

Chapter 2

Fluorescent Biosensors: Design and Application to Motor Proteins

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Abstract Reagentless biosensors are single molecular species that report the concentration of a specific target analyte, while having minimal impact on the system being studied. This chapter reviews such biosensors with emphasis on the ones that use fluorescence as readout and can be used for real-time assays of concentration changes with reasonably high time resolution and sensitivity. Reagentless biosensors can be designed with different types of recognition elements, particularly specific binding proteins and nucleic acids, including aptamers. Different ways are described in which a fluorescence signal can be used to report the target concentration. These include the use of single, environmentally sensitive fluorophores; FRET pairs, often used in genetically encoded biosensors; and pairs of identical fluorophores that undergo reversible stacking interactions to change fluorescence intensity. The applications of these biosensors in different types of real-time assays with motor proteins are described together with some specific examples. These encompass regulation and mechanism of motor proteins, using both steady-state assays and single-turnover measurements.

Keywords Biosensor • Fluorescence • Assay • ATPase • Helicase • Kinesin • Myosin

Abbreviations

DCC-SSB	(G26C)SSB adduct with <i>N</i> -[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence in situ hybridization

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FP	Fluorescent protein
FRET	Förster (or fluorescence) resonance energy transfer
MDCC	7-diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl)coumarin
MDCC-PBP	(A197C) Phosphate binding protein adduct with MDCC
PNA	Peptide nucleic acid
SELEX	Systematic evolution of ligands by exponential enrichment
TIRFM	Total internal reflection microscopy

2.1 Introduction

This chapter reviews different types of biosensors and how some of them are being applied to probe molecular motors. The biosensors field is wide, in part because of a somewhat diffuse definition of biosensor, which means different things to different people. This chapter is restricted to what are often called reagentless biosensors, essentially a single species in solution that reports the concentration of a target molecule. The term “reagentless” implies that no other reagent, apart from the biosensor, needs to be added to the system being studied. This is in contrast with, for example, a coupled enzyme assay, in which several extra components may need to be present. This chapter focuses on reagentless biosensors that can be applied to continuous, time-resolved measurements with fluorescence as the readout, as these have found widespread use in assays with motor proteins to measure either the movement, ATP hydrolysis, or such aspects as regulation.

There are two essential elements of the reagentless biosensors of the type described here. The example of a biosensor for inorganic phosphate (P_i) [1, 2] in Fig. 2.1 will be used to illustrate these concepts, as this is a simple entity and has been widely used in the motility field [3–5]. Firstly, a recognition element senses the target analyte with some degree of selectivity over similar molecules. The recognition element can be an enzyme that catalyses a reaction of the analyte, a macromolecule, such as protein or nucleic acid, or a small molecule that binds the analyte. In the case of the P_i biosensor, this element is a phosphate binding protein that binds P_i tightly and with a high degree of selectivity. The second element is a reporter, which produces a signal, in response to interaction of the analyte with the recognition element that can be measured without interference from the rest of the assay components. The signal can be electrons or light, for example. The P_i biosensor has a coumarin fluorophore covalently bound to a single cysteine on the protein surface. At this position, the label responds with a ~tenfold increase in fluorescence intensity when P_i binds. The signal is transduced by a protein conformation change on binding P_i , thereby changing the environment of the fluorophore and hence its fluorescence: in this case, there is no separate transducing element as the protein itself does this. In some biosensors, such an element needs to be included within the biosensor to couple the recognition and reporter elements.

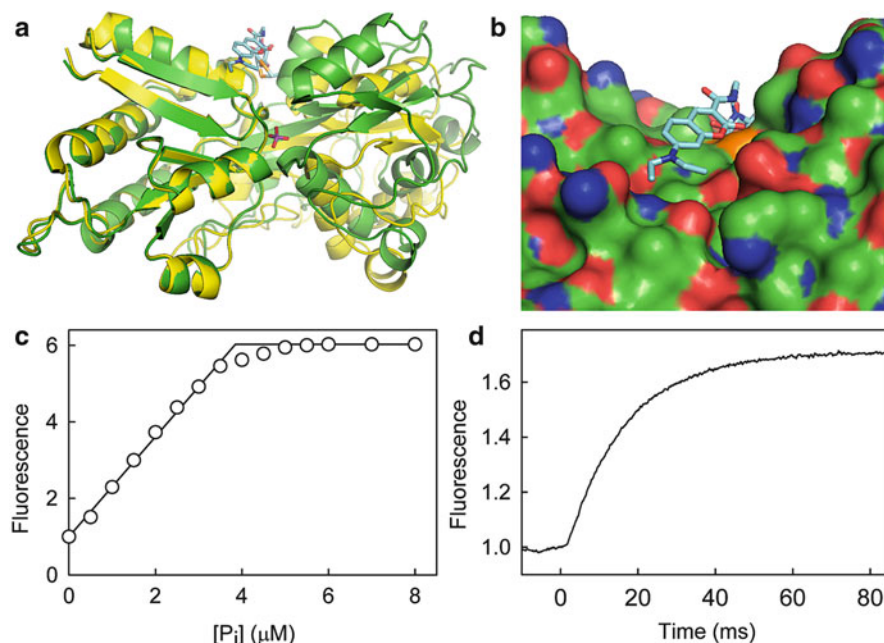


Fig. 2.1 P_i biosensor, MDCC-PBP. (a) Structure, showing P_i-bound form (*green/dark*) overlaid with unlabelled apo-PBP (*yellow/pale*). P_i is bound between the two domains. One domain (*left*) is overlaid to show movement of the other (*right*) on P_i binding [31, 77]. (b) Diethylaminocoumarin sits in a cleft between the two domains in the P_i-bound structure. In the apo form, it is likely that the coumarin position is flexible and pointing into the medium [31]. (c) Titration of P_i into 5 μM MDCC-PBP, to show region of linear response, capacity and calibration [2]. (d) Measurement of binding kinetics by mixing 0.25 μM P_i with 0.1 μM MDCC-PBP in a stopped-flow apparatus [2]

A major advantage of reagentless biosensors is that only a single component is added to the system being studied, so it causes only a minimal modification or interference. Essentially, reagentless biosensors can be “impactless” on a solution of proteins, etc. being assayed. For example, the P_i biosensor has been used to measure P_i produced by the ATP hydrolysis of myosins, kinesins, helicases, etc. In measuring such a product, the motility system itself is not directly altered: the biosensor interacts with P_i only after it dissociates from the motor protein. This is in contrast to some other fluorescence techniques, such as labelling components of the system itself (e.g. proteins or substrates), which potentially affects the studied reaction significantly. However, it should be pointed out that binding products of a reaction does limit the reversibility of that reaction, should that be an important feature: for example, P_i can inhibit muscle ATPase activity.

2.2 Types of Reagentless Biosensor

Reagentless biosensors can be classified according to the nature of their recognition element, which interacts with the target molecule. This can be, for example, a peptide, protein, nucleic acid, or a small synthetic molecule.

2.2.1 Protein-Based Biosensors

Protein-based biosensors rely mostly on ligand-binding proteins that selectively interact with the target molecule, as outlined above. If necessary, selectivity can be further enhanced or modified by mutating the binding site, based on structural information. This has, for example, been done on an ADP biosensor, based on a bacterial actin homologue, ParM [6]. The wild-type protein is an ATPase that binds both ADP and ATP, but introducing mutations in the active site strongly decreases the affinity for ATP and results in a biosensor with >400-fold preference for ADP. The high specificity, together with potentially tight binding, is an advantage over most other types of biosensors, where the scaffold for specific recognition has to be developed first. However, it largely restricts such biosensors to target molecules for which a suitable, specific binding protein is available. There are only a few examples of protein-based biosensors, where a new ligand specificity has been generated, for example, by computational design [7, 8].

