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Quantitative Monitoring Spatiotemporal Activation of Ras and PKD1 Using Confocal Fluorescent Microscopy

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Abstract

Receptor activation upon ligand binding induces activation of multiple signaling pathways. To fully understand how these signaling pathways coordinate, it is essential to determine the dynamic nature of the spatiotemporal activation profile of signaling components at the level of single living cells. Here, we outline a detailed methodology for visualizing and quantitatively measuring the spatiotemporal activation of Ras and PKD1 by applying advanced fluorescence imaging techniques, including multichannel, simultaneous imaging and Förster resonance energy transfer (FRET).

Keywords

Confocal fluorescence microscopy; Ras activation; Protein kinase D 1 (PKD1); Phosphorylation of PKD1; Förster resonance energy transfer (FRET)

1 Introduction

Chemotaxis, a chemoattractant gradient-directed cell migration, plays critical roles in many physiological processes such as recruitment of neutrophil to the sites of inflammation, neuron patterning, immune response, angiogenesis, and metastasis of cancer cells. Eukaryotic cells detect many chemoattractants by different receptors, including G protein-coupled receptors (GPCRs). Ras GTPases are one of the most evolutionarily conserved proteins among eukaryotes and play essential roles in many signaling pathways. By coupling with many different receptors, Ras GTPases are universal molecular switches that act as kinetic timers of signal transduction events upon extracellular stimulation. The engagements of various chemoattractants to their GPCRs generally trigger Ras activation in neutrophil and neutrophil-like HL60 cells [1]. It is important to measure the spatiotemporal dynamics of Ras activation in response to various fields of chemoattractant stimuli in neutrophils.

The protein kinase D family of serine/threonine kinases plays critical roles in many physiological processes, including cell growth, protein trafficking, and lymphocyte biology [2]. A recent study has shown that PKD1 phosphorylates SSH1 and regulates cofilin to mediate F-actin depolymerization in breast cancer cells [3]. PKD isoforms are also known to be highly expressed in immune cells, including neutrophils [4]. We have shown that PKD and its spatiotemporal activation play essential roles in neutrophil chemotaxis [5]. Though

several methods had been developed to measure the activation profile of PKDs [6–8], it is becoming increasingly important to measure the spatiotemporal activation of PKD levels in single live cells from different tissue origins.

Here, we report two imaging methods that have been successfully applied to measure spatiotemporal changes of signaling events in single live cells in real time. First, we describe how to simultaneously visualize multiple signaling events upon application of different fields of chemoattractant stimulation. Next, we describe how to apply the Förster resonance energy transfer (FRET) imaging technique to monitor and measure PKD1 activation in single live cells using two different intramolecular FRET probes, DKAR and CFP-PDK1-YFP (hereafter referred to as “PKD1-CY”).

2 Materials

2.1 HL60 Cell Culture

1. RPMI 1640 medium GlutaMAX™ Supplement (Life Technologies, Grand Island, NY, #61870-036).
2. Fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA).
3. HEPES 1 M sterile solution, pH 7.3 (Quality Biological Inc. Gaithersburg, MD).
4. Penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution (Gibco, Invitrogen Life Science).
5. RPMI 1640 complete culture media (RPMI 1640 medium GlutaMAX™ Supplement with 20 % FBS, 25 mM HEPES, and 1 % Penicillin/Streptomycin).
6. RPMI 1640 starving media (RPMI 1640 medium GlutaMAX™ Supplement with 25 mM HEPES).
7. T75 flask (BD Biosciences, Falcon).
8. HL-60 cell line (ATCC, Manassas, VA).
9. Cell culture incubator, 37 °C, 5 % CO₂.
10. Cell culture hood (NUNR Class II biological safety cabinet type A/B3, Plymouth, MN).
11. *N*-Formyl-Met-Leu-Phe (fMLP) (Sigma, St. Louis, MO). A 1 mM stock is made in DMSO and frozen at –20 °C in 10 µl aliquots. The working concentration of fMLP is 100 nM–1 µM.
12. Alexa 594 (Molecular Probes, Eugene, OR). A 1 mg/ml stock is made in water and frozen at –20 °C in 10 µl aliquots. The working concentration is 0.1 mg/ml.

