Biotechnology Journal
www.biotechnology-journal.com

Biotechnol. J. 2014, 9, 253–265

DOI 10.1002/biot.201300196

Review

Fluorescent biosensors for high throughput screening of protein kinase inhibitors

Camille Prével, Morgan Pellerano, Thi Nhu Ngoc Van and May C. Morris

CRBM-CNRS-UMR 5237, Chemical Biology and Nanotechnology for Therapeutics, Montpellier, France

Received 24 JUL 2013
Revised 24 JUL 2013
Accepted 30 OCT 2013

High throughput screening assays aim to identify small molecules that interfere with protein function, activity, or conformation, which can serve as effective tools for chemical biology studies of targets involved in physiological processes or pathways of interest or disease models, as well as templates for development of therapeutics in medicinal chemistry. Fluorescent biosensors constitute attractive and powerful tools for drug discovery programs, from high throughput screening assays, to postscreen characterization of hits, optimization of lead compounds, and preclinical evaluation of candidate drugs. They provide a means of screening for inhibitors that selectively target enzymatic activity, conformation, and/or function in vitro. Moreover, fluorescent biosensors constitute useful tools for cell- and image-based, multiplex and multiparametric, high-content screening. Application of fluorescence-based sensors to screen large and complex libraries of compounds in vitro, in cell-based formats or whole organisms, requires several levels of optimization to establish robust and reproducible assays. In this review, we describe the different fluorescent biosensor technologies which have been applied to high throughput screens, and discuss the prerequisite criteria underlying their successful application. Special emphasis is placed on protein kinase biosensors, since these enzymes constitute one of the most important classes of therapeutic targets in drug discovery.

Keywords: Drug Discovery · Fluorescent Biosensor · High Throughput Screening · Inhibitor · Protein Kinase

1 Introduction

One of the major challenges in biological sciences consists in devising novel strategies to detect and visualize biomolecules in their natural environment so as to gain insight into their behavior in physiological settings but also to provide and harness information from pathological settings for developing better therapeutics and targeting strategies. To this aim, several classes of imaging probes have been developed – from small ligands, peptides, and drugs to larger macromolecules including engineered fluorescent protein reporters and antibodies and nanotechnology-derived systems, such as dye-doped organic and inorganic particles [1].

The discovery and engineering of autofluorescent proteins (AFPs) and the chemical synthesis of a vast palette of small fluorescent probes over the past decade, have led to the design of a wealth of imaging probes, which, together with the escalation of fluorescent imaging technologies, currently offer numerous possibilities for imaging biomolecules in living cells and organisms [2–5]. Amongst the broad spectrum of imaging probes that have been developed over the last decade, and which are generally used to label a biomolecule of interest so as to visualize it and track it through space and time, a particular class of tools known as fluorescent biosensors has gained

Correspondence: Dr. M. C. Morris, CRBM-CNRS-UMR 5237, Chemical Biology and Nanotechnology for Therapeutics, 1919 Route de Mende, 34293 Montpellier, France
E-mail: may.morris@crbm.cnrs.fr

Abbreviations: AFP, autofluorescent protein; **CDK,** cyclin-dependent kinase; **CHEF,** chelation-enhanced fluorescence; **CML,** chronic myelogenous leukemia; **ELISA,** enzyme-linked immunosorbent assay; **FLIK,** fluorescent labels in kinases; **FRET,** fluorescence resonance energy transfer; **HCS,** high content screening; **HTS,** high throughput screening; **MAPK,** mitogen-activated protein kinase; **NES,** nuclear exclusion sequence; **NLS,** nuclear localization sequence; **PAABD,** phosphoamino acid binding domain; **PKA,** cAMP-dependent protein kinase A; **SOX,** sulfonamide-oxine; **S/N,** signal-to-noise

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

in potency and popularity, thanks to the additional ability of fluorescent biosensors to transduce information on the target in a quantitative, continuous, and reversible fashion. Fluorescent biosensors typically consist of a receptor moiety that recognizes the target (generally a substrate or binding domain), which is coupled genetically, chemically, or enzymatically to one or several fluorescent probes, which act as bio-transducers that output a measurable signal, proportional to the abundance, activity or conformational status of the target. Whether genetically-encoded or based on peptide, protein, or polymer-based scaffolds, fluorescent biosensors constitute potent tools for the detection of biomolecules in complex biological samples, in living cells and even in animal models [6–10]. These tools provide sensitive means of monitoring dynamic changes in biomolecular behavior in response to natural stimuli or to stress, to pathological conditions, or to therapeutics. Moreover, fluorescence imaging is a sensitive and non-destructive process which allows to visualize dynamic processes in living cells in real time with high spatial and temporal resolution, thereby providing precious information on the subcellular localization and behavior of intracellular targets. As such, fluorescent biosensors offer a wealth of opportunies to address fundamental issues in life sciences, and are perfectly suited for development of biomedical applications [11–13].

1.1 Fluorescent biosensors for drug discovery programs

Fluorescent biosensors are attractive tools for drug discovery programs, and have been widely used throughout the drug discovery pipeline, from primary high throughput screening (HTS) and high content screening (HCS) assays which integrate additional phenotypic and cellular imaging information, for hit validation and postscreen characterization, lead optimization, and preclinical evaluation (Fig. 1). Indeed, fluorescent biosensors are particularly well suited to screen large complex libraries in high throughput formats due to the inherent high sensitivity of fluorescence in response to compounds that affect the enzymatic activity or function of target enzymes. Moreover, since fluorescent reporters allow the monitoring of biomolecular processes in real time and in a continuous fashion, they allow the study of the kinetics of compound interference with target activity rather than simply providing a snapshot of the effect at a given time-point. Hence, fluorescent biosensors are perfectly well suited to study the dynamics of response and the mechanism of action of compounds, to study their differential effects on target activity and dynamics of activation, and to undertake pharmacological studies and kinetic profiling of agonists and antagonists [14]. As such, they allow for comparative mechanistic studies between different drugs, whilst also providing new opportunities for development of more effective drugs.

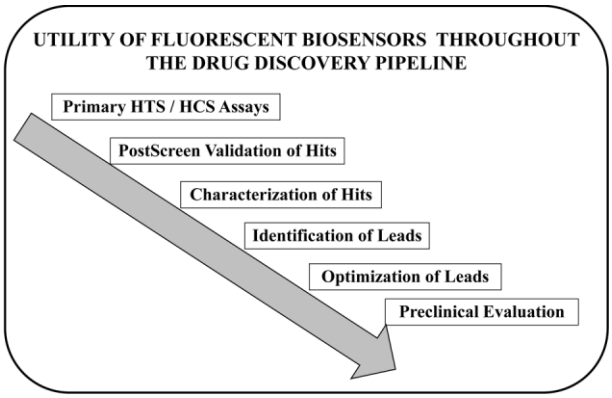


Figure 1. Utility of fluorescent biosensors throughout the drug discovery pipeline. Fluorescent biosensors constitute powerful tools for drug discovery purposes, from primary HTS/HCS assays, hit validation and postscreen characterization, lead optimization, and preclinical evaluation.

Last but not least, fluorescent biosensors constitute tools for screening in living cells, thereby offering a means of assaying inhibitor potential in a more physiological environment than that of a single purified recombinant target in buffer [15–20]. Moreover, with the development of cell-and image-based screens, it has become possible to perform multiparametric screens to seek for hits that interfere specifically with the target whose activity is probed by a fluorescent biosensor, and which also meet several predefined criteria with respect to cellular morphology, cell cycle progression, apoptosis, and cytotoxicity [21–23].

