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Cell-Cycle Markers and Biosensors

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Since the first schematic illustrations of dividing cells, we have come a long way in characterising eukaryotic cells and defining their cell-cycle status thanks to a number of complementary approaches. Although most of these approaches rely on cell-fixation procedures to identify molecular components in cell lysates, cultured cells or tissues, the development of GFP technology has enabled visualisation of virtually any fusion protein in cellulo and in vivo, and the exploitation of functional elements with well-defined spatiotemporal characteristics has enabled the development of genetically encoded fluorescent markers of cell-cycle phases, thus providing novel means of characterising the status of living cells in real time with high resolution. Together with technological advances in fluorescence chemistry and imaging approaches, the more recent development of fluorescent biosensors has provided direct means of probing cell-cycle regulators and of studying their dynamics with high spatial and temporal resolution. Here we review classical approaches that rely on cell fixation to characterise the cell-cycle status and its regulatory enzymes, and we describe the more recent development of cell-cycle markers based on genetically encoded fusions of fluorescent proteins with characteristic cell-cycle features, and of fluorescent biosensor technology to probe cell-cycle regulators in living cells. Biosensors not only provide a means of characterising the behaviour of cell-cycle regulators in their natural environment, they are also very useful for comparative studies of biological processes in healthy and pathological conditions, and can be further applied to diagnostic approaches to assess the status of a specific target, and to monitor response to therapeutic intervention.

1. Introduction

The first description of cell division can be traced back to 1765, soon after the development of the first microscopic lenses, when Abraham Trembley published drawings of cell division in protozoans and algae. During the following century, a number of illustrations of cell division were published, but the first real report of eukaryotic cell division, revealing a thread-like nuclear structure termed chromatin and describing the distribution of chromosomes from mother to daughter nuclei, was published in 1882 by the German anatomist Walther Flemming who investigated this process and coined the term "mitosis" (from the Greek "mitos" for thread) in reference to the morphological changes of chromatin during division of the cell nucleus.[1] Although the process of cell growth and division, by which all unicellular and multicellular organisms duplicate a pre-existing cell into two identical cells, was first established in the middle of the nineteenth century, scientists have remained captivated by the mystery and complexity of events leading to cell division and necessary for reproduction, known as the cell cycle ever since. Thanks to technological advances in cell culture, in optical and video-microscopy, our view of the cell cycle as a gross description of morphological changes cells undergo has evolved considerably.[2] Until the late 1940s research on cell division was restricted to fixed cells that were stained with contrast-generating dyes. The development of contrast-producing optics in the 1950s (Nobel Prize Zernicke 1955) allowed for studies of living cells with greater resolution, which spurred a number of studies on cell division; however, they remained restricted to morphological observation. Introduction of the electron microscope a decade later allowed for further resolution in characterisation of subcellular structures. Yet another major breakthrough for the study of mitosis was the development of video technology in the 1980s, which allowed time-lapse imaging and visualisation of complex motions of subcellular structures and dynamic processes during cell division in living cells to be observed. However, these studies provided little if any information on the complex interplay of signalling proteins that organise cell-cycle progression and division.

In the late 1960s and 1970s, studies from several independent groups aiming to understand the process of oocyte maturation in xenopus oocytes on the one hand, and to decipher the genetics of cell division in yeast on the other, culminated in the identification of MPF, "maturation-promoting factor" also known as "mitosis-promoting factor", the universal molecular component responsible for cell division.[3–7] Following the discovery of MPF, the identification of other members of the family of cyclin-dependent kinases and of the network of molecular regulators of cell-cycle progression led scientists to focus on the biological behaviour and subcellular localisation of these enzymatic regulators, and to characterise their temporal and spatial dynamics, so as to unravel the molecular mechanisms underlying cell-cycle regulation.[8] Over the years we have come a long way in our understanding of the regulatory mechanisms that orchestrate cell-cycle progression. Together with the development of fluorescent dyes, cell-cycle-specific

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antibodies and indirect immunofluorescence approaches, it became possible to characterise the subcellular location of specific cell-cycle components and subcellular structures.[9] Towards the end of the 20th century, the development of GFP technology combined with video-microscopy provided a powerful means of imaging cell-cycle regulators in living cells with high spatial and temporal resolution.[10–13] Together with this technological breakthrough, the more recent design and application of fluorescent biosensor strategies has opened a unique means of probing endogenous enzyme levels and activities in real time in living cells.[14–17]

2. The Cell-Cycle Machinery: The Molecular Players of Cell-Cycle Progression

Cell growth and division are essential processes for unicellular and multicellular organisms alike, ensuring survival and propagation of a species through faithful division of a mother cell into two identical daughter cells with the same genetic complement.[8] The cell cycle can be described as the set of successive transitions between four well-resolved cellular phases, with clear morphological differences and specific biochemical features: A phase of cell growth (G1), followed by a phase of DNA synthesis and replication (S-phase), another phase of cell growth and active synthesis of factors (G2) required for cell division or mitosis (M). Cell-cycle progression is regulated by a complex molecular machinery and driven by a family of heterodimeric kinases, termed cyclin-dependent kinases, which consist of a CDK kinase subunit associated with a regulatory cyclin subunit.[18–20] CDK–cyclin complexes are responsible for phosphorylation of a myriad of substrates involved in cell growth and division, DNA replication and chromatin condensation, assembly of the mitotic spindle and disassembly of the nuclear envelope.[21–24] In mammalian cells more than ten different CDKs and cyclins form complexes with unique substrate specificity, which are responsible for coordination of distinct cell-cycle transitions.[20,25,26] Cyclins play a major role in promoting activation of CDKs by inducing significant conformational changes to the kinase subunit, in defining substrate specificity, and in targeting their associated CDK to well-defined subcellular locations.[27–29] In cellulo, the spatiotemporal patterns of expression and degradation of cyclins dictate their availability for CDK binding, but the specificity of recognition between different CDK and cyclin partners is equally defined by molecular features that are inherent to their sequence and structure.[30] As such, progression through G1 is coordinated by cyclin D isoforms (D1, D2, D3) complexed to Cdk4 and Cdk6, whilst the G1/S transition and progression through S-phase are ensured by Cdk2–cyclin E and Cdk2–cyclin A, respectively. Cdk2 and Cdk1 both participate in preparation of the G2/M transition, together with cyclin A and cyclin B, with Cdk1–cyclin B1, also known as MPF, "Mitosis-Promoting Factor", as the master regulator of mitotic progression.[25,26] Studies in mouse models provided additional insights into the physiological relevance and function of CDKs and cyclins in vivo.[20,26,31] Single cyclin knockout mice are all viable, with the exception of cyclin A2 and cyclin B1, which lead to embryonic lethality; this is indicative of widespread compensatory mechanisms among cyclins. Likewise all single CDK knockout models are viable, with the exception of Cdk1. This would tend to suggest that most CDKs are dispensable for mammalian cell-cycle regulation, Cdk1 being sufficient to drive cell-cycle progression in association with a small subset of cyclins (A2 and B1). Nevertheless, the absence of individual CDKs or cyclins was found to be associated with more or less severe cell-type-specific anomalies, thereby pointing to distinctive functional properties for each of these protein family members. Table 1 summarises the different phenotypes for single CDK and cyclin knockout mouse models; more detailed descriptions can be found in recent literature reviewing these in vivo studies.[20,26,31]

Table 1. Phenotypes associated with deletion of genes encoding CDKs or cyclins.