A fluorescent reporter is coupled to the ligand-binding protein either by chemical labelling of the protein with small fluorescent probes or by genetic fusion to fluorescent proteins like GFP (green fluorescent protein) and its variants to produce genetically encoded biosensors. Chemical labelling at specific sites is often achieved via cysteine-reactive probes, mostly iodoacetamide or maleimide derivatives, which are commercially available for many fluorescent dyes. This enables the label to be positioned specifically on the surface. Small synthetic dyes generally give larger signal changes and may be more suitable for applications in vitro, whereas genetically encoded biosensors are the method of choice for measurements in cells or in vivo [9, 10]. There are also approaches combining the two strategies by genetic fusion with protein tags, such as SNAP-tag or CLIP-tag [11, 12], which can then be chemically modified with fluorescent dyes [13, 14], as outlined in Sect. 2.3. The chemical reaction is specific enough for labelling of a genetically encoded protein scaffold on a cell surface or even within cells, and thus, the functional biosensor can be generated in situ.

Another advantage of using a protein framework is that the normal specificity and activity of the protein may be harnessed as the transducing element. That is particularly so, when using a protein conformation change, linked to ligand binding, to change the environment of an attached fluorophore. This was outlined above for the P_i biosensor (Fig. 2.1). However, a number of different strategies have been

used to transduce the binding event into a fluorescence change of the reporter in these types of biosensors, some of which are discussed in Sect. 2.3.

2.2.2 Nucleic Acid-Based Biosensors

Oligonucleotide-based sensors fall into two groups: firstly, hybridization probes that anneal to complementary sequences of DNA and RNA and, secondly, aptamers that recognize targets other than nucleic acids.

In most cases, hybridization probes are made up of synthetic nucleic acids, which are complementary to their target sequence and linked to a fluorescent reporter group [15, 16]. Many such probes are used in techniques requiring either the analyte or the hybridization probe to be surface bound, as is the case in single-molecule studies, microarrays and FISH (fluorescence in situ hybridization). Hybridization probes can also be applied in solution, for example in quantitative PCR or kinetic assays. The reporter element is formed by labels attached to the 3'- and/or 5'-ends of the probe(s), either as a single fluorophore or as a FRET (fluorescence resonance energy transfer) or fluorophore–quencher pair, depending on the strategy [15] (also see Sect. 2.3).

Aptamers form a versatile subclass of nucleic acid probes. In contrast to hybridization probes, aptamers are generated through “systematic evolution of ligands by exponential enrichment” (SELEX) specifically to bind small molecule ligands, metal ions, proteins, or even cell surfaces [17, 18]. The wide range of target molecules, which can be addressed by aptamer-based biosensors, is an advantage over the protein-based biosensors described above, where obtaining new ligand specificities usually requires a new recognition protein. Due to this, aptamers have found widespread applications in therapeutics and medical screens [19, 20], as well as in basic research [21]. While showing similar affinity and selectivity to antibody-based systems (see Sect. 2.2.3), single-stranded DNA or RNA aptamers have several advantages over the former class of biosensors. Their wide range of targets, small size, high thermostability, low immunogenicity, ease of synthesis and modification have led to an increase in their popularity [20–22].

Aptamers often undergo conformational changes when binding their target. Such structural changes can be exploited by strategically introducing chromophores to report on the structural alterations in different ways. One aptamer, making use of a conformational change to detect ATP with a >threefold selectivity over guanosine and pyrimidines, consists of a 26-bp DNA molecule, labelled with two pyrenyl groups [23]. In the absence of a ligand, the two pyrene rings are thought to interact with neighbouring bases on the single-stranded DNA (ssDNA), resulting in a low fluorescence quantum yield. Upon binding two ATPs, the DNA folds back on itself, becoming double stranded. The newly formed base interactions release the pyrenes, which become free to form a bis-pyrene excimer and so have an increased fluorescence [24]. While excimers and FRET pairs have been used in the development of a number of aptamers, the most common approach consists of a fluorophore and a

quencher moiety attached to the 3'- and 5'-ends [22]. These “molecular beacons” are a large class of biosensors that are mostly based on nucleic acids but some also on peptides or PNA (peptide nucleic acid). They possess a typical stem–loop structure, which is disrupted by target association, resulting in the separation of the fluorophore–quencher pair [25]. A comprehensive description of the various types of aptamer sensors and their applications has been covered by several recent reviews [15, 26–28].

2.2.3 Antibody-Based Biosensors (Immunosensors)

Immunosensors use antibodies or antibody fragments as recognition elements to detect antigens with high specificity and high affinity. A wide range of reporters have been used, including those giving a fluorescence signal. Most antibody-based probes require other molecules as part of the biosensor and are therefore not reagentless. However, due to their importance in research, the principles of some immunosensors are outlined here.

Most immunoassays are heterogeneous with recognition taking place at the surface of a solid matrix. This is achieved either by direct binding of a fluorescently labelled antibody to an immobilized target or by secondary labelling, whereby a fluorescent molecule binds to an unlabeled, surface-bound antibody. Alternatively, there is a sandwich approach, in which an antigen–antibody complex, attached to a surface, is recognized by a labelled antibody for detection, as is used in the enzyme-linked immunosorbent assay, ELISA [29]. All these assays require the target or antibody to be separated from a homogeneous sample via interaction with the complementary component, fixed to a solid matrix. The accumulation of fluorescent molecules at a surface provides the readout. In a homogeneous format, target and sensor interact in solution, dispensing with a separation step. This interaction must result in a fluorescence change, and so these are similar in principle to other protein-based biosensors (Sect. 2.2.1). For example, two pools of antibodies raised against two different sites on an antigen can be labelled with compatible fluorescent dyes and used in a FRET-based manner [29]. To date the majority of immunosensors are used in surface-immobilized assays, which typically have low time resolution, rather than being suitable for time-resolved measurements [22].

2.2.4 Other Recognition Elements

Chemosensors, based on small synthetic molecules, form a large group of probes and include what are often simply called indicators [30]. Their design principles are largely very different from those of the biosensors described here. However, they often target biological molecules and are used similarly to biosensors, based on

proteins or nucleic acids. An example, to measure calcium ions, is given in Sect. 2.5.

2.3 Design Principles: How Target Recognition Is Coupled to a Fluorescence Signal

A critical step in biosensor development is to couple target recognition to a fluorescence change of the reporter. There are a number of different designs, depending in part on the nature of the sensing element. Here, the main focus is on protein-based biosensors, but some design principles also apply to biosensors based on nucleic acid aptamers.

