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Investigating signaling consequences of GPCR trafficking in the endocytic pathway

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Abstract

Ligand-dependent regulation of adenylyl cyclase by the large family of seven-transmembrane G protein-coupled receptors (GPCRs) represents a deeply conserved and widely deployed cellular signaling mechanism. Studies of adenylyl cyclase regulation by catecholamine receptors have led to a remarkably detailed understanding of the basic biochemistry of G protein-linked signal transduction and have elaborated numerous mechanisms of regulation. Endocytosis of GPCRs plays a significant role in controlling longer-term cellular responses, such as under conditions of prolonged or repeated receptor activation occurring over a course of hours or more. It has been more challenging to investigate regulatory effects occurring over shorter time intervals, within the minutes to tens of minutes spanning the time course of many acute cAMP-mediated signaling processes. A main reason for this is that biochemical methods used traditionally to assay changes in cytoplasmic cyclic AMP (cAMP) concentration are limited in spatiotemporal resolution, and typically require perturbing cellular structure and / or function for implementation. Recent developments in engineering genetically encoded cAMP biosensors linked to optical readouts, which can be expressed in cells or tissues and detected without cellular disruption or major functional perturbation, represent a significant step toward overcoming these limitations. Here we describe the application of two such cAMP biosensors, one based on enzyme complementation and luminescence detection and another using Förster resonance energy transfer and fluorescence detection. We focus on applying these approaches to investigate cAMP signaling by catecholamine receptors, and then on combining these analytical approaches with manipulations of receptor endocytic trafficking.

Keywords

Endosome; cyclic AMP; clathrin; dynamin; fluorescence microscopy; luminescence imaging

1. Introduction

Much of what is presently known about cellular GPCR signaling has been inferred from analysis of semi-intact cells, cell extracts, or isolated membrane fractions using biochemical methods. Such approaches have provided extremely powerful mechanistic insight, and have

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led to the appreciation of a complex set of regulatory processes that affect GPCR signaling activity over a wide temporal range. Endocytic membrane trafficking processes have been recognized for many years to impact GPCR-mediated signaling responsiveness after prolonged or repeated exposure to agonist ligands and drugs, typically over a period of hours or more. With increasing interest in more rapid regulatory effects, and toward elucidating the subcellular localization and dynamics of particular protein interactions mediating GPCR function and regulation, there is a need to investigate signaling processes in intact cells and with spatiotemporal resolution exceeding that typically available using conventional biochemical assays.

Considerable recent progress has been made in engineering genetically encoded biosensors to specifically detect a wide variety of metabolites and signaling mediators in unperturbed, or minimally perturbed, cells and tissues. A number of useful biosensors of cytoplasmic cAMP are now available, most based on linking specific cAMP binding domains to conformation-dependent readouts such as enzyme complementation (Fan et al., 2008) or Förster resonance energy transfer (FRET) (Lohse et al., 2012; Zhou et al., 2012). The present chapter will discuss application of one example of each approach to investigate regulation of cytoplasmic cAMP by catecholamine receptors in cultured cells. We then discuss experimental manipulations of the endocytic pathway, and of specific GPCR engagement with the endocytic pathway, which can be combined with optical biosensor technology to investigate the impact of receptor endocytic trafficking on the cellular cAMP response. Studies using this combination of experimental approaches have revealed a previously unanticipated role of endocytic membranes in supporting canonical GPCR - Gs - adenylyl cyclase signaling, and suggest that endocytosis may significantly impact acute as well as longer-term G protein-linked cellular signaling responses (Calebiro et al., 2009; Feinstein et al., 2011; Ferrandon et al., 2009; Kotowski et al., 2011; Mullershausen et al., 2009; Werthmann et al., 2012).

2. Luminescence-based assay of acute cAMP regulation in cell populations

Intramolecular enzyme complementation has emerged as a powerful approach for detecting many signaling mediators and metabolites in intact cells. Split luciferase linked to various AMP binding domains can provide a convenient way to detect increases in cytoplasmic cAMP concentration in a cell population (Fan et al., 2008). Typically these sensors are engineered so that binding of cAMP stabilizes a conformational change that complements the active site and results in increased luciferase activity. A problem with early versions of such biosensors was poor reversibility, limiting temporal resolution and thus obscuring the regulation of acute signaling effects. We have had good experience with a commercially marketed sensor (pGloSensor-20F, Clontech), based on cyclic-permuted split luciferase fused to a cAMP binding domain modified from the RIIβB regulatory domain of protein kinase A (Fan et al., 2008). We have found that the cAMP-stimulated luciferase activity produced by this sensor can be reversed within tens of seconds in intact cells (J Tomshine, unpublished). We describe a relatively economical system, taking advantage of an existing electron-multiplying charged coupled device (EM-CCD) camera that is used otherwise in the laboratory for fluorescence microscopy, to detect luminescence changes representing regulation of cytoplasmic cAMP concentration in cell populations aliquoted into multi-well

