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Contribution of Fluorophores to Protein Kinase C FRET Probe Performance

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Intracellular signaling is frequently monitored by using fluorescent molecules, especially when high temporal and spatial resolution is required.[1,2] The various designs include simple fusion proteins that translocate from the membrane to the cytosol or vice versa,[3–5] sensors that change in fluorescence intensity after binding to a ligand,[6] or probes with two fluorophores that exhibit Förster resonance energy transfer (FRET).[2,7,8] The last kind can be used for ratiometric imaging, which has the advantage of being fairly independent of probe concentration.[9] Many FRET probes consist of a construct with two genetically encoded fluorescent proteins, most often ECFP and EYFP.[10–15] All naturally occurring fluorescent proteins have the tendency to oligomerize, with varying affinities and distinct stoichiometry. Concern has been raised that this characteristic could negatively influence probe performance.[16] Fluorescent proteins from the *Aequorea* family, including CFP and YFP, have been widely used in probe design, because of their relatively low tendency to dimerize. Introduction of a single point mutation, A206K, renders these fluorescent proteins monomeric while retaining the same photophysical properties, thus making them ideal for use in many biological applications.[17] Alternatively, extensive modifications of the originally tetrameric *Discosoma* sp. red fluorescent protein DsRed have generated a number of monomeric proteins fluorescing in the red area of the optical spectrum.[18,19]

Recently, we developed two FRET probes, KCP-1 and KCP-2, for monitoring protein kinase C activities in living cells. Both are based on a pleckstrin fragment sandwiched between two fluorescent proteins, GFP^2 and EYFP.[20,21] The probes comprise a N-terminal pleckstrin homology domain (PH1) linked to a DEP domain by a PKC-sensitive substrate loop and a C-terminal linker region (KCP-1: aa 1-239; KCP-2: aa 1-221; Figure 1A). In in vivo measurements, the EYFP/GFP^2 emission ratio is followed over time. The longer construct, KCP-1, responds to phosphorylation by protein kinase C with an increase in emission ratio of about 10–15% which is fully reversible by adding the PKC inhibitor Gö6983 (Figure 1B). Mutants in which all PKC-sensitive phosphorylation sites ^{113}Ser, ^{114}Thr, and ^{117}Ser were replaced by alanine residues were nonresponsive.[20] The shorter version, KCP-2, lacking the C-terminal linker region, displays a decrease in emission ratio of 15% upon PKC activation. The dimerization constant of the two fluorophores in solution is expected to be around 110 μM.[17] Intramolecularly, the effective concentration of fluorophores is much higher and dimerization is more relevant. It was previously shown that the use of monomeric fluorescent proteins resulted in improved FRET probe performance, for example for monitoring mechanotransduction with a Src sensor.[22] However, in other cases, the dimerization of fluorophores might be beneficial, as was shown for the protease sensor CLY9.[23] Therefore in this work, we explored the effect of dimerization on PKC probe performance.

We first equipped both constructs, KCP-1 and KCP-2, with nondimerizing fluorophores by introducing an A206K mutation. The fluorescent proteins carrying the mutation were named mGFP^2 or mYFP,[17] respectively. Surprisingly, mutants carrying A206K mutations in both or only one fluorescent protein were close to nonresponsive. As depicted in Figure 1B, the original KCP-1 probe showed an increase of emission ratio whereas the original KCP-2 probe responded with a decrease in emission ratio upon PKC stimulation by a phorbol ester (PMA). Nondimerizing KCP-1, however, resembled KCP-2 in its response pattern, albeit with a smaller amplitude. Monomeric sensors based on KCP-2 did not change the direction of the signal, but gave a much weaker signal than the original KCP-2 probe (Figure 1B). These observations were independent of which of the fluorescent proteins (or both) was monomeric. In addition, when the monomeric fluorescent proteins mOrange and mCherry[19] were used, no significant FRET change was observed in either the KCP-1 or KCP-2 constructs (Figure 1C). These results suggest that fluorophore dimerization significantly contributes to probe performance of KCP-1 and KCP-2. The mOrange/mCherry FRET pairs exhibited low starting FRET (Figure S1C), which probably further prevents significant ratio changes by this FRET pair.

