Utilization of Green Fluorescent Proteins for *In vitro* Biosensing

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Due to green fluorescent proteins considerable stability and possibility to be readily permutated or mutated, they may be exploited in multiple ways to enhance a functionality of *in vitro* biosensors. Many possibilities such as formation of chimeras with other proteins or antibodies, as well as Förster resonance emission transfer performance may be used for very sensitive and specific detection of target molecules. The review considers the high potential of green fluorescent proteins as the fluorescent probing or recognition biomolecule in various *in vitro* biosensors application, as well as the obstacles connected with their utilization.

Keywords: *Aequorea victoria*; Biosensor; Chimera; Emission; Förster Resonance Emission Transfer

Introduction

According to the definition, firmly established in the lexicon of analytical chemistry "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 also must be able to accurately detect analyte in complex sample, maximally eliminating interference from background (Campbell, 2009; Lim et al., 2005). 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, originated from 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, characterized by a highly stable 11-stranded β-barrel structure (Ormo et al., 1996; Yang et al., 1996). GFP is widely established as a superlative biological macromolecule prevalent in basic research and applied sciences due to its autofluorescence and high stability (Tansila et al., 2007). Unique architecture of fluorescent proteins aids in both the formation and stabilization of the conjugated ring systems that are responsible for their spectral properties (Pouwels et al., 2008; Wachter, 2007). 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 it is mentioned 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 − 520 nm), yellow (YFPs; λ_{em} = 521 − 550 nm), orange (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 summarized in Tab. 1.

Because of GFP remarkable stability and versatility, it can be used and manipulated in multiple ways to enhance sensor´s functionality. In this review, we will summarize the potential of green fluorescent proteins as a recognition agent of *in vitro* biosensors, based on fluorescence shifts, enhancement or quenching.

Table 1. Selected fluorescent proteins useful in 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	(Pedelacq 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)

FRET-based biosensors using GFP as an acceptor

Förster (or Fluorescence) resonance emission transfer (FRET) has been widely used as a technique in various areas such as 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 nonradiative 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 versatile acceptor molecule in FRET configuration. The polypeptide sequence can be genetically modified to include structural and functional elements needed for signal transduction, and/or probe assembly. Standard molecular biology techniques can be easily 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 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 and Peng, 2002). FRET pairs comprising GFP-like FPs and QDs exhibit high energy transfer efficiencies and enable ratiometric measurements, resulting in heightened 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 a FRET generally based on quenching of GFP due to an enzyme cleavage of peptide linker containing an enzyme cleavage site. Due to cleavage and subsequent increase of distance between acceptor and donor, emission of quantum dot occurs pointing at the enzyme presence.

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 (Dennis and Bao, 2008). The polyhistidine sequence was inserted at the *N*-terminus of the protein followed by three glycines acting as a linker between the polyhistidine sequence and the barrel structure of GFP. It was demonstrated that polyhistidine linker can be applied as a straightforward and effective provider of GFP conjugation with QDs.

