

High-throughput Flow Cytometry Compatible Biosensor Based on Fluorogen Activating Protein Technology

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• Abstract

Monitoring the trafficking of multiple proteins simultaneously in live cells is of great interest because many receptor proteins are found to function together with others in the same cell. However, existing fluorescent labeling techniques have restricted the mechanistic study of functional receptor pairs. We have expanded a hybrid system combining fluorogen-activating protein (FAP) technology and high-throughput flow cytometry to a new type of biosensor that is robust, sensitive, and versatile. This provides the opportunity to study multiple trafficking proteins in the same cell. Human beta2 adrenergic receptor (β 2AR) fused with FAP AM2.2 and murine C-C chemokines receptor type 5 fused with FAP MG13 was chosen for our model system. The function of the receptor and the binding between MG13 and fluorogen MG-2p have been characterized by flow cytometry and confocal microscopy assays. The binding of fluorogen and the FAP pair is highly specific, while both FAP-tagged fusion proteins function similarly to their wild-type counterparts. The system has successfully served as a counter screen assay to eliminate false positive compounds identified in a screen against NIH Molecular Libraries Small Molecule Repository targeting regulators of the human β 2AR. © 2013 International Society for Advancement of Cytometry

• Key terms

high-throughput; flow cytometry; fluorogen activating protein; live cell; protein trafficking

INTRODUCTION

Seven transmembrane G-protein coupled receptors (GPCRs) are the largest receptor family in the human genome and the dominant family for drug targets. Currently, 19 of the top 50 drugs sold in the US target GPCRs (1,2). GPCRs can function as monomers, dimers, or oligomers and can also crosstalk with other receptor molecules (3–5). The crosstalk between receptors may play a role in heart disease (6) and different forms of cancer (7,8). However, the mechanism of action of many receptor pairs is not known, and the capability of monitoring the behavior of different receptors in the same cell is of great interest.

Current methods to monitor multiple targets trafficking in a single cell are usually based on the fusion between the target protein and a fluorescent tag, such as cyan, green, or yellow fluorescent protein due to their low cytotoxicity. However, the limited expression of GFP on extracellular sites can limit the utility to intracellular trafficking processes.

We have previously reported a high-throughput compatible approach using fluorogen activating protein (FAP) as the reporter protein (9). The binding of specific fluorogens induces a fluorescence signal that can be enhanced up to 10,000 fold (10). In this study, we have characterized cells transfected with AM2.2-beta2

adrenergic receptor (AM2.2- β 2AR) fusion protein, where AM2.2 is a FAP that binds to the fluorogen thiazole orange (TO1) and its derivatives with excitation and emission maxima of 509 and 530 nm, respectively, which is similar to that of enhanced green fluorescent protein. AM2.2- β 2AR was chosen as our model system because the receptor has been studied for more than 50 years, and extensive tools are available to validate the biological relevance of the FAP-tagged receptor with its wild-type counterpart. We previously demonstrated that the fusion FAP tag does not interfere with the normal function of β 2AR. The receptor internalizes normally when it binds to an agonist, and the ligand-induced internalization can be blocked by known β 2AR antagonists. A cell membrane impermeable fluorogen allows us to identify receptors expressed on the cell surface, and thus makes it possible to measure receptor trafficking by flow cytometry. Moreover, the hybrid platform combines high throughput flow cytometry with FAP technology. Together, they provide a complementary toolset to study protein trafficking and to search for new ligands of the target protein with high sensitivity. The model system enabled us to perform high-throughput screening against a 340,000 compound library in 8 days, and it helped us to understand the connection between receptor internalization and downstream signal pathways.

Here we introduced an advanced system that was transfected with both AM2.2- β 2AR and MG13-C-C chemokines receptor type 5 (MG13-CCR5) fusion protein, where MG13 is another FAP that binds to the fluorogen malachite green (MG) and derivatives with excitation and emission maxima of 609 and 649 nm (10,11). We chose these two receptors in our model system because the function of both receptors has been well studied, providing tools and references to compare the function of the fusion proteins with wild-type counterparts (12,13). In addition, the two GPCRs are not known to share any common ligands, and activate different G proteins primarily. This provides us the opportunity to focus on the validation of multiple FAP-tagged targets rather than the potential cross-talk between receptors.

