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REVIEW

Use of green fluorescent proteins for in vitro biosensing

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Due to the considerable stability of green fluorescent proteins and their capacity to be readily permutated or mutated, they may be exploited in multiple ways to enhance the functionality of in vitro biosensors. Many possibilities, such as the formation of chimeras with other proteins or antibodies, as well as Förster resonance emission transfer performance, may be used for the highly sensitive and specific detection of the target molecules. This review considers the great potential of green fluorescent proteins as the fluorescent probing or recognition biomolecule in various in vitro biosensors applications, as well as obstacles associated with their use.

Keywords: *Aequorea victoria*, biosensor, chimera, emission, Förster resonance emission transfer

Introduction

According to the well-established definition in the analytical chemistry lexicon, a "biosensor" is a detection system that relies on a biomolecule for molecular recognition and a transducer to produce an observable output. These platforms must not only be sensitive and specific, but must also be able to accurately detect the analyte in a complex sample, maximally eliminating interference from the background (Lim et al., 2005; Campbell, 2009). In conventional biosensors the molecular recognition component is formed by a protein. For such applications, genetically encoded fluorophores, members of the green fluorescent proteins (GFP) family, originating from the jellyfish *Aequorea victoria*, are suitable.

A. victoria GFP is the first discovered member of a family of fluorescent proteins derived from several bioluminescent marine organisms, characterised by a highly stable 11-stranded β-barrel structure (Ormö et al., 1996; Yang et al., 1996). GFP is widely established as a superlative biological macromolecule extensively used in fundamental research and the applied sciences due to its autofluorescence and high stability (Tansila et al., 2007). The unique architecture of fluorescent proteins assists both the formation and stabilisation of the conjugated ring systems which are responsible for their spectral properties (Wachter, 2007; Pouwels et al., 2008). In the case of wild-type *A. victoria* GFP composed of the single-chain 238 amino acid polypeptide (27 kDa), green fluorescence is emitted under 488 nm excitation light (Coumans et al., 2014) as a result of highly fluorescent *p*-hydroxybenzylidene-5-imidizolinone (*p*-HBI) species formation from the Ser65-Tyr66-Gly67 tripeptide (Zhang et al., 2006).

As noted in reviews by Shaner et al. (2007) and Day and Davidson (2009), GFP-family members may be divided into seven classes based upon their emission maxima. These include proteins emitting in the blue (abbreviated as BFPs; with λ_{em} = 440–470 nm), cyan (CFPs; λ_{em} = 471–500 nm), green (GFPs; λ_{em} = 501–20 nm), yellow (YFPs; λ_{em} = 521–550 nm), orange

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Table 1. Selected fluorescent proteins with biosensor applications

Protein	Origin	λ_{ex}/nm	λ_{em}/nm	Structure	Reference
mPlum	*Q. striata*	590	649	Monomeric	Wang and Tsien (2006)
mKeima	*Montipora*	440	620	Monomeric	Kogure et al. (2006)
eqFP670	*E. quadricolor*	605	670	Dimeric	Shcherbo et al. (2010)
mRFP1	*Q. striata*	584	607	Monomeric	Campbell et al. (2002)
mApple	*Q. striata*	568	592	Monomeric	Shaner et al. (2008)
mTangerine	*Q. striata*	568	585	Monomeric	Shaner et al. (2004)
mKOk	*Cerianthus* sp.	551	563	Monomeric	Tsutsui et al. (2008)
OFP	*Cerianthus* sp.	548	573	Tetrameric	Ip et al. (2007)
TurboRFP	*E. quadricolor*	553	574	Monomeric	Merzlyak et al. (2007)
Ypet	*A. victoria*	517	530	Monomeric	Nguyen and Daugherty (2005)
mCitrine	*A. victoria*	516	529	Monomeric	Griesbeck et al. (2001)
PhiYFP	*Hydrozoa* sp.	525	537	Monomeric	Shagin et al. (2004)
Superfolder	*A. victoria*	488	510	Monomeric	Pédelacq et al. (2006)
mWasabi	*Clavularia*	493	509	Monomeric	Ai et al. (2008)
TurboGFP	*Copepoda* sp.	482	502	Monomeric	Shagin et al. (2004)
Cerulean	*A. victoria*	433	475	Monomeric	Rizzo et al. (2004)
CyPet	*A. victoria*	435	477	Monomeric	Nguyen and Daugherty (2005)
mTFP1	*Clavularia*	462	492	Monomeric	Ai et al. (2008)
TagBFP	*E. quadricolor*	399	456	Monomeric	Subach et al. (2008)
Sirius	*A. victoria*	355	424	Monomeric	Tomosugi et al. (2009)
SBFP2	*A. victoria*	380	446	Monomeric	Kremers et al. (2007)

