Biochimica et Biophysica Acta 1834 (2013) 1387–1395

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

Fluorescent biosensors — Probing protein kinase function in cancer and drug discovery ☆

May C. Morris *

Chemical Biology and Nanotechnology for Therapeutics, CRBM-CNRS-UMR5237,1919 Route de Mende, 34293 Montpellier, IFR122, France

ARTICLE INFO

Article history:
Received 5 January 2013
Accepted 24 January 2013
Available online 1 February 2013

Keywords:
Fluorescent biosensor
Protein kinase
Biomarker
Drug discovery
CDK/cyclin

ABSTRACT

One of the challenges of modern biology and medicine is to visualize biomolecules in their natural environment, in real-time and in a non-invasive fashion, so as to gain insight into their physiological behavior and highlight alterations in pathological settings, which will enable to devise appropriate therapeutic strategies. Fluorescent biosensors constitute a class of imaging agents which have provided major insights into the function and regulation of enzymes in their cellular context. GFP-based reporters and genetically-encoded FRET biosensors, have been successfully applied to study protein kinases in living cells with high spatial and temporal resolution. In parallel, combined efforts in fluorescence chemistry and in chemical biology have enabled the design of non-genetic, polypeptide biosensors coupled to small synthetic fluorescent probes, which have been applied to monitor protein kinase activities in vitro and in more complex biological samples, with an equally successful outcome. From a biomedical perspective, fluorescent biosensor technology is well suited to development of diagnostic approaches, for monitoring disease progression and for evaluating response to therapeutics. Moreover it constitutes an attractive technology for drug discovery programs, for high content, high throughput screening assays, to assess the potency of new hits and optimize lead compounds, whilst also serving to characterize drugs developed through rational design. This review describes the utility and versatility of fluorescence biosensor technology to probe protein kinases with a specific focus on CDK/cyclin biosensors we have developed to probe abundance, activity and conformation. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

© 2013 Elsevier B.V. All rights reserved.

1. Introduction — imaging in modern biology & medicine

One of the challenges of modern biology consists in attempting to visualize biomolecules in their natural environment, in real-time and in minimally invasive conditions. Ideally one seeks to monitor changes in their expression profile, in their spatio-temporal localization and in their biological activity in a physiological context, in response to specific stimuli, as well as in clinically-relevant pathological settings. Moreover, the development of personalized medicine and targeted therapies calls for sensitive and selective means of detecting specific biomarkers, that further allow to monitor their status during disease progression and in response to the administration of therapeutics.

With the development of chemical biology and fluorescence technologies, a wide array of imaging agents have been developed over the last decade, including small molecules such as peptides, drugs, small ligands, and environmentally sensitive fluorescent probes, larger macromolecules such as polymers recombinant proteins and engineered antibodies, and dye-doped fluorescent nanoparticles [1,2]. These molecular probes have provided major advances in our understanding of protein activity and regulation in their natural environment, allowing to track the dynamics of molecules in motion and to study biological processes in four dimensions.

2. Fluorescent biosensors

Amongst the different classes of probes designed for molecular imaging, fluorescence-based reporters and biosensors constitute a class of tools which has undoubtedly provided the most promising advances and perspectives for imaging, biomedical and drug discovery applications. Fluorescent biosensors are small biological or biomimetic scaffolds onto which one or several fluorescent probes are coupled (enzymatically, chemically or genetically) through a receptor moiety which recognizes a specific analyte or target, thereby transducing the recognition process into a fluorescent signal which can be readily detected and measured [3–5] (Fig. 1).

Fluorescent biosensors provide a sensitive means of probing ions, metabolites, and protein biomarkers and can report on the presence, activity or conformational status of a specific target in complex solutions such as serum and cell extracts, as well as living cells. They offer

Abbreviations: AFP, autofluorescent protein; CDK, cyclin-dependent kinase; CHEF, chelation-enhanced fluorescence; FRET, fluorescence resonance energy transfer; KAR, kinase activity reporter; PAABD, phosphoamino acid binding domain; HCS, high content screening; HTS, high throughput screening

☆ This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

* Tel.: +33 4 34 35 95 24; fax: +33 4 34 35 95 10.
E-mail address: may.morris@crbm.cnrs.fr.

1570-9639/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2013.01.025

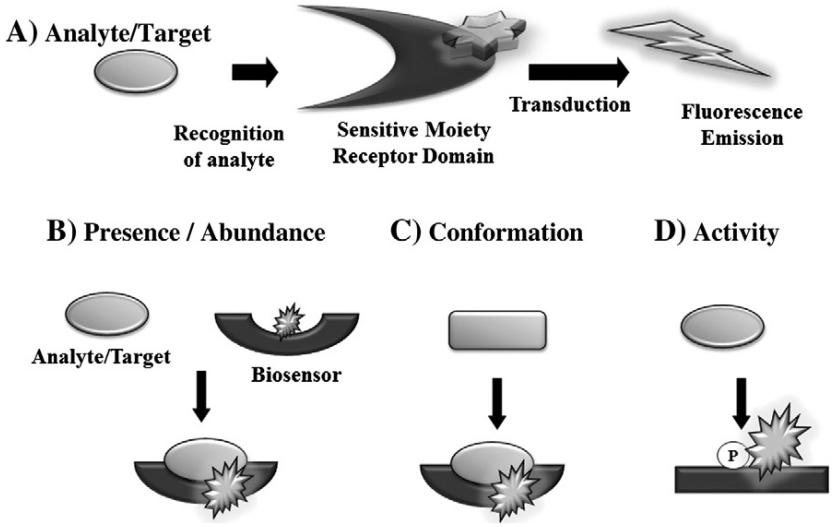

Fig. 1. Fluorescent biosensors. A) Fluorescent biosensors are analytical devices which combine a sensor moiety involved in recognition of a specific analyte or target, with a physiochemical transducer, a fluorescent probe that emits a detectable and measurable signal upon binding to the target. B) A ligand-protein biosensor may report on the relative abundance of a target or biomarker C) A conformational biosensor — or conformation sensitive biosensor may report on a specific conformation of a target. D) An activity-protein biosensor may report on enzymatic activity such as phosphorylation or proteolytic cleavage.

means of monitoring dynamic processes in real-time, and are therefore particularly well suited to study the behavior of enzymes to gain insight into their function and regulation in a quantitative fashion, in space and in time. Fluorescent biosensors are commonly employed in fundamental studies to probe gene expression, protein localization, activity, function and conformation in a wide variety of fields including signal transduction, transcription, cell cycle and apoptosis. Moreover, they constitute a sensitive means of reporting on characteristic biomarkers of several pathological conditions, including arthritis and inflammatory diseases, cardiovascular and neurodegenerative diseases, viral infection, cancer and metastasis [5,6] (Fig. 2).