MATERIAL AND METHODS

Materials

Human monocyte U937 cells were transfected with AM2.2- β 2AR and MG13-CCR5 cells at Carnegie Mellon University (CMU) as described (11,14); fluorogens (TO1-2p and MG-2p) are synthesized as described (10); chemokines are from ProSpec-Tany TechnoGene (East Brunswick, NJ); all other chemical biological and are reagents from Sigma if not stated elsewhere.

Production of AM2.2- β 2AR and MG13-CCR5 Expressing Cells

A plasmid expressing the surface displayed fusion protein of FAP AM2.2 to the extracellular N-terminus of the human β 2AR, and another plasmid expressing surface displayed fusion protein of FAP MG13 to the extracellular N-terminus of the murine CCR5 were generated as described previously

(10–11,15). A similar retroviral transfection protocol was applied to stably express AM2.2- β 2AR and MG13-CCR5 in the U937 human monocytic cell line. U937 cells are known to show robust internalization of agonist-stimulated GPCRs and are well-suited for high-throughput flow cytometry assays, being a suspension cell line. The human single chain antibody AM2.2 binds the thiazole orange derivative TO1-2p, while MG13 binds to the malachite green derivative MG-2p respectively with high affinity. The binding increases the fluorescent signal from the fluorogens over 2000-fold (10,15). Both TO1-2p and MG-2p are cell membrane impermeable.

Cell Culture

Cells transfected with both AM2.2- β 2AR and MG13-CCR5 and parental U937 cells were maintained in sterile filtered RPMI 1640 media containing 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, pH 7.4, 20 μ g/mL ciprofloxacin, 2 mM L-glutamine, at 37°C in a water jacketed incubator with 5% CO₂ and 95% air (Forma Scientific, Marietta, OH) in tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at a maximum cell density of 400,000/mL until the day of harvest.

Kinetic and Equilibrium Binding of Fluorogen to Cells

Both parental U937 cells and FAP-tagged receptor transfected cells were harvested and resuspended in serum free RPMI 1640 medium at a final density of 5×10^5 /mL at least an hour before the experiment. An aliquot of 500 μ L of cells was added to sample tubes and the fluorescent signal was collected by an Accuri C6 flow cytometer (BD Accuri Cytometers, MI) equipped with 488 nm and 640 nm lasers and 530/50 nm (FL1) and 675/25 nm (FL4) band pass filters. The signal from TO1-2p bound to AM2.2- β 2AR was detected in the FL1 channel (with 488 nm excitation), and the signal from MG-2p bound to MG13-CCR5 was collected from the FL4 channel (with 640 nm excitation). For kinetic binding, up to 150 nM fluorogen was added to the sample after collecting baseline fluorescence for 30 secs. The experiment is done at 25°C under gentle mixing. For equilibrium binding, both parental U937 cells and dual transfected cells were kept at 4°C under refrigeration for 30 min before exposing to MG-2p ranging from 0.3 nM to 100 μ M. The cells were allowed to bind to the fluorogen for at least 60 min before collecting the fluorescent signal with the Accuri C6 flow cytometer.

Dose Response of Dual Transfected Cells

Freshly harvested dual transfected cells were resuspended in serum free RPMI medium at a final density of 1.5×10^6 /mL. 9 μ L of cells were added to each well of a 384 well plate, followed by the addition of 1 μ L buffer or drug solution. The plate was vortexed and kept in a 37°C tissue culture incubator in 5% CO₂ for 90 min, after which the plate was transferred to a 4°C refrigerator for 30 min before the addition of 3 μ L of 650 nM TO1-2p/220 nM MG-2p fluorogen mixture. The cells were allowed to bind to the fluorogen for a minimum of 30