By performing acceptor photobleaching experiments in living cells,[24] we confirmed that KCP-1 has higher FRET efficiency after phosphorylation (27%). Before this event, FRET is reduced to the level of nondimerizing fluorophores (17%; Figure 2). Original KCP-2 gave higher starting FRET efficiencies than after phosphorylation or in its monomeric versions, likely due to initial dimerization of the fluorophores. The original dimerizing KCP-2 has a FRET efficiency of 28%, which decreases to 17% after phosphorylation. Monomeric constructs of KCP-2 exhibited FRET levels of 19–20% and no significant change following phosphorylation. This corresponds well with the idea of a sensor molecule in which fluorophore dimerization serves as a clamp pulling both ends of the molecule together. Phosphorylation breaks the pulling force and the fluorophores separate from each other leading to a decrease in FRET (Figure 3C). In KCP-1, however, the fluorophores are initially unable to dimerize in a fashion favorable for FRET (see below). Only after phos-

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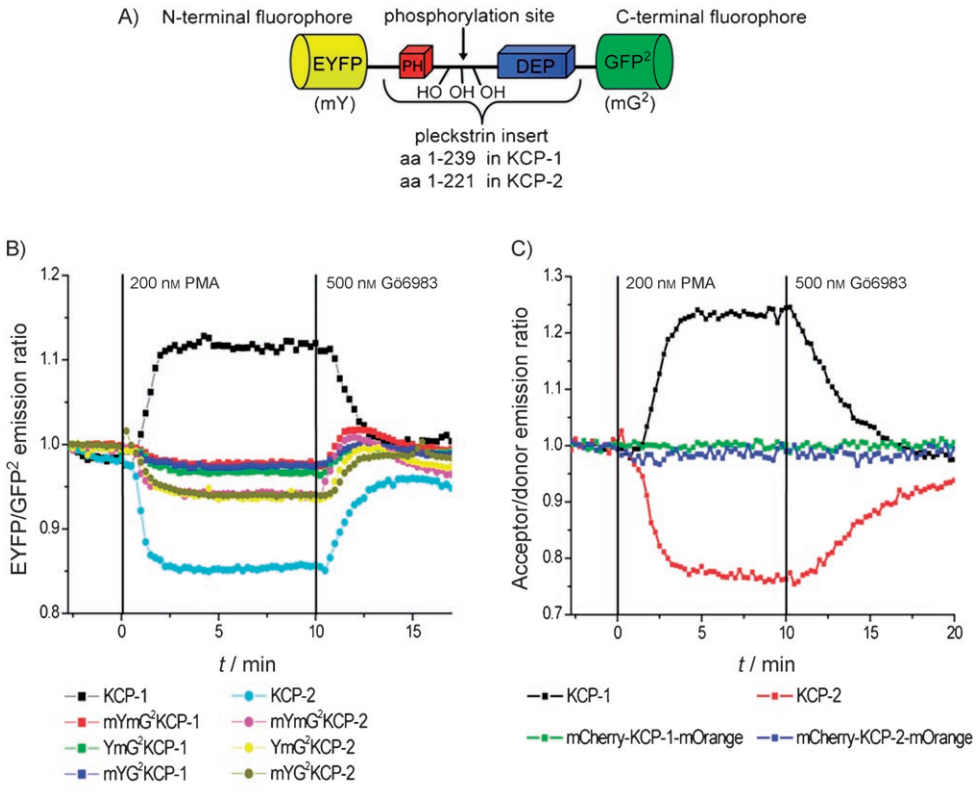

