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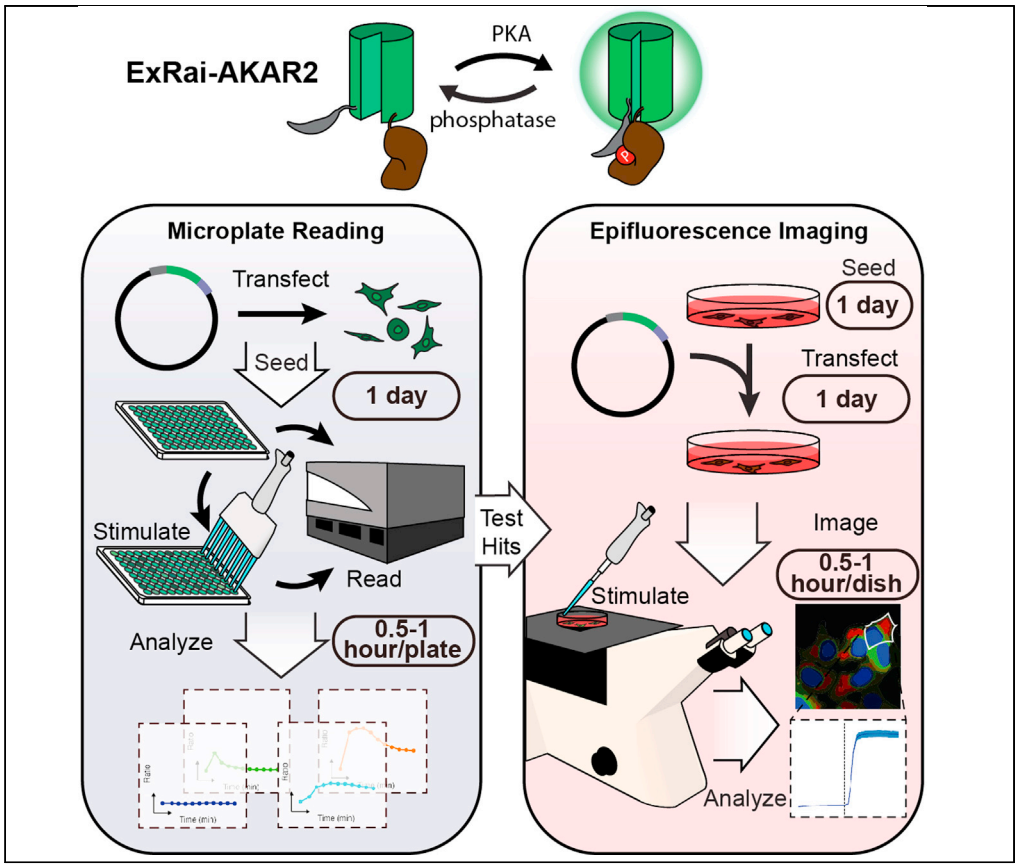
Protocol for reading and imaging live-cell PKA activity using ExRai-AKAR2



Fluorescent protein (FP)-based kinase activity biosensors are powerful tools for probing the spatiotemporal dynamics of signaling pathways in living cells. Yet, the limited sensitivity of most kinase biosensors restricts their reliable application in high-throughput detection modalities. Here, we report a protocol for using an ultrasensitive excitation-ratiometric PKA activity reporter, ExRai-AKAR2, to detect live-cell PKA activity via fluorescence microplate reading and epifluorescence microscopy. The high sensitivity of ExRai-AKAR2 is well suited to these high-throughput applications.

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Highlights

An ultra-sensitive biosensor for measuring PKA activity in living cells

Steps for high-throughput screening of G protein-coupled receptor modulators

Details for epifluorescence imaging with acute drug treatment and image analysis

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Protocol for reading and imaging live-cell PKA activity using ExRai-AKAR2

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SUMMARY

Fluorescent protein (FP)-based kinase activity biosensors are powerful tools for probing the spatiotemporal dynamics of signaling pathways in living cells. Yet, the limited sensitivity of most kinase biosensors restricts their reliable application in high-throughput detection modalities. Here, we report a protocol for using an ultrasensitive excitation-ratiometric PKA activity reporter, ExRai-AKAR2, to detect live-cell PKA activity via fluorescence microplate reading and epifluorescence microscopy. The high sensitivity of ExRai-AKAR2 is well suited to these high-throughput applications.

For complete details on the use and execution of this protocol, please refer to Mehta et al. (2018) andZhang et al., 2021a) .