plates. A PMT-based plate luminometer could be used as well, provided that a unit with sufficient sensitivity and acquisition speed is available. In our experience the EM-CCD approach is advantageous for detecting changes in cytoplasmic cAMP accumulation simultaneously in multiple samples, and with temporal resolution sufficient to reliably report increases in cytoplasmic cAMP occurring on the order of seconds.

2.1 Materials

2.1.1 Cell culture—

1. HEK-293 cells passage 20–50 (ATCC:CRL-1573)
2. 10 cm and 24 well tissue culture plates (Costar)
3. Dulbecco's Modified Eagle's Medium-high glucose (DMEM) supplemented with 10% fetal bovine serum (Sigma)
4. Dulbecco's Modified Eagle's Medium-high glucose (DMEM) Phenol Red free Imaging media supplemented with 30mM HEPES (Invitrogen)
5. Lipofectamine 2000 (Invitrogen)
6. Opti-MEM (Invitrogen)
7. Poly-D-Lysine (Sigma P0899)
8. (−)Isoproterenol (Sigma or RBI)
9. pGloSensor-20F (Promega)
10. Luciferin (Biogold)
11. Forskolin (Sigma-Adlrich)

2.1.2 Imaging equipment and settings—

1. Electron multiplying CCD sensor (Hamamatsu C9100-13) fitted with 8.5mm f/1.3 portrait lens (Edmund Scientific).
2. Light-proof gel documentation cabinet with internal heater to achieve 37°C internal temperature (we use a simple resistive heater and thermostatic controller purchased from Omega Scientific, mounted well outside of the camera's field of view to reduce glare in the luminometry image due to infrared emission that is generated by the heater and can be detected by the camera).
3. PC running image acquisition software interfaced to camera (we use Micromanager, micromanager. org, running as a plug-in to ImageJ, rsb.info.nih.gov/ij/).

2.2 Methods

2.2.1 Cell preparation—

1. Plate HEK293 cells on 10cm dishes.

2. Transfect pGloSensor-20F (figure 1A) using lipofectamine 2000, following manufacturer's protocol, 24 h prior to luminescence assay.
3. On the day of luminescence assay, dissolve poly-D-lysine in sterile water (50 mg/ml) and place1 ml in each 24 well culture dish for 15 min at room temperature. Wash away poly-D-lysine with sterile water (3 washes) and dry the culture dishes.
4. Plate ~200,000 cells / well in 500μl DMEM onto the freshly coated wells and let them seed for 5 h.
5. Equilibrate cells for 1 h in the presence of 250μg /ml luciferin (Biogold) in 250μl DMEM without phenol red and no serum supplemented with 30mM HEPES (Invitrogen) (figure 1B).

2.2.2 Imaging—

1. Turn on the camera and the temperature controlled lightproof cabinet (37°C) 30 min before data acquisition.
2. Place the plates containing cells from section 2.2.1(5) and focus the plate on the EM-CCD sensor, with EM gain set to zero, using room light and video rate readout.
3. Close the light-proof cabinet, increase EM gain to 100 and record basal frames by sequential 10 s exposure. Close camera shutter and open cabinet to add agonist as desired to particular wells (e.g. varying concentrations of Isoproterenol) diluted in 250μl DMEM without phenol red and no serum supplemented with 30mM HEPES (Invitrogen).
4. In each multi-well plate measure a reference value of luminescence in the absence of any ligand addition (basal) and in the presence of 5 μM Forskolin (forskolin-stimulated signal); In our HEK293 cells this stimulates a moderate amount of receptor-independent activation of adenylyl cyclase that is equivalent (in peak luminescence intensity) to approximately two-thirds of the signal elicited by full activation (i.e., in the presence of a saturating isoproterenol concentration) mediated by the endogenous complement of β2ARs present in these cells.
5. Close the light-proof chamber and continue to collect sequential luminescence images every 10 s. Acquisition times and EM gain can be adjusted as needed for the experiment and to empirically optimize signal-to-noise of the data. With our camera and for 10 s acquisition we typically use an EM gain of 100.
6. Using ImageJ, determine mean luminescence values as a function of time for each well (figure 1B).

