Chapter 9

FRET-Based Biosensors: Genetically Encoded Tools to Track Kinase Activity in Living Cells

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http://dx.doi.org/10.5772/intechopen.71005

Abstract

Fluorescence microscopy is widely used in biology to localize, to track, or to quantify proteins in single cells. However, following particular events in living cells with good spatio-temporal resolution is much more complex. In this context, Forster resonance energy transfer (FRET) biosensors are tools that have been developed to monitor various events such as dimerization, cleavage, elasticity, or the activation state of a protein. In particular, genetically encoded FRET biosensors are strong tools to study mechanisms of activation and activity of a large panel of kinases in living cells. Their principles are based on a conformational change of a genetically encoded probe that modulates the distance between a pair of fluorescent proteins leading to FRET variations. Recent advances in fluorescence microscopy such as fluorescence lifetime imaging microscopy (FLIM) have made the quantification of FRET efficiency easier. This review aims to address the different kinase biosensors that have been developed, how they allow specific tracking of the activity or activation of a kinase, and to give an overview of the future challenging methods to simultaneously track several biosensors in the same system.

Keywords: kinase, biosensor, FRET, multiplex, protein conformation, fluorescence microscopy

1. Introduction

Investigating kinase activity in living cells remains a challenge, and usual methods are limited when one wishes to study cellular dynamic events. For a large panel of kinases, the phosphorylation state of the kinase or its substrate has become the main indicator of its activity [1]. One of the most common methods to study this activity is to perform Western Blot analysis on cell extracts by targeting the phosphorylated kinase residue or the phosphorylated substrate residue with an antibody. However, this semi-quantification of the activity state of the kinase

applies only for the whole population of the cells [2]. Thus, the other most frequent way to investigate kinase activity is to perform immunofluorescence by targeting the phosphorylated kinase or the phosphorylated substrate with a fluorescent antibody for microscopy observation. This method allows localization of the proteins phosphorylation state in a single cell. However, these two methods suffer from one major limitation: the inability to track this activation state both in space and in time to track dynamic events in living cells. Indeed, this requires lysing the cells or fixing them and permeabilizing them, which prevents sufficient spatio-temporal resolution to investigate intracellular events [3].

To overcome this limitation, new tools have been developed including Forster resonance energy transfer (FRET) biosensors [4]. FRET is a nonradiative transfer of energy of one donor fluorophore to an acceptor fluorophore and relies on (i) an overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor, (ii) an adequate orientation between the two fluorophores, and (iii) a distance less than 10 nm between the two [5]. This feature has been used to investigate various cellular events such as protein–protein interactions by genetically tagging the two proteins of interest with a donor and an acceptor fluorescent protein [6], the intra-cellular Ca^{2+} signal by using calmodulin biosensor [7], proteases activity where the substrate is flanked by two fluorescent proteins, and the decrease of FRET indicates a cleavage of the protein [8], Rho GTPases for cytoskeleton dynamics [9, 10], and mechanical forces at adherent junctions [11, 12, 13]. The first kinase biosensor has been developed for cAMP-dependant protein kinase A (PKA) [14]. From this example and by taking advantage of the FRET characteristics and the conformational modifications of the phosphorylated sensors, several tools to monitor the kinase activity in space and time in living cells have been developed.

By conception, these tools are genetically encoded providing an invaluable advantage to endogenously producing the biosensor in live samples. In this review, we will first present genetically encoded FRET biosensors to monitor kinase activities based on phosphorylated peptide substrate. We will then introduce an alternative way of designing biosensors based on a conformational change of the kinase itself. Finally, we will present new methodological challenges such as multiplex FRET measurements in the same cell, thus allowing simultaneous monitoring of several kinase activities in time and space.