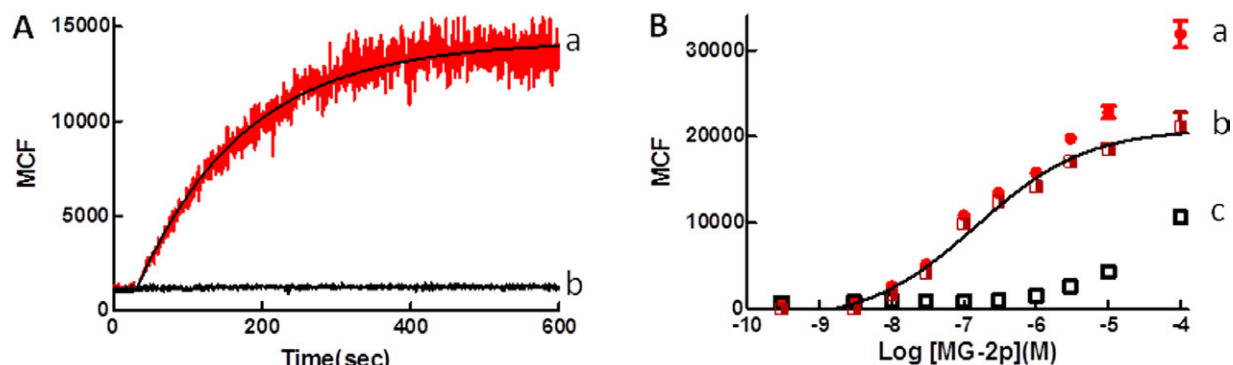


Figure 1. Flow cytometric characterization of MG-2p and surface displayed MG13-CCR5 in AM2.2- β 2AR/MG13-CCR5 dual transfected cells. (A) Kinetic binding of MG-2p to cell surface MG-13. The median channel fluorescence (MCF) in FL4 channel was plotted over time. MG-2p binds specifically to cell surface expressed FAP MG13 with a half-time of approximately 100 s (line a). No fluorescent signal was detected from parental U937 cells in the presence of 100 nM MG-2p (line b). (B) Equilibrium binding of MG-2p to surface displayed MG-13. The specific binding (line b) was obtained by subtracting the nonspecific signal from parental U937 cells (line c) from the total binding (line a). An EC_{50} of ~ 150 nM was obtained under these experimental conditions. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

min on ice, and fluorescent signals from FL1 and FL4 channels were recorded by Accuri C6 cytometer.

High-Throughput Screening Assay

The high-throughput screening protocol was described elsewhere (9). Briefly, cells were harvested, centrifuged, and resuspended in fresh serum-free RPMI medium at a final concentration of 4×10^6 /mL. Five microliters of serum-free RPMI or 14 μ M isoproterenol (ISO) plus 128 nM RANTES (positive controls), 100 nL of test compound, and 3 μ L cells were added to the microtiter plates sequentially by Microflow microplate dispenser (BioTek instruments, VT), and Biomek FX^P laboratory automation work station from Beckman Coulter. The plates were removed from the deck and kept in the 37°C humidified incubator for 90 min before transferring the plate to the 4°C refrigerator for 15 min. Three microliters of a mixture of 650 nM TO1-2p and 220 nM MG2p were added to the cells by Microflow and allowed to incubate for 30 min at 4°C. Fluorescent signals from the plate were immediately detected by CyanTM ADP flow cytometer (Beckman Coulter, CA) connected to a HyperCytTM autosampler (IntelliCyt, Albuquerque, NM).

Confocal Microscopic Imaging

Live cell confocal microscopy images were taken with a Zeiss 510 Meta Confocal Laser Scanning Microscope (Carl Zeiss, Thornwood, NY) equipped with Argon (488 nm) and HeNe (633 nm) lasers and a 63 $\times$ oil immersion objective. Cells were exposed to 1 μ M TO1-2p and 1 μ M MG-2p in the absence or presence of desired compounds for up to 60 min before imaging with the microscope. The green fluorescent signals were collected using a 525/50 nm band pass filter, while the red fluorescent signals were collected using a 680/60 nm band pass filter.

RESULTS AND DISCUSSION

AM2.2- β 2AR/MG13-CCR5 dual transfected cells were characterized by flow cytometry and confocal microscopy