2.2.3 Analysis—

1. Calculate integrated luminescence intensity detected from each well after background subtraction. Our camera-based system also requires correction of intensity values for vignetting, an optical artifact caused by the limited aperture afforded by the (relatively inexpensive) lens used to focus the image on the EM-CCD sensor. This is determined based on position of each well relative to the

center of field, computed using the imaging toolbox of Matlab (Math Works). A better solution would be to use a wider-aperture lens or a sufficiently sensitive plate-based luminometer, if available, for which vignetting is either less of an issue or not an issue at all.

2. Normalize the average luminescence value measured across duplicate wells to the forskolin-stimulated value measured on that plate (figure 2).

3. We typically use the forskolin-normalized luminescence signal as the cAMP readout directly, and find that this value is reliable across experiments. In principle this arbitrary readout scale could be calibrated to an absolute cAMP concentration, but we have not found a practical way to accomplish this without introducing significant additional experimental error.

3. FRET imaging of acute cAMP regulation in individual cells

cAMP biosensors are also available that couple a conformational change stabilized by AMP binding to a change in Förster resonance energy transfer (FRET) between linked protein labels. Here we describe application of a FRET-based cAMP sensor, Epac1-cAMPs, which is based on a cAMP binding domain derived from Epac1 fused to spectrally shifted green fluorescent protein variants (Calebiro et al., 2009). cAMP binding to this biosensor results in a decreased FRET signal and, in our hands, this approach offers slightly better temporal resolution than the luminometry-based biosensor described above. Epac1-cAMPs also allows single cell analysis of cAMP changes using a conventional epifluorescence microscope. We describe application of this FRET-based cAMP biosensor for single-cell analysis of FRET using a basic sensitized emission fluorescence microscopy setup.

3.1 Materials

3.1.1 Cell culture—

1. HEK-293 cells passage 20–50 (ATCC:CRL-1573)

   10 cm conventional tissue culture dishes (Costar) and 3.5 cm glass bottom dishes (MatTek)

2. Dulbecco's Modified Eagle's Medium-high glucose (DMEM) supplemented with 10% fetal bovine serum (Sigma)

3. Dulbecco's Modified Eagle's Medium-high glucose (DMEM) Phenol Red free imaging media supplemented with 30mM HEPES (Invitrogen)

4. Effectene (Qiagen)

5. Poly-D-Lysine (Sigma P0899)

6. Plasmid encoding Epac1-cAMPs, a FRET-based cAMP sensor based on the Epac1 binding domain cloned into pcDNA3 (Calebiro et al., 2009)

7. Plasmid encoding the donor fluorophore (pECFP, Clontech)

8. Plasmid encoding the acceptor fluorophore (pEYFP, Clontech)

3.1.2 Imaging equipment—

1. Inverted epifluorescence microscope (we use a Nikon TE2000) fitted with 20x NA0.4 objective, electronically shuttered mercury arc lamp, JP4 PC dichroic mirror (Chroma 104947), and motorized excitation and emission filter wheels (Lambda 10 system, Sutter Instruments) containing the following dichroic bandpass filters: Excitation filter wheel - S436/10 (CFP exciter, Chroma 51232), S500/20 (YFP exciter, Chroma 51724); Emission filter wheel- S470/30 (CFP emitter, Chroma 52479), S535/30 (YFP emitter, Chroma 51706).
2. Temperature/CO2-controlled imaging chamber (We use a homemade unit but there are various commercial vendors).
3. Cooled CCD camera (Roper CoolSnap or equivalent)
4. PC running image acquisition software (e.g., Micromanager) interfaced to the Lambda 10 controller and camera.

3.2 Methods

3.2.1 Cell preparation—

1. Plate HEK293 cells on 10cm dishes.
2. Transfect Epac1-cAMPs plasmid using Effectene, following manufacturer's protocol, 48 h prior to assay. Also transfect cells singly with pECFP and pEYFP for direct excitation and bleedthrough controls (see below). 24 hours before assay, plate transfected cells onto Matek dishes (10^4 - 10^5 / cm^2).
3. On the day of experiment, transfer cells to phenol red-free imaging medium.

