Luminescent kinase activity biosensors based on a versatile bimolecular switch

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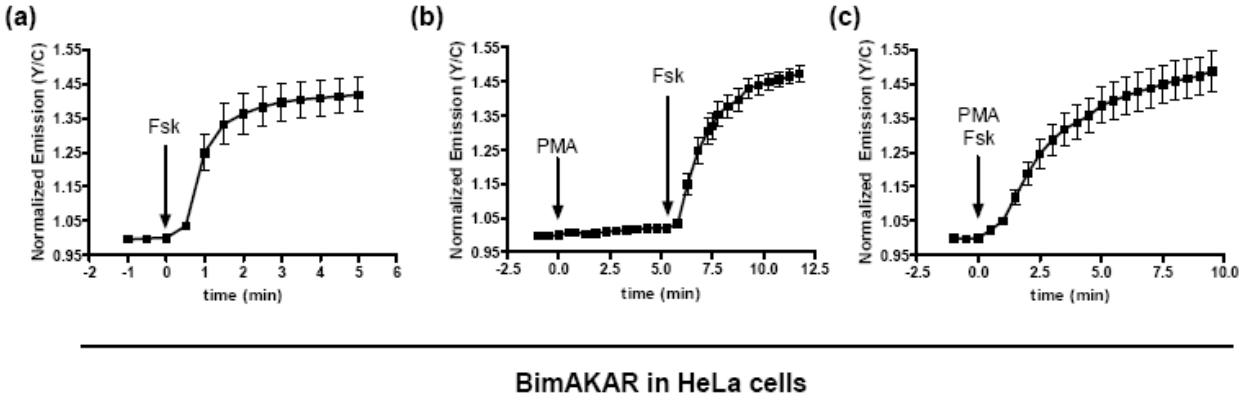
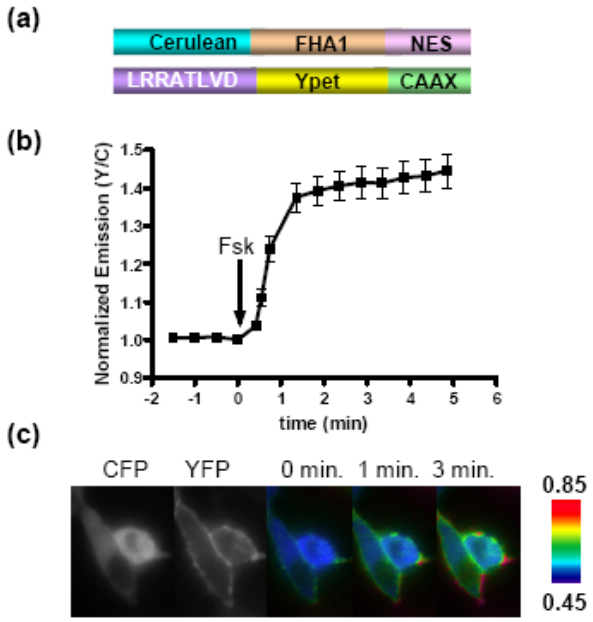
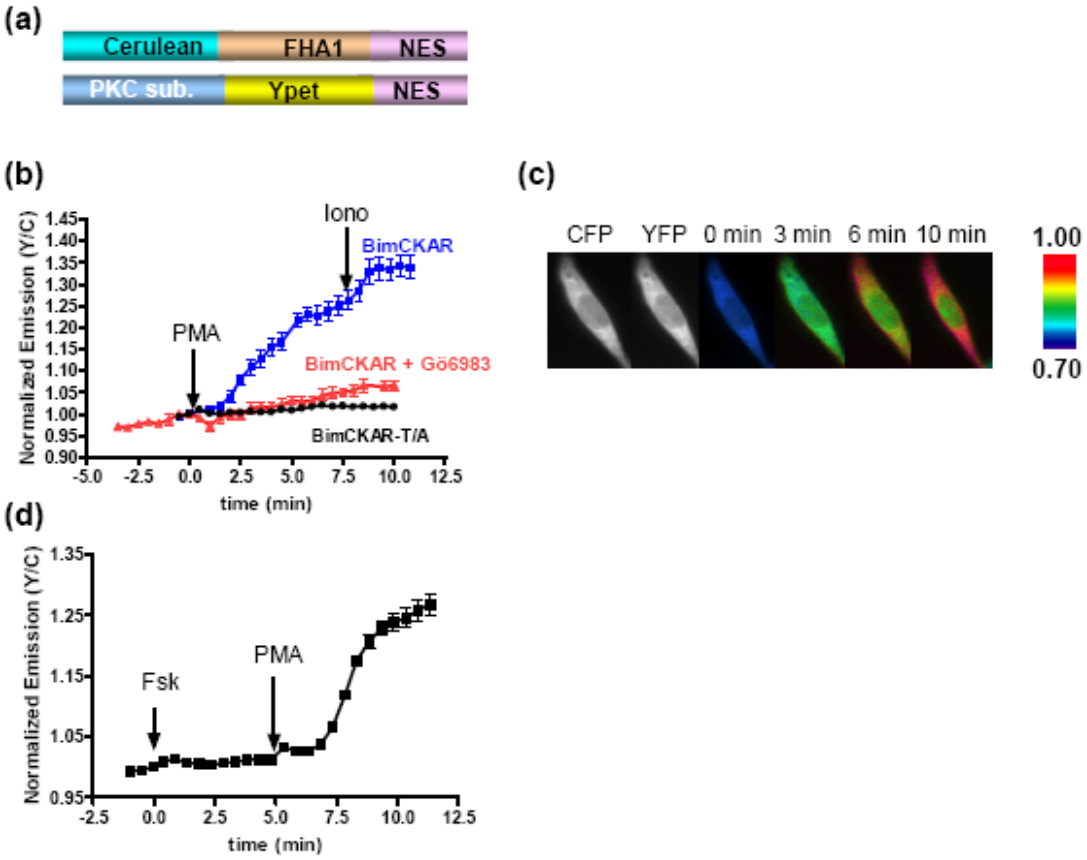