Figure 1. A) Scheme of the PKC-sensitive FRET probes KCP-1 and KCP-2. In both probes, the dimerizing fluorophores, EYFP and GFP^2, were replaced by monomeric mutants by introducing a single A206K mutation (mY and mG^2, respectively) and compared to the original probes in live cell imaging experiments. B) Experiments were performed in N1E cells on a Zeiss/Visitron widefield microscope. PKC activity was stimulated by addition of PMA and inhibited by addition of Gö6983 10 min later, as indicated. The EYFP/GFP^2 emission ratio of different probes was plotted against time. Probes unable to dimerize showed very little FRET changes. C) In similar experiments, sensors with EYFP replacing mCherry and EGFP^2 replacing mOrange, were compared to KCP-1 and KCP-2. Both constructs showed no change in FRET, probably due to a lack of fluorophore dimerization and/or the small FRET efficiency (Figure S1). These experiments were performed in HeLa cells on a Leica SP2 AOBS confocal microscope. Each graph is an average of at least ten cells from at least three independent experiments.

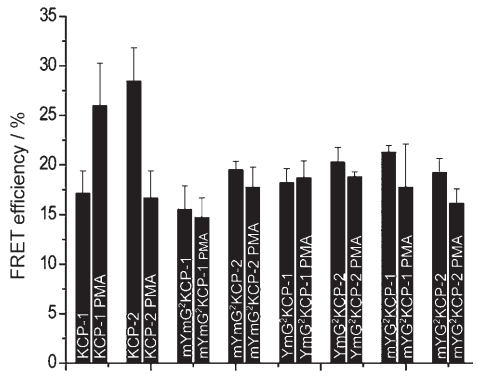

Figure 2. Comparison of FRET efficiencies of KCP-1 and KCP-2 and their monomeric or partially monomeric constructs, as measured by acceptor photobleaching. FRET efficiencies were compared in at least ten different untreated or PMA-stimulated HeLa cells and subsequently fixed. mY indicates the A206K mutant of EYFP, mG^2 the same mutant of GFP^2.

phorylation is steric restriction absent and fluorophore dimerization possible (Figures 3 A and B).

In essence, in the two cases where we find high FRET efficiency around 27 % (Figure 2), dimerization of the fluorophores is likely. In all other cases dimerization is absent or inefficient, leading to an insensitivity of these constructs to conformational changes induced by the phosphorylation event.

Two models (Figure 3) describing the performance of KCP-1 are conceivable: A) In its nonphosphorylated form KCP-1 has an opened structure which does not permit fluorophore dimerization. Only phosphorylation by PKC allows the fluorophores to reside in close proximity. B) The molecule initially forms a closed structure with significant interaction between the PH and DEP domains. These interactions, however, prevent the fluorophores to dimerize, potentially for steric reasons. Phosphorylation results in a rearrangement of the DEP–PH interaction, permitting the fluorophores to adapt a proximal position that permits FRET. For all monomeric KCP-1 molecules the starting FRET levels are similar to the original probe but lack the FRET increase upon phosphorylation, because in this case the structure is entirely opened. The stretching of the substrate linker then leads to a slight decrease in FRET. This explains why fluorophore dimerization is essential for the performance of KCP-1. We favor the second model because preliminary NMR data of the nonphosphorylated DEP–PH construct without fluorophores show a closed conformation (Simon, Stier, Sattler, unpublished results). This would be in opposition to model A.

It appears that removal of the C-terminal amino acid sequence 222–239 (the difference between KCP-1 and KCP-2) will avoid the steric hindrance and thus permit initial fluorophore dimerization (Figure 3 C). The difference likely relates to the fact that in KCP-2 the PH–DEP domain interaction is prevented because several amino acids at the C terminus of the DEP domain fold (aa222–229) are missing. The presence of these residues may place the C-terminal YFP domain in a different position in KCP-1 compared to KCP-2.[25,26]

In summary, KCP-1 appears to have a mechanism of action opposite to that of KCP-2. Both sensor actions rely mainly on the dimerization of fluorophores and the concomitant conformational changes (Figure 3 B) or the opening and closing of the structure (Figure 3 C). KCP-1 action is governed by an addi-