BEFORE YOU BEGIN

Design of ExRai-AKAR2

Signaling pathways form a carefully coordinated network for cellular communication and information-processing, through which cells sense extracellular cues and coordinate specific functional responses. The reversible phosphorylation of proteins on Ser, Thr, or Tyr residues by protein kinases is of particular importance in signaling, regulating almost all cellular functions. Understanding the architecture of kinase signaling has been greatly improved by the development of optical tools such as genetically encoded fluorescent biosensors, which enable direct visualization of the spatial and temporal dynamics of kinase activities in live cells (Zhang et al., 2021b). Among these, fluorescence resonance energy transfer (FRET)-based kinase biosensors are the most widely used tools to visualize kinase activities. These probes commonly incorporate a sensing unit consisting of a surrogate substrate for a given kinase and a phospho-amino acid binding domain. Phosphorylation then drives binding between these two, leading to a rearrangement of the sensor conformation to modulate FRET between a pair of fluorescent proteins (FPs) (Greenwald et al., 2018). This design was first applied by Zhang and colleagues to generate A Kinase Activity Reporter (AKAR), using a PKA-specific substrate peptide and forkhead-associated 1 (FHA1) domain as the sensing unit, and CFP and YFP as the FRET pair (Zhang et al., 2001, 2005). Over the past two decades, increasingly optimized versions of AKAR have greatly expanded our understanding of PKA signaling (Depry et al., 2011; Ma et al., 2018; Ni et al., 2011; Shelly et al., 2010; Moore et al., 2016; Chen et al., 2017; Mo et al., 2017). Nevertheless, further improvements in the design of AKAR to increase sensitivity and signal-to-noise ratio (SNR) remain highly desirable to enable wider applications, such as high-throughput screening of compound libraries, which are currently beyond the reach of most FRET-based kinase sensors.

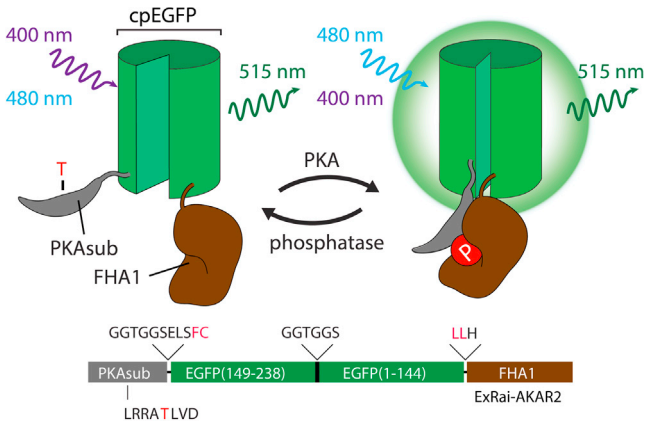

Figure 1. Design and mechanism of ExRai-AKAR

The design of ExRai-AKAR features cpEGFP as the reporting unit sandwitched between a PKA-specific substrate (PKAsub) and FHA1 phosphoamino acid-binding domain as the sensing unit. The fluorescence of cpEGFP is modulated by the phosphorylation-dependent interaction between the PKAsub and FHA1, with the resulting conformational change shifting the maximum excitation wavelength of cpEGFP from ~400 nm to ~480 nm.

An increasingly common alternative to FRET-based biosensor designs uses a conformational switch to modulate the fluorescence of a single, circularly permuted FP (cpFP). This design was popularized by the calcium indicator GCaMP, which integrates a calcium-sensing molecular switch directly into cpEGFP and whose fluorescence intensity dramatically increases in response to calcium binding, achieving significantly higher sensitivity and SNR compared with FRET-based calcium sensors (Nakai et al., 2001). To enhance the sensitivity of kinase biosensors, we similarly switched to a cpFP-based design by inserting the PKA substrate and FHA1 domain from FRET-based AKAR into cpEGFP (Figure 1, Mehta et al., 2018). The resulting sensor, excitation ratiometric AKAR (ExRai-AKAR), is dimly fluorescent under 480-nm excitation and bright under 400-nm excitation, with phosphorylation of the sensor by PKA inducing a conformational change that increases fluorescence intensity at 480-nm excitation and decreases intensity at 400-nm excitation. Measured as the ratio of fluorescence intensities at these two excitation wavelengths, the PKA-stimulated response from ExRai-AKAR is up to 3-fold higher than optimized FRET-based AKARs, making ExRai-AKAR the most sensitive kinase sensor at the time. Much like the original AKAR spawned a large family of FRET-based KARs, the generalizable design of ExRai-AKAR has also yielded a series of highly sensitive reporters for other kinases, such as PKC and Akt (Chen et al., 2021; Mehta et al., 2018). At the same time, we realized that ExRai-AKAR can be further optimized through directed evolution by targeting its short linker regions, given the importance of linker sequences in biosensor engineering (Akerboom et al., 2012; Dana et al., 2019; Miyawaki et al., 1997; Hires et al., 2008; Nakai et al., 2001; Patriarchi et al., 2018). By randomizing the two amino acids immediately preceding and following cpEGFP, we were able to successfully generate an improved version, ExRai-AKAR2, with a nearly ~11-fold dynamic range, a 7-fold improvement over the original (Zhang et al., 2021a). The ultrasensitivity of ExRai-AKAR2 provides new capabilities for elucidating the spatiotemporal dynamics of PKA signaling across diverse experimental modalities, including high-throughput fluorescence detection.