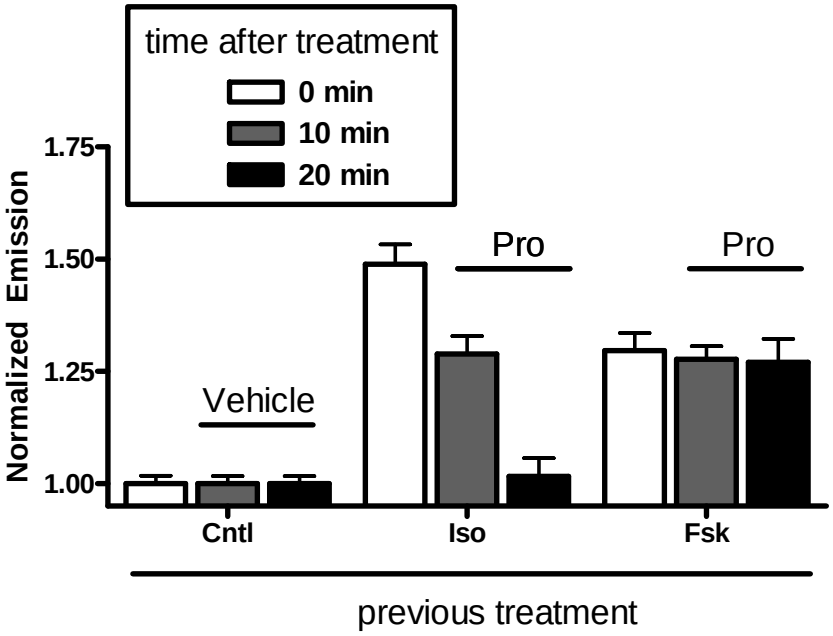
Supporting Figure S1. Characterization of BimAKAR in HeLa cells. (a) Fsk (50 μM) (n = 6) (b) but not PMA (50 ng/mL) (n = 7) generates an increase in yellow over cyan emission ratio. (c) Simultaneous activation of PKA and PKC generates a BimAKAR response comparable to that observed when PKA is activated alone, showing that the presence of additional endogenously phosphorylated substrates does not hinder the response of BimAKAR (n = 7). Data is depicted as mean ± SEM.



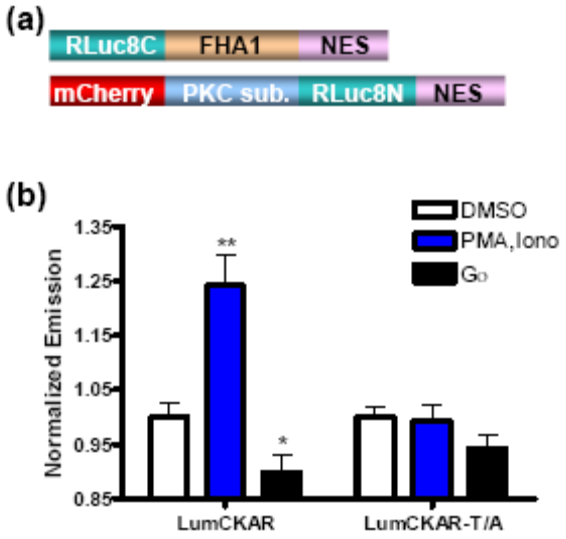
Supporting Figure S2. BimAKAR detects subcellular PKA activity. (a) Schematic representation of BimAKAR-CAAX. (b) Average response of BimAKAR-CAAX to Fsk (50 μM) in HEK293T cells (n = 9). (c) CFP and YFP images show the localization of each portion of the PM-targeted BimAKAR, and pseudocolor images show the response of BimAKAR-CAAX to Fsk. Data is depicted as mean ± SEM.