2.2 Plasmids for Ras Activation and PKD1 FRET Probe

1. Plasmid encoding an active Ras binding domain (RBD) tagged with GFP (Addgene, plasmid # 18664).

2. Plasmid encoding Ras tagged with RFP (John F. Hancock, University of Texas Medical School at Houston).
3. Plasmids encoding CFP-CXCR1 (single donor control) and CXCR1-YFP (single acceptor control) (T. Jin, NIAID/NIH).
4. Plasmid encoding the FRET probe PKD1-CY (Q. Jane Wang, University of Pittsburg).

2.3 Transfection of HL60 Cells

1. Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland).
2. Plastic bulb sterile pipette included in Nucleofector® Kit.
3. 1.6 ml tube (Neptune, #4445.S.X).

2.4 Coating Cover-Glass Surface of 4-Well Chamber

1. 10 % Gelatin solution (Sigma-Aldrich, St. Louis, MO, #G1393).
2. HBSS (Hanks' Balanced Salt Solution, Life Technologies, Grand Island, NY).
3. Single- and four-well Lab-Tek II cover-glass chambers (Nalge Nunc International, Naperville, IL).

2.5 MDA-MB-231 Cell Culture

1. DMEM Medium with high glucose, l-glutamine, and phenol red (Gibco, Invitrogen).
2. Fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA).
3. Penicillin/streptomycin stock solution (Gibco, Invitrogen Life Science, #15140). Penicillin (10,000 U/ml) and streptomycin 10 mg/ml solution is frozen at -20 °C in 5 ml aliquots.
4. DMEM complete culture medium. 500 ml DMEM Medium with high glucose, l-glutamine, and phenol red containing DMEM Medium with 10 % FBS and 5 ml penicillin/streptomycin stock solution.
5. Trypsin-EDTA (0.05 %) (Gibco, Life Technologies, Cat# 25300-054).
6. PBS 10× (KD Medical, Cat# RGF-3210).
7. DEPC Treated Water (Quality Biological, Cat# 351-068-131).
8. 1× PBS. Combine 50 ml of 10× PBS with 450 ml of DEPC-treated water.
9. T75 flask (BD Biosciences, Falcon).
10. Cell lines: MDA-MB-231 cells.
11. Cell culture incubator, 37 °C, 5 % CO₂.
12. Cell culture hood (NUNR Class II biological safety cabinet type A/B3, Plymouth, MN).

2.6 Transfection of MDA-MB-231 Cells by Lipofectamine 2000

1. Lipofectamine 2000 Reagent (Invitrogen, Cat# 11668-027).
2. Opti-MEM (Invitrogen, Cat#51985091).
3. Plasmids: CFP, YFP, CFP-PKD1-YFP (Q. Jane Wang, University of Pittsburg).
4. DMEM transfection medium (DMEM medium with 10 % FBS).
5. DMEM complete culture medium.
6. 6-Well cell culture plate (Costar, Corning Incorporated, Cat# 07-200-80).
7. Trypsin–EDTA (0.05 %) (Gibco, Life Technologies, Cat# 25300-054).
8. 1× PBS. Combine 50 ml of 10× PBS with 450 ml of DEPC-treated water.
9. 4-well chamber with cover glass (Lab-Tek, Nunc, Cat# 155383).
10. Dimethyl Sulfoxide (Sigma-Aldrich).
11. Phorbol 12-Myristate 13-Acetate (Sigma-Aldrich).