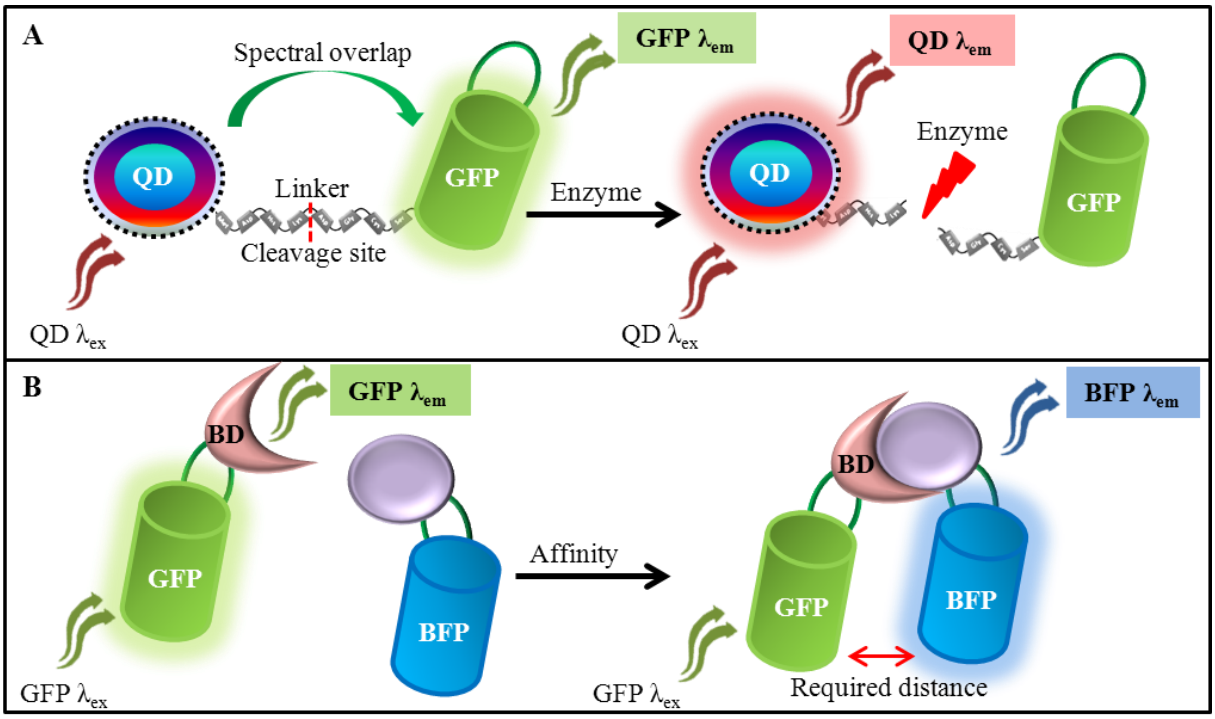

Fig. 1. (**A**) General principle of FRET based on a cleavage of peptide linker *via* enzyme causing a turn of GFP emission maxima into QD emission maxima. (**B**) General principle of FRET based on affinity of binding domain to a molecule, modified on a fluorescent protein. GFP stays for green fluorescent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

Genetic engineering has triggered the new wave of interest of FRET techniques carried out between two differently colored fluorescent proteins; however, a lot of the issues generally associated with FRET are particularly acute for FP-FRET (Piston and Kremers, 2007). Firstly, due to the breadth of the excitation and emission spectra of numerous FPs, there may occur a significant cross-talk (Patterson et al., 2000), which could be an issue of the FRET performance. Further, fluorescent proteins of large size (4.2 nm with barrel diameter of 2.4 nm (Ormo et al., 1996; Yang et al., 1996)) occupies much of the useful FRET distance, significantly decreasing its efficiencies (Patterson et al., 2000). It is shown in Fig. 1B 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 are still more used to reveal the molecular dynamics in living cells. A lack of publications with implication for the development of FRET sensors for various *in vitro* biosensors points at obstacles, connected with GFP utilization for these purposes, such as need of sufficient excitation spectra

separation, or acquirement of efficient energy transfer (Piston and Kremers, 2007). On the other hand, the potential of GFP especially in FRET pair with suitable QDs for measurements of e.g. enzymatic activities with enzyme-cleavable sequences used as linkers between acceptor and donor, might impact the field of biomedical application of FRET-based *in vitro* biosensors established on GFP behavior in near future.

GFPAbs chimeras

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

Pavoor *et al.* (2009) demonstrated that it is possible to form fluorescent dual-loop inserted GFPAbs scaffolds capable of binding to various antigens with nanomolar affinity. In their study they utilized directed evolution with yeast surface display using a surrogate loop approach, and a family of GFP scaffolds capable of accommodation of two proximal binding loops was obtained. Moreover, it was revealed that some mutations as F64L increasing fluorescence of GFP and shifting λ_{ex} to 488 nm, or Y39H and N105T able to improve refolding kinetics and stability, may be beneficial for the forming of 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.