Two large classes of biosensors have been developed: genetically-encoded and non-genetic fluorescent biosensors. The discovery of the

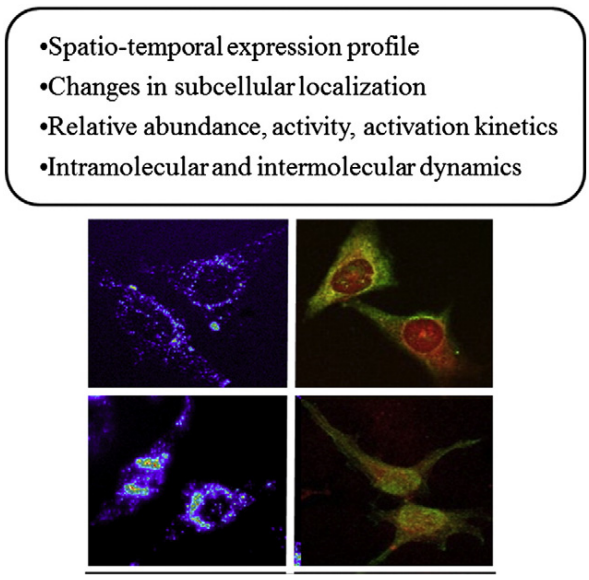

Fig. 2. Fluorescent Biosensor Applications in Fundamental Science. Fluorescent biosensors are useful tools for fundamental studies in life sciences, to probe the function, regulation and spatio-temporal dynamics of biomolecules in a wide variety of biological processes. Left panels show differential localization of CDK/cyclins in different cell cycle stages thanks to a CDK/cyclin biosensor. Right panels show differential localization of cyclin B in the same cell cycle stages as in left panels.

Green Fluorescent Protein (GFP) and the subsequent development of autofluorescent protein (AFP) variants and genetic fusion reporters paved the way for development of genetically-encoded biosensors [7–14]. This class of biosensors is user-friendly, easy to engineer, manipulate and transfect into cells. Although several subtypes of genetically-encoded biosensors have been designed, the larger part are single-chain FRET biosensors, consisting of a pair of AFPs which can transfer fluorescence resonance energy between one another when brought into close proximity in response to target activity. Different strategies may be employed to monitor changes in FRET signals based on intensity, ratio or lifetime of the AFPs. However genetically-encoded biosensors rely on ectopic expression of autofluorescent protein fusions in the cell and are therefore somewhat limited with respect to control over expression time and levels. The development of chemical biology strategies to probe biological processes and the synthesis of a wide array of fluorescent chemical probes with particularly attractive photophysical properties, such as environmental sensitivity and metal-ion selectivity, have lead to the design of a different class of fluorescent biosensors, based on peptide or protein scaffolds, onto which fluorescent probes may be coupled [3,4,15–17]. Environmentally-sensitive probes respond to changes in the polarity of their environment, exhibiting poor fluorescence in a fairly polar, aqueous solution, high fluorescence in apolar solvents, or when bound to a hydrophobic protein pocket or membrane [15]. Peptide and protein biosensors are easily generated through synthetic chemistry or recombinant protein technology followed by chemical or enzymatic labeling with synthetic fluorophores. Since these biosensors do not rely on genetically-encoded autofluorescent proteins, they may be readily employed to monitor target activity, and constitute attractive alternatives in that they offer a high degree of control and versatility. Peptide and protein biosensors further allow for chemical modifications to improve signal-over-noise and sensitivity of response, through the introduction of chemical quenchers and photoactivatable groups. Although these biosensors are readily applicable in vitro, they require appropriate technologies to facilitate their intracellular delivery for application in living cells.

3. Fluorescent biosensors for probing protein kinases

Protein kinases are involved in a wide variety of signaling pathways and regulatory processes. Moreover, these enzymes are most often

deregulated, hyperactivated or overexpressed, as a result of genetic alterations in human pathologies, including cardiovascular diseases, neurodegenerative and endocrinological disorders, immune deficiency and viral infection, psoriasis, cancer and diabetes, thereby contributing to establish the pathological disorder. As such they constitute attractive targets for the development of therapeutics, as well as relevant biomarkers of disease, and several small molecule inhibitors have been developed to interfere with their function [18–25].

Standard approaches to monitor protein kinase activity classically rely on radioactive or antigenic assays. However, these techniques can merely be applied in vitro in endpoint assays, and do therefore not convey any information concerning the kinetic parameters of kinase activity. Studying the behavior of endogenous protein kinases in their natural environment remains very challenging. Indeed, measuring a specific kinase activity amongst the myriad of enzymatic activities that coordinate biological processes in a cell is like trying to find a needle in a haystack, and requires probes with a high degree of sensitivity and selectivity. The development of fluorescent biosensors that report on protein kinase activity has provided a means of studying the protein kinase function in real time and of imaging the behavior of these enzymes in living cells, with high spatial and temporal resolution. Both genetically-encoded and polypeptide-based biosensors have been developed to report on protein kinase activity. Single-chain FRET biosensors have been successfully applied to study protein kinases in living cells with high spatial and temporal resolution. Likewise, a wide variety of nongenetic biosensors have been applied to monitor protein kinase activities in vitro and in more complex biological samples with an equally successful outcome [26–33] (Fig. 3).

3.1. Genetically-encoded fluorescent kinase biosensors

Genetically-encoded biosensors designed to probe protein kinase activities are single-chain FRET biosensors, commonly known as kinase activity reporters (KARs). The basic structure of KARs consists of a kinase-specific substrate sequence including a consensus phosphorylation site and a matching phosphoamino acid binding domain (PAABD), separated by a flexible linker, and flanked by a FRET pair of AFPs (Fig. 3A). KARs provide a direct readout of kinase activity through phosphorylation-induced changes in FRET between two AFPs, associated with an intramolecular conformational change upon binding of the PAABD to the phosphorylated substrate sequence [for review 30,32–34]. In some instances, a docking domain distinct from the substrate sequence is included in the KAR, so as to increase both specificity for the target kinase, as well as phosphorylation efficiency [35,36]. In other cases, KARs include a targeting sequence to promote enrichment in a specific subcellular compartment (cytoplasm, nucleus, Golgi, the endoplasmic reticulum, mitochondria, or plasma membrane) revealing differences in kinase activities and dynamics, as well as differences attributable to differential localization of discrete subpopulations, or specific kinase isoforms [37–40].

The first genetically-encoded fluorescent reporter of kinase activity was engineered by A. Ting and coll. and successfully employed to probe activity of phospho-tyrosine kinases Src, Abl and EGFR [41]. This biosensor is based on a phosphotyrosine kinase-specific substrate sequence, phosphorylation of which promotes binding to an intramolecular SH2 domain, thereby bringing together a CFP and a YFP. A wide variety of FRET biosensors have been designed according to the same principle. Of particular interest, the Picchu biosensor was constructed based on an SH2–SH3 domain derived from the CrkII adaptor protein, to sense specific phosphorylation by c-Abl [42]. This biosensor was further optimized to generate Pickles, which reports on tyrosine kinase activity of Bcr-Abl in a sensitive fashion [43], and has more recently been applied to monitor Bcr-Abl activity in cells from patients that have CML, to evaluate response to therapy and to identify drug-resistant cells within a heterogeneous population [44,45].

A detailed overview of genetically-encoded biosensors which have been developed so far to probe the activities of protein kinases is provided elsewhere [32,33].

3.2. Fluorescent peptide/protein biosensors

Several strategies have been devised to generate environmentally-sensitive peptide and polypeptide biosensors of protein kinase activity.