3.2.2 Imaging—

1. Turn on microscope and temperature/CO_2-controlled environment to achieve 37°C and 5% CO_2 prior to imaging.
2. Place a 3.5cm dish of Epac1-cAMPs transfected HEK293 cells on the stage, focus on a field with transfected cells and empty space.
3. Collect sequential images at the desired frequency for the desired duration. Acquire the following 3 images at every time point:
    a. FRET image (for determination of $\mathbf{I_{FRET}}$): CFP exciter, YFP emitter
    b. CFP image (for determination of $\mathbf{I_{CFP}}$): CFP exciter, CFP emitter
    c. YFP image (for determination of $\mathbf{I_{YFP}}$): YFP exciter, YFP emitter

    Note that optimal camera and arc lamp intensity settings will vary depending on equipment. Images must be collected in the linear range of camera detection and at the lowest practical illumination intensity and exposure settings to minimize photobleaching and toxicity.

4. Agonist can be added to the dish at any point in the time-lapse sequence, though acquisition of at least 2 baseline (untreated) frames is recommended.
5. Steps 2-4 can be repeated for multiple dishes of Epac1-cAMPs transfected HEK293 cells on a given day of imaging.
6. Place a 3.5cm dish of pECFP (only) transfected HEK293 cells on the stage, focus on a field with transfected cells and empty space.
7. Collect 1 set of the following images to determine a factor to correct for bleedthrough of donor (CFP) fluorescence emission into the YFP emission bandpass (BT_{DONOR}):
    a. $\mathbf{I_{FRET\ (CFP\ ONLY)}}$: CFP exciter, YFP emitter
    b. $\mathbf{I_{CFP\ (CFP\ ONLY)}}$: CFP exciter, CFP emitter Intensity and acquisition settings should be the same as those used above.
8. Place a 3.5cm dish of pEYFP (only) transfected HEK293 cells on the stage; focus on a field with transfected cells and empty space.
9. Collect 1 set of the following images to determine a factor to correct for direct excitation of acceptor by the CFP excitation ($DE_{ACCEPTOR}$):
    a. $\mathbf{I_{FRET\ (YFP\ ONLY)}}$: CFP exciter, YFP emitter
    b. $\mathbf{I_{YFP\ (YFP\ ONLY)}}$: YFP exciter, YFP emitter

    Intensity and acquisition settings should be the same as those used above.

3.2.3 Analysis—

1. Using ImageJ, create a stack of I_{FRET} images for a given time lapse series.
2. Draw an ROI around each cell and measure integrated intensity at each time point to obtain a numerical value for I_{FRET}.
3. Move the ROI to an area on the image stack where no cells are present and measure integrated intensity at each time point to obtain a numerical value for background (BG_{FRET})
4. Repeat this for all cells within the field.
5. Repeat steps 1-5 with I_{CFP} and I_{YFP} image series to obtain numerical values for I_{CFP}, BG_{CFP}, I_{YFP}, and BG_{YFP}.
6. Open the $\mathbf{I_{FRET\ (CFP\ ONLY)}}$ and $\mathbf{I_{CFP\ (CFP\ ONLY)}}$ images. Create a stack.
7. Draw an ROI around a cell and determine BT_{DONOR} constant values by measuring the ratio of integrated FRET intensity to integrated CFP intensity in cells expressing only CFP (BT_{DONOR}= $\mathbf{I_{FRET\ (CFP\ ONLY)}}$/ $\mathbf{I_{CFP\ (CFP\ ONLY)}}$).
8. Open the $\mathbf{I_{FRET\ (YFP\ ONLY)}}$ and $\mathbf{I_{YFP\ (YFP\ ONLY)}}$ images. Create a stack.

9. Draw an ROI around a cell and determine $DE_{ACCEPTOR}$ constant values by measuring the ratio of integrated FRET intensity to integrated YFP intensity in cells expressing only YFP ($DE_{ACCEPTOR}$= $\mathbf{I_{FRET\ (YFP\ ONLY)}}$/ $\mathbf{I_{YFP\ (YFP\ ONLY)}}$).