2. Substrate-based kinase activity biosensors

The first genetically encoded FRET biosensor for kinase activity was called A-kinase activity reporter (AKAR) and was designed to investigate the activity of PKA [14]. The idea was to follow a conformational change by FRET in a fusion protein composed of a substrate peptide sequence and a phosphorylated recognition domain. In this foundational work, the biosensor was composed of two fluorescent proteins, CFP and YFP (cyan and yellow fluorescent proteins). Between them sits a first domain, “the peptide substrate,” containing a sequence phosphorylated by PKA, followed by a second domain, “the phosphorylation recognition domain,” that binds to the peptide substrate when phosphorylated, these two domains are separated by an elastic linker. In the presence of active PKA, the peptide substrate becomes

phosphorylated, triggering its affinity for the phosphorylation-binding domain. This association between the two domains induces a conformation change of the biosensor that brings closer both fluorophores and increases FRET efficiency between CPF donor and YFP acceptor (**Figure 1**). The efficiency of FRET can be detected by ratiometric measurements between the intensity signal of the donor and the acceptor. This biosensor can be expressed in cell and is able to provide a response to cell treatment such as forskolin that raises the level of cAMP-activating PKA [14]. This tool has then been improved several times by using a better reversible phospho-binding domain called FHA1 [15] or by changing the fluorophore couple to improve the ratiometric measurements [16, 17]. The AKAR biosensor has been used to report the activity of PKA in neurons of mouse brain slices, showing its value in neurosciences [18].

Based on this concept, several new kinase FRET biosensors were developed. The E-kinase activity reporter (EKAR) biosensor is a FRET-based probe to study ERK activity [19]. The fluorophore pair is composed of the green donor eGFP and the red acceptor mRFP1. The consensus substrate peptide originates from Cdc25c, a member of the MAPK family. As other kinases from this family could phosphorylate the substrate, an ERK binding domain has been inserted to ensure ERK specificity. A WW domain (containing 2 tryptophans separated by around 20 aa) was used to bind the phosphorylated substrate [20], and a flexible linker allows a conformational change, when the Cdc25C peptide substrate is phosphorylated. This biosensor has also experienced several steps of optimization by modifying the fluorophores or the flexible linker [21, 22]. Among all these biosensors, the reversibility of the conformational modification is a major feature to study variations of kinase activation states [23].

Kinase biosensors are such powerful tools to investigate the dynamic of kinase activity events in cells that several of these biosensors have been created to study mitotic kinases activity through the cell cycle, including cyclin B1-Cdk1 [24]. A kinase biosensor has also been used to study PKC (protein kinase C) activation which is involved in tumor promotion. CKAR (C-kinase activity reporter) is composed of the CFP/YFP fluorophore pair, a specific peptide substrate for PKC, and the FHA2 domain of Rad53p that can bind to the phosphorylated substrate [25]. In this particular case, the unphosphorylated biosensor harbors a maximum

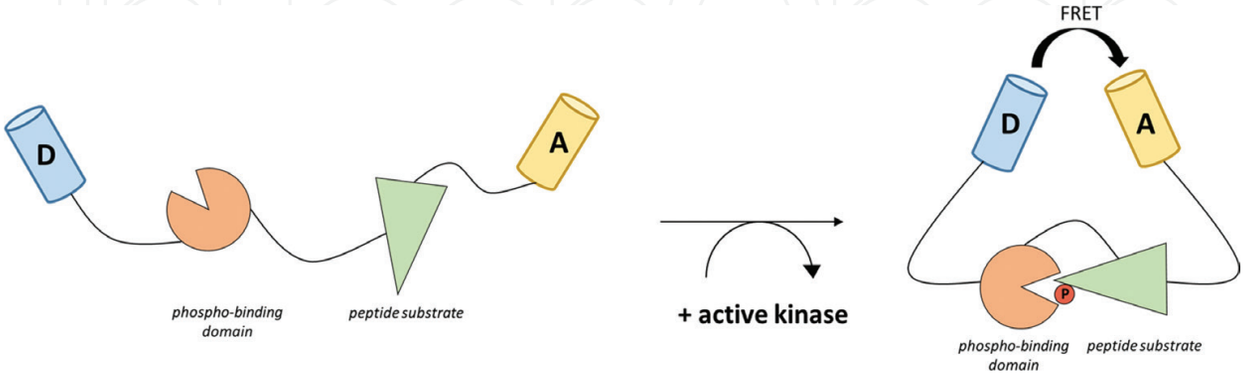

Figure 1. Mechanism of a substrate-based FRET biosensor. When the biosensor is not phosphorylated, it adopts an opened conformation keeping away the donor fluorophore D, and the acceptor fluorophore, A. After the peptide substrate phosphorylation by the kinase, a phospho-binding domain can bind to it gathering the fluorophore pair and allowing FRET.