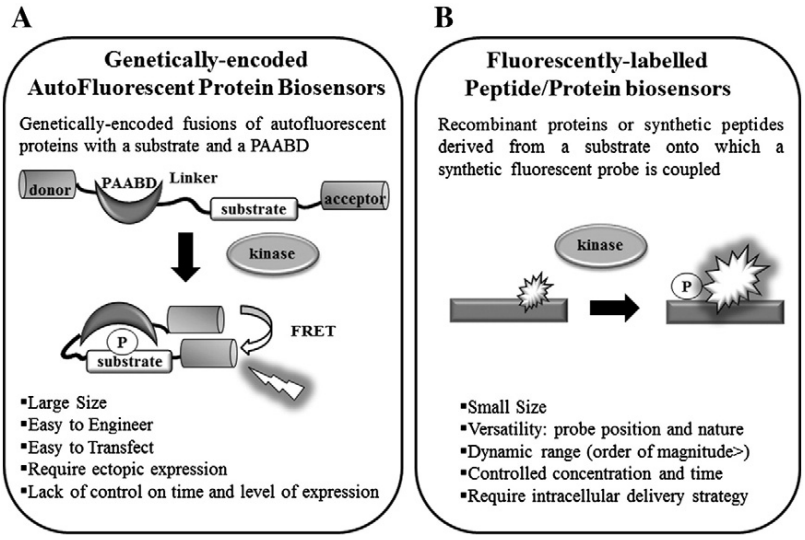

Fig. 3. Fluorescent protein kinase biosensors. Fluorescent protein kinase biosensors consist of substrate scaffolds that respond to phosphorylation by a protein kinase activity by emitting a corresponding fluorescent signal. A) Genetically-encoded kinase biosensors also known as kinase activity reporters (KARs) generally consist of single chain FRET biosensors. These are plasmid constructs that encode a pair of AFPs together with an intervening kinase substrate sequence and a PAABD, which are ectopically expressed as a single molecule in living cells. The AFPs are brought together upon phosphorylation of the substrate sequence, which triggers a conformational change by promoting the binding of the neighboring PAABD, consequently leading to fluorescence resonance energy transfer between the donor and the acceptor. B) Environmentally-sensitive peptide-based kinase biosensors are protein domains or peptides that are site-specifically labeled with a synthetic fluorophore whose spectral properties are directly or indirectly affected by proximal phosphorylation. In their simplest form, the fluorescent dye is coupled directly at or proximal to the phosphorylation site, and its fluorescence is enhanced by phosphorylation itself.

In their simplest form, environmentally-sensitive kinase biosensors are derived from kinase substrate sequences that bear a small synthetic fluorescent dye on the phosphorylation site itself, or proximal to it, that responds to phosphorylation through changes in its spectral properties (intensity or wavelength shift) (Fig. 3B) [for review 27–29, 31, 32].

One of the first set of fluorescent peptide probes based on this principle, the propinquity effect, was developed by D.S. Lawrence and coll. to probe PKC and monitor its activity in cell lysates and spatiotemporal dynamics in living cells [46]. This and other studies reveal that the nature and the position of the fluorescent dye coupled onto the peptide backbone have a major effect on the dynamic range of the response. This probe was further optimized to generate a light-activatable variant thereby offering a precise means of controlling biosensor availability in living cells through photoactivation [47].

Self-reporting tyrosine kinase biosensors are based on the principle that tyrosine quenches the fluorescence of organic dyes coupled at a proximal position, until phosphorylation disrupts the stacking interaction between the dye and the tyrosine group. Proof-of-concepts of this principle have been established with self-reporting biosensors of Src, Lyn and Abl kinases [48–50]. A similar strategy termed "Deep Quench" was developed for Ser/Thr kinases, and successfully applied to probe PKA activity. This approach involves shielding of the fluorophore by a quencher in solution, which is displaced upon phosphorylation and binding of a PAABD to the phosphorylated serine or threonine [51,52].

Chelation-enhanced dyes constitute a particular subset of environmentally-sensitive dyes, that have the ability to coordinate metal ions ($Ca2+$, $Mg2+$, $Zn2+$ or lanthanides) in response to proximal phosphorylation of a peptide substrate, which consequently affects the electronic structure and spectral properties of the dye. Chelation-enhanced fluorescence (CHEF) has been best described by B. Imperiali and coll. for biosensors based on the Sox dye (sulfonamide-oxine), a derivative of 8-hydroxyquinoline, that chelates $Mg2+$ and undergoes fluorescent enhancement upon the coordination of a phosphate group on a phosphopeptide [53,54]. Sox-based biosensors have been successfully developed to probe PKC, PKA and Abl kinases and applied to probe a variety of different kinases in multiplex fluorescence-based assays in cell lysates [55,56]. Improved generations of these biosensors have been developed to maximize the specificity of kinase recognition through extension of binding sequences or incorporation of docking domains distal from the phosphorylation site [57–59].

A more complex class of environmentally-sensitive kinase biosensors undergo changes in fluorescence in response to the binding of the phosphorylated peptide sequence to a PAABD, or through phosphorylation-induced conformational changes that promote binding of an intramolecular PAABD, such as 14-3-3, SH2 or WW domains [27–29,60]. Yet another class of biosensors, exemplied by the Src merobody biosensor undergo fluorescence enhancement upon recognition and binding of the active conformation of their target kinase, at a site which does not interfere with its ability to catalyze phosphorylation [61].

A detailed overview of environmentally-sensitive biosensors developed to probe protein kinase activities is provided elsewhere [31,32].

4. New tools for old targets: CDK/cyclin biosensors

Cancer is a deadly disease which currently suffers from an overall lack of efficient targeted therapies, despite progress made in this field. But perhaps more importantly, from a lack of early and personalized diagnostics. After more than a decade since the first insights into the molecular basis of cancer were provided by Hanahan & Weinberg [62], we have come a long way in understanding the pathogenesis of this disease and in identifying the features which are inherent characteristics of cancer cell proliferation. These hallmarks constitute attractive targets for the development of therapeutic and strategies — amongst these, mechanisms that drive cell cycle progression and cell proliferation. Indeed, aside from mutations that promote upregulation of oncogenes or downregulation of tumor suppressor genes, alterations that lead to amplification, overexpression and/or hyperactivation of enzymes involved in regulation cell cycle progression allow cells to bypass the controls that restrict cell proliferation, thereby conferring self-sufficiency with respect to growth signals together with an overall insensitivity to anti-growth signals.

Cyclin-dependant kinases (CDK/cyclins) are heterodimeric protein kinases that play a central role in coordinating cell growth and division and are considered as molecular engines that drive cell cycle progression [63,64]. In mammalian cells there are ten different CDKs and about the same number of cyclins, that assemble in a well-defined fashion, to perform specific functions in timely and coordinated fashion in physiological conditions [25,65,66]. CDK/cyclin levels and activities are frequently altered in human cancers, and contribute to sustain aberrant proliferation in cancer cells. As such, they constitute attractive pharmacological targets for the development of anti-cancer, antiviral and anti-parasitic drugs [67–70]. Several anticancer compounds isolated from natural substances target CDK/cyclin kinases by competing with the ATP-binding pocket, and inhibitors derived from these compounds have been designed through rational derivatization [24,69–72]. However, despite the oncological relevance and pharmacological attractivity of CDK/cyclins, there are currently very few means of probing these kinases in their natural environment, aside from a genetically-encoded FRET biosensor of CDK1/cyclin B [73]. Indeed, as for most other intracellular kinases, detection of CDKs and cyclins remains essentially limited to antigenic approaches which require cell or tissue extraction and fixation, whilst the determination of their activity remains limited to endpoint assays based on radioactivity or on antigenic recognition of phosphorylated substrates. This not only restricts our full understanding of the dynamic function of CDK/cyclins in a physiological context, it also limits the development of preclinical and clinical studies, since little information can be obtained with respect to specificity, selectivity, efficiency and kinetics of inhibitors targeting these kinases in cellulo and in vivo. Moreover, the lack of tools to probe the structure/function relationship and dynamics of CDK/cyclin behavior leaves little chance for the identification of compounds targeting other steps in their mechanism of activation.