(OFPs; λ_{em} = 551–575 nm), red (RFPs: λ_{em} = 576–610 nm), and far-red (FRFPs; λ_{em} = 611–660 nm). The details of some representatives are summarised in Table 1.

Due to the remarkable stability and versatility of GFP, there are many ways in which it can be used and manipulated to enhance the sensor's functionality. This review summarises the potential of green fluorescent proteins as a recognition agent of in vitro biosensors, based on fluorescence shifts, enhancement or quenching.

FRET-based biosensors using GFP as an acceptor

Förster (or fluorescence) resonance emission transfer (FRET) has been widely used as a technique in areas such as the structural elucidation of biological molecules and their interactions, in vitro assays, or in vivo monitoring in cellular research (Chen et al., 2013). FRET is based on a non-radiative process in which an excited dye donor transfers its energy to a dye acceptor in the ground state through long-range dipole–dipole interactions (Sapsford et al., 2006a).

GFP forms a versatile acceptor molecule in FRET configuration. The polypeptide sequence can be genetically modified to include the structural and functional elements needed for signal transduction and/or probe assembly. Standard molecular biology techniques can be readily applied to modify the protein to include the polyhistidine tag, a variety of linkers and/or amino acid sequences, contributing to the FRET functionality.

Quantum dots (QDs) were shown to be one of the most suitable donors in the FRET configuration due to their exceptional brightness and high quantum yields (Sapsford et al., 2006b), their capacity to bind multiple acceptor molecules (Medintz et al., 2003) and the unique qualities of their characteristic excitation and emission spectra (Qu & Peng, 2002). FRET pairs comprising GFP-like FPs and QDs exhibit high energy transfer efficiencies and enable ratiometric measurements, resulting in enhanced sensitivity by eliciting opposing changes in fluorescence emission at two wavelengths while maintaining an internal control (Dennis et al., 2010). In Fig. 1A it is suggested that a FRET is generally based on the quenching of GFP due to an enzyme cleavage of a peptide linker containing an enzyme cleavage site. Due to cleavage and the subsequent increase in distance between acceptor and donor, the emission of the quantum dot occurs indicating the enzyme presence.

A His-tagged green fluorescent protein was employed by Dennis and Bao (2008) to perform a FRET with CdSe/ZnS core-shell QD, coated with lipid-PEG. The polyhistidine sequence was inserted at the *N*-terminus of the protein followed by three glycine molecules acting as a linker between the polyhistidine sequence and the barrel structure of GFP. It was demonstrated that a polyhistidine linker could be applied as a straightforward and effective provider of GFP conjugation with QDs.

Genetic engineering has triggered the new wave of interest in FRET techniques carried out between two differently coloured fluorescent proteins; however, many of the issues generally associated with FRET are particularly acute for FP-FRET (Piston & Kremers, 2007). First, due to the breadth of the excitation and emission spectra of a number of FPs, a significant cross-talk may occur (Patterson et al., 2000), which