Fluorescent biosensors are ideal for the qualitative and quantitative evaluation of inhibitor efficacy in living cells at a postscreening stage, allowing researchers to gain insight into the pharmacokinetics of hits, to monitor both their fate following administration and in particular their absorption and distribution profiles, as well as their pharmacodynamics, in order to characterize their effect, biochemical, and physiological consequences and mechanisms of action. Furthermore, when standardized assays are established with lead compounds, fluorescent biosensors provide means of screening for derivatives capable of supporting structure–activity relationships, so as to optimize candidates for therapeutic applications. Finally, biosensor technology can play a central role in preclinical evaluation trials, to assess the efficacy of candidate drugs, to monitor response over time and emergence of resistance, in parallel or in combination with early evaluation of ADME (absorption, distribution, metabolism, excretion) and toxicity profiles. For example, intravital imaging of Src activity, thanks to a Src-specific genetically-encoded fluorescent biosensor, allowed investigators to monitor the targeting efficacy and clearance of dasatinib in a transgenic p53-mutant mouse model of pancreatic cancer. This study revealed a spatial gradient of Src activity within invading tumor cells and enhanced activity at the

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

invasive borders of live tumors relative to the cortex, as well as a threshold of drug penetrance in vivo, which allowed the mapping of areas of poor drug-targeting efficiency [24]. Another illustration of fluorescent biosensor application to preclinical evaluation in cancer therapy is the fluorescent biosensor developed to probe c-Myc activity in living mice, and used to monitor response to drugs by non-invasive molecular imaging. This approach revealed that inhibition of c-Myc activity could be imaged in vivo, well before changes in tumor size were observed in mouse xenografts and liver tumor models [25].

1.2 Criteria for successful application of fluorescent biosensors to HTS

The successful application of fluorescence-based sensors to screen large and complex libraries of compounds in high throughput and high content screening formats depends on the quality of the assay. Ideally, a performant assay should yield robust signals with a high signal-to-noise ratio, which is not lost upon miniaturization, and does not suffer from off-target effects. Hence several levels of optimization are required to establish robust and reproducible assay conditions. Prior to screening, several criteria must be considered and met to obtain robust and sustained responses in high throughput formats, since several parameters may affect the quality and intensity of the signals, in particular when downscaling the conditions and miniaturizing the assay to reduce the amount and cost of material used for the screen. Aside from the obvious specificity and sensitivity expected from the biosensor itself, as well as its ability to respond in a dynamic and reversible fashion, it is essential to ensure maximal differences between the signals associated with response to positive and negative controls, and minimal variability between these. Differences in these signals may vary with the kinetics and parameters of acquisition. As such it is important to measure the signal-to-noise ratio, the dynamic range and the variability of the response over time, since these features are critical for the establishment of optimal screening conditions (Fig. 2).

1.2.1 *Robustness, reproducibility, performance, and statistical value*

The statistical value and performance of an assay can be established thanks to several parameters, in particular the signal-to-noise (S/N) ratio, assay variability, and the difference between positive and negative controls, which are all taken into account to calculate the Z-factor [26–31]. Z-factor is a screening coefficient that reflects both the dynamic range and data variation of control signal acquisitions and is widely used to evaluate the quality of the screening assay. It is calculated based on the differences in the average values of positive and negative controls and of their standard deviations as follows: $Z = 1 - [(3O^+ + 3O^-)/u^+ - u^-)]$, where O^+ and O^- correspond to the standard deviations of positive and negative controls, whilst u^+ and u^- are the average responses for positive and negative controls. For an assay to be considered suitable for a screen the Z factor should be greater than 0.5 [26, 27].

The S/N ratio can be defined as the difference between positive and negative controls, or between the positive response and background fluorescence, due to the presence of cell extracts or to autofluorescence of living cells. In this respect, several factors may influence the variability and noise of the assay. Aside from the sensitivity/selectivity performance of the biosensors themselves, sources of variation with genetically-encoded biosensors may result from differences in transfection efficiency or expression. Hence the use of stable cell lines is preferred to minimize heterogeneity. Variability associated with nongenetic biosensors is more often due to protein instability and/or loss of a preferred biosensor conformation.

Table 1 reports several examples of fluorescent biosensors which have been validated and/or applied for

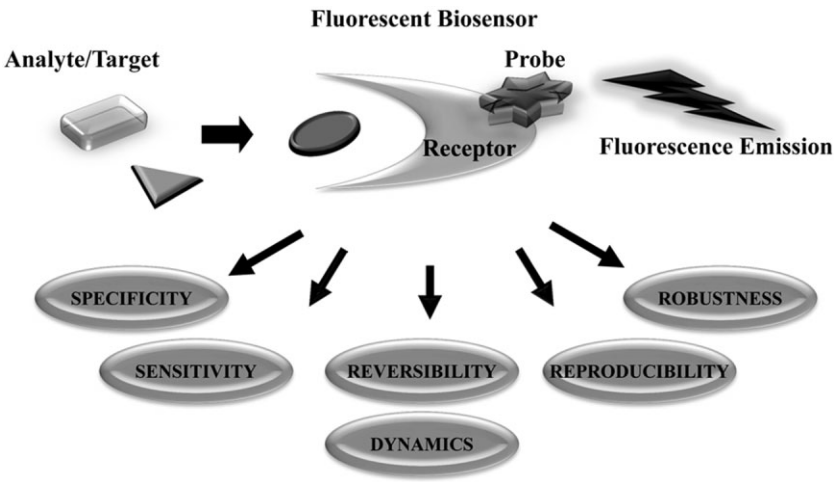


Figure 2. Criteria for successful application of fluorescent biosensors. Successful application of fluorescent biosensors requires a high degree of specificity and sensitivity, the ability to respond in a dynamic and reversible fashion, robust, and reproducible signals.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Fluorescent biosensors validated and/or implemented in HTS Assays

Biosensor Name	Biosensor type	Target	Z factor	Dye/probe	Comments	Reference
PKA	Genetic, positional	PKA		GFP-NLS-NES	Cellular assay	[68]
GPCR	Genetic, positional	GPCR		GFP	Cellular assay	[70]
PPIB	Genetic, positional	P53/hDM2	0.64	GFP-NLS-p53/ mRFP-NLS-NES-hDM2	High content screen for protein/ protein interaction inhibitors	[71]
EGFRB	Genetic, positional	EGFR	0.56	GFP-EGFR	High content screen for modulators of EGFR function	[72]
AKAR 2	Genetic, FRET	PKA	<0.5	FRET couple of AFPs	HTS	[29]
AKAR 3	Genetic, FRET	PKA	0.84	FRET couple of AFPs	HTS	[29]
Pickles	Genetic, FRET	Bcr-Abl		FRET couple of AFPs	Proposed for HTS / HCS	[74, 75]
C3	Genetic, FRET	Caspase 3	0.87	FRET couple of AFPs	HTS performed to identify anticancer compounds from Chinese medicine	[101]
Kinase Chemosensors	Environmentally-sensitive	Akt, MK2, PKA		SOX (sulfonamide oxine)	Validation for multiplex screening using cell extracts	[77]
Sox Chemosensor	Environmentally-sensitive	Serine/ threonine PKs		SOX (sulfonamide oxine)	Proposed for HTS	[78]
ERK1/2 chemosensor	Environmentally-sensitive	ERK1/2		SOX (sulfonamide oxine)	Proposed for HTS using cell extracts	[79]
p38α chemosensor	Environmentally-sensitive	p38 alpha		SOX (sulfonamide oxine)	Proposed for HTS	[80]
Bcr-Abl & Lyn Biosensors	Environmentally-sensitive	Bcr-Abl & Lyn			Proposed for HTS	[81]
DFG Biosensor	FLIK	P38a		Acrylodan	Validation with focused library of pyrazolourea derivatives	[82]
DFG Biosensor	FLIK	P38a		Acrylodan	HTS performed on 35 000 compounds	[83]
P-loop Biosensor	FLIK	P38a		Acrylodan	Validation with library of DFG-in or -out conformation ligands	[84]
MAPK Insert Biosensor	FLIK	P38a	>0.7	Acrylodan	Validation with library of 2-arylquinalozines	[86]
DFG Biosensor	FLIK	cSrc		Acrylodan		[85]
Myristate Pocket	FLIK	Abl	>0.84	Acrylodan	Amenable to HTS	[88]

HTS together with their Z′, when available, and comments with respect to their sensitivity/selectivity performance.

1.2.2 *Choice of fluorescent probes and optimization of the dynamic range for HTS/HCS*

Autofluorescent proteins have been used for development of FRET-based assays as well as for screens based on gene reporter assays, which involve changes in expression levels or on the subcellular localization of fusion proteins. To avoid heterogeneity of expression associated with ectopic expression of AFP fusions, stable cell lines are generally used for high throughput screening assays. Moreover, the brightness of the AFPs and FRET efficiency between the donor and acceptor couples are amongst the major features sought to be improved to optimize assay conditions. In particular the dynamic range of genetically-encoded FRET-based biosensors may be improved by optimizing the change in distance and orientation between the FRET couple of AFPs. In several instances, this has been successfully achieved by reengineering the modules involved in intramolecular interactions so as to influence the conformational changes that ultimately affect FRET between AFPs [29].