In order to study CDK/cyclins in their natural environment and gain insight into their behavior in physiological and pathological conditions, we developed three families of fluorescent peptide/polypeptide biosensors to probe the relative abundance, activity and conformational dynamics of CDK/cyclins, respectively [74–76].

CDKSENS biosensors are biligand peptides that recognize both the CDK and the cyclin subunit partners, thereby reporting on the presence of heterodimeric CDK/cyclin complexes [74]. Indeed, the presence of both a CDK-binding sequence and a cyclin-binding sequence within the biosensor, allows it to dock onto the CDK/cyclin complex with high affinity and specificity, thereby prompting fluorescent enhancement of an environmentally-sensitive probe coupled central to these moieties (Fig. 4A). CDKSENS biosensors report on the relative abundance of CDK/cyclin complexes in vitro and in living cells, thereby providing information which is not conveyed by antibodies which recognize individual CDKs or cyclins. As such, these biosensors allow to monitor alterations in CDK/cyclin levels when tampering with a single CDK or cyclin upon treatment with siRNA or with a drug, and between different healthy and cancer cell lines (Fig. 4B).

CDKACT biosensors are activity-based probes developed to monitor CDK/cyclin activity in real-time. They are modular biosensors constituted of a peptide substrate, onto which a fluorescent probe is coupled proximal to the phosphorylation site, and a phosphoamino acid binding domain that recognizes the phosphorylated peptide sequence, separated by a short linker (Fig. 4C). Upon phosphorylation by active CDK/

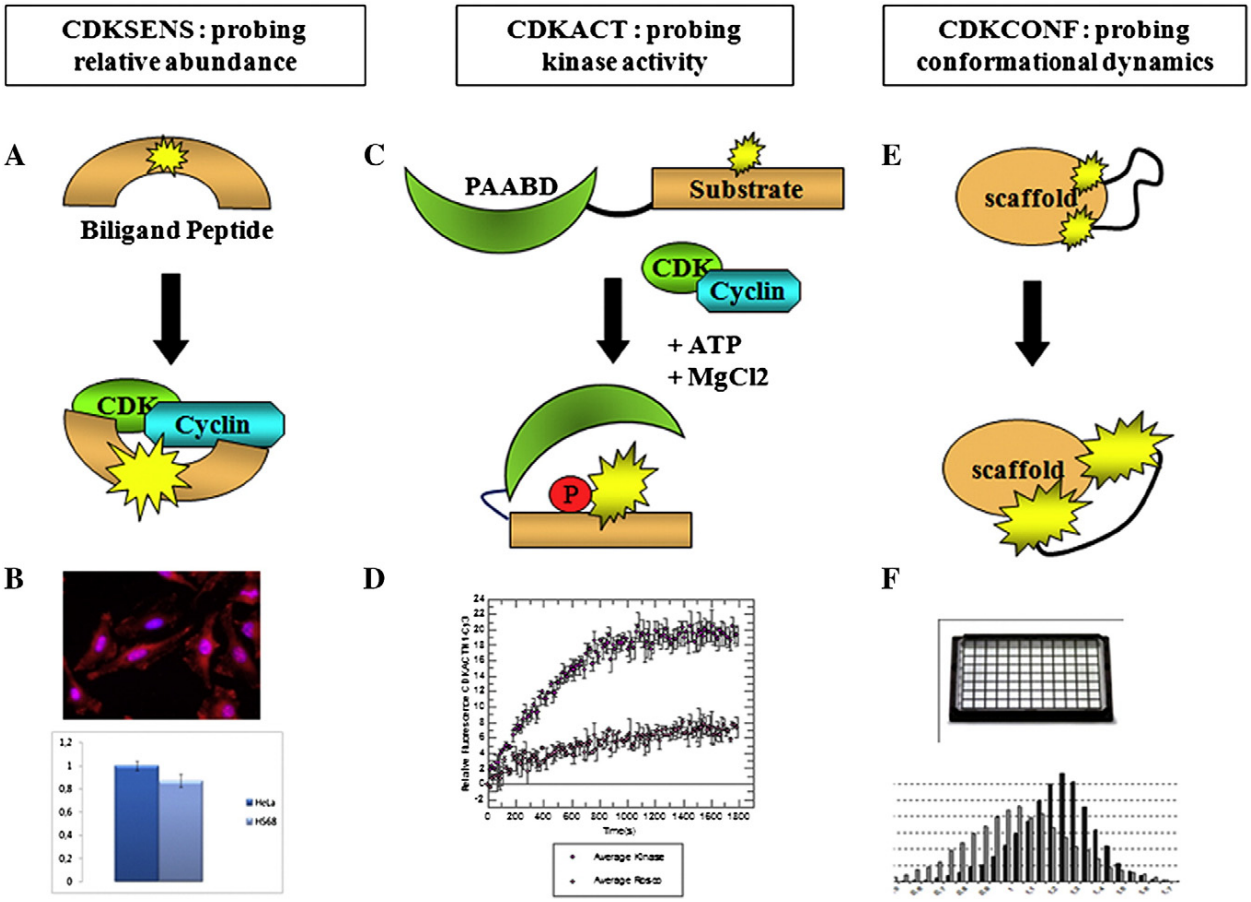

Fig. 4. CDKSENS, CDKACT and CDKCONF biosensors. CDK/cyclin biosensors developed to monitor these heterodimeric kinases. A) CDKSENS biosensor design B) CDKSENS reports on the relative abundance of CDK/cyclin complexes C) CDKACT biosensor design D) CDKACT reports on the kinase activity of CDK/cyclins E) CDKCONF biosensor scaffold F) CDKCONF reports on conformational changes associated with activation of the CDK, allowing for the screening of allosteric CDK inhibitors in high throughput formats.

cyclin complexes, these biosensors undergo changes in fluorescence intensity of an environmentally-sensitive probe, thereby reporting on kinase activity in real-time, in a fully reversible fashion. CDKACT biosensors allow us to monitor differences in CDK/cyclin activities between different cell types, throughout the cell cycle and in response to drugs (Fig. 4D) [75].

CDKCONF biosensors are protein-based biosensors that probe the conformational dynamics of the CDK associated with its activation. This sensor bears probes that serve as molecular hinges in sensing conformational changes of the activation loop of this kinase, for example associated with partner, substrate or inhibitor binding (Fig. 4E). As such CDKCONF can report on compounds that induce conformational changes, modulators that indirectly affect its catalytic activity, thereby constituting a potent tool for screening for allosteric inhibitors that target CDK/cyclin complexes through a mechanism of action which differs from competition with ATP binding in high throughput screening formats (Fig. 4F) [76].

Overall, these fluorescent biosensors constitute potent technologies for fundamental and mechanistic studies in biochemistry and cell biology. Moreover, they offer promising perspectives for the development of fluorescence-based cancer diagnostics, for monitoring progression of disease and response to therapeutics, and present strong potential for drug discovery programs. Indeed, CDKSENS, CDKACT and CDKCONF constitute sensitive tools for reporting on CDK/cyclin levels, activity and conformation in vitro and in cell extracts, and can further be introduced into living cells thanks to cell-penetrating peptides [77–79], thereby allowing to monitor CDK/cyclins in their natural environment through live-cell fluorescence imaging. These biosensors allow us to identify differences in CDK/cyclin complexes between different healthy and cancer cell lines, and for example to distinguish between cells that express high levels or activities of CDK/cyclin kinases, from cells that present decreased or defective assemblies. Furthermore, they can be applied to monitor the status of CDK/cyclins in tumor xenografts ex vivo, using biopsies or in blood samples by FACS, and can further be applied to probe CDK/cyclin levels and activities in vivo, and to monitor response to drugs targeting these kinases in animal tumor models. Additionally, these biosensors are directly applicable to high throughput, high content screening, which makes them most attractive for drug discovery programs.

