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REVIEW ARTICLE

Fluorescent Biosensors of Intracellular Targets from Genetically Encoded Reporters to Modular Polypeptide Probes

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Abstract With the escalation of drug discovery programmes, it has become essential to visualize and monitor biological activities in healthy and pathological cells, with high spatial and temporal resolution. To this aim, the development of probes and sensors, which can report on the levels and activities of specific intracellular targets, has become essential. Together with the discovery of the Green Fluorescent Protein (GFP), and the development of GFP-based reporters, recent advances in the synthesis of small molecule fluorescent probes, and the explosion of fluorescence-based imaging technologies, the biosensor field has witnessed a dramatic expansion of fluorescence-based reporters which can be applied to complex biological samples, living cells and tissues to probe protein/protein interactions, conformational changes and posttranslational modifications. Here, we review recent developments in the field of fluorescent biosensor technology. We describe different varieties and categories of fluorescent biosensors together with an overview of the technologies commonly employed to image biosensors in cellulo and in vivo. We discuss issues and strategies related to the choice of synthetic fluorescent probes, labelling, quenching, caging and intracellular delivery of biosensors. Finally, we provide examples of some well-characterized genetically encoded FRET reporter systems, peptide and protein biosensors and describe biosensor applications in a wide variety of fields.

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Abbreviations

CDK	Cyclin-dependent kinase
CPP	Cell-penetrating peptide
CRS	Cytoplasmic retention sequence
FACS	Fluorescence-activated cell sorting
FCS	Fluorescence correlation spectroscopy
FP	Fluorescent protein
FRET	Fluorescence resonance energy transfer
FLIM	Fuorescence life-time imaging
GFP	Green fluorescent protein
IRFP	Infra-red fluorescent protein
Mant	Methylanthraniloyl
MMP	Matrix metalloproteinase
NIRF	Near-infrared fluorescence
NLS	Nuclear localization sequence
PBD	p21-Derived binding domain
PTD	Protein transduction domain
RBD	RhoA-Binding domain

Introduction

With the escalation of drug discovery programmes, it has become essential to visualize biological activities in healthy and pathological cells, and to monitor their spatial and temporal dynamics for a comprehensive understanding of their behaviour. Together with the development of fluorescent probes and evergrowing advances in imaging technologies, biosensor systems capable of probing and reporting on the presence and activity of specific targets and biomarkers have come of age.

Biosensors: Definition and General Concepts

Biosensors are defined as "analytical devices" which combine a biological component derived from biological or biomimetic material (e.g. tissue, microorganism, receptor, enzyme, antibody, nucleic acid, etc.) with a physicochemical transducer (optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical). Whilst the biological component is responsible for sensing a specific analyte or target, the physicochemical transducer converts recognition of the analyte into a detectable and measurable signal, a suitable quantitative output (Fig. 1) [1]. Biosensors offer a convenient, rapid, specific and sensitive means of monitoring analytes and of reporting on the presence of specific target substrates or enzymes, through a signal which is generally proportional to the concentration or activity of the target.

Whilst the type of biosensor will really be adapted to meet the needs of an individual application, its specificity and sensitivity is paramount for its utility and applicability within a complex physiological ensemble or solution. Although such criteria are often inherently defined by the biological molecules, the sensors derive from, they may be improved to optimize the selectivity of target recognition thanks to molecular biology and protein engineering approaches.

Over the years the development of biosensors has evolved together with technologies available and the applications requiring this type of device. The first biosensor can undoubtedly be traced down to the invention of the electrochemical oxygen biosensor by Clark [2], closely followed by the development of a glucose biosensor through combination of glucose oxidase as an enzyme transducer to an oxygen electrode in 1962 [3]. In 1969, the first potentiometric enzyme electrode was developed by Guilbault and Montalvo [4], the urea sensor, based on immobilization of urease onto an ammonium-selective liquid membrane electrode. Thermal enzyme probes also known as thermistors were next developed by Mosbach et al. [5, 6]. In 1975, the first "microbial electrodes", based on the use of bacterial microorganisms were developed for alcohol measurement by Divis [7], whilst Lubbers and Oppitz [8, 9] developed the first fibre-optic sensors or "optodes" which were applied to measure carbon dioxide, oxygen and alcohol, respectively. In 1976, Clemens et al. [10] introduced an electrochemical glucose biosensor into an artificial pancreas, which would later be improved and commercialized for generalized use in the clinic. A real breakthrough for in vivo applications was the development of needle-type enzyme electrodes for subcutaneous implantation by Shichiri et al. [11]. In 1983, an altogether novel type of biosensor was developed by Liedberg et al. [12], the immunosensor, precursor of the widely used BIAcore system, based on immobilization of antibodies to a piezoelectric or potentiometric transducer, thereby enabling immuno-recognition of analytes by surface plasmon resonance. Ever since biosensor technology has expanded to meet the needs of a variety of applications and analytical problems in medicine, environment, food and process industries, security and defence [13]. Over the past decade, a number of optical biosensors based on surface plasmon resonance, waveguides and resonant mirrors have been developed to monitor a variety of biomolecular interactions between label-free targets in vitro, with important applications in the field of drug discovery [14]. In parallel, thanks to significant advances in the development of fluorescent probes, in particular green fluorescent protein (GFP)-based systems by Roger Tsien, and more recently of small fluorescent dyes with enhanced spectral properties for in vivo imaging, and the concomitant explosion of fluorescent imaging technologies, the biosensor field has witnessed a dramatic expansion of fluorescence-based biosensors for probing protein activities, conformational changes and postranslational modifications in living cells with high spatial and temporal resolution [15–27].

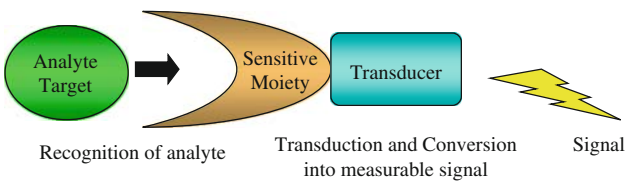

Fig. 1 Generic definition of biosensors. Biosensors can be defined as analytical devices which combine a biological component, a biologically derived or a biomimetic material, responsible for sensing a specific analyte or target, with a physiochemical transducer which converts recognition of the analyte into a detectable and measurable signal

Fluorescence-Based Biosensors

Fluorescence-based biosensors are useful tools for detection of biomolecules both in vitro and in vivo. Fluorescence is sensitive, specific and lends itself to non-destructive imaging. As such high-resolution imaging and real-time measurements of fluorescent biosensors have provided precious information on the spatial and the temporal localization of a wide variety of intracellular targets and have proven a successful means of visualizing dynamic processes and of monitoring enzymatic activities in living cells, as well as useful tools for drug discovery [23, 24, 28–36]. In order to transduce ligand binding into a detectable optical signal, fluorescence-based biosensors bear a sensing moiety that is either genetically or chemically coupled to

(a) fluorescent probe (s) whose spectral properties undergo measurable variations upon target recognition.

Fluorescent biosensors come in all sorts of flavours, which are commonly distinguished based on their chemical nature, i.e. genetically encoded, peptide-, protein, or nucleotide-based, or on the fluorescence technology applied to monitor target recognition, i.e. fluorescence resonance energy transfer (FRET)-based or environmentally sensitive. Moreover, fluorescent biosensors may be designed to probe an enzymatic activity, or to recognize a specific target or conformation, and are consequently referred to as activity-, ligand- or affinity-based, respectively. These different families of biosensors overlap; as such activity-based biosensors may be either genetically encoded, peptide- or nucleotide-based; peptide-based biosensors may be FRET-based or environmentally sensitive, activity- or affinity-based. The characteristics of some of the most common fluorescent biosensors are briefly described below and schematized in Fig. 2.