analyses. The binding between fluorogens and their associated FAPs and the biological function of the receptors were tested individually. The interaction between the cell membrane impermeable fluorogen TO1-2p and AM2.2 has been characterized elsewhere (9). TO1-2p binds to cell surface expressing FAP AM2.2 with ~ 2 nM affinity and a $t_{1/2}$ of less than 5 s, where $t_{1/2}$ represents the time for 50% occupancy of the cell surface fluorogen binding sites. Both kinetic and equilibrium binding between cell membrane impermeable fluorogen MG-2p and FAP MG13 are plotted in Figure 1. As illustrated, the binding between MG-2p and its FAP MG13 is also highly specific with essentially no nonspecific binding. At room temperature (25°C), the addition of 100 nM MG-2p resulted in an immediate fluorescent signal increase in MG13-CCR5 transfected cells with $t_{1/2} \sim 100$ s (line a in Fig. 1A). Signals from wild type U937 cells remain unchanged (line b in Fig. 1A). The equilibrium binding curve plotted in Figure 1B revealed that the EC_{50} between MG-2p and surface displayed MG13 is ~ 150 nM. (While the reported MG-2p/cell surface MG13 affinity from the MSDS is ~ 3.2 nM, the result is sensitive to experimental conditions and time after sample preparation (16)). In combination with the TO1-2p/AM2.2 binding results reported earlier, our data suggest that the fluorogen-FAP reporter system is robust with a fast on-rate, adequately high affinity, and low background fluorescence.

In order to validate the biological function of the FAP-receptor fusion protein, we designed a set of high-throughput flow cytometry compatible experiments and validated the results with confocal microscopy.

Dual transfected cells were treated with nine different concentrations of β 2AR agonist isoproterenol (ISO) (17) or CCR5 agonist RANTES (18) alone as controls, or with a mixture of the two drugs. Figure 2A shows the average fluorescence signal from both FL1 (green filled circles, left Y axis, represents the signal from β 2AR) and FL4 (red filled squares, right Y axis, represents the signal from CCR5) channels from the labeled wells. The first quartile of the plate (wells A1 to A12) contains cells treated with increasing concentrations of