FRET efficiency conformation, and FRET signal decreases once phosphorylated. By rapidly acquiring FRET efficiency, oscillations of PKC phosphorylation in a range of a minute were highlighted [25].

Other biosensors have been derived from CKAR. Polo-like kinase-1 (PLK1) is a major mitotic kinase that activates Cdc25C phosphatase, which abrogates the inhibitory phosphorylation of proteins controlling the entry to mitosis. A FRET-based biosensor has been created by replacing the peptide substrate of PKC with a peptide substrate of PLK1, the use of which revealed that the timed-control activation of PLK1 depends on Aurora A [26]. The choice of the kinase peptide substrate to construct the biosensor is a key point to improve its specificity. For Plk1, a c-Jun substrate-based biosensor was developed [27], since the previous version based on Myt1 substrate sequence was also sensitive to Mts1 activity [28]. The c-jun–based version was then used to demonstrate that Plk1 activity is required for commitment to mitosis during cell cycles [29].

A biosensor to study Aurora B activation has also been developed [30]. But in this work, authors wanted to monitor the kinase activity at a specific location, since it has been postulated that an activity gradient of Aurora B at the mitotic spindle may play a role for mitotic progression. If one considers a conventional version of the FRET biosensor, its diffusion throughout the cell is too fast, and it is not possible to reveal a precise localization of the activation. Thus, Fuller and co-workers have added different localization sequences to target the biosensor either to the centromere using a peptide from CENP-B or to the chromatin using histone H2B [30].

FRET-based substrate kinase biosensors are good tools to investigate kinase activity, but they have some limitations and present three major challenges: (i) the biosensor relies on the endogenous kinase phosphorylating the substrate peptide, and thus, FRET variation is observed only when the kinase is particularly abundant or heavily stimulated, (ii) the sequence flanking the phosphorylation residue(s) targeted by the kinase must be known and selective for the kinase under study, and (iii) these biosensors only explore the catalytic activity of the kinase toward a specific substrate at once and not the activation process of the kinase itself. To solve this last issue, a new set of kinase FRET biosensors has been developed based on conformational changes of the kinase when active.

3. Conformational kinase-based biosensor

An alternative way of genetically encoded FRET-based substrate kinase biosensors has been developed by directly using the full-length kinase peptide sequence. Activation of a kinase frequently relies on a conformational opening of the enzymatic pocket. The idea is then to tag the whole kinase at its N- and C-terminus with a FRET pair of fluorescent proteins to be able to monitor this kinase activation related to the conformational change (**Figure 2**). To our knowledge, the first kinase FRET biosensor using this concept was developed to study c-Raf conformation [31]. This biosensor called Prin-c-Raf uses the CFP/YFP pair to flank c-Raf. A flexible linker has been added between the acceptor fluorophore and the kinase to enhance