2.7 Imaging with Carl Zeiss Zen710 Microscope

1. Four-well Lab-Tek II coverglass chambers (Nalge Nunc International, Naperville, IL).
2. FemtoJet microcapillary pressure supply (Eppendorf, Germany).
3. TransferMan NK2 micromanipulator (Eppendorf, Germany).
4. Zen 710 or equivalent with 40× 1.3 NA objective lens.
5. Other microscope accessories:
  - a. Heating Insert P Lab-Tek S1 (Pecan, Part# 131-800 029).
  - b. TempModule S1 (Pecan, Part# 800-800 000).
  - c. Zen 2010 Software, Macro FRET 55.
  - d. Carl Zeiss Immersol 518 F (Zeiss).
  - e. Lasos Ar-Ion Laser Series (Zeiss, Model LGN 4000, Remote Control RMC 7812 Z2).
  - f. Controller pad (Wienecke & Sinske GmbH, Serial# 12-0902305, Gleichen, Germany).

3 Methods

3.1 Simultaneous Monitoring of Ras Activation in Response to Various Visible Fields of Chemoattractant Stimulation

3.1.1 Cell Culture

1. Culture HL60 cells in RPMI 1640 medium with 20 % FBS, 1 % Penicillin and streptomycin containing 25 mM HEPES and passage them every 2 or 3 days (*see Note 1*).

3.1.2 Coating the Cover-Glass Surface of the 4-well Chamber

1. Dilute 10 % Gelatin Stock Solution with 1× HBSS to final concentration of 0.2 % gelatin.
2. Add 1 ml 0.2 % gelatin in HBSS to the two middle wells of a 4-well chamber bottomed with cover glass. Incubate the chamber at 37 °C for 1 h.
3. Just before seeding cells, remove the gelatin solution from 4-well chamber (*see Note 2*).

3.1.3 Transfection of HL60 Cells

1. Differentiate HL60 cells in 1.3 % DMSO for 5 days. Cell density is measured before transfection (*see Note 3*).
2. Centrifuge the cells at $200 \times g$ for 5 min.
3. After removing the supernatant, suspend the cells in transfection reagent according to the manufacturer's recommendation (usually two million cells in 100 μ l of reagent per transfection) and add the mixture to electroporation cuvette.
4. Use the manufacturer's program T-019 to perform the electroporation.
5. Without delay, gently add 500 μ l RPMI 6140 complete culture medium to the electroporated cells in the cuvette.
6. Gently transfer the cells from the cuvette to a 1.6 ml tube with the provided plastic pipettes.
7. Place the tube into a cell culture incubator for 30 min.
8. Seed a 100 or 500 μ l of cells into pre-coated wells of 4-well or 1-well chamber, respectively (*see Note 4*).

¹It is not recommended that HL60 cells are passaged more than 30 times.

²About 5–10 % differentiated cells adhere to gelatin coated cover glass. We suggest a thorough removal of excess gelatin since we often saw less HL60 cells adhere to the cover glass containing floating gelatin.

³After 5 days of the differentiation, cell densities vary from 1 to 2.5×10^6 cells/ml. The best chemotactic cells might be found at days 5 or 6 after differentiation. Best guess bases on the morphological changes from the round-shape undifferentiated cells to an oval-like polarized, chemotactic cells.

⁴ $3\text{--}5 \times 10^5$ HL60 cells in an appropriate volume are usually seeded into one well of four-well chamber for the experiments. Up to 1 ml of medium is added to one well of 4-well chamber for slightly longer time of incubation or starvation.

9. Add culture medium up to a final volume of 1 or 4 ml to one well of a four-well chamber or a one-well chamber, respectively.
10. After allowing the cells to recover for 3 h in the incubator, gently remove all of the culture medium from the well of chamber and replace it with RPMI 1640 starving medium (*see* Note 5).
11. One hour later, the cells are ready for imaging experiments.
12. Meanwhile, prepare 100 nM or 1 μ M fMLP stimuli (*see* Note 6).