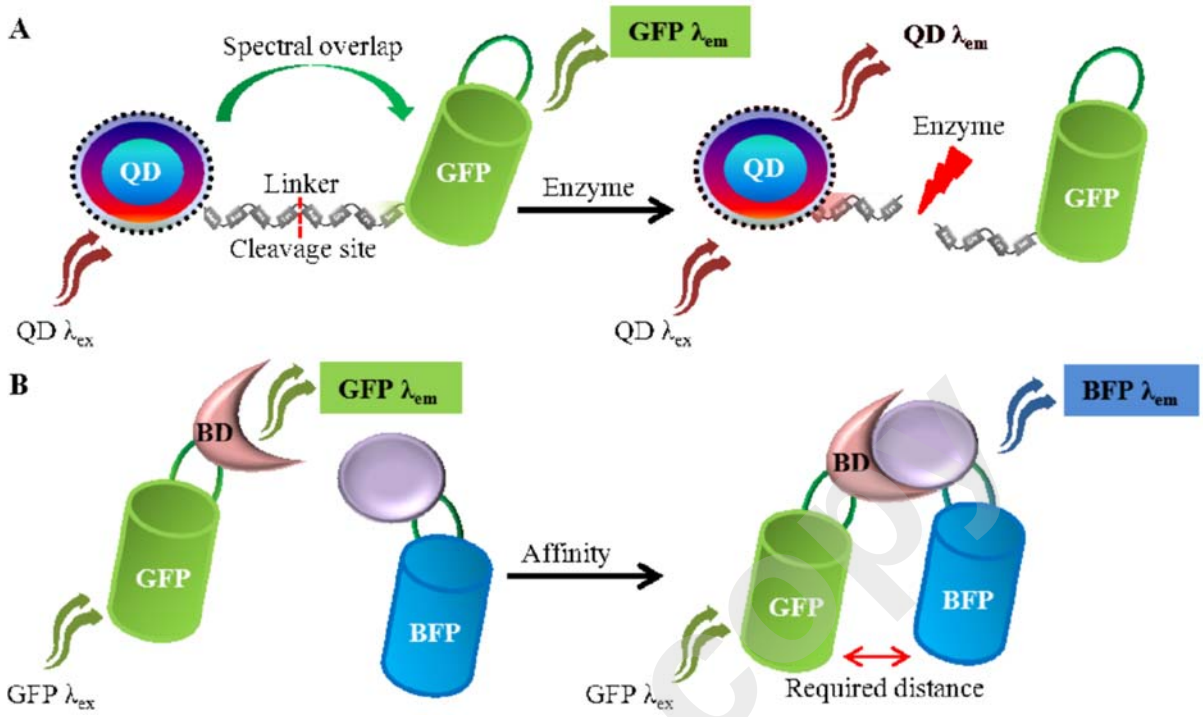

Fig. 1. General principle of FRET based on cleavage of peptide linker via enzyme causing GFP emission maxima to turn into QD emission maxima (A). General principle of FRET based on affinity of binding domain to a molecule, modified on a fluorescent protein (B). GFP denotes green fluorescent protein, QD quantum dot, BD binding domain and BFP blue fluorescent protein.

could represent an issue for the FRET performance. Further, fluorescent proteins of a large size (4.2 nm with barrel diameter of 2.4 nm (Ormö et al., 1996; Yang et al., 1996)) occupy much of the useful FRET distance, significantly decreasing its efficiencies (Patterson et al., 2000). Fig. 1B shows how FP-FRET can be based on the binding domain affinity.

Although FRET experiments, based on green fluorescent proteins in the role of both acceptor and/or donor, offer tremendous potential, they continue to be used to reveal the molecular dynamics in living cells. The dearth of publications, with implications for the development of FRET sensors for various in vitro biosensors, points to obstacles associated with GFP utilisation for these purposes, such as the need for sufficient excitation spectra separation, or the acquisition of efficient energy transfer (Piston & Kremers, 2007). On the other hand, the potential of GFP, especially in FRET pairs with suitable QDs for measurements of, for example, enzymatic activities with enzyme-cleavable sequences used as linkers between acceptor and donor might have an impact on biomedical applications of FRET-based in vitro biosensors established on GFP behaviour in the near future.