10. Obtain a normalized FRET value for each cell at each time point using the following equation (figure 3):

$$nFRET = [\ (I_{FRET} - BG_{FRET}) - (I_{CFP} - BG_{CFP})BT_{DONOR} - (I_{YFP} - BG_{YFP})DE_{ACCEPTOR})\ ] / I_{CFP}$$

4. Experimental manipulation of GPCR endocytic trafficking

The biosensors described above have many potential applications, and are not specific to examining the effects of endocytosis. However, these biosensors are compatible with various experimental manipulations of GPCR endocytic trafficking, and thus facilitate studies of the signaling consequences of GPCR endocytic trafficking. Some experimental manipulations that we have found useful are briefly described below.

4.1 Receptor mutation

For some GPCRs, such as the β2 adrenergic receptor (β2AR), we know enough about structural determinants engaging the endocytic machinery that it is possible to manipulate receptor trafficking properties selectively by mutation, and without affecting other cargoes. One concern with receptor mutation is the effect of specific mutations on other (non-endocytic) receptor functions. A second concern with the use of receptor mutation is that it requires study of recombinant rather than endogenous receptors, with associated potential complications of over-expression. We usually address this by generating stably transfected cell clones selected for a defined level of receptor expression (we typically strive for 100 – 300 pmol / mg cell protein, determined by saturating radioligand binding assay), and taking care to compare mutations in multiple cell clones all selected for comparable expression. A third concern with the use of recombinant receptors is resolving the signaling consequences of their activation from the effects of endogenous receptors present in the same cells. Our HEK293 express endogenous β2ARs but not D1 dopaminergic receptors (D1Rs). Therefore one approach is to focus on GPCRs such as the D1R in HEK293 cells, where the recombinant receptor can be examined unambiguously using D1R-selective agonists. Another approach is to use a knockdown / replacement strategy. Based on our experience using HEK293 cell clones expressing recombinant β2ARs at >100 pmol / mg, we find that recombinant receptors become the dominant determinant of cellular cAMP accumulation. This makes it feasible to assess mutational effects by simply neglecting the endogenous pool, as a first approximation, but caution is advised and this approach is probably not sufficient for detailed examination of mutational effects. In this case, one should search for cells that do not endogenously express the receptor of interest, or devise a sufficient knockout or knockdown strategy to deplete the endogenous receptor.

4.1.1 Inhibiting β2AR endocytosis—Regulated endocytosis of β2ARs can be inhibited by mutating a small cluster of phophorylatable residues in the receptor's proximal cytoplasmic tail (Hausdorff et al., 1991). This strongly inhibits β2AR internalization but does so by reducing receptor recruitment of β-arrestins. Accordingly, signaling effects of

such mutation are not necessarily specific to endocytosis, and could represent other effects such as inhibited desensitization and / or arrestin-linked signaling.

4.1.2 Manipulating β2AR trafficking after endocytosis—β2AR membrane trafficking after endocytosis can also be manipulated by mutation. Lysine mutation of the β2AR, presumably by preventing receptor ubiquitination and association with ubiquitin-dependent sorting proteins on the endosome membrane (Henne et al., 2011), has been reported to impair long-term down-regulation of receptors (Shenoy et al., 2001). A C-terminal PDZ motif present in the distal cytoplasmic tail of the β2AR is required for efficient recycling of receptors after endocytosis and, when mutated, internalized receptors are effectively re-routed for lysosomal down-regulation (Cao et al., 1999). There are also caveats of these manipulations. For example lysine mutation may produce pleiotropic effects or affect other ubiquitin-dependent regulatory processes (Shenoy, 2007) and there is evidence that the β2AR PDZ motif functions in other receptor signaling and regulatory processes distinct from endocytic sorting (Romero et al., 2011).

4.2 Genetic manipulation of the endocytic pathway

Manipulations of the endocytic machinery allow GPCR trafficking to be altered without requiring structural modification of the receptor itself. This has advantages with respect to avoiding the various potential complications of receptor mutation, but has potential disadvantages because the trafficking of many other cellular proteins is also affected.

4.2.1 Inhibiting clathrin-dependent endocytosis—Many GPCRs undergo regulated endocytosis primarily via clathrin-coated pits. The GTP binding protein dynamin is an essential component of this machinery in animal cells, and mutations of dynamin isoforms have been described that inhibit clathrin-dependent endocytosis. For example K44A mutant dynamin 1 produces a dominant negative effect by blocking the scission of clathrin-coated pits from the plasma membrane (van der Bliek et al., 1993). K44A mutant dynamin has been used successfully to block regulated endocytosis of a number of GPCRs including the β2AR (Zhang et al., 1996). Depletion of cellular clathrin heavy chain by RNA interference is an alternative strategy that blocks endocytosis by disrupting formation of clathrin-coated pits (Motley et al., 2003). For example, clathrin knockdown strongly inhibits regulated endocytosis of the D1 dopamine receptor (Kotowski et al., 2011) and the β2AR (J Tomshine, unpublished).