3.1.4 Simultaneous Monitoring Application of Chemoattractant Stimuli and Ras Activation in Response to Chemoattractant Stimulation—Chemotactic cells are able to respond to small spatiotemporal differences in concentrations of chemoattractants. We have developed a method that allows us to indirectly visualize the spatiotemporal changes of an applied cAMP stimulus around cells by mixing the chemoattractant with a fluorescent dye and measuring the changes in fluorescence intensity [9]. This method also allows us to measure the difference of a chemoattractant gradient to which cells are exposed or to measure the spatiotemporal application of stimuli exposed to cells. Moreover, by using cells expressing a different emission fluorescent marker, we are able to simultaneously measure multiple events, including application of chemoattractant stimuli and cellular dynamics in response to the stimuli. Here, we present different procedures to apply several kinds of fMLP stimuli to neutrophil like cells, HL60, using live cell imaging experiments.

1. Sudden exposure of a cell to uniform fMLP stimulation. To generate a uniform stimulation, 200 μ l of a mixture of fMLP and Alexa 633 (0.1 μ g/ μ l) is applied to the cells covered with 800 μ l buffer and placed in a four-well chamber (Fig. 1a, b). Under this condition, cells are normally exposed to a uniform fMLP stimulation that was about 2.5-fold of the final concentration.
2. Exposure of a cell to a steady fMLP gradient. To establish a fMLP gradient, a micropipette (Eppendorf Femtotips) is filled with 30 μ l solution of fMLP and Alexa 633 and then attached to a FemtoJet and micromanipulator 5171 (Eppendorf). FemtoJet is set at $P_c = 70$ and $P_i = 70$ to inject a constant and tiny volume of a mixture of fMLP and Alexa 633 into a one-well chamber that is covered with 5 ml buffer. Under this condition, a gradient can be established with 100 μ m around the tip of the micropipette and is usually stable for more than 1 h (Fig. 1c; *see* Note 7).

⁵For stimulation experiment, cells are much more responsive after a short period of starvation. In order to starve cells, RPMI1640 complete culture medium is replaced by RPMI1640 starving medium without FBS. Caution: to remove medium from the cells already seeded in either 4-well or 1-well chamber, we tried several different ways to figure out a best way to exchange the medium without washing away the adhering cells. We found that directly flipping the chamber to dump the excess medium works well.

⁶To visualize the application of chemoattractant stimuli to the cells, a final concentration of 100 nM fMLP is mixed with Alexa 633 (final concentration is 0.1 μ g/ μ l). For gradient experiment, a final concentration of 1 μ M fMLP is mixed with Alexa 633 (0.1 μ g/ μ l). The selected dyes should be compared under the experimental conditions. It is important to select proper fluorescence dyes to monitor a chemoattractant. Because the delivery procedures depend on the diffusion, diffusion properties of a chemoattractant, the selected dyes should be compared with the chemoattractant under experimental conditions. The dye with similar diffusion efficiency as that of the chemoattractant should be used in order to accurately measure the concentrations of the chemoattractant in live cell experiments.

⁷We often experience blockage of the needle of microinjector during chemotaxis assay of HL60 cells. To avoid this problem, we suggest to remove the non-adhering cells by change the medium prior to the experiments.

We presented three channel setting (DIC, red, and far red) here and observed a clear membrane localization of H-Ras in HL60 cells as previously reported [10]. If necessary, it can be set up to five channels by adding blue and green emission channels [9]. Although these procedures are well established, it is recommended to optimize the channel distribution by the abundance and biological relevance of the proteins, the options and photon yields of the labeling fluorophores, and other necessary considerations for the experiments. For the fluorescent dye, it is has to be ensured that chemoattractant stimuli are properly delivered in each experiment. It is also important to select an appropriate fluorescent dye to monitor a chemoattractant. Because the delivery procedures depend on the diffusion properties of a chemoattractant, the selected dyes should be compared with the chemoattractants under the experimental conditions. The dye with a diffusion coefficient similar to that of the chemoattractant should be used in order to accurately measure the concentrations of the chemoattractant in live cell experiments.