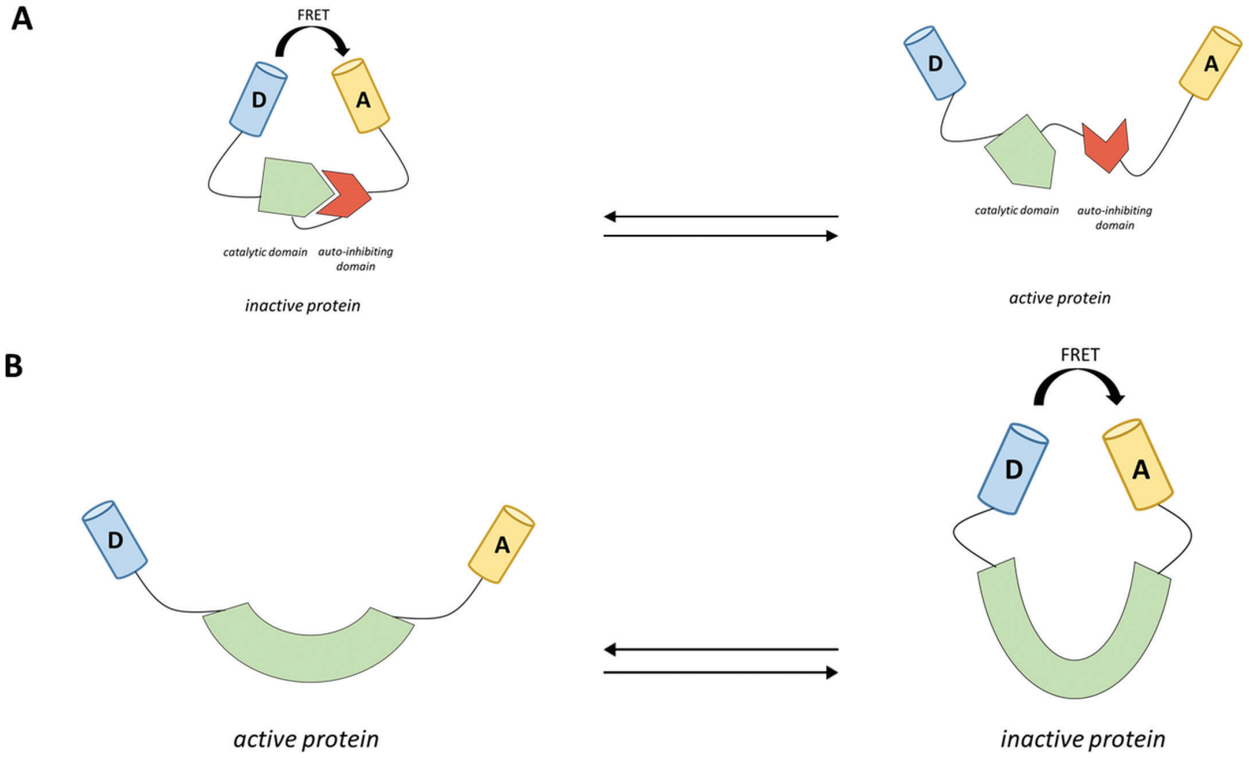

Figure 2. Mechanisms of a conformational-based FRET biosensor. (A) The auto-inhibiting domain can bin the catalytic domain of the kinase bringing closer the donor fluorophore D and the acceptor fluorophore A, allowing FRET. When the kinase is activated, the auto-inhibiting domain unbinds and the kinase adopts an opened and active conformation with a FRET decrease. (B) A lot of proteins adopt a new conformation when activated that modulates the distance between the pair of fluorophores.

FRET efficiency. Mutation of the residues Ser259 and Ser261 preventing c-RAF phosphorylation and mimicking the active state of the kinase leads to an open conformation of Prin-c-RAF as FRET ratio is decreased. When a constitutively active mutant of AKT that negatively regulates c-RAF is expressed, the wild-type version of c-RAF shows high FRET signal consecutive to a closed inactive conformation, while the mutated version S259A and S261A stays open with a lower FRET ratio. By using this biosensor, authors were able to show that the constitutively active H-RasV12 localized at the plasma membrane binds and opens the wild-type biosensor in an active conformation, inducing the recruitment of MEK at the plasma membrane.

A biosensor for PKCγ consisting of the kinase flanked by the donor super cyan fluorescent protein 3 (SCFP3A) and the acceptor YFP has also been developed [32]. The kinase displays a pseudosubstrate domain that is able to bind and inhibit catalytic activity. In this work, they compared a different mutated form of the biosensor PKCγ-A24E, where the pseudosubstrate cannot bind to PKCγ and observed a decrease of FRET that they are associated with an opened active conformation.