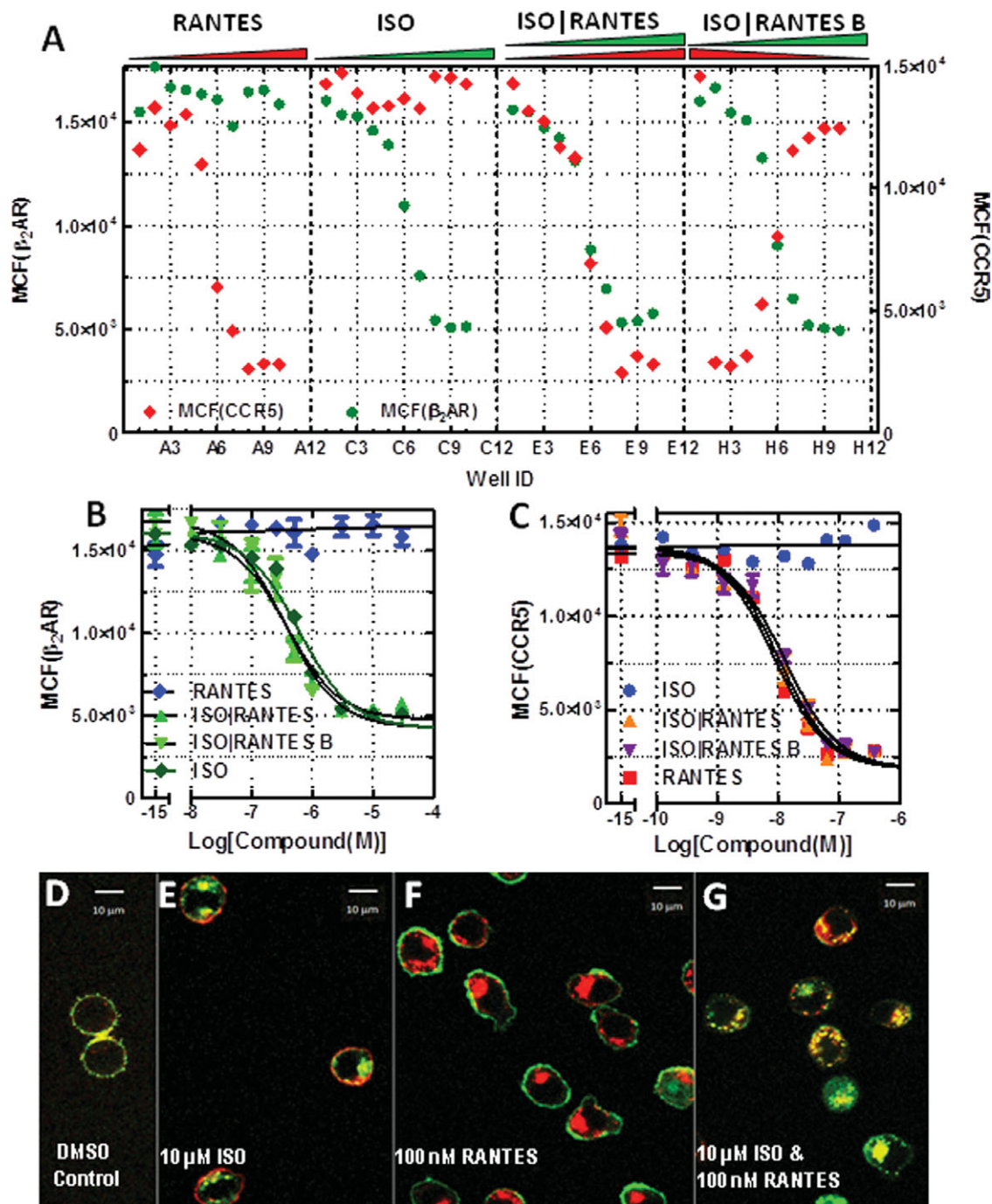


Figure 2. Functional characterization of AM2.2- β 2AR/MG13-CCR5 dual transfected cells. (A) Mean channel fluorescence signal from selected wells of a 96-well plate. The FL1 signal from AM2.2- β 2AR is plotted on the left Y axis and the FL4 signal from MG13-CCR5 is plotted on the right Y axis. As indicated at the top of the graph, cells from wells A1 to A12 were treated with increasing concentrations of RANTES; wells C1 to C12 contained increasing concentrations of ISO; wells E1 to E12 contained increasing concentrations of both ISO and RANTES (labeled as ISO|RANTES); and wells H1 to H12 contained increasing concentrations of ISO and decreasing concentration of RANTES (labeled as ISO|RANTES B). The average concentration dependent curves of the three experimental setups are shown for ISO induced β 2AR internalization (B) and RANTES induced CCR5 internalization (C). Live cell confocal microscopic images confirmed that both AM2.2- β 2AR (green) and MG13-CCR5 (red) are located on the plasma membrane of cells receiving no drug treatment (D). 40 min of 10 μ M ISO treatment induced β 2AR internalization (E), and treatment with 100 nM RANTES only induced CCR5 internalization (F). The combination of both drugs induced the internalization of both receptors (G).

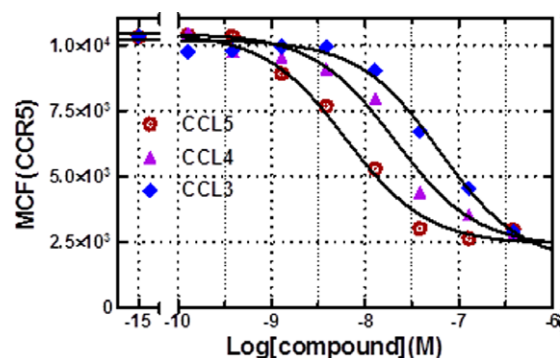


Figure 3. Comparison of dose—response curves of murine CCR5 internalization induced by human chemokines CCL3 (blue diamonds), CCL4 (purple triangles), and CCL5 (red open circles). The measured EC_{50} values are approximately 70 nM (CCL3), 20 nM (CCL4), and 7 nM (CCL5). The fluorescent signal from the cells was plotted against the concentration of test compounds on a log scale. [Color figure can be viewed in the online issue which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com)]

RANTES (128 pM to 38 nM); the 2nd quartile (C1 to C12) contains cells treated with 10 nM to 30 μ M ISO; cells in the 3rd quartile were treated with increasing concentrations of both RANTES and ISO; cells in the last quartile were simultaneously treated with increasing concentrations of ISO and decreasing concentrations of RANTES. The experiment was done in triplicate. For clarity, only one representative set of data from each quartile is plotted in the graph, and the average for all four sets of experiments is presented in Figures 2B and 2C. The fluorescence signal from both receptors decreased in a dose-dependent manner when exposed to the receptor-specific ligand, and no cross-talk was observed between these two GPCRs. The measured EC_{50} was very similar regardless of the set up of these experiments, and yielded $0.45 \pm 0.15 \mu$ M for ISO induced β 2AR internalization and 11 ± 2 nM for RANTES induced CCR5 internalization. The amount of off-target drug does not appear to affect the behavior of the other receptor. These values are in close agreement with reported affinities for ISO- β 2AR binding (17) or RANTES-CCR5 binding (high and low affinity 0.022 and 18 nM (19)).