Gene deleted	Associated phenotype in vivo
CDK1	embryonic lethality in the first cell divisions
CDK2	viable; sterility associated with defective meiosis; no defect in mitotic cells
CDK3	viable; no defects
CDK4	viable; diabetes and defective postnatal proliferation of endocrine cells such as pancreatic β-cells or pituitary hormone-producing cells
CDK5	perinatal lethality; severe neurological defect
CDK6	viable; anaemia associated with defective development of haematopoietic cells
CDK11	embryonic lethality at blastocyst stage accompanied with mitotic defects
cyclin A1	viable; male sterility
cyclin A2	embryonic lethality
cyclin B1	embryonic lethality
cyclin B2	viable; no abnormalities
cyclin D1	lethality within 3 weeks after birth. Reduced body size, hypoplastic cerebella
cyclin D2	viable; defective B-lymphocyte proliferation, pancreatic β-cell proliferation, cerebellar development, adult neurogenesis and hypoplastic thymus; female sterility
cyclin D3	viable; defects in T-lymphocyte development
cyclin E1	viable; no abnormalities
cyclin E2	viable; reduced male fertility
cyclin F	viable; reduced male fertility

Extracellular growth signals and intracellular cues feed into the cell-cycle network to coordinate sequential activation of the different molecular players, whilst inhibitory signals and checkpoint mechanisms restrict their inappropriate or untimely activation in conditions, which could be detrimental to cell survival. Regulation of cyclin-dependent kinases exemplifies the complexity of the regulatory mechanisms that underlie cell-cycle progression. Sequential activation of CDK–cyclin complexes is a complex multistep process and is governed by protein–protein interactions, post-translational modifications and conformational changes.[27,32] Indeed, CDK–cyclin complexes are subject to both activating and inhibitory regulation, in particular inhibitory phosphorylation by Wee1 and Myt1 kinases,[33] which prevents ATP binding, counteracted by activating dephosphorylation by Cdc25 phosphatases,[34] followed by acti-

vating phosphorylation by the CDK-activating kinase (CAK), which consists of Cdk7–cyclin H, which favours substrate binding.[35–37] In addition, activating kinases such as Polo-like kinases play a critical role in CDK–cyclin activation through coordinate phosphorylation of the cyclin subunit and of Cdc25 phosphatases.[38,39] The complexity of CDK–cyclin regulation is further emphasised by regulatory feedback mechanisms. In particular, active MPF is involved in a negative feedback loop that inactivates Wee1 and Myt1, and a positive feedback loop that leads to further activation of Cdc25 phosphatases, thereby amplifying MPF activity to a threshold level, which triggers unconditional and irreversible entry into mitosis.[40–42] Moreover, the CKS (CDK Subunit) and the cyclin-dependent kinase inhibitors (CKIs), are involved in modulation of CDK–cyclin activity in a noncatalytic fashion. CKS proteins stabilise CDK–cyclin complexes and participate in recruitment of phosphoprotein regulators such as Cdc25, Wee1 and Myt1.[43,44] CKIs include members of the p21, p27 Cip/Kip and of the Ink4 families, structural inhibitors that maintain CDKs in an inactive conformation throughout normal cell-cycle progression, as well as in response to stress, growth factor deprivation, cell contact inhibition or checkpoint activation.[45,46] Last, but not least, the different players of this intricate network are subject to controlled mechanisms of degradation by the ubiquitin/proteasome system, notably cyclins, the degradation of which is triggered by ubiquitinylation-dependent mechanisms.[47] Figure 1 provides a schematic representation of the cell-cycle phases and of the basic machinery that orchestrates cell-cycle progression.

An additional layer of complexity to these regulatory mechanisms is brought about by the spatiotemporal dynamics that cell-cycle players are subject to, in particular very dynamic shuttling between the cytoplasm and the nucleus, which leads to compartmentalisation, confinement or conversely brings two partners closer at subcellular structures that behave as platforms such as the centrosomes. Spatiotemporal dynamics have a direct effect on regulatory mechanisms, favouring molecular cooperation or competition, mechanisms of dependency, of autoamplification, or of positive or negative feedback.

3. Cell-Cycle Biomarkers and Therapeutic Targets

Cancer is a complex multicausal pathology that is notably characterised by self-sufficiency with respect to growth signals, resistance to anti-growth signals and limitless replicative po-