2.3.1 *Single Fluorophore*

Most simple designs rely on the fluorescence intensity change of a single, environment-sensitive fluorescent reporter that is attached to the recognition element. A conformation change in the recognition element upon target molecule binding is translated into a change of the local environment of the fluorophore (Fig. 2.2a). This can lead to a fluorescence intensity change by several mechanisms, such as changing the solvent accessibility and thus dynamic quenching, a change in polarity of the surrounding environment, or a change of specific interactions of the fluorophore with the protein. The last mechanism has been shown to be the main contribution for the phosphate biosensor, MDCC-PBP (Fig. 2.1). In this case, there is a specific mechanism by which the diethylaminocoumarin has high fluorescence in the phosphate-bound state, namely, by becoming coplanar due to an interaction with the protein [31]. A large number of other examples for this type of design, based on periplasmic binding proteins similar to PBP, exist, where ligand binding induces a large movement of two subdomains relative to each other leading to closure of the ligand-binding cleft [1, 32, 33].

Alternatively, a direct interaction of the fluorophore with the bound target molecule can cause a change in fluorescence intensity (Fig. 2.2b). An example is a biosensor for single-stranded DNA (DCC-SSB), based on the single-stranded DNA-binding protein (SSB). DNA binding causes an increase in the fluorescence of a single, environment-sensitive fluorophore (diethylaminocoumarin) in the absence of a recognized conformation change [34]. A third method of obtaining a fluorescence change is by ligand-induced modification of the biosensor, and an example is the biosensor that responds to ATP/ADP ratio [35]. A fluorophore-modified nucleoside-diphosphate kinase emits fluorescence depending on the degree of phosphorylation of the protein. Normally such phosphorylation is a transient part of the natural enzyme mechanism, but the process was harnessed so the biosensor responds to phosphorylation by ATP and dephosphorylation by ADP.

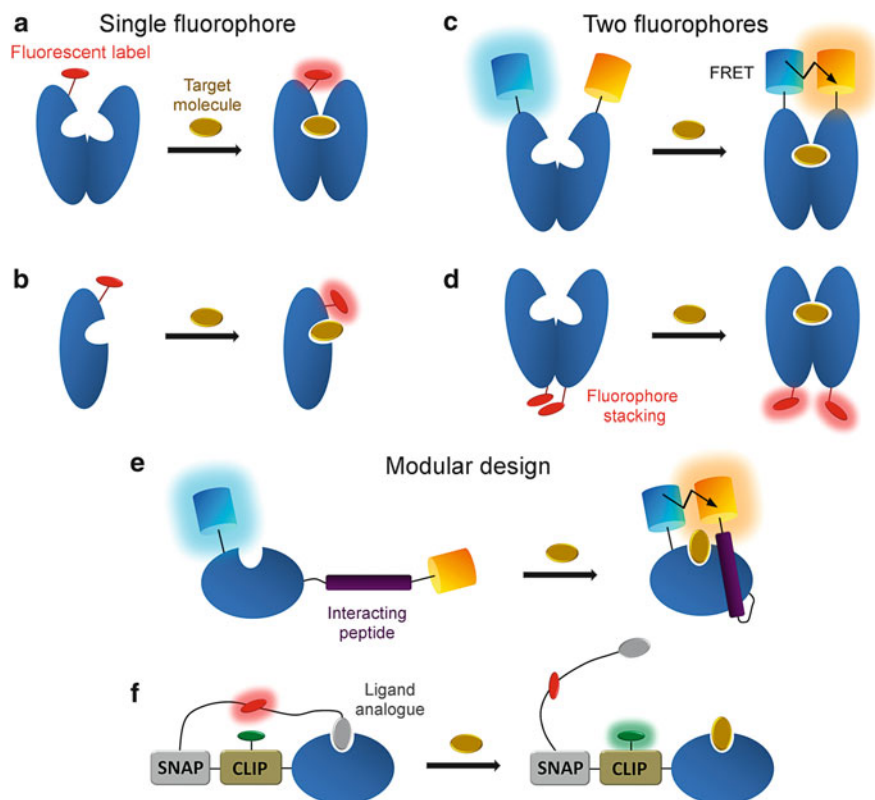


Fig. 2.2 Different designs of fluorescent reagentless biosensors. These design features mainly are applicable to protein-based biosensors. *Single-fluorophore biosensors*: conformation change (a) or interaction with target (b) alters the fluorophore environment. *Biosensors with two fluorophores*: readout is by FRET between two different fluorophores, shown here as fluorescent proteins (c), or by disruption of stacking between two identical fluorescent dyes (d). *Examples of modular designs*: part of the fused system interacts with the recognition element in either the target-bound (e) or target-free state (f), so transducing the signal when the target binds. In both types shown, the readout is by FRET. See Sect. 2.3 for details

In contrast to small synthetic dyes, in genetically encoded biosensors that use fluorescent proteins (FP), the chromophore is shielded inside the protein structure, which renders it less susceptible to environmental changes. The strategy for signal transduction is thus different. Several genetically encoded biosensors rely on the intensity change of a single FP, for example, biosensors reporting on the ATP/ADP ratio [36], Ca^{2+} [37, 38], $\text{Ins}(1,3,4,5)\text{P}_4$ [39] or glutamate [40]. By inserting FPs into a structural element that undergoes a conformation change upon ligand binding, this can alter the local structure or protonation state of the chromophore and thus the fluorescence intensity. Often circularly permuted versions of FPs are used, in which the N- and C-termini of the FP are in close proximity to the chromophore. In this way, when fused to the recognition protein, circularly permuted FPs are more likely

to give a fluorescence change than the normal FP version. The dependence of fluorescence on the protonation state has also been exploited to use FPs as pH biosensors. Mutations around the chromophore have yielded variants that respond to changes in different pH ranges and with different spectral properties [41].

2.3.2 *Two Fluorophores*

A large number of biosensor designs are based on FRET (Fig. 2.2c). Here, a pair of fluorophores, specifically a FRET donor and acceptor, are fused to the recognition element. If a ligand-induced conformational change alters the distance or relative orientation of the FRET pair, this can result in a change in FRET. In comparison to probes based on fluorescence intensity of a single fluorophore, FRET provides a ratiometric signal, the ratio of acceptor to donor emission. This is an advantage for quantitative measurements in a complex environment, such as in cells, as the ratio should be independent of biosensor concentration. Due to the practical difficulty of specific labelling, a FRET-based design is not easily implemented with two different chemical dyes. In contrast, FRET-based mechanisms are very common among genetically encoded biosensors, such as for Ca^{2+} [42–44], cAMP [45, 46] and cGMP [47, 48], sugars [49–51], phosphate [52] and ATP [53].

In a similar way, a fluorophore–quencher pair of labels can be used, whereby the fluorescence depends on the proximity of the quencher to the fluorophore. Fluorescence quenching occurs by FRET or static quenching or, mostly, by a combination of both. Static quenching involves a physical interaction between fluorophore and quencher and thus requires the pair to be closer to each other than in a FRET pair. This principle is realized in “molecular beacons”, as described in Sect. 2.2.2.

A more straightforward approach is to take advantage of stacked dimer formation with two identical fluorescent labels. Stacking can occur with various aromatic dyes, such as xanthenes, which show strongly quenched fluorescence in the stacked state (Fig. 2.2d). This requires labelling the protein with only one type of fluorophore, but at two positions. The strategy has successfully been applied to develop protein-based biosensors for phosphate and ADP/GDP [54–56]. These consist of a specific binding protein labelled with two tetramethylrhodamines, positioned so they can form a stacked dimer in the ligand-free state with a shift in absorbance wavelength from monomeric to dimeric state. A conformation change upon ligand binding causes disruption of the dimer and thereby a fluorescence increase. While the stacked rhodamine dimer forms in the ground state of the molecules, other dyes form excited state dimers (excimers). Excimers show a shift in emission wavelength and a signal increase can be observed upon target binding when monitoring the dimer emission wavelength. An example of the application of an excimer label is given by the bis-pyrene-labelled aptamer, described in Sect. 2.2.2.