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Small synthetic probes may be selected based on a rational choice, or through a diversity-oriented approach using a fluorescent dye library. Fluorescent dyes that respond to different conformations or to biologically relevant changes constitute useful probes. In this respect, combinatorial chemistry and high throughput screening have allowed to generate optimized versions of biosensors by profiling changes in their fluorescence spectra upon recognition of their target or its activity. The dynamic range of signals may be improved by replacing a fluorescent probe by another and modulating its position [32–34].

1.2.3 *False positives and hit validation*

When screening for small molecule compounds in fluorescence based assays, it is important to keep in mind that their heterocyclic nature and associated intrinsic fluorescence may contribute significantly to the assay signals if they overlap with the spectral properties of the biosensor. Therefore it is always important to perform a screen of the compounds themselves, to verify whether they exhibit autofluorescence at the wavelength predefined to monitor biosensor fluorescence in the assay. Moreover, to ensure that compounds are not interfering with signals from the assay itself, it is often wise to retest hits in an alternative assay, or with a different set of fluorescent probes.

1.2.4 *Cell-based and image-based screens*

Cell-based assays offer the advantage that the target is within its natural environment rather than in a purified and isolated form in buffer. However there are several drawbacks to cell-based assays due to the complexity of the system, including variability, which can be minimized to a certain extent by using stable cell lines, artefacts related to toxicity of certain compounds, and interferences due to off-target hits that inhibit activities unrelated to the target.

High throughput screens based on imaging have a level of complexity that requires appropriate programs for analysis of data. The development of light microscope imaging technologies, together with multiparametric acquisition and analysis software lead to the development of high content screening, first introduced in 1997, in which the performance of high throughput screening of spectral emission intensity readouts is combined with the acquisition of quantitative imaging data concerning cellular morphology. High content screening therefore allows to analyze large numbers of cells in an automated fashion with subcellular resolution, combining screening of biological activities with acquisition of phenotypic features [15–20]. Moreover, thanks to advances in multiphoton imaging technologies which improve the depth of penetration and minimize photodamage, high throughput image-based screens have also become widely applicable to tissues and organisms, such as zebrafish, nematode, or thale cress [35–38].

1.2.5 *Multiplex, multicolor, and multiparameter screens*

Multiplex screening with fluorescent biosensors involves the use of different sets of fluorescent probes, whose spectral properties are sufficiently distinct, to allow the discrimination of fluorescent signals related to different biosensors. The development of imaging systems that allow the acquisition of multispectral data sets has allowed researchers to devise assays in which the behavior of several targets can be monitored by combining the simultaneous use of several biosensors [21–23]. Although multiparametric imaging remains challenging, it is particularly informative in drug discovery programs, as it allows researchers to gain information on the specificity of pharmacological inhibitors, to characterize differences in potency and inhibitory kinetics, and to identify off-target effects. Indeed, acquisition of phenotypic information and cytotoxicity profiles together with target-specific information, thanks to the use of a fluorescent biosensor, will allow researchers to distinguish inhibitors that interfere directly with the target of interest from drugs with inhibitory potential that are completely unrelated or have an indirect influence on target function. Moreover, the combination of several fluorescent biosensors that report on closely related yet distinct targets in the same assay enables identification of target-specific and selective hits. Multispectral imaging modalities are also becoming increasingly used in preclinical studies in animal models, to image disease and therapeutic response in vivo. Moreover, new generations of molecular imaging probes that combine several imaging modalities based on the electromagnetic properties of different wavelengths, multifunctional or multiplexed characteristics are being developed, thereby offering promising perspectives for diagnostics and drug discovery programmes [39].

2 Protein kinases – therapeutic targets in disease

2.1 Protein kinases in disease

Protein kinases are central to regulation of most signalling pathways and biological processes [40, 41] and constitute one of the major, yet most challenging, classes of therapeutic targets for drug discovery programs, due to their implication in disease. Whereas in healthy cells, kinase activity is generally tightly controlled both spatially and temporally, in pathological conditions they are found to be overexpressed and/or subject to mutations that directly perturb their function, thereby wreaking havoc in cellular homeostasis [42–44]. As such, protein kinase dysregulation has been documented in cardiovascular diseases, neurodegenerative and endocrinological disorders, immune deficiency and viral infection, cancer and diabetes.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

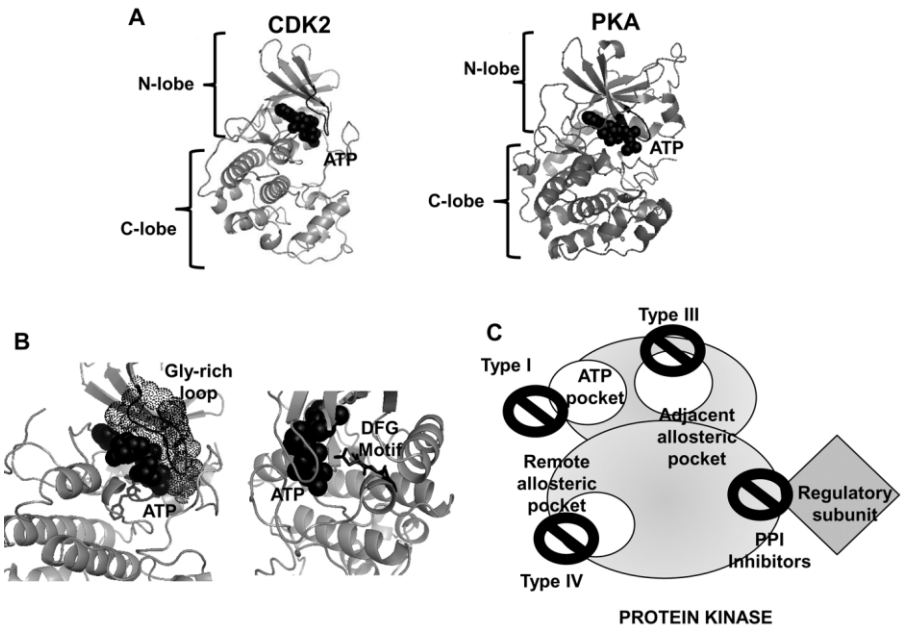


Figure 3. Strategies for targeting protein kinases. (**A**) Protein kinases display very similar structural folds consisting of two lobes, an N-terminal lobe that harbors an ATP-binding pocket, and a C-terminal lobe. The catalytic cleft where the substrate binds and the phosphotransfer reaction occurs lies between these two lobes. The structure of PKA and of CDK2 (PDB 1B38) are in gray, ATP and the Gly-rich loop in black. (**B**) ATP binds a conserved pocket in the Nterminal lobe of CDK2 kinase where it forms hydrogen bonds with the hinge region just above the ATP-binding site (left panel) and interacts with the Asp side chain of the conserved DFG motif (right panel). CDK2 is in gray – ATP is in black – the Gly-Rich loop is in black. (**C**) Cartoon of the different strategies devised to inhibit protein kinases: Type I inhibitors that bind the hinge region, and compete with ATP binding; Type II inhibitors bind the hinge region, compete with ATP, and extend deep into a proximal allosteric site; Type III inhibitors bind exclusively within an allosteric pocket masked by the DFG loop thereby maintaining the kinase in an inactive state; Type IV inhibitors bind an allosteric pocket remote from the ATP pocket thereby affecting conformational transitions associated with kinase activation; additional strategies involve targeting critical protein/protein interactions-PPI Inhibitors.

The 518 protein kinases of the human genome present very similar structural folds, yet display a high degree of structural plasticity [45–48]. Furthermore, the mechanisms that regulate substrate binding and kinase activity vary largely from one kinase to another, conditioned by posttranslational modifications, as well as binding to cofactors and regulatory subunits which affect their conformation [49–52]. Overall, protein kinase activation is associated with a net conformational change of the activation loop, thereby providing an accessible binding site for the substrate [47–51] (Fig. 3).