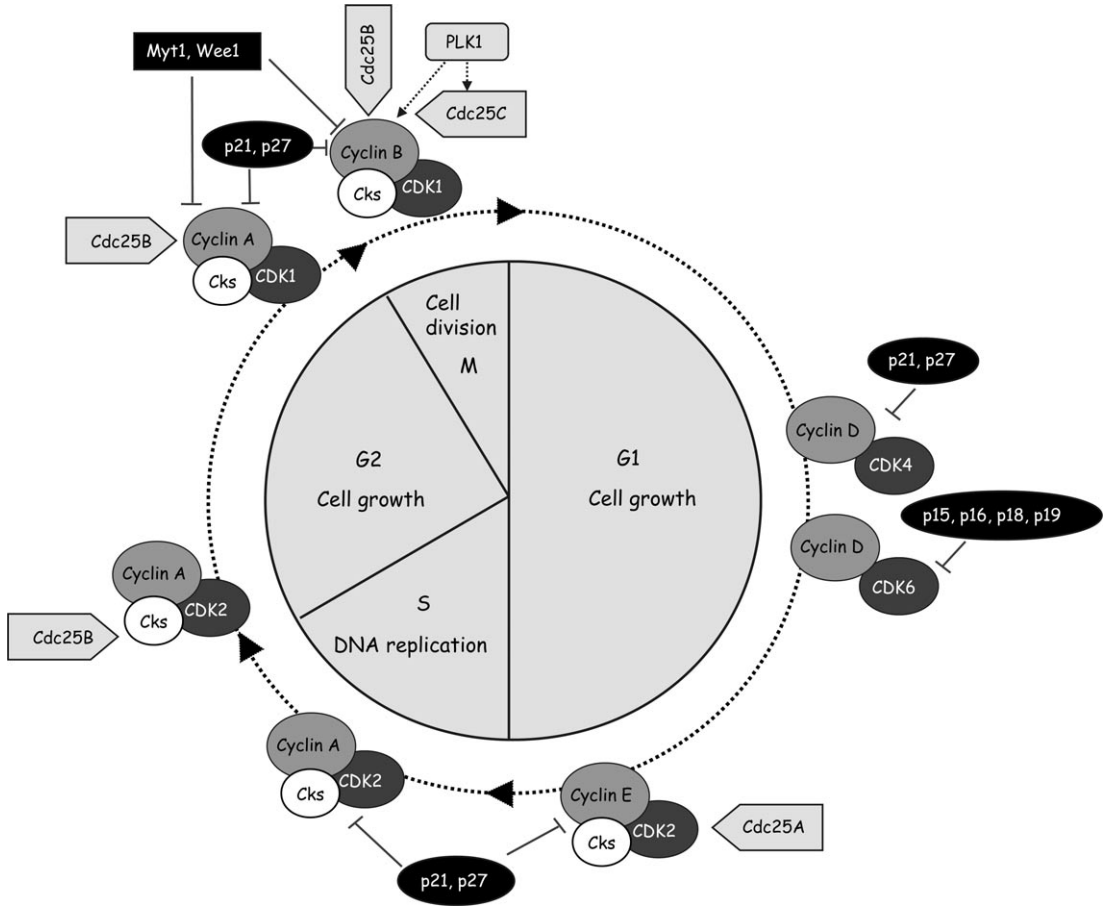

Figure 1. The cell division cycle phases and basic molecular machinery. The cell cycle consists of four distinct cellular phases: G1, a stage of cell growth, S-phase, during which DNA synthesis and replication takes place, G2, a second phase of cell growth and active synthesis of factors required for cell division or mitosis (M). Cell-cycle progression is driven by the cyclin-dependent kinases, heterodimeric complexes consisting of a catalytic cdk subunit associated with a regulatory cyclin subunit. Sequential assembly and activation of cdk–cyclin complexes is regulated by a complex network of activators, including Cdc25 phosphatases and Plk kinases, and inhibitors, in particular Wee1 and Myt1 kinases, and structural inhibitors p21, p27 Cip/Kip and members of the Ink4 family p15, p16, p18, p19.

tential.[48] One of the major hallmarks of cancer is the ability of cells to proliferate in an uncontrolled fashion through loss of control of the mechanisms that coordinate timely and orderly cell-cycle progression, thanks to the transforming potential of oncogenes, and the cooperative action of cell-cycle regulators the amplification, overexpression or hyperactivation of which sustains aberrant proliferation.[48–51] With the escalation of drug discovery programmes, a large effort has been made to identify biomarkers of cell proliferation that constitute relevant therapeutic targets so as to design or screen for efficient drugs to treat hyperproliferative disorders. Although most of the currently defined biomarkers are serum proteins or cell-surface receptors or antigens, the explosion of proteomics-based approaches over the past years has lead to identification of a myriad of intracellular biomarkers, including several cell-cycle regulators.[52,53] Moreover gene profiling studies have revealed the existence of "proliferation signatures" constituted in large part of cell-cycle-regulated genes, including genes involved in DNA metabolism, DNA synthesis, regulation of cell-cycle progression, cell proliferation and cell division.[54–56] A number of different genetic alterations in cell division control genes have been found to correlate with uncontrolled cell proliferation, and their increased expression in tumours is associated with poor prognosis in cancer patients. Aside from mutations in proto-oncogenes, tumour suppressor and checkpoint control genes, one of the most frequent alterations in human cancer concerns misregulation of cyclin-dependent kinase activity.[51,57,58] Although genetic alterations of CDKs themselves are not very frequent, mutations leading to hyperactivation of CDK activity provide a selective growth advantage, such as mutations in CDK4 and CDK6 that result in loss of CKI binding, and overexpression of CDK1, CDK2 and CDK4 in a small proportion of cancer subtypes.[51,57,58] However, most genetic alterations responsible for CDK hyperactivation occur in genes that control CDK activity, in particular cyclins, Cdc25 phosphatases, Plk kinases and CKIs, which therefore equally constitute biomarkers and pharmacological targets of interest for the development of anticancer drugs. Indeed, alterations in the levels and/or activities of these cell-cycle regulators have been documented in a wide range of cancers, including breast and ovarian cancer, prostate, colorectal and lung cancer, lymphoma, myeloma and sarcoma. Gene amplification, protein overexpression, mislocalisation or expression of truncated variants of several cyclins appear to be responsible for exacerbated CDK activity in several cancers and are associated with poor prognosis in patients.[58,59–72] Alterations in levels and/or the subcellular localisation of cyclins that associate with CDKs from different cell-cycle phases have been reported in a widespread number and variety of cancers.[60–62] D-type cyclins associated with CDK4 and CDK6 are responsible for loss of control between mitogenic stimuli and cell proliferation.[58,64,65] Cyclin A deregulation in cancer cells is associated with its overexpression and mislocalisation.[62,63,66] Likewise, cyclin B1 is overexpressed throughout the cell cycle and resides predominantly in the cytoplasm of cancer cells, whereas it is otherwise expressed at very low levels, only accumulating at the G2/M transition concomitantly with its nuclear translocation in healthy cells.[67,68] Cyclin E is processed to low-molecular-weight forms in cancer cells, which appear to be associated with tumour progression.[69–72] Cdc25 phosphatases, in particular Cdc25A and B, are either expressed at high levels or as splice variants in various types of human cancers, and are often associated with aggressive phenotypes and poor survival prognosis.[73,74] Plk1 kinase is highly expressed in malignant cells, and constitutes a prognostic marker in several human cancer types.[75,76]

As such, given the documented alterations of cyclin-dependent kinases, and of their activators in human cancers, their central role in cell-cycle regulation and their prognostic value, they constitute established pharmacological targets.[77] Moreover, these cell-cycle regulators can be considered to be invaluable biomarkers of cell proliferation for diagnostic purposes. However, their intracellular location has hindered the development of tools to probe their levels and activities directly in situ. Indeed, current approaches for detecting alterations of these enzymes in human tumours remain invasive and rely on biopsies; furthermore they are indirect methods that involve more or less lengthy procedures such as immunohistochemistry, RT-PCR or mass spectrometry. Hence this lack of appropriate methods to monitor cell-cycle biomarkers in a pathological context calls for new strategies to assess their levels and activities in situ. Together with the development of fluorescence chemistry and imaging technologies, the design of novel tools such as fluorogenic sensors has provided an extremely sensitive means of probing the levels and activities of intracellular targets in a reliable fashion, of interrogating intracellular biochemical pathways in space and time, and of developing sensitive diagnostic applications.