2.3.3 Modular Designs

In all biosensor types described so far, target recognition and signal transduction are in a single protein or nucleic acid recognition element. However, another option is a modular design, in which additional transducing elements are introduced into the same molecule to create a functional signal transduction mechanism. This is realized, for example, with a peptide or protein domain that binds to the recognition element only in the ligand-bound form (Fig. 2.2e). Target molecule binding brings the recognition domain and the interacting domain in close proximity, which can be converted to a fluorescence signal, for example, by a FRET pair. This principle is the basis for a group of genetically encoded, Ca^{2+} biosensors (cameleons) [42, 57]. They consist of calmodulin (CaM) as the recognition element and a calmodulin-binding peptide derived from myosin light chain kinase (M13), which are fused to either BFP/GFP or CFP/YFP as a FRET pair. Binding of Ca^{2+} to CaM induces wrapping of CaM around the M13 peptide. This conformation change brings the FPs into closer proximity and increases FRET. Calmodulin has four Ca^{2+} binding sites with a large difference in affinity (>100 -fold) between the binding sites in the N- and C-terminal domain. This enables the biosensor to report a very wide range of Ca^{2+} concentrations ($<10^{-7}$ to $>10^{-4}$ M), which could be further increased by mutations in CaM to create versions with different affinities [42]. An example of these Ca^{2+} biosensors being applied to a motility system is a study in live *Caenorhabditis elegans*, where they have been used to measure Ca^{2+} transients in pharyngeal muscle [58]. Since the first cameleons were described, several new versions of CaM-based biosensors have been developed with improved characteristics such as increased brightness and dynamic range, improved pH-stability, folding efficiency and photostability [59, 60].

Another type of modular biosensor design has been described using an intramolecular ligand analogue together with the recognition domain [14] (Fig. 2.2f). In these semisynthetic sensors, the ligand analogue is coupled to the recognition protein using a genetically encoded SNAP-tag. In the absence of a target, the ligand analogue occupies the binding site of the recognition element giving the biosensor a closed conformation. The target molecule competes off the intramolecular ligand leading to an open conformation. The signal readout is by FRET, whereby one fluorophore is introduced on the linker of the ligand analogue and a second fluorophore is either an FP or a dye coupled to another self-labelling tag (CLIP-tag). Several examples of these biosensors, including ones for glutamate and GABA, have been described [13, 61, 14, 62].

2.4 Other Considerations: Suitability to Particular Types of Assay

Most of the description of biosensor development so far has concentrated on the overall design process. Some of the basic properties of fluorescent biosensors, which are important in terms of their applicability, are discussed below.

2.4.1 *Fluorophore*

Important properties of the fluorophore are the wavelength and whether it is (or they are) suitable for the selected type of readout, such as environmental change of fluorophore or FRET. Interference with the fluorescence signal can be a problem, and this may depend on the type of assay intended. For example, are the wavelengths compatible with other components of the assay, or do other components absorb light and so may be damaged by the high intensity of exciting light? Generally, fluorophores with longer excitation and emission wavelengths are less prone to artefacts due to optical interference.

The sensitivity of the assay is in part dependent on the choice of fluorophores, the inherent signal change and the intensity of exciting light. An inherently large fluorescence change, usually severalfold, is desirable with a preference for a fluorescence increase rather than decrease. For a biosensor with a single fluorophore, a large change in fluorescence quantum yield is needed to report on a change in the environment of the fluorophore. Choice of FRET pairs depends on the overlap of the spectra, and the literature cited in this review gives a number of suitable pairs of fluorescent proteins. However, as pointed out already, labelling a protein with two different chemical dyes can be a challenge.

Fluorophores with high extinction coefficient and high quantum yield (at least in the high fluorescence state) will increase the photons available for detection. High intensity of exciting light can also increase sensitivity, but the usable intensity may depend on the photostability of assay components, particularly the fluorophore, together with the time that the irradiation must be maintained. In single-molecule experiments where laser excitation is used, many fluorophores are photobleached after a few seconds. On the other hand, when exciting by, for example, mercury lamps used in some fluorimeters and stopped-flow apparatuses, the same fluorophore may be stable for many minutes: with lower power xenon lamps stability may be many hours.

Finally, the size of the fluorophore might be an important factor. Fluorescent dyes that are bright and absorb and emit at long wavelengths are generally larger than shorter wavelength variants and this might affect the recognition element negatively. For example, a large (and hydrophobic) fluorophore may limit the solubility of the biosensor.

2.4.2 Recognition Element

In the case of a protein framework, in particular, a good starting point is using a protein which has a known high-resolution structure to aid design and is robust to ensure stability. Important general properties must also be considered, including the concentration range that a biosensor can measure, the sensitivity of the detection and how fast it can respond to changes in concentration. A titration under the experimental conditions of the actual assay will give information about these factors, as shown as an example in Fig. 2.1c for the P_i biosensor.

Another point to consider is whether other components in the assay may produce a response by interacting with the recognition element. The ADP biosensor mentioned above is an example in which this problem has been overcome to allow measurement of ADP in the presence of structurally similar ATP [6].

Different types of assay require measurements over different concentration ranges: this might be “as low as possible” for many measurements *in vitro*, but much higher to reproduce cellular conditions. In the case of protein-based biosensors, in particular, there is a rational approach to vary the concentration range by varying the affinity of the protein for the target. This was demonstrated, for example, for a P_i biosensor [52] or Ca^{2+} biosensors [42, 44]. An issue then becomes reagent usage and cost, versus sensitivity. A tight-binding biosensor may bind the target stoichiometrically under assay conditions, and it needs to be in excess over the target analyte. By weakening the affinity, the biosensor can be used sub-stoichiometrically, as it now responds to changes in target concentration on either side of the dissociation constant [56]. Going sub-stoichiometric reduces sensitivity but also reduces reagent usage and cost.

2.4.3 Time Resolution

For time-resolved measurements, the speed of response is important. For simple reagentless biosensors, based on ligand binding, the speed depends largely on the kinetics of association and dissociation. Real-time measurements of motor proteins require biosensors that have a rapid response: many motor proteins, for example, hydrolyze ATP at $>100\text{ s}^{-1}$ and move in $>100\text{ steps s}^{-1}$. Ideally a biosensor should have a response severalfold faster than the process being assayed to ensure the observed rate is purely that of the motor protein. If a high speed of response is required, then characterization may need to include the rate of binding: an example trace is shown in Fig. 2.1d for P_i binding to the P_i biosensor in tens of milliseconds.

2.4.4 Biosensors for Cellular and Single Molecule Studies

Apart from hybridization probes, which are applied in situ, and genetically encoded sensors, most biosensors are currently used mainly in vitro. This has several practical reasons, including difficulty of getting a functional biosensor into a cell. The choice of label is furthermore limited by the fact that many different, physical environments exist within a single cell. These could affect the behaviour of fluorophores leading to high background and rendering environmentally sensitive fluorophores inapplicable. In addition, discrimination may be needed against many molecules present within the cytoplasm, and there may be a wide range of concentrations of the target molecule in different subcellular compartments. It thus remains a major challenge to develop probes for measuring precise concentrations in live cells.