Using ExRai-AKAR2 for GPCR agonist screening

The cAMP-PKA signaling pathway plays a pivotal role in bridging extracellular stimuli with key cellular functions. In particular, PKA activity has been implicated as a major integrator downstream of various G protein-coupled receptors (GPCRs), most notably during neuromodulatory signaling in the brain (Chen et al., 2014; Ma et al., 2018). Thus, a PKA activity-based screening platform will be helpful for studying GPCR modulators (e.g., agonists, antagonists and allosteric modulators). As a proof of concept, we have already demonstrated the use of ExRai-AKAR2 to screen a series of known GPCR agonists in a 96-well plate format, revealing dose-dependent PKA activation or inhibition in response to multiple Gαs or Gαi agonists, respectively (Zhang et al., 2021a). Rather than providing

simple endpoint measurements of PKA activity, ExRai-AKAR2 can reveal the dynamics of PKA activity changes, enabling detailed comparisons across various timescales. Any hit compounds highlighted through screening are further tested using epifluorescence imaging to achieve higher sensitivity and spatiotemporal resolution. In addition to providing a necessary validation step for screening results, epifluorescence imaging can also uncover additional details, particularly single-cell behaviors such as incoherent or pulsatile PKA responses that would be averaged out across an entire well in the plate reader. Here, we use GPCR agonist screening as an application example to provide step-by-step protocols and considerations for using ExRai-AKAR2 in both plate reader and microscopy formats.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
PolyJet	SignaGen Laboratories	Cat# SL100688
Poly-D-Lysine	Sigma-Aldrich	Cat# P6407
Boric acid	Sigma-Aldrich	Cat# 10043-35-3
Sodium tetraborate	Sigma-Aldrich	Cat# 1330-43-4
HEPES	Sigma-Aldrich	Cat# H3375
Forskolin (Fsk)	Calbiochem	Cat# 344281
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	Cat# I7018
35-mm glass-bottom imaging dish	Cellvis	Cat# D35-20-1.5-N
96 well glass bottom plate	Cellvis	Cat# P96-1.5H-N
Experimental models: Cell lines		
HEK293T	ATCC	ATTC Cat# CRL-11268
Recombinant DNA		
pcDNA3.1(+)-ExRai-AKAR2	Zhang et al. (2021a)	Addgene Plasmid #161753
pcDNA3.1(+)-ExRai-AKAR2 (T/A)	Zhang et al. (2021a)	Addgene Plasmid #161754
Software and algorithms		
PRISM	GraphPad	https://www.graphpad.com/scientific-software/prism/
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/download.html
METAFLUOR 7.7	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#Resources
Spark Control	TECAN	https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab–4
Excel	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
Other		
Zeiss AxioObserver Z1 microscope	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/light-microscopes/axio-observer-for-biology.html#features
Lambda 10-2 Filter-changer	Sutter Instruments	LB10-B/IQ
Fluorescence microplate reader	TECAN	SPARK 20M
Evolve 512 EMCCD Camera	TELEDYNE PHOTOMETRICS	Evolve 512 EMCCD Camera

MATERIALS AND EQUIPMENT

Hank's Balanced Salt Solution (HBSS) for Imaging: Imaging Buffer (pH 7.4)		
Reagent	Final concentration	Amount
HEPES	20 mM	238.3 mg
NaCl	137 mM	400.3 mg
KCl	5.4 mM	20.1 mg
$Na_2HPO_4 \cdot 7H_2O$	0.25 mM	3.4 mg
KH_2PO_4	0.44 mM	5.6 mg

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
$CaCl_2 \cdot 2H_2O$	1.3 mM	9.6 mg
$MgSO_4 \cdot 7H_2O$	1 mM	12.3 mg
$NaHCO_3$	4.2 mM	17.6 mg
Glucose	5.5 mM	49.6 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**
Store at 4°C and use within 3 months		

Boric acid buffer for PDL-coating: Boric acid buffer (pH 8.5)		
Reagent	Final concentration	Amount
Boric acid	50 mM	154.6 mg
Sodium tetraborate	25 mM	251.5 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**
Store at 4°C and use within 1 month		

STEP-BY-STEP METHOD DETAILS

Initial calculations and assay setup for fluorescence microplate reading

Timing: 1 day

This step defines the initial set-up for the plate reader assay, including the layout of wells in a 96-well plate, the total cell numbers, and the amount of transfection reagent to be used. Readers can passage cells according to the experimental design and the corresponding calculation.

1. Prior to the assay, determine the number of experimental conditions (e.g., compounds to be tested), X.
2. Include positive and negative controls: Fsk/IBMX treatment and no-treatment conditions. This brings the total number of conditions to X + 2.

   Note: For every plate reader assay, we recommend including wells treated with Fsk (50 μM)/IBMX (100 μM) as a positive control condition. Fsk directly activates transmembrane adenylyl cyclases, which produce cAMP, and IBMX inhibits cAMP-degrading phosphodiesterase, thus maximally elevating cellular cAMP levels and PKA activity. Fsk/IBMX-treated wells can be used to confirm whether the sensor is working properly and to assess the dynamic range of ExRai-AKAR2 under different conditions (e.g., cell type, transfection rate, etc.). We also recommend including a set of non-treated wells as a negative control to make sure that there is no significant baseline drifting during the assay.

3. Also include a non-transfected condition to correct for background fluorescence.

   Note: The fluorescence from non-transfected wells will reflect cellular autofluorescence, and it will be subtracted from the fluorescence readings obtained from transfected cells.

