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Using FRET-Based Reporters to Visualize Subcellular Dynamics of Protein Kinase A Activity

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Abstract

The ubiquitous Protein Kinase A (PKA) signaling pathway is responsible for the regulation of numerous processes including gene expression, metabolism, cell growth, and cell proliferation. This method details how to monitor real-time PKA activity dynamics in mammalian cells using fluorescence resonance energy transfer (FRET)-based reporters.

Keywords

Protein kinase A; Kinase activity reporter; Fluorescence resonance energy transfer; Live-cell imaging

1. Introduction

1.1. Protein Kinase A

Protein kinase A (PKA), also known as cAMP-dependent protein kinase, is ubiquitously expressed and regulates key cellular functions including gene expression, metabolism, growth, and proliferation (1). The PKA holoenzyme is tetrameric and consists of a regulatory subunit dimer and two catalytic subunits. Upon cAMP binding to the former, the catalytic subunits are released and able to phosphorylate numerous substrate proteins throughout the cell (2). Given that PKA regulates a myriad of different signaling events, it is vital that proper phosphorylation occurs in a specific temporal and spatial pattern. Four regulatory subunit isoforms (RI α , RI β , RII α , and RII β) and three catalytic subunit isoforms (C α , C β , C γ), which are differentially expressed in cells and have distinct biological and physical properties, play a role in achieving signaling specificity (1, 3). Additionally, A-kinase anchoring proteins (AKAPs) assemble signaling complexes containing PKA and its specific substrates and regulators at distinct subcellular locations, thereby facilitating specific phosphorylation and regulation of PKA substrates (4–6). All AKAPs anchor PKA via its regulatory domain, bind other signaling molecules to form multiprotein complexes, and target these signaling complexes to distinct subcellular locations (6).

1.2. FRET-Based Protein Kinase A Activity Reporter

Using fluorescence microscopy, genetically encodable fluorescence resonance energy transfer (FRET)-based A-Kinase Activity Reporters (AKARs) allow for live-cell visualization of endogenous PKA activity dynamics with high spatiotemporal resolution. AKARs consist of a molecular switch sandwiched between a FRET pair, which undergoes a conformational change when phosphorylated by PKA, leading to a change in FRET (Fig. 1). The molecular switch is comprised of a surrogate substrate for PKA and a phospho-amino acid-binding domain (PAABD) (e.g., forkhead-associated domain, FHA). Upon phosphorylation of the surrogate substrate by PKA, the PAABD binds the phosphorylated substrate.

The FRET response generated by kinase activity reporters depends on the fluorescent proteins used. FRET takes place when an excited donor fluorophore (e.g., Cyan Fluorescent Protein, CFP) transfers energy to an acceptor fluorophore (e.g., Yellow Fluorescent Protein, YFP) in close molecular proximity (i.e., <10 nm). For FRET to occur, the donor emission spectrum must overlap with the acceptor excitation spectrum (7, 8). CFP and YFP are commonly used as a FRET pair because the emission spectrum of CFP significantly overlaps with the excitation spectrum of YFP, while their excitation spectra have minimal overlap.

The energy transfer process causes donor emission intensity to be quenched and acceptor emission intensity to increase. As the stoichiometry between the donor and acceptor is fixed in AKAR (i.e., since it is a unimolecular reporter), the changes in emission ratio directly correlate to changes in FRET (7). On the other hand, FRET efficiency can be directly determined by acceptor photobleaching. This technique destroys the acceptor, thus abolishing FRET, which results in dequenched donor fluorescence intensity (7, 8).

For all experiments using AKAR it is important to use a negative control to ensure that the observed changes in FRET are due to phosphorylation of the reporter. The negative control AKAR is mutated at the phosphorylation site and can no longer be phosphorylated.