For transferring assays to single-molecule formats, such as total internal reflection fluorescence microscopy (TIRFM), immobilization of the fluorophore on the surface may be sufficient to obtain the readout, rather than actual fluorescence enhancement. Such a strategy was used to measure helicase unwinding (Sect. 2.5.6), whereby the environmentally sensitive coumarin fluorophore used for solution studies was replaced by a cyanine dye with relatively constant high quantum yield for TIRFM [63]. This also had the advantage of two other properties favourable for TIRFM, namely, a much more stable fluorophore and a higher wavelength.

2.5 Applications to Motor Proteins

2.5.1 Regulation: Calcium

A distinct application of biosensors and similar probes to motor proteins has been to monitor levels of molecules involved in regulation. Possibly the most-used class of small molecule indicators are those for calcium ions. A series of papers from Tsien described a range of such indicators with improved performances, such as the indo and fura series [64, 65]. These are based on an aromatic core containing multiple carboxyl groups, which form a complex with the calcium ion, inducing a fluorescent conformation in the indicator molecule. A key feature is their selectivity for calcium over magnesium, together with an affinity for calcium that allows the indicators to measure concentrations relevant for cell signalling, ranging from sub-micromolar to micromolar. These probes have been widely used, and early examples of their application in the muscle field were to obtain time-resolved information, especially in smooth muscle, in order to understand the control of contraction by calcium [66, 67]. An example is work that combined the use of the calcium indicator, fluo-3 [68], with caged compounds (Fig. 2.3a). The timed sequence of activation events for permeabilized smooth muscle was measured

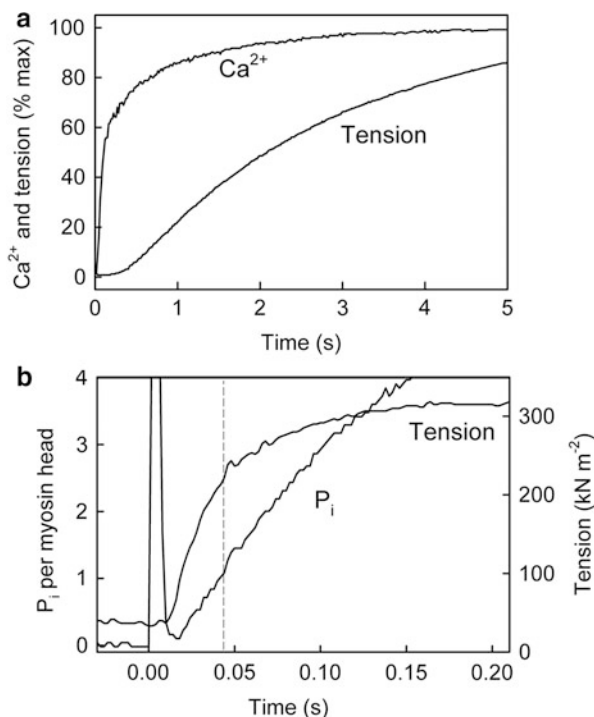


Fig. 2.3 Use of sensors to measure changes in target molecule concentration during activation of muscle contraction. (a) A Ca^{2+} sensor, fluo-3, was used to measure a Ca^{2+} transient leading to activation of smooth muscle force generation after release of inositol trisphosphate by photolysis of caged IP3 [69]. (b) A P_i biosensor, used to measure ATP usage during generation of force in striated muscle. Activation of the glycinerated muscle cell is by photolysis of caged ATP [70]. The spike at zero time is the flash photolysis artefact. The vertical, dashed line represents turnover of one ATP per myosin head ($\sim 150 \mu\text{M}$) within the fibre, showing that most tension is generated concurrently with that first turnover

following rapid release of inositol trisphosphate (IP3) from its caged precursor [66, 69]. The IP3 concentration jump was followed by an increase in calcium, then in turn by muscle contraction.

2.5.2 Mechanism

Biosensors have also provided a way to get detailed, time-resolved information about motor protein mechanisms. Although some questions are likely to vary between different types of motors, there are some shared questions, particularly for linear motors, in which ATP hydrolysis drives movement along a track. A large number of such linear motors comprise those that move along DNA, such as

helicases, and those that move along protein tracks, especially myosins on actin filaments, and kinesins and dyneins along microtubules. One common question for such linear motors is that of the processivity: how far the protein moves along the track before dissociation. A second question relates to the step size and how it is coupled to ATP hydrolysis. “Step size” can have various meanings, including what distance is moved for each discrete step: often the smallest discernible step comes out of single-molecule measurements. However, another type of step size describes how far the motor moves for each ATP used, and this can be designated as the “ATP-coupling step size”. That leads to considerations of the efficiency of the motor and how movement is affected by load. An additional question, common to the different types of motor, linear or rotary, concerns mechanochemical coupling that understands how the ATPase cycle relates to movement, especially through protein conformation changes.

As described in Sect. 2.1, reagentless biosensors are particularly suited to measure reaction products, as they have only minimal impact on the system being studied. These types of measurements have played a significant part in understanding motor protein mechanism and defining their properties, particularly in respect to measuring the products of ATP hydrolysis. However, helicases unwind their nucleic acid track, making it possible to measure the extent of unwinding using a biosensor which binds the single-stranded DNA product of unwinding (see Sect. 2.5.6). In the case of protein tracks, such a measurement would be more difficult, because the motor proteins generally do not alter the track but also because it is more challenging to obtain protein tracks of uniform length in comparison with nucleic acids, where production of defined lengths is possible.

Because of its high speed, variants of the phosphate biosensor [1, 54] have been widely used to probe the ATP hydrolysis: under some conditions, its application gives the ATP usage relative to the extent of movement [5]. It also allows investigation of the P_i release step of the ATPase cycle of myosins [70, 4], kinesins [3] and helicases [71], which may be a key step in the transduction to produce movement and which otherwise is a difficult step to measure. Some specific examples of this detailed probing of ATP hydrolysis are discussed below.

2.5.3 Time-Resolved Measurements: Steady-State Kinetics

The simplest type of kinetic measurement is often to measure steady-state rates with the enzyme undergoing multiple turnovers. In the case of motor proteins, the concept is not quite straightforward as there is both movement and ATP hydrolysis that each can reach a steady state at different times. Although detailed interpretation of such measurements may be limited, steady-state measurements can provide a simple, reproducible measure of activity and a way to get basic kinetic parameters and information on the effectiveness of inhibitors. An example is given in Fig. 2.4a, in which the P_i biosensor is used to measure the ssDNA-activated ATPase activity of the helicase PcrA. The dependence on ATP concentration gives the basic