Activity-based reporters are amongst the most common fluorescent biosensors developed for monitoring specific enzymatic activities. They consist of enzyme substrates, typically polypeptide sequences which undergo a specific physico-chemical modification, such as phosphorylation, methylation, ubiquitinylation or enzymatic cleavage by the target enzyme, which in turn affects the spectral properties of the associated fluorescent probe(s), leading to measurable changes in fluorescence (Fig. 2a–c). Typical examples include genetically encoded biosensors that monitor kinase activity through phosphorylation-induced intramolecular FRET (Fig. 2a), peptide-based biosensors that monitor proteolytic activities through cleavage-induced abrogation of intramolecular FRET (Fig. 2b), and peptide-based biosensors that monitor phosphorylation-induced enhancement of a fluorescent probe adjacent to the phosphorylated residue (Fig. 2c).

Nucleotide-based sensors consist of fluorescently labelled nucleotide analogs whose fluorescent properties are environmentally sensitive, and modified upon incorporation into the nucleotide-binding pocket of target enzymes, such as methylantraniloyl-GTP or -ATP (Fig. 2d). In particular, this strategy has been widely applied to study ATPase and GTPase activities, the dynamics and conformational changes of protein kinases and motor proteins. Although nucleotide-based probes remain very useful for in vitro studies of enzyme activities, their application to living cells

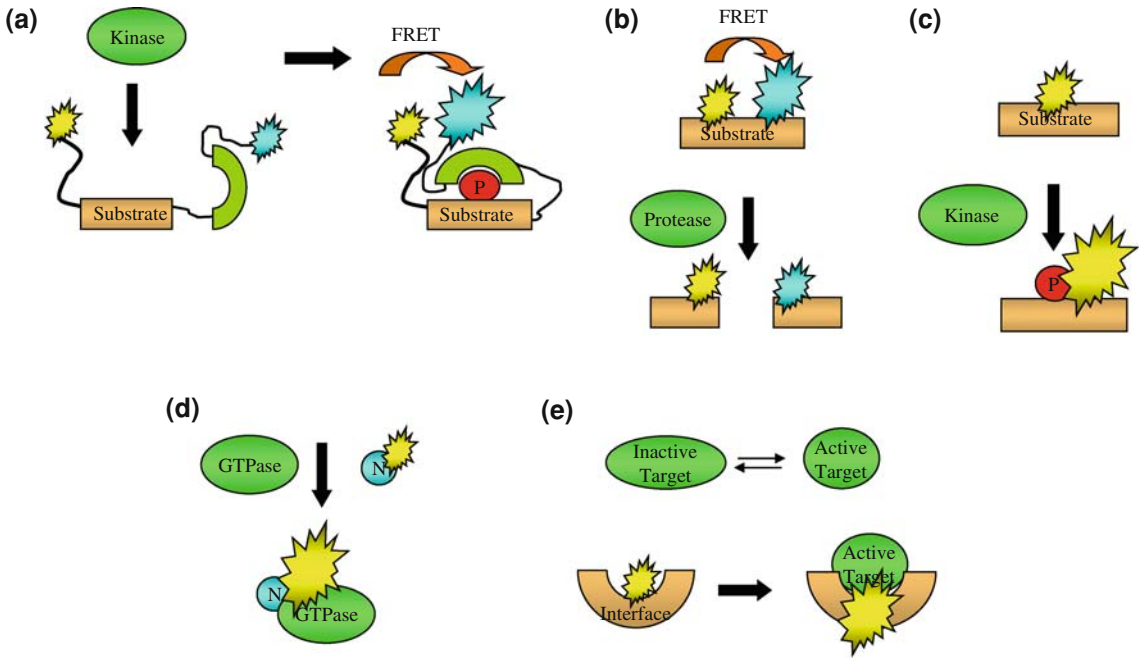

Fig. 2 Common examples of fluorescence-based biosensors. Schematic representation of different varieties of fluorescence-based biosensors. **a**, **b**, **c** Activity-based reporters consist of polypeptide sequences which undergo a specific physico-chemical modification by the target enzyme which affects fluorescence of the probe. **a** FRET-based genetically encoded single-chain biosensor of protein kinase A activity through phosphorylation-induced intramolecular FRET, **b** peptide-based biosensor of proteolytic activity through cleavage-induced abrogation of intramolecular FRET, **c** peptide-based biosensor of protein phosphorylation through enhancement of adjacent probe fluorescence, **d** Nucleotide-based sensors consist of fluorescently labelled nucleotide analogs whose fluorescence is generally enhanced upon incorporation into the nucleotide-binding pocket of target enzymes, **e** interfacial biosensors consist of polypeptide domains or sequences that recognize unique interfaces or bind a specific conformation of the target, thereby generating a measurable variation of associated probe fluorescence

is limited, due to an inherent lack of specificity, as they cannot discriminate between different enzymes requiring these nucleotide cofactors for their activity.

Affinity-based or interfacial biosensors can be described as reporters harbouring a polypeptide sequence or domain that recognizes a unique interface or which binds a specific conformation of a target protein, thereby generating a measurable variation of associated probe fluorescence (Fig. 2e). Affinity-based biosensors can provide a high degree of specificity for target recognition, and are excellent alternatives to activity-based probes for monitoring active enzyme conformations in vitro and in cellulo.

Genetically Encoded Versus Non-Genetic Biosensors

The first fluorescent biosensors of protein function were derived from proteins, peptides or nucleotides which were chemically labelled [28]. Although some of these were introduced into living cells, essentially through microinjection or electroporation, relatively few were applied to monitor dynamic activities in cellulo, let alone in vivo, due to limitations associated with internalization, biodistribution and imaging technologies. The discovery of GFP and the development of GFP-based systems by Roger Tsien truly revolutionized fluorescence technology, allowing for genetic engineering of cell lines that report on the spatiotemporal localization and dynamics of ectopically expressed GFP-fusion products [15–25]. Moreover, this technology has provided a solid basis for developing genetically encoded fluorescent reporters, to probe enzymatic activities, protein interfaces or conformational changes directly in living cells with high spatial and temporal resolution [29–33]. The GFP fluorophore and its variants are excellent tools for probing biological components and activities in living cells, and genetically encoded GFP-based biosensor technology provides unique opportunities of engineering constructs to suit the specificity of the target. However, this technology is subject to certain limitations, notably the control over the timing and levels of expression within cells, as well as the behaviour of the GFP-fusions, whose size may affect the behaviour of the sensing element, its localization and ability to recognize the intracellular target. As such, there is a growing demand for non-genetic biosensors designed to report on specific biological activities, with maximal resolution, specificity and sensitivity.

Genetically Encoded FRET Biosensors

Genetically encoded single-chain FRET biosensors constitute one of the most widespread classes of fluorescent biosensors. They consist of plasmid constructs engineered to express a target reporter sequence (typically a peptide substrate), flanked by a FRET pair of fluorescent proteins derived from GFP (FPs), typically CFP and YFP. Modification of the target sequence upon activation of the target enzyme induces a conformational change that modifies the proximity of the FPs, consequently favouring or disrupting FRET between the donor and the acceptor. The first reporters to provide a valid proof-of-concept of this technology were the fluorescent Ca2+ indicators, dubbed "cameleons", consisting of tandem fusions of GFP variants with calmodulin and the calmodulin-binding peptide M13 [37, 38]. Binding of Ca2+ to calmodulin promotes its binding to the M13 domain, thereby bringing together the FRET pair of FPs and favouring their intramolecular FRET (Fig. 3a). Ever since a wide variety of genetically encoded single-chain FRET biosensors have been developed. Genetically encoded FRET-based biosensors of kinase activity have been generated through incorporation of a kinase-specific substrate sequence and a matching phosphoaminoacid-binding domain in a plasmid construct, together with a FRET pair of GFP variants (Fig. 3b) [39–47]. A similar approach has been employed to design fluorescent reporters of histone methylation by incorporating a sequence encoding a substrate peptide from the N-terminus of histone H3 fused to a matching chromodomain, between a FRET pair of GFP variants [48]. More recently a biosensor of histone acetylation "Histac" has been developed, consisting of an acetylation-binding bromodomain and a substrate histone H4 [49]. Several elegant FRET-based biosensors have been developed to monitor GTPase activation, bearing a GTPase together with a binding domain which only recognizes the conformationally active form of the enzyme [50, 51] (Fig. 3c). The converse strategy has been applied to generate fluorescent protease reporters to probe proteolytic activities, by sandwiching specific substrate sequences between GFP variants, whose ability to FRET is disrupted by proteolytic cleavage [52–55] (Fig. 3d) (further details are provided in "Biosensor applications").

