FRET-Based Enzyme Activity Reporter: Practical Hints for Kinases as Indicators of Virulence

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Chapter 12

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http://dx.doi.org/10.5772/intechopen.72388

Abstract

Modulation of protein kinases activity is often requested for pathogenicity or virulence. This chapter provides several hints for one who might be interested in using FRET-based kinase activity reporters. The archetypes of these reporters, which are now within the arsenal of biosensors, were devoted to the detection and characterization of the activity of the cAMP-Protein kinase A pathway. Based on the principle of this biosensor, other FRET-based kinase activity reporters emerged. Here, the choice of the kinase to be monitored, the artifacts that might be met, and the flexibility and amenability of the FRET-based kinase activity reporters both for high-throughput analysis and dissection of protein kinase functions are discussed.

Keywords: genetically encoded biosensor, KAR, fret, MAPK, ERK

1. Introduction

Biological signatures of parasitic diseases may (1) involve the production and release of specific proteases, which are called to promote host invasion, to evade host defenses or to provide nutrients from the local environment [1] or (2) rely on the modulation of specific protein kinases activity such as mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (Erk, *Toxoplasma gondii* [2], *Leishmania* spp. and *Trypanosoma cruzi* [3]). The abovementioned enzymes have been regarded from two angles, leading either to the development of inhibitory strategies or biosensors development [4].

Herein, we discuss several aspects related to use of biosensors in living cell contexts, which are of high interest in the perspective of biosensing in living organisms. Nevertheless, we restrain our talk to signaling pathways and focus on protein kinases. One shall note that biosensor is a generic term describing the various analytical devices incorporating a biological sensing element. Back in

the 1980s, biosensors were mainly either sophisticated laboratory machines or amenable portable devices [5] based on electric currents [6] or conductivity [7]; optical properties [8] or other physico-chemical measurements. In the 1990s, emerged a plethora of new tools, conforming to the biosensor definition, and reporting enzymes activities. The latter were built and developed in different contexts (living cells, lysates), aiming at benefiting either from high sensitivity or selectivity. To these extents, devices like amperometric biosensors [9], bioluminescent-based sensors *in vivo* [10, 11] and functionalized nanoparticles were used [12, 13], exhibiting high sensitivity and selectivity, which are mandatory for diagnosis, especially in case of pathogens [14].

Among biosensors, genetically encoded Förster Resonance Energy Transfer (FRET) biosensors raised hope to focus on both enzymatic activities and ion concentration with high spatiotemporal resolution in both living cells and organisms. It relies on Förster Resonance Energy Transfer, or FRET, a radiationless coupling from a donor fluorophore to an acceptor molecule. Several conditions must be met for this transfer to occur (spectral overlap between fluorophore, dipole relative orientation or distance). The most useful property is that the donor and acceptor molecules must be in close vicinity (for commonly used fluorophore pairs, <10 nm) and that the FRET level depends on the sixth power of the distance between fluorophores. FRET biosensors are thus built to switch between two configurations where the distance between donor and acceptor are above and below this threshold distance (**Figure 1**). They are made of an adapted

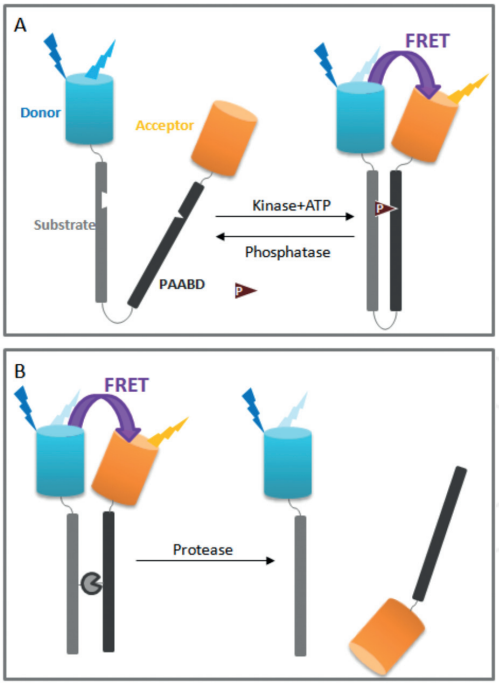

Figure 1. Scheme representing two categories of FRET-based biosensors. A, Kinase activity reporters reflect the balance between specific kinase and phosphatase; protease reporters (B) rely on an irreversible cleavage. Regarding interpretation, an increase in kinase activity will be reflected by an increase in FRET level, while the protease activation will induce a loss in the original FRET signal.

bioreceptor tagged on both end with a donor and acceptor. The biosensor configuration will be specifically altered by the presence of either a second messenger or the action of an enzyme, inducing either an increase or decrease in FRET efficiency. A FRET event will induce changes in most properties of light such as fluorophores excitation and emission or donor fluorescence polarization or lifetime. A variety of fluorescence-based methods are then derived from these changes to quantify biosensors' response with associated fluorescence microscopy benefits (selectivity, low toxicity, high temporal and spatial resolution, optical sectioning, etc.).

