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A Multiparameter Live Cell Imaging Approach to Monitor Cyclic AMP and Protein Kinase A Dynamics in Parallel

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Abstract

Parallel detection of signaling activities allows us to correlate activity dynamics between signaling molecules. In this review, we detail a multiparameter live cell imaging method to monitor 3′,5′-cyclic adenosine monophosphate (cAMP) levels and protein kinase A (PKA) activities in parallel.

Keywords

Parallel imaging; FRET; cAMP; Protein kinase A; Signal transduction

1 Introduction

Signal transduction is a way for cells to communicate internally or externally to respond to various stimuli. At the heart of such communication are the signaling nodes that integrate diverse signals into specific outputs [1], resulting in desired functional responses. Rather than being linear, signaling pathways exist as structured networks, with multiple layers of regulation that ensure signal specificity and amplification. Signaling molecules involved in these pathways are, thus, coupled to one another in spatiotemporally synchronized manners. Knowledge of such dynamic relationships can be critical in assessing the information flow within the cells [2] or in identifying new therapeutic targets [3]. To comprehend inter-regulation of multiple signaling molecules at a systems level, it is important to be able to watch them in parallel, over the course of their cellular actions, and to correlate their activity dynamics.

1.1 cAMP/PKA Signaling

Protein kinase A (PKA), a ubiquitous protein kinase, is central to many signaling pathways. It is involved in regulating major biological processes, such as metabolism, differentiation and growth. Upon ligand binding and activation of seven transmembrane G-protein coupled receptors (GPCRs), associated G-proteins activate adenylyl cyclases that produce cyclic 3′, 5′-monophosphate (cAMP). cAMP then binds to PKA in its tetrameric holoenzyme form, triggering the dissociation of catalytic subunits from the latter's regulatory subunits [4]. The catalytic subunits then phosphorylate diverse substrates, altering various aspects of cellular physiology. Although PKA is the most well-known mediator of cAMP signaling, it is not the sole effector downstream of cAMP. Exchange proteins activated by cAMP (Epacs)

represent another family of major cAMP effectors that channels most of the PKA-independent cAMP signaling inside cells [5].

1.2 FRET-Based Reporters

Genetically encodable fluorescent protein (FP)-based reporters are useful tools for monitoring signaling activities inside living cells [6]. In these reporters, fluorophores from two spectrally distinct FPs serve as a pair of donor and acceptor for fluorescence resonance energy transfer (FRET). These FPs are fused to a signal-dependent molecular switch constructed via semirational protein engineering (Fig. 1a). Upon signal activation, conformational changes in the molecular switch lead to a shift in relative distance and orientation of the fluorophores from the two FPs, generating a change in FRET. This allows FRET change to be used as a readout to detect signaling dynamics in real time and in the native cellular environment.

1.3 Orthogonal FRET Pairs with a Shared FRET Acceptor

A typical and widely used FRET pair contains a cyan fluorescent protein (CFP) as a donor and a yellow fluorescent protein (YFP) as an acceptor. Emergence of fluorescent proteins across the visible spectrum made parallel FRET detection possible when spectrally distinct donors and acceptors were put together to generate orthogonal FRET pairs [7]. Various laboratories have developed methods to monitor two or more signaling activities, taking advantage of these newly developed FPs (reviewed in refs. 7, 8).

Recently, we have reported a parallel imaging method, where mCherry [9], a red fluorescent protein (RFP) derived from *Discosoma* sp., serves as a shared FRET acceptor for two FRET donors, CFP and YFP [10]. This method relies on the distinct absorption peaks of CFP and YFP. More importantly, our experiments showed that FRET was efficient between CFP and RFP [11], and YFP and RFP with miminal cross-excitation of RFP upon YFP excitation, which can be easily corrected (*see* Subheading 3.7 for correction methodology). The advantages of the shared acceptor imaging are several-fold. First, FPs from any established CY-based FRET reporters can be easily replaced with mCherry, requiring minimal reporter characterization. In addition, imaging can be conveniently achieved by addition of an RFP filter set in an existing CY-FRET protocol.