Peptide- and Polypeptide-Based Biosensors and Probes

Together with the development of new generations of fluorescent probes with enhanced photostability and optimal photophysical properties for cellular applications, quenching and caging strategies for reducing basal fluorescence, as well as technologies enabling direct enzymatic labelling of specific sequences in living cells, the improvement of chemical strategies to synthesize membrane-permeant analogs and the development of suitable and efficient cellular internalization technologies, non-genetically encoded peptide- and polypeptide-based reporters have become attractive alternatives to GFP-based genetically encoded systems (Fig. 4).

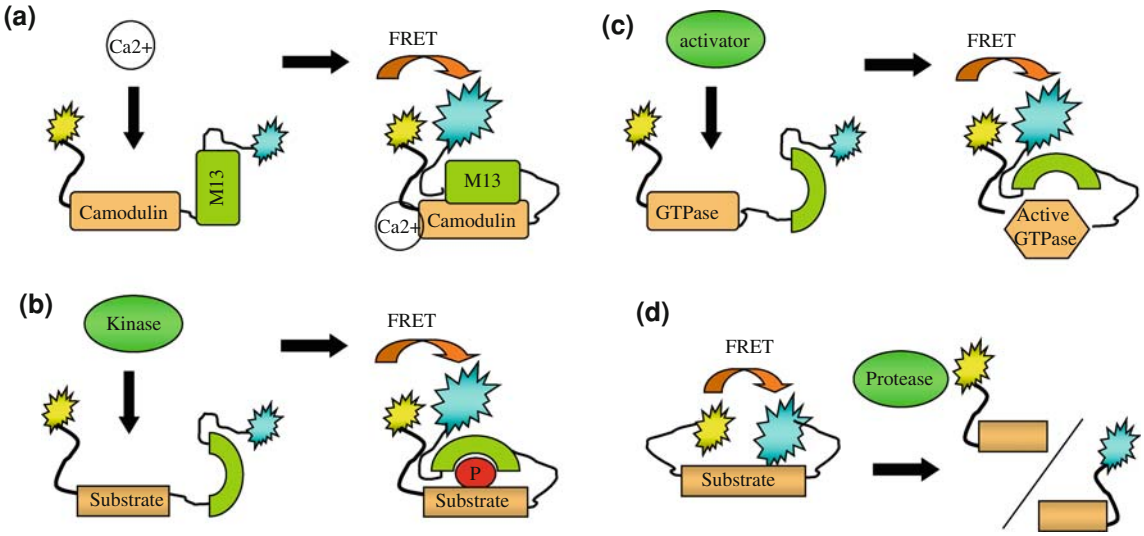

Fig. 3 Genetically encoded single-chain FRET-based biosensors consist of plasmid constructs engineered to express a target reporter sequence, flanked by a FRET pair of fluorescent proteins (FPs) derived from GFP, typically CFP and YFP. Modification of the target sequence upon activation of the target enzyme induces a conformational change that modifies the proximity of the FPs, consequently favouring or abrogating FRET between the donor and the acceptor. **a** Fluorescent Ca2+ indicators or "cameleons", consisting of tandem fusions of GFP variants with calmodulin and the calmodulin-binding peptide M13, **b** FRET-based biosensors of kinase activity consist of tandem fusions of FPs with a kinase-specific substrate sequence and a matching phosphoaminoacid-binding domain, **c** fluorescent reporters of histone methylation bear a sequence encoding a substrate peptide from the N-terminus of histone H3 and a matching chromodomain, between a FRET pair of GFP variants, **d** FRET-based biosensors of GTPase activation bear a GTPase together with a binding domain which only recognizes the conformationally active form of the enzyme, **e** FRET-based protease reporters harbour the specific substrate sequence between GFP variants, whose ability to FRET is abrogated upon proteolytic cleavage

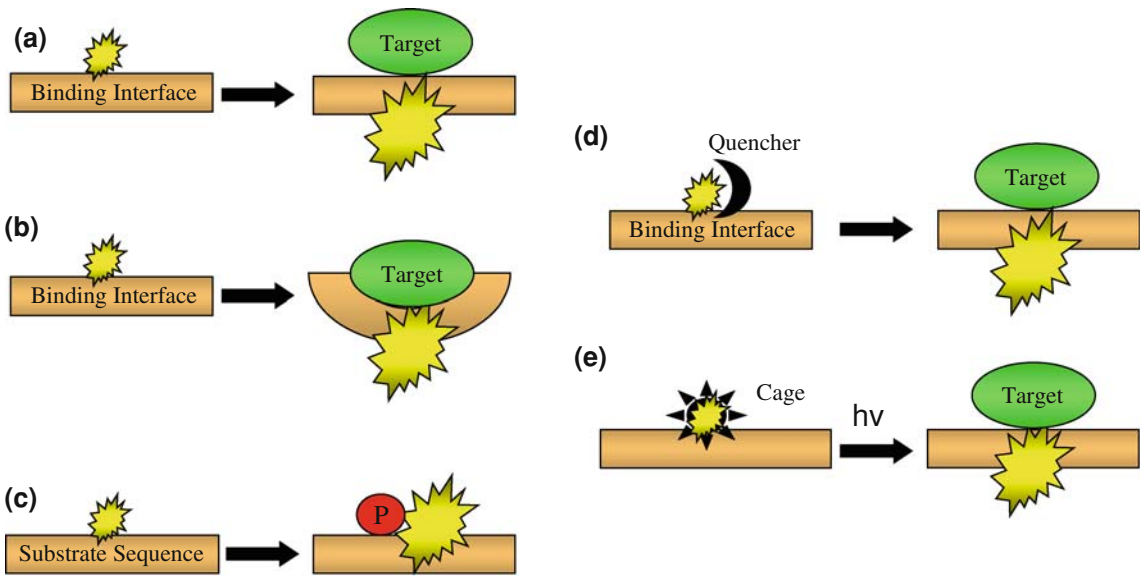

Fig. 4 Peptide- and polypeptide biosensors. Non-genetically encoded biosensors are generated by chemical coupling or enzymatic incorporation of a fluorescent label into a peptide or polypeptide sensing sequence, generally derived from a substrate or a unique-binding interface. Target binding (**a**), target-induced conformational change (**b**) and/or posttranslational modification (**c**) will affect the spectral properties of environmentally sensitive fluorescent probes. Moreover, probes can be internally quenched to minimize noise to signal (**d**) or caged, so as to control release of an active biosensor through selective photoactivation (**e**)

Peptide- and polypeptide biosensors are generated by chemical coupling or enzymatic incorporation of (a) fluorescent label(s) into a peptide or polypeptide sensing sequence, generally derived from a substrate or a unique-binding interface. In principle, a peptide or polypeptide derived from a protein substrate or a partner-binding

interface can function as a sensor, reporting on its interaction with the target enzyme thanks to an "environmentally sensitive" fluorescent probe coupled to a neighbouring amino acid, whose spectral properties will be affected by changes in the local environment, whether these involve interactions with the target, enzyme-induced conformational change, or a chemical modification, such as a phosphorylation. As for genetically encoded systems, the specificity of protein/substrate or protein/partner interactions can be refined, so as to achieve optimal selectivity for target recognition in a complex solution. In addition, biligand strategies can be devised, in which multiple interfaces are combined to recognize a single target, or a heterodimeric complex. Moreover, this technology allows for the use of a wide variety of small molecule fluorescent labels, which can be internally quenched to minimize noise to signal, or caged, so as to control the release of the active biosensor through selective photoactivation. Although a wide variety of peptide- and protein-biosensors have been developed to probe activities of kinases, phosphatases and proteases (see "Biosensor applications" for a more detailed description), few have actually been applied to living cells or in vivo, due to cellular permeability issues associated with their chemical nature. However, advances in the field of cell-penetrating peptides (CPPs) and protein transduction domains (PTDs) have provided efficient means of introducing these biosensors into living cells and in vivo.