4. Classical Approaches to Characterise the Cell-Cycle Status

The first studies on cell division, provided very little insight into the cell-cycle status per se, because they were essentially limited to the morphological description of cellular features, such as cell rounding and changes in chromatin condensation upon entry into mitosis (see Figure 2A). Since these first schematic illustrations of dividing cells, we have come a long way in characterising the cell-cycle status of eukaryotic cells thanks to a number of complementary approaches. The combination of different staining procedures on fixed cells, including the use of DNA-intercalating dyes and of antibodies for indirect immunofluorescence generally provides sufficient information to determine whether cells are in interphase or undergoing mitosis. Wide-field imaging of fixed cells stained with fluorescent DNA-intercalating dyes such as Hoechst or diaminido phenyl indole (DAPI) allows the evaluation of chromatin condensation to be made, whilst incorporation of the deoxyribonucleotide analogue bromodeoxyuridine (BrDu) into chromatin enables us to assess whether active DNA synthesis has taken place (Figure 2B). Fluorescence-activated cell sorting (FACS) of cells stained with intercalating dyes such as propidium iodide (PI) is employed to determine whether cells have a 2N or 4N DNA content, and is equally useful to identify polyploidy, and cells with fragmented DNA, in apoptosis (Figure 2C). FACS can also

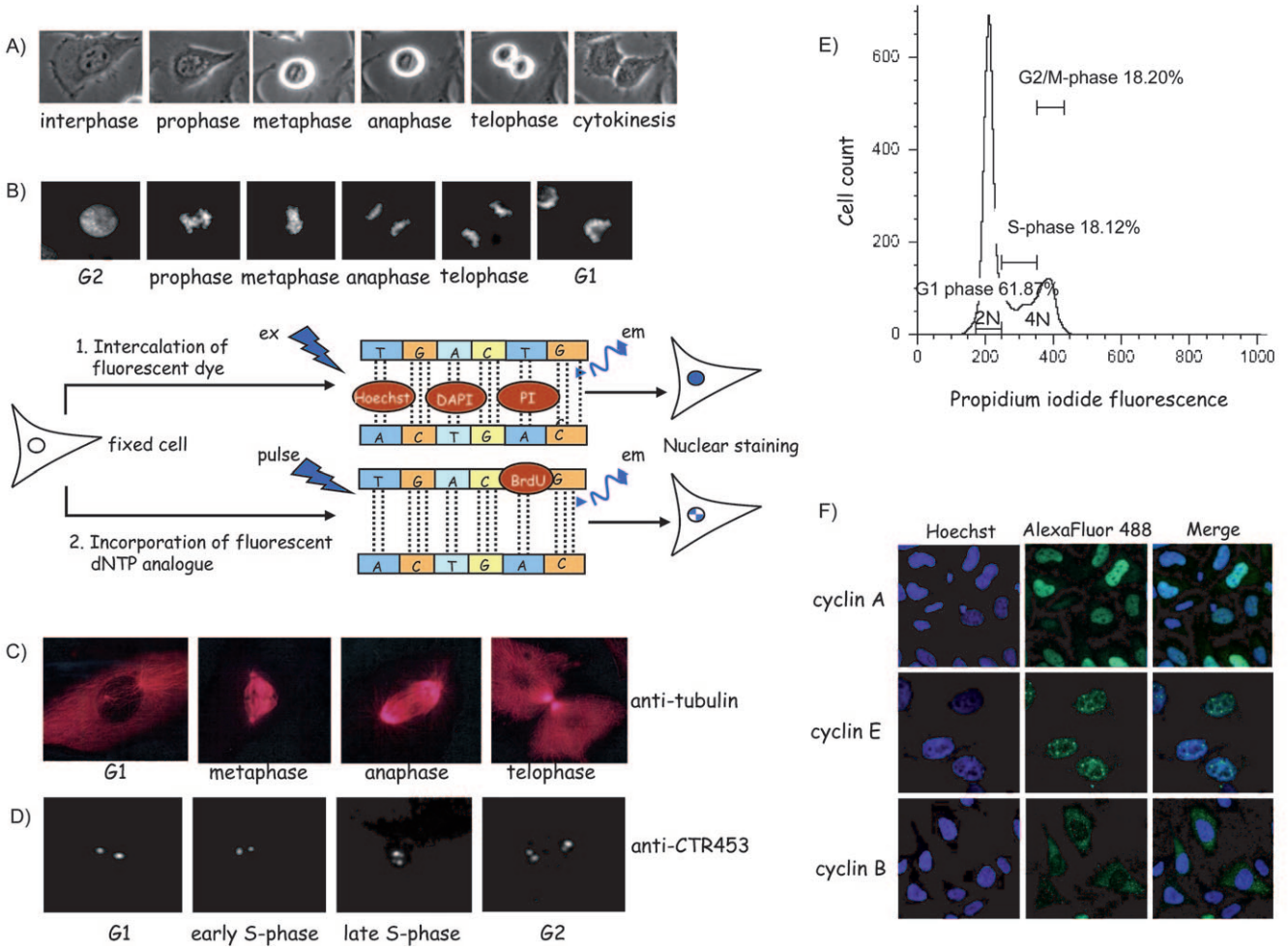

Figure 2. Classical approaches for characterising the cell-cycle status. A) Phase-contrast micrographs of HeLa cells undergoing cell division. B) DNA staining of chromatin in HeLa cells at different cell-cycle stages with Hoechst 33342; the cartoon depicts the difference between DNA-intercalating dyes, which are classically used to stain the nucleus and monitor chromatin condensation, and fluorescent dNTP analogues, which are incorporated into replicating DNA during S-phase. C) FACS analysis of an asynchronous population of HeLa cells stained with propidium iodide allows for the discrimination of cells with a 2N or 4N DNA content. D) Indirect immunofluorescence with antibodies directed against tubulin allow for visualisation of the mitotic spindle E) Indirect immunofluorescence with antibodies directed against AKAP-450 (CTR-453) allow for direct visualisation of centriole duplication in living cells throughout the cell cycle. F) Indirect immunofluorescence of cyclins A, B and E reveals distinct nuclear localisation of cyclin A, staining of replication foci with cyclin E, and cytoplasmic localisation of cyclin B, in the S-phase.