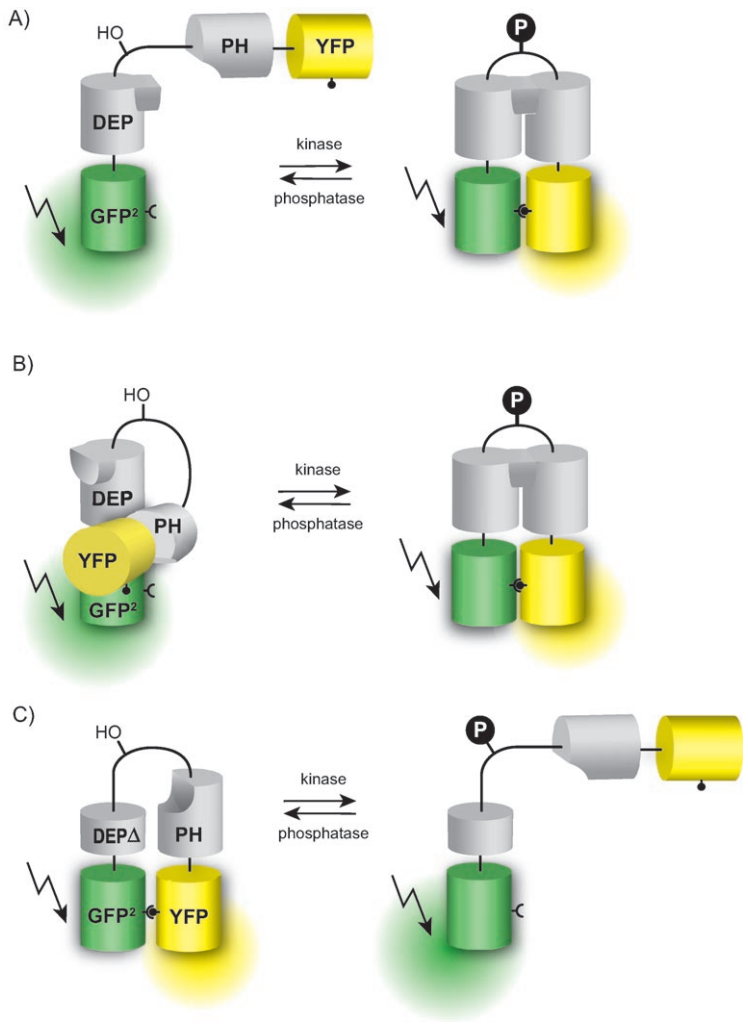

Figure 3. Proposed mechanisms of action of KCP-1 and KCP-2. For KCP-1 two models are conceivable: A) Two interactions are crucial for the mechanism of action, the dimerization of the genetically encoded fluorophores and an interaction between the PH and DEP domains of the pleckstrin. Only upon phosphorylation, is the PH–DEP interaction efficient enough to permit dimerization and an increase in FRET. B) Alternatively, there is initially close interaction between the PH and DEP domains but in the nonphosphorylated state the adopted conformation prevents parallel fluorophore dimerization resulting in a lower starting FRET efficiency. Phosphorylation leads to intramolecular rearrangement and an increase in FRET efficiency. C) The model for KCP-2: The compromised DEP domain (DEPΔ) permits parallel fluorophore arrangement, but phosphorylation breaks the protein–protein interaction and the fluorophores turn further apart, resulting in a decrease in FRET. With nondimerizing fluorophores the DEP–PH interaction is too weak to keep the fluorophores in close proximity.

tional interaction between the PH and DEP domains which apparently leads to an orientation of the fluorophores unfavorable for dimerization, resulting in a lower starting FRET. Phosphorylation results in an intramolecular rearrangement, which then improves the orientation of the transition dipoles of the fluorophores (Figure 3B). In cases of monomeric fluorophores, where dimerization is impossible, phosphorylation leads to a slightly larger separation of fluorophores similar to KCP-2 and a minor reduction in FRET.