Synthetic Fluorescent Probes and Labelling Strategies

In order to obtain a high signal-to-noise ratio in living cells, and to limit the autofluorescence of biological samples, it is important to use fluorophores with long-wave excitation and emission spectral properties. Over the recent years efforts have been made to generate novel generations of fluorescent probes with enhanced photostability and photophysical properties suited to live-cell imaging (for review [26, 27, 56, 57]). In particular, infra-red fluorescent proteins (IRFPs) and near-infra-red fluorescent probes (NIRFs) are of particular interest for cellular and in vivo application as they yield far less tissular damage upon in vivo imaging [57–59] Moreover, the development of

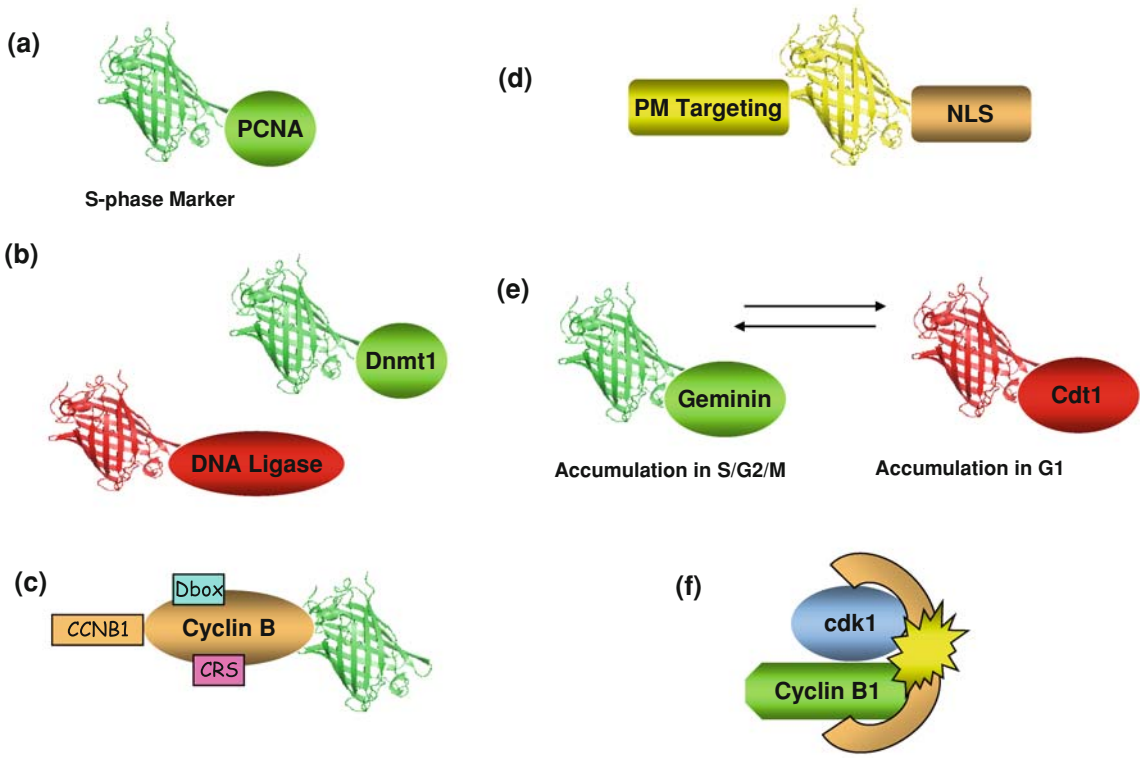

Fig. 5 Cell cycle sensors. (**a**) GFP-PCNA (proliferating cell nuclear antigen) was proposed as a marker of S-phase, as it accumulates in DNA replication foci during S-phase [175–177], **b** the combination of RFP-DNA ligase with GFP-Dnmt1 DNA methyltransferase have been employed to distinguish all cell cycle phases [178], **c** GFP-cyclin B uses functional elements from cyclin B1 including its endogenous promoter (CCNB1) allowing for expression from S phase through G2 and mitosis, its destruction box (D-box), leading to its degradation at the end of mitosis, and its cytoplasmic retention signal (CRS) inactivation of which promotes cyclin B1 translocation into the nucleus [181, 182], **d** genetically encoded YFP associated with a plasma membrane (PM) targeting domain and a nuclear localization sequence (NLS) allows to visualize nuclear envelope breakdown at mitosis [179], **e** the FUCCI system combines GFP-Geminin which accumulates in S/G2/M and is degraded in G1 and RFP-Cdt1 with accumulates in G1 [180], **f** the biligand peptide biosensor of cdk/cyclins recognizes heterodimeric cdk/cyclin complexes [186]

quantum dots, semi-conductor nanocrystals with exceptional optical and spectroscopic properties, have provided an attractive alternative to both small-molecule and protein fluorophores for the high-resolution imaging of intracellular processes and in vivo imaging [60–64]. Quantum dots have been successfully coupled to peptides, proteins and biosensors, through standard chemical approaches, can serve as excellent FRET donors, and have been delivered into cells for intracellular imaging applications thanks to cell-penetrating peptides [62–65]. Small fluorescent labels may be incorporated into the peptide or polypeptide sensor during synthesis or covalently coupled postsynthesis through classical chemical coupling to amino, sulfhydryl, hydroxy or carboxy groups. Alternative approaches have been developed for site-specific fluorescent labelling of recombinant proteins in living cells. The FlAsH system, developed by Tsien and coworkers [66, 67], involves incorporation of a membrane-permeant biarsenical fluorescein derivative into a polypeptide sequence at a specific tetracysteine motif. The transglutaminase-based strategy, developed by Weiss and coworkers [68], enables enzymatic labelling of a reactive glutamine in a specific recognition sequence. The SNAP-tag and the CLIP-tag, developed by Johnsson et al. [69–71] are derived from the human DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) and are used to label proteins in living cells with fluorescent derivatives of O6-benzylguanine and O6-benzylcytosine, respectively. Finally incorporation of genetically encoded fluorescent amino acids during polypeptide synthesis in *E.coli* has been proposed as an alternative to postsynthetic coupling of fluorescent labels [72].

Quenching and Caging Strategies

One of the major limitations of most fluorescent probes is their general fluorescence both in solution and when bound to a target, which largely restricts overall changes in intensity upon recognition of the target. Therefore in an attempt to control and optimize the sensitivity and selectivity of the response so as to obtain a robust fluorescent signal upon target recognition, strategies have been devised to quench and/or cage the fluorophore. Strategies of intramolecular fluorophore quenching have proven to be effective in reducing background fluorescence prior to target binding to fluorescent peptide biosensors both in vitro and in living cells [73–76]. Molecular caging allows the conversion of a biomolecule into an inert form through chemical modification with a photolabile protective group. Selective photoactivation of the caged molecule allows for its release into a form which is readily accessible for its biological function, enzymatic activity or ability to bind a partner. In the case of a biosensor, this provides a means of controlling biosensor/target recognition in space and in time. This approach has been largely developed for selective activation of enzymes and peptides, and provides a means of fine-tuning new generations of fluorescent biosensors [77–82]. An alternative strategy involves direct modification of probes, as exemplified by the esterase-reactive fluorophores developed by Raines and coworkers [83], whose fluorescence is masked by "trimethyl locks", which can be hydrolyzed to yield a robust fluorescent signal. Another noteworthy example is the application of the photoreactive light oxygen voltage (LOV) domain from phototropin which has recently been used to sterically block interactions of the Rac1 GTPase until photoactivation, in an entirely reversible fashion [84].