Using the common acceptor approach, we have constructed a cyan and red FP-based PKA activity reporter called CR-AKAR (*C*FP/*R*FP-based *A*-*k*inase *A*ctivity *R*eporter) based on a previously developed, widely used CY-based AKAR [12]. In CR-AKAR, a phosphothreonine binding domain, forkhead associated domain 1 (FHA1) and a surrogate PKA substrate motif serve together as a signal-dependent switch that Cerulean (a CFP) and mCherry are flanking (Fig. 1b). Upon PKA activation and phosphorylation of the surrogate substrate, a phosphorylation-dependent conformational switch results in an increase in cyan to red FRET. We have also engineered a yellow and red FP-based cAMP sensor called YR-ICUE (*Y*FP/*R*FP-based *I*ndicator of *c*AMP *u*sing *E*pac), by replacing the CFP in the original CY-ICUE biosensor [13] with mCherry. In this reporter, the cAMP sensing domain of exchange protein activated by cAMP-1 (Epac1) is sandwiched between Venus (a YFP) and

mCherry (Fig. 1b). Conformational changes in Epac1 domain, upon cAMP binding, result in a FRET decrease from Venus to mCherry.

Expressing both of these reporters in single living cells, we observed differential dynamics of cAMP and PKA upon stimulation with different G-protein coupled receptor agonists (Fig. 2). This has opened up the possibility to study and characterize the pathway parameters such as feedback loops and cross-regulation in a more systematic approach. Below, we outline the detailed method for parallel monitoring of cAMP and PKA activity dynamics using YR-ICUE and CR-AKAR.

2 Materials

2.1 Cell Culture and Transfection

1. Cell lines: Human Embryonic Kidney with SV40 T Antigen (HEK293T).
2. Dulbecco's phosphate-buffered saline without Mg^{2+} and Ca^{2+} (DPBS).
3. T-25 cm^2 tissue culture flasks.
4. Imaging dish: 35 mm glass bottom petri dishes for live cell imaging (MatTEK).
5. HEK293T culture medium: Dulbecco's Modified Eagle's Medium (DMEM, low glucose) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin to culture HEK293T cells. Other suitable tissue culture medium for additional cell lines of interest.
6. Solution of trypsin (0.05 %) and ethylenediamine tetraacetic acid (EDTA, 0.53 mM) or relevant trypsinization reagents.
7. Constructs: CR-AKAR and YR-ICUE biosensors.
8. Calcium phosphate-mediated transfection reagents: 2×HBS (50 mM HEPES, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2PO_4), pH adjusted to 7.05 using KOH; and 2 M $CaCl_2$; both filter-sterilized with 0.22 μm filters.

2.2 Preparation for Imaging

1. Hanks' Balanced Salt Solution for Imaging (HBSS*): 1× Hanks' Balanced Salt Solution (Gibco) with 2.0 g/L D-glucose; pH-adjusted to 7.4 using NaOH and filter sterilized using a 0.22 μm filter. Store at 4 °C and bring to room temperature prior to imaging.
2. 1,000× stock of stimuli: forskolin (FSK; Calbiochem), prostaglandin E1 (PGE1; Sigma), ritodrine (RITO; Sigma), isoproterenol (ISO; Sigma), and H89 (Sigma) (*see* Note 1) are prepared in DMSO and stored at −20 °C.

2.3 Epifluorescence Microscopy

1. Microscope: Axiovert 200M microscope; 40×/1.3NA oil-immersion objective lens (Zeiss).

[1]Additional stimuli can be prepared at desired concentration (preferably 1,000× for easier addition during imaging; *see* Note 11).

2. Camera: MicroMAX BFT512 cooled charge-coupled device camera (Roper Scientific).
3. Xenon lamp: XBO 75W (Zeiss).
4. Neutral density (ND) filters 0.6 and 0.3 (Chroma Technology).
5. Filtersetsforindividualchannels(AllfromChromaTechnology):

   CR-FRET—420DF20 excitation filter, 450DRLP dichroic mirror, 653DF95 emission filter.

   CFP—420DF20 excitation filter, 450DRLP dichroic mirror, 475DF40 emission filter.

   RFP—568DF55 excitation filter, 600DRLP dichroic mirror, 653DF95 emission filter.

   YFP—495DF10 excitation filter, 515DRLP dichroic mirror, 535DF25 emission filter.

   YR-FRET—495DF10 excitation filter, 515DRLP dichroic mirror, 653DF95 emission filter.
6. Lambda 10-2 filter changer (Sutter Instruments).
7. Immersol® 518F fluorescence free immersion oil (Zeiss).