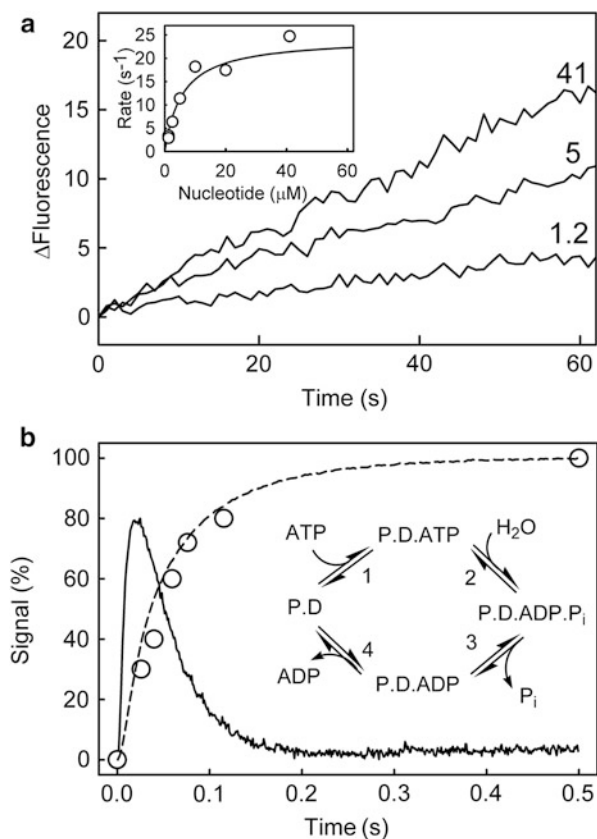
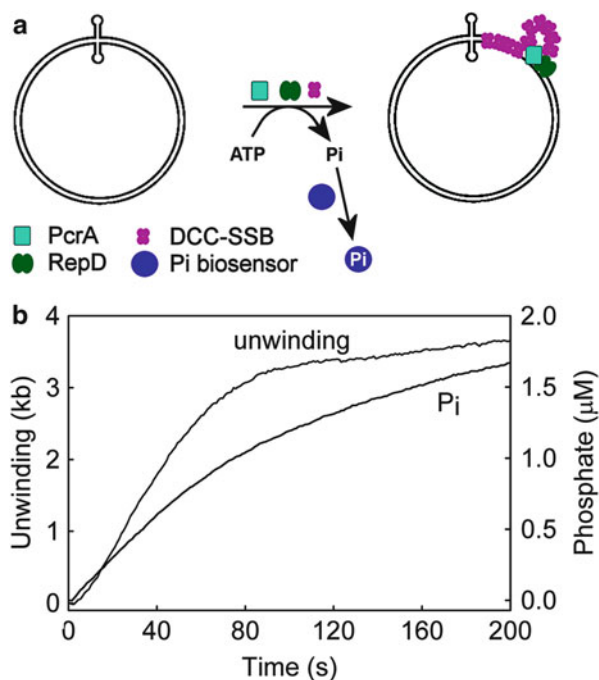


Fig. 2.4 P_i sensor used to measure PcrA helicase activity. (a) Steady-state ATPase assay with ssDNA, showing rate at micromolar ATP concentrations. The *inset* shows the concentration dependence of the ATPase rate, normalized to PcrA concentration (C.P. Toseland and M. R. Webb, unpublished). (b) Single-turnover measurement of mantATP binding (step 1 of inset with P. D being PcrA.DNA complex), cleavage (step 2), P_i release (step 3) and mantADP release (step 4) during dsDNA unwinding with RepD and PcrA. *Solid line*: mant fluorescence, showing mantATP binding (fluorescence increase) and subsequent mantADP release (decrease). *Circles*: quench-flow/HPLC measurement of mantATP cleavage. *Dashed line*: P_i release, measured using a P_i biosensor [71]

Michaelis–Menten parameters. The maximum rate gives a k_{cat} value of $\sim 20 s^{-1}$. However, this rate constant does not relate to ATPase activity during movement, but rather is limited by recycling the PcrA off the end of ssDNA and rebinding to another DNA strand. The ATP hydrolysis rate during translocation, obtained by a measurement analogous to that in Sect. 2.5.4, is severalfold faster [5].

Fig. 2.5 P_i sensor used to measure PcrA unwinding plasmid. (a) Cartoon showing partial unwinding by PcrA and initiator protein, RepD in the presence of DCC-SSB and the P_i biosensor, MDCC-PBP [72]. (b) Single-turnover measurement of plasmid unwinding and P_i production



2.5.4 Single-Turnover Measurements

For reasons outlined above, well-defined tracks in terms of length can be produced for experiments involving nucleic acid motors. The phosphate biosensor [1] enabled the measurement of ATP usage in real time, while a DNA helicase, PcrA, translocated along a single-strand track of known length [5]. This method was then adapted to measure ATP usage during complete plasmid unwinding by this helicase (Fig. 2.5) [72]. The P_i produced can be correlated with the precise length of DNA: in this case the ATP-coupled step size is that one ATP is required for each base pair unwound.

A similar measurement cannot be done with a protein track, but nevertheless, measurement of ATP usage in a glycerinated muscle fibre was achieved [73]. In such preparations, the membrane of the muscle cell is disrupted, allowing introduction of the P_i biosensor and caged ATP. Both tension and P_i production could be measured in real time, following laser flash photolysis to release ATP (Fig. 2.3b). These types of measurement allowed the relationship between P_i release and force generation to be investigated: in skeletal muscle, there is a close correlation of these processes.

Although P_i measurements form the large majority of single-turnover measurements, ADP biosensors have also been used. Analogous to the use of the P_i biosensor, an ADP biosensor, or more correctly ADP/ATP ratio biosensor [35],

has been used to measure ADP release from myosin within muscle fibres [74]. A different ADP biosensor [6] has been applied to the SecA kinetic mechanism: this protein moves relative to a peptide chain and is responsible for exporting proteins through bacterial membranes [75].

2.5.5 *Mechanochemical Coupling*

Particularly because of the correlation of P_i release with force generation in muscle, it has been important to consider P_i release as a potentially important process in the ATPase cycle of motor proteins, when aiming to understand the detailed mechanochemical coupling. Both ADP and P_i biosensors have been featured in studies of individual steps in the biochemical cycle of ATP hydrolysis. However, other methods such as fluorescent ADP analogues are available for measuring ADP release. An example of a P_i release measurement is shown in Fig. 2.4b for PcrA helicase. The experiment shows a single ATPase cycle of PcrA in the presence of double-stranded DNA, whereby a combination of measurements gave kinetic data on ATP binding, cleavage, P_i release and ADP release [71]. A fluorescent ATP analogue was used to obtain signals for ATP binding and ADP release. Quench-flow measurement gave a direct measure of cleavage. P_i release, in this case, had the same kinetics as cleavage, and ADP release was also similar, indicating that ATP cleavage is the rate-limiting step followed by rapid release of both products, ADP and P_i .

2.5.6 *Measurement of Movement*

As indicated above, biosensors are less often useful to measure motor protein movement. However, because helicases unwind double-stranded nucleic acids to give a single-stranded product, this change can be monitored. One way has been to use dsDNA-binding dyes to monitor this reaction [76], although there are potential problems as the substrate is altered, and the dye may affect the movement of the helicase. SSB binds highly selectively to ssDNA, a property which has been harnessed to probe DNA helicase activity. A fluorescent version, therefore, acts as an ssDNA biosensor, giving a fluorescence increase on binding to ssDNA [34]. This type of approach has been used to measure DNA unwinding in solution [72] (Fig. 2.5) and in a single-molecule format using TIRFM [63].

2.6 Summary and Future Directions

Fluorescent biosensors have facilitated new types of measurements for motor proteins, as well as for other systems, and so have greatly helped our understanding of such systems. However, often developments of biosensors have been driven by commercial applications, such as high-throughput drug screening, so the ease of preparation and the cost of production are likely to be important design considerations. Commercial applications, even more than in basic research, require simplicity and robustness, as well as affordability. Simplicity comes from both ease of application and large signal size. There are an ever increasing number of ways to design biosensors, some aimed at simplicity, but others, such as aptamers, having the advantage of being easily adapted to new targets. In most cases, there is unlikely to be, at least for a while, a universally applicable biosensor for any target, because different types of assays diverge greatly in what biosensor properties are most suitable.