Intracellular Delivery Strategies

Introduction of molecules with biological activity into living cells, tissues and animals is a major challenge for basic research, as well as for therapeutic and for diagnostic applications. For application in living cells, both genetically encoded reporters and non-genetically encoded fluorescent probes must be delivered through the cell membrane in a non-invasive fashion and be able to reach their intracellular target at the appropriate subcellular location. Whilst genetically encoded biosensors may be introduced into cells thanks to standard transfection, electroporation or microinjection procedures, peptides and polypeptides may rely on a different set of approaches for efficient internalization into cells and tissues, which will preserve their integrity and stability. The first approaches classically employed for introduction of peptides and proteins into cells relied on mechanical or physical techniques, such as microinjection, electroporation or "cell-loading" techniques, which apply a mechanical stress that affects the plasma membrane permeability [85]. Although microinjection may be applied to introduce of polypeptides into a subset of cultured cells, this approach is not applicable to larger populations of cells, tissues or animals. As such, several alternative strategies for delivery of polypeptides and peptides into living cells and animal models have been developed, including lipid-based formulations, cell-penetrating peptides and carbon-based nanoparticles or nanotubes. Although each of these approaches presents specific advantages and limitations, the critical issue is to ensure maximal uptake, whilst preserving cargo integrity and stability. Uptake efficiency is directly related to the chemical nature of the cargo, to the physicochemical characteristics of the carrier and to its mechanism of cellular internalization, and dependent on the cell type, due to differences in membrane components.

PTDs and CPPs constitute a family of polypeptide domains and peptides which can insert into and cross-biological membranes to penetrate into living cells whose

potential has been harnessed to deliver a wide variety of biomolecules, including nucleic acids, polypeptides and synthetic drugs into living cells. Two classes of PTD/CPPs have been developed, the first requiring genetic or chemical linkage between the carrier, or protein transduction domain, and the cargo for cellular internalization, the second based on formation of stable but non-covalent carrier/cargo complexes. CPPs of the first class include protein transduction domains from HIV-1 Tat, the third helix of the homeodomain of Antennapaedia (Penetratin), and oligomers of arginine, which are covalently attached or synthesized as fusions with the molecule to be delivered [86, 87]. Non-covalent CPPs constitute a growing class of peptide carriers, which have been successfully applied for ex vivo and in vivo delivery of therapeutic molecules and several CPP-based strategies are currently being evaluated in clinical trials for viral infection and cancers [86–89]. In particular, cell-penetrating peptide carriers of the Pep family are amphipathic, membranotropic peptides that can mediate cellular uptake of small peptides and large proteins including antibodies, nucleic acid analogs (Peptide Nucleic Acids) and therapeutic drugs with high efficiency [90–92]. Pep carriers present several advantages including rapid delivery of cargoes into cells with very high efficiency, stability in physiological buffers, lack of toxicity and of sensitivity to serum. Moreover, unlike many covalent CPPs, Pep carriers enter cells independently of the endosomal pathway and have been shown to deliver cargoes in a fully biologically active form into a wide variety of cell types, including challenging primary, neuronal and pancreatic cell lines, macrophages and hepatocytes and in animal models [92–94]. As such Pep-based technologies constitute excellent alternatives to covalent strategies, and powerful tools for protein and peptide biosensor applications.

Targeting strategies have been associated to intracellular delivery, so as to favour delivery of cargo to a specific cell or tumour type. Selective delivery of molecules to tumour cells can involve homing strategies or coupling with antibodies targeting to cancer-specific receptors. An increasingly employed strategy for selective targeting of molecules to tumours is the covalent attachment of antibodies or of ligands (e.g. folic acid, avidin, antibody fragments, homing antigens) that recognize cell-surface tumour-associated antigens or receptors [95, 96].

Technologies for Imaging Biosensors In Cellulo and In Vivo

Monitoring intracellular activities thanks to fluorescent biosensors require application of specific fluorescence imaging approaches, which really depend on the nature of the biosensor and on the spectral properties of the associated fluorescent probe. Several technologies to study fluorescence-based processes are available to characterize the information signalled by fluorescence-based biosensors in vitro and in cellulo, in particular fluorescence intensity-based approaches, FRET, fluorescence life-time imaging (FLIM) and fluorescence correlation spectroscopy (FCS).

Intensity-based approaches involve direct measurement of changes in fluorescence intensity, which may be related to target concentration, conformational changes or post-translational modifications. This approach is however somewhat limited when the changes in probe fluorescence are weak, and barely detectable, or when the probe is sensitive to environmental factors other than target activity or binding, such as local pH in the environment of a subcellular structure or within a subcellular compartment such as lysosomes. Moreover such approaches are equally dependent on the initial concentration of biosensor/probe applied and therefore require some form of standardization over an inert or "passive" probe to obtain a ratiometric signal [97–99].

Förster or fluorescence resonance energy transfer is the non-radiative transfer of energy absorbed by a "donor" to an "acceptor" fluorophore, whose absorption spectrum overlaps with the emission spectrum of the donor, when they are brought into close proximity (<10 nm) and their dipoles are oriented correctly [100–102]. Although FRET technology was largely developed in the 1970s for in vitro studies of protein/protein interactions, its applications in modern biology associated with GFP reporters have brought this technology back into fashion. FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein/protein interactions, protein/DNA and conformational changes [103–106]. FRET-based biosensors generally transduce target activity or biological stimulus through a change in distance or orientation between a couple of fluorophores as a consequence of a posttranslational modification, proteolytic cleavage or protein/protein interaction, which affects the ability of the donor to transfer energy to the acceptor. FRET leads to a decrease in donor intensity and an increase in acceptor intensity, and can therefore be determined through an intensiometric or a ratiometric approach by measuring intensities of absorption and emission of the donor and the acceptor fluorophores using wide-field or confocal imaging [29, 32, 33, 106, 107]. Whilst the intensiometric approach suffers certain limitations, the ratiometric approach is inherently quantitative. FRET also leads to a decrease in donor lifetime and can therefore be measured by FLIM.

FLIM is a very useful approach for biomedical tissue imaging. Indeed, changes in the lifetime or in the exponential decay rate of the fluorescence of a fluorophore indicate a change in its local environment, such as an interaction with a partner. FLIM may be measured for

single fluorophores or applied in conjunction with FRET, when ratiometric imaging is problematic, as energy transfer from the donor to the acceptor affects its fluorescence lifetime [107, 108]. FLIM is particularly useful for measuring FRET as it is altogether independent of probe concentration. Moreover, FLIM is particularly useful for imaging multiple probes simultaneously (multiparametric imaging) as it places little constraints on the spectra of fluorophores [33]. However, the equipment required for this technology is less accessible than standard widefield or confocal microscopes, thereby limiting its application.

FCS is a technique which is widely applied in vitro and which has recently become applicable in cellulo that relies on measurements of fluorescence intensity fluctuations associated with protein dynamics in solution (or in the cellular milieu) which are affected by changes in molecular size, typically upon binding with partners [109, 110]. FCS measurements are commonly acquired through confocal or two photon microscopy and then analysed by temporal analysis of small spontaneous deviations from the average fluorescence of the sample (temporal autocorrelation) to obtain quantitative information on the kinetics and thermodynamics of processes associated with reversible changes in fluorescence, such as diffusion coefficients and flow rates, hydrodynamic radii, particle concentration, aggregate formation, kinetic rate constants of chemical reactions and triplet lifetimes and populations. Although this approach is extremely sensitive for the fine characterization of biosensor behaviour in an intracellular environment, it is also very sensitive to fluctuations associated with noise due to scattering, autofluorescence, photobleaching and cell damage. However, most problems can be overcome by selecting probes absorbing at high wavelengths (>550 nm) with high quantum yields and photostability.