Supporting Figure S3. Design and characterization of BimCKAR. (a) Schematic representation of BimCKAR. (b) Time courses of PMA (50 ng/mL) plus Iono (1 μM) treated HeLa cells expressing BimCKAR (n = 6, blue), BimCKAR in the presence of Gö6983 (1 μM) (n = 5, red), and BimCKAR-T/A (n = 3, black). Values are mean ± SEM. (c) CFP and YFP images show the localization of each portion of BimCKAR, and pseudocolor images show the response of BimCKAR to PMA. (d) In HeLa cells expressing BimCKAR, Fsk did not generate a FRET increase, indicating that BimCKAR specifically detects PKC, but not PKA activity.



Supporting Figure S4. In HEK293T cells expressing LumAKAR, treatment with Pro (10 μM) completely reverses the bioluminescence signal detected in cells previously treated with Iso, but it does not alter the signal in cells that were previously treated with Fsk. Data is depicted as mean ± SEM (n = 7-8 wells per treatment).



Supporting Figure S5. Design and characterization of LumCKAR. (a) Schematic representation depicting the modular design of LumCKAR. mCherry serves as a transfection marker. (b) Responses of LumCKAR or LumCKAR-T/A to vehicle (DMSO), PMA (50 ng/mL) plus Iono (1 μM), or Gö6983 (1 μM), 20 minutes after drug addition (n = 5 for all treatments). ** indicates $p < 0.002$; * $p < 0.02$. Values are mean ± SEM.

Supporting Methods

Gene construction: Cerulean-FHA1 was PCR amplified and subcloned into pCDNA3 (Invitrogen) containing the 3'-NES (LPPLERLTL). Ypet was PCR amplified with forward primers that introduced the PKA-specific substrate (LRRATLVD), PKC-specific substrate (RFRRFQTLKDKAKA), or the appropriate threonine to alanine mutation for the PKA or PKC substrate sequence, and these were subcloned into pCDNA3 containing a 3'-NES or the plasma membrane targeting sequence derived from K-Ras (KKKKKSKTKCVIM). RLuc8C was PCR amplified and subcloned 5' to FHA1-NES in pCDNA3. RLuc8N was PCR amplified with a forward primer encoding the substrate sequence for PKA, PKC, PKA-T/A, or PKC-T/A, and subcloned into pCDNA3 between a 5'-mCherry and a 3'-NES.

Cell Culture and Transfection. HEK293T cells and HeLa cells were grown in DMEM cell culture media (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin at 37^{o}C with 5% CO_2. All biosensors were transfected into HEK293T cells via calcium phosphate at 60% confluency. HeLa cells were transfected via Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. 35 mm glass bottom imaging dishes were used for single cell experiments and 10 cm plates were used for cell population studies.

Fluorescence imaging and analysis. Cells were washed with Hank's Balanced Salt Solution (HBSS) and maintained in the dark at room temperature. Phorbol-12-myristate-13-acetate (PMA; Sigma), forskolin (Fsk; Calbiochem), Gö6983 (Sigma), H89 (Sigma), and ionomycin (Iono; Sigma) were added as indicated. Cells were imaged on a Zeiss Axiovert 200M microscope with a cooled charge-coupled device camera (MicroMAX BFT512, Roper Scientific, Trenton, NJ) controlled by METAFLUOR 6.2 software (Universal Imaging, Downingtown, PA). Yellow/cyan emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and 475DF40 emission filter for CFP or 535DF25 for YFP. These filters were alternated by a filter-changer Lambda 10-2 (Sutter Instruments, Novato, CA). Exposure time was 50-500 ms and images were taken every 30 sec. Fluorescence images were background-corrected by subtracting the fluorescence intensity of background with no cells from the emission intensities of cells expressing fluorescent reporters. The ratios of yellow/cyan emissions were then calculated at each time point. The values of all time courses were normalized to the value before drug addition.

Live cell plate reading. After an 18-24 hour transfection period in a 10 cm dish, cells were plated at 50,000 cells/well into white-walled, clear-bottom 96 well plates (Corning) coated with 0.1 mg/mL poly-D-lysine. After 24 hours, media was replaced with HBSS supplemented with 5 μM benzyl-coelenterazine (Nanolight Technology) and allowed to incubate for 15-25 min. Luminescence measurements were taken on a FLUOstar OPTIMA microplate reader (BMG Labtechologies, Inc.) without an emission filter. A baseline was established in 3-5 cycles. Cells were then treated as indicated and luminescence was detected immediately after addition for 30-40 min. Bioluminescence signals were normalized by dividing all acquired values by the average baseline value in each well and then normalized to the average control (Cntl) reading at each time point.

Bioluminescence imaging and analysis: Cells were imaged on a Zeiss Axiovert 200M microscope with a Evolve EM cooled charge-coupled device camera (Photometrics) controlled by METAFLUOR software (Universal Imaging, Downingtown, PA). Using a 475DF40 emission filter and 450DRLP

dichroic mirror, images were acquired immediately after benzyl-coelenterazine addition. Acquisition time was 90 seconds for LumAKAR and 4 minutes for LumAKAR-T/A, and images were acquired every 2-4 minutes. Fsk and IBMX (Sigma) were added directly to the imaging dish. Images were background corrected and processed on Image J software (NIH).