Targets that are widely assayed are likely to continue to be addressed by new biosensors. Examples include important signalling molecules, such as glutamate, and important products of enzymes that are drug targets, such as ADP for kinases. Within the motility field, faster biosensors would aid real-time measurements. New biosensors for regulatory molecules other than calcium are desirable, especially if suitable for intracellular measurements. In the broader sense of motor proteins, assays for polymerases would benefit from a biosensor that is specific for pyrophosphate. While ingenuity can potentially lead to a wide array of biosensors with varying complexity, it is likely that the ones that can be applied simply and easily to assays for such molecules will also have widespread appeal.

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References

1. Brune M, Hunter JL, Corrie JET, Webb MR (1994) Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry* 33:8262–8271
2. Brune M, Hunter JL, Howell SA, Martin SR, Hazlett TL, Corrie JET, Webb MR (1998) Mechanism of inorganic phosphate interaction with phosphate binding protein from *Escherichia coli*. *Biochemistry* 37:10370–10380
3. Gilbert SP, Webb MR, Brune M, Johnson KA (1995) Pathway of processive ATP hydrolysis by kinesin. *Nature* 373:671–676
4. White HD, Belknap B, Webb MR (1997) Kinetics of nucleoside triphosphate cleavage and phosphate release steps by associated rabbit skeletal actomyosin, measured using a novel fluorescent probe for phosphate. *Biochemistry* 36:11828–11836

5. Dillingham MS, Wigley DB, Webb MR (2000) Demonstration of unidirectional single-stranded DNA translocation by PcrA helicase: measurement of step size and translocation speed. *Biochemistry* 39:205–212
6. Kunzelmann S, Webb MR (2009) A biosensor for fluorescent determination of ADP with high time resolution. *J Biol Chem* 284:33130–33138
7. Marvin JS, Hellinga HW (2001) Conversion of a maltose receptor into a zinc biosensor by computational design. *Proc Natl Acad Sci USA* 98(9):4955–4960
8. Looger LL, Dwyer MA, Smith JJ, Hellinga HW (2003) Computational design of receptor and sensor proteins with novel functions. *Nature* 423(6936):185–190
9. Frommer WB, Davidson MW, Campbell RE (2009) Genetically encoded biosensors based on engineered fluorescent proteins. *Chem Soc Rev* 38(10):2833–2841
10. VanEngelenburg SB, Palmer AE (2008) Fluorescent biosensors of protein function. *Curr Opin Chem Biol* 12(1):60–65
11. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat Biotechnol* 21(1):86–89
12. Gautier A, Juillerat A, Heinis C, Correa IR Jr, Kindermann M, Beaufile F, Johnsson K (2008) An engineered protein tag for multiprotein labeling in living cells. *Chem Biol* 15(2):128–136
13. Brun MA, Griss R, Reymond L, Tan KT, Piguet J, Peters RJ, Vogel H, Johnsson K (2011) Semisynthesis of fluorescent metabolite sensors on cell surfaces. *J Am Chem Soc* 133(40):16235–16242
14. Brun MA, Tan KT, Nakata E, Hinner MJ, Johnsson K (2009) Semisynthetic fluorescent sensor proteins based on self-labeling protein tags. *J Am Chem Soc* 131(16):5873–5884
15. Juskowiak B (2011) Nucleic acid-based fluorescent probes and their analytical potential. *Anal Bioanal Chem* 399(9):3157–3176
16. Morrison LE, Halder TC, Stols LM (1989) Solution-phase detection of polynucleotides using interacting fluorescent labels and competitive hybridization. *Anal Biochem* 183(2):231–244
17. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature* 346(6287):818–822
18. Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249(4968):505–510
19. Fang X, Tan W (2010) Aptamers generated from cell-SELEX for molecular medicine: a chemical biology approach. *Acc Chem Res* 43(1):48–57
20. Keefe AD, Pai S, Ellington A (2010) Aptamers as therapeutics. *Nat Rev Drug Discov* 9(7):537–550
21. Iliuk AB, Hu L, Tao WA (2011) Aptamer in bioanalytical applications. *Anal Chem* 83(12):4440–4452
22. Liu Y, Matharu Z, Howland MC, Revzin A, Simonian AL (2012) Affinity and enzyme-based biosensors: recent advances and emerging applications in cell analysis and point-of-care testing. *Anal Bioanal Chem* 404(4):1181–1196
23. Yamana K, Ohtani Y, Nakano H, Saito I (2003) Bis-pyrene labeled DNA aptamer as an intelligent fluorescent biosensor. *Bioorg Med Chem Lett* 13(20):3429–3431
24. Yamana K, Iwai T, Ohtani Y, Sato S, Nakamura M, Nakano H (2002) Bis-pyrene-labeled oligonucleotides: sequence specificity of excimer and monomer fluorescence changes upon hybridization with DNA. *Bioconjug Chem* 13(6):1266–1273
25. Tyagi S, Bratu DP, Kramer FR (1998) Multicolor molecular beacons for allele discrimination. *Nat Biotechnol* 16(1):49–53
26. Wang RE, Zhang Y, Cai J, Cai W, Gao T (2011) Aptamer-based fluorescent biosensors. *Curr Med Chem* 18(27):4175–4184
27. Mascini M, Palchetti I, Tombelli S (2012) Nucleic acid and peptide aptamers: fundamentals and bioanalytical aspects. *Angew Chem Int Ed* 51(6):1316–1332
28. Song KM, Lee S, Ban C (2012) Aptamers and their biological applications. *Sensors* 12(1):612–631