be employed to obtain a synchronised population of cells with specific biological features. Likewise the application of centrifugal elutriation to separate cells based on their size, of nutritional deprivation, or the use of drugs to block cells at specific cell-cycle stages constitutes a means of synchronising cells to observe their morphological and biological features at a well-defined point of the cell cycle. Some of the most commonly used drugs include hydroxyurea and (deoxy)thymidine, which block cells in S-phase by affecting production and incorporation of deoxyribonucleotides, the microtubule destabiliser nocodazole, which interferes with microtubule polymerisation and assembly of the mitotic spindle and therefore blocks cells at the G2/M transition.[78,79] Staining with antibodies against cytoskeletal filaments, such as tubulin, provides complementary information on the tubulin network and on spindle assembly (Figure 2D), whereas antibodies against nuclear lamins stain the nuclear membrane and therefore allow us to determine whether nuclear membrane disassembly has taken place; antibodies that recognise centrosomal proteins allow us to monitor the centrosomal cycle, which is tightly associated with the cell-division cycle (Figure 2E). Further details on the cell-cycle status can be obtained by indirect immunofluorescence thanks to specific antibodies that recognise cell-cycle regulators with well-defined spatial and temporal patterns of expression and subcellular localisation, such as cyclins (Figure 2F). For example evaluation of levels and subcellular localisation of cyclins A, B and E allows the S-phase, G2 and M phases to be distinguished, because cyclin E appears at nuclear foci at the onset of replication, closely followed by nuclear accumulation of cyclin A during S-phase, whereas cyclin B resides mainly in the cytoplasm and at centrosomes, and accumulates in the nucleus during late G2.[80,81]

Despite the wealth of information provided by these different approaches, their reliance on cell fixation and permeabilisation procedures makes them inapplicable to monitor dynamic processes such as chromatin condensation/decondensation, spindle assembly/disassembly and spatiotemporal dynamics of subcellular structures and cell-cycle regulators in real time. Indeed fixation procedures only provide a "snapshot" of a truly dynamic process.

5. Genetically Encoded Fluorescent Markers

The purification and characterisation of GFP in the 1960s by Osamu Shimomura from the jellyfish *Aequorea victoria*, its molecular cloning and expression as a recombinant protein in *E. coli* by Martin Chalfie, and its engineering into different autofluorescent protein variants (AFPs) and into a genetically encoded reporter system by Roger Tsien introduced a new era in fluorescence biology, providing a means of visualising virtually any gene product that can be genetically encoded as a fusion to the GFP gene or one of its variants and ectopically expressed in living cells, tissues or animal models.[10–13,82–91] The application of GFP technology to the cell-cycle field allowed for the re-examination of the subcellular localisation of cell-cycle regulators and subcellular structures and the characterisation of their dynamic nature thanks to time-lapse imaging in living cells transfected with genetically encoded GFP fusions.[92–98] Noteworthy examples include studies of GFP–centrin to characterise centrosomal dynamics[94] and the characterisation of the spatiotemporal localisation of cyclins[95,96] and cdc25 phosphatases.[97,98] Moreover, several fluorescence technologies have been applied to characterise genetically encoded GFP fusions, such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) to monitor the dynamics of proteins of interest within cells,[13,99–100] FRET to monitor intracellular interactions between two ectopically expressed GFP fusions,[101–105] fluorescence lifetime imaging (FLIM) to monitor changes in the local environment of a GFP fusion, such as an interaction with a partner[104,105] and fluorescence correlation spectroscopy (FCS) which can provide information on the kinetics and thermodynamics of processes associated with reversible changes in fluorescence, and typically changes in protein dynamics upon interaction with partners.[106,107]

The power of GFP technology has been harnessed to develop fluorescent markers to discriminate between different cell-cycle phases with high precision directly in living cells. These fluorescent reporters are based on genetically encoded fusions of AFPs with proteins, the functional elements of which encode well-defined spatiotemporal characteristics, such as cell-phase-dependent expression, nuclear translocation or proteolytic degradation. Noteworthy examples include cell-cycle markers of S-phase constituted of fusions of proliferating cell nuclear antigen (PCNA) to GFP and of DNA ligase I to DsRed1, which accumulate in DNA replication foci during S-phase (Figure 3A).[108–111] A truncated version of human DNA helicase B was fused to an AFP to generate a G1 biosensor the cell-cycle-dependent translocation from the nucleus into the cytoplasm of which marks the end of G1 phase (Figure 3B).[112] The combination of the genetically encoded DsRed1-DNA ligase I marker with GFP-Dnmt1 DNA methyltransferase allows differences in nuclear distribution during cell-cycle progression to be used for the further discrimination of all cell-cycle phases[111] (Figure 3C). Likewise the combination of two genetically encoded translocation-based biosensors, YFP–PCNA and a truncated version of the human DNA helicase B were employed to analyse cell-cycle progression, and the precision of individual cell-cycle phase durations in different cell types at the single cell resolution[113] (Figure 3D). An ingenious system based on a genetically encoded YFP fusion to both a plasma-membrane-targeting domain (PM) and a nuclear localisation sequence (NLS) that localises to the nucleus throughout interphase and relocates to the plasma membrane upon nuclear envelope breakdown was employed to monitor the kinetics between nuclear envelope breakdown and reformation in mitosis under physiological conditions and upon treatment with the mitotic inhibitor taxol (Figure 3E).[114] Another elegant system based on the combination of a set of AFP fusions with antiphase oscillating proteins, that mark cell-cycle transitions through differential accumulation and degradation patterns, was developed to monitor cell-cycle progression in real time and successfully used to characterise the spatiotemporal patterns of cell-cycle dynamics during different biological processes in cultured cells, brain slices and in live mice. The so-called "FUCCI" (fluorescent ubiquitination-based cell-cycle indicator) system, combines RFP-Cdt1, a substrate of SCF^{Skp2} that accumulates in G1, with GFP–geminin, a substrate of APC^{Cdh1} that accumulates in S/G2/M and is degraded in G1 (Figure 3F).[115] Likewise functional elements of cyclin B1 were taken advantage of to generate a cell-cycle marker to probe the G2/M transition in living cells. GFP was genetically fused to a fragment of cyclin B harbouring a functional destruction box (D-box) and a cytoplasmic retention signal (CRS), under control of its endogenous promoter (CCNB1); this allowed for expression of the fluorescent marker from the S-phase through mitosis, its nuclear translocation at the G2/M transition upon inactivation of the CRS by phosphorylation and its degradation at the end of mitosis (Figure 3G).[116–118]

Although GFP technology provides precious information by allowing studies of dynamic processes in living cells, it is subject to certain limitations, in particular a certain lack of control over the time required for expression of the GFP fusion and over the levels of ectopic expression, as well as the size of GFP, which might affect the behaviour, localisation and function of the gene fusion. It should also be kept in mind that genetically encoded GFP fusions encoding cell-cycle components are ectopically expressed and might therefore not necessarily reflect the behaviour of the endogenous population. Moreover, although the genetically encoded cell-cycle markers described in this review provide very elegant means of defining the cell-cycle position at the single cell level, none reports directly on the levels and activities of cell-cycle enzymes. To this aim one must resolve alternative strategies, such as the development of fluorescent biosensors that recognise endogenous enzymes and provide a direct readout of their levels or activities.