2.4 Image Acquisition and Data Analysis

1. METAFLUOR 6.2 software (Molecular Devices).
2. Microsoft Office Excel.

3 Methods

3.1 Cell Culture

HEK293T cells are maintained in T-25 cm^2 flasks at 37 °C with 5 % CO_2. Upon reaching about 80 % confluency (about every 2–3 days), the cells were subject to passage in the following steps:

1. Wash cells twice with DPBS buffer (*see* Note 2). This step removes general debris as well as facilitates cell detachment.
2. Trypsinize the cells with 0.05 % trypsin (+EDTA) at room temperature (RT) for 1 min.
3. Gently tap the flask and neutralize the trypsin with desired volume of cell culture medium (*see* Note 3).
4. Split a desired volume of cells to a new flask or imaging dishes containing fresh medium.

[2]Gentle washing is required since HEK293T cells are very easy to detach. Use a 10 mL pipette to gently load the wash buffer in a corner of the flask.
[3]Typically, three volumes of serum-containing culture medium should completely neutralize one volume of trypsin.

3.2 Preparation for Transfection

Bring solutions to room temperature before use.

1. Aliquot 100 μL of 2× HBS (solution 1).
2. Mix 500 ng each of CR-AKAR and YR-ICUE in another eppendorf tube and add 8 μL of 2 M $CaCl_2$ into the mixture. Bring the volume to 100 μL with deionized, distilled water (solution 2).
3. Add solution 2 drop-wise onto solution 1 while the latter is being vortexed and incubate for 2–40 min at RT (*see* Note 4).

3.3 HEK293T Cells Transfection

1. (*see* Note 5) Replace the medium in the imaging dishes with fresh medium. Gently add the DNA complex drop-wise into the center of imaging dish (*see* Note 6).
2. Culture for additional 18–48 h before imaging.

3.4 Preparation for Imaging (Microscope)

1. Turn on the lamp, microscope, filter changer, camera and computer sequentially. Place a drop of immersion oil on the 40× objective.
2. Load METAFLUOR software and set a protocol for image acquisitions in CR-FRET, CFP, RFP, YFP and YR-FRET channels every 30 s. Check to make sure proper filters are in place for each channel.

3.5 Preparation for Imaging (Cells)

All steps are performed at room temperature unless otherwise noted.

1. For each imaging experiment, prepare a 2 μL aliquot of the desired stimulus in a new 1.5 mL eppendorf tube and leave it on ice until ready to use.
2. Aspirate the culture medium from an imaging dish and wash the cells twice gently with HBSS* to remove dead cells and general debris (*see* Note 7).
3. Add 2 mL of HBSS* into the dish and mount the dish securely on the microscope for imaging.

3.6 Image Acquisition and Data Analysis

1. Focus the cells properly using the bright field setting.
2. Switch to fluorescence mode and look for healthy (*see* Note 8), co-transfected cells with optimal CFP and YFP levels (*see* Note 9).

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[4]200 μL of total DNA–calcium phosphate solution should be prepared for each imaging dish.
[5]About 20–40 % confluency of cells is desired at this point to achieve optimal transfection efficiency.
[6]Check for even distribution of DNA complex (as tiny black particles) on top of cells under the microscope.
[7]HEK293T cells are very easy to detach. Thus, extra caution is necessary at this step so as not to disturb the attached HEK293T cells. Gently rock the dish side to side.
[8]Healthy cells have a distinct morphology compared to cells under stress. Healthy cells will adhere well and have a flat appearance.

3. Draw a region of interest (ROI) on each of the fluorescent cells in the field. Also choose a region of interest on a region with an untransfected cell or no cell for background correction.

4. Log the intensity data and save the images from all five channels for each acquisition (*see* Note 10).

5. Acquire a series of images to establish the baseline before stimulus addition.

6. Stimulate the cAMP/PKA pathway by addition of a desired activator (FSK) or a GPCR agonist (ISO, PGE or RITO) (*see* Notes 11 and [12]). Draw ~500 μL of imaging buffer from the dish and add it to the eppendorf tube prepared during **step 1** of Subheading 3.5. Mix thoroughly and pipette drop-wise into the periphery of the imaging area. Gently pipette up and down several times without touching or disrupting the imaging area. Resume image acquisition immediately afterward.