Shortcomings of Fluorescence-Based Studies

Each of these methodologies comes with its set of advantages and limitations, providing useful information for different types of applications. Accurate quantification of fluorescent signals requires one to pay attention to the different parameters which may affect the precision and quality of the measurement, including background signal, noise and focus, autofluorescence, bleedthrough or crosstalk, photobleaching, fluorophore quenching, specimen preparation and instrumentation [99, 106]. As such, it is preferable to work with live versus fixed cells to limit artefacts due to the fixation procedure itself, autofluorescence and fluorophore quenching and to substract background signal. High signal-to-noise ratio and limited autofluorescence are preferentially obtained with fluorophores with long-wave excitation and emission spectral properties. Probe photobleaching may limit live cell and in vivo imaging, and provide false information in a ratiometric analysis, as the rate of probe photobleaching will differ from one fluorophore to another. It is therefore wise to choose probes with high quantum yields and photostability in the design of biosensors for live-cell imaging and in vivo applications and to correct for photobleaching when seeking an accurate quantitation of fluorescence intensity over time. Last but not least, phototoxicity may arise through intense light exposure and/or long time periods of irradiation, and is therefore associated with weak fluorescent signals emitting at lower wavelengths which require high energy exposure. This is a major issue when dealing with whole-body, tumour or tissue imaging, which can be significantly reduced using NIRFs such as indocyanine green, and applying dual-photon microscopy for in vivo imaging [57, 58]. Near-infrared fluorescence is currently applied to image vasculature and tissue perfusion and has become a very useful means of guiding oncologic surgery [58, 59].

Applications of Fluorescence-Based Biosensors

Faced with the limitations of indirect approaches, such as ectopic expression, gene knockout or siRNA-mediated knockdown, there has been a growing demand for the development of more direct strategies to probe and characterize cellular activities, protein dynamics and function in living cells. As such we have witnessed a real explosion of fluorescent biosensor technologies in a wide variety of fields over the past decade, notably in signal transduction pathways, cell cycle division and proliferation, cancer and metastasis, apoptosis and proteolytic systems, cardiovascular medicine, neuroscience and viral infection.

Fluorescence-based biosensors have a broad utility both in vitro and in vivo, and can further be applied for development of screening and diagnostic strategies. In vitro, fluorescence-based biosensors may be applied to monitor activities of enzymes in solution, used in high-throughput screening assays, to identify cofactors, partners or inhibitors that affect the enzymatic activities or conformations of the target, or even applied to proteomic profiling. In vivo, biosensors allow for direct visualization of endogenous targets and/or activities in real time, and in principle several dynamic processes can be monitored simultaneously through multiparametric spectral imaging. Finally biosensor technology can be adapted to endoscopy or tomographic optical imaging methods in vivo for screening, molecular profiling and oncological surgery.

Sensors of cytoskeletal proteins were amongst the first protein biosensors introduced into living cells for characterizing the dynamics assembly and disassembly of the

actin cytoskeleton [111, 112], of microtubule dynamics [113], of myosin [114] and of several intermediate filaments [115–118]. More recently a peptide biosensor of 17 residues which binds F-actin but does not interfere with actin dynamics, coined "Lifeact", was applied to visualize actin dynamics in vitro and in vivo [119]. LifeAct was fused to GFP and used to study of actin remodelling in living cells without perturbing actin polymerization.

Sensors of second messengers and metabolites such as cAMP and Ca2+, phosphate and glutamate, respectively, have been developed based on recognition domains either fused to FPs or coupled with sensitive probes. Noteworthy examples include the genetically encoded pleckstrin-homology (PH) domain which probes 3′-phosphoinositides [120] and domains derived from PKC to monitor Ca2+ and diacylglycerol [121–123]. The first sensor of cAMP consisted of fluorescently labelled PKA subunits, whose cAMP-mediated dissociation disrupted FRET between the attached fluorophores [124]. This system was then optimized and genetically encoded together with a FRET pair of FPs to monitor cAMP levels in living cells [125]. In the early 1990s, Hahn and colleagues generated a protein biosensor of calcium, MeroCaM, generated through covalent attachment of a merocyanine fluorophore to calmodulin, the spectral properties of which are significantly affected by calcium binding, thereby reporting on changes in calcium concentration in vitro, with high photostability and brightness for cellular applications [126]. A similar approach was later devised by Tsien and colleagues [37, 38] to develop "cameleons", genetically encoded calcium biosensors based on tandem fusions of a FRET pair of FPs, calmodulin and the calmodulin-binding peptide M13. A different strategy has consisted in inserting conformationally responsive motifs such as calmodulin into FPs, as exemplified by the Ca2+ sensors termed "camgaroos" whose fluorescence increases several fold upon Ca2+ binding [127, 128]. Alternatively modified FPs, such as circularly permuted GFP (in which the original amino and carboxy termini are joined by a flexible linker and new termini are introduced near the chromophore [127, 128]), have been introduced between calmodulin and M13 peptide thereby generating highly sensitive Ca2+ indicators termed "GcaMP" or "pericams" [129, 130].

Nucleotide sensors to probe ATPase activities have been developed to monitor activities and conformational changes of enzymes that rely on nucleotide binding, including motor proteins, protein kinases and GTPases. Fluorescently labelled nucleotides have applied for probing the function of motor proteins such as the Eg5 kinesin and actomyosin [131–134]. Fluorescently labelled ATP analogs, such as mant-ATP, have been used to monitor nucleotide binding to cyclin-dependent protein kinases [135] and cyclic nucleotide analogs to probe enzyme activities such as PKA, PKG, phosphodiesterases and cyclic-nucleotide-regulated ion channels. A similar strategy based on fluorescently labelled GTP derivatives has been applied to the study of GTPases [136, see below].

Protein kinases constitute important regulators of signal transduction pathways and many constitute pharmacological targets in a variety of human diseases. Three strategies have been developed to probe protein kinase activities: nucleotide analogs, genetically encoded FRET-based activity biosensors and peptide-based reporters. Since protein kinases rely on ATP binding and hydrolysis for their function, fluorescent nucleotide analogs are routinely employed to probe their activity in vitro [135; cyclic nucleotide analogs have been used to probe enzyme activities such as PKA, PKG, phosphodiesterases and cyclic-nucleotide-regulated ion channels]. However, nucleotide-based probes have very little specificity in a cellular context and therefore call for alternative strategies to monitor protein kinases in living cells. Genetically encoded single-chain FRET biosensors of PKA and PKC, combining the consensus kinase substrate sequence with a matching phosphoaminoacid-binding domain, sandwiched between a FRET pair of GFP variants, were amongst the first kinase reporters developed to characterize their endogenous activity in living cells, together with reporters of tyrosine kinases Src, Abl and epidermal growth factor receptor, and a biosensor of insulin receptor phosphorylation [39–42]. Over the following years, this strategy has been widely applied to engineer fluorescent probes that report on intracellular activities and dynamics of the entire kinome [45]. Such genetically encoded biosensors have enabled the characterization of the subcellular dynamics of PKA and PKC [42–44], the study of protein phosphorylation by extracellular signal-regulated kinase [46] and of the intracellular activity of the ATM DNA damage sensing protein kinase [47]. An alternative approach is based on peptide-based fluorescent biosensors devised to report on protein kinase activity [137–144,], which constitute very promising new tools, in association with efficient intracellular delivery strategies. Lawrence and coworkers [73, 139–141] have developed a series of "environmentally sensitive", "deep-quench" and "self-reporting" peptide sensors of protein kinases which can be applied in vitro as well as for live-cell imaging. A phosphorylation-driven protein/protein-interaction-based protein kinase sensing system is based on the premise that the fluorescence of a labelled peptide kinase substrate will be enhanced if it becomes embedded in a hydrophobic environment following phosphorylation and subsequent recognition by a protein-binding domain [140]. These environmentally sensitive fluorescently labelled peptide probes display severalfold changes in fluorescence intensity in the presence of their target, compared to only 30% FRET change for genetically encoded FRET-based

sensors of kinase activity. The self-reporting strategy consists in engineering peptide substrates with a fluorescent probe proximal to a critical tyrosine residue, phosphorylation of which disrupts pi–pi stacking with the fluorophore, thereby leading to enhanced fluorescence [141]. A similar principle is applied in the deep-quench strategy, through introduction of a quencher into the peptide biosensor which silences probe fluorescence until it is phosphorylated by its corresponding kinase [73]. An ingenious hybrid protein biosensor developed by Anai et al. [144], bearing a natural WW phosphoprotein binding domain and a fluorescent phosphate binding chemosensor, was demonstrated to exhibit high sensing selectivity for bis-phosphorylated substrates and applied to monitor real-time phosphorylation of RNA polymerase II C-terminal Domain by CDK9/cyclin T1. Finally, several light-activated probes of intracellular protein kinase activities have been developed [145, 146]. For example a light-activatable sensor of PKC beta was applied in conjunction with several inhibitors to probe intracellular kinase activity throughout the cell cycle [146].