4. Each condition should be performed in triplicate (e.g., 3 wells per condition). Thus, the total number of wells to be seeded will be N = 3(X + 2 + 1).

   Note: Figure 2 provides an exemplary layout for one reading plate.

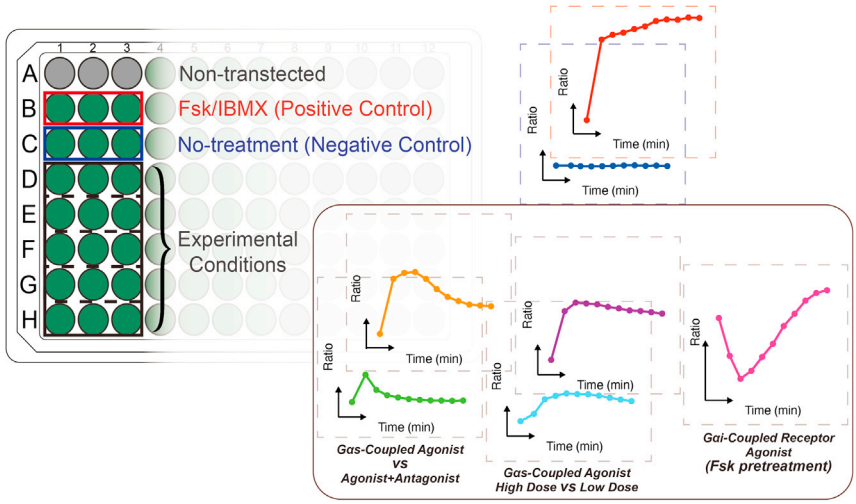

Figure 2. Layout of ExRai-AKAR2 microplate reading for GPCR modulators screening

Left: A single 96-well plate is seeded with ExRai-AKAR2-transfected HEK293T cells, setting aside 3 wells to be seeded with non-transfected cells for background correction. One set of triplicate transfected wells is treated with Fsk/IBMX as a positive control for maximal PKA activation and ExRai-AKAR2 response. Another triplicate set of transfected wells should be left untreated as a negative control for baseline recording. Each experimental condition is similarly performed in triplicate. Right: Example time course recordings illustrating the response of ExRai-AKAR2 to various stimuli, including positive and negative controls (upper curves), as well as Gαs-coupled agonist vs agonist + antagonist (lower left), different doses of a Gαs-coupled receptor agonist, (lower middle), and Gαi-coupled receptor agonist treatment following Fsk induced-PKA activation (lower right).

5. Calculate the total cell number for seeding. For HEK293T cells, the seeding density is 5×10^4 cells/well, thus the total cell number is $5 \times 10^4 \times N$.
6. Determine the quantity of plasmid DNA and PolyJet to be used for transfection (8×10^5 to 1×10^6 cells/3 μL PolyJet/1 μg DNA).

   Note: For example, to seed one 96-well plate, we use $5 \times 10^4 \times 96 = 4.8 \times 10^6$ cells. The ratio of cell number/transfection reagent/DNA amount is: 4.8×10^6 cells/18 μL PolyJet/6 μg DNA

   Alternatives: Other transfection reagents such as Lipofectamine may suffice as well, and longer incubations times might be required for protein expression depending on the specific cell type. For difficulties with transfection, we refer readers to problem 1 in the trouble-shooting section.

7. Passage HEK293T cells for seeding and transfection.

Plate coating with poly-D-lysine

Timing: 1 day

This step describes the procedure for coating the 96 well plate with poly-D-lysine to enhance cell attachment, which is helpful to avoid artifacts from cell detachment after drug addition.

8. Prepare boric acid buffer: 50 mM boric acid, 25 mM sodium tetraborate, pH 8.5.
9. Dissolve poly-D-lysine (PDL) in boric acid buffer to a final concentration of 0.1 mg/mL.
10. Add 50 μL of 0.1 mg/mL PDL solution to N wells (calculated above) of a black-walled, clear-bottom 96-well assay plate (CellVis) and incubate for 24 h at 20°C–25°C in a biosafety cabinet. Afterwards, wash all wells once with HBSS.

Alternatives: Other coating reagents such as lamin, fibronectin, collagen, etc., may also be used as appropriate depending on the specific cell type chosen for the experiments. Pre-coated plates may be purchased commercially as well.

Cell seeding and transfection

Timing: 1 day

This step describes preparation of a 96-well plate to obtain sufficient cell density and ExRai-AKAR2 expression for the following screening assay.

11. Dilute calculated amount of ExRai-AKAR2 plasmid DNA into high-glucose DMEM to a concentration of 1μg DNA/50 μL high-glucose DMEM (e.g., 6 μg DNA/300 μL high-glucose DMEM for one plate).
12. Dilute calculated amount of PolyJet reagent into high-glucose DMEM to a concentration of 3 μL PolyJet/50 μL high-glucose DMEM (e.g., 18 μL PolyJet/300 μL high-glucose DMEM for one plate).
13. Mix and incubate the DNA/lipid complex for 5 min at 20°C–25°C.
14. Harvest cultured HEK293T cells, collecting at least $5 \times 10^4 \times N$ cells (see step 5 above).
15. Resuspend the harvested HEK293T cells in culture media to a final concentration of 5×10^4 cells/100 μL.
16. Prepare 3(X + 2) × 100 μL of cell suspension for transfection; prepare another 300 μL of cell suspension for the non-transfected control wells.
17. Transfect the cells by mixing 3(X + 2) × 100 μL of cell suspension with the DNA/lipid complex.
18. Seed both transfected and non-transfected cells by adding 100 μL of cell suspension into the corresponding wells of a PDL-coated 96-well plate.
19. Incubate the seeded cells for 24 h at 37°C to express the biosensor.