7. Export the logged data to an excel file and calculate the FRET emission ratio change using the following formulae for each construct.

   For CR-AKAR,

$$\text{Emission Ratio} = \frac{(\text{CR}-\text{FRET channel intensity of ROI} - \text{CR}-\text{FRET channel intensity of background})}{(\text{CFP channel intensity of ROI} - \text{CFP channel intensity of background})}$$

   For YR-ICUE,

$$\text{Emission Ratio} = \frac{(\text{YFP channel intensity of ROI} - \text{YFP channel intensity of background})}{(\text{YR}-\text{FRET channel intensity of ROI} - \text{YR}-\text{FRET channel intensity of background})}.$$

8. Set the time point immediately after stimulus addition to time 0 and adjust subsequent time points accordingly using simple subtraction. Plot the emission ratio versus time on a graph (*see* Note 13). Emission ratio should increase upon signal activation.

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[9] Optimal intensity for each FP varies under different microscope settings. Thus, it is necessary to adjust the neutral density (ND) filters and exposure times of individual channels accordingly in order to achieve the desired intensity for each FRET pair and to avoid photobleaching of each FP. Maintain the same exposure time for donor and FRET channels of each FRET pair.

[10] The saved images can later be used to reanalyze the data, if necessary, or to make pseudocolor images or movies for visual purposes.

[11] The final concentration should be 50 μM and 1 μM for FSK and ISO respectively, and should be 10 μM for both PGE1 and RITO. If a higher volume of stock solution is required, prepare the stock solution using imaging buffer (HBSS*). Avoid adding high volume of DMSO into the imaging dishes to minimize DMSO-induced cytotoxicity.

[12] Pre-incubate cells with the PKA inhibitor H89 to check for pathway specificity. Be sure to use H89 containing imaging buffer for the subsequent steps.

[13] Sometimes it is desirable to normalize the emission ratio at a particular time point to the ratio at time 0 so as to easily analyze the percent change in signal. For example, normalized ratio change at time point A can be calculated as,

$$\text{Normalized Emission Ratio (at time point } A) = \frac{\text{Emission Ratio (at time point } A)}{\text{Emission Ratio (at time 0)}}.$$

3.7 Correction for Cross-Excitation of RFP Following YFP Excitation

If necessary, the contribution from cross-excitation of RFP can be corrected using the following steps:

1. Use an RFP construct (for example mCherry in pcDNA3.1) to obtain the correction factor, defined by YR/RR, where YR and RR represent readings in the RFP intensity following excitation of YFP and RFP, respectively;
2. Acquire the RFP emission to obtain the total RFP intensity in cells where correction is required (RR′) (*see* Note 14); and
3. Multiply the correction factor (YR/RR) by the total RFP direct emission (RR′) to determine the contribution of cross-excited RFP following YFP excitation (YR_x),
4. Subtract YR_x from the experimental reading in the YR-FRET channel (YR_{exp}). Thus, after correction, the actual YR-FRET signal (YR_{actual}) can be calculated as

$$\mathrm{YR_{actual}} = \mathrm{YR_{exp}} - \mathrm{YR_x},$$

where YR_x= RR′×(YR/RR).

References

1. Jordan JD, Landau EM, Iyengar R. Signaling networks: the origins of cellular multitasking. Cell. 2000; 103:193–200. [PubMed: 11057893]
2. Howe AK. Cross-talk between calcium and protein kinase A in the regulation of cell migration. Curr Opin Cell Biol. 2011; 23:554–561. [PubMed: 21665456]
3. Papa S, Bubici C, Zazzeroni F, Franzoso G. Mechanisms of liver disease: cross-talk between the NF-kappaB and JNK pathways. Biol Chem. 2009; 390:965–976. [PubMed: 19642868]
4. Kim C, Cheng CY, Saldanha SA, Taylor SS. PKA-I holoenzyme structure reveals a mechanism for cAMP-dependent activation. Cell. 2007; 130:1032–1043. [PubMed: 17889648]
5. Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev. 2005; 85:1303–1342. [PubMed: 16183914]
6. Zhou X, Herbst-Robinson KJ, Zhang J. Visualizing dynamic activities of signaling enzymes using genetically encodable FRET-based biosensors from designs to applications. Methods Enzymol. 2012; 504:317–340. [PubMed: 22264542]
7. Carlson HJ, Campbell RE. Genetically encoded FRET-based biosensors for multiparameter fluorescence imaging. Curr Opin Biotechnol. 2009; 20:19–27. [PubMed: 19223167]
8. Schultz C, Schleifenbaum A, Goedhart J, Gadella TW Jr. Multiparameter imaging for the analysis of intracellular signaling. Chembiochem. 2005; 6:1323–1330. [PubMed: 16010697]
9. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nat Biotechnol. 2004; 22:1567–1572. [PubMed: 15558047]
10. Aye-Han NN, Allen MD, Ni Q, Zhang J. Parallel tracking of cAMP and PKA signaling dynamics in living cells with FRET-based fluorescent biosensors. Mol Biosyst. 2012; 8:1435–1440. [PubMed: 22362165]
11. Allen MD, Zhang J. A tunable FRET circuit for engineering fluorescent biosensors. Angew Chem Int Ed Engl. 2008; 47:500–502. [PubMed: 18058965]