2. Kinase activity reporter archetypes

Being two FRET-based biosensors for protein kinase A activity, protein kinase A activity reporter (AKAR) and exchange proteins activated by cAMP (Epac) are considered as the archetypes for genetically encoded FRET reporters. Activity of protein kinase A (PKA) is controlled by cyclic adenosine monophosphate (cAMP) levels, which behaves as a second messenger for many cellular responses driven by external stimuli. The tandem cAMP-PKA is considered to play many essential functions within cellular life like cell cycle [15]. cAMP concentration is regulated by the activity of adenylyl cyclase, the latter being activated by G protein coupled receptor (GPCR), upon the specific interaction with its ligand. Under its inactive state, PKA is made up of regulatory subunit dimers associated with catalytic subunit dimers. The activation of PKA requests the fixation of four molecules of cAMP that are catalyzed on the regulatory subunit. Such fixation of the cAMP leads the catalytic dimer to dissociate (**Figure 2**). Counteracting the activity of adenylyl cyclase and phosphodiesterase downregulates PKA activity through cAMP degradation.

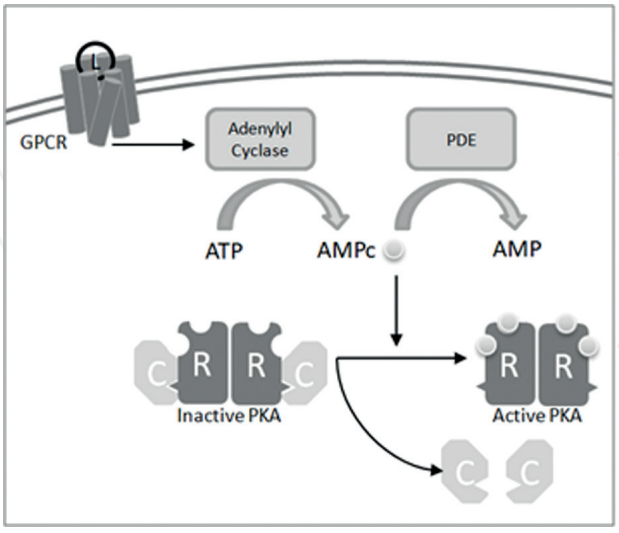

Figure 2. Focus on the PKA and cAMP signaling node. GPCR (G protein coupled receptor), R (regulatory subunit), C (catalytic subunit) of protein kinase A and PDE (PhosphoDiEsterase).

As mentioned earlier, two FRET-based biosensors have been developed and devoted to study the dynamics of c-AMP-PKA, mainly to overcome the shortcomings of the classical biochemical methodologies and to monitor individual cellular responses, which can either be sub-localized or transient. Both biosensors were based upon a similar structure: a specific phosphorylable peptide and a phosphoamino acid binding domain (PAABD), standing together between two fluorophores [16]. When phosphorylated, the peptide sequence interacts with PAABD, driving a conformational change bringing the fluorescent proteins in close vicinity. The latter enables the FRET to occur and provides measurable changes acknowledging for the activity of the considered enzyme, here PKA in case of AKAR. While AKAR mirrors the activities of kinase/phosphatases on a specific substrate of PKA [17], Epac proteins aimed at measuring the changes in