5. Fluorescent biosensors in biomedical applications

Whilst fluorescent biosensors are commonly employed in fundamental studies, they constitute equally potent imaging probes in biomedicine, for monitoring health and disease. They constitute useful and potent tools for the early detection of biomarkers in molecular and clinical diagnostics, for monitoring disease progression and response to treatment/therapeutics, for intravital imaging and image-guided surgery [6,80–82] (Fig. 5).

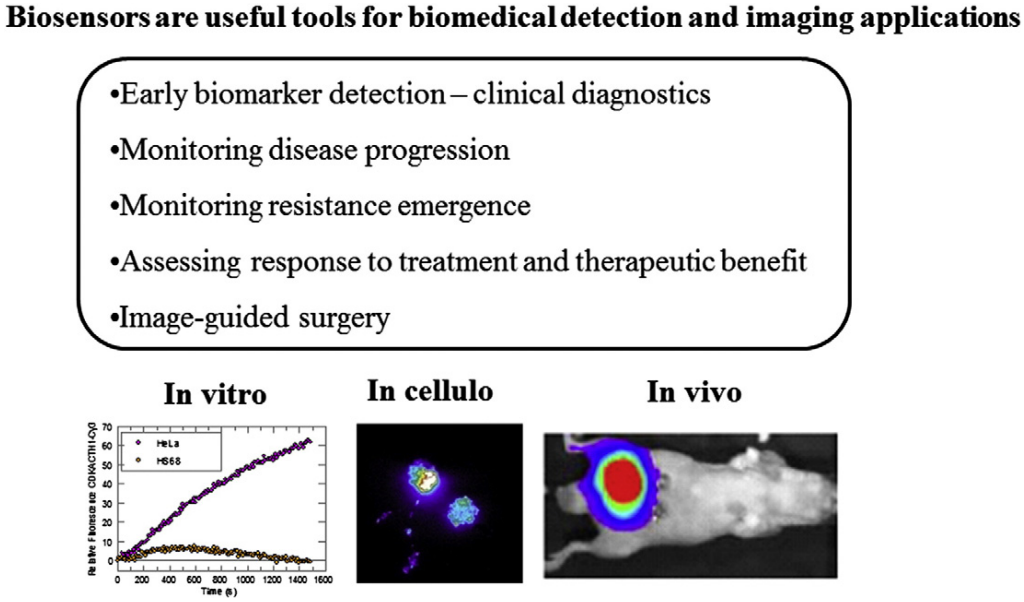

Fig. 5. Fluorescent Biosensor Applications in Biomedicine. Fluorescent biosensors are useful tools for the detection of disease biomarkers, monitoring disease progression, response to therapeutics and the emergence of resistance through molecular imaging, endoscopy, intravital or tomographic optical imaging.

Fluorescent probes allow us to visualize targets which cannot be detected by the human eye and which would normally require extraction of a biopsy or histological studies for ex vivo detection of biomarkers by classical antigenic approaches. In particular, fluorescent biosensors allow us to detect biomarkers in cancer models, thereby providing means of imaging the diseased tissue or tumor, disease progression and response to therapeutics. As such they offer a wealth of opportunities for development of personalized medicine [80].

Protein kinases are central to a wide variety of signaling pathways involved in physiological processes, and their implication in disease has been widely documented. As such they constitute established pharmacological targets and attractive biomarkers for monitoring appearance and progression of disease, as well as response to therapeutics [18–25]. Visualizing and quantifying protein kinase activities allow first to detect dysregulations in a pathological setting, second to justify the administration of a given set of inhibitors to a patient and assess their efficacy over time. From a biomedical perspective, fluorescent biosensors therefore constitute potent tools for the development of clinical diagnostics associated with aberrant protein kinase activation. Besides providing the means of imaging specific disease biomarkers and monitoring their status during disease progression, fluorescent biosensors allow to assess the benefits of therapy or resistance to drugs, and to devise alternatives to target disease-related deregulations if necessary.

Several fluorescent biosensors applied to the detection of protein kinases in the context of cancer constitute well documented and particularly relevant examples. A genetically-encoded FRET biosensor developed for the detection of Bcr-Abl kinase activity was applied to assess Bcr-Abl activity from cancer patient cells and further employed to establish a correlation with the disease status in chronic myeloid leukemia (CML). This probe was further employed to monitor response to therapy, and to detect the onset of drug-resistant cells, thereby allowing to predict the necessity for alternative therapeutics [43–45]. Likewise, a set of fluorescent peptide biosensors were developed to monitor Bcr-Abl and Lyn tyrosine kinase activities, and employed to probe these kinases simultaneously through multicolor imaging in imatinib-sensitive or -resistant CML cell lines [50].

Multiplex detection of different biomarkers through fluorescence biosensor technology would provide a more complete reflection of a disease state or stage. Multisensing approaches are particularly relevant for personalized medicine, since they offer a means of assessing the status or behavior of distinct targets, of a given set of biomarkers. In addition, multiplex detection strategies are particularly relevant for theragnostic approaches as they would allow to couple tailored therapeutic intervention through the selection of an appropriate cocktail of inhibitors to neutralize a pathological condition.

6. Fluorescent biosensors in drug discovery

Fluorescent biosensors are widely used in drug discovery programs for the identification of drugs by high throughput, high content screening approaches, for postscreening evaluation of hits and optimization of leads. Moreover fluorescent biosensors constitute very potent tools for preclinical evaluation and clinical validation of the therapeutic potential, biodistribution and pharmacokinetics of candidate drugs (Fig. 6) [83–87].

Protein kinases constitute one of the major classes of therapeutic targets for drug discovery programs. To date, most strategies employed in drug discovery for screening novel inhibitors of protein kinases rely on activity-based assays, which introduce a strong bias towards the identification of nucleotide-binding pocket inhibitors. Indeed, to date most kinase inhibitors identified in high throughput screens are essentially ATP analogs or substrate competitors which directly affect the catalytic step. However few ATP-competing, nucleotide-based compounds actually inhibit their targets in a selective fashion, explaining their fairly poor success rate in clinical trials. Consequently important efforts are being made to develop original assays that allow for the identification of new and potent hits from high throughput and high content screens. In this respect, and given their high sensitivity and selectivity, fluorescent biosensors are particularly well suited to screen libraries of small molecule compounds in search of inhibitors that affect kinase activity or function in high throughput formats in vitro or in cell-based assays [88]. The gold standard for high throughput formats is an assay which can be easily miniaturized without losing signal intensity or signal-to-noise ratio, and which does not suffer from off-target effects. Moreover, fluorescent peptide/protein biosensors can be designed to screen for