⚠ CRITICAL: Check the fluorescence signal for the experimental groups and non-transfected controls 24 h after transfection. A transfection efficiency of approximately 50%–70% should be observed in the wells for the experimental groups to achieve a good SNR, and little or no fluorescence should be detected in the non-transfected wells. If the transfection rate is low, we refer readers to problem 1 in the troubleshooting section.

Fluorescence microplate reading of ExRai-AKAR2-expressing cells

Timing: 1 day

This section provides a step-by-step walkthrough for performing fluorescence microplate reading with acute drug addition. The resulting data reveal the dynamics of PKA activity changes in response to various GPCR modulators.

20. Prepare 10× solutions of all drugs by diluting drug stocks with HBSS imaging buffer.
21. Set up the fluorescence microplate reader to perform dual-excitation measurements of ExRai-AKAR2 fluorescence intensity.

⚠ CRITICAL: Appropriate excitation and emission, Z position, flashes number, camera gain and time lapse settings are required to collect enough fluorescence and achieve good SNR. For example, on our TECAN SPARK 20M, we use 480 nm and 400 nm excitation with 10 nm bandwidth, and 520 emission with a 20 nm bandwidth, and both channels use 30 flashes and 180 gain. The read applies bottom-read mode, and the optimal Z position is automatically determined from a transfected well. A 2-min time lapse is required for our protocol to read an entire plate, though this value can be modified depending on the

hardware and the read settings. We recommend performing a pilot read to test how long it takes to complete one round and then set an appropriate interval between each round of reading.

22. Carefully remove the culture media from each well, wash the wells once with HBSS Imaging Buffer, and refill each well with 45 μL of HBSS Imaging Buffer.
23. Acquire a baseline recording (cycle 0) by reading the plate for a single cycle.
24. Remove the plate from the reader and carefully add 5 μL of 10× drug solution to each well.

Note: Although adding 5 μL of drug solution will increase the total assay volume from 45 μL to 50 μL, this should have little impact on the fluorescence intensity and excitation ratio of the sensor, as these measurements are recorded from cells adhered to the bottom of each well, rather than from the assay solution.

Note: Adding drug to each well individually may lead to a significant lag in the responses from different wells, potentially making kinetic comparisons between wells more difficult. To avoid this, we recommend performing drug addition using a multichannel pipette if possible. Before plate reading, pipette all of the 10× drug stocks into a separate 96-well plate, arraying the drugs to match the arrangement of the experimental conditions in the test plate. For drug addition, transfer the drugs row by row using a P10 multi-channel pipette.

⚠ CRITICAL: Some drugs may exhibit strong fluorescence that overlaps with the spectrum of ExRai-AKAR2, leading to false-positive responses. Therefore, the fluorescence of any new drug should be assessed before inclusion in the assay. To examine the intrinsic fluorescence of a given drug, we recommend testing it using a plate of cells transfected by ExRai-AKAR2 (T/A), which is a negative-control mutant that cannot be phosphorylated. Similar to the ExRai-AKAR2 plate reader assay, add 45 μL of Imaging Buffer to perform a baseline read, and then 5 μL of 10× drug solution or Imaging Buffer to read the fluorescence under 480-nm excitation/520-nm emission and 400-nm excitation/520-nm emission. Special attention should be given to any drug that shows significantly stronger fluorescence than a control well with no drug added under either 480-nm or 400-nm excitation. Corrections can be made using drug fluorescence readings, though compounds with very strong fluorescence may need to be excluded from the experiment.

25. Re-insert the plate and resume reading for the desired number of cycles to obtain a time-course recording.
26. Repeat steps 24 and 25 for any subsequent drug additions.

Cell culture and transfection for epifluorescence imaging

Timing: 2 days

This step describes the seeding and transfection of cells for epifluorescence imaging of ExRai-AKAR2.

27. Passage HEK293T cells into 35-mm glass-bottom dishes at approximately 30% confluency.
28. The following day, determine the amount of ExRai-AKAR2 plasmid DNA and PolyJet to be used for transfection (500 ng DNA/1.5 μL PolyJet/35-mm dish).
29. Dilute the calculated amount of ExRai-AKAR2 plasmid DNA into high-glucose DMEM to a concentration of 500 ng DNA/50 μL high-glucose DMEM.
30. Dilute the calculated amount of PolyJet reagent into high-glucose DMEM to a concentration of 1.5 μL PolyJet/50 μL high-glucose DMEM.
31. Mix and incubate the DNA/lipid complex for 5 min at 20°C–25°C.