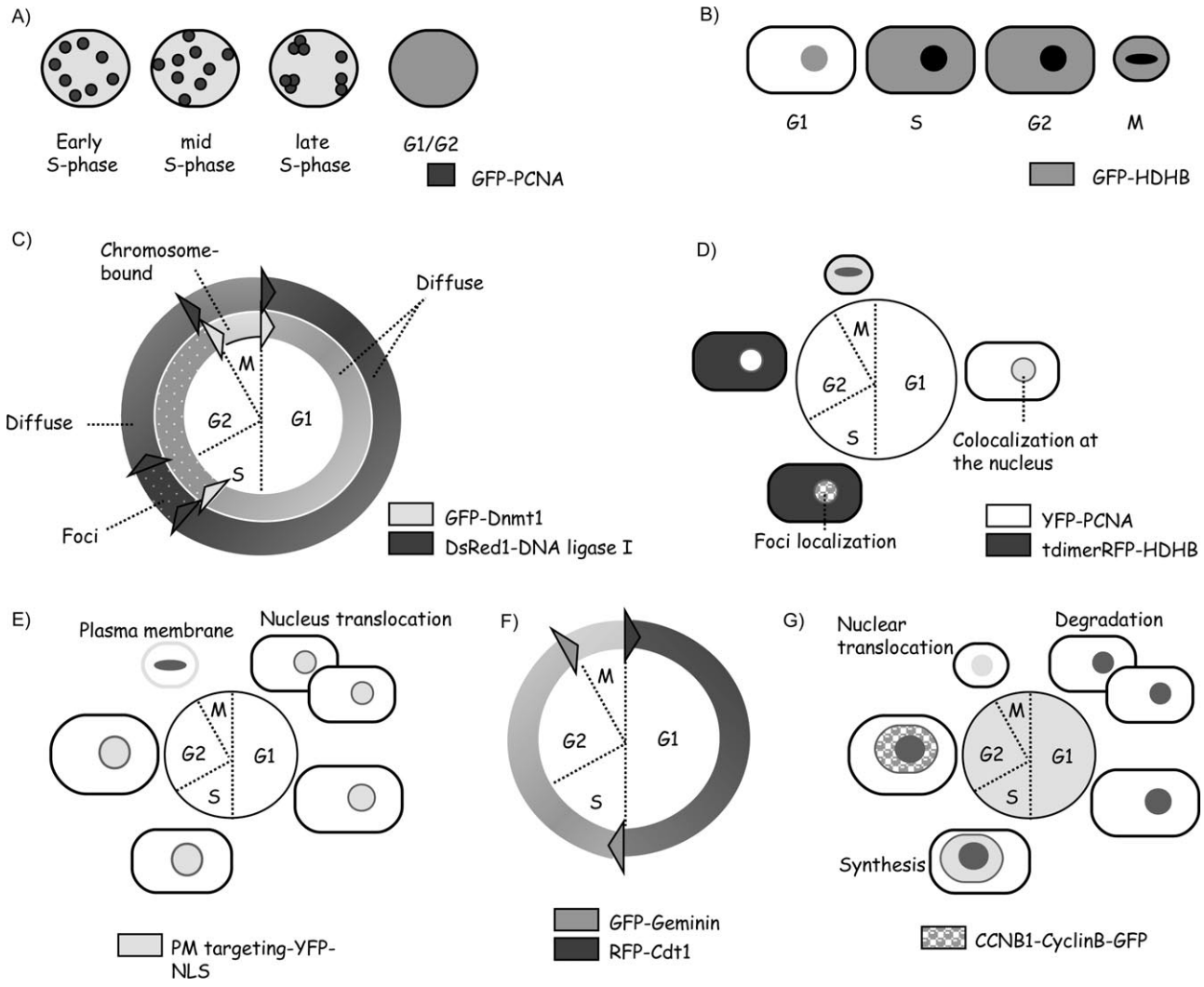

Figure 3. Genetically encoded reporters of cell-cycle phases. A) GFP–PCNA accumulates in nuclear DNA replication foci during S-phase.[108–110] B) A truncated version of human DNA helicase B fused to GFP translocates from the nucleus into the cytoplasm at the end of G1 phase.[112] C) The combination of the genetically encoded DsRed1-DNA ligase I marker with GFP-Dnmt1 DNA methyltransferase, present differences in nuclear distribution that have been employed to distinguish all cell-cycle phases[111] D) The combination of YFP–PCNA and a truncated version of the human DNA helicase B fused to RFP allows to discriminate between individual cell-cycle phase durations[113] E) Genetically encoded YFP associated with a plasma membrane (PM)-targeting domain and a NLS localises in the nucleus until the nuclear envelope breakdown at mitosis[114] F) The FUCCI system combines GFP-Geminin, which accumulates in S/G2/M and is degraded in G1 and RFP-Cdt1, which accumulates in G1[115] G) A truncated version of EGFP–cyclin B reports on cell-cycle progression thanks to functional elements of cyclin B1 including its endogenous promoter (CCNB1), which leads to expression from S-phase through G2 and mitosis, its cytoplasmic retention signal (CRS), which retains the fluorescent fusion in the cytoplasm until the end of G2, and its destruction box (D-box), leading to its degradation at the end of mitosis[116–118]

6. Fluorescent Biosensors

Over the past decade, a significant interest has been taken in designing biosensors to probe cell-cycle activities in living cells. A biosensor is commonly defined as an integrated analytical device of biological or biomimetic nature that converts recognition of an analyte into a detectable physicochemical signal.[119] Biosensors therefore offer a convenient means of reporting on the presence of specific analytes, targets or biomarkers within a complex physiological solution such as plasma, blood and living cells. Since the first biosensor applications were developed in the late 1950s and 1960s to measure oxygen, glucose and urea in solution,[120–122] different technologies have been harnessed to develop a wide variety of biosensors including electrochemical, mechanical, potentiometric, thermal and optical biosensors, including fluorescence-based biosensors.[17]