2.2 Targeting protein kinases

Although several protein kinases constitute established biomarkers in human diseases, they have not always been considered attractive and druggable pharmacological targets. Indeed, 20 years ago they were considered too difficult to target, given their conserved structural features and in particular the similarities of their ATP-binding pocket, which made it seem impossible to design compounds with sufficient specificity to inhibit a single protein kinase amongst the 518 encoded by the human genome. Over the past 15 years however, protein kinases have become one of the major classes of drug targets in particular in the field of cancer. Thanks to major advances in structure-guided design of protein kinase inhibitors, some 20 drugs have been successfully developed and approved for clinical use over the past decade, including Gleevec/Imatinib, and hundreds more are undergoing clinical trials [44, 53]. This being said, developing potent and specific protein kinase inhibitors still remains a challenge, and lack of efficacy, limited selectivity, and the emergence of acquired drug resistance still represent major bottlenecks [44, 53].

One of the major strategies developed so far has consisted in targeting the ATP-binding pocket of kinases so as to interfere with catalytic activity by competing with ATP binding. ATP binds a conserved pocket in the N-terminal lobe of CDK2 kinase where the adenine moiety forms hydrogen bonds with the hinge region of the kinase, just above the ATP-binding site, whilst the beta and gamma phosphates are coordinated by ionic and hydrogen interactions with Mg^{2+} or Mb^{2+} ions, the Asp chain of the conserved DFG motif at one extremity of the activation segment and the glycine-rich loop positioned above the ATP-binding cleft [54] (see Fig. 3A and B). The vast majority of small molecule kinase inhibitors target this highly conserved ATP-binding pocket by forming hydrogen bonds with the hinge region, and consequently

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

exhibit poor specificity [55]. Considering that the ATP-binding pocket of the 518 kinases in the human genome is highly conserved and that several other cellular enzymes also bind ATP, it rapidly became obvious that targeting the ATP-binding pocket was not a good strategy, and alternative targeting sites were sought to overcome limited selectivity and drug resistance, including interfering with substrate recognition or targeting essential protein/protein interactions [53]. More recent efforts, expected to yield inhibitors with superior selectivity profiles have focused on the design of inhibitors that bind inactive kinase conformations and allosteric sites [53, 56, 57] (Fig. 3). One strategy has consisted in targeting sites outside the ATP-binding pocket, for example compounds that bind hydrophobic pockets close to the ATP binding site which are unique to a particular protein kinase. An alternative strategy involves targeting sites which are remote from the ATP-pocket, so as to stabilize enzymatically inactive conformations, or interfere with conformational transitions, so as to prevent kinase activation. For example, targeting the "DFG-out" conformation of protein kinases through an unconserved allosteric site adjacent to the ATP-binding site which becomes exposed as a result of a conformational change of the activation segment [58, 59].

Since allosteric modulators target essential regulatory features and bind sites that are barely conserved across the kinome and which are accessible in specific conformations only, they are expected to become leads for development of potent and selective drugs. However, so far they have proven extremely difficult to identify in high throughput screens, due to an overall lack of tools for their selection.

3 Screening for protein kinase inhibitors in HTS/HCS assays

Several strategies have been developed for high throughput screening of chemical compound libraries in view of identifying novel inhibitors of protein kinases [15–17, 29–31]. The first of these were essentially based on conventional activity-based assays relying on radioactive or antigenic approaches to measure and quantify perturbations in substrate phosphorylation, such as gel-based assays, filter-binding assays, and enzyme-linked immunoabsorbant assays (ELISA). However these approaches were limited to in vitro assays using enzymatically active kinases, required laborious and time-consuming postreaction treatment steps and essentially yielded inhibitors that bind the ATP-binding pocket or compete with substrate binding. To replace these assays, several fluorescence-based approaches, which are straightforward "mix-and-measure" and homogeneous, have been developed for high throughput screening formats, including scintillation proximity, FRET, and fluorescence polarization, luminescence and alphascreen assays [30, 31]. The development of fluorescent biosensors that report on protein kinase activity has also provided a new source of technologies for high throughput screening formats, and high content screening assays [60–67]. Four classes of fluorescent biosensors have been developed for HTS/HCS

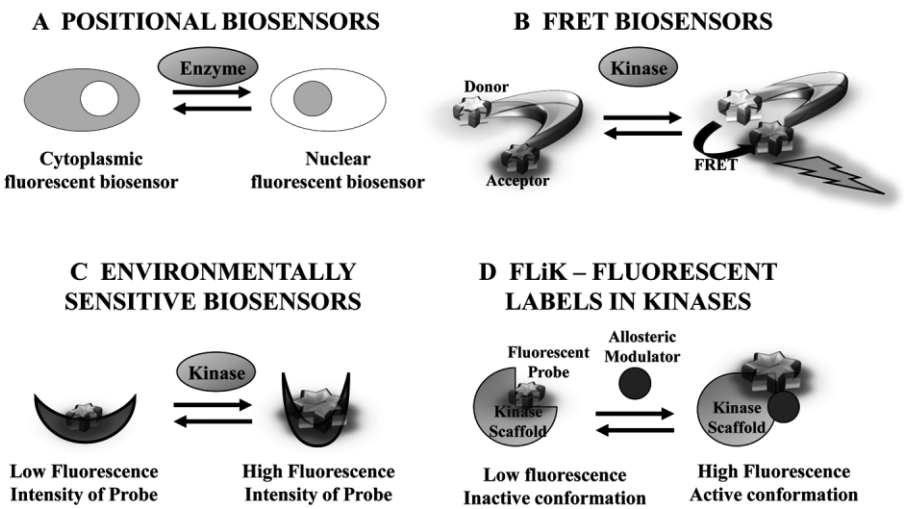


Figure 4. Fluorescent Biosensors for high throughput screening assays. Four types of fluorescent biosensors have been developed for HTS/HCS applications. (**A**) Positional Biosensors convey information on the activity of the target through changes in their subcellular localization. (**B**) FRET-based biosensors are genetically-encoded single-chain biosensors that report on target activity through changes in fluorescence transfer between a FRET pair, whose proximity varies in response to target activity. (**C**) Environmentally-sensitive biosensors report on changes in target activity through changes in fluorescence intensity or wavelength emission. (**D**) FLiK (fluorescent labels in kinases)-based approach involves direct incorporation of fluorescent probes into kinase scaffolds, thereby yielding conformation-selective sensors.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Pros and cons of different classes of biosensors

Class of biosensor	Positional biosensors	FRET-based biosensors	Environmentally sensitive biosensors	FLiK – fluorescent labels in kinases
Nature	Genetic fusions of an AFP that bear a subcellular localization sequence (generally NLS or NES), exposure of which is modulated in response to target activity.	Genetic constructs that encode a couple of AFPs separated by a substrate sequence and a complementary interacting module, specifically a phosphoamino acid binding domain in the case of kinase activity biosensors	Peptide substrate sequences or protein domains that bind a select analyte or interface and which serve as platforms for site-selective coupling of one or more fluorescent probe(s)	Direct incorporation of fluorescent dyes into specific positions of the kinase scaffold
Mechanism	Convey information on the activity/inhibition of the target through changes in their subcellular localization	Report on kinase activity through changes in FRET efficiency between a couple of AFPs	Report on changes in kinase activity through changes in fluorescence intensity or emission wavelength of a probe conjugated to the biosensor receptor moiety	Fluorescent kinase derivatives which can sense and report on ligands that bind and induce specific active or inactive conformational states
Pros	Easy to engineer and transfect; application in cellulo	Easy to engineer and transfect; application in cellulo	Versatility in design and engineering; choice of the nature and position of incorporation of the fluorescent probe; allow for controlled use (no variation in expression levels / no time required for maturation); application in vitro, in cell extracts and in cellulo	Easily applicable to HTS assays in vitro – conformation-selective kinase sensors – offer means of screening for allosteric inhibitors
Cons	Require a mechanism that promotes or disrupts shuttling between cytoplasm and nucleus; large size – essentially due to the size of the autofluorescent proteins; ectopic expression required; stable transfection preferred for screening assays	Large size – essentially due to the size of the autofluorescent proteins; ectopic expression required ; lack of control over expression levels and timing; heterogeneous expression in a population of cells; stable transfection preferred for screening assays	Require facilitated delivery approaches for their application in living cells or in vivo; require homogeneous and efficient delivery for cellular screening applications	Not applicable to cell-or image-based assays
Examples and references	PKA, Caspase, p53/hDM2 interaction, GPCR, EGFR [68–72]	AKAR and Bcr-Abl [29, 64, 74, 75]	Akt, MK2, PKA, ERK1, 2, p38a, Ser/Thr PKs [77–80]	MAPK, Src, Abl [82–88]

applications – positional, genetically-encoded FRET biosensors, environmentally-sensitive biosensors and FLiK (Fluorescent Labels in Kinases). Figure 4 provides a schematic illustration of these different biosensors and Table 2 reports on the pros and cons of these tools and technologies.