Considering the importance of fluorophore dimerization for probe performance, the experiments shown above could also be interpreted as fluorophore interaction between different probe molecules.[22] To determine whether fluorophore dimerization in KCP-1 and KCP-2 happened intra- or intermolecularly, we designed a set of experiments in which FRET was not possible within a molecule but only between different probe molecules. In the first experiment, KCP-1 and KCP-2 were equipped with one nonfluorescent (blinded) mutant (Y66S) of GFP^2 or EYFP, respectively (Figure S1A). Alternatively, we used homotagged KCP-1 and KCP-2 constructs which contained either two GFP^2 or two EYFP molecules (Figure S1B). When co-expressing both versions of the blinded or the homotagged constructs in HeLa cells, no FRET change was detectable after PKC stimulation (Figure 4A and B). Accordingly, FRET between two probe molecules was not observed in acceptor photobleaching experiments (Figure S1C). Therefore, contributions by intermolecular FRET could be excluded.

We wondered whether significantly reducing the size of the construct would overcome the need for dimerization and result in a useful sensor independent of fluorophore dimerization. Shortening of the construct is difficult because of the rigid domain structure (Figure 1A) required for responses to phosphorylation. A solution was to replace one of the bulky fluorescent proteins with a small molecule dye. A method providing a small, yet specific tag for protein labeling is Roger Tsien's FlAsH technology.[27,28] FlAsH, a bisarsenite fluorescein derivative, recognizes and binds specifically to a tetracysteine motif which can easily be introduced into proteins. We replaced GFP^2 with a tetracysteine sequence, FLNCCPGCCMEP, at the C terminus of the ECFP-pleckstrin construct aa1-229 (Figure 5C).[29] This novel construct is about the size of KCP-1 without the second fluorescent protein, contains all amino acids necessary for full DEP domain folding,[26] and is called KCP-F. It was expressed in various cell lines and labeled with FlAsH-EDT_2 following a known protocol.[28] KCP-F was homogeneously expressed in the cytosol and the nucleus similar to KCP-1. Background staining of nonexpressing cells was determined to be approximately 6% of intensity compared to specific staining in KCP-F expressing cells (Figure S4). Stimulation of PKC activity with phorbol ester in HEK293 cells resulted in a decrease in the FRET ratio of about 20%, roughly the same amount that was observed with KCP-2 (Figure 5A). This result supports the hypothesis that PH–DEP domain interaction plus fluorophore dimerization is necessary to yield an increase in FRET. Introduction of a triple alanine mutation replacing the PKC-sensitive phosphorylation sites (KCP-F-AAA) showed no change in the FRET ratio, as expected (Figure S3). KCP-F exhibited substantial FRET efficiencies of 35–45% (Figure 5B) as could be shown by addition of the thiol-reactive compound BAL, which released FlAsH from the construct (Figure S2). The fluorophores seem to be close enough to the Förster radius (R_0) to monitor subtle changes in distances even without the