The first fluorescent biosensors were developed in the 1970s by chemical labelling of small synthetic probes to proteins peptides or nucleotides that specifically recognised specific ions or metabolites.[123] Although chemical coupling of small synthetic fluorescent probes provided the first real means of generating protein-based biosensors, relatively few were applied to monitor dynamic activities in cellulo, let alone in vivo, due to limitations associated with internalisation, biodistribution and imaging technologies. However, the development of GFP-based reporter systems paved the road for the develop-

ment of genetically encoded fluorescent biosensors that can be expressed in living cells to probe enzymatic activities, protein–protein interactions, conformational changes and post-translational modifications in real time with high spatial and temporal resolution.[14,15,124–127] Although several types of genetically encoded biosensors have been developed, including single AFP-based biosensors, split GFP-based biosensors and dual AFP–FRET biosensors, the latter present a number of advantages, which have been exploited in a wide variety of applications. Genetically encoded single-chain FRET biosensors bear a target reporter sequence (typically a substrate or a peptide ligand) and most often a specific recognition domain, separated by a linker, and flanked by a FRET pair of autofluorescent proteins, such as EGFP and mRFP or CFP and YFP. Modification of the biochemical properties of the target sequence by an enzyme or binding of a cofactor leads to its recognition by the corresponding intramolecular domain, thereby promoting a conformational change which brings together (or disrupts) the AFPs, consequently favouring (or disrupting) the transfer of fluorescence resonance energy. The first genetically encoded FRET biosensors, the so-called "cameleons" were designed to probe Ca^{2+} levels thanks to the calcium-binding protein calmodulin, the binding of which to intracellular calcium promotes binding to an intramolecular M13 domain.[128,129] Thereafter, a wide variety of biosensors have been designed and successfully applied to probe the molecular dynamics and activities of protein kinases, GTPases and proteases in living cells.[14–17,125–128] Genetically encoded protein kinase biosensors bearing a consensus phosphorylation site for the kinase of interest and a matching phosphoamino acid recognition domain, have been developed to probe protein tyrosine kinases, PKA and PKC;[130–136] conformationally sensitive biosensors have been designed to probe active forms of GTPases involved in cell motility;[137–140] and protease biosensors have been developed to monitor proteolytic activities of caspases.[141–143]

Despite the opportunities offered by GFP technology, it does not provide any control over the concentration of the biosensor expressed in cells and requires a certain length of time for its expression. Because of these limitations alternative fluorescent biosensors have been developed based on peptide and proteins labelled with small synthetic probes that can be directly introduced into living cells through physical approaches such as microinjection or electroporation, or thanks to protein transduction domains (PTD) or cell-penetrating peptide (CPP) carriers.[16,17] Protein-based biosensors are generally derived from a protein substrate or a partner-binding interface, which will report on its interaction with its ligand thanks to an "environmentally sensitive fluorescent probe", incorporated into the protein itself, the spectral properties of which are directly affected upon ligand binding if the probe is close to the ligand-binding site, or indirectly, through an allosteric-coupled mechanism if ligand binding promotes a conformational change which affects the probe. Protein biosensors have been designed to characterise reversible cofactor or ligand binding, protein–protein interactions with partners or modulators of activity, conformational changes, post-translational modifications and catalytic activities (enzyme-based fluorescent protein biosensors of catalytic activity). To name but a few, noteworthy examples that have been applied to living cells include the GTPase biosensors derived from the Cdc42 effector protein WASP,[144,145] the ratiometric biosensor of Zn^{2+} derived from carbonic anhydrase,[146] Lifeact, the 17-mer peptide biosensor of actin,[147] and enzymatically activated probes for tumour imaging that are sensitive to matrix metalloproteases.[148]

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 kinases and phosphatases involved in regulation of cell-cycle progression in living cells. Genetically encoded FRET-based reporters of protein kinases have been recently developed to probe the intracellular activity of DNA-damage-sensing checkpoint kinase ATM,[149] to study the dynamics of protein phosphorylation by the mitotic kinase aurora B during anaphase[150] and to probe Plk1 kinase activity upon checkpoint recovery.[151] More recently, we have developed an original strategy to evaluate the relative levels of cyclin-dependent kinases in living cells and in tumours based on the design of a fluorescent bi-ligand peptide biosensor coupled to an environmentally sensitive probe that is sensitive to recognition of CDK–cyclin complexes in vitro and in cellulo (L. Kurzawa, M. Pellerano, M. C. Morris, personal communication, 2010). This biosensor is efficiently delivered into living cells in the form of noncovalent nanoparticle formulations with cell-penetrating peptide carriers,[152,153] and allows for direct visualisation of endogenous CDK–cyclin complexes within their cellular environment through live-cell imaging. This technology can be employed to monitor alterations in levels of cyclin-dependent kinases associated with cancer. Moreover, this strategy can potentially be applied to develop cell-cycle biosensors of other cell-cycle regulators, thereby offering promising perspectives for diagnostic approaches, which might be further coupled to tailored therapeutic intervention. Figure 4 depicts several common examples of genetically encoded, protein and peptide, activity- and affinity-based biosensors that have been successfully applied in living cells.

7. Conclusions

Despite our understanding of the molecular mechanisms underlying cell-cycle progression, this field truly suffers from an overall lack of tools to probe the levels, activities and spatiotemporal dynamics of cell-cycle regulators in living cells. Since the early days of gross morphological descriptions of cell division and characterisation of subcellular structures and molecular components requiring fixation procedures, the development of GFP technology has provided a powerful opportunity to visualise ectopically expressed fusions of cell-cycle regulators in living cells, further leading to the design of elegant reporter systems to characterise distinct cell-cycle phases. Although these genetically encoded fluorescent markers of cell-cycle phases based on fusions of functional elements with characteristic spatiotemporal features provide an ingenious means of determining the status of living cells, they do not provide any insight into the behaviour of cell-cycle regulators