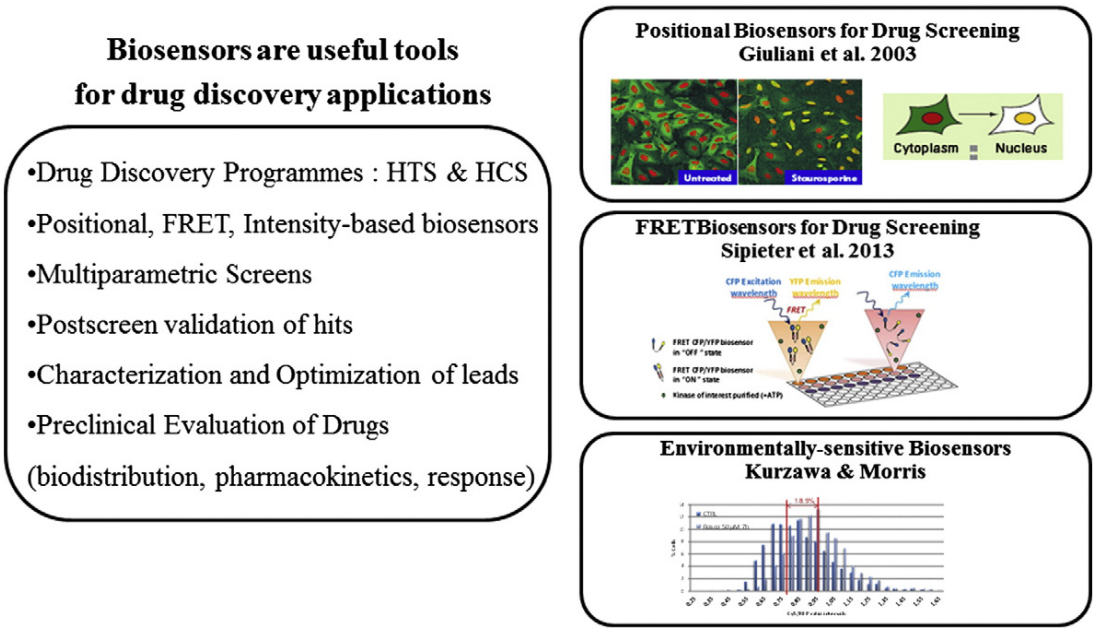

Fig. 6. Biosensors in drug discovery. Fluorescent biosensors are widely used in drug discovery programs for the identification of drugs by HTS, HCS approaches, for postscreening evaluation of hits, optimization of leads, preclinical evaluation and clinical validation of candidate drugs.

compounds which target different mechanistic steps of kinase activation, including allosteric inhibitors or compounds that modulate expression, interaction with regulatory activities or subcellular localization thanks to the design of smart strategies, more generally, in search of novel inhibitors that do not compete with ATP-binding.

Three strategies have been developed for drug discovery approaches. Genetically-encoded FRET-based approaches — several high throughput screening assays have been developed for screening small molecule compounds from complex libraries, based on genetically-encoded FRET biosensors that report on kinase activity [88]. Likewise, non-genetic, environmentally-sensitive peptide biosensors are perfectly well suited to HTS assays. Sox biosensors have been readily applied to probe kinase activities in HTS formats using cell lysates [56]. Positional biosensors undergo changes in subcellular localization in response to the activity or inhibition of target enzymes, thereby providing a very easy readout of kinase activity [83].

Fluorescent biosensors allow to characterize kinase inhibitors in a qualitative and quantitative fashion during the postscreening characterization process, thereby providing critical information concerning the efficacy, pharmacokinetics and pharmacodynamics of hit compounds, and further enabling comparative studies during the optimization process. Finally, fluorescent biosensors are appropriate for preclinical evaluation trials, to monitor response to candidate drugs, disease progression and emergence of resistance over time.

7. Concluding remarks — perspectives and challenges

Fluorescent biosensors undeniably constitute a potent and sensitive class of tools for the detection of biomolecules in vitro, in cellulo and in vivo, for monitoring dynamic molecular events, and imaging biological processes with high precision in time and in space. The versatility and potential of these tools will certainly prompt their application in a wide variety of fields, including the early-stage diagnostics, multiplexed detection of biomarkers and biomedical applications based on molecular imaging, including image-guided surgery, theragnostics and preclinical evaluation of drugs in drug discovery programs. Notwithstanding, such developments require overcoming major challenges with respect to technological bottlenecks, in particular sensitivity, signal-to-noise ratio and reliability, as well as targeted delivery and selective activation of biosensors. Optimization of these criteria is a sine qua none for the development of biomedical applications, as well as for a more widespread application of biosensor technology to drug discovery programs. In this respect, developments in the chemistry of fluorescent probes, in quenching and photoactivation strategies can be expected to improve the sensitivity and signal-to-noise ratio. Furthermore, the use of near-infrared fluorescent probes which are compatible with in vivo applications will pave the way for studying biomolecular activities in vivo. Finally, the development of cell- or disease-specific targeting or activation strategies should provide means of imaging biomarker alterations in a more localized fashion, with greater precision and selectivity.

Acknowledgements

Research in M.C. Morris group is supported by the CNRS (Centre National de la Recherche Scientifique) and grants from the Association de Recherche contre le Cancer (ARC), the Region Languedoc-Roussillon (Subvention “Chercheuse d'Avenir”) and the Institut National du Cancer (INCA) to MCM.

References

[1] R. Weissleder, M.J. Pittet, Imaging in the era of molecular oncology, Nature 452 (2008) 580–589.
[2] E.A. Lemke, C. Schultz, Principles for designing fluorescent sensors and reporters, Nat. Chem. Biol. 7 (2011) 480–483.
[3] H. Wang, E. Nakata, I. Hamachi, Recent progress in strategies for the creation of protein-based fluorescent biosensors, ChemBioChem 10 (2009) 2560–2577.
[4] M.C. Morris, Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes, Cell Biochem. Biophys. 56 (2010) 19–37.
[5] M.C. Morris, Fluorescence-based Biosensors — From Concepts to Applications, Prog. Mol. Biol. Transl. Sci.Elsevier Press, 2013.
[6] M.C. Morris, Fluorescent biosensors for cancer cell imaging and diagnostics, in: V. Preedy, J. Hunter (Eds.), Biosensors and Cancer, CRC Press, ISBN: 978-1-57808-734-1, 2012, pp. 101–124.
[7] J. Zhang, R.E. Campbell, A.Y. Ting, R.Y. Tsien, Creating new fluorescent probes for cell biology, Nat. Rev. Mol. Cell Biol. 3 (2002) 906–918.