4.2.2: Inhibiting recycling—Some GPCRs engage specific recycling machinery that can be targeted by RNA interference. The β2AR, for example, undergoes rapid recycling by PDZ motif-directed sorting on the endosome membrane by a mechanism that requires sorting nexin 27 (SNX27) and the retromer complex. Depleting either SNX27 or the retromer component VPS35 inhibits β2AR recycling after regulated endocytosis and effectively re-routes internalized receptors to lysosomes (Lauffer et al., 2010; Temkin et al., 2011). β1 adrenergic receptors are also sensitive to these manipulations but it is not yet clear how many other GPCRs are sorted by this machinery and some (such as the D1R) are not. Therefore, manipulations of the recycling mechanism by knockdown of such specific trans-acting components need to be validated on a case-by-case basis.

4.3 Acute chemical inhibition of endocytosis

A significant concern of using genetic manipulations of endocytosis is the relatively prolonged time course (typically several days) over which they are imposed. This increases the probability of secondary effects impacting cellular signaling responses and thus adds caveats to interpretation. It is possible to circumvent this using temperature-sensitive alleles to produce more rapid induction of genetic inhibition (Damke et al., 1994). However, a number of chemical inhibitors of endocytosis are now available and are being used more commonly. Several small molecule inhibitors of clathrin-mediated endocytosis have been described (Hill et al., 2009; Macia et al., 2006; von Kleist et al., 2011). We presently use Dyngo-4a, a chemical inhibitor of dynamin, to impose acute endocytic inhibition. This compound is commercially available (Abcam Biochemicals). It is dissolved in dry DMSO at 30 mM (aliquots can be stored under dry nitrogen or argon at -80C) and delivered to cells by 1:1000 dilution from this DMSO stock into the culture medium. Cells should be washed and equilibrated in serum-free medium prior to application of Dyngo-4a because this drug binds avidly to serum proteins. Typically we find that 15 min pre-incubation with 30 μM Dyngo-4a is sufficient to achieve strong endocytic inhibition, and we identify effects by comparison to exposing cells to 0.1% DMSO under the same conditions (vehicle control). Potential disadvantages of chemical endocytic inhibition include off-target effects, which are still poorly understood and may vary across cell types or experimental conditions.

5. Summary

The availability of improved cAMP biosensors, combined with advances in manipulating the endocytic machinery and specific GPCR engagement with this machinery, is revealing previously unexplored spatial and temporal features of GPCR signaling as they occur in intact, living cells. One intriguing hypothesis that has emerged from these studies is that endosomes may represent a membrane surface from which GPCRs are able to elicit classical G protein-mediated signaling. The strategy of perturbing GPCR trafficking processes and then observing associated effects on cellular cAMP accumulation, as described in the present chapter, suggests the occurrence of such signaling from endosomes but remains an indirect approach. A logical next step is to develop biosensors that can directly detect the operation of signaling machineries in intact cells, and allow precise subcellular resolution of their location.