GFPAbs chimeras

Chimeric proteins (also known as fusion proteins) are hybrids formed by the substitution of fragments between two parent proteins ranging from short peptides to entire domains (Shanmugaratnam et al., 2012). GFP-antibodies (GFPAbs) chimeric proteins (Fig. 2 shows a general scheme) provide a possibility to convert a multi-step experimental workflow for the detection of molecules via antibodies and enzyme-linked secondary antibodies into a one-step workflow process. Pavoor et al. (2009) showed that this complex might be further employed as a detection system in fluorescence-based ELISAs. While GFP affords easy detection, attempts to insert multiple binding loops into its structure to impart affinity for a specific target have met with only limited success. This phenomenon is caused by the structural sensitivity of the GFP chromophore, which responds to various modifications by a crucial, undesired reduction in yields of fluorescence. Several attempts have been made to grant the binding capacity to GFP. This has mainly been done by inserting the binding loops into various solvent-exposed turns; the regions most amenable to amino acids insertion have been determined as Gln-157-Lys-158 and Glu-172-Asp-173 (Abedi et al., 1998; Doi & Yanagawa, 1999).

Pavoor et al. (2009) demonstrated that it was possible to form fluorescent dual-loop inserted GFPAbs scaffolds capable of binding to various antigens with nanomolar affinity. In their study, they used directed evolution with a yeast surface display using a surrogate loop approach, thereby obtaining a family of GFP scaffolds capable of accommodating two proximal binding loops. Moreover, it was revealed that

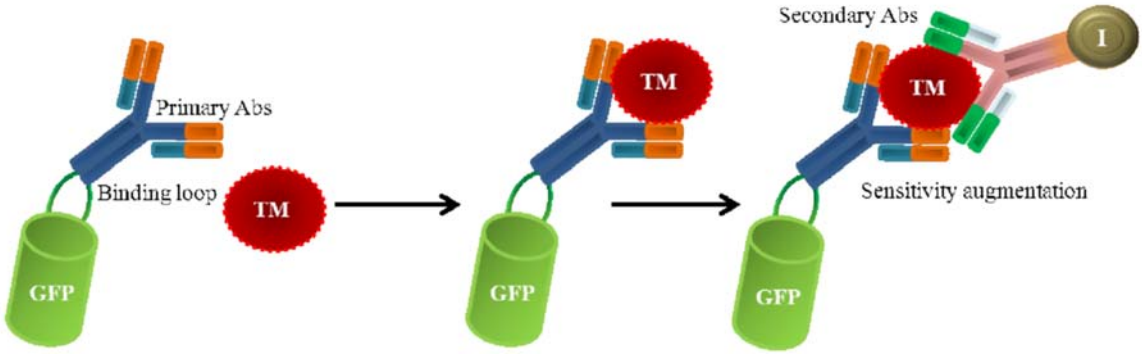

Fig. 2. General scheme of GFPAbs chimera comprising both fluorescence of GFP and specificity for binding target molecules (TM). I denotes indicator and Abs denotes antibodies. Substrate created by fluorescent protein forms the platform for rapid increase in sensitivity of secondary antibodies.

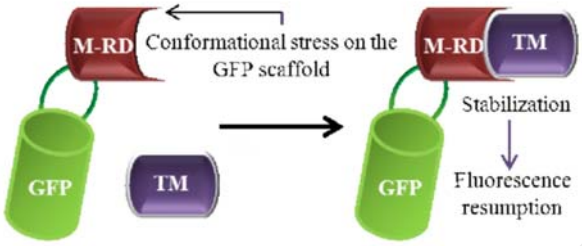

Fig. 3. Allosteric GFP-based biosensor. In the absence of the target molecule (TM), conformational influence of the molecular-recognition domain (M-RD) or receptor protein puts stress on GFP and thus reduces fluorescence yields. After binding with TM a stabilisation of conformation occurs resulting in a resumption of the GFP fluorescent ability.

some mutations such as F64L increasing the fluorescence of GFP and shifting λ_{ex} to 488 nm, or Y39H and N105T capable of improving refolding kinetics and stability, might be beneficial for formation of the GFPAbs chimera (Pavoor et al., 2009). This approach offers a wide range of applications, because of many GFP spectral variants (Zhang et al., 2002). Moreover, the surrogate loop may be applied to other structurally homologous monomeric fluorescent proteins.