Another conformational biosensor has been developed to study FAK (Focal Adhesion Kinase) activity by taking advantages of the conformational changes associated with the activation state of the kinase being controlled by an inhibitory domain [33]. For that, a biosensor was constructed with the full-length kinase containing a FERM domain (F for 4.1

protein, E for ezrin, R for radixin, and M for moesin) for membrane localization and a kinase domain using the CFP/YFP FRET pair, the donor at the N-terminus of the protein and the acceptor directly between the two domains. When the FERM domain binds to the catalytic domain of FAK, it inhibits the kinase activity and FRET occurs. On the contrary, the absence of FRET corresponds to an active and thus open conformation. It is then possible to monitor FAK activity at the focal adhesion of living cells by expressing the biosensor transiently in living cells.

A biosensor of maternal embryonic leucine zipper kinase (MELK) has been created consisting of the MELK sequence flanked by the CFP/YFP pair [34]. As well as FAK, MELK has an auto-inhibited domain at the C-terminus that can bind to the catalytic domain of the kinase. This biosensor was expressed in *Xenopus* embryos, and conformational changes were monitored in dividing cells. It has been demonstrated that the biosensor exhibits a closed conformation in the cytosol and an open conformation at the cleavage furrow. But here again, as for previous conformational sensors, only conclusions on the conformational change of the kinase could be made. Its direct link to the kinase activation (and activity) was not tackled.

Recently, we have developed an Aurora A biosensor based on conformational changes [35]. It is composed of the full-length kinase flanked by a GFP donor and a mCherry acceptor. To be functional, Aurora A undergoes a conformational change following autophosphorylation on the T288 residue [36, 37]. By exploiting this mechanism, we designed a biosensor that directly associates the conformational change of the kinase with its state of activation. Indeed, *in vitro* treatment with ATP leads to a closed conformation when treatment with phosphatase leads to an opened conformation. The activation state is also monitored by fluorescence lifetime imaging microscopy (FLIM) in living cells. Through this work, we show that the biosensor was able to functionally replace the endogenous Aurora A depleted by siRNA. Thus, by replacing the endogenous kinase, this biosensor is a direct reporter of the activation state of Aurora A at endogenous levels in stable cell lines with a good spatio-temporal resolution. With this tool, by dissociating the quantity and the activation state of the kinase, we were able to highlight a new nonmitotic role of Aurora A in G1 phase [35].

This kind of biosensor can also be adapted to other enzymatic activities. As an example, a BRET (bioluminescence resonance energy transfer)-based biosensor of the PTEN (phosphatase and tensin homolog) phosphatase has been developed and is composed of the full-length PTEN protein flanked by a donor Rluc and the acceptor YFP [38]. PTEN biosensor immunoprecipitated from cells displays the same phosphatase activity on PIP3 (phosphatidylinositol 3,4,5 trisphosphate) and AKT (or Protein kinase B) as the wild-type PTEN. This biosensor can also be expressed at endogenous levels in human embryonic kidney (HEK) cells. The mutation of four residues Ser380, Thr382, Thr383, and Ser385 favoring a closed conformation leads to a strong decrease of BRET signal. The association between conformational changes and the activity state has allowed the monitoring of PTEN regulation in living cells. Authors have thus been able to correlate inhibition of the known activation pathway of PTEN using a CK2 (Casein Kinase 2) inhibitor, with its change of conformation or inversely by co-expressing S1PR2, an activator of PTEN. Once the biosensor was validated, the authors used it to identify new GPCRs (G protein–coupled receptors) activating PTEN.