Sensors of Signal Transduction Pathways

Small GTPases of the Ras superfamily are important components of signal transduction pathways and are central to the morphological changes involving reorganization of the actin cytoskeleton and adhesion dynamics in living cells. In order to gain insight into the complex pattern of GTPase activation, different biosensor strategies have been devised to investigate their function and characterize their dynamics of activation in vitro and in cellulo. The development of fluorescent biosensors for probing GTPase activities exemplifies the variety of strategies that can be employed to monitor and image enzymatic behaviour. Fluorescently labelled guanine nucleotides have been used as fluorescence reporter groups to investigate interactions of GTPases of the Rho family, such as RhoA, Rac1 and Cdc42 in vitro [136]. Hahn and colleagues have developed a number of protein and genetically encoded biosensors to probe the active conformation of several GTPases involved in cell motility. A FRET-based live-cell imaging approach termed fluorescence activation indicator for Rho proteins (FLAIR) was applied to quantify spatio-temporal dynamics of growth-factor induced Rac1 activation in living cells [147]. This approach relies on a fluorescent protein biosensor derived from p21-activated kinase 1 (PAK1), which binds the active GTP-bound form of Rac1. The protein biosensor was introduced into living cells expressing a GFP–Rac1 fusion through microinjection, and allowed for mapping of GTPase activation, revealing a gradient of activation at the leading edge of motile cells in membrane ruffles. Genetically encoded FRET biosensors of Rho and Rac have been developed encoding the GTPase itself, together with the CRIB (Cdc42 and Rac-interactive motif), the PBD (p21 binding domain) or the RBD (Rho-binding domain of rhotekin) which only recognizes the conformationally active form of Cdc42, Rac and RhoA, respectively, flanked by a FRET pair of FPs which are brought together upon GTPase activation [50, 51, 148]. An alternative to FRET-based approaches involves the application of environmentally sensitive dyes that report on conformational changes or protein interactions such as the merocyanine dyes developed by Hahn and coworkers [149]. This strategy was undertaken to generate a protein biosensor of active Cdc42 based on the WASP (Wiskott Aldrich Syndrome Protein) effector domain, “Mero-CBD” to investigate Cdc42 activation in cellulo, revealing activation kinetics well coordinated with cell extension and retraction, localized in the trans-Golgi apparatus, at the cell periphery [150, 151]. The power and extreme precision of these biosensors is evidenced by their recent application to characterize GTPase coordination during cell protrusion in mouse embryonic fibroblasts. In a very elegant study, the Mero-CBD protein biosensor of Cdc42, the PAK1-based protein biosensor of Rac1, and a genetically encoded biosensor of RhoA were combined to characterize the activation profiles of these different GTPases during cell membrane protrusion and retraction and a computational multiplexing approach enabled to establish a tight correlation between cell morphological dynamics and individual GTPase activities in space and in time [152].

Fluorescent protease reporters based on consensus polypeptide substrate sequences whose fluorescent properties are affected upon proteolytic cleavage have been generated to probe the activities of extracellular and lysosomal proteases, of caspases and metalloproteases, of the ubiquitin/proteasome system and of viral proteases (for review [54]). Moreover, deregulation of proteolytic mechanisms is linked to several diseases including cancer, inflammation, arteriosclerosis, neurodegeneration and infection. As such protease reporters have not only been developed to probe intracellular proteolytic activities, but have also been applied with a diagnostic purpose, and to monitor treatment, in response to therapeutic drugs. In particular, fluorescent biosensors have been designed to probe caspase activities in vitro, in cellulo and in vivo, thereby providing a direct readout of the kinetics of apoptosis [52, 55, 153]. Several reporters of cathepsins have been developed and applied to monitor their proteolytic activation in rheumatoid arthritis and in atherosclerosis [154–158]. A NIRF reporter of cathepsin K has been shown to probe osteoclast activity in vitro and in vivo, and potentially provides a sensitive means to diagnose bone resorption [159]. A near-infrared probe was developed to monitor thrombin activation, thereby providing insight into thrombin regulation in vivo, and novel means of diagnosis

of arterial and venous thrombosis [160]. Several probes have been designed to image cell surface matrix metalloproteinase activity, which is associated with progression of tumorigenesis, thereby allowing for detection of small early stage tumours [161–163]. Likewise, since many lysosomal proteases are upregulated in tumours, NIRF quenched peptide biosensors have been developed and successfully applied to image their activities with both a diagnostic purpose, and to monitor treatment efficacy [74, 164–166]. Both fluorogenic peptide substrates and genetically encoded fluorescent reporters based on GFP fusions of constitutively active degradation signals which behave as genuine substrates of the ubiquitin/proteasome system have been developed, thereby enabling characterization of individual proteasomal activities, evaluation of the functional status of the ubiquitin/proteasome in cellulo and establishment of mouse models [167–170]. Many viral proteases play an essential role in viral infection and therefore constitute important targets for the development of antiviral drugs. In particular, the human immunodeficiency virus protease is a major target for therapeutic strategies to treat AIDS. However, given the continual emergence of inhibitor-resistant strains, a diagnostic strategy was devised to monitor treatment efficacy and progression of viral infection, based on a genetically encoded GFP-protease precursor of HIV-1 [171, 172].

Cell Cycle Sensors

Since the early studies on cell division, characterization of the cell cycle status has essentially been limited to the description of morphological criteria with little resolution, such as cell rounding upon entry into mitosis and to methods which require cell fixation and permeabilization, such as fluorescence-activated cell sorting (FACS), following labelling with a DNA intercalating dye, imaging of bromo-deoxyuridine incorporation, and the development of indirect immunofluorescence approaches for the detection of subcellular structures and cell cycle regulators by indirect immunofluorescence, which is highly dependent on specific antibodies. Although these approaches provide precious information at single cell resolution, they do not allow gaining insight into the dynamics of protein function and localization. The more recent application of GFP technology to express fusions of the cell cycle proteins has allowed these issues to be addressed for many cell cycle regulators; however, this approach does not enable the study of endogenous proteins themselves [173, 174]. Since many cellular processes involve cell-cycle-dependent changes in the subcellular localization and dynamics of proteins, new strategies have been devised to distinguish different cell cycle stages based on fusions of cell cycle proteins with well-defined spatio-temporal characteristics (Fig. 5). Cell cycle markers that identify S-phase have been developed by generating genetically encoded fusions of fluorescent proteins to proliferating cell nuclear antigen (PCNA) [175–177], or to DNA Ligase I [178]. Jones et al. developed a genetically encoded live-cell fluorescent biosensor bearing a reversible plasma membrane-targeting domain fused to an NLS was developed to monitor the time between nuclear envelope breakdown and reformation, and applied to "probe the precision of the "mitotic clock" in physiological conditions and upon treatment with the mitotic inhibitor taxol [179]. Another elegant system was recently developed by Sakaue-Sawano et al., "fluorescent ubiquitination-based cell cycle indicator" (FUCCI) system, consisting of a set of genetically encoded fluorescent protein fusions of substrates of the APC^{Cdh1} and SCF^{Skp2}, geminin and Cdt1, respectively, whose proteolysis oscillates inversely, thereby marking different cell cycle transitions. This system was applied to real-time observation of cell cycle progression in cultured cells and to characterize the spatiotemporal patterns of cell cycle dynamics during different biological processes in cultured cells, brain slices and live mice [180]. Thomas developed a "Cell Cycle Phase Marker" to probe the G2/M transition in living cells by fusing GFP to a fragment of cyclin B harbouring a functional destruction box and a cytoplasmic retention signal (CRS), which translocates from the cytoplasm to the nucleus [181, 182].