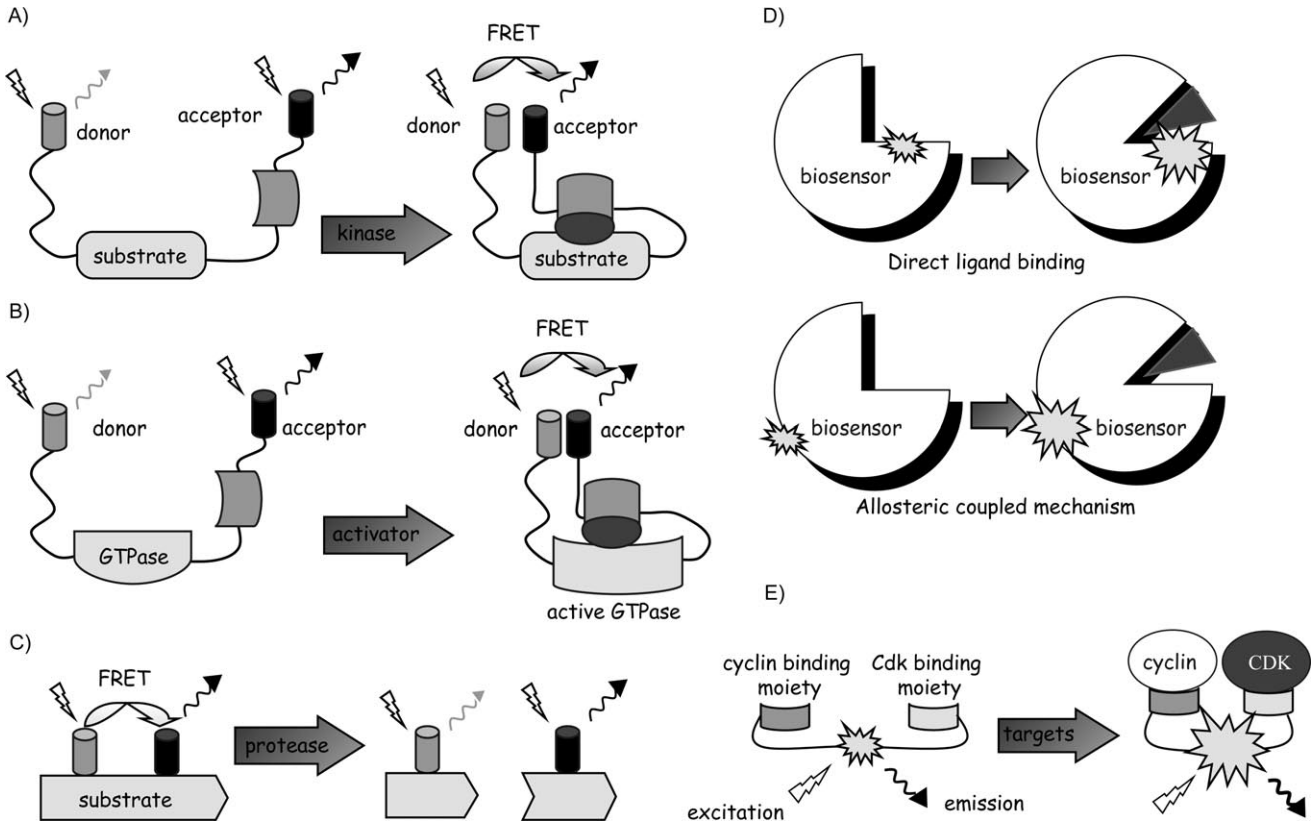

Figure 4. Fluorescent biosensors. Common examples of activity-based biosensors include genetically encoded single-chain FRET biosensors that consist of a FRET pair of AFPs and a substrate sequence which is modified by an enzyme of interest, such as A) protein kinase biosensors, phosphorylation of which promotes recognition of the substrate sequence by an adjacent phosphobinding domain, thereby bringing together the AFPs and favouring FRET.[130–136] B) Conformationally sensitive biosensors of GTPase activation, that harbour a GTPase together with a binding domain which only recognises the conformationally active form of the enzyme.[137–140] C) Protease biosensors, such as caspase biosensors whose ability to FRET is disrupted upon proteolytic cleavage of the substrate sequence.[141–143] D) Affinity-based biosensors consist of fluorescently labelled peptide or protein sensing sequences, derived from substrate sequences or binding interfaces, which exhibit changes in fluorescence upon analyte recognition, such as the LifeAct peptide biosensor of actin,[147] and the GTPase biosensor derived from the Cdc42 effector domain WASP;[144,145] the fluorophore can either be directly involved in analyte recognition or indirectly through an allosteric coupled mechanism. E) The CDK/cyclin biosensor is a bi-ligand peptide that bears two recognition moieties linked by a sequence bearing a fluorescent probe. A modification in the environment of the probe occurs upon recognition of the CDK–cyclin complex, leading to a significant and measurable variation in the spectral properties of the probe.[153].

themselves. The explosion of fluorescent biosensor technologies over the past decade, notably applied to the study of signal transduction pathways, has led to the more recent development of fluorescence-based biosensors to probe cell-cycle regulators and characterise their dynamics with high spatial and temporal resolution.

Biosensors not only provide a means of studying the behaviour of enzymes in their natural environment, they are also very useful for comparative studies of biological processes in healthy and pathological conditions, and may be further applied to diagnostic approaches to assess the status of a specific target, to highlight molecular and cellular alterations associated with pathological disorders, and to monitor response to therapeutic intervention. This is of particular relevance in the cell-cycle field given the central role of many cell-cycle regulators, their documented alterations in human cancers and their prognostic value. However, despite the pharmacological importance of a large panel of cell-cycle regulators, their intracellular localisation has limited the development of diagnostic approaches to monitor alterations in their levels or activities associated with human cancer. Hence, ongoing efforts to engineer fluorescent biosensors to probe these intracellular biomarkers of cell proliferation in cellulo should provide precious information regarding their cellular behaviour in physiological and in pathological conditions, thereby allowing insight into the signal transduction pathways underlying cell-cycle progression to be gained. Moreover, biosensors are directly applicable to screening and diagnostic strategies, further allowing for coupled therapeutic intervention. Finally, fluorescent biosensors have proven useful for endoscopy or tomographic optical imaging methods in vivo, as well as for screening, molecular profiling and oncological surgery.

Abbreviations

AFP: autofluorescent proteins, BrDu: bromodeoxyuridine, CDK: cyclin-dependent kinase, CRS: cytoplasmic retention signal, DAPI: diaminido phenyl indol, FACS: fluorescence-activated cell sorting, FLIP: fluorescence loss in photobleaching, FRAP: fluorescence recovery after photobleaching, FLIM: fluorescence

life-time imaging, GFP: green fluorescent protein, MPF: maturation- or mitosis-promoting factor, NLS: nuclear localisation sequence, PI: propidium iodide, PM: plasma membrane

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