Specificity	Sensor name	References
Abl (Abelson murine leukemia viral oncogene homolog 1)	Crk indicator	*(Ting et al. 2001) [18]*
	EGFR indicator	*(Ting et al. 2001) [18]*
	PICCHU	*(Kurokawa et al. 2001) [19]*
Akt/PKB (Protein Kinase B)	AktAR	*(Gao and Zhang 2008) [20]*
	Aktus	*(Sato and Umezawa 2004) [21]*
	Akind	*(Yoshizaki et al. 2006) [22]*
	BKAR	*(Kunkel et al. 2005) [23]*
	GFP-PKB-RFP	*(Calleja et al. 2007) [24]*
	ReAktion	*(Ananthanarayanan et al. 2007) [25]*
AMPK (AMP-activated protein kinase)	AMPKAR	*(Tsou et al. 2011) [26]*
Aurora B kinase	Aurora B sensor	*(Chu et al. 2011) [27]*
ATM kinase	ATOMIC	*(Johnson, You, and Hunter 2007) [28]*
CyclineB1/cdk1	CyclineB1/cdk1 sensor	*(Gavet and Pines 2010) [29]*
EGFR (Epidermal Growth Factor Receptor)	EGFR-ECFP/PTV-EYFP	*(Offterdinger et al. 2004) [30]*
ERK (Extracellular signal-regulated kinase)	EKAR	*(Harvey et al. 2008) [31]*
	EAS	*(Green and Alberola 2005) [32]*
	Miu2	*(Fujioka et al. 2006) [33]*
FAK (Focal adhesion kinase)	FAK sensor	*(Seong et al. 2011) [34]*
Glucokinase	mCer-GCK-mVenus	*(Ding et al. 2011) [35]*
Histone H3 phosphorylation	H3 Reporter	*(Lin and Ting 2004) [36]*
Insulin receptor	Phocus	*(Sato and Umezawa 2004) [21]*
JNK	JNKAR	*(Fosbrink et al. 2010) [37]*
MK2 (MAP kinase activated protein kinase 2)	EGFP-MK2-EBFP	*(Neininger, Thielemann, and Gaestel 2001) [38]*
MARK (Microtubule affinity regulating kinase)	MARK sensor	*(Timm et al. 2011) [39]*
PLK1 (Polo like kinase 1)	Plk sensor	*(Macůrek et al. 2008) [40]*
PKA (Protein kinase A)	AKAR	*(Zhang et al. 2007) [41]*
	ART	*(Nagai et al. 2000) [42]*
PKC (Protein kinase C)	CKAR	*(Violin et al. 2003) [43]*
RSK (Ribosomal s6 kinase)	Eevee-RSK	*(Komatsu et al. 2011) [44]*
	Eevee-S6K	*(Komatsu et al. 2011) [44]*
Stress-activated protein kinase kinase kinase (SAP3K)	SAP3K activity reporter	*(Tomida et al. 2009) [45]*
Src (Proto-oncogene tyrosine-protein kinase)	Srcus	*(Sato and Umezawa 2004) [21]*
	Src indicator	*(Ting et al. 2001) [18]*

Table 1. Kinase activity reporters and associated references.

concentration of cAMP. These biosensors unfold their structure upon the fixation of cAMP and break the vicinity of the donor and acceptor fluorescent proteins. Thus, while FRET increase is related to an increase in PKA activity in case of AKAR, a decrease in FRET activity is related to the increase of cAMP levels. The two biosensors provided complementary information regarding the levels of cAMP and PKA, being two angles of a same pathway.

From the initial development of kinase-specific biosensor for cAMP and kinases, several derivatives have been built as illustrated by the extracellular signal regulated kinase activity reporter (EKAR) variants (see **Table 1**) [18–45]. Indeed, kinase activity reporters (KAR) follow fluorophores' optimizations for FRET assay, thereby increasing their sensitivity and robustness. Furthermore, microscopy measurements allow sub-localization of kinase activity, which may be mandatory for the understanding of signaling nodes. KAR versions directed toward the different subcellular compartments were thus developed.

3. Choosing the right kinase activity to report: the needle in a haystack

Biological messages are mediated by intracellular signaling pathways, whose dynamics and interplays have not yet been fully deciphered. Biosensors are focused on specific elements of the networks conveying the information and interpretation shall be carried out accordingly. One has to carefully consider the complexity of pathways where protein kinases could be nodes within networks (**Figure 3**) [46]. Monitoring the phosphorylation of one particular sequence within the sensors will not necessarily reflect its involvement in all functions of the considered kinase.