32. Transfect the cells by adding 100 μL of DNA/lipid complex into each 35-mm glass-bottom dish. Incubate the cells for 24 h at 37°C to allow for protein expression.

Validating screening hits using live-cell epifluorescence imaging of ExRai-AKAR2

Timing: 1 day

This step describes a step-by-step walkthrough of live-cell epifluorescence imaging of ExRai-AKAR2, and all screening hits will be validated by testing PKA responses from single cells with enhanced sensitivity and temporal resolution.

33. Prepare drugs at 1000× stock concentration. Aliquot 2 μL of 1000× drug stocks into separate 1.5-mL tubes.

Note: Store drug aliquots properly during imaging. Most drugs are best kept on ice, but this will depend on the specific compound.

34. Perform real-time imaging with acute stimulation.

⚠ CRITICAL: Multiple factors need to be considered when performing real-time imaging with ExRai-AKAR2. Appropriate excitation and emission filters for dual-excitation imaging, exposure times, ND filters and acquisition intervals are required to obtain enough fluorescence signal and avoid photobleaching. For example, we use a 480DF20 excitation filter and 535DF50 emission filter for the 488-nm excitation channel (GFP480) and a 405DF20 excitation filter and 535DF50 emission filter for the 400-nm excitation channel (GFP400), along with 500 ms exposure time for both channels, a 0.6 ND filter, and a 15–30 s acquisition interval. In addition, higher-magnification objectives (≥40×) are recommended to achieve greater spatial resolution during imaging.

a. Carefully aspirate the culture media from the imaging dish and wash the cells twice with 1 mL of HBSS Imaging Buffer. Add 2 mL of Imaging Buffer for imaging.
b. Mount the prepared imaging dish on the microscope stage.
c. Focus the cells and begin acquiring images in both the GFP480 and GFP400 channels.
d. Image the cells for at least 5 min prior to any stimulation to acquire a baseline signal.

Note: A minimum baseline recording of 5 min is required to see the effect from drug addition. Longer times may be required if a drifting baseline is observed initially. For issues with drifting baseline, we refer readers to the Problem 4 of the troubleshooting section.

e. Pause the image acquisition to perform the first drug addition: Leaving the dish fixed on the microscope stage, use a P1000 pipette to transfer ~0.5 mL of Imaging Buffer from the imaging dish into a 1.5 mL tube containing an aliquot of 1000× drug stock. Mix well by pipetting up and down, and then carefully add the mixture back to the imaging dish. GENTLY mix within the imaging dish by pipetting at least 3 more times to ensure homogeneous distribution of the drug.

⚠ CRITICAL: Drug addition and mixing may cause significant shear stress on the cell surface. When pipetting the drug and Imaging Buffer, keep the pipette tip away from the center of the dish to avoid strong turbulence on the cells being recorded. For cells with relatively weak attachment to the imaging dish, such as HEK293T cells, using imaging dishes coated with PDL (or another substrate) can help reduce the risk of cell detachment. Diluting the drug aliquot into a smaller volume of Imaging Buffer (i.e., using a P200 pipette) may also help reduce turbulence during mixing. In addition, avoid touching the imaging dish and

the microscope stage with the pipette tip, as this may cause the imaging field to shift and/or go out of focus.

f. Resume imaging and continue acquisitions until the fluorescence signal reaches a plateau. Repeat for any subsequent drug additions.

Note: ExRai-AKAR2 has been used to report changes in PKA activity induced by various stimuli. We recommend performing Fsk/IBMX stimulation at the end of each imaging experiment as a positive control. Including Fsk/IBMX addition is helpful for verifying biosensor function, normalizing observed ratio changes caused by test compounds against the maximum sensor response, thus facilitating quantitative comparisons across different compounds and experimental replicates, and also verifying that the selected drug concentrations are not saturating the biosensor response. For any compounds whose responses are close to the Fsk/IBMX-induced maximum, we recommend that readers test a range of drug concentrations to identify an appropriate, sub-saturating dose.

Note: To verify that ratio changes observed in cells expressing ExRai AKAR2 are due to phosphorylation of the sensor, we recommend testing any new and unknown compounds identified from plate-reader experiments via epifluorescence imaging using the non-phosphorylatable ExRai-AKAR2 (T/A) mutant as a negative control. Cells expressing ExRai-AKAR2 (T/A) should exhibit no ratio change in response to stimulation. For any excitation ratio changes observed using ExRai-AKAR2 (T/A), please refer to problem 5 in the troubleshooting section.