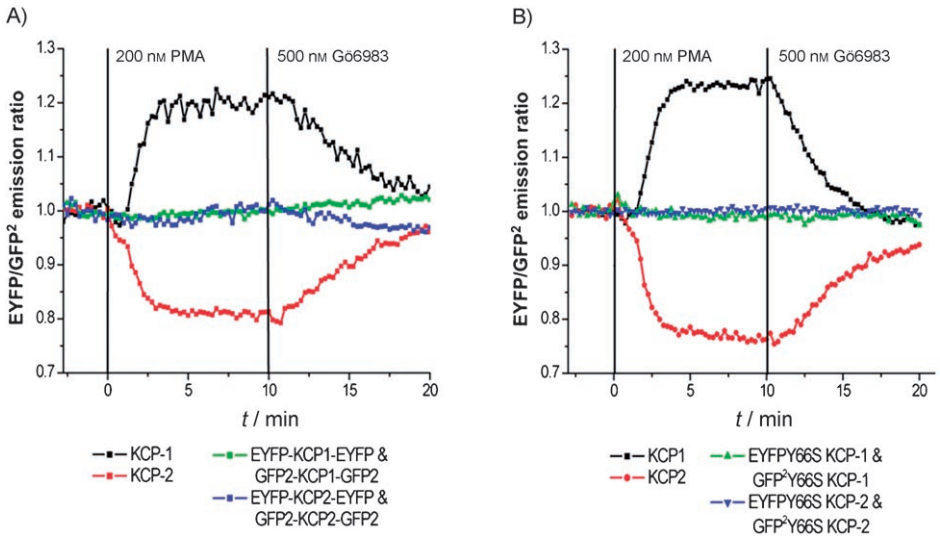

Figure 4. To discriminate between inter- and intramolecular FRET, we designed two experiments, in which FRET can only occur between different molecules, not within the same molecule (see Figure S1 for the experimental set-up). A) KCP-1 and KCP-2 were modified to code for identical fluorophores at both termini. EYFP-KCP-1-EYFP and GFP^2-KCP-1-GFP^2 or EYFP-KCP-2-EYFP and GFP^2-KCP-2-GFP^2 were co-transfected into N1E cells and imaged on a Leica SP2 confocal microscope. Cells were chosen that expressed both individual constructs at the same level. PKC activity was stimulated as indicated. Original traces from KCP-1 and KCP-2 probes are plotted as controls. B) In a second experiment, a single Y66S mutation was introduced to either EYFP or GFP^2. This mutation renders the fluorophores nonfluorescent. Constructs were co-transfected into HeLa cells with the respective reciprocal constructs: EYFP-Y66S-KCP-1 and GFP^2-Y66S-KCP-1 or EYFP-Y66S-KCP-2 and GFP^2-Y66S-KCP-2. Cells were chosen that expressed both individual constructs to a similar degree and imaged on a Leica SP2 confocal microscope. PKC activity was stimulated as indicated.

dramatic effect of breaking fluorophore dimers. These results suggest that FRET in KCP-F is due to the nearness of the acceptor to the fluorescent protein, made possible by the small size of one fluorophore. The changes in FRET ratio in KCP-2 and KCP-F both rely on an alteration of the distance between the two fluorophores: phosphorylation results in a stretching of the molecule, thereby increasing the distance between both fluorophores. In KCP-2, however, this effect is only possible through fluorophore dimerization which serves as a clamp and increases the difference between the phosphorylated and nonphosphorylated states. When nondimerizing A206K fluorophores were used, stretching of the molecule is barely detectable because the two fluorophores are already in a more random orientation. The size of KCP-F is below 50 kDa, which will potentially allow observation of its mode of function in response to phosphorylation using NMR in the future.

It should be mentioned that KCP-F is one of the first FRET sensors using FlAsH technology.[30] The FlAsH technology might be generally useful to overcome size problems in FRET sensors,[31] especially when the latter arise from the dimensions of the fluorescent protein β-barrels. In addition, the development of new biarsenical dyes with higher quantum yield, better photostability, and new colors will increase the applicability of FlAsH labeling for probe development even further.[32]

In conclusion, we have demonstrated that probe performance depends on subtle changes in the sensor structure. It would be interesting to investigate the effect of other structural alterations, such as circulatory permutations, in the future.[33] We showed that fluorophore dimerization can be beneficial and even necessary for probe performance in some of our protein kinase C probes. It has yet to be determined whether dimerization is sufficiently predictable to be established as an additional tool in FRET probe design, but it increases the options.

Experimental Section

Determination of FRET efficiencies through acceptor photobleaching: Experiments were performed on a Leica AOBS SP2 equipped with a strong 514 nm laser for photobleaching and using the FRET acceptor photobleaching module provided by the Leica software.