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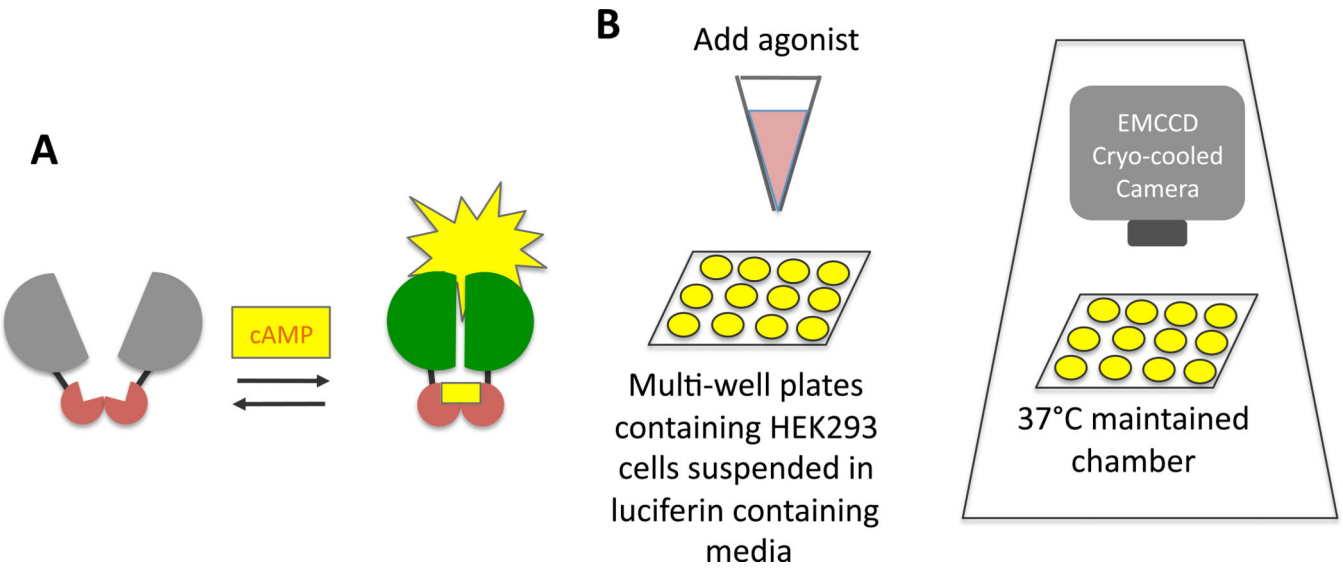

Figure 1. Schematic summarizing luminescence-based method used to measure cAMP accumulation in intact cells

A. Increased cytoplasmic cAMP (cAMP) concentration is detected by increased enzyme (luciferase) activity of the genetically encoded biosensor that is caused by intramolecular protein complementation. **B.** Multi-well plates containing HEK293 cells suspended in luciferin containing media are placed on a temperature-controlled cabinet (37°C) after agonist application and images are taken every 10 sec using EMCCD camera (Hamamatsu C9100-13).