Although these genetically encoded cell cycle sensors provide a means of identifying the cell cycle position, none report directly on the levels and activities of endogenous cell cycle enzymes themselves, in particular of protein kinases and phosphatases that regulate progression through the different cell cycle transitions. Indeed, the task of developing reporters of cell cycle enzymes is not obvious, given their in-built redundancy and the close similarity of their consensus substrate sequences. Moreover, the dynamic, cyclic and often transient activities of these enzymes call for strategies which enable their rapid and specific detection. Therefore, in spite of the advances in the understanding of the molecular mechanisms underlying regulation of the cell cycle machinery, this field truly suffers from a lack of biosensors to probe the levels and activities of its molecular components. Moreover, in spite of the pharmacological importance of many cell cycle enzymes in hyperproliferative disorders such as cancer [183–185], there is an overall lack of diagnostic approaches for detecting alterations in their levels and activities, aside from indirect and somewhat lengthy procedures, relying on biopsies, such as RT-PCR, immunohistochemistry or mass spectrometry. This lack of appropriate methods to detect cell cycle biomarkers in a pathological context is in large part related to their intracellular localization, and therefore calls for novel and sensitive means of tracing these targets

and monitoring their activity in situ. We have engineered a novel generation of peptide-based fluorescent biosensors that report on the levels and alterations of mitotic kinases and phosphatases that regulate cell cycle progression. The design of these reporters is based on modules that recognize specific molecular interfaces at the surface of the cell cycle targets of interest. Such modules are incorporated in a multi-ligand peptide sensor bearing an environmentally sensitive probe which is affected when it encounters the target (Fig. 5e). These polypeptide biosensors are efficiently delivered into cells through non-covalent association with nanoparticles of cell-penetrating carriers [90]. The first of these original biosensors is a biligand sensor of cyclin-dependent kinases, the engines that drive cell cycle progression, which exhibits a robust increase in fluorescence upon binding to the heterodimeric target both in vitro and in living cells and allows for standardized quantification of cdk-cyclin levels in different cell lines [186]. A second family of modular biosensors targets Cdc25 phosphatases, activators of cyclin-dependent kinases and established therapeutic targets. Ongoing efforts to engineer fluorescent biosensors to probe endogenous cell cycle regulators should provide precious information regarding their cellular behaviour in physiological and in pathological conditions. Moreover, such biosensors are potentially applicable to non-invasive diagnostic approaches to highlight cellular alterations of cell cycle regulators associated with tumoral evolution.

Imaging Cancer: Detection and Diagnosis and Drug Development

Optical imaging of tumours and of biological processes related to cancer, has made considerable progress thanks to the development of novel fluorescent probes and biosensors, together with ingenious strategies devised to maximize signal-to-noise ratio in tumour cells versus healthy tissues, to target fluorescent probes specifically to tumour cells and to promote their selective activation in cancer cells [57–59, 187–189]. Fluorescent tracers have been used for intravital imaging of the vasculature during angiogenesis, and several fluorescent probes have been employed to study cancer cell growth, tumour cell mobility, invasion and metastasis. New generations of "smart sensors" have been designed to optimize sensitivity and specificity in tumour imaging to recognize targets with high specificity and selectivity. Enzymatically activated fluorescent probes developed by several groups are a noteworthy example of ingenious sensing strategies applied to tumour imaging. Tsien and colleagues [190] developed probes for tumour imaging termed "activatable CPPs" (ACPPs) based on proteolytic activation of a polyarginine cell-penetrating peptide by extracellular matrix metalloproteinases. This strategy is potentially applicable to any pathology involving extracellular proteolytic activities. Urano and colleagues [191, 192] designed a strategy for specific tumour imaging based on pH-activatable fluorescent probes conjugated to cancer-targeted monoclonal antibody, which are sensitive to lysosomal acidification following internalization into viable cancer cells. They showed that this strategy could be applied to visualize submillimeter tumours in mouse models, thereby providing a means of diagnosing and monitoring tumours. Weissleder and colleagues [155] developed a probe to image tumour-associated protease activity thanks to an autoquenched near-infrared fluorescence probe bound to a long circulating graft copolymer consisting of poly-L-lysine and methoxypolyethylene glycol succinate whose cleavage accumulation in tumours lead to release of the quenched fluorophore by lysosomal proteases, yielding a 12-fold increase in signal in vivo. Hama et al. [193] took advantage of a similar strategy by developing an avidin-rhodamine self-quenching probe with affinity for lectin on cancer cells, which is activated through dequenching following endocytosis and lysosomal degradation.

Moreover, fluorescent biosensors are tools of major interest for drug discovery and development, as they allow for direct screening of compounds that affect the target or the activity recognized by the probe, and can therefore be applied to the high throughput screening of libraries of synthetic and natural compounds [35, 36, 194–197]. Fluorescent probes can also be employed for molecular imaging of drugs in vivo in real time to characterize their biodistribution and pharmacokinetics. As such fluorescent biosensors can be applied to the preclinical evaluation of anticancer drugs in high-throughput, cell-based screens or yet in vivo to visualize the behaviour of a drug in real time, and to monitor treatment response.

Concluding Remarks and Perspectives

Biosensors constitute a convenient means of probing and reporting on the presence and activity of specific analytes, targets or biomarkers within a complex physiological solution such as living cells. The explosion of fluorescence-based imaging technologies, together with the development of GFP technologies, of small fluorescent labels with exceptional spectral properties, and the growing information on mechanisms of enzyme regulation, on molecular interfaces and structural characteristics of protein targets, has provided an appropriate environment for the design of fluorescent reporter systems to monitor dynamic biological activities, with minimal invasiveness, but maximal spatial

and temporal resolution, specificity and sensitivity, in living cells and tissues.

Here, we describe some of the features of the different fluorescent biosensors that have been developed over the past decade, in particular genetically encoded biosensors, as well as more recent efforts to develop peptide- and polypeptide-based reporters of enzymatic activities, conformational changes or protein/protein interactions. Such tools not only provide information on target enzymes in their natural environment for a comprehensive understanding of their dynamic behaviour, but are also very useful for comparing biological processes in healthy and pathological conditions, and will no doubt spur new discoveries in many fields. One major advantage of fluorescent biosensors is their ability to provide a direct readout of intracellular levels and activities, thereby avoiding the use of indirect technologies involving ectopic expression of the gene product of interest, or siRNA-mediated knock-downs, genetic knockouts or immunodepletion, which may further induce compensatory effects. It is clear that virtually any field in biological and medical sciences will benefit from the development of fluorescent biosensors either to gain insight into the behaviour of poorly characterized targets, or to aid in diagnostic and therapeutic approaches. The application of these original technologies will be particularly beneficial when conventional genomic and proteomic analyses are difficult. In particular, the cell cycle field, which suffers from a lack of sensing technologies to monitor the dynamics of protein kinases and phosphatases responsible for regulation of cell cycle progression. Hence, ongoing efforts to engineer fluorescent biosensors enabling detection of these enzymes in cellulo should provide precious information regarding their cellular behaviour. Moreover, given the central role of many of these enzymes in physiological conditions, and their established value for therapeutic intervention, they constitute excellent biomarkers of hyperproliferative disorders. Finally, fluorescent biosensors are applicable to screening programmes for identification of small molecule inhibitors and have significant potential for development of diagnostic approaches. Needless to say, the combination of fluorescence-based biosensors with non-invasive strategies of delivery into tissues and tumours would benefit greatly to patients, by potentially circumventing the current requirement for biopsies and providing precious information for the development and administration of tailored therapeutic treatments.

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