The reliability of these biosensors, consisting of the full-length protein flanked by a pair of fluorophores, was recently applied to study any protein function associated with a conformational change. For example, a study of the conformational change of the Tau protein has been tackled using the protein flanked by a CFP/YFP pair [39]. The use of this biosensor led to the demonstration that the binding of Tau to the microtubules induces a switch to a hairpin conformation of Tau. In addition, it has been shown that mutations of Tau responsible of Frontotemporal dementia with parkinsonism-17 (FTDP-17) disorder alter this conformation change. This method has also been used to study vinculin conformation by using a biosensor consisting of the protein flanked by an mTurquoise donor and a NeonGreen acceptor [40]. Vinculin displays an auto-inhibited state, when the tail domain and the head domain are binding, increasing FRET signal. A mutated version of the biosensor that is unable to bind to talin showed a decreasing FRET signal and a disruption in the vinculin localization at focal adhesions. In contrast, paxillin knock-down or mutations leading to a decrease in actin binding did not modify FRET signal.

Thus, these genetically encoded biosensors are efficient at monitoring protein activation at cellular levels when expressed in living cells. A lot of proteins are known to adopt different conformation states according to their activation, and this is why FRET or BRET biosensors are best suited for tracking their activity in space and in time in living cells. It is likely that this tool will be used intensively to study protein conformation linked to activity in the next few years.

One can thus follow the activation of a kinase by following its conformational change using a conformation-based biosensor and follow its catalytic activity using a substrate-based biosensor. It would be of great interest to simultaneously follow activation and activity in a single living cell, a pursuit that calls for methods able to monitor the two different FRET biosensors simultaneously.

4. New methodological insights for multiplexing kinase biosensors

Owing to complex crosstalk between signaling pathways, multi-parameter biosensing experiments have become essential to correlate biochemical activities without lag time during a dedicated cellular process. A very exciting challenge has thus been to follow several FRET biosensors on the same sample at the same time and in the same location [41]. Commonly, FRET is measured by the fluorescence intensity ratio of the acceptor to the donor. In that case, whatever the two fluorescent protein FRET pairs chosen, CFP/YFP and mOrange/mCherry [42], mTFP1/mCitrine and mAmetrine/tdTomato [43, 44], mTagBFP/sfGFP and mVenus/mKok [45], the multiplex approach suffers from two limitations: (i) a spectral bleed-through of the first acceptor in the second donor emission band that depends directly on the respective quantities of the two biosensors and (ii) the multiple excitation wavelength which requires sequential acquisition that does not adequately follow fast signal dynamics or signal changes in highly motile samples.

To overcome the first limitation, a meroCBD (merocyanine–Cdc42-binding domain) biosensor modified with a far-red organic fluorophore (Alexa750) was used for probing Cdc42

simultaneously with a genetically encoded CFP/YFP FRET-based biosensor for Rho A [46]. This approach prevents spectral bleed-through but cannot be generalized to all genetically encoded FRET biosensors, where organic fluorophores cannot easily replace fluorescent proteins. The same team also developed an environment-sensing dye called mero199 [47]. This dye can bind to the active form of Cdc42 leading to a shift of its excitation/emission ratio. In combination with a Rac1 biosensor, they were able to simultaneously monitor activation of both proteins and to correlate it with retraction or velocity of migrating MEF (mouse embryonic fibroblasts) cells. Very recently, an elegant method based on linear unmixing of 3D excitation/emission fingerprints applied to three biosensors simultaneously was published [48]. This type of approach based on image calculation is often limited by the different biosensors expression levels and a poor signal-to-noise ratio after complex image corrections.

To overcome the second limitation, the two FRET pairs CFP/YFP and Sapphire/RFP in combination with a single violet excitation were used [49], resulting in no lag time in biochemical activity recording. But again, in this case, the spectral bleed-through and excitation crosstalk necessitate linear unmixing. Another interesting approach for simultaneously multiplexing two FRET activities was developed using a "Large Stokes Shift" orange fluorescent protein, LSSmOrange [50]. The authors used a CFP-YFP together with LSSmOrange-mKate2 biosensors enabling imaging of apoptotic activity and calcium fluctuations in real time using intensity-based methods. Other studies were carried out utilizing FLIM instead of ratio imaging to measure FRET. When FRET occurs, donor fluorescence lifetime decreases. This method requires measurement of the donor fluorescence only and is independent of emission from the acceptor. By using CFP and YFP as donor and the same red acceptor (tHcRed), FLIM of CFP and YFP donors allow the two different FRET signals to be distinguished [51]. Combination of FLIM-FRET of a red-shifted TagRFP/mPlum pair with ratio imaging of a CFP/Venus pair allows maximal the spectral separation while, at the same time, overcoming the low quantum yield of the far-red acceptor mPlum [52]. The two last examples alleviated the spectral bleed-through but not the limitation associated with multiple excitations.