To further confirm the action of the receptors we performed confocal microscopy to monitor receptor trafficking in live cells. As shown in Figure 2D, both β 2AR (green) and CCR5 (red) are visualized on the plasma membrane of untreated cells; β 2AR was internalized after 40 min treatment with 10 μ M ISO (Fig. 2E) while CCR5 remain on the surface; when cells were treated with 100 nM RANTES, CCR5 was detected inside the cells and β 2AR remained on the cell surface (Fig. 2F). However, both receptors were observed in the cytoplasmic compartment of the cells when treated with a mixture of the two drugs (Fig. 2G). It is worthwhile noting that it required 60 min incubation with 10 μ M ISO for ~90% of surface β 2AR to internalize (9), whereas 30 min incubation is sufficient to induce maximal internalization of CCR5 in the presence of RANTES. This observation is consistent with the rapid binding of RANTES to CCR5 and the fast internalization rate of CCR5 reported in literature (20,21). Together,

these confirm that the MG13 tag does not interfere with the normal function of CCR5.

The function of cell surface expressed FAP- β 2AR has been previously characterized (9). We further investigated the function of MG13-CCR5 by comparing receptor internalization induced by three different human chemokines: CCL3 (MIP-1 α), CCL4 (MIP-1 β) in addition to RANTES (CCL5), dose-dependent receptor internalization data is presented in Figure 3. As illustrated, the three human chemokines induced murine CCR5 internalization with EC_{50} between 7 nM for RANTES to 70 nM for MIP-1 α . The reported affinity of human chemokine binding to human CCR5 is between 0.2 and 10 nM, with MIP-1 α < MIP-1 β < RANTES (20). However, Mack et al. (22) reported that the affinity between human chemokines and murine CCR5 is an order of magnitude lower measured by a CCR5 down-regulation assay in murine natural killer cells. These results are in overall agreement with our observations, indicating that the transfected murine FAP-CCR5 functions normally compared to its wild-type counterpart.

The feasibility of high-throughput flow cytometry with the dual transfected cells was validated by screening against the Prestwick Chemical Library (PCL) that contains ~1,200 off-patent drugs, including 10 active β 2AR agonists but no chemokine agonists. Fourteen molecules, including all 10 β 2AR agonists, induced β 2AR internalization in a dose-response manner. The measured EC_{50} of these molecules inducing β 2AR internalization in dual transfected cells are in close agreement with that obtained from U937 cells transfected with β 2AR alone (9). On the other hand, none of the PCL compounds appeared to induce CCR5 internalization. These results were not surprising because the PCL does not contain any chemokine-like molecules.

The characterized cell line was then used in a high-throughput flow cytometry-based approach to validate the activity of ~1,100 molecules previously identified from a 340,000 compound library as reported in PubChem (PubChem AID: 588765). These molecules were cherry picked because they were “active” in an HTS assay for β 2AR agonists (PubChem AIDs: 504448 (project summary) and 504454 (primary screen)). Since there is no ligand shared between adrenoceptor β 2AR and chemokine receptor CCR5 reported in the literature, a compound that simultaneously reduces the fluorescence signal from both β 2AR and CCR5 is most likely to be a false positive hit.