1.3. Studying Various PKA Activities

1.3.1. Basics of Studying PKA Activity with AKARs—AKAR is most commonly used to visualize changes in PKA activity dynamics over time. Such changes are typically induced by drugs or other perturbations to stimulate or inhibit PKA under various conditions (see Note 1). For example, AKAR was used to demonstrate that chronic insulin treatment induces a delay in β -adrenergic receptor (β -AR) stimulated PKA activity in adipocytes. The study used isoproterenol, a β -AR stimulant, and forskolin, an adenylyl cyclase activator, in the presence and absence of insulin to show that the time delay is specific to β -AR-stimulated PKA (9).

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¹It is important to verify effective drug concentrations and conditions that could affect their function. For example, when stimulating the PKA pathway using a G-Protein-Coupled Receptor agonist, first make certain that the specific receptor is expressed in the cell line being used. Western blots using antibodies against the receptor of choice can determine its presence. Additionally, western blots employing an antiphospho- PKA substrate antibody (Cell Signaling) can be used to determine effective drug concentrations.

1.3.2. Studying Discrete Domains of PKA Activity with Subcellularly Targeted AKARs

PKA activity dynamics at specific subcellular locations can be monitored using AKAR. In order to study PKA activity at a discrete location, AKAR may be targeted to that location. Subcellularly targeted AKARs are created by adding N- or C-terminal localization motifs, and then verified with colocalization studies using specific subcellular markers. For instance, proper targeting of AKAR to the nucleus can be validated by checking the colocalization of the reporter with a DNA stain. A study demonstrating the ability of AKAR to monitor PKA activity in discrete subcellular locales targeted AKAR: (1) to the plasma membrane via the addition of a C-terminal lipid modification, (2) to the nucleus via a C-terminal nuclear localization signal, (3) to the cytoplasm via a C-terminal nuclear export signal, and (4) to the outer membrane of mitochondria via an N-terminal localization sequence derived from a mitochondria-targeted protein. Subsequently, the mitochondria-targeted AKAR was used to show that PKA activity at mitochondria and global PKA activity are differentially regulated (10).

1.3.3. Studying Spatially Localized PKA Activity—AKAR can be used to study the spatial organization of PKA activity during different cellular processes. For example, using a plasma membrane targeted AKAR it was found that PKA activity is spatially organized in migrating Chinese Hamster Ovary (CHO) cells. In this study, cell migration was initiated by scratching a wound into a monolayer of CHO cells. Increased PKA activity was observed at the leading edge of migrating cells, but not at the trailing edge (11).

The following method details how to maintain Human Embryonic Kidney (HEK) 293T and Chinese Hamster Ovary (CHO) cells, transfect these cells with AKAR plasmid DNA, prepare the cells for imaging, prepare the imaging setup, image live-cell kinase activity, and analyze acquired data to quantify observed changes in FRET.

2. Materials

2.1. Cell Culture and Transfection

1. Cell lines: Human Embryonic Kidney – SV40 T Antigen (HEK 293T) and Chinese Hamster Ovary (CHO) (American Type Culture Collection).
2. Dulbecco's phosphate-buffered saline without Mg^{2+} and Ca^{2+} (DPBS)
3. T-25 cm² tissue culture flasks.
4. 35 mm glass-bottom imaging dishes (MatTEK).
5. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (DMEM-HEK 293T) to use with HEK 293T cells. Supplement this medium with 1% nonessential amino acids (DMEM-CHO) to use with CHO cells (see Note 2).
6. Solution of trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA, 0.53 mM).

²All solutions should be made under sterile conditions in a tissue culture hood and cell culture media should be warmed to 37°C before using with cells.

7. Fibronectin from human plasma lyophilized powder is dissolved in DPBS to 5 $\mu\text{g/mL}$ and stored in 200 μL aliquots at -20°C .
8. Bovine Serum Albumin lyophilized powder (BSA) is dissolved in DPBS to make a 1% BSA solution and then heat-denatured by boiling for 5 min. Be sure to let this solution cool to room temperature before using.
9. Lipofectamine 2000 (Invitrogen)
10. OPTI-MEM I Reduced Serum Medium (Opti-MEM; Gibco).
11. AKAR and pm-AKAR plasmid DNA.