3.2 Spatiotemporal Monitoring PKD1 Activation by Förster Resonance Energy Transfer

Dynamic changes in protein conformation or protein–protein interaction can be monitored in single living cells in real time using FRET imaging methodologies. FRET detection and its efficiency depend upon the distance between the donor and acceptor fluorophores, which ranges between 10 and 100 Å. A good fret pair would have an efficient overlapping of donor CFP emission spectrum with the excitation spectrum of acceptor YFP (>30 %). Previously, we presented a spectral-resolved FRET measurement of heterotrimeric G protein activation in subcellular level in real time [9, 11]. Below, we describe the two different FRET probes designed by different principles to spatiotemporally measure PKD1 activation upon extracellular stimuli.

3.2.1 MDA-MB-231 Cell Culture

1. Culture the MDA-MB-231 cells in DMEM complete medium containing DMEM medium, 10 % FBS and 1 % Penicillin/Streptomycin.
2. Monitor the cells daily. When they reach a density of 85–95 % confluency, passage the cells every 2–4 days as follows.
3. Remove culture medium using vacuum.
4. Rinse the cell layer with 10 ml of PBS 1×.
5. Remove the PBS with vacuum.
6. Rinse the cell layer with 2 ml of 0.05 % Trypsin–EDTA. To facilitate dissociation of the cell layer, place the flask in the 37 °C cell culture incubator for 1 min.
7. Lightly tap the flask and confirm that the cells have detached from the flask under the microscope.
8. Add 10 ml of fresh DMEM growth medium and gently pipette up and down to prevent clumping of cells.

9. Aliquot the MDA-MD-231 cells to new T75 flasks at sub-cultivation ratios of 1:2 to 1:6 as desired.
10. Incubate the cell cultures in the 37 °C cell culture incubator with 5 % CO₂.

3.2.2 Transfection of MDA231 Cells with CFP, YFP, and CFP-PKD1-YFP

1. Cell cultures should be prepared so that a T75 flask of MDA-MB-231 cells is 90 ~ 95 % confluent 3 days before the planned imaging experiment.
2. Remove the culture medium from T75 flask using vacuum and gently rinse the cell layer with 10 ml of 1× PBS and remove the PBS using vacuum.
3. To dissociate the cell layer, transfer 2 ml of 0.05 % Trypsin–EDTA and place the flask in the 37 °C cell culture incubator for 1 min. Lightly tap the flask and confirm that the cells have detached from the flask under the microscope. Then, add 6 ml of fresh DMEM culture medium and gently pipette up and down to prevent clumping of cells.
4. Add 1 ml of the cell suspension to each well of a 6-well cell culture plate and incubate the plated cells in the 37 °C cell culture incubator with 5 % CO₂ overnight.
5. In three 1.5 ml Eppendorf tubes, dilute 25 µl of Lipofectamine 2000 Reagent (room temperature; mix gently before use) in 250 µl of Opti-MEM (room temperature).
6. In another three tubes, dilute 10 µl of CFP, YFP, or PKD1-CY plasmids in 250 µl of Opti-MEM (room temperature). After the 5 min of incubation, combine the diluted Lipofectamine 2000 and diluted DNA. Gently pipette to mix and Incubate for 20 min at room temperature.
7. Add the DNA-Lipofectamine complexes drop by drop into 6-well plates of cells. As the solution is added, gently swirl the plate to evenly distribute to the cells.
8. After 3–4 h of incubation in 37 °C cell culture incubator, change the medium to fresh DMEM growth medium (10 % FBS and 1 % Penicillin/Streptomycin). Continue incubation overnight in 37 °C cell culture incubator.
9. Aliquot the cells obtained from one well of the 6-well plates into 4-well chambers.
10. Gently pipette out the DMEM medium from each well of the 6-well plate.
11. Wash each well with 2 ml of PBS 1× into each well and add 0.5 ml of 0.05 % Trypsin–EDTA into each well and place in 37 °C cell culture incubator for 1 min. Tap gently and view under microscope to confirm all cells have detached and then add 1 ml of culture medium into each well.
12. Centrifuge the cells at 300 rpm for 5 min and discard the supernatant.
13. Suspend the cells with 1.5 ml fresh DMEM culture medium and aliquot 180 µl into the center of each well of the 4-well Lab-Tek Chambers (only the middle