3.1 Positional biosensors

These biosensors convey information on the activity/inhibition of the target through changes in their subcellular localization (Fig. 4A). More generally speaking, these biosensors are genetic fusions of an AFP that bear a sub-

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

cellular localization sequence (generally NLS or NES), exposure of which is modulated in response to target activity. Positional biosensors thereby provide a very easy readout of activity profiles and are particularly well suited to high content image-based cellular screens [68, 69]. For example, positional biosensors of protease activity have been designed so as to remain tethered to the cytoskeleton until protease activation induces proteolytic cleavage, thereby releasing an NLS-tagged AFP, which translocates to the nucleus [68]. A positional biosensor of PKA activity was designed as a GFP fusion incorporating an NES, and an NLS, adjacent to a binding domain that recognizes the catalytic subunit of PKA. Upon cellular activation of PKA dissociation of the inhibitory regulatory subunit releases the catalytic subunit which binds the biosensor binding domain, thereby masking the NLS and favoring nuclear export of the GFP-tagged biosensor [68]. A GFP-tagged positional biosensor of G-protein coupled receptors was developed through incorporation of an NLS within the GPCR structure, to identify ligand-induced conformational changes that prevented nuclear translocation [70]. A positional biosensor system was developed and implemented to identify disruptors of the interaction between hDM2 and p53 thanks to expression of a GFP-NLS-p53 and an mRFP-NLS-NES-hDM2 construct [71]. A biosensor based on expression of a GFP-tagged cytosolic, soluble version of EGFR was developed to screen for modulators of its function, which prevent its aggregation and formation of visible granules upon EGF stimulation [72].

3.2 FRET-based biosensors

FRET-based biosensors are essentially genetically-encoded single-chain biosensors that report on target activity through changes in FRET efficiency between a couple of AFPs which are separated by a substrate sequence and a complementary interacting module, specifically a phosphoamino acid binding domain (PAABD) in the case of kinase activity biosensors. Upon phosphorylation of the substrate sequence, the PAABD interacts with the phosphosubstrate, thereby inducing a major conformational change of the biosensor and consequently bringing together the FRET pair of AFPs [64, 66] (Fig. 4B). Fluorescence energy transfer between AFPs promotes an increase in fluorescence intensity and lifetime of the acceptor AFP together with a decrease in intensity and in lifetime of the donor. As for positional biosensors, these biosensors are easy to transfect and to engineer, but depend on the ectopic expression and maturation of the plasmid construct. This may consequently lead to heterogeneous expression levels within a field of cells. Therefore application of this type of biosensor to high throughput screening formats requires establishment of stable cell lines.

Cyclic-AMP-dependent protein kinase (PKA) is involved in a wide variety of essential biological processes and signalling pathways, including gene expression, metabolism, cell growth, and cell proliferation [73]. In order to probe the activity of this kinase, AKAR biosensors were developed and successfully applied in different settings [29, 64].

Bcr–Abl is a chimeric oncogenic non-receptor tyrosine-kinase generated from the formation of the Philadelphia chromosome via the fusion between Abelson (Abl) and Breakpoint cluster (Bcr) genes, which is implicated in the pathogenesis of chronic myelogenous leukemia (CML). In order to probe the activity of Bcr-Abl in living cells, genetically-encoded FRET biosensors Picchu and Pickles were developed, based on CrkII and CrkL-derived substrates placed between a YFP/CFP and Venus/ECFP couple, respectively [74, 75]. These have been successfully applied to probe Bcr-Abl activity in cells from CML patients and have further been used to monitor response to first and second generation therapeutics, to evaluate kinase inhibitor efficacy and emergence of resistance [74–76]. Bcr-Abl FRET biosensors are much more sensitive than classical antibody-based approaches to study the potency of inhibitors in living cells, and therefore constitute potent tools for high throughput screening assays.

3.3 Environmentally-sensitive biosensors

Environmentally-sensitive biosensors report on changes in kinase activity through changes in fluorescence intensity or emission wavelength of a probe conjugated to the biosensor receptor moiety (Fig. 4C). Environmentally-sensitive biosensors have been essentially engineered from peptide/protein or polymeric scaffolds which are coupled to synthetic dyes whose fluorescence is sensitive to their environment. Peptide and protein biosensors are designed by exploiting peptide substrate sequences or protein domains that bind a select analyte or interface and which serve as platforms for site-selective coupling of one or more fluorescent probe(s). These non-genetic biosensors are based on the principle of solvatochromism: changes in the environment of the fluorescent probe, associated with detection of the target or its activity, have a direct consequence on its photophysical properties, namely intensity, life-time, emission wavelength, associated with changes in local solvent polarity which affects the energetic gap between ground and excited states of the fluorophore [5]. Hence environmentally-sensitive probes can report on switches between active/inactive conformations, binding events which expose or bury a probe and intramolecular conformational changes and interactions within a biosensor scaffold.

Peptide and protein biosensors constitute tools of choice for probing enzymes, biomarkers or analytes due to their modularity and versatility both in their design and in their applicability. They allow for incorporation of the fluorescent probe of choice, the choice of position of the labelling site within the sensing domain, so as to yield optimal response to changes in its molecular environ-

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ment. They further allow for controlled use (no variation in expression levels/time/maturation) but require facilitated delivery approaches for their application in living cells or in vivo. These features make non-genetic, environmentally-sensitive peptide biosensors very attractive for high throughput screening assays and several examples have already been applied successfully. Peptide biosensors based on chelation-enhanced fluorescence (CHEF) of SOX (sulfonamide-oxine) dyes coupled proximal to the phosphorylation site have been readily applied to probe ERK and MAPK activities in fluorescence-based assays and HTS formats using cell lysates [77–80].

Wang et al. developed fluorescent peptide biosensors that are enzymatically and photophysically distinct, allowing to simultaneously monitor Bcr-Abl and Lyn tyrosine kinase activity through multicolor imaging. This combination of orthogonal probes allowed to assess differences in Lyn and Abl kinase activities in CML cell lines that are sensitive to imatinib or which develop resistance to this drug [81].

3.4 New tools for old targets – fluorescent labels in kinases

As an alternative to strategies that provide a direct readout of kinase activity, based on substrate phosphorylation, which classically lead to an enrichment of ATP competitive inhibitors in high throughput screens, a fluorescence-based approach based on conformational changes between active and inactive forms of protein kinases has been developed and successfully applied to select for inhibitors that bind inactive conformations or intermediate transitional states, rather than ATP competitive inhibitors. This approach known as FLiK, for Fluorescent Labels in Kinases, relies on the direct incorporation of fluorescent dyes into specific positions of the kinase scaffold, thereby generating fluorescent kinase derivatives which can sense and report on ligands that bind and induce specific active or inactive conformational states [82–88] (Fig. 4D). These tools constitute conformation-selective kinase sensors which offer an original means of screening for allosteric inhibitors. FLiK assays are well suited for direct application to high throughput fluorescence binding assays in view of identifying ligands that bind and/or stabilize specific kinase conformations and have been successfully applied to screen for inhibitors of p38α MAPK, Src and Abl [82–88].

Most ATP-competitive inhibitors bind the active "DFG-in" conformation of protein kinases in which the regulatory activation loop is open and extended so as to allow substrate and ATP binding [54, 55]. Since the activation loop and the glycine-rich loop of protein kinases are dynamic segments subject to crosstalk, fluorophore labelling of the activation loop or of the glycine-rich loop of the serine/threonine p38alpha mitogen-activated protein kinase (MAPK) with acrylodan have enabled develop assays for identifying compounds that induce conformational changes, and in particular inhibitors that bind and stabilize the enzymatically incompetent "DFG-out" conformation [82–84]. This assay was also successfully developed to identify allosteric inhibitors that stabilize the inactive "DFG-out" conformation of tyrosine kinase cSrc [85]. A similar strategy was applied to identify allosteric inhibitors that bind the myristate pocket of Abl tyrosine kinase [88].