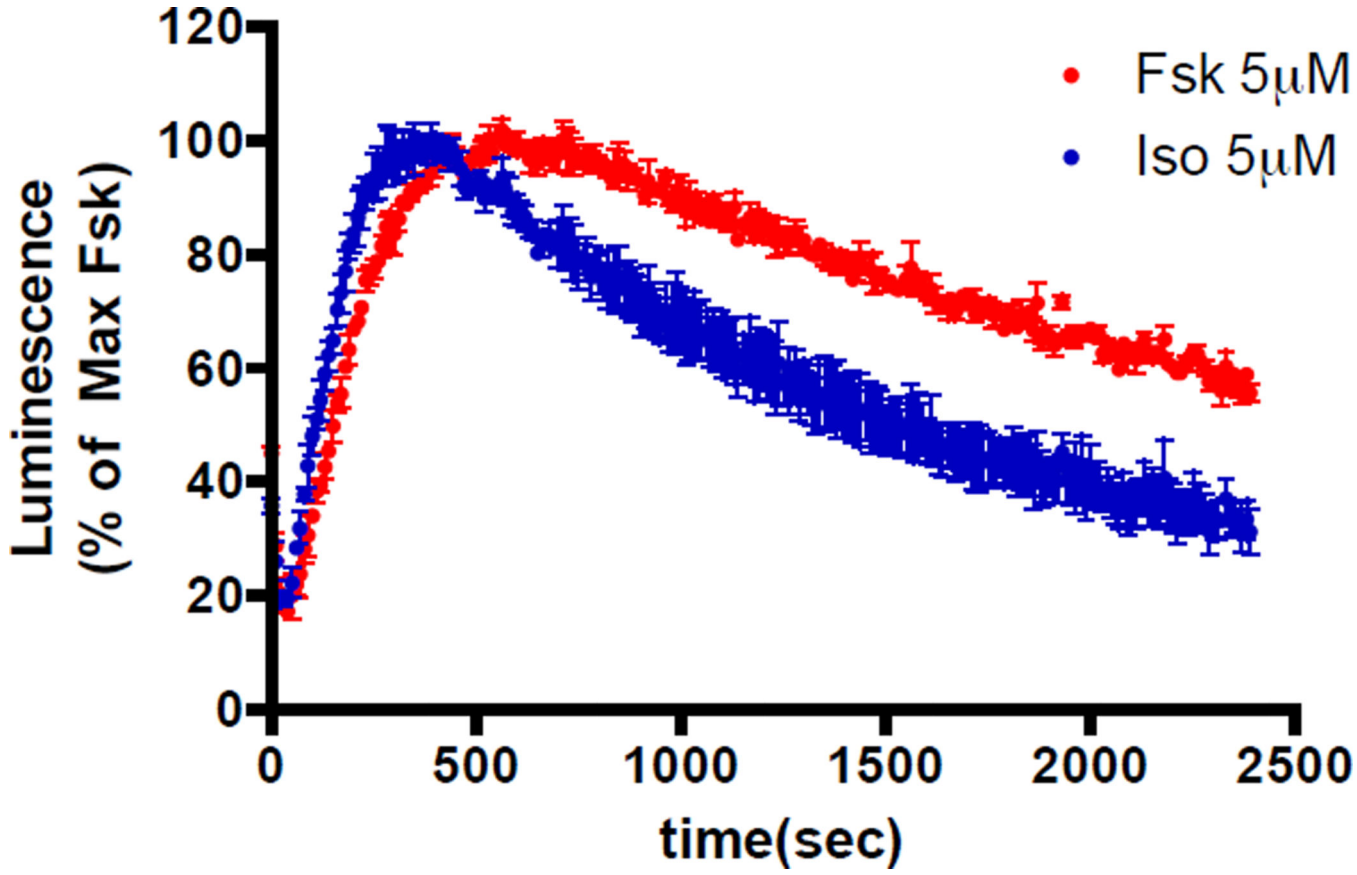

Figure 2. Kinetics of the cAMP response detected using the luminescence assay

A representative example of luminescence imaging from triplicate wells after application of 5 μM isoproterenol (blue) or forskolin (red) at t = 0. Data shown are normalized to the percentage of maximum forskolin response.

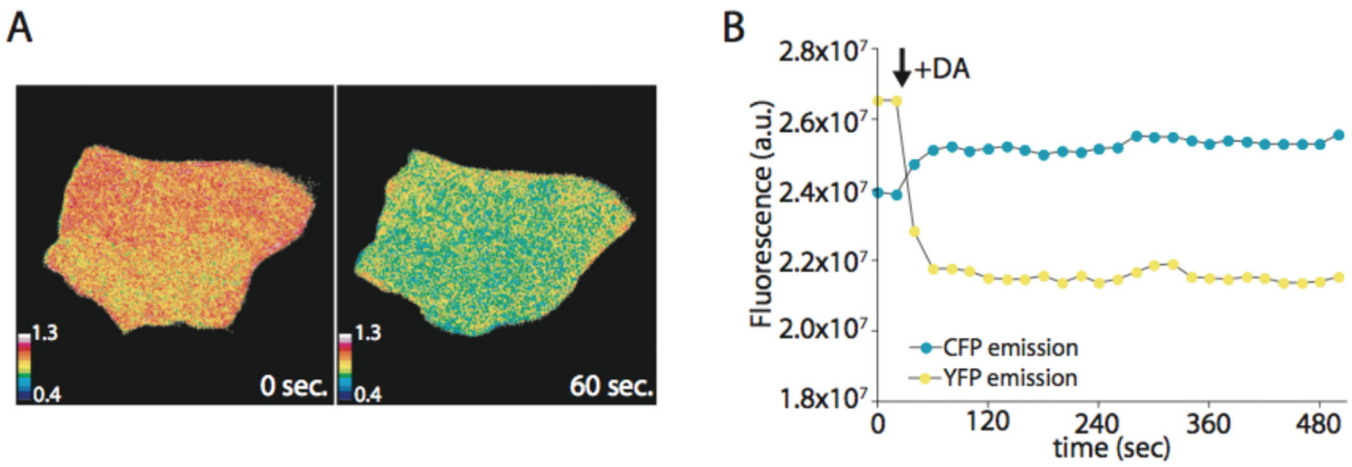

Figure 3. FRET-based detection of D1R-mediated cAMP accumulation

A. Pixel-by-pixel calculation of nFRET in a representative HEK293 cells expressing Flag-tagged D1R before (left) and 60 sec after (right) application of 10μM dopamine. **B.** Time course of integrated whole-cell CFP and YFP fluorescence intensity changes on which the nFRET calculation is based.