[14]It is necessary to use the same setting for the microscope, camera, ND, and exposure time in **steps 1** and **2**.

12. Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. Biochem Biophys Res Commun. 2006; 348:716–721. [PubMed: 16895723]

13. DiPilato LM, Zhang J. The role of membrane microdomains in shaping beta2-adrenergic receptor-mediated cAMP dynamics. Mol Biosyst. 2009; 5:832–837. [PubMed: 19603118]

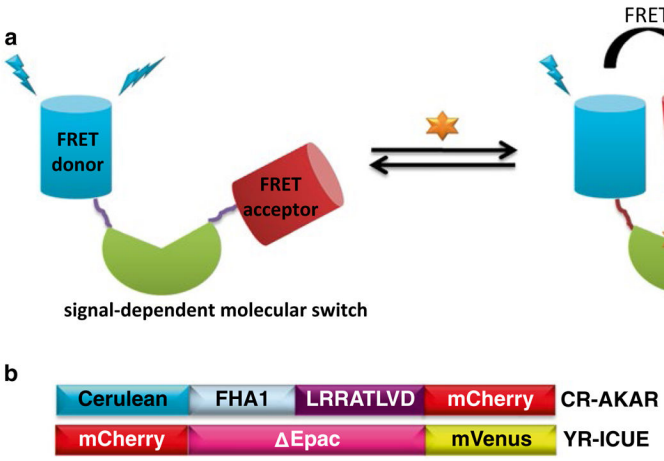

Fig. 1.

(**a**) General scheme of FRET-based activity reporters. A signal-dependent conformational change induces a change in FRET. We have constructed red-shifted reporters, in which CR-FRET and YR-FRET change can be efficiently detected. (**b**) Domain structures of CR-AKAR and YR-ICUE. These reporters can be conveniently generated by replacing a fluorescent protein from the CY-based reporters with mCherry

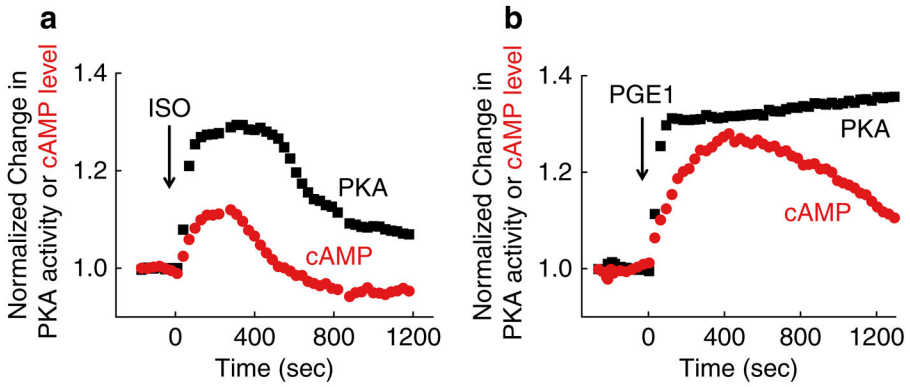

Fig. 2.

Parallel detection of differential cAMP and PKA dynamics upon a GPCR-agonist stimulation. Representative timecourses of PKA activity (*black*) and cAMP level dynamic (*red*) in HEK293T cells upon stimulation with (**a**) isoproterenol (ISO) and (**b**) prostaglandin E1 (PGE1)