To overcome both limitations, a novel red-shifted fluorophore mCyRFP1 has been developed with a high Stokes shift [53]. This fluorophore has an excitation spectrum in the range of the GFP emission spectrum (around 500 nm), but its emission spectrum is shifted compared to GFP. An emission dichroic filter allows simultaneous detection of the GFP fluorescence lifetime and the mCyRFP1 fluorescence lifetime. The authors were able to perform two-photon fluorescence lifetime imaging by using only one excitation laser at 920 nm with a Rhoa biosensor and a CaMKIIα biosensor. Furthermore, while the RhoA biosensor uses the pair mCyRFP1/mMaroon1, the CaMKIIα biosensor uses mEGFP and dimVenus which is a dark fluorophore preventing bleed-through with mCyRFP1.

Recently, our team has developed a similar method by taking advantages of the LSSmOrange (Large Stoke Shift) and the dark fluorophore ShadowG [54]. We modified two substrate kinase biosensors, EKAR2G and AKAR4 (E-Kinase Activity Reporter type 2G for ERK and A-Kinase Activity Reporter type 4 for PKA), with a new pair of fluorophores mTFP1/ShadowG and LSSmOrange/mKate2, respectively. LSSmOrange and mTFP1 are both excitable by using a single 440 nm wavelength. By single excitation wavelength dual-color FLIM, we are able to

simultaneously monitor the activity of ERK and PKA in living cells at the same location. Thus, the activity of each kinase in response to forskolin or EGF treatment can be imaged simultaneously. This approach overcomes the limitations of the multiple excitation wavelengths and bleed-through.

Because FLIM is now a widely used microscopy approach, the decrease of the donor lifetime is sufficient to quantify FRET, and fluorescence of the acceptor is not mandatory, as it is still the case when one uses ratiometric FRET. Changing the fluorescent acceptor with a nonfluorescent acceptor leads to the development of a new kind of single-color FRET biosensor (**Figure 3A**). It is then perfectly adapted for simultaneous monitoring of kinase FRET biosensing.

Another method to get a single-color biosensor to perform multiplex could be based on homoFRET measured by anisotropy [55, 56]. HomoFRET occurs when a fluorophore transfers its energy to a closely identical fluorophore. However, it is impossible to measure homoFRET by ratiometric or fluorescence lifetime measurements. Fluorescence anisotropy can be measured by detecting the parallel and the perpendicular light emitted by a fluorophore excited with a polarized light [57], and this anisotropy decreases when FRET occurs between nonparallel fluorescent dipoles. This method was already used to study protein oligomerization [58]. For example, a study has used fluorescence anisotropy to determine the degree of clustering of proteins such as GPI or EGFR fused to GFP in living cells [59]. This approach has been investigated to multiplex at the same time a conventional calcium heteroFRET biosensor using FLIM with the oligomerization of pleckstrin homology domains of Akt (Akt-PH) labeled