two wells of each Lab-Tek Chamber were used for convenience during imaging). Note: eight total wells per plasmid were used. And then incubate overnight in 37 °C cell culture incubator.

14. Four hours before imaging, add 820 µl of fresh DMEM culture medium to each well and prepare 2 µM PMA stimuli for imaging experiment.

3.2.3 Sensitized Emission FRET Imaging Monitors PKD1 Activation Using

Intramolecular FRET Probe DKAR—FRET Sensitized Emission is used for measuring the actual FRET efficiency. Same as the spectrally resolved FRET measurement, FRET Sensitized Emission is widely used to measure protein–protein interaction in live cells. Particularly, FRET Sensitized Emission provides a powerful approach to detect protein–protein interaction (FRET measurement) in fixed cells or tissue samples in which a dynamic of FRET is either not possible or necessary.

Same as spectral resolved FRET detection, sensitized emission FRET measurement also requires three types of cells: donor cells containing only the donor fluorophore; receptor cells containing only the acceptor fluorophore; and FRET cells containing both donor and acceptor fluorophores. FRET measurement starts with the acquisition of images in three channels under Channel Mode: (1) Donor channel: Excitation with the donor's excitation wavelength and detection with donor's parameter settings. (2) FRET channel: Excitation with donor's excitation wavelength and detection with acceptor's parameter settings. (3) Acceptor channel: Excitation with acceptor's excitation wavelength and detection with acceptor's parameter settings. The two single labeled samples provide the reference values to correct the optical bleed through of the donor (donor emission bleeds into acceptor emission) and the possible excitation of the acceptor through the wavelength of the donor. The third sample is the actual FRET sample on which the measurement is performed and measured.

3.2.4 Acquisition Configuration of Sensitized Emission FRET Measurement by

Applying CFP/YFP FRET Pairs—Sensitized emission FRET measurement calculates the bleed through of donor and cross-talk of acceptor which must be obtained in cells expressing single fluorophore, as an example here are cells expressing CFP or YFP, respectively. The key point of Sensitized Emission FRET image acquisition is that all the imaging parameters (laser output, zoom, pinhole, detector gain, and amplifier offset and so on) should be kept constant.

1. Set the system to the Channel acquisition mode and configure Donor CFP channel, FRET channel, and Acceptor YFP channel as follows:
  - a. *CFP channel*: Excitation with 458 nm laser and application of NFT 515 nm to split the signal and detection with parameter settings of donor (BP 480–520 nm).
  - b. *FRET channel*: Excitation with 458 nm laser and detection with parameter settings of acceptor (BP 500–550 nm).

- c. *YFP channel*: Excitation with 514 nm laser and detection with parameter settings of acceptor (BP 500–550 nm).
2. Adjust the pinhole size for each channel as an optical slice of the specific type of cells. Here, we set it to about 3 μm .
3. To image single CFP expressing cells, adjust the detector gain and amplifier offset of the donor and acceptor channels to obtain a decent signal. Imaging parameters should be kept constant. FRET channel will show the bleed-through from the donor CFP emission channel using donor CFP excitation wavelength (Fig. 2b, upper panel) (*see* Note 8).
4. For single YFP expressing cells, the FRET channel will show the cross talk from the acceptor YFP emission channel using donor CFP excitation wavelength (Fig. 2b, second panel) (*see* Note 9).
5. For CFP/YFP double expressing cells, select those with both CFP and YFP expression (*see* Note 10).
6. Set up time series acquisition as detailed in **step 1**.
7. After two or three scans, add stimuli homogenously to the cells and continue the acquisition (*see* Notes 11 and 12).