[8] J. Lippincott-Schwartz, G.H. Patterson, Development and use of fluorescent protein markers in living cells, Science 300 (2003) 87–91.
[9] N.C. Shaner, P.A. Steinbach, R.Y. Tsien, A guide to choosing fluorescent proteins, Nat. Methods 2 (2005) 905–909.
[10] R.Y. Tsien, Breeding and building molecules to spy on cells and tumors, FEBS Lett. 579 (2005) 927–932.
[11] B.N. Giepmans, S.R. Adams, M.H. Ellisman, R.Y. Tsien, The fluorescent toolbox for assessing protein location and function, Science 312 (2006) 217–224.
[12] A. Ibraheem, R.E. Campbell, Designs and applications of fluorescent protein-based biosensors, Curr. Opin. Chem. Biol. 14 (2010) 30–36.
[13] B. Wu, K.D. Piatkevich, T. Lionnet, R.H. Singer, V.V. Verkhusha, Modern fluorescent proteins and imaging technologies to study gene expression, nuclear localization, and dynamics, Curr. Opin. Cell Biol. 23 (2011) 310–317.
[14] N.-N. Aye-Han, N. Qiang, J. Zhang, Fluorescent biosensors for real-time tracking of post-translational modification dynamics, Curr. Opin. Chem. Biol. 13 (2009) 392–397.
[15] G.S. Loving, M. Sainlos, B. Imperiali, Monitoring protein interactions and dynamics with solvatochromic fluorophores, Trends Biotechnol. 28 (2010) 73–83.
[16] L.D. Lavis, R.T. Raines, Bright ideas for chemical biology, ACS Chem. Biol. 3 (2008) 142–155.
[17] E. Pazos, O. Vázquez, J.L. Mascareñas, M.E. Vázquez, Peptide-based fluorescent biosensors, Chem. Soc. Rev. 38 (2009) 3348.
[18] J. Brognard, T. Hunter, Protein kinase signaling networks in cancer, Curr. Opin. Genet. Dev. 21 (2011) 4–11.
[19] R. Kumar, V.P. Singh, K.M. Baker, Kinase inhibitors for cardiovascular disease, J. Mol. Cell. Cardiol. 42 (2007) 1–11.
[20] K. Blease, Targeting kinases in asthma, Expert Opin. Investig. Drugs 14 (2005) 1213–1220.
[21] H. Ben-Bassat, Biological activity of tyrosine kinase inhibitors: novel agents for psoriasis therapy, Curr. Opin. Investig. Drugs 2 (2001) 1539–1545.
[22] P. Cohen, Protein kinases — the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. 1 (2002) 309–315.
[23] L. Johnson, Protein kinases and their therapeutic exploitation, Biochem. Soc. Trans. 35 (2007) 7–11.
[24] S. Lapenna, A. Giordano, Cell cycle kinases as therapeutic targets for cancer, Nat. Rev. Drug Discov. 8 (2009) 547–566.
[25] M. Malumbres, Physiological relevance of cell cycle kinases, Physiol. Rev. 91 (2011) 973–1007.
[26] M.K. Tarrant, A.P. Cole, The chemical biology of protein phosphorylation, Annu. Rev. Biochem. 78 (2009) 797–825.
[27] C.A. Chen, R.H. Yeh, X. Yan, D.S. Lawrence, Biosensors of protein kinase action: from in vitro assays to living cells, Biochim. Biophys. Acta 1697 (2004) 39–51.
[28] D.S. Lawrence, Q. Wang, Seeing is believing: peptide-based fluorescent sensors of protein tyrosine kinase activity, ChemBioChem 8 (2007) 373–378.
[29] V. Sharma, Q. Wang, D.S. Lawrence, Peptide-based fluorescent sensors of protein kinase activity: design and applications, Biochim. Biophys. Acta 1784 (2008) 94–99.
[30] J. Zhang, M.D. Allen, FRET-based biosensors for protein kinases: illuminating the kinome, Mol. Biosyst. 3 (2007) 759–765.
[31] J.A. Gonzalez-Vera, Probing the kinome in real time with fluorescent peptides, Chem. Soc. Rev. 41 (2012) 1652–1664.
[32] T.N.N. Van, M.C. Morris, Fluorescent sensors of protein kinases: from basics to biomedical applications, Prog. Mol. Biol. Transl. Sci. 113 (2013) 217–274.
[33] F. Sipieter, P. vandame, C. Spriet, A. Leray, P. Vincent, D. Trinel, J.F. Bodart, F.B. Riquet, L. Heliot, From FRET imaging to practical methodology for kinase activity sensing in living cells, Prog. Mol. Biol. Transl. Sci. 113 (2013) 145–216.
[34] F. Gaits, K. Hahn, Shedding light on cell signaling: interpretation of FRET biosensors, Sci STKE (2003) PE3.
[35] P.M. Holland, J.A. Cooper, Protein modification: docking sites for kinases, Curr. Biol. 9 (1999) R329–R331.
[36] N. Fernandes, D.E. Bailey, D.L. Vanvranken, N.L. Allbritton, Use of docking peptides to design modular substrates with high efficiency for mitogen-activated protein kinase extracellular signal-regulated kinase, ACS Chem. Biol. 2 (2007) 665–673.
[37] J.D. Violin, J. Zhang, R.Y. Tsien, A.C. Newton, A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C, J. Cell Biol. 161 (2003) 899–909.
[38] K. Sasaki, M. Sato, Y. Umezawa, Fluorescent indicators for Akt/protein kinase B and dynamics of Akt activity visualized in living cells, J. Biol. Chem. 278 (2003) 30945–30951.
[39] M.T. Kunkel, Q. Ni, R.Y. Tsien, J. Zhang, A.C. Newton, Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter, J. Biol. Chem. 280 (2005) 5581–5587.
[40] M. Sato, Y. Kawai, Y. Umezawa, Genetically encoded fluorescent indicators to visualize protein phosphorylation by extracellular signal-regulated kinase in single living cells, Anal. Chem. 79 (2007) 2570–2575.
[41] A.Y. Ting, K.H. Kain, R.L. Klemke, R.Y. Tsien, Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 15003–15008.
[42] K. Kurokawa, N. Mochizuki, Y. Ohba, H. Mizuno, A. Miyawakiand, M. Matsuda, A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo, J. Biol. Chem. 276 (2001) 31305–31310.
[43] T. Mizutani, T. Kondo, S. Darmanin, M. Tsuda, S. Tanaka, M. Tobiume, M. Asaka, Y. Ohba, A novel FRET-based biosensor for the measurement of BCR-ABL activity and its response to drugs in living cells, Clin. Cancer Res. 16 (2010) 3964–3975.
[44] S. Lu, Y. Wang, Fluorescence resonance energy transfer biosensors for cancer detection and evaluation of drug efficacy, Clin. Cancer Res. 16 (2010) 3822–3824.
[45] A. Tunceroglu, M. Matsuda, R.B. Birge, Real-time fluorescent resonance energy transfer analysis to monitor drug resistance in chronic myelogenous leukemia, Mol. Cancer Ther. 9 (2010) 3065–3073.
[46] R.H. Yeh, X. Yan, M. Cammer, A.R. Bresnick, D.S. Lawrence, Real time visualization of protein kinase activity in living cells, J. Biol. Chem. 277 (2002) 11527–11532.
[47] W.F. Veldhuyzen, Q. Nguyen, G. McMaster, D.S. Lawrence, A light-activated probe of intracellular protein kinase activity, J. Am. Chem. Soc. 125 (2003) 13358–13359.
[48] Q. Wang, S.M. Cahill, M. Blumenstein, D.S. Lawrence, Self-reporting fluorescent substrates of protein tyrosine kinases, J. Am. Chem. Soc. 128 (2006) 1808–1809.
[49] Q. Wang, Z. Dai, S.M. Cahill, M. Blumenstein, D.S. Lawrence, Light-regulated sampling of protein tyrosine kinase activity, J. Am. Chem. Soc. 128 (2006) 14016–14017.
[50] Q. Wang, E.I. Zimmerman, A. Toutchkine, T.D. Martin, L.M. Graves, D.S. Lawrence, Multicolor monitoring of dysregulated protein kinases in chronic myelogenous leukemia, ACS Chem. Biol. 5 (2010) 887–895.
[51] V. Sharma, R.S. Agnes, D.S. Lawrence, Deep quench: an expanded dynamic range for protein kinase sensors, J. Am. Chem. Soc. 129 (2007) 2742–2743.
[52] R.S. Agnes, F. Jernigan, J.R. Shell, V. Sharma, D.S. Lawrence, Suborganelle sensing of mitochondrial cAMP-dependent protein kinase activity, J. Am. Chem. Soc. 132 (2010) 6075–6080.
[53] M.D. Shults, D.A. Pearce, B. Imperiali, Modular and tunable chemosensor scaffold for divalent zinc, J. Am. Chem. Soc. 125 (2003) 10591–10597.
[54] M.D. Shults, B. Imperiali, Versatile fluorescence probes of protein kinase activity, J. Am. Chem. Soc. 125 (2003) 14248–14249.
[55] M.D. Shults, D. Carrico-Moniz, B. Imperiali, Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases, Anal. Biochem. 352 (2006) 198–207.
[56] M.D. Shults, K.A. Janes, D.A. Lauffenburger, B. Imperiali, A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates, Nat. Methods 2 (2005) 277–283.
[57] E. Luković, J.A. González-Vera, B. Imperiali, Recognition-domain focused chemosensors: versatile and efficient reporters of protein kinase activity, J. Am. Chem. Soc. 130 (2008) 12821–12827.
[58] E. Lukovic, E. Vogel Taylor, B. Imperiali, Monitoring protein kinases in cellular media with highly selective chimeric reporters, Angew. Chem. Int. Ed. Engl. 48 (2009) 6828–6831.
[59] C.I. Stains, E. Lukovic, B. Imperiali, A p38α-selective chemosensor for use in unfractionated cell lysates, ACS Chem. Biol. 6 (2011) 101–105.
[60] Q. Wang, D.S. Lawrence, Phosphorylation-driven protein–protein interactions: a protein kinase sensing system, J. Am. Chem. Soc. 127 (2005) 7684–7685.
[61] A. Gulyani, E. Vitriol, R. Allen, J. Wu, D. Gremyachinskiy, S. Lewis, B. Dewar, L.M. Graves, B.K. Kay, B. Kuhlman, T. Elston, K.M. Hahn, A biosensor generated via high-throughput screening quantifies cell edge Src dynamics, Nat. Chem. Biol. 7 (2011) 437–444.
[62] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57–70.
[63] D.O. Morgan, Cyclin-dependent kinases: engines, clocks, and microprocessors, Annu. Rev. Cell Dev. Biol. 13 (1997) 261–291.
[64] M. Malumbres, M. Barbacid, Mammalian cyclin-dependent kinases, Trends Biochem. Sci. 30 (2005) 630–641.
[65] A.J. Obaya, J.M. Sedivy, Regulation of cyclin-Cdk activity in mammalian cells, Cell. Mol. Life Sci. 59 (2002) 126–142.
[66] A. Satyanarayana, P. Kaldis, Regulation of cyclin-Cdk activity in mammalian cells, Oncogene 28 (2009) 2925–2939.
[67] M. Malumbres, M. Barbacid, Cell cycle kinases in cancer, Curr. Opin. Genet. Dev. 17 (2007) 60–65.
[68] M. Malumbres, M. Barbacid, Cell cycle, CDKs and cancer: a changing paradigm, Nat. Rev. Cancer 9 (2009) 153–166.
[69] M. Noble, P. Barrett, J. Endicott, L. Johnson, J. McDonnell, G. Robertson, A. Zawaira, Exploiting structural principles to design cyclin-dependent kinase inhibitors, Biochim. Biophys. Acta 1754 (2005) 58–64.
[70] W.F. De Azevedo, S. Leclerc, L. Meijer, M. Strand, S.H. Kim, Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine, Eur. J. Biochem. 243 (1997) 518–526.
[71] L.T. Vassilev, C. Tovar, S. Chen, D. Knezevic, X. Zhao, H. Sun, D.C. Heimbrook, L. Chen, Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 10660–10665.
[72] A. Huwe, R. Mazitschek, A. Giannis, Small molecules as inhibitors of cyclin-dependent kinases, Angew. Chem. Int. Ed. 42 (2003) 2122–2138.
[73] O. Gavet, J. Pines, Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis, Dev. Cell 18 (2010) 533–543.
[74] L. Kurzawa, M. Pellerano, J.B. Coppolani, M.C. Morris, Fluorescent peptide bioprobe for quantification of cyclin-dependent kinases in living cells, PLoS One 6 (10) (2011) e26555.
[75] S. Tcherniuk, C. Perals, M. Pellerano, T.N.N. Van, E. Garcin, F. Mahuteau-Betzer, M.P. Teulade-Fichou, M.C. Morris, Conformation-sensitive biosensor of CDK2 applied to a high throughput screen leads to identification of allosteric T-loop inhibitors, Nat. Biotech. (submitted for publication).
[76] S. Tcherniuk, M. Pellerano, C. Perals, TNN Van, E. Garcin, F. Mahuteau, M.P. Teulade, M.C. Morris, Conformation-sensitive biosensor of CDK2 applied to a high throughput screen leads to identification of allosteric inhibitors, in preparation.
[77] M.C. Morris, S. Deshayes, F. Heitz, G. Divita, Cell-penetrating peptides: from molecular mechanisms to therapeutics, Biol. Cell 100 (2008) 201–217.
[78] F. Heitz, M.C. Morris, G. Divita, Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, Br. J. Pharmacol. 157 (2009) 195–206.