2.2. Epifluorescence Microscopy

1. All the described experiments are performed on an Axiovert 200 M microscope using a $40\times/1.3\text{NA}$ oil-immersion objective lens equipped with an Aqua Stop to prevent liquid from running down the objective (Zeiss). Images are captured using a MicroMAX BFT512 cooled charge-coupled device camera (Roper Scientific).
2. Xenon lamp: XBO 75W (Zeiss).
3. Neutral density filters 0.6 and 0.3 (Chroma Technology).
4. Filter sets for individual channels (All from Chroma Technology):
  - FRET – 420DF20 excitation filter, 450DRLP dichroic mirror, 535DF25 emission filter.
  - CFP – 420DF20 excitation filter, 450DRLP dichroic mirror, 475DF40 emission filter.
  - YFP – 495DF10 excitation filter, 515DRLP dichroic mirror, 535DF25 emission filter.
  - YFP photobleaching – 525DF40 excitation filter, 560DRLP dichroic mirror.
  - A Lambda 10-2 filter changer (Sutter Instruments) alternates the filters being used.
5. Immersol® 518F fluorescence free immersion oil (Zeiss).
6. METAFLUOR 6.2 software (Molecular Devices).

2.3. Preparing Cells for Imaging

1. Hanks' Balanced Salt Solution for Imaging (HBSS*): $10\times$ Hanks' Balanced Salt Solution (Gibco), 20 mM HEPES, 2.0 g/L d-glucose; adjust pH to 7.4, then filter sterilize using a 0.22 μm filter. Keep a 50 mL aliquot at room temperature in the microscope room and store the rest at 4°C (see Note 3).

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³All solutions should be prepared with water that has an 18.2 M Ω cm resistivity unless otherwise noted.

2.4. Cell Stimulation and Image Acquisition

1. Forskolin (Fsk) dissolved at 50 mM in dimethyl sulfoxide (DMSO) and stored at -20°C .
2. 200 μL pipet tip to scratch and wound CHO cell monolayer.

2.5. Image and Data Analysis

1. Spreadsheet application (e.g., Microsoft Office Excel).

3. Methods

3.1. Cell Culture and Transfection

1. The cells are maintained in T-25 cm^2 flasks at 37°C with 5% CO_2 and passaged when they are 85–95% confluent (every 2–3 days) into flasks or 35 mm imaging dishes.
2. HEK293T cells can be plated on uncoated imaging dishes, but CHO cells must be plated on fibronectin-coated dishes. To coat the imaging dishes, add 200 μL of 5 $\mu\text{g}/\text{mL}$ fibronectin solution to the glass cover-slip in the imaging dish and incubate at room temperature for 30–45 min. Then aspirate off the fibronectin solution and add 200 μL of 1% BSA solution to the glass cover-slip of the imaging dish for 1 h at room temperature (see Note 4).
3. To passage cells, aspirate the culture medium from the flask and wash cells with 2 mL of DPBS. Add 300 μL of trypsin/EDTA solution and gently rock the dish from side to side to disperse the solution, then let it sit for 2–5 min (see Note 5). Add 4.7 mL of fresh medium (make certain to use cell line appropriate medium) into the flask and mix well. Perform a 1:10 split of CHO cells and a 1:20 split of HEK 293T cells into 35 mm glass-bottom imaging dishes. Both cell lines should reach 60–70% confluence in approximately 24 h (see Note 6). Transfect the cells at this confluence.
4. For each 35 mm dish to be transfected, prepare two separate microcentrifuge tubes. Tube 1 contains 1 μg AKAR or pm- AKAR plasmid DNA and 50 μL Opti-MEM. Tube 2 contains 2 μL Lipofectamine 2000 and 50 μL Opti-MEM. Let these tubes incubate at room temperature for 5 min. Then add Tube 1 drop-wise to Tube 2 and mix well with a pipet (see Note 7). Incubate the transfection solution at room temperature for 20 min.
5. Gently add the AKAR transfection solution to HEK293T cells and the pm-AKAR transfection solution to CHO cells drop-wise and lightly rock the dish from side to side to get even distribution. Incubate at 37°C with 5% CO_2 for 18–24 h.