Figure 4 shows the analyzed results from a complete 384 well plate. Signal from the FL1 channel (β 2AR) is plotted on the left Y axis, and signal from the FL4 channel (CCR5) is plotted on the right Y axis. Each dot in the graph represents the average channel fluorescence of cells from a single well, where green half circles and red half diamonds represent the FL1 signal from AM2.2- β 2AR and the FL4 signal from MG13-CCR5, respectively. Open dark green and dark red circles represent FL1 and FL4 signals from the negative control (NCntrl) wells when cells only received DMSO. Open diamonds represent FL1 and FL4 signals from positive control (PCntrl) wells where cells were treated with 10 μ M ISO and 13 nM RANTES. In this

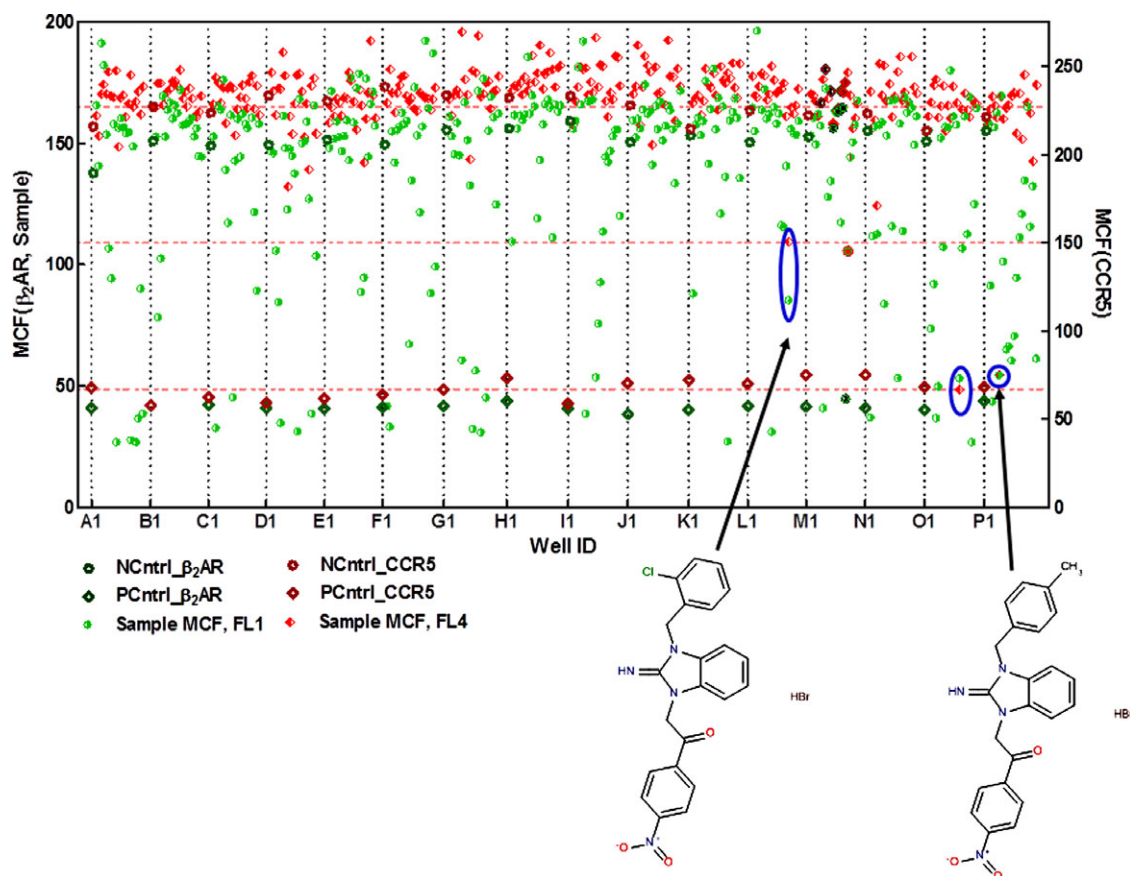


Figure 4. Analyzed screening data from a complete 384 well plate collected with Cyan^{ADP} flow cytometer connected to HypercytTM auto-sampler. The signal from AM2.2- β 2AR is plotted on the left Y axis, and the signal from MG13-CCR5 is plotted on the right Y axis. Each dot in the graph represents the mean channel fluorescence (MCF) of cells from a single well containing 10 μ M test compounds (green half circles and red half diamonds) except for the controls. Cells from the first row of the plate were treated with 10 μ M ISO (open green diamonds) and 13 nM RANTES (open red diamonds) and served as the positive controls. Cells from the 2nd row of the plate were exposed to 1% DMSO and served as negative controls (open green and red circles). Dotted pink lines from top to bottom indicate the RVs of 100, 50 and 0 for the CCR5 signals, respectively. The blue circles marked the wells where cell surface fluorescence from both receptors were significantly decreased. Chemical structures of test compounds in the marked wells were similar as shown in the insets. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