[79] L. Kurzawa, M. Pellerano, M.C. Morris, PEP and CADY-mediated delivery of fluorescent peptides and proteins into living cells, Biochim. Biophys. Acta (2010), http://dx.doi.org/10.1016/j.bbamem.2010.02.027.
[80] M.C. Morris, Promises for personalized medicine, J. Biosens. Bioelectron. 3 (2012).
[81] V. Ntziachristos, Fluorescence molecular imaging, Annu. Rev. Biomed. Eng. 8 (2006) 1–33.
[82] S. Keereweer, J.D. Kerrebijn, P.B. van Driel, B. Xie, E.L. Kaijzel, T.J. Snoeks, I. Que, M. Hutteman, J.R. van der Vorst, J.S. Mieog, A.L. Vahrmeijer, C.J. van de Velde, R.J. Baatenburg de Jong, C.W. Löwik, Optical image-guided surgery — where do we stand? Mol. Imaging Biol. 13 (2011) 199–207.
[83] K.A. Giuliano, D.L. Taylor, Fluorescent-protein biosensors: new tools for drug discovery, Trends Biotechnol. 16 (1998) 135–140.
[84] M. Wolff, J. Wiedenmann, G.U. Nienhaus, M. Valler, R. Heilker, Novel fluorescent proteins for high-content screening, Drug Discov. Today 11 (2006) 1054–1060.
[85] P. Lang, K. Yeow, A. Nichols, A. Scheer, Cellular imaging in drug discovery, Nat. Rev. Drug Discov. 5 (2006) 343–356.
[86] W.S. El-Deiry, C.C. Sigman, G.J. Kelloff, Imaging and oncologic drug development, J. Clin. Oncol. 24 (2006) 3261–3273.
[87] J.K. Willmann, N. van Bruggen, L.M. Dinkelborg, S.S. Gambhir, Molecular imaging in drug development, Nat. Rev. Drug Discov. 7 (2008) 591–607.
[88] M.D. Allen, L.M. DiPilato, M. Rahdar, Y.R. Ren, C. Chong, J.O. Liu, J. Zhang, Reading dynamic kinase activity in living cells for high-throughput screening, ACS Chem. Biol. 1 (2006) 371–376.