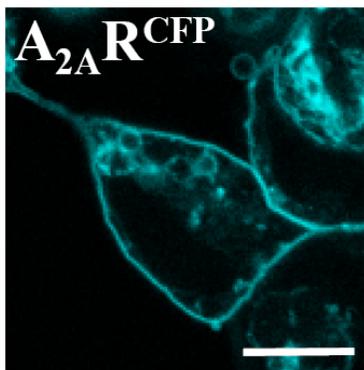
4. Polychrome V (TILL Photonics)
5. Avalanche photodiodes (TILL Photonics)
6. Focal drug application system (ALA Scientific Instruments, OCTAFLOW™)
7. Digidata 1440A analog/digital converter (Molecular Devices)

### **Software**

1. pCLAMP (Molecular Devices)
2. GraphPad Prism (GraphPad Software)

### **Procedure**

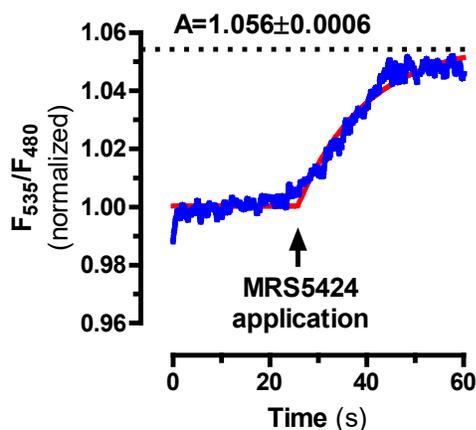
1. Two days before the experiment, the cells (*i.e.* HEK-293 cells) were seeded onto 18 mm diameter glass coverslips and transiently transfected with an A<sub>2A</sub>R construct tagged with the CFP at its N-terminal tail (A<sub>2A</sub>R<sup>CFP</sup>) (Figure 1).



**Figure 1. Cell surface localisation of the A<sub>2A</sub>R<sup>CFP</sup> construct.** HEK-293 cells were transiently transfected with A<sub>2A</sub>R<sup>CFP</sup>, fixed and analyzed by confocal microscopy. The A<sub>2A</sub>R<sup>CFP</sup> was mainly targeted to the cell surface and scarcely accumulated at the intracellular level (Fernández-Dueñas *et al.*, 2013). Scale bar: 10 μm

2. The day of the experiment the transiently transfected cells were mounted in an Attofluor holder and placed on an inverted Axio Observer microscope equipped with a 63x oil immersion objective and a dual-emission photometry system.
3. Then, cells were continuously superfused with a FRET-compatible A<sub>2A</sub>R fluorescent ligand (*i.e.* MRS5424) (Fernández-Dueñas *et al.*, 2012) dissolved in HBSS and applied with the aid of a focal drug application system.
4. A Polychrome V was used as the light source in our dual-emission photometry system. Upon excitation with the corresponding donor excitation wavelength (*i.e.* A<sub>2A</sub>R<sup>CFP</sup>) the

- fluorescent signals of the donor and acceptor fluorophores were detected by avalanche photodiodes and digitized using a Digidata 1440A analog/digital converter.
5. pCLAMP and GraphPad Prism softwares were used for data collection and analysis.
  6. Accordingly, a FRET signal was measured upon donor (*i.e.*  $A_{2A}R^{CFP}$ ) excitation at  $430 \pm 10$  nm [beam splitter dichroic long-pass (DCLP) 460 nm] and an illumination time set to 10 ms at 10 Hz. Then, the emission light intensities were determined at  $535 \pm 15$  nm ( $F_{535}$ ; MRS5424 emission) and  $480 \pm 20$  nm ( $F_{480}$ ;  $A_{2A}R^{CFP}$  emission) with a beam splitter DCLP of 505 nm. No corrections for spillover between channels or direct MRS5424 excitation were made.
  7. The increase in FRET ratio ( $F_{535}/F_{480}$ ) was fitted to the equation:  $r(t) = A \times (1 - e^{-t/\tau})$ , where  $\tau$  is the time constant (in seconds) and  $A$  is the magnitude of the FRET signal (Figure 2). When necessary for calculating  $\tau$ , agonist-independent changes in FRET due to photobleaching were subtracted (Fernández-Dueñas *et al.*, 2012, Fernández-Dueñas *et al.*, 2013).



**Figure 2. Example of FRET ratio fitting.** Time-resolved changes in  $A_{2A}R^{CFP}$  and MRS5424 fluorescence emission signals in single cells transfected with  $A_{2A}R^{CFP}$  (see Figure 1). The ratio (blue trace) of the emission intensities of the MRS5424 ( $F_{535}$ ) and CFP ( $F_{480}$ ) in response to MRS5424 application was recorded from single HEK293 cells expressing the  $A_{2A}R^{CFP}$  (see Figure 1). Shown are the changes induced by rapid superfusion with 2  $\mu$ M MRS5424. The increase of the ratio  $F_{535}/F_{480}$  was fitted by a simple monoexponential curve ( $r(t) = A \times (1 - e^{-t/\tau})$ ) using the GraphPad Prism software which gave a time constant ( $\tau$ ) in this experiment of  $14 \pm 1$  s. This assay is well suited for competitive ligand binding experiments using non-fluorescent compounds (Fernández-Dueñas *et al.*, 2012, Fernández-Dueñas *et al.*, 2013).

## **Recipes**

### 1. HBSS

137 mM NaCl  
 5.4 mM KCl  
 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>  
 0.4 mM KH<sub>2</sub>PO<sub>4</sub>  
 4.2 mM NaHCO<sub>3</sub>  
 1.3 mM CaCl<sub>2</sub>  
 0.5 mM MgCl<sub>2</sub>  
 0.6 mM MgSO<sub>4</sub>  
 5.6 mM glucose  
 pH 7.4

### 2. Cell culture medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with:

1 mM Sodium pyruvate  
 2 mM L-glutamine  
 100 U/ml streptomycin  
 100 mg/ml penicillin  
 5% (v/v) fetal bovine serum

## **Acknowledgments**

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## **References**

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