Since GFPAbs also offers the possibility of use of the recombinant antibodies, able to provide significantly enhanced specificity and sensitivity using site-directed mutagenesis or chains-shuffling (Hudson & Souriau, 2003), these protein chimeras exhibit great potential as recognition and detection agents, applicable in highly sensitive and specific GFP-based immunosensors.

Allosteric-based chimeric biosensors

Another way of combining GFP with another protein to form chimeras for biosensors development is to insert a receptor protein into a surface loop of GFP (Fig. 3). The resulting combination of an optical signal-transduction mechanism of GFP with a specificity provided by a ligand-binding site of the receptor protein creates the allosteric GFP biosensors that may be used in a wide range of applications including biochemistry and environmental or analytical chemistry.

Baird et al. (1999) proposed a sensor formed by the domain insertion of calmodulin (Ca^{2+} binding protein) inserted into a GFP molecule. Upon binding with Ca^{2+} the calmodulin domain undergoes a large conformational change, resulting in a 7-fold increase in the fluorescence intensity (Baird et al., 1999). This phenomenon is caused by a change in the protonation state of the fluorophore, thereby increasing its fluorescence. In addition, fusion between calmodulin and GFP was also employed to detect the anti-depressant drug phenothiazine (Dikici et al., 2003). This approach was further improved by Puckett et al. (2004) by the incorporation of an assay into a centrifugal microfluidic platform. To do this, the biological reagents were dried on the platform and rehydrated to carry out the analysis. The ability to pre-aliquot reagents on the platform should enhance its versatility and portability and this biosensor may be useful in designing analytical systems for high-throughput screening of pharmaceuticals.

Doi & Yanagawa (1999) described a method whereby a protein domain containing a desired molecular-binding site (TEM1 β-lactamase) was initially inserted into a GFP surface loop. Using the random mutation of the inserted fusion protein, entirely new molecular-recognition sites for detection of the β-lactamase-inhibitory protein (BLIP) were formed on GFP (Doi & Yanagawa, 1999). As a result, a novel allosteric protein–ligand system undergoing fluorescence changes upon the binding of target molecules was obtained. Following conformational changes after protein–ligand binding, the fluorescence is increased and the surface loop-bearing receptor is fairly close in space to the fluorophore. Using this approach, a double mutant was identified that was shown to detect BLIP in vitro with micromolar affinity. In principle, all these methods could be used to generate a sensor

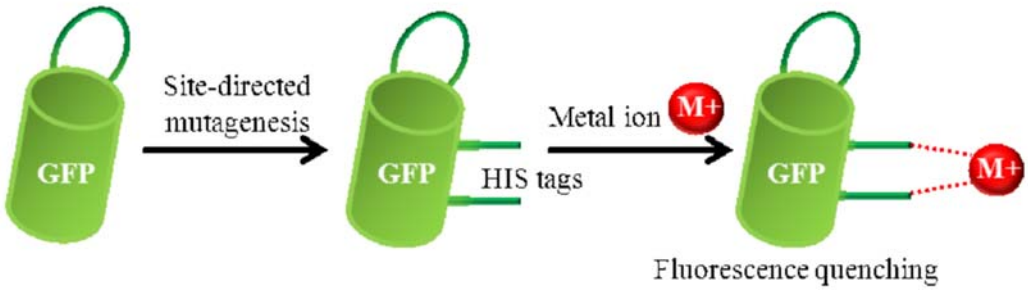

Fig. 4. General scheme of GFP-based metal ions biosensor. Due to site-directed mutagenesis, the affinity of GFP towards metal ions may be elevated. Due to binding, the metal ion is close enough to quench the GFP chromophore by energy transfer.

for any ligand that can be added exogenously, as well as for ligands expressed in bacteria.