EXPECTED OUTCOMES

A good transfection with ExRai-AKAR2 can achieve 50%–70% transfection efficiency per well (96-well plate) or 35-mm dish. Positive-control treatment with Fsk/IBMX should induce a maximum response from ExRai-AKAR2 that remains sustained throughout the time-course, and the non-treated negative control should exhibit a flat baseline signal. For Gαs-coupled receptors, agonist stimulation will lead to a significant increase in the ExRai-AKAR2 excitation ratio. For Gαi-coupled receptors, stimulation should produce a decrease in the ExRai-AKAR2 excitation ratio, reflecting an inhibition of basal PKA activity. Alternatively, cells can first be treated with a low dose of Fsk to moderately activate PKA, followed by Gαi agonist treatment to inhibit PKA (Figure 2). For instance, we have used 100 nM Fsk prestimulation in HEK293T cells to test Gαi agonist effects (Zhang et al., 2021a). This will be reflected by an initial increase in the ExRai-AKAR2 excitation ratio upon Fsk treatment, followed by a decrease in the excitation ratio upon Gαi agonist treatment. We recommend performing pilot experiments to identify an optimal Fsk dose that will induce a robust but submaximal ExRai-AKAR2 response. Candidate compounds identified via screening can be further validated using epifluorescence imaging. Valid hits should produce consistent results between the two imaging modalities.

QUANTITATIVE AND STATISTICAL ANALYSIS

Plate reader data analysis

Data acquired from the plate reader assay should be analyzed by calculating the 480-nm/400-nm fluorescence excitation ratio for each well. The average 480 nm- and 400 nm-excited fluorescence intensities from the non-transfected control wells are regarded as background fluorescence, $F_{EX480\text{-}background}$ and $F_{EX400background}$. The raw fluorescence readings for each transfected well will then be corrected by subtracting the corresponding background fluorescence value. For each experimental well, the corrected 480 nm- and 400 nm-excited fluorescence are calculated as:

$$F_{EX480} = F_{EX480raw} - F_{EX480background}$$

$$F_{EX400} = F_{EX400raw} - F_{EX400background}$$

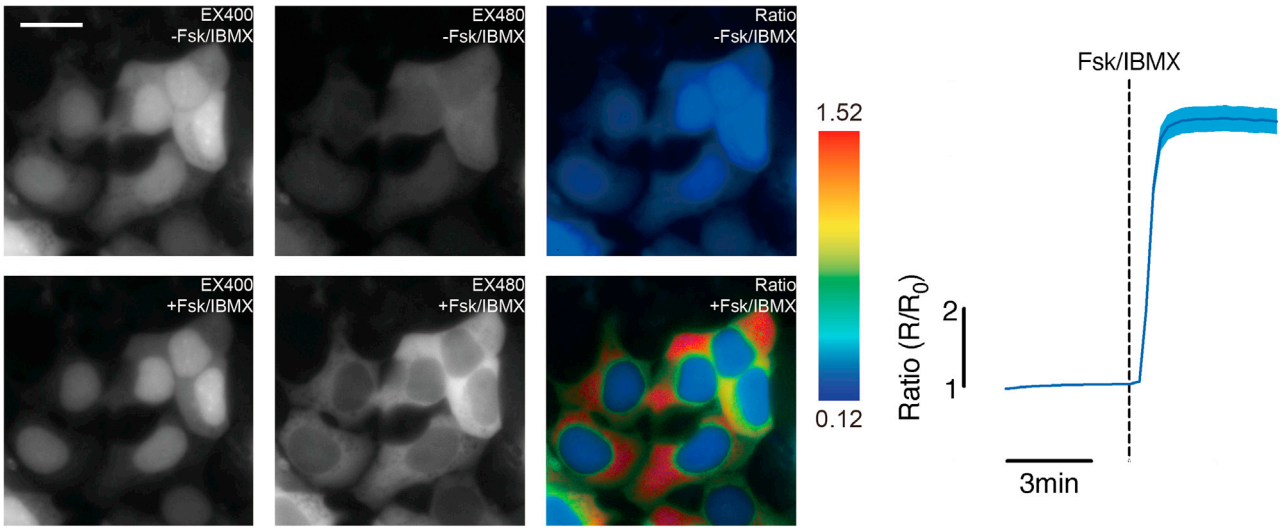

Figure 3. Epifluorescence imaging of ExRai-AKAR2 response to Fsk/IBMX stimulation

An example experiment for HEK293T cells transfected with ExRai-AKAR2 and stimulated with Fsk/IBMX. Left: Representative epifluorescence images of ExRai-AKAR2-expressing HEK293T cells showing the 400 nm- (left) and 480 nm-excited (middle) fluorescence intensity, as well as the 480 nm/400 nm excitation ratio (right; pseudocolored) before and after stimulation. Warmer colors indicate higher ratios. Scale bar, 10 μm. Right: Representative time-course showing the normalized 480 nm/400 nm excitation ratio change (R/R_0) in HEK293T cells (n = 40) stimulated with Fsk/IBMX. Solid line indicates the mean, and the shaded area indicates SEM.

Calculate the 480 nm/400 nm fluorescence excitation ratio (R) for each well:

$$R = \frac{F_{EX480}}{F_{EX400}} = \frac{F_{EX480raw} - F_{EX480background}}{F_{EX400raw} - F_{EX400background}}$$

Ratio values calculated from triplicate wells should be pooled to calculate the mean and standard error of the mean (SEM). Data can be visualized by plotting the mean and SEM values from each condition as a time course or by generating a heatmap of the mean values. Each plate should be subjected to at least 3 independent replications to ensure the robustness of the data.