3.2.5 Quantitative Calculation of Sensitized Emission FRET Using Carl Zeiss Zen FRET plus 55—We followed the data analysis procedures of Zeiss Zen FRET Plus 55. During the data analysis, we found that following key points should be carefully followed for accurate data analysis:

1. Be aware that whole sets of data for sensitized emission FRET data analysis should be acquired with the exact same acquisition parameters.
2. To set up background threshold of donor single, acceptor single, or FRET samples, choose the areas without any signal.
3. To set up bleed through ratio in donor/FRET/acceptor channels, choose the areas without any saturated pixels. We recommend a medium range intensity for this setup.
4. We recommend choosing the ROIs (regions of interest) based on the biology of the interested protein.

⁸While a shorter wavelength laser would provide an excitation line closer to the optimal excitation wavelength of CFP, effective FRET measurements can be made using the 458 nm line from the Argon laser.

⁹Choose cells for FRET analyses that have a balanced CFP and/or YFP signal. Make adjustments for relative signal strength of each fluorophore depending on the limitations of the available filter sets on your system. For example, we visually inspect for strong CFP signal with the mercury lamp using a CFP (or DAPI) filter set. We use a GFP filter set to inspect for the cells co-expressing YFP and choose those that have a slightly higher YFP signal relative to the CFP signal for analysis.

¹⁰Due to the very close emission spectrum of GFP and YFP, it is normal to use a GFP filter cube to observe YFP expressing cells.

¹¹Since whole sets of comparable experiments have to be acquired with exact same acquisition condition, it is recommended to use CFP/YFP double expressing cells to set up acquisition condition instead of using single control cells. We often choose those cells with a similar level of CFP and YFP expression instead of those highly expressing only one fluorescent protein.

¹²We noticed that a high dose of PMA treatment often triggers a strong apoptosis of MDA-MB-231 cells. In the current chapter, we use a range of stimuli ranging from 100 nM to 1 μM PMA as indicated in the figures.

3.2.6 FRET Loss of KDAR Indicates PKD1 Activation upon Stimulation—The protein kinase D family of serine/threonine kinases plays critical roles in many physiological processes, including cell growth, protein trafficking, and lymphocyte biology [2]. It is important to measure the activation profile of PKDs in different type of mammalian cells. There are several methods had been developed to detect PKD activation [6 – 8]. Confocal imaging approaches, including applying FRET technique, had been used for PKD activation as well. An intramolecular FRET probe designed by inserting a substrate peptide of PKD1 between CFP/YFP FRET pairs (Fig. 2a) [6, 8]. Using sensitized emission FRET measurement, we also measured a FRET loss of DKAR and confirmed the feasibility of FRET measurement in MDA-MD-231 cells at the level of whole cell (Fig. 2b–d). Consistent with previously reported [6, 8], we measured a FRET loss of DKAT upon PMA treatment while its negative control DKAR-TA, in which serine residue is replace by arginine, did not display a FRET loss (Fig. 2d). The advantage of this FRET probe is able to measure kinase activity of the endogenously expressed PKD1 upon stimulation. However, there are two major disadvantages of DKAR probe: (1) the specificity issue of the substrate peptide in the construct, (2) the lack of spatial information of the measurement due to the diffusion nature of this FRET probe. These two disadvantages do not meet the increasing demands on the understanding of the temporal-spatial activation and regulation of PKDs.