⁴It is unlikely that fibronectin coating of the imaging dish will be 100% effective, meaning uncoated spots will be present for other secreted adhesion proteins to bind to. In order to control for these situations 1% BSA is used to block the potential non-fibronectin adhesion sites.

⁵Be sure the cells are fully detached before continuing. Gently swaying the flask side to side should help.

⁶This protocol can be adapted for other cell lines by following recommended cell culture and transfection guidelines for the cell line of choice. Additionally, cell growth rates may vary, so it is important to verify doubling times for each cell line used.

⁷Be sure to mix gently. Do not vortex the solution.

3.2. Preparing the Epifluorescence Microscope

1. Turn on the lamp, microscope, filter changer, camera, and computer. Load the METAFLUOR 6.2 application and a protocol to acquire a time series of sets of images for the FRET, CFP, and YFP channels (see Note 8). Check that all of the appropriate filters are in place.
2. Set the excitation exposure times for the FRET, CFP, and YFP channels to 500, 500, and 50 ms, respectively. The time lapse between each set of acquisitions is set between 10 and 120 s, typically 30 s.
3. Apply a small drop of immersion oil directly onto the objective. Make sure not to use an excess amount (i.e., 1 drop from the attached applicator should suffice).

3.3. Preparing Cells for Imaging

3.3.1. HEK 293T Cells

1. Aspirate the medium from transfected cells in the imaging dish and wash twice with 1 mL HBSS*.
2. Gently add 1–2 mL HBSS* to the imaging dish, while holding the dish on a slight angle. Slowly return the dish to a level position and place securely on microscope stage (see Note 9).
3. Raise the objective until the drop of the oil comes into full contact with the glass cover-slip and then examine the cells using the eyepiece and focus.
4. In the dark, use the FRET or CFP channel to select cells with good morphology and good AKAR expression, meaning intermediate to high emission intensity and a uniformly distributed fluorescence (see Note 10).

3.3.2. CHO Cells

1. Using a 200 μ L pipet tip, scratch the glass cover-slip in the imaging dish with the transfected monolayer of CHO cells (see Note 11). Carefully wash the cells twice with 1 mL of HBSS* to remove cell debris. Gently add 1–2 mL HBSS* to the imaging dish, securely fasten dish on microscope stage, raise the objective, focus on cells near the scratch, and let cells migrate for 15 min before imaging.

⁸The FRET channel logs YFP emission intensity when CFP is excited, the CFP channel logs CFP emission intensity upon direct CFP excitation, and the YFP channel logs YFP emission intensity upon direct YFP excitation. The YFP channel serves to control for YFP photobleaching and is not used to determine emission ratios.

⁹Securing the dish to the stage is important as it minimizes slight movement of the dish that may occur while imaging.

¹⁰Key criteria for proper cell selection: first, cell morphology is important to verify before starting an experiment as healthy cells are required for successful imaging experiments. For instance, when imaging HEK 293 cells, select cells that are spread out and lying flat rather than balled-up and rounded, as the latter could indicate unhealthy cells. Second, the fluorescence intensity level of AKAR should be closely monitored, though a recommended range cannot be given as the intensity values will vary with microscope setups. However, cells with a moderate- to high-intensity level are typically used. Cells with very dim fluorescence intensities will have a low signal-to-noise ratio, thus changes in FRET will be difficult to visualize, whereas cells with very high fluorescence intensities may have perturbed endogenous signaling pathways because of excessive expression of AKAR. Third, if using a targeted AKAR to look at specific effects at a subcellular locale, verification of proper AKAR distribution within the cell is critical. For example, diffusible AKAR should fluoresce uniformly throughout cells, whereas fluorescence of pm-AKAR should be limited to the plasma membrane of cells.

¹¹It is important to verify that the CHO cells have formed a confluent monolayer before inflicting the wound.