Epifluorescence imaging analysis

Start by selecting regions of interest (ROIs), such as the whole cell or a specific cytosolic region. Also select a cell-free ROI as the background. Calculate the excitation ratio (R) at each time point as follows:

$$R = \frac{F_{EX480ROI} - F_{EX480background}}{F_{EX400ROI} - F_{EX400background}}$$

Normalize the resulting ratio values by dividing the ratio at each time point by the basal ratio value at time zero (R/R_0), which is the time point immediately before the first drug addition (R_0). Data can be plotted as single-cell time courses to visualize cell-to-cell variations, or responses from all cells in a dish can be averaged and plotted as the mean and SEM. Figure 3 shows the results of an example experiment for HEK293T cells transfected with ExRai-AKAR2 and stimulated with Fsk/IBMX. We recommend repeating imaging experiments at least 3 times (e.g., 3 independent 35-mm dishes across different cell preparations).

LIMITATIONS

ExRai-AKAR2 functions as a surrogate substrate for PKA, and excessive levels of ExRai-AKAR2 expression may therefore buffer PKA activity towards endogenous substrates. The expression level of ExRai-AKAR2 should thus be carefully controlled to minimize any potential buffering effect. Furthermore, the sequence motif and PKA affinity of ExRai-AKAR2 may not precisely reflect the

specific phosphorylation of various endogenous PKA substrates. As a result, complementary methods such as western blotting for phosphorylated substrate may be needed to quantify the phosphorylation levels of a specific PKA substrate.

When using ExRai-AKAR2-based GPCR agonist screening, we should also note that assaying PKA activity alone may not be sufficient to provide insights into G protein-signaling specificity, given its role in integrating signals from multiple G-protein classes such, as Gi and Gs. Additional, complementary approaches may thus be required when screening compounds to discover novel GPCR modulators. For example, readers might need to knock out specific G proteins or treat with selective inhibitors to test their contribution to PKA activities detected by ExRai-AKAR2.

TROUBLESHOOTING

Problem 1

Poor transfection of cells seeded into 96-well plates or 35-mm dishes. Related to "cell seeding and transfection steps 11–19" and "cell culture and transfection for epifluorescence imaging steps 27–32".

Potential solutions

Optimize the transfection method for the chosen cells. For HEK293T cells, we find that PolyJet achieves a better transfection rate compared to Lipofectamine 2000.

Increase the amount of plasmid DNA and/or the transfection reagent.

Generate a stable cell line expressing ExRai-AKAR2.

Problem 2

No response from the positive control in the 96-well plate. Related to "fluorescence microplate reading of ExRai-AKAR2-expressing cells steps 20–26."

Potential solutions

Increase the seeding density to achieve enough fluorescence signal from transfected cells.

Improve the expression of the sensor by incubating the cells for longer after transfection.

Optimize plate-reading settings such as gain, flashes, band width of excitation/emission and try multi-position reading to get enough fluorescence signal.

Problem 3

Little or no response from a given GPCR agonist in fluorescence microplate reading. Related to "fluorescence microplate reading of ExRai-AKAR2-expressing cells steps 20–26."

Potential solutions

Try a higher dose for stronger stimulation.

Test the agonist by epifluorescence imaging of ExRai-AKAR2-expressing cells to achieve higher sensitivity.

Check the the expression level of targeting receptor in the cells selected for microplate reading. If not expressed, we recommend testing another cell line that is known to express the receptor under study or generating a stable line expressing the receptors for the agonist.

Problem 4

Significant baseline drifting before the first drug addition. Related to "Validating screening hits using live-cell epifluorescence imaging of ExRai-AKAR2 steps 33–34".

Potential solutions

Pre-incubate the cells in HBSS Imaging Buffer for at least 15 min to stabilize the cells.

Wait longer before adding the first drug to allow the baseline reading to stabilize.

Reduce the exposure time to minimize photobleaching.

Use higher ND filters to minimize photobleaching.

Increase the time interval between each frame to decrease light damage.

For a baseline that is drifting consistently throughout the entire imaging time course, perform a baseline correction by fitting a straight line to the baseline before addition of the first drug and subtracting the value of the linear fit from the recorded value at each time point.

Problem 5

Responses observed in ExRai-AKAR2 (T/A) imaging. Related to "Validating screening hits using live-cell epifluorescence imaging of ExRai-AKAR2 step 34."

Potential solutions

Morphological changes may cause cells to shift out of the ROI, causing a significant decrease in of fluorescence intensity and resulting changes in the 480 nm/400 nm fluorescence excitation ratio. To overcome that, we recommend readers to re-select ROI according to the morphology of the cells and keep the whole cell within the ROI.

Some drugs may be fluorescent at the wavelengths used to image ExRai-AKAR2. To determine if ratio changes are caused by drug fluorescence, check the fluorescence intensities of untransfected cells and the cell-free background in each acquisition channel. Significant but non-reciprocal changes in fluorescence intensity from either channel are indicative of autofluorescence from the drug.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jin Zhang (jzhang32@ucsd.edu).

Materials availability

All plasmid DNA constructs described in the protocol are available through Addgene.

Data and code availability

Not applicable.

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AUTHOR CONTRIBUTIONS

J.F.Z. wrote the manuscript. S.M. and J.Z. edited the manuscript. J.Z. supervised the project. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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