Cyclin-dependent kinases (CDK/Cyclins) form a family of heterodimeric kinases whose function is essential to drive cell cycle progression in a timely and controlled fashion [89]. Constitutive or deregulated hyperactivity of these kinases, associated with their overexpression, amplification, or mutation has been shown to contribute to proliferation of cancer cells [90]. As such these kinases constitute biomarkers of proliferation as well as attractive pharmacological targets for the development of anticancer drugs and a wide variety of ATP-competitive inhibitors of CDKs have been identified from natural substances, in high throughput screening assays, or through structure-guided approaches [91–95]. Alternative strategies have been explored to inhibit CDK/Cyclin function, including interfering with substrate recognition, or targeting essential protein/protein interfaces and/or residues required for structural organization [96–99]. More recently fluorescent-based approaches have been devised in an attempt to identify novel allosteric inhibitors of these kinases. An assay based on displacement of 8-anilino-1-naphthalene sulfonate (ANS) fluorophore from an allosteric pocket adjacent to the ATP-binding site of CDK2 was implemented to screen for novel CDK inhibitors [100]. So far, no allosteric inhibitors that target the activation loop of CDKs have been designed or identified in screens. However tampering with T-loop dynamics would provide an original and selective means of preventing conformational activation of these kinases. To this aim, our own group has developed a biosensor that reports on conformational changes of the activation segment of CDK2 associated with its activation, but which is insensitive to ATP-pocket ligands. We have further applied this technology to screen a library of small chemical compounds for inhibitors that interfere with the conformational activation of this kinase, and have successfully identified a family of structural analogs that dock across the T-loop, consequently preventing access to the substrate, and which constitute potent inhibitors of cancer cell proliferation (unpublished data).

4 Concluding remarks

Fluorescent biosensors constitute sensitive tools for biomarker detection and can be expected to serve for development of quantification strategies and diagnostic strategies – to improve early cancer detection, and to provide better means of determining cancer origin, stage, and grade. Moreover, sensing technologies provide a opportu-

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

nity to monitor disease progression, as well as therapeutic response and benefit, thereby paving the way for personalized diagnostics.

Fluorescent biosensors also constitute powerful tools for drug discovery purposes, from early stage primary HTS/HCS assays and throughout the drug discovery pipeline. They allow for postscreen characterization and kinetic profiling of inhibitors, and are useful tools for optimization of lead compounds, and preclinical evaluation of drug candidates therapeutics.

The design or identification of selective inhibitors of protein kinase function remains a major challenge in chemical biology and medicinal chemistry. One of the issues at the forefront of kinase inhibitor research currently consists in identifying ligands which bind to sites which are distinct from ATP-pockets, and targeting non-catalytic functions of protein kinases. Allosteric inhibitors and compounds that interfere with critical protein/protein interactions are thought to constitute promising strategies to overcome limited selectivity and emergence of drug resistance. To this aim, new generation technologies with high sensitivity and specificity are needed, and the development of different types of fluorescent biosensors offer new avenues for exploration and promising perspectives for drug discovery programmes.

Last but not least, beyond their diagnostic function, fluorescent biosensors can be expected to contribute to cancer theranostics. The combination of molecular imaging approaches with targeting strategies would provide a means of coupling biomarker detection with tailored therapeutic intervention, the efficiency of which could in turn be determined by monitoring response to treatment. A remaining challenge will then concern the in vivo application of fluorescent biosensors for molecular imaging and theragnostics.

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"), Institut National du Cancer (INCA) and Agence Nationale de Recherche (ANR). CP is supported by a fellowship from CBS2-University Montpellier. TNNV is recipient of a fellowship from the USTH. MP is funded by a grant from the INCA.

The authors declare no conflict of interest.

5 References

[1] Weissleder, R., Pittet, M. J., Imaging in the era of molecular oncology. *Nature* 2008, *452*, 580–589.
[2] Zhang, J., Campbell, R. E., Ting, A. Y., Tsien, R. Y., Creating New Fluorescent Probes for Cell Biology. *Nat. Rev. Mol. Cell. Biol.* 2002, *3*, 906–918.
[3] Chudakov, D. M., Matz, M. V., Lukyanov, S., Lukyanov, K. A., Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* 2010, *90*, 1103–1163.
[4] Lavis, L. D., Raines, R. T., Bright ideas for chemical biology. *ACS Chem. Biol.* 2008, *3*, 142–155.
[5] Loving, G. S., Sainlos, M., Imperiali, B., Monitoring protein interactions and dynamics with solvatochromic fluorophores. *Trends Biotechnol.* 2010, *28*, 73–83.
[6] Ibraheem, A., Campbell, R. E., Designs and applications of fluorescent protein-based biosensors. *Curr. Opin. Chem. Biol.* 2010, *14*, 30–36.
[7] Palmer, A. E., Qin, Y., Park, J. G., McCombs, J. E., Design and application of genetically encoded biosensors. *Trends Biotechnol.*. 2011, *29*, 144–152.
[8] Wang, H., Nakata, E., Hamachi, I., Recent progress in strategies for the creation of protein-based fluorescent biosensors. *Chembiochem* 2009, *10*, 2560–2577.
[9] Pazos, E., Vázquez, O., Mascareñas, J. L., Vázquez, M. E., Peptide-based fluorescent biosensors. *Chem. Soc. Rev.* 2009, *38*, 3348–3359.
[10] Morris, M. C., Fluorescent biosensors of intracellular targets from genetically encoded reporters to modular polypeptide probes. *Cell. Biochem. Biophys.* 2010, *56*, 19–37.
[11] Morris, M. C., *Fluorescent Biosensors for Cancer Cell Imaging and Diagnostics. Biosensors and Cancer*, Preedy, V., Hunter, J. (Eds.), CRC press, Science Publishers, 2012, pp. 101–124.

Short Biography – Cell Cycle Biosensors and Inhibitors Group
The "Cell Cycle Biosensors and Inhibitors" group focuses on development of protein kinase inhibitors and fluorescent polypeptide biosensors to probe cell cycle kinases for biomedical imaging applications and drug discovery programs. Camille PREVEL studied chemistry at the National School of Chemistry and Chemical Engineering of Montpellier, before joining the group in 2011 for her MSc and PhD. Thi Nhu Ngoc VAN graduated from the National University of Hanoi in biochemistry and molecular biology, then joined the group and obtained her PhD in 2013. Morgan PELLERANO studied biochemistry and specialized in protein purification and fluorescence-based studies of protein/protein interactions. He joined the group in 2007 and is currently a CNRS engineer. May C. MORRIS is a CNRS Research Director with a background in biochemistry and molecular biology, a PhD in Biology and Health Sciences from the University of Montpellier, and further postdoctoral training at the Scripps Research Institute, La Jolla, CA. She was awarded the CNRS Bronze Medal in 2006 and the "Chercheuse d'Avenir" award from Languedoc-Roussillon Region in 2009.