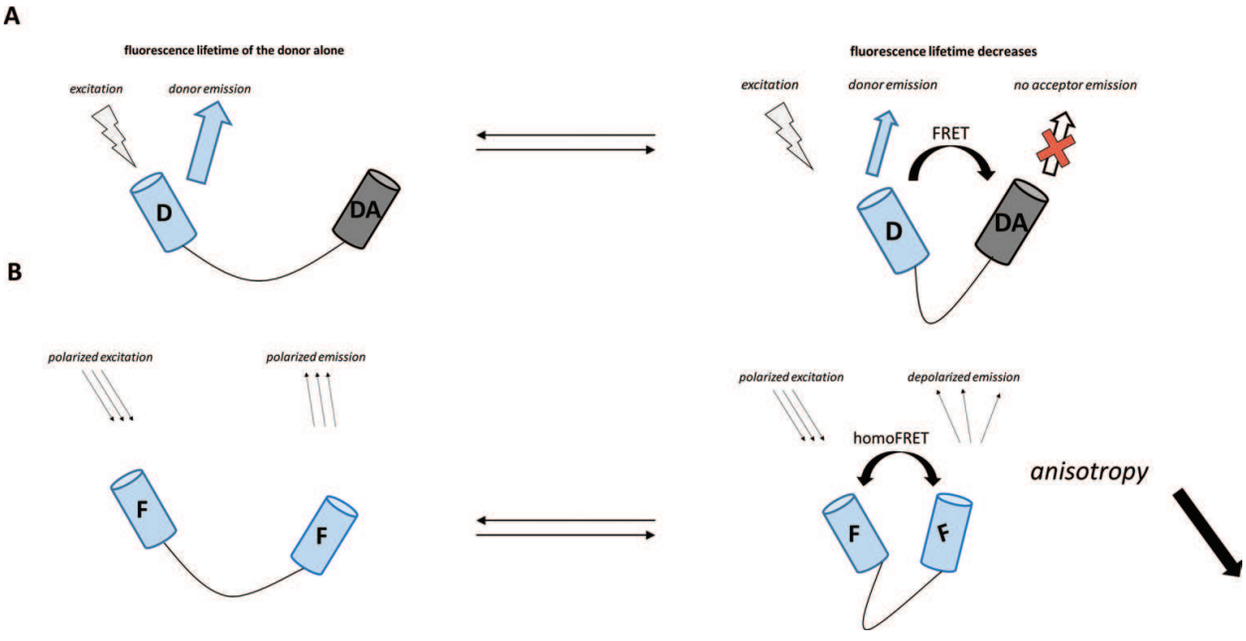

Figure 3. Single-color genetically encoded FRET biosensor. (A) When a fluorophore F is excited, it can transfer its energy to a dark acceptor DA by FRET. Even excited, the dark acceptor emits no detectable light, and FRET is measured by the measurement of donor fluorescence lifetime. It constitutes a single-color FRET biosensor. (B) When a fluorophore F is excited by a polarized light, it emits polarized fluorescence. When homoFRET occurs between two identical fluorophores, it can leads to the depolarization of fluorescence emission decreasing the anisotropy. Again, it constitutes a single-color FRET biosensor.

with mCherry [60]. From our knowledge, the development of an intramolecular homoFRET biosensor to follow a biochemical activity was not yet developed but has very interesting potential. Adapting this method to kinase biosensors would provide a new methodology to simultaneously follow multiple biosensors.

5. Concluding remarks

Kinases have multiple functions in cells, and their mechanisms are very dynamic in both space and time. We have focused our review on two types of kinase biosensors. The substrate-based kinase biosensors are good tools to specifically monitor the activity of a kinase, but it requires to have a good knowledge of the substrate peptide sequence, particularly for its specificity, and a biological system where the activity of the kinase is sufficient to detect FRET. The conformation-based biosensors provide information about the activation state of the kinase itself; however, they do not provide information about its catalytic activity that can be further regulated by other post-translational modifications. Gathering these different tools with a multiplex methodology by using the approaches of single-color FRET biosensor would provide new mechanistic insight to investigate kinase functions with an adequate spatio-temporal resolution.

Acknowledgements

We wish to apologize to the authors whose works were not cited in this review. We wish to thank G. Bertolin, G. Herbomel, and C. Demeautis for their many helpful discussions about FRET biosensors and D. Fairbass for critical reading the manuscript. Work in the laboratory is supported by the CNRS and the University of Rennes 1 and grant from the "Ligue Nationale Contre le Cancer" (LNCC, region Grand Ouest). F. S. is fellow of the Région Bretagne and University of Rennes 1.

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