3.2.7 Monitoring Spatiotemporal PKD1 Activation Using Intramolecular FRET Probe PKD1-CY—Phosphorylation mediates activation and conformational change of PKD1 [2, 12, 13]. The underlying mechanisms of PKD1 activation depend on the relief of autoinhibition by the PH domain following activation-loop phosphorylation [12, 14]. Hence, we construct an intramolecular FRET probe by inserting PKD1 protein between CFP/YFP FRET pairs (CFP-PKD1-YFP, also PKD1-CY) to measure the tempo-spatial dynamics of PKD1 activation (Fig. 3a). Upon PMA stimulation PKD1-CY displays a clear membrane translocation (Fig. 3c), confirming the functionality of FRET probe and, more importantly, the subcellular localization of PKD1. Along with membrane translocation, we detected a clear FRET gain of PKD1-CY, which is inhibited by PKD specific inhibitor CID755673 (Fig. 3d; *see Note 13*). This result indicates that FRET gain can be used to measure PKD1 activation. More importantly, simultaneous monitoring of the subcellular location of PKD1-CY and FRET change upon PMA stimulation provides an efficient way to visualize and quantitatively measure the spatiotemporal dynamics of PKD1 activation at the subcellular level upon extracellular stimulation.

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¹³There might be other possibilities resulting in a FRET increase of PKD1-CY, such as a local accumulation of PKD1-CY. However, we observed that a PKD inhibitor abolished the FRET signal without affecting the membrane accumulation of PKD1 [5], suggesting that the gain of FRET is most likely due to PKD activation resulting to/from the intramolecular conformational changes. The detailed activation mechanisms will require further investigation.

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Fig. 1. GPCR-mediated Ras activation in HL60 cells monitored by active Ras probe. **(a)** Cellular localization of H-Ras in HL60 cells. HL60 cells express mRFP-HRas(G12V). **(b)** Activation of Ras in HL60 cells upon chemoattractant fMLP stimulation. HL60 cells expressing 100 nM fMLP is mixed with Alexa 644. **(c)** An enriched activation of Ras in the leading edge of a chemotaxing HL 60 cells. A final concentration of 1 μM fMLP is mixed with Alexa 644 (0.1 μg/ml)

Fig. 2. PKD1 activation monitored by spectrum FRET measurement of an intramolecular FRET probe CFP-PKD1-YFP. **(a)** Scheme shows the principle of the applied FRET probe DKAR for PKD1 activation. The peptide of PKD1 substrates is inserted between FRET pair CFP and YFP. Phosphorylation of the peptide by PKD1 results in loss of FRET between CFP and YFP. DKAR-TA encodes PKD1 substrate peptide with mutation and serves as a negative control for DKAR. **(b)** Configuration and channel settings of CFP, FRET, and YFP for sensitized emission FRET measurement of CFP single control, YFP single control, DKAR, and DKAR-TA. **(c)** Configuration and channel settings of CFP, FRET, and YFP for sensitized emission FRET measurement of CFP single control, YFP single control, DKAR, and DKAR-TA. **(d)** Graph showing Normalized N-FRET vs Time (s).

and DKAR-TA using confocal fluorescent microscopy. **(e)** N-FRET image of PKD1 is processed and obtained by Zen710 FRET plus 55. **(d)** The dynamic change of the normalized N-FRET upon stimulation is presented in **(d)**. Mean $\pm$ SD is shown

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Fig. 3. PKD1 activation monitored by sensitized emission FRET measurement of an intramolecular FRET probe CFP-PKD1-YFP. **(a)** Scheme shows the possible mechanism of the applied FRET probe CFP-PKD1-YFP for PKD1 activation. PKD1 is inserted between FRET pair CFP and YFP and is designated as PKD1-CY. Phosphorylation of PKD1 results in its conformational change which cause the gain of FRET between CFP and YFP. **(b)** Configuration and channel settings of CFP, FRET, and YFP for sensitized emission FRET measurement of CFP single control, YFP single control, and PKD1-CY using confocal

fluorescent microscopy. **(c)** N-FRET image of PKD1 is processed and obtained by Zen710 FRET plus 55. The dynamic change of the normalized FRET efficiency upon stimulation with or without PKD1 inhibitors is presented in **(d)**. Mean $\pm$ SD is shown