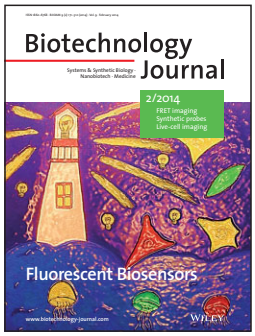
© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[12] Morris, M. C., Fluorescent biosensors – promises for personalized medicine. *J. Biosensors Bioelectron.* OMICS, 2012, *3*, 3.
[13] Morris, M. C., *Fluorescence-Based Biosensors – concepts and applications*, Morris, M. C. (Ed.), *Progr. Mol. Biol. Trans. Science*, Vol. 113, Elsevier Press, 2013, pp. 1–414.
[14] Giuliano, K. A., Taylor, D. L., Fluorescent-protein biosensors: New tools for drug discovery. *Trends Biotechnol.* 1998, *16*, 135–140.
[15] Hanson, G. T., Hanson, B. J., Fluorescent probes for cellular assays. *Comb. Chem. High Throughput Screen.* 2008, *11*, 505–513.
[16] Taylor, D. L., A personal perspective on high-content screening (HCS): From the beginning. *J. Biomol. Screen.* 2010, *15*, 720–725.
[17] Wolff, M., Wiedenmann, J., Nienhaus, G. U., Valler, M. et al., Novel fluorescent proteins for high-content screening. *Drug Discov. Today.* 2006, *11*, 1054–1060.
[18] Lang, P., Yeow, K., Nichols, A., Scheer, A., Cellular imaging in drug discovery. *Nat. Rev. Drug Discov.* 2006, *5*, 343–356.
[19] Carpenter, A. E., Image-based chemical screening. *Nat. Chem. Biol.* 2007, *3*, 461–465.
[20] Zanella, F., Lorens, J. B., Link, W., High content screening: seeing is believing. *Trends Biotechnol.* 2010, *28*, 237–245.
[21] Piljic, A., Schultz, C., Simultaneous recording of multiple cellular events by FRET. *ACS Chem. Biol.* 2008, *3*, 156–160.
[22] Carlson, H. J., Campbell, R. E., Genetically encoded FRET-based biosensors for multiparameter fluorescence imaging. *Curr. Opin. Biotechnol.* 2009, *20*, 19–27.
[23] Welch, C. M., Elliott, H., Danuser, G., Hahn, K. M., Imaging the coordination of multiple signalling activities in living cells. *Nat. Rev. Mol. Cell Biol.* 2011, *12*, 749–756.
[24] Nobis, M., McGhee, E.J., Morton, J.P., Schwarz, J.P., Intravital FLIM-FRET imaging reveals dasatinib-induced spatial control of src in pancreatic cancer. *Cancer Res.* 2013, *73*, 4674–4686.
[25] Fan-Minogue, H., Cao, Z., Paulmurugan, R., Chan, C. T., Massoud, T. F. et al., Noninvasive molecular imaging of c-Myc activation in living mice. *Proc. Natl. Acad. Sci. USA* 2010, *107*, 15892–15897.
[26] Zhang, J. H., Chung, T. D., Oldenburg, K. R., A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* 1999, *4*, 67–73.
[27] Inglese, J., Shamu, C. E., Guy, R. K. Reporting data from high-throughput screening of small-molecule libraries. *Nat. Chem. Biol.*, 2007, *3*, 438–441.
[28] Sui, Y., Wu, Z. Alternative statistical parameter for high-throughput screening assay quality assessment. *J. Biomol. Screen.* 2007, *12*, 229–234.
[29] Allen, M. D., DiPilato, L. M., Rahdar, M., Ren, Y. R. et al., Reading dynamic kinase activity in living cells for high-throughput screening. *ACS Chem. Biol.* 2006, *1*, 371–376.
[30] Zaman, G. J., Garritsen, A., de Boer, T., van Boeckel, C. A., Fluorescence assays for high-throughput screening of protein kinases. *Comb. Chem. High Throughput Screen.* 2003, *6*, 313–320.
[31] von Ahsen, O., Bömer, U., High throughput screening for kinase inhibitors. *ChemBioChem* 2005, *6*, 481–490.
[32] Vendrell, M., Zhai, D., Er, J. C., Chang, Y. T., Combinatorial strategies in fluorescent probe development. *Chem Rev.* 2012, *112*, 4391–4420.
[33] Ljosa, V., Carpenter, A. E., High-throughput screens for fluorescent dye discovery. *Trends Biotechnol.* 2008, *26*, 527–530.
[34] Inglese, J., Johnson, R. L., Simeonov, A., Xia, M. *et al.*, High-throughput screening assays for the identification of chemical probes. *Nat Chem Biol.* 2007, *3*, 466–479.
[35] Berg, R. H., Beachy, R. N., Fluorescent protein applications in plants. *Methods Cell Biol.* 2008, *85*, 153–177.
[36] Nicholson, R. L., Welch, M., Ladlow, M., Spring, D. R., Small-molecule screening: advances in microarraying and cell-imaging technologies. *ACS Chem. Biol.* 2007, *2*, 24–30.
[37] O'Rourke, E. J., Conery, A. L., Moy, T. I., Whole-animal high-throughput screens: the *C. elegans* model. *Methods Mol. Biol.* 2009, *486*, 57–75.
[38] Blackburn, J. S., Liu, S., Raimondi, A. R., Ignatius, M. S. et al., High-throughput imaging of adult fluorescent zebrafish with an LED fluorescence macroscope. *Nat. Protoc.* 2011, *6*, 229–241.
[39] Kobayashi, H., Longmire, M. R., Ogawa, M., Choyke, P. L., Rational chemical design of the next generation of molecular imaging probes based on physics and biology: Mixing modalities, colors and signals. *Chem. Soc. Rev.* 2011, *40*, 4626–4648.
[40] Manning, G., Whyte, D. B., Martinez, R., Hunter, T. et al., The protein kinase complement of the human genome. *Science* 2002, *298*, 1912–1934.
[41] Hunter, T., A thousand and one protein kinases. *Cell.* 1987, *50*, 823–829.
[42] Cohen, P., Protein kinases – the major drug targets of the twenty-first century? *Nat. Rev. Drug Discov*. 2002, *1*, 309–315.
[43] Johnson, L., Protein kinases and their therapeutic exploitation. *Biochem. Soc. Trans.* 2007, *35*, 7–11.
[44] Zhang, J., Yang, P. L., Gray, N. S., Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer.* 2009, *9*, 28–39.
[45] Taylor, S. S., Radzio-Andzelm, E., Three protein kinase structures define a common motif. *Structure* 1994, *2*, 345–355.
[46] Véron, M., Radzio-Andzelm, E., Tsigelny, I., Taylor, S. S., Protein kinases share a common structural motif outside the conserved catalytic domain. *Cell Mol. Biol*. 1994, *40*, 587–596.
[47] Johnson, L. N., Noble, M. E., Owen, D. J., Active and inactive protein kinases: structural basis for regulation. *Cell* 1996, *85*, 149–158.
[48] Huse, M., Kuriyan, J., The conformational plasticity of protein kinases. *Cell* 2002, *109*, 275–282.
[49] Ubersax, J. A., Ferrell, J. E. Jr., Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol*. 2007, *8*, 530–541.
[50] Turk, B. E., Understanding and exploiting substrate recognition by protein kinases. *Curr. Opin. Chem. Biol.* 2008, *12*, 4–10.
[51] Nolen, B., Taylor S., Ghosh, G., Regulation of protein kinases: controlling activity through activation segment conformation. *Mol. Cell* 2004, *15*, 661–675.
[55] Taylor, S. S., Yang, J., Wu, J., Haste, N. M. et al., PKA: A portrait of protein kinase dynamics. *Biochim. Biophys. Acta* 2004, *1697*, 259–269.
[53] Cohen, P., Alessi, D. R., Kinase drug discovery – what's next in the field? *ACS Chem. Biol*. 2013, *8*, 96–104.
[54] Aimes, R. T., Hemmer, W., Taylor, S. S., Serine-53 at the tip of the glycine-rich loop of cAMP-dependent protein kinase: role in catalysis, P-site specificity, and interaction with inhibitors. *Biochemistry* 2000, *39*, 8325–8332.
[55] Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H. et al., Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*. 1991, *253*, 414–420.
[56] Fang, Z., Grütter, C., Rauh, D., Strategies for the Selective Regulation of Kinases with Allosteric Modulators: Exploiting Exclusive Structural Features. *ACS Chem. Biol*. 2013, *8*, 58–70.
[57] Eglen, R. M., Reisine, T., Human kinome drug discovery and the emerging importance of atypical allosteric inhibitors. *Expert Opin. Drug. Discov*. 2010, *5*, 277–290.
[58] Liu, Y., Gray, N. S., Rational design of inhibitors that bind to inactive kinase conformations. *Nat. Chem. Biol*. 2006, *2*, 358–364.
[59] Pargellis, C., Tong, L., Churchill, L., Cirillo, P. F. et al., Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat. Struct. Biol*. 2002, *9*, 268–272.
[60] Tarrant, M. K., Cole, A. P., The chemical biology of protein phosphorylation. *Annu. Rev. Biochem*. 2009, *78*, 797–825.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[61] Chen, C. A., Yeh, R. H., Yan, X., Lawrence, D. S., Biosensors of protein kinase action: From in vitro assays to living cells. *Biochim. Biophys. Acta* 2004, *1697*, 39–51.