2. In the dark, use the FRET or CFP channel to select cells with good morphology and good AKAR expression, meaning intermediate to high emission intensity and plasma membrane-restricted fluorescence distribution.

3.4. Cell Stimulation and Image Acquisition

3.4.1. Chemically Induced PKA Activity in HEK 293T Cells

1. Select several regions of interest to follow during the course of the experiment (see Note 12). A background region consisting of an untransfected cell must also be selected to correct for cell autofluorescence and other background fluorescence.
2. Acquire 3–5 min of data (all three channels) from the unstimulated cells to establish a baseline for the experiment. Pipet ~300 μ L of HBSS* out of the imaging dish, mix with a 1–2 μ L aliquot of 50 mM Fsk in a 1.5 mL tube, then gently pipet this solution back into the imaging dish. The final concentration of Fsk should be 50 μ M. Be sure to note the time of the drug addition (see Note 13). The yellow to cyan emission ratio (FRET channel emission/CFP channel emission) should rapidly increase, indicating a change in PKA activity.
3. At the end of the experiment remove all neutral density filters, use the YFP photobleaching excitation filter, and then excite for 5 min. This should sufficiently photobleach YFP, but it is important to verify this by acquiring the YFP channel. The acquired data can be used to calculate absolute FRET efficiency using the following formula:

$$\text{FRET efficiency} = 1 - \frac{\text{CFPEmission}(\text{before YFP photobleaching})}{\text{CFPEmission}(\text{after acceptor photobleaching})}$$

3.4.2. Localized PKA Activity in Migrating CHO

1. Select several regions of interest that are specific to the leading edge and trailing edge of a migrating cell to follow during the course of the experiment. Regions within a nonmigrating cell should also be selected to compare PKA activity between these two types. A background region must also be selected to correct for cell autofluorescence and other background fluorescence.

3.5. Image and Data Analysis

1. Use METAFLUOR 6.2 to generate pseudo-colored images for each acquisition where a pseudocolor is used to indicate the yellow to cyan emission ratio (FRET channel emission/ CFP channel emission) (see Note 14). These images can be strung together in a movie clip or a selection of them can be used to visually represent the observed real-time changes. An example is shown in Fig. 2.

¹²The selected regions of interest will need to remain in the same cellular region throughout the time series, and thus may be adjusted should the cells move. Alternatively, cell tracking software (e.g., Imaris Track) can be used to overcome this problem.

¹³All different types of PKA agonists and antagonists can be used, either alone or together in a single imaging dish.

¹⁴If the software being used does not have this feature, an image processing application (e.g., ImageJ) can be used to create pseudo-colored ratiometric images using the raw emission intensity images from the individual channels.

2. Using a spreadsheet application, calculate emission ratios from the logged data using the following formula for each time point:

Yellow to Cyan Emission Ratio=

$$\frac{\text{FRETchannel Emission Intensity} - \text{FRETchannel Emission Intensity of Background}}{\text{CFPchannel Emission Intensity} - \text{CFPchannel Emission Intensity of Background}}$$

3. Plot the ratio time course (ratios vs. time).

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Fig. 1.

Design and Mechanism of AKAR. **(a)** AKAR uses CFP as the donor FP and YFP as the acceptor FP with the phospho-amino phospho-amino acid-binding domain, FHA1, and a PKA substrate sandwiched in between. The star indicates the phosphorylation site. **(b)** Once PKA phosphorylates AKAR, FHA1 binds the phosphorylated substrate, inducing a conformational change that brings the FPs into closer proximity. This action is reversed by phosphatases. The *triangle* in the closed conformation represents the phosphorylated threonine.

Fig. 2. AKAR response in live cells. HEK 293 cells were transfected with AKAR and imaged 24 h later. The cells were stimulated with 50 μ M Fsk, an adenylyl cyclase activator. **(a)** Yellow to cyan emission ratios plotted against time represent changes in PKA activity over time. **(b)** Pseudocolor images showing AKAR response.