First, pictures of the two fluorophores were taken at their excitation maxima (GFP^2: 405 nm, EYFP: 514 nm). Subsequently, the acceptor was bleached at 514 nm until fluorescence could not be distinguished from background. Finally, images of both fluorophores were taken again at their excitation maxima. Each picture always consisted of an average of four scans.

Data were analyzed using an ImageJ plugin that takes the pre- and postbleaching pictures of the donor, subtracts the background, and then runs a median filter with two iterations to remove noise. Finally, the FRET efficiency was calculated as the percentage of the total excitation energy transferred from donor to acceptor, as measured by the emitted fluorescence.

FlAsH labeling: FlAsH labeling was performed according to protocols published before.[28] Transfected cells were grown on coverslips for 24–48 h. Cells were washed twice with Hank's balanced salt solution (HBSS) containing 1 g L^{-1} glucose and incubated with 500 nM FlAsH-EDT$_2$ in HBSS and 12.5 μM EDT at 37 °C for 1 h. After incubation, cells were washed twice with HBSS and incubated with HBSS containing 250 μM EDT for 10 min. Cells were washed again twice with HBSS to reduce nonspecific labeling. Cells were kept at 37 °C and 5% CO_2 until imaging. FlAsH binding to the tetracysteine motif was destroyed by adding 10 mM British-Anti-Lewisite (BAL, 2,3-dimercaptopropanol). Addition of BAL enables determination of FRET efficiency. FRET efficiency was calculated as the percentage of the total excitation energy transferred from donor to acceptor, as measured by the emitted fluorescence. FlAsH was prepared by A. Schleifenbaum or was purchased as Lumino Green from Invitrogen.

Fluorescence microscopy: Cell medium was replaced by HEPES buffer 1 h prior to the experiments. Cells were kept at 37 °C and 5% CO_2. Compounds for addition were prepared as 1000× stock solutions in DMSO or water according to the protocol of the supplier. The compounds were predissolved in 50 μL buffer immediately before addition.