high-throughput assay, we evaluated the activity of each compound by calculating its response value (RV):

$$RV = \frac{MCF_{Sample} - MCF_{NCntrl,avg}}{MCF_{PCntrl,avg} - MCF_{NCntrl,avg}} \times 100$$

where MCF represents mean channel fluorescence. RV indicates the level of fluorescent signal depletion, and an RV of 0 indicates that the compound behaves like the negative control DMSO which has no affect on the surface receptor. An RV of 100 indicates that the compound reduced cell surface fluorescence to the same level as the positive controls, ISO and RANTES. In the approach described, we defined an active compound for β 2AR with an RV of > 42 , and an active compound for CCR5 when the RV is > 50 . Red dotted lines in the figure show RVs of 0, 50, and 100 for the CCR5 signal.

As illustrated in the figure, many compounds appeared to significantly reduce the fluorescent signal from β 2AR, but only a handful of the compounds had a significant impact on the

CCR5 signal. The three wells that have an RV of 50 or higher for the CCR5 signal all show reduced fluorescence signal from β 2AR (marked with red ellipses). From all 1,100 compounds screened by this assay, only 9 of them simultaneously reduced the fluorescence signal from β 2AR and CCR5. Because of the low possibility of ligand cross talk between β 2AR and CCR5, all 9 compounds were considered false positives and removed from our list of potential β 2AR agonists. It is noteworthy that there is observed chemical similarity in this small group of compounds that affected both receptors, such as two of the compounds identified in the plate shown in Figure 4, marked with red circles (see insets in Fig. 4). The only difference between these two compounds is an ortho -Cl versus a para-CH₃ endgroup. This not only demonstrates the successful application of the two color/dual receptor/single cell system in a high-throughput flow cytometry format, but again validates the biological relevance of the assay.

These experiments indicate that the two FAP-tagged receptors functioned in a remarkably similar fashion to their

natural counterparts. The system can be used to quantitatively measure the amount of both β 2AR and CCR5 ligands, and to serve as a model system for FAP-based biosensors. The concept can easily be adapted to other receptor pairs of interest such as GPCR/epidermal growth factor receptor pair with crosstalk activity, the virus entry receptor/coreceptor pair CD4/CCR5, receptors that form hetero-dimers such as human serotonin 5-HT_{2A} receptor and dopamine D₂R receptor, and the chemokine receptor complexes CCR5/CXCR4, CXCR5/CXCR4, CXCR3/CXCR7, etc. (4). The system could also be adapted to track the behavior of a receptor pair due to drug synergy effects to understand the mechanism of action.

In conclusion, after the successful development of a FAP-based high-throughput flow cytometry platform with a single receptor, we extended our analysis to a two color/dual receptor single cell system. The system has been validated by confocal microscopy imaging and flow cytometry. Both fluorogens bound to a specific FAP with no detectable cross-reaction. The FAP tag that fused to the N-terminus of the receptors does not interfere with the normal function of the receptor, and the EC₅₀ values of ligand induced receptor internalization measured by the system are consistent regardless of the amount of non-associated ligand in solution. We adapted the dual transfected cell system in a high-throughput flow cytometry assay to eliminate compounds that reduced the fluorescent signal from both β 2AR and CCR5, and to verify compounds that have an impact on the β 2AR signal, only. Of the ~1,200 compounds screened, nine compounds significantly decreased the fluorescent signal from both receptors, including two that share similar chemical structure. These results suggested the two color/dual receptor/single cell system reported herein is biologically pertinent, sensitive and high-throughput ready. In addition to the obvious advantage of increasing the throughput of drug screens, this system opens the door for simultaneously monitoring the trafficking and interaction of multiple receptors in the same cell. In addition to the TO1-2p-AM2.2 and MG-2p-MG13 fluorogen-FAP binding pairs discussed here, other fluorogen-FAP binding pairs with different excitation/emission profiles are also under investigation, such as fluorogen dimethylindole red and its specific FAP named K7 (23). The unique feature of low fluorescent background of fluorogens and FAP alone and high yield after binding also increase the possibility of studying the behavior of low expression receptors.

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