29. Zeng X, Shen Z, Mernaugh R (2012) Recombinant antibodies and their use in biosensors. *Anal Bioanal Chem* 402(10):3027–3038
30. Terai T, Nagano T (2013) Small-molecule fluorophores and fluorescent probes for bioimaging. *Pflugers Arch* 465(3):347–359
31. Hirshberg M, Henrick K, Haire LL, Vasisht N, Brune M, Corrie JE, Webb MR (1998) Crystal structure of phosphate binding protein labeled with a coumarin fluorophore, a probe for inorganic phosphate. *Biochemistry* 37(29):10381–10385
32. De Lorimier RM, Smith JJ, Dwyer MA, Looger LL, Sali KM, Paavola CD, Rizk SS, Sadigov S, Conrad DW, Loew L, Hellinga HW (2002) Construction of a fluorescent biosensor family. *Protein Sci* 11(11):2655–2675
33. Dwyer MA, Hellinga HW (2004) Periplasmic binding proteins: a versatile superfamily for protein engineering. *Curr Opin Struct Biol* 14(4):495–504
34. Dillingham MS, Tibbles KL, Hunter JL, Bell JC, Kowalczykowski SC, Webb MR (2008) Fluorescent single-stranded DNA binding protein as a probe for sensitive, real time assays of helicase activity. *Biophys J* 95:3330–3339
35. Brune M, Corrie JET, Webb MR (2001) A fluorescent sensor of the phosphorylation state of nucleoside diphosphate kinase and its use to monitor nucleoside diphosphate concentrations in real time. *Biochemistry* 40:5087–5094
36. Berg J, Hung YP, Yellen G (2009) A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat Methods* 6(2):161–166
37. Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nat Biotechnol* 19(2):137–141
38. Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. *Proc Natl Acad Sci USA* 96(20):11241–11246
39. Sakaguchi R, Endoh T, Yamamoto S, Tainaka K, Sugimoto K, Fujieda N, Kiyonaka S, Mori Y, Morii T (2009) A single circularly permuted GFP sensor for inositol-1,3,4,5-tetrakisphosphate based on a split PH domain. *Bioorg Med Chem* 17(20):7381–7386
40. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen TW, Bargmann CI, Orger MB, Schreiter ER, Demb JB, Gan WB, Hires SA, Looger LL (2013) An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nat Methods* 10(2):162–170
41. Bizzarri R, Serresi M, Luin S, Beltram F (2009) Green fluorescent protein based pH indicators for in vivo use: a review. *Anal Bioanal Chem* 393(4):1107–1122
42. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* 388:882–887
43. Mank M, Reiff DF, Heim N, Friedrich MW, Borst A, Griesbeck O (2006) A FRET-based calcium biosensor with fast signal kinetics and high fluorescence change. *Biophys J* 90(5):1790–1796
44. Heim N, Griesbeck O (2004) Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein. *J Biol Chem* 279(14):14280–14286
45. Nikolaev VO, Bunemann M, Schmitteckert E, Lohse MJ, Engelhardt S (2006) Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined beta2-adrenergic receptor-mediated signaling. *Circ Res* 99(10):1084–1091
46. DiPilato LM, Cheng X, Zhang J (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci USA* 101(47):16513–16518
47. Honda A, Adams SR, Sawyer CL, Lev-Ram V, Tsien RY, Dostmann WR (2001) Spatio-temporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. *Proc Natl Acad Sci USA* 98:2437–2442
48. Nikolaev VO, Gambaryan S, Lohse MJ (2006) Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nat Methods* 3(1):23–25

49. Fehr M, Frommer WB, Lalonde S (2002) Visualization of maltose uptake in living yeast cells by fluorescent nanosensors. *Proc Natl Acad Sci USA* 99(15):9846–9851
50. Fehr M, Lalonde S, Lager I, Wolff MW, Frommer WB (2003) In vivo imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J Biol Chem* 278(21):19127–19133
51. Lager I, Fehr M, Frommer WB, Lalonde S (2003) Development of a fluorescent nanosensor for ribose. *FEBS Lett* 553(1–2):85–89
52. Gu H, Lalonde S, Okumoto S, Looger LL, Scharff-Poulsen AM, Grossman AR, Kossmann J, Jakobsen I, Frommer WB (2006) A novel analytical method for in vivo phosphate tracking. *FEBS Lett* 580(25):5885–5893
53. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H (2009) Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci USA* 106(37):15651–15656
54. Okoh MP, Hunter JL, Corrie JET, Webb MR (2006) A biosensor for inorganic phosphate using a rhodamine-labeled phosphate binding protein. *Biochemistry* 45:14764–14771
55. Kunzelmann S, Webb MR (2011) Fluorescence detection of GDP in real time with the reagentless biosensor rhodamine-ParM. *Biochem J* 440(1):43–49
56. Kunzelmann S, Webb MR (2010) A fluorescent, reagentless biosensor for ADP based on tetramethylrhodamine-labeled ParM. *ACS Chem Biol* 5(4):415–425
57. Miyawaki A, Griesbeck O, Heim R, Tsien RY (1999) Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *Proc Natl Acad Sci USA* 96(5):2135–2140
58. Kerr R, Lev-Ram V, Baird G, Vincent P, Tsien RY, Schafer WR (2000) Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* 26(3):583–594
59. Palmer AE, Qin Y, Park JG, McCombs JE (2011) Design and application of genetically encoded biosensors. *Trends Biotechnol* 29(3):144–152
60. Mank M, Griesbeck O (2008) Genetically encoded calcium indicators. *Chem Rev* 108(5):1550–1564
61. Brun MA, Tan KT, Griss R, Kielkowska A, Reymond L, Johnsson K (2012) A semisynthetic fluorescent sensor protein for glutamate. *J Am Chem Soc* 134(18):7676–7678
62. Masharina A, Reymond L, Maurel D, Umezawa K, Johnsson K (2012) A fluorescent sensor for GABA and synthetic GABA(B) receptor ligands. *J Am Chem Soc* 134(46):19026–19034
63. Fili N, Mashanov G, Toseland CP, Batters C, Wallace MI, Yeeles JTP, Dillingham MS, Webb MR, Molloy JE (2010) Visualizing DNA unwinding by helicases at the single molecule level. *Nucleic Acids Res* 38:4448–4457
64. Grynkiewicz G, Poenie M, Tsien R (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260(6):3440–3450
65. Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19(11):2396–2404
66. Somlyo AP, Somlyo AV (1990) Flash photolysis studies of excitation-contraction coupling, regulation, and contraction in smooth muscle. *Annu Rev Physiol* 52:857–874
67. Himpens B, Somlyo AP (1988) Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J Physiol* 395(1):507–530
68. Minta A, Kao JP, Tsien RY (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* 264(14):8171–8178
69. Somlyo AV, Horiuti K, Trentham DR, Kitazawa T, Somlyo AP (1992) Kinetics of Ca²⁺ release and contraction induced by photolysis of caged D-myo-inositol 1,4,5-trisphosphate in smooth muscle. The effects of heparin, procaine, and adenine nucleotides. *J Biol Chem* 267(31):22316–22322
70. He Z-H, Chillingworth RK, Brune M, Corrie JET, Trentham DR, Webb MR, Ferenczi MA (1997) ATPase kinetics on activation of rabbit and frog permeabilized isometric muscle fibres: a real time phosphate assay. *J Physiol* 501:125–148

71. Toseland CP, Martinez-Senac MM, Slatter AF, Webb MR (2009) The ATPase cycle of PcrA helicase and its coupling to translocation on DNA. *J Mol Biol* 392:1020–1032
72. Slatter AF, Thomas CD, Webb MR (2009) PcrA helicase tightly couples ATP hydrolysis to unwinding double-stranded DNA, modulated by the replication initiator protein, RepD. *Biochemistry* 48:6326–6334
73. He Z-H, Chillingworth RK, Brune M, Corrie JET, Webb MR, Ferenczi MA (1999) The efficiency of contraction in rabbit skeletal muscle fibres, determined from the rate of release of inorganic phosphate. *J Physiol* 517:839–854
74. West TG, Hild G, Siththanandan VB, Webb MR, Corrie JET, Ferenczi MA (2009) Time course and strain dependence of ADP release during contraction of permeabilized skeletal muscle fibers. *Biophys J* 96(8):3281–3294
75. Das S, Grady LM, Michtav J, Zhou Y, Cohan FM, Hingorani MM, Oliver DB (2012) The variable subdomain of *Escherichia coli* SecA functions to regulate SecA ATPase activity and ADP release. *J Bacteriol* 194(9):2205–2213
76. Eggleston AK, Rahim NA, Kowalczykowski SC (1996) A helicase assay based on the displacement of fluorescent, nucleic acid-binding ligands. *Nucleic Acids Res* 24(7):1179–1186
77. Ledvina PS, Yao N, Choudhary A, Quirocho FA (1996) Negative electrostatic surface potential of protein sites specific for anionic ligands. *Proc Natl Acad Sci USA* 93:6786–6791

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