[62] Lawrence, D. S., Wang, Q., Seeing is believing: Peptide-based fluorescent sensors of protein tyrosine kinase activity. *Chembiochem* 2007, *8*, 373–378.
[63] Sharma, V., Wang, Q., Lawrence, D. S., Peptide-based fluorescent sensors of protein kinase activity: Design and applications. *Biochim. Biophys. Acta*. 2008, *1784*, 94–99.
[64] Zhang, J., Allen, M. D., FRET-based biosensors for protein kinases: Illuminating the kinome. *Mol. Biosystems* 2007, *3*, 759–765.
[65] Gonzalez-Vera, J. A., Probing the kinome in real time with fluorescent peptides. *Chem. Soc. Rev.* 2012, *41*, 1652–1664.
[66] Van, T. N. N., Morris, M. C., Fluorescent sensors of protein kinases: From basics to biomedical applications. *Prog. Mol. Biol. Transl. Sci*. 2013, *113*, 217–274.
[67] Morris, M. C., Fluorescent Biosensors – Probing protein kinase function in cancer and drug discovery. *Biochim. Biophys. Acta*. 2013, *1834*, 1387–1395.
[68] Giuliano, K. A., Chen, Y. T., Haskins J. R., Positional biosensors: A new tool for high content screening. *Modern Drug Discov.*. 2003, *6*, 33–37.
[69] Kurzawa L, Morris M. C. Cell-cycle markers and biosensors. *Chembiochem* 2010, *11*, 1037–1047.
[70] O'Dowd, B. F., Alijaniaram, M. Ji. X., Nguyen, T., Eglen, R. M., George, S. R. Using ligand-induced conformational change to screen for compounds targeting G-protein-coupled receptors. *J. Biomol. Screen.* 2007, *12*, 175–85.
[71] Dudgeon, D. D., Shinde, S., Hua, Y., Shun, T. Y. et al., Implementation of a 220,000-compound HCS campaign to identify disruptors of the interaction between p53 and hDM2 and characterization of the confirmed hits. *J. Biomol. Screen.* 2010, *15*, 766–782.
[72] Antczak, C., Mahida, J. P., Bhinder, B., Calder, P. A., Djaballah, H. A high-content biosensor-based screen identifies cell-permeable activators and inhibitors of EGFR function: Implications in drug discovery. *J. Biomol. Screen.* 2012, *17*, 885–899.
[73] Taylor, S. S., Zhang, P., Steichen, J. M., Keshwani, M. M. et al., PKA: Lessons learned after twenty years. *Biochim. Biophys. Acta* 2013, *1834*, 1271–1278.
[74] Mizutani, T., Kondo, T., Darmanin, S., Tsuda, M. et al., A novel FRET-based biosensor for the measurement of BCR-ABL activity and its response to drugs in living cells. *Clin. Cancer Res*. 2010, *16*, 3964–3975.
[75] Lu, S., Wang, Y., Fluorescence resonance energy transfer biosensors for cancer detection and evaluation of drug efficacy. *Clin. Cancer Res.* 2010, *16*, 3822–3824.
[76] Tunceroglu, A., Matsuda, M., Birge, R. B., Real-time fluorescent resonance energy transfer analysis to monitor drug resistance in chronic myelogenous leukemia. *Mol. Cancer Ther*. 2010, *9*, 3065–3073.
[77] Shults, M. D., Janes, K. A., Lauffenburger, D. A., Imperiali, B., A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat. Methods* 2005, *2*, 277–283.
[78] Shults, M. D., Carrico-Moniz, D., Imperiali, B., Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. *Anal. Biochem.* 2006, *352*, 198–207.
[79] Lukovic, E., Vogel Taylor, E., Imperiali, B., Monitoring protein kinases in cellular media with highly selective chimeric reporters. *Angew. Chem. Int. Ed .Engl*. 2009, *48*, 6828–6831.
[80] Stains, C. I., Lukovic, E., Imperiali, B., A p38α-selective chemosensor for use in unfractionated cell lysates. *ACS Chem. Biol.* 2011, *6*, 101–105.
[81] Wang, Q., Zimmerman, E. I., Toutchkine, A., Martin, T. D. et al., Multicolor monitoring of dysregulated protein kinases in chronic myelogenous leukemia. *ACS Chem. Biol*. 2010, *5*, 887–895.
[82] Simard, J. R., Getlik, M., Grütter, C., Pawar, V. et al., Development of a Fluorescent-tagged kinase assay system for detection and characterization of allosteric kinase inhibitors. *JACS* 2009, *131*, 13286–13296.
[83] Simard, J. R.,et al. High-throughput screening to identify inhibitors which stabilize inactive kinase conformations in p38alpha. *J. Am. Chem. Soc.* 2009, *131*, 18478–18488.
[84] Simard, J. R., Getlik, M., Grütter, C., Schneider, R. et al., Fluorophore labelling of the glycine-rich loop as a method of identifying inhibitors that bind to active and inactive kinase conformations. *JACS* 2010, *132*, 4152–4160.
[85] Simard, J. R., Klüter, S., Grütter, C., Getlik, M. et al., A new screening assay for allosteric inhibitors of cSrc. *Nat. Chem. Biol.* 2009, *5*, 394–396.
[86] Getlik, M., Simard, J. R., Termathe, M., Grütter, C. et al., Fluorophore labelled kinase detects ligands that bind within the MAPK insert of p38a kinase. *PLoS One* 2012, *7*, e39713.
[87] Zhang, J., Adrian, F. J., Jahnke, W., Cowan-Jacob, S. W. et al., Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* 2010, *463*, 501–506.
[88] Schneider, R., Becker, R., Simard, J. R., Getlik, M. et al., Direct binding assay for the detection of Type IV allosteric Inhibitors of Abl. *JACS* 2012, *134*, 9138–9141.
[89] Morgan, D. O., Cyclin-dependent kinases: Engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol*. 1997, *13*, 261–291.
[90] Malumbres M, Barbacid M. To cycle or not to cycle: A critical decision in cancer. *Nat. Rev. Cancer* 2001, *1*, 222–231.
[91] Lapenna, S., Giordano, A., Cell cycle kinases as therapeutic targets for cancer. *Nat. Rev. Drug Discov*. 2009, *8*, 547–566.
[92] Noble, M., Barrett, P., Endicott, J., Johnson, L. et al., Exploiting structural principles to design cyclin-dependent kinase inhibitors. *Biochim. Biophys. Acta* 2005, *1754*, 58–64.
[93] Lolli, G., Structural dissection of cyclin dependent kinases regulation and protein recognition properties. *Cell Cycle* 2010, *9*, 1551–1561.
[94] De Azevedo, W. F., Leclerc, S., Meijer, L., Strand, M. et al., Inhibition of cyclin-dependent kinases by purine analogues: Crystal structure of human cdk2 complexed with roscovitine. *Eur. J. Biochem*. 1997, *243*, 518–526.
[95] Vassilev, L. T., Tovar, C., Chen, S., Knezevic, D. et al., Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 10660–10665.
[96] Mendoza, N., Fong, S., Marsters, J., Koeppen, H. et al., Selective cyclin-dependent kinase 2/Cyclin A antagonists that differ from ATP site inhibitors block tumor growth. *Cancer Res*. 2003, *63*, 1020–1024.
[97] Canela, N., Orzaez, M., Fucho, R., Mateo, F. et al., Identification of an Hexapeptide that binds to a surface pocket in cyclin A and inhibits the catalytic activity of the complex cyclin-dependent kinase 2-cyclin A. *J. Biol. Chem*. 2006, *281*, 35942–35953.
[98] Bagella, L., Sun, A., Tonini, T., Abbadessa, G. et al., A small molecule based on the pRb2/p130 spacer domain leads to inhibition of cdk2 activity, cell cycle arrest and tumour growth reduction in vivo. *Oncogene* 2007, *26*, 1829–1839.
[99] Gondeau, C., Gerbal-Chaloin, S., Bello, P., Aldrian-Herrada, G. et al., Design of a novel class of peptide inhibitors of cyclin-dependent kinase/cyclin activation. *J. Biol. Chem*. 2005, *280*, 13793–13800.
[100] Martin, M. P., Alam, R., Betzi, S., Ingles, D. J. et al., A novel approach to the discovery of small-molecule ligands of CDK2. *Chembiochem* 2012, *13*, 2128–2136.
[101] Tian, H., Ip, L., Luo, H., Chang, D. C., Luo, K. Q. ,A high throughput drug screen based on fluorescence resonance energy transfer (FRET) for anticancer activity of compounds from herbal medicine. *Br. J. Pharmacol.* 2007, *150*, 321–334.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



The Fluorescent Biosensor special issue of *Biotechnology Journal* is edited by Dr. May Morris and Prof. Marc Blondel. The cover image is an artistic interpretation of how fluorescent biosensors function as molecular beacons for scientists navigating a sea of molecules. Image courtesy of L. Divita and R. Wintergerst.

Biotechnology Journal – list of articles published in the February 2014 issue.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim