



HAL
open science

A Toolbox of Fluorescent Peptide Biosensors to Highlight Protein Kinases in Complex Samples: Focus on Cyclin-Dependent Kinases

May C Morris

► **To cite this version:**

May C Morris. A Toolbox of Fluorescent Peptide Biosensors to Highlight Protein Kinases in Complex Samples: Focus on Cyclin-Dependent Kinases. *European Journal of Organic Chemistry*, 2022, 2022 (21), pp.e202200120. 10.1002/ejoc.202200120 . hal-03808056

HAL Id: hal-03808056

<https://hal.science/hal-03808056>

Submitted on 10 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

A Toolbox of Fluorescent Peptide Biosensors to Highlight Protein Kinases in Complex Samples : focus on cyclin-dependent kinases

May C. Morris*^[a]

[a] Dr. M.C.Morris
Cellular Pharmacology,
Institut des Biomolécules Max Mousseron, Pole Chimie Balard Recherche, University Montpellier
1919 Route de Mende, 34293 Montpellier, France
E-mail: may.morris@umontpellier

Abstract: Development of sensitive and selective strategies for detection of disease biomarkers constitutes a major challenge for diagnostics and companion assays. New strategies are required to advance personalized and point-of-care medicine. Protein kinases (PK) are hyperactivated in many human cancers thereby constituting relevant biomarkers and attractive pharmacological targets. Yet quantification of PK expression levels alone does not convey an accurate readout of their activity, since PKs are subject to numerous interactions and post-translational regulations. Hence technologies that report on PK function are essential for complete appreciation of their behaviour and dysregulation in physio-pathological settings. This review focuses on fluorescent peptide biosensors designed to report on PK activities, and in particular on a toolbox of biosensors tailored to monitor cyclin-dependent kinases. These selective optical probes offer a straightforward means of quantifying differences in kinase activities between healthy and cancer cells, and can report on alterations in response to therapeutics *in vitro*, in living cells and *in vivo*.

1. Introduction

Detection of disease biomarkers constitutes a major challenge for development of diagnostics and companion assays. A wide variety of genetic, antigenic and proteomic approaches have been implemented to detect and quantify biomarkers of interest, whose expression levels are affected in pathological contexts. However together with our growing understanding of the dynamic complexity of biological signalling pathways and their rewiring in disease, new technologies are required to obtain functional information which will further guide therapeutic intervention. In this respect, fluorescent biosensors constitute attractive and versatile tools that offer means of monitoring dynamic processes and in particular enzymatic activities in complex samples, thereby offering promises for biomedical and diagnostic applications. The

intrinsic sensitivity of fluorescence is perfectly suited for detecting biomarkers of interest within living cells, and a large class of genetically-encoded fluorescent biosensors have been widely used to image biological processes with high spatial and temporal resolution^[1-7]. A somewhat less popular yet growing class of synthetic biosensors, engineered through combination of peptides and synthetic fluorophores provides further means of detecting biomarkers *ex vivo* in complex samples with exquisite sensitivity and strong potential for diagnostic and biomedical applications^[8-18]. Indeed, plasmids encoding genetically-encoded biosensors are routinely transfected into cultured cells through lipid-based or viral strategies, thereby enabling imaging studies following ectopic expression of the fluorescent protein biosensor. Synthetic biosensors require facilitated intracellular delivery, which can be mediated by a wide panel of available cell-penetrating peptides, or following conjugation to nanomaterial-based vectors, such as carbon nanotubes that readily penetrate mammalian cells^[19-22]. Biosensor expression following transfection of plasmids may require some time (12-24h or more), unless stable transfectants are generated, and the concentration of biosensor expressed from plasmid constructs is not always well determined. In contrast, the concentration of synthetic peptides can be predetermined and quantified following cellular internalization, offering a better control of biosensor in a given experiment. Last but not least, synthetic biosensors can be readily implemented for *ex vivo* applications, for instance in cell extracts or human biopsies, whereas genetically-encoded biosensors have to be expressed and purified prior to *in vitro* application. It should also be noted that genetically-encoded biosensors are rather large proteins, in the 40-80kDa range, since they include one or two large autofluorescent proteins, whereas synthetic biosensors are much smaller, generally 1-3 kDa.

Biography: May C. Morris obtained her PhD in Biology and Health Sciences at the University of Montpellier, France and completed her postdoctoral training at the Scripps Research Institute, La Jolla, USA. She is CNRS Research Director at the Institute of Biomolecules Max Mousseron, where she heads the "Kinase Biosensors and Inhibitors" group in the team of Cellular Pharmacology. She holds a strong background on cell cycle kinases and cancer with longstanding interest in the biological functions and molecular mechanisms of regulation of protein kinases, and a strong interest in development of biosensing technologies and therapeutic targeting of kinase dysregulation in cancer.



2. Fluorescent Peptide Biosensors – synthetic tools for biomarker detection

2.1. How to design and engineer synthetic biosensors ?

Peptides are endowed with functional and structural properties of particular interest for design of new generations of therapeutics, delivery strategies such as cell-penetrating peptides and biomaterials, and further constitute attractive scaffolds for biosensing applications [8-18].

Design of peptide biosensors should account for the biological context and the biomarker of interest, to define the specific needs and requirements, for instance whether they are meant for *in vitro* applications or for imaging purposes (Figure 1) [23,24]. As for any biosensor, the first issue to address concerns the bioreceptor, i.e. the moiety that will enable recognition of the biomarker of interest and confer selectivity/specificity. In some instances, it may be desirable to improve selectivity by incorporating a docking sequence that will facilitate binding to the target of interest. The second task consists in selecting a fluorescent probe for conjugation onto the peptide scaffold, depending on the intended /anticipated mechanism of biosensor response, but also bearing in mind the kind of application, which will often define the working window of fluorescence excitation and emission. For instance *in vivo* applications require red-shifted or near-infra-red dyes to minimize phototoxicity, whereas blue, green or yellow dyes are perfectly suited for *in vitro* applications. The choice of the dye is critical as it will define sensitivity, ie the limits of detection and dynamic range of response, the robustness of response and signal-to-noise ratio. Finally, a cell-penetrating peptide sequence may be included in the design, when peptide biosensors are engineered for imaging studies.

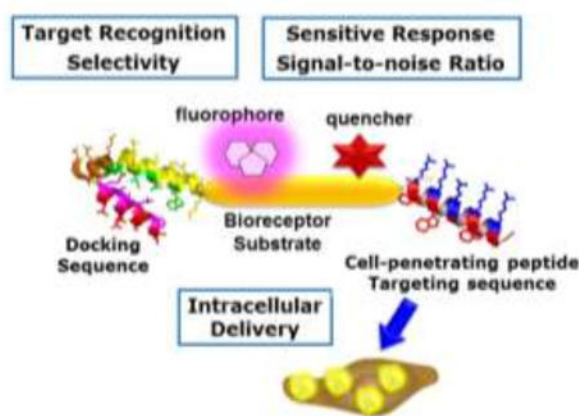


Figure 1. Peptide biosensor design involves three features : (1) a bioreceptor or substrate sequence and in some cases a docking sequence to improve selective target recognition (2) an appropriately selected fluorophore associated or not with a quencher, to optimize the sensitivity of response and the signal-to-noise ratio (3) a cell-penetrating peptide or a targeting sequence enabling intracellular delivery for imaging applications

Small organic dyes/fluorophores can be readily used to label peptide of interest and monitor their interactions with partner proteins, their posttranslational modification by enzymes such as PKs or proteases for instance, as well as conformational changes that affect the fluorescence properties of the probe. A wide palette of organic fluorophores have been synthesized by chemists and long been used as fluorescent probes to label molecules of interest and monitor biomolecular behaviour (Figure 2A). Amongst the wide range of these polycyclic aromatic compounds, heterocycles such as naphthalene and pyrene derivatives, coumarins, quinolines and oxazines, indoles and imidazole, xanthene dyes such as fluorescein and rhodamine, BODIPY dyes (boron difluoride dipyrromethane) and cyanine dyes (polymethine derivatives) are some of the most widely used dyes that present photophysical properties of interest for engineering synthetic biosensors [25, 26]. Although these "first generation" fluorophores are widely available and generally characterized by quite reasonable quantum yields, they suffer several limitations for biosensing applications, including poor sensitivity to their environment, lack of photostability and are not sufficiently red-shifted for biological imaging experiments.

New generations of "smart dyes" have been synthesized with features that are much better suited to the requirements sought for sensitive biosensors (Figure 2B). These include solvatochromic dyes which are sensitive to their environment, and in particular to the local polarity around the fluorophore exposed to the biological medium, as opposed to when it is buried in a peptide or protein environment following recognition of target of interest [27, 28]; red-shifted dyes that are better suited for biological experiments in living cells or *in vivo* since less near infra-red or infra-red illumination is less phototoxic and allows for deeper penetration into tissues; fluorogenic dyes that may be activated by changes in pH or following protease-specific cleavage; dyes with enhanced brightness and photostability such as SiRhodamines developed by Kai Johnsson's group [29] and tetraalkyl rhodamines developed by Luke Lavis' group [30, 31]; dyes that behave like On/Off switches that can self-aggregate and quench or remain silent in the absence of the biosensing target [32-34].

Last but not least, lanthanides offer very attractive photophysical properties for biosensing applications, such as long luminescence

lifetimes and narrow emission bands, and have been successfully employed for the development of several peptide biosensors, as described below.

In summary, the ideal probe for biosensing applications should be red-shifted, exhibit large Stoke's shifts and be "silent" or "quenched" in the absence of its target, fluorescence emission being activated, unquenched or significantly enhanced upon target recognition, and exhibit a high quantum yield, brightness and photostability. Needless to say, the best choice offers a compromise between some, but never all of these features.

Fluorescent dyes can be incorporated into peptide backbones through chemical, biochemical or enzymatic processes, including post-synthetic conjugation onto thiol or NHS groups, through semi-synthetic incorporation thanks to self-labelling protein tags (SNAP, CLIP, FIAsh), as fluorescent amino acids, or through site-specific enzymatic incorporation thanks to fluorophore ligases [27, 18].

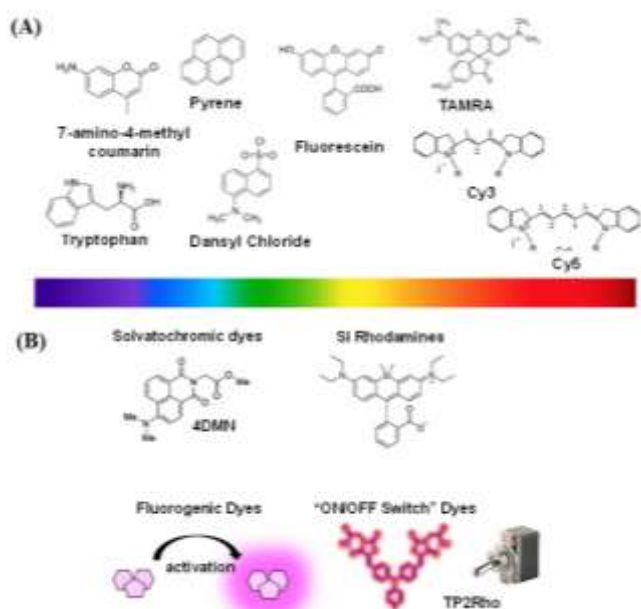


Figure 2. Fluorescent Probes for Peptide Biosensors (A) commonly used organic fluorophores (B) smart dyes with attractive photophysical properties for biosensing applications

2.2. Successful Examples of Peptide Biosensors

Peptide biosensors come in all kinds of "shapes and sizes". A wide variety of synthetic and semi-synthetic fluorescent biosensors have been developed and different strategies have been implemented to improve and optimize their ability to recognize and respond to detection of their target in a selective and sensitive fashion. Several comprehensive reviews cover the diversity of biosensor designs [8-14] and specific examples will be described below and in section 3.

In the simplest design an environmentally sensitive fluorophore will be conjugated onto a peptide scaffold that will simply emit fluorescence upon recognition of the active target (**Figure 3A**). In a second design, biosensor fluorescence will undergo sensitive changes upon target modification, for instance phosphorylation. This implies that the fluorophore is both sufficiently sensitive to addition of a phosphate group onto the peptide substrate, but also

that it is positioned close enough to sense this posttranslational modification (**Figure 3A**). A third design involves binding of an extrinsic domain (peptide or protein) such as a phosphoamino acid binding domain (PAABD) that binds the phosphorylated peptide substrate, thereby altering the local environment of the fluorophore and consequently inducing changes in fluorescence emission. In the case of solvatochromic fluorophores this may also promote a shift in fluorescence emission (**Figure 3A**). Further biosensor designs comprising two fluorophores that may undergo fluorescence energy resonance transfer between the donor and the acceptor upon conformational change induced by target binding or posttranslational modification such as phosphorylation, or conversely (**Figure 3B**). These designs may further be optimized by including a quencher group close to the fluorophore, such as an aromatic amino acid (tyrosine or tryptophan) or a pyrene group, which quench fluorescence until the biosensors are phosphorylated, thereby disrupting internal quenching, as will be described in section 3.

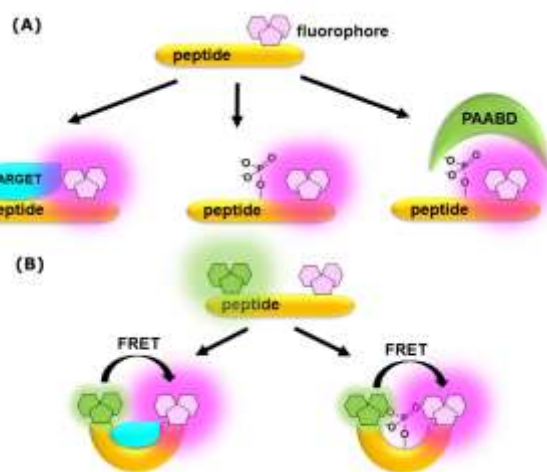


Figure 3. Peptide Biosensor Strategies. (A) In the absence of the target, a peptide biosensor labelled with a single fluorophore emits basal fluorescence, which will be altered (enhanced) according to one of the following mechanisms : (1) in response to target binding (2) or to addition of a phosphate group (3), to binding of a phospho-amino acid binding domain (PAABD) following phosphorylation (B) a peptide biosensor labelled with two fluorophores may undergo conformational changes that brings these fluorophores in close proximity following target binding or posttranslational modifications, enabling FRET between the donor and the acceptor.

The meroCBD biosensor of CDC42 developed by Klaus Hahn's group was derived from the CRIB domain of WASP, that specifically binds active CDC42, labelled with merocyanine dye, which is highly sensitive to changes in its environment. This biosensor was successfully implemented to image endogenous CDC42 activation dynamics in living cells following microinjection [35] (**Figure 4A**). Semi-synthetic SNAP- and CLIP-tag metabolite biosensors developed by Kai Johnsson's group incorporate two organic fluorophores, one donor and one acceptor of a FRET couple, fused to a metabolite bioreceptor protein, maintained in close proximity in the closed conformation, resulting in high FRET efficiency until metabolite binding displaces the intramolecular ligand yielding an open biosensor conformation with no FRET between donor and acceptor [36] (**Figure 4B**). Semi-synthetic biosensors dubbed LUCIDs for Luciferase Indicators of Drugs were developed by Kai Johnsson's group to monitor drugs present in plasma and/or urine. LUCIDs comprise a luciferase responsive element combined with a SNAP-tag labelled with Cy3

and a drug receptor. This biosensor adopts a closed conformation in the absence of drug, due to receptor binding of a drug-mimicking ligand attached to the SNAP-tag, thereby enabling BRET between luciferase and cyanine. In the presence of drug, which displaces the ligand from the receptor, the biosensor adopts an open conformation, which disrupts BRET between the Cy3 dye and luciferase, luminescence of which becomes maximal [37] (**Figure 4C**). LUCIDs enable dose-dependent quantification of drug concentration and have been proposed for point-of-care therapeutic drug monitoring.

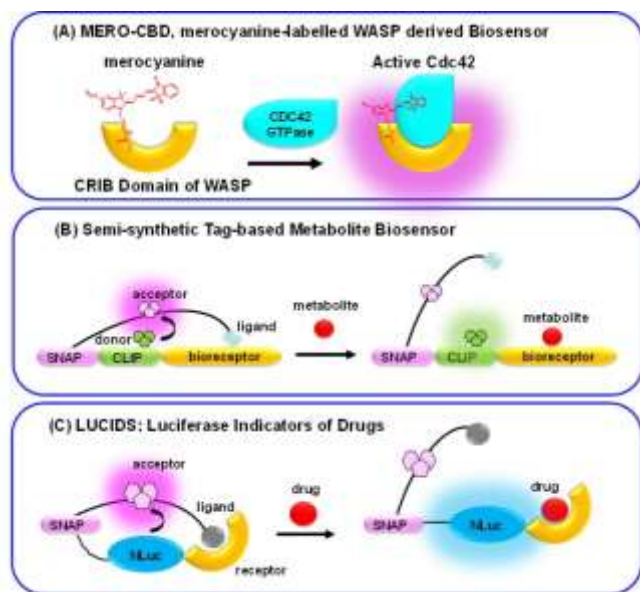


Figure 4. Successful examples of Synthetic Fluorescent Biosensors (A) CDC42 biosensor derived from the CRIB domain of the WASP effector of CDC42, that binds the active form of CDC42, developed by K.Hahn's group, (B) Semi-synthetic metabolite biosensors developed by K.Johnsson's group include fluorescently-labelled SNAP- and CLIP-tags together with a bioreceptor that binds a ligand linked to the SNAP-tag in the closed conformation, thereby enabling FRET, which is disrupted upon metabolite binding to the bioreceptor, due to displacement of the ligand (C) LUCIDs developed by K.Johnsson's group are bioluminescent biosensors that combine a SNAP-tag labeled with a cyanine dye, with luciferase and a receptor for a drug: in the absence of drug, the closed conformation of the biosensor emits Cy3 fluorescence due to BRET between luciferase and cyanine, whereas drug binding promotes an open conformation which disrupts BRET, resulting in strong luminescence emission of luciferase.

3. Protein Kinases : cancer biomarkers and pharmacological targets of interest for biosensing applications

3.1. Protein Kinase Activities

Protein kinases (PK) form a large class of enzymes that phosphorylate protein substrates on Ser/Thr or Tyr residues and which are hyperactivated in many human cancers, thereby constituting relevant biomarkers and attractive pharmacological targets in oncology, with 75 FDA-approved drugs in 2021 [38-41]. Yet quantification of PK expression levels alone does not provide an accurate readout of their activity, since PKs are subject to post-translational regulation as well as interactions with activating and inhibitory partners [42, 43]. Despite the wealth of information gained from genetic, transcriptomic and proteomic characterization of PK signalling pathways over the past decade, technologies that report on their functional activities remain poorly developed for

biomedical applications, thereby limiting our appreciation of their dysregulation in physio-pathological settings.

Fluorescent biosensor technologies that can report on PK activity provide essential information, which enables us to gain insight into their role and regulation, broadening our understanding of their function and overall behaviour in response to trigger signals, drugs and disease.

3.2. Fluorescent Peptide Biosensor reporters kinases

Given the importance of phosphorylation in regulation of biological processes and signal transduction pathways, it is not surprising that a wide variety of kinase activity reporters have been designed and engineered for in vitro and imaging applications. David Lawrence's group pioneered the development of "self-reporting" and "deep quench" peptide biosensors [44-46] (**Figure 5A**). The former are based on the environmental sensitivity of organic fluorophores that respond to phosphorylation of a peptide substrate through binding of PAABDs, such as SH2 and 14-3-3 domains for tyrosine and serine/threonine kinases, respectively. The deep-quench strategy was tailored to design highly sensitive and responsive PKA biosensors, in which fluorescence emission is silenced by a quencher, but enhanced by phosphorylation which disrupts the interaction between fluorophore and quencher and further promotes binding of a PAABD. Using these technologies they further developed a multicolour Bcr-ABL and Lyn kinase biosensing strategy using peptide biosensors applied to cells from CML patients [47] (**Figure 5A**).

B.Imperiali's group developed a smart and sensitive strategy based on chelation-enhanced fluorescence. A sulfonamido oxine (Sox) dye was introduced into peptide substrates to engineer biosensors that respond to phosphorylation through chelation of the Mg(II) ion involved in the reaction [48-51]. To further optimize recognition of the substrate moiety by specific kinases, her group developed a "recognition-domain focused" approach which enabled engineering of Sox-labelled substrate peptides with additional determinants that facilitate selective recognition of the biosensor onto the kinase of interest. Sox-biosensors for a wide variety of serine/threonine and tyrosine kinases (PKCs, Pim2, Akt1, MK2, PKA, IRK, Src, Abl) have been engineered and have further been implemented to multiplexed kinase assays in cell extracts. A highly selective ERK1/2 biosensor was further engineered through introduction of a docking domain derived from the Ets-1 transcription factor that is recognized by these kinases (**Figure 5B**).

Luminescent reporters of phosphorylation based on chelation of lanthanides have been developed by several groups. N.J. Zondlo developed a kinase-inducible motif derived from an EF hand calcium-binding loop that binds metals, the structure of which is dependent on phosphorylation. When associated with recognition sequences for serine/threonine kinases PKA, PKC and ERK, or adapted for tyrosine kinases such as Abl, these biosensors complex Tb3+ and exhibit strong luminescence following phosphorylation by the cognate kinases [52, 53]. Dalimor Sames group also developed phosphorylation-responsive lanthanide peptide conjugates, luminescence of which is enhanced through Tb3+ or Eu3+ binding following phosphorylation [54]. Laurie Parker's group developed several Tb3+-based tyrosine biosensors for Syk, ALK and Abl and further established a pipeline

dubbed KINATEST-ID for phosphorylation-dependent Terbium-sensitizing kinase assays. This group further engineered a set of orthogonal biosensors of Lyn and Syk through conjugation of small fluorophores (fluorescein and Cy5) on the phosphotyrosine peptides, which were excited by Tb³⁺ luminescence. Time-resolved luminescence studies with these biosensors revealed their potential and further led this group to develop self-cell penetrating luminescent probes bearing the sequence of the cell-penetrating peptide TAT applied to fluorescence life-time imaging in cultured cells [55-60]. (Figure 5C).

Another strategy of interest concerns the use of artificial Zn (II) dipicolylamine (Dpa) complexes for detection of phosphate anion derivatives [61]. Hamachi's group further designed a hybrid biosensor derived from the WW phosphoprotein binding domain of Pin1, coupled with a Zn (II) Dpa stilbazole chemosensor that selectively recognizes bis-phosphorylated over mono-phosphorylated peptides or ATP, thanks to cooperative phosphate binding of the WW motif and of the Zn(II) Dpa [62].

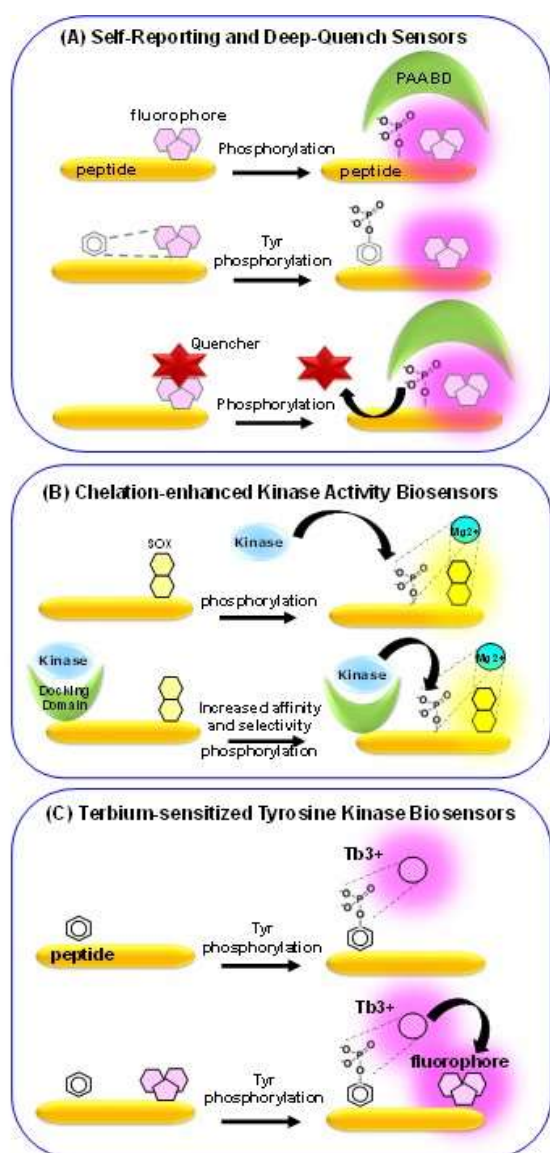


Figure 5. (A) Self-reporting reporters of phosphorylation and deep-quench peptide biosensors whose fluorescence is quenched either by tyrosine or by an extrinsic quencher in the absence of phosphorylation, developed by D.Lawrence's group. (B) Chelation-enhanced SOX biosensors and

Recognition-domain focused derivatives developed by B.Imperiali's group, (C) Terbium-sensitizing phospho-tyrosine biosensors and luminescent biosensors conjugated with organic fluorophores developed by L.Parker's group to yield orthogonal probes.

3.3. Fluorescent Peptide Biosensor reporters of Cyclin-dependent kinases

Cyclin-dependent kinases (CDK/cyclins) play a central role in coordination of cell growth and division and participate in several essential biological processes. They are frequently hyperactivated in human cancer and constitute established biomarkers and attractive pharmacological targets for anticancer therapeutics [63-72]. In order to monitor CDK kinase activities in complex biological samples, such as cell extracts, tissue or tumour biopsies, we have developed a toolbox of fluorescent biosensors through conjugation of environmentally-sensitive probes to synthetic modular peptide scaffolds/bioreceptors. [73-79] (Figure 6).

CDKACT biosensors are modular bipartite peptides comprising a CDK-specific substrate moiety onto which an environmentally-sensitive dye is conjugated, and a PAABD that folds onto the substrate sequence phosphorylated by an active CDK. This intramolecular conformational change alters the local environment of the fluorescent probe, consequently promoting fluorescence enhancement. By tailoring the substrate moiety for specific recognition by different CDKs, we generated a highly selective panel of synthetic fluorescent peptides that report on the specific activity of CDK1, CDK2, CDK4, CDK5 and CDK6, in a sensitive, direct and quantitative fashion through changes in fluorescence emission. Throughout design and characterization of these different CDKACT biosensors and incrementing complexity to improve selectivity and reduce their size, we have established a solid proof-of-concept for this technology, which has been implemented to report on kinase activities in cancer cell lysates and tumour biopsies, as well as in living cells and in vivo.

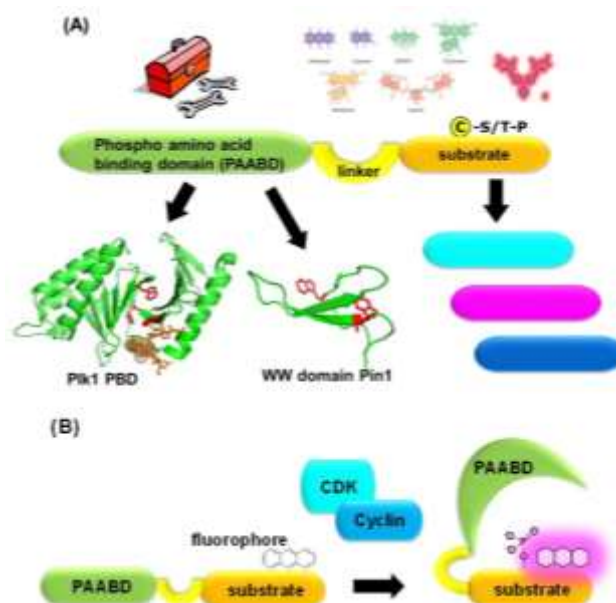


Figure 6. CDKACT ToolBox comprises a panel of biosensors tailored to respond to different CDKs. (A) Specific substrate sequences for cognate CDKs bear a unique cysteine for site-specific labelling with environmentally-sensitive fluorophores. PAABD sequences ranging from the PBD of PIK1 (shown in green complexed with a phosphopeptide from Cdc25C (orange); Trp residues in red; PDB 20JS) to the WW domain of Pin1 (PDB 2ZQT; Trp residues in red) and a

shorter derivative of the latter (not shown). (B) Upon phosphorylation by an active CDK/Cyclin, CDKACT biosensors undergo an intramolecular conformational change, as the PAABD folds onto the phosphorylated substrate sequence, altering the local environment of the fluorophore, promoting changes in fluorescence emission.

The first CDKACT was a generic biosensor derived from the PAABD of PIK1 PBD and Histone H1, a substrate commonly phosphorylated by many CDKs [73]. This peptide biosensor was labelled with Cy3 and shown to respond to several recombinant active CDKs as well as to CDKs in lysates from cultured cancer cell lines in a dose-dependent fashion and to report on kinase inhibition by ATP-competitive kinase inhibitors. We further engineered an RFP-CDKACT derivative which was labelled with Cy5, so as to generate a protein biosensor that could be internalized into cultured cells using cell-penetrating peptides for imaging purposes. Ratiometric quantification of changes in Cy5 fluorescence emission relative to RFP allowed to validate response of this protein biosensor to CDK activity within living cells, essentially prior to cell division, when CDK1 and CDK2 are most active, but not in non-dividing cells or cells treated with the CDK inhibitor Roscovitine.

CDKACTRb and CDKACT4 biosensors were tailored by selecting a substrate sequence from p107Rb (Retinoblastoma protein) specifically phosphorylated by CDK4 kinase. With the aim of shortening the biosensor design relative to the first CDKACT [73], we replaced the PBD of PIK1 by the WW domain of Pin1, thereby generating CDKACTRb biosensor that could be expressed in *E. coli* and purified by FPLC prior to labelling and use for biosensing purposes. We further replaced the native WW domain of Pin1 by a shorter sequence that comprises the residues directly involved in the interface with the phosphopeptide substrate moiety to yield CDKACT4. This biosensor responds to recombinant CDK4 and reports on differences in CDK4 activity between different cell lines, in particular to its hyperactivity in melanoma cell lines, and to its inhibition by Palbociclib, Ribociclib and Abemaciclib. CDKACT4 was further implemented to measure CDK4 activity in skin, revealing greater activity in epidermis, where cells are rapidly dividing, than in the dermis. Finally CDKACT4 was used to measure differences in CDK activity in A375 xenografts implanted in mice, and treated with Palbociclib or Roscovitine [74]. In a different study, we investigated the implication of CDK2 and CDK4 in the skin condition psoriasis by combining CDKACT peptide biosensors, Western blotting and immunohistochemical analyses and showed that CDK2 was upregulated, whereas CDK4 activity was inhibited by p16INK4 [76]. CDKACT4 technology was further improved by developing a lanthanide derivative [75]. Specifically, a DOTA[Tb(III)] complex was conjugated onto the Rb substrate moiety of the CDKACT4 design and the tryptophan group within the PAABD served as an antenna to sensitize the lanthanide. This luminescent derivative of CDKACT4 proved to be 3-4 times more sensitive to phosphorylation as compared to CDKACT4 labelled with a variety of other fluorophore. Moreover we showed that Tb-CDKACT4 could report on dose-dependent inhibition of CDK4 activity by Abemaciclib in melanoma cell extracts, in a highly similar fashion to inhibition of A375 cell proliferation.

A CDKACT6 biosensor was engineered by synthesizing several peptides derived from CDK6-specific substrates from the glucose metabolism pathway, ie phosphofructokinase and pyruvate kinase M2 [78]. This rational approach to select the most appropriate substrate revealed that only peptides derived from phosphofructokinase were recognized by CDK6 and be best

candidate displayed complete selectivity for CDK6 over CDK4, its most closely related kinase. Combined with the PAABD derived from Pin1 which was optimized for CDKACT4, we engineered a peptide biosensor labelled with TAMRA, which responds in a dose-dependent fashion to CDK6 activity from cultured cell lysates. In this study we further combined CDKACT6 and CDKACT4 biosensors to profile the kinase activities of CDK4 and CDK6 in cell lines derived from patients with mesothelioma, thereby revealing different patterns of response.

A panel of CDK5 biosensors were designed based on the literature and in particular a comparative phosphoproteomics study of substrates in mice expressing or lacking CDK5 [77]. As for other CDKs, these substrate sequences were combined with the PAABD of CDKACT4 and 6 and screened *in vitro* against recombinant CDK5 and CDK2, one of its most closely related kinase. Interestingly, only one of these biosensors responded selectively to CDK5 over CDK2, and this peptide was therefore further characterized using cell extracts from glioblastoma cell line U87 in which CDK5 is hyperactivated. Moreover, we designed a self-cell-penetrating derivative to facilitate cellular internalization of CDKACT5 and image CDK5 activity in U87 cells by fluorescence microscopy. These studies allowed us to visualize activation of CDK5 at the cell membrane prior to its diffusion into the cytoplasm and further allowed us to demonstrate that CDK5 activity occurs in parallel to calcium oscillations in U87 cells.

More recently we engineered a hybrid CDKACT1 nanobiosensor by immobilizing CDK1-specific peptide biosensor onto the surface of multiwall carbon nanotubes (MWCNTs) [79]. This hybrid biosensor responds to recombinant CDK1 but not to CDK2 and displays much greater sensitivity to kinase activity *in vitro* than CDKACT1 peptide alone. Moreover MWCNT-CDKACT1 penetrates readily into living cells and was successfully implemented to quantify differences in cultured cells treated with CDK1 inhibitor, as well as to *in vivo* imaging in mouse models.

CDKACT biosensors therefore translate CDK kinase activity into sensitive changes in fluorescence intensity and their synthetic nature enables quantification of CDK activity *in vitro* and in living cells, as well as *in vivo*, thanks to different strategies facilitating their cellular uptake.

We have more recently combined our ensemble of fluorescent peptide biosensors to profile several CDK activities simultaneously in lysates from cell lines derived from human cancer patients and in human biopsies from lung adenocarcinoma and lymphoma in collaboration with several partners associated with French hospitals (C. Blanquart, INSERM-CHU Nantes; CRB-CHU Montpellier). Our studies reveal that patients with different CDK signatures may be stratified into different groups and may be predisposed to different therapeutic responses [78 and personal communication]. This multiplex optical biosensing approach enables characterization of several distinctive PK signatures from one same sample, thereby providing functional information of potential utility for diagnostic purposes to guide therapeutic decisions.

4. Conclusions & Perspectives

Fluorescent peptide biosensors constitute selective optical probes that offer straightforward means of sensing and quantifying biomarkers of interest *in vitro*, in living cells and *in vivo*, following microinjection or facilitated delivery strategies. The intrinsic sensitivity of environmentally-sensitive fluorophores incorporated in synthetic biosensors enables detection and

quantification of biomarker alterations such as PK activities in pathological settings and in response to stimuli or drugs. As such, they constitute attractive tools for cancer diagnostics, for evaluating response to therapeutics and development of sensitive assays for drug discovery programmes [15-17].

Several examples from our lab and others highlight the potential of synthetic biosensors for detection and quantification of PKs in a complex physio-pathological environment. Moreover, given their synthetic, controllable nature, fluorescent peptide biosensors are potentially amenable to predictive molecular diagnostics, monitoring of disease progression and response to therapeutics. For instance synthetic fluorescent biosensors have already been developed to detect dysregulated PK biomarkers in leukemia [37] or for point-of-care monitoring of therapeutic drugs in plasma and urine [47].

Several smart strategies have been devised to engineer more selective, sensitive and responsive biosensors thanks to new generations of highly sensitive fluorophores or through strategic incorporation of quenchers to silence fluorescence in the absence of the target. The sensitivity and selectivity of fluorescent biosensors allows for their implementation to detect subnanomolar concentrations of biomarkers and report on enzymatic activities of interest in highly complex samples.

CDKACT biosensors offer straightforward means of quantifying differences CDK/Cyclin kinase activities between healthy and cancer cells, and can report on alterations in response to therapeutics *in vitro*, in living cells and in mice, following cellular internalization mediated by cell-penetrating peptides or through functionalization of carbon nanotubes. Given the central role and implication of this family of kinases in disease pathways, and the recent FDA-approval of several inhibitors targeting CDKs for clinical therapeutics, CDKACT biosensors constitute attractive tools for developing diagnostics and companion assays. These selective optical probes have further been implemented to multiplexed detection of several CDKs in a variety of cancer cell extracts and are readily applicable to human biopsies. We have taken further advantage of CDKACT technology to profile several CDK activities simultaneously in human biopsies. This multiplex approach has enabled us to highlight distinct PK signatures in samples derived from lymphoma, lung and skin cancers, thereby providing critical information for diagnostic purposes. As such they constitute attractive tools for cancer diagnostics and for evaluating response to therapeutics in drug discovery programmes.

In the future immobilization of fluorescent peptide biosensors onto chips or nanomaterials to generate arrays for multiplex signaling should provide efficient means of analyzing complex samples in a high throughput fashion and profiling biomarker signatures in pathological samples. Development of point-of-care devices and companion assays would also greatly improve and speed up data collection. Personalized and point-of-care approaches based on synthetic biosensors that can be readily combined with targeted therapeutics will offer significant promises for advancing personalized medicine.

Acknowledgements

M.C. Morris thanks all the members of her group who have contributed to developing CDKACT peptide biosensors, as well as collaborators who have shared critical discussions and advice on biosensor improvement and implementation. Recent work on CDKACT biosensing applications was supported by the French Region Occitanie and the European Regional Development Fund (READYNOV/FEDER no. 2018-003539-01), La Ligue Régionale contre le Cancer and Montpellier University Site of Excellence (MUSE) Key Initiative Biomarkers & Therapy.

Keywords: Biosensor • Cancer • Fluorescence • Kinase • Peptide

Conflict of Interest

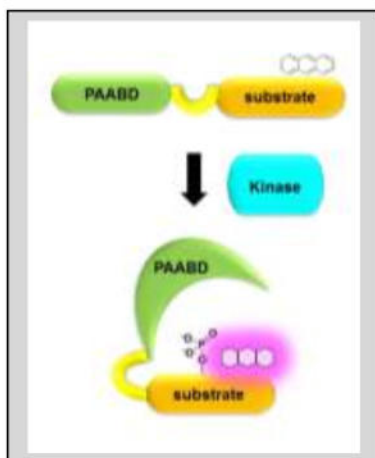
The author declares no conflict of interest

References

- [1] E. C. Greenwald, S. Mehta, J. Zhang, *Chem. Rev.* **2018**, *118*, 11707–11794
- [2] J. Zhang, R.E. Campbell, A.Y. Ting, R.Y. Tsiens, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906-918.
- [3] W. Lin, S. Mehta, J. Zhang, *J.Biol.Chem.* **2019**, *294*, 14814-14822
- [4] L. Oldach, J. Zhang, *Chem. Biol.* **2014**, *21*, 186–197
- [5] A. Ibraheem, R.E. Campbell, *Curr Opin Chem Biol.* **2010**, *14*, 30-6.
- [6] A.E. Palmer, Y. Qin, J.G. Park, J.E. McCombs, *Trends Biotechnol.* **2011**, *29*, 144-152.
- [7] S.B. VanEngelenburg, A.E. Palmer, *Curr Opin Chem Biol.* **2008**, *12*, 60–65.
- [8] H. Wang, E. Nakata, I. Hamachi, *ChemBiochem.* **2009**, *10*, 2560-2577
- [9] E. Pazos, O.Vázquez, J.L. Mascareñas, M.E. Vázquez, *Chem. Soc. Rev.* **2009**, *38*, 3348
- [10] M.K. Tarrant, P.A. Cole, *Ann.Rev.Biochem.* **2009**, *78*, 797-825
- [11] M.C. Morris, *Cell Biochem Biophys.* **2010**, *56*, 19-37
- [12] J.A. González-Vera, *Chem Soc Rev.* **2012**, *41*, 1652-1664.
- [13] J.A. González-Vera, M.C. Morris, **2015**, *Proteomes*, *3*, 369-410
- [14] T.N.N. Van, M.C. Morris, *Prog Mol Biol Transl Sci.* **2013**, *113*, 217-74.
- [15] M.C.Morris, *ACS Med Chem Lett.* **2013**, *5*, 99-101.
- [16] C.Prével, L.Kurzawa, T.N.N. Van, M.C. Morris, *Eur. J. Med.Chem.* **2014**, *88*, 74-88
- [17] C. Prével, M. Pellerano, T.N.N. Van, M.C. Morris, *Biotechnol.J.* **2014**, *9*, 253-265
- [18] T.Tamura, I.Hamachi, *ACS Chem. Biol.*, **2014**, *9*, 2708–2717
- [19] M.C. Morris, S.Deshayes, F. Heitz, G. Divita, *Biol. Cell*, **2008**, *100*, 201-217
- [20] F.Heitz, M.C.Morris, G. Divita, *Br J Pharmacol.* **2009**, *157*, 195-206.
- [21] S.B.Fonseca, M.P. Pereira, S.O.Kelly, *Adv. Drug Deliv.Rev.* **2009**, *61*, 953-964.
- [22] C.M. Tilmaciu, M.C. Morris, *Front.Chem.*, **2015**, *3*, 59.
- [23] E.A. Lemke, C. Schultz, *Nat Chem. Biol.* **2011**, *7*, 480-483
- [24] E.J. New, *ACS Sensors*, **2016**, *1*, 328-333
- [25] L.D.Lavis, R.T. Raines, *ACS Chem. Biol.* **2008**, *3*, 142-155.
- [26] L.D.Lavis, R.T. Raines RT. *ACS Chem. Biol.* **2014**, *9*, 855-866.
- [27] G.S. Loving, M. Sainlos, B. Imperiali, *Trends Biotechnol.* **2010**, *28*, 73-83
- [28] A.S. Klymchenko, *Acc.Chem.Res.* **2017**, *50*, 366-375
- [29] G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I.R. Jr Corrêa, Z.G. Luo, C. Schultz, E.A. Lemke, P. Heppenstall, C. Eggeling, S.Manley, K. Johnsson K. *Nat Chem.* **2013**, 132-9
- [30] J.B. Grimm, B.P. English, J. Chen, J.P. Slaughter, Z. Zhang, A. Revyakin R. Patel, J.J. Macklin, D. Normanno, R.H. Singer, T. Lionnet, L.D. Lavis, *Nat Methods.* **2015**, *12*, 244-50
- [31] J.B. Grimm, A.K. Muthusamy, Y. Liang, T.A. Brown, W.C.Lemon, R. Patel, R. Lu, J.J. Macklin, P.J. Keller, N. Ji, L.D. Lavis, *Nat Methods.* **2017**, *14*, 987-994.
- [32] B. Dumat, G. Bordeau, A.I. Aranda, F. Mahuteau-Betzer, Y. El Harfouch, G. Metgé, F. Charra, C. Fiorini-Debuisschert, M.P. Teulade-Fichou, *Org Biomol Chem.* **2012**, *10*, 6054-61.
- [33] M. Auvray, F. Bolze, G. Clavier, F. Mahuteau-Betzer, *Dyes Pigm.*, **2021**, *187*, 109083.

- [34] F. Mahuteau-Betzer, M. Auvray, F. Bolze, D. Naud-Martin, M. Poulain, M. Bossuat, G. Clavier, *Chemistry*, **2022**, *28*, e202104378
- [35] P. Nalbant, L. Hodgson, V. Kraynov, A. Toutchkine, K.M. Hahn, *Science*, **2004**, *305*, 1615-19
- [36] M.A. Brun, K-T. Tan, E. Nakata, M.J. Hinner, K. Johnsson, *J.Am.Chem Soc.* **2009**, *131*, 5873-5884.
- [37] R. Griss, A. Schena, L. Reymond, L. Patiny, D. Werner, C.E. Tinberg, D.Baker, K. Johnsson *Nat Chem Biol.* **2014**, *10*, 598-603.
- [38] E.D. Fleuren, L. Zhang, J. Wu, R.J. Daly, *Nat Rev Cancer*, **2016** *16*, 83-98.
- [39] P. Wu, T.E. Nielsen, M.H. Clausen, *Trends Pharmacol Sci.* **2015**, *36*, 422-39.
- [40] R. Roskoski Jr. *Pharmacol Res*, **2021**, *165*, 105463
- [41] P. Cohen, D. Cross, P.A. Janne *Nat Rev Drug Discov*, **2021**, *20*, 551-569
- [42] W.C. Hahn, R.A. Weinberg, *Nat. Rev. Cancer*, **2002**, *2*, 331-341
- [43] D. Fabbro S.W. Cowan-Jacob, H. Moebitz, *Br J Pharmacol.* **2015**, *172*, 2675-2700.
- [44] C.A. Chen, R.H. Yeh, X. Yan, D.S. Lawrence, *Biochim. Biophys. Acta* **2004**, *1697*, 39-51.
- [45] V. Sharma, V., Q. Wang, D.S. Lawrence, *Biochim Biophys Acta.* **2008**, *1784*, 94-99.
- [46] D.S.Lawrence, Q. Wang, *Chembiochem.* **2007**, *8*, 373-378.
- [47] Q. Wang, E.I. Zimmerman, A. Toutchkine, T.D. Martin, L.M. Graves, D.S. Lawrence, *ACS Chem Biol.* **2010**, *5*, 887-95.
- [48] E. Luković, J.A. González-Vera, B. Imperiali, *J. Am. Chem. Soc.* **2008**, *130*, 12821-12827
- [49] E. Lukovic, E. Vogel Taylor, B. Imperiali, *Angew.Chem.Int.Ed.Engl.* **2009**, *48*, 6828-6831
- [50] M.D. Shults, B. Imperiali, *J Am Chem Soc.* **2003**, *125*, 14248-9.
- [51] M.D. Shults, K.A. Janes, D.A. Lauffenburger, B. Imperiali, *Nat Methods.* **2005**, *2*, 277-83.
- [52] S. Balakrishnan, N. J. Zondlo, *J. Am. Chem. Soc.* **2006**, *128*, 5590-5591
- [53] S.C. Zondlo, F. Gao, N.J. Zondlo, *J. Am. Chem. Soc.* **2010**, *132*, 5619-5621
- [54] M.S. Tremblay, M. Lee, D. Sames, *Org. Lett.* **2008**, *10*, 5-8
- [55] A.M. Lipchik, L.L. Parker, *Anal.Chem.* **2013**, *85*, 2582-2588.
- [56] A.M. Lipchik, M. Perez, S. Bolton, V. Dumrongprechachan, S.B. Ouellette, W. Cui, L.L. Parker, *J.Am.Chem.Soc.* **2015**, *137*, 2484-2494.
- [57] A.M. Lipchik, M. Perez, W. Cui, L.L. Parker, *Anal. Chem.* **2015**, *87*, 7555-7558.
- [58] S. Jena S., N.P. Damayanti, J. Tan, E.D. Pratt, J.M.K. Irudayaraj, L.L. Parker *Chem.Commun.* **2020**, *56*, 13409-13412.
- [59] N.P. Damayanti, L.L. Parker, J.M. Irudayaraj, *Angew Chem Int Ed Engl.* **2013** *52*,3931-4..
- [60] S. Jena, L.L. Parker, *Methods Mol Biol.* **2022**, *2394*,133-162.
- [61] T. Sakamoto, A. Ojida, I. Hamachi, *Chem. Commun.*, **2009**, 141-152
- [62] T. Anai, E. Nakata, Y. Koshi, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2007**, *132*, 5619-5621
- [63] S. Lim, P.Kaldis, *Development* **2013**, *140*, 3079-93
- [64] M. Malumbres, *Physiol Rev.* **2011** *91*, 973-1007
- [65] D.O. Morgan, *Annu Rev Cell Dev Biol.* **1997** *13*, 261-291.
- [66] A.J. Obaya, J.M. Sedivy, *Cell. Mol. Life Sci.* **2002** *59*, 126-142.
- [67] A.Satyanarayana, P. Kaldis, *Oncogene* **2009** *28*, 2925-2939.
- [68] P. Hydbring, M. Malumbres, P. Sicinski, *Nat Rev Mol Cell Biol.* **2016** *17*, 280-92
- [69] M. Peyressatre, C. Prével, M. Pellerano, M.C. Morris *Cancers* **2015**, *7*, 179-237.
- [70] M. Malumbres, M. Barbacid, *Curr. Opin. Genet. Dev.* **2007** *17*, 60-65.
- [71] U. Asghar, A.K. Witkiewicz, N.C. Turner, E.S. Knudsen, *Nat. Rev. Drug Discov.*, **2015**, *14*, 130-146
- [72] S. Lapenna, A. Giordano, *Nat Rev Drug Discov.* **2009** *8*, 547-66.
- [73] T.N.N. Van, M. Pellerano, S. Lykaso, M.C. Morris, *ChemBioChem*, **2014**, *15*, 2298-305
- [74] C. Prével, M. Pellerano, J.A. Gonzalez-Vera, P. Henri, L. Meunier, J. Vollaire, V. Josserand, M.C.Morris, *Biosens. Bioelectron.* **2016**, *85*,371-380.
- [75] J.A. Gonzalez-Vera, D. Bouzada, C. Bouclier, E.M. Vazquez, M.C.Morris, *Chem.Commun.* **2017**, *53*,6109-6112.
- [76] P. Henri, C. Prevel, M. Pellerano, J. Lacotte, P.E. Stoebner, M.C. Morris, L.Meunier, *Br J Dermatol.*, **2019**, *182*, 678-689.
- [77] M. Peyressatre, A.Laure, M. Pellerano, H. Boukhaddaoui, I. Soussi, M.C. Morris, *Biotechnol. J.* **2020**, e1900474.
- [78] J.Soamalala, S.Diot, M. Pellerano, C. Blanquart, M. Galibert, M. Jullian, K.Puget, M.C. Morris, *ChemBioChem.* **2020**, *22*, 1065-1071
- [79] C.M. Tilmaciu, B. Dinesh, M. Pellerano, S. Diot, M. Guidetti, J. Vollaire, A. Bianco, C. Ménard-Moyon, V.Josserand, M.C.Morris, *Small*, **2021**, *17*, e2007177

Entry for the Table of Contents



This review reports on Fluorescent Peptide Biosensors designed to report on PK activities, highlighting a toolbox of biosensors tailored to report on cyclin-dependent kinases. These selective optical probes enable quantification of differences in kinase activities between healthy and cancer cells and constitute attractive and powerful chemical tools for diagnostic applications.