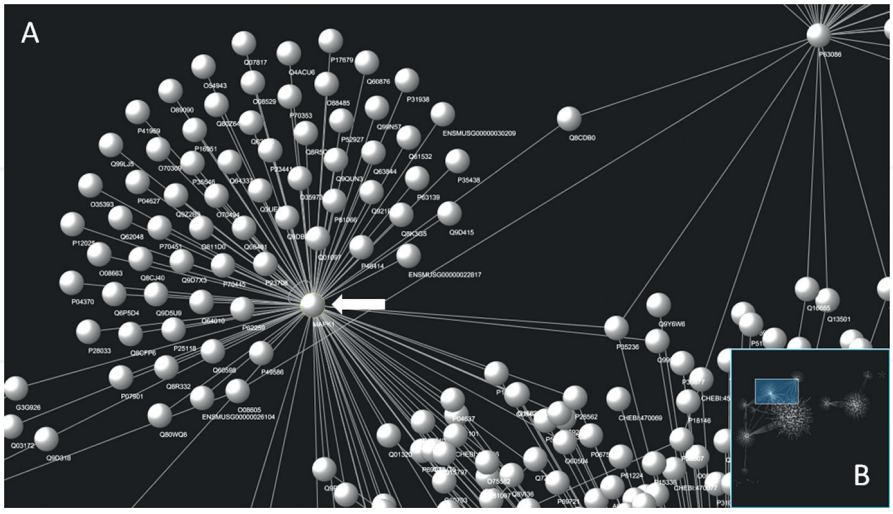

Figure 3. Scheme of MAPK1 interactom generated through Cytoscape. MAPK1 is highlighted by the white arrow, spheres correspond to interactors. A focus on MAPK1 node and B represent the overall network.

Modulation of protein kinase activities might be requested for pathogenicity or virulence [47]. Mitogen-activated protein kinase (MAPK)/extracellular regulated kinases (Erk) can be taken as a school case, since the latter activity is solicited in many different aspect of cellular life, that is, proliferation, migration and differentiation. On the one hand, MAPK are inhibited by several pathogens such as anthrax [48, 49], mycobacteria [50], *Vibrio parahaemolyticus* [51], herpes simplex virus 1 (HSV-1) [52] or *Yersinia* spp. [53]. Activation of MAPK/Erk can also be manipulated by diverse families of virus to favor their replication. For example, enteropathogenic coronavirus like the porcine epidemic diarrhea virus are infecting cells, due to the activity of MAPK/Erk [54]. Impairing the activation of MAPK drives the suppression of viral progeny production. As well, MAPK activity might be enrolled in human immunodeficiency virus of type 1 (HIV-1) replication [55]. Increase in other protein kinase activities may be requested for life cycle of other pathogens. A recent meta-analysis of data from different ribonucleic acid interference (RNAi) screening revealed a potential role for the members of Polo-like kinase for *Influenza A* virus infections [56]. Therefore, the latter Polo-kinase (Plk), which was more known for its pivotal role in cell cycle regulation, appeared as a therapeutic target and was extracted likely as a needle out of a haystack. Nevertheless, the function of Plk in this context remains elusive, but Plk might be required for the *Influenza* viral infection through the creation of an optimal environment for viral replication by balancing the apoptotic and antiapoptotic signaling pathways [57].

In this context, after identifying the hijacked node, researcher needs to monitor the pathogenic modulation of the kinase/phosphatase balance. FRET-based biosensors are thus optimal tool for dissecting these subtle alterations, far from binary modifications.

4. Discarding artifacts: chemical inhibitors and dead reporters

Insights gained by genetically engineered enzyme reporters are solely validated through adequate controls. Any response gathered using biosensors shall be carefully considered and fully analyzed: what you might see may not be what you will get as a response at the end of the analysis procedure [58]. Among other parameters, consensus sequence of the phosphorylable peptide, expression levels, kinetics and dynamic ranges have, for example, to be taken in account.

The choice of the peptide substrate is crucial and has to be defined accordingly to the specificity of the kinase, if known. For example, there is a current failure to determine a consensus site for p38MAPK. The latter inability to determine a sequence consensus hinders the amenability to construct any KAR for this particular kinase. The process of the KAR design can be optimized through a screening strategy for the best phosphopeptide sequence [59] or the linkers between the different segments and/or the fluorophores [17].

One shall also take a particular care to discriminate a specific response from the noise within the crowded environment of the intracellular compartments. The cellular noise depends upon the biophysical properties of the chosen cell lines to work with, as well as results from cell autofluorescence, intracellular pH and biosensors expression levels. In case of KAR, morphological changes are likely not to alter the signals, as observed for monitoring cyclin-dependent kinase 1 (Cdk1) activity during cell rounding at the beginning of mitosis [60].

To discriminate the noise, several options might be undertaken to determinate the specificity and dynamical range of response. First is the use of chemical inhibitors to separate the balance of kinase/phosphatase activity from the cellular noise (*i.e.*, Cdk1/2 inhibitor of RO3306 for Cdk1 KAR [60] or U0126 for EKAR [17]). Second is the use of a dead reporter. The latter must be mandatory for any FRET-based enzyme reporter. A dead-reporter can be built upon a mutation that replaces, for example, a phosphorylable residue by another one, which cannot be phosphorylated. Thereby, the conformational change of the biosensor is never achieved, and the FRET changes shall be minimal, due to intrinsic flexibility of the structure, either in relaxed form or in a conformation with the fluorophores in close neighborhood. In case of KAR, dead reporter can be built, where the phosphorylable threonine of the phosphorylable peptide is substituted by an alanine. Thus, a control "baseline" can be monitored in these conditions (PKA [15–17], Erk [17]).

Though time-consuming, these steps of artifacts controls and intrinsic properties characterization of sensors are mandatory for proper analysis of KAR spatiotemporal profiles (**Figure 4**).

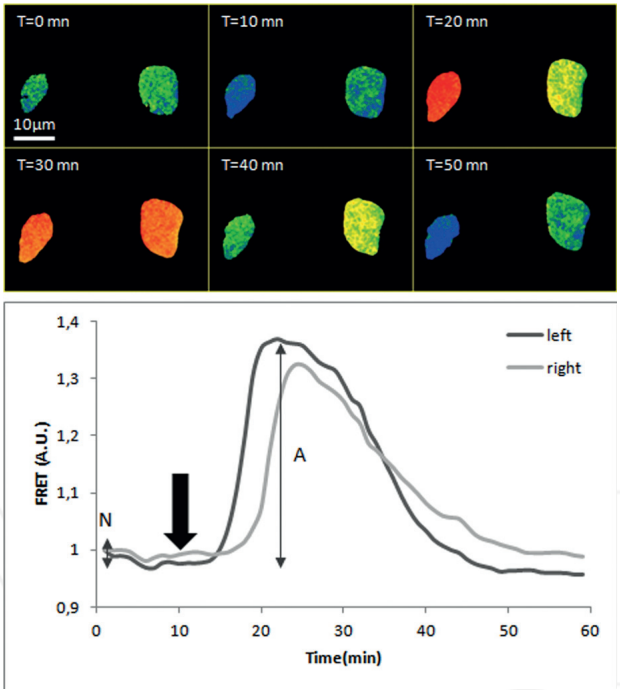

Figure 4. Time-lapse FRET measurement applied on MCF-7 cells expressing EKAR biosensor after EGF activation of the ERK pathway at 10 mn. The upper panel corresponds to FRET level color-coded from dark grey (low activity) to white (high activity). The bottom graph corresponds to the mean FRET measurement of both cells with N the biological "noise" and "A" the maximum amplitude achieved after induction. This state-of-the-art experiment illustrates the advantages of cell by cell analysis. Indeed, even two cells treated exactly in the same way can behave differently upon network activation. In this case, the maximum amplitude, the time needed to reach maximum activation and the duration before returning to the basal activity are different. Averaging these behaviors upon a large amount of cells can smooth or mask the individual response to stimuli and make it difficult to dissect regulatory networks.

5. A dynamic and flexible tool

Among the FRET-based biosensors, several categories exist and might have an impact on data interpretation. Especially, the change in FRET level can be due to either a configuration change or a cleavage of the sensor. In the first case, the sensor will be reversible as it is the case for most kinase activity reporters. Thus, the sensor will not monitor the kinase activity, but the balance between the kinase and its phosphatase counterpart. Cleavage-based reporters will have an irreversible response. In this case, the cumulative effect of the enzyme will be measured. Both behaviors are represented in **Figure 5**. In **Figure 5A**, a cyclic alternation of kinase and phosphatase

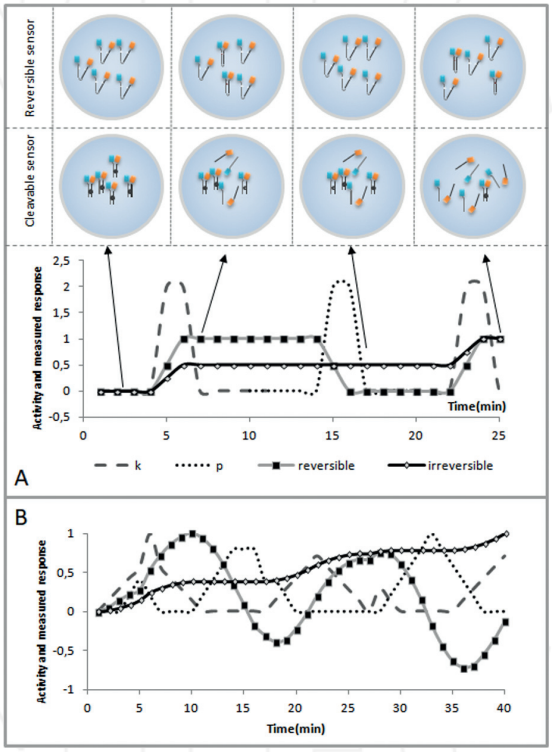

Figure 5. Illustration of sensor response to the balance between a kinase (k) and a phosphatase (p). (A) Sequential activation of a kinase and its phosphatase counterpart and associate response measured with either a reversible or an irreversible sensor. Upper panel: scheme representing both biosensors behavior after each activation step. Bottom panel: Associated activity and measured response. (B): Illustration of a more complex kinase/phosphatase oscillatory behavior with both sequential and simultaneous activation with different amplitudes and duration of activation. Irreversible sensors present a smooth response to kinase activity until cleavage of all available sensors. Reversible sensors are impacted by both kinase and phosphatase. While it offers a more realistic view of regulatory nodes, one shall keep in mind for interpretation that (i) the measure depends on the global state of the sensor. Kinase activation after a strong phosphatase period will take some time to change KAR conformation and thus to restore a positive response (T = 37 min). Thus, interpretation of time-lapse measurements is way easier than single acquisition. (ii) No difference can be made between no activity and a balance between kinase and phosphatase. Both will result in a constant behavior of the sensor (T = 18 min). (iii) An increase in the phosphatase activity can also result in activity measurements below the equilibrium value (T = 15 min).

action and associated biosensor response is depicted. While both behave in a similar manner upon the first kinase action, measurements diverge after the first phosphatase effect. Indeed, reversible sensors will then return to their basal level where the irreversible sensor will not be altered. Thus, while the second kinase activation will induce the same increase for both sensors, the final level will be different due to the cumulative effect observed for the irreversible version. More complex behavior is illustrated in **Figure 5B**.

From these simple schemes, it seems obvious that dissecting a node regulated by a kinase will be way easier with reversible sensors. Nevertheless, one should keep in mind that despite the name of sensors like KAR, reversibility mirrors the equilibrium of two enzymes. Thus, the measure corresponds to the kinase/phosphatase balance and biological interpretation should be made accordingly.

6. Amenability of FRET-based biosensors for high throughput

Perspectives are on different battleground for KAR use: (1) detection on environment or within living organisms and/or (2) untangling the host-pathogen interaction and the hijacking of host metabolism and signaling pathways (either to benefit from them or to mask host presence). Requested tools have therefore to be chosen accordingly to the purpose and to face the demand for high-throughput strategies or to face the complexity of molecular interactions within living organisms.

Energy transfer biosensors' sensitivity has been increased by the numerous multidisciplinary advances in the fields of photophysics, instrumentation and even nanomaterials. Above-mentioned advantages of KAR have thus made these tools amenable for high throughput [61] and led the kinase sensors to be cited as best biosensors in physiology [62].

Abbreviations

AKAR	Protein kinase A activity reporter
AktAR	Akt activity reporter
AMPK	AMP-activated protein kinase
AMPKAR	AMP-activated protein kinase activity reporter
ATM	Ataxia Telangiectasia mutated
ATOMIC	ATM observation method in cell
BKAR	B kinase activity reporter
cAMP	Cyclic adenosine monophosphate
Cdk1	Cyclin dependent kinase 1
EAS	ERK activity sensors

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EKAR	Extracellular signal regulated kinase activity reporter
Epac	Exchange proteins activated by cAMP
Erk	Extracellular regulated kinase
FAK	Focal adhesion kinase
FRET	Förster Resonance Energy Transfer
GPCR	G protein coupled receptor
JNK	c-Jun N-terminal kinase
JNKAR	JNK activity reporter
KAR	Kinase activity reporters
MAPK	Mitogen activated protein kinase
MARK	Microtubule affinity regulating kinase
MK2	MAP kinase activated protein kinase 2
PAABD	Phosphoamino acid binding domain
PICCHU	Phosphorylation indicator of CrkII chimeric unit
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
Plk	Polo-kinase
RNAi	Ribonucleic acid interference
RSK	p90 Ribosomal S6 kinase
SAP3K	Stress-activated protein kinase

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