GFP-based biosensors for ions detection

Fluorescent proteins have been shown to be sensitive not only to pH changes but also to the concentration of certain ions (Fig. 4). Mazzola et al. (2006) studied the GFP stability in chlorinated water for injection (WFI) and chlorinated buffered solutions at various pH ranges, in order to measure the exposure time required for chlorine to decrease 90 % of the GFP fluorescence intensity. It was shown that the GFP fluorescence decreased abruptly when contacting the chlorine in concentrations greater than 150 ppm (μM). The recovery of GFP fluorescence due to renaturation was observed at between 30 ppm and 100 ppm of chlorine. It was concluded that, due to that property, GFP may serve as a suitable fluorescent recognition molecule for biosensors applicable for the monitoring of disinfection effectiveness. Subsequently, Arosio et al. (2010) developed a ratiometric biosensor based on a highly chloride-sensitive *A. victoria* GFP spectral variant (E^2GFP). The GFP-based ion biosensor was shown to be appropriate for the detection of pH changes and chloride ions levels. E^2GFP was chosen because it contains two excitation and emission maxima, whereas biosensor analysis is based on the ratio between green and cyan fluorescence, using the longer wavelength emission that is pH-dependent.

Metal ions in close proximity to GFP chromophores are known to quench fluorescence in a distance-dependent fashion (Cubitt et al., 1995). Richmond et al. (2000) identified the metal-binding sites on the surface of GFP and designed mutants exhibiting fluorescence- quenching. Under the influence of Cu^{2+}, Ni^{2+} or Co^{2+}, the fluorescence of mutants was quenched at a much lower concentrations when compared with wild-type GFP (approximate binding constant in the low micromolar range). The affinity was enhanced due to the conversion of either residue 202 or 223 to aspartic or glutamic acid, resulting in a third potential metal ligand on the putative metal-binding site. These GFP mutants represent a new class of possible GFP-based metal biosensors, but their sensitivity needs to be increased via directed evolution.

Tansila et al. (2007) described the approach in which a site-directed mutagenesis was exploited for the construction of solvent-exposed analyte channels on the GFP surface without negative effects on the fluorescent properties and protein stability. The channel permits the passage of analytes into the β-barrel. Cu^{2+} or Zn^{2+} ions were shown to quench the fluorescence as well as H_2O_2 that, unlike the quenching phenomenon by metal ions, was shown to be pore size-dependent. All of these approaches hold great potential for the future, for further design and development of highly-sensitive GFP-based biosensors for various applications.

Living bacteria cell-based assays

One special group of GFP biosensors (summarised in Table 2) is based on the response of living organisms to the presence of chemical substances (Fig. 5). While most chemical sensors respond only to molecular binding, living organism-based biosensors can provide functional measurements such as bioavailability, genotoxicity or general toxicity (McFadden, 2002). Kuang et al. (2004) developed a biosensor composed of a high-density living bacterial cell array, fabricated by inserting bacteria into a microwell array formed at the end of an imaging fibre bundle. Inside the biosensor, *Escherichia coli* cells carrying arecA fusion with GFP were used as the recognition agent for genotoxin detection. Promising parameters such as high sensitivity, short incubation times (1 ng mL^{-1} mitomycin C for 90 min), active sensing lifetime of more than 6 h and a shelf-life of two weeks show that this array could be employed for high-throughput drug screening where only small quantities of an analyte are present.

E. coli, comprising L-arabinose (L-ara)-producing GFP was also employed in a microfluidic analysis of antibiotics such as tetracycline and erythromycin (Sun et al., 2011). Microfluidic devices constructed in this manner serve to investigate the effect of antibiotics on the amounts of GFP expression, which represents bacterial cell growth states, hence providing a framework for developing new research methods involving bacteria-based diagnostics and antibiotic drug-

Table 2. Overview of biosensors, exploiting GFP as reporter or recognition biomolecule