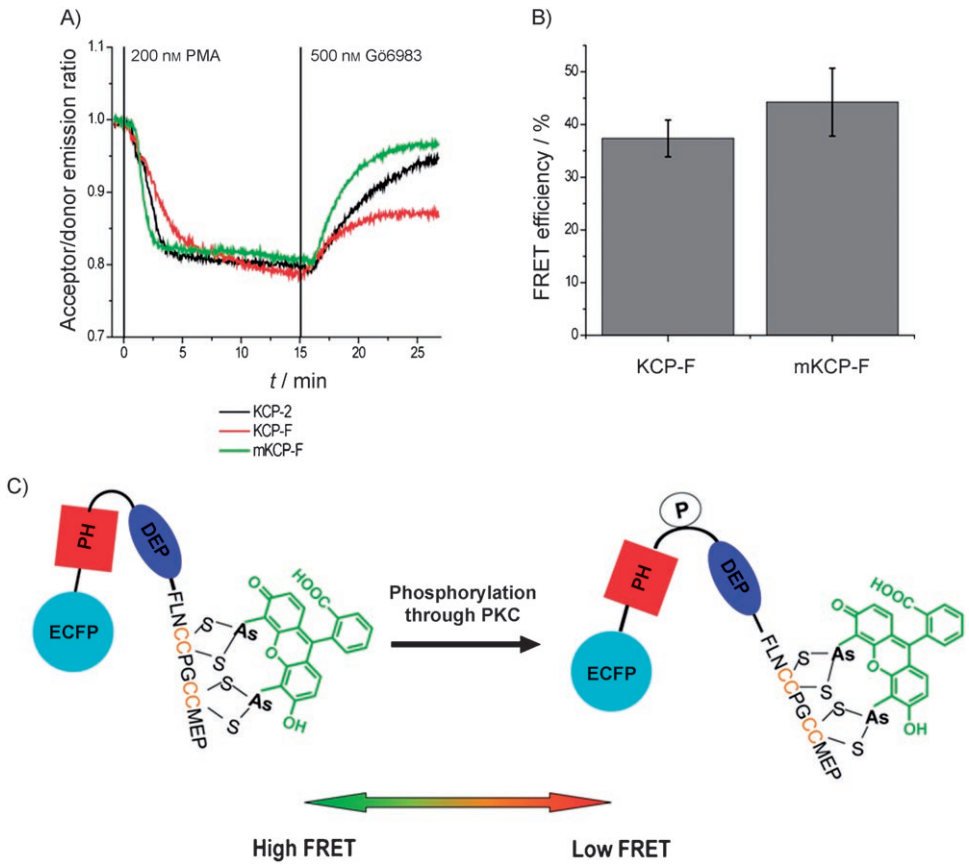

Figure 5. A protein kinase C FRET probe based on KCP-2 was designed, in which the acceptor fluorescent protein was replaced by a tetracysteine motif (FLNCCPGCCMEP). The latter was labeled by FlAsH in living cells. CFP or nondimerizing mCFP served as a donor fluorophore. A) HEK cells were transiently transfected with KCP-2, KCP-F (CFP-KCP2-FlAsH), or mKCP-F (mCFP-KCP2-FlAsH). PKC activity was stimulated as indicated. The FlAsH/CFP emission ratio was plotted against time. B) Comparison of FRET efficiency of KCP-F and mKCP-F: FRET efficiency was calculated as the percentage of the total excitation energy transferred from donor to acceptor, as measured by the emitted fluorescence (see Figure S2). C) Proposed function of the FlAsH-based PKC probe KCP-F: Phosphorylation of the probe by PKC results in stretching of the pleckstrin insert, thereby increasing the distance between the fluorophores and decreasing the high initial FRET efficiency.

Confocal imaging was performed on a Leica SP2 AOBS system at room temperature. Cell dishes were allowed to equilibrate to room temperature 10 min prior to the experiments. All KCP-1 and KCP-2 samples were excited with a 405 nm laser (50 mW). Emission of GFP^2 was measured at 490–510 nm, and EYFP at 520–540 nm. EYFP was directly excited using the 514 nm laser line. The FRET pair mOrange/mCherry was imaged using a 532 nm laser for excitation. Emission of mOrange was measured from 560 to 600 nm; mCherry from 610 to 650 nm.

Wide field microscopy of monomeric constructs was performed on a Zeiss Axiovert microscope with a PH2 Acrostigmat 40x/0.65 objective, automated stage, and fast emission and excitation filter wheels (Visitron Systems GmbH, Germany) at 37 °C in a heated chamber. Images were recorded with a CCD camera and processed using Metamorph software 6.2r4. GFP^2 in all KCP-1 and KCP-2 samples was excited through a DAPI filter (405/20 nm). Excitation and emission light were separated through a 425 dclp beam splitter. GFP^2 fluorescence was detected through a 500/20 nm filter; EYFP fluorescence through a 535/30 nm filter. As expression levels and fluorescence intensities were usually high, a 90% neutral grey filter was used to dim the excitation light.

Fluorescence imaging of FlAsH labeled probes was performed on a Zeiss Axiovert 135 inverted microscope equipped with a Zeiss Plan-Neofluar 100×/1.3 Oil objective at room temperature. Samples were excited at 420/20 nm (dichroic 450 nm) with light from a polychrome IV (Till Photonics). The light source settings were controlled by Till pmc communications software version 1.0.5. The emission ratio (FlAsH over ECFP) was measured with emission filters 480/40 nm (ECFP) and 535/30 nm (FlAsH), beam splitter dclp 505 nm. Signals detected by avalanche photodiodes were digitized using an AD converter (Digidata1322 A, Axon Instruments) and stored on a PC using Clampex 8.1 software (Axon Instruments).

The emission ratio was corrected for bleed-through of ECFP into the FlAsH channel to give a corrected emission ratio (bleed-through of FlAsH into the ECFP channel is negligible). FlAsH emission at 490 nm was determined in order to subtract direct excitation of FlAsH.

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