Analyte	Sensor category	Limit of detection	Sensing principle	Assay format	Reference
Enzyme (concept)	FRET sensors	–	Quenching of GFP – cleavage of enzyme cleavage site	–	Dennis and Bao (2008)
Antigen (concept)	Chimeric protein	–	Augmentation of sensitivity of fluorescence detection	–	Pavoor et al. (2009)
Ca^{2+}	Allosteric chimera	–	Calmodulin graft, enhancing fluorescence after analyte binding	Homogenous	Baird et al. (1999)
Phenothiazine-type of drugs	Allosteric chimera	0.1×10^{-6}–7×10^{-6} M	Quenching of GFP due to interaction of calmodulin graft with drug	Homogenous	Dikici et al. (2003)
Trifluoperazine	Allosteric chimera	6×10^{-7} M	Quenching of GFP due to interaction of calmodulin graft with analyte	Homogenous	Puckett et al. (2004)
β-Lactamase-inhibitory protein	Allosteric chimera	Units μM	Restoration of GFP fluorescence upon binding of target molecule to domain	Homogenous	Doi and Yanagawa (1999)
Chlorine	Ions detection	150 ppm	Alteration of structure, resulting in quenching	Homogenous	Mazzola et al. (2006)
Cu^{2+}, Co^{2+}, Ni^{2+}	Ions detection	–	Quenching of chromophore due to close proximity of his-bound metal	Homogenous	Richmond et al. (2000)
Cu^{2+}, Zn^{2+}	Ions detection	4.88×10^{-6}–33.3×10^{-6} M	Metal-channelling effect towards chromophore *via* artificial pores	Homogenous	Tansila et al. (2007)
Mitomycin C	Bacterial cell-based assay	1 ng mL^{-1}	Fluorescence increase, due to overexpression of GFP	Homogenous	Kuang et al. (2004)
Tetracycline, erythromycin	Bacterial cell-based assay	–	Decrease in fluorescence due to alteration in growth rates and bacterial morphology	Homogenous	Sun et al. (2011)
Methyl-methanesulphonate	Bacterial cell-based assay	–	Fluorescence increase, under effect of analyte	Homogenous	García-Alonso et al. (2009)

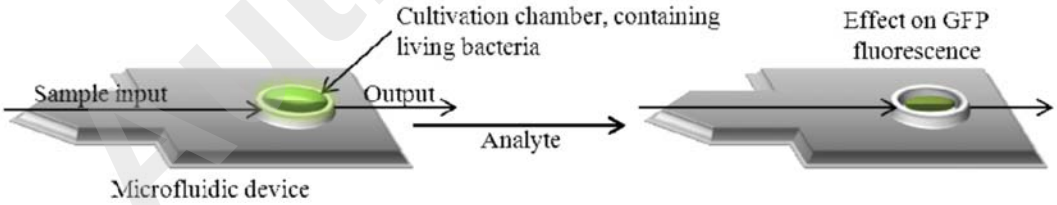

Fig. 5. Overall scheme of microfluidic device where living organisms in the form of bacteria or yeasts are placed in high-density cultivation chamber. Under an effect of analyte, bacteria may either overexpress or down-regulate their GFP formation.

screening, as well as bacterial cell-based biosensor development.

The recombinant yeast *Saccharomyces cerevisiae*, which expresses GFP when exposed to genotoxins, was exploited in development of a microfluidic chip for environmental screening (García-Alonso et al., 2009). Treatment with methyl-methanesulphonate (MMS) causes organisms to respond by way of increase in fluorescence. The device has the potential for use by industrial manufacturers to detect toxic compounds, as well as to characterise already polluted environments.

Conclusions

The unique properties of GFP, such as its great stability and capacity to be readily permutated or mutated, make GFP a highly promising biomolecule in the field of biosensors' development. The signal of a GFP-based biosensor may be provided via quenching caused by protein unfolding under the influence of certain ions, pH changes or via FRET performance. A major advantage is that GFP can be immobilised and even dried while retaining its structure and thus its biosensor function. In order to become generally useful, green fluorescent proteins should be produced with low costs and low demands on storage prior to use. Although a few attempts have been made to develop the field on GFP-based in vitro biosensors, clearly more work is needed to exploit the full potential of these powerful molecules. This will involve not only refinements of the current devices, but also the development of new approaches that combine high temporal

and spatial resolution with increased sensitivity. Arrays based on GFP may in the future become available for households, serving as a rapid and cheap diagnostic tool, as well as a biosensor applicable in screening, providing rapid and accurate information on environmental contamination.

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