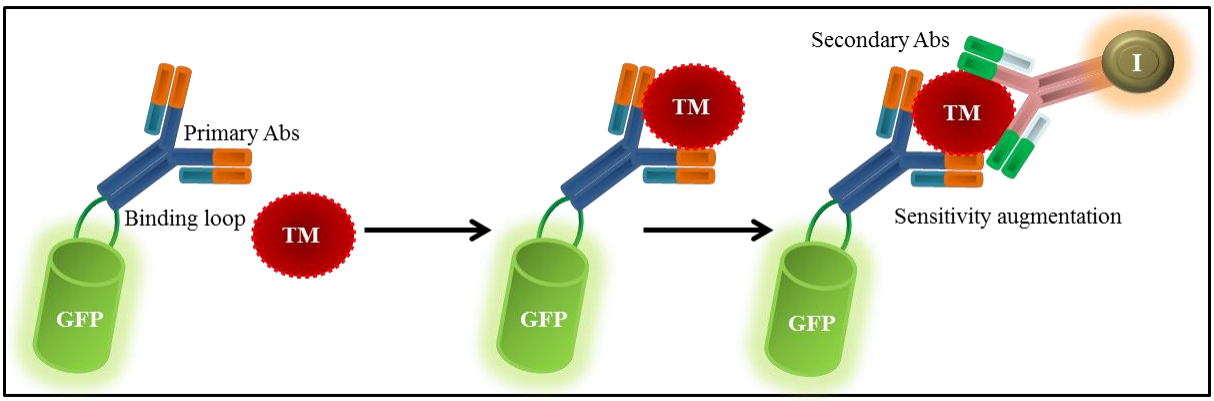

Fig. 2. General scheme of GFPAbs chimera comprising both fluorescence of GFP and specifity for binding target molecules (TM). I stays for indicator and Abs for antibodies. Substrate created by fluorescent protein forms the platform for rapid increase of sensitivity of secondary antibodies.

In view of the fact that GFPAbs offers also a possibility of utilization of recombinant antibodies, able to provide significantly enhanced specifity and sensitivity using site-directed mutagenesis or chains shuffling (Hudson and Souriau, 2003), these protein chimeras show large potential as the recognition and detection agents, applicable in very sensitive and specific GFP-based immunosensors.

Allosteric-based chimeric biosensors

Another way how to combine GFP with another protein to form chimeras for biosensors development is to insert a receptor protein into a surface loop of GFP (Fig. 3). Resulting combination of optical signal-transduction mechanism of GFP with a specifity provided by a ligand-binding site of 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) suggested sensor formed by domain insertion of calmodulin (Ca^{2+} binding protein) inserted into GFP molecule. Upon binding of Ca^{2+} 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 employed also to detect the anti-depressant drug phenothiazine (Dikici et al., 2003). Moreover, this approach was further improved by Puckett *et al.* (2004) by the incorporation of an assay into a centrifugal microfluidic platform (Puckett et al., 2004).

To do this, the biological reagents were dried on the platform and rehydrated to carry out the analysis. The ability to prealiquot reagents on the platform should enhance its versatility and portability and this biosensor may be useful for designing of the analytical systems for high-throughput screening of pharmaceuticals.

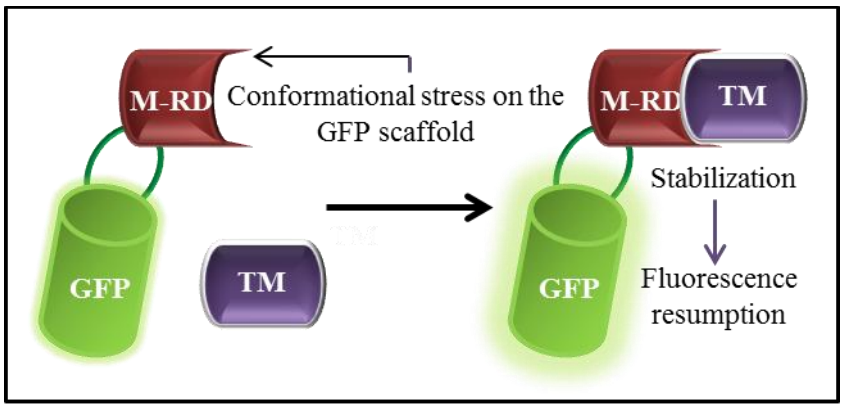

Fig. 3. Allosteric GFP-based biosensor. In the absence of the target molecule (TM), conformational influence of the molecular-recognition domain (M-RD) or receptor protein put stress on GFP and thus reduce a fluorescence yields. After binding with TM a stabilization of conformation occurs resulting in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

Doi and Yanagawa (1999) described a method, where a protein domain containing a desired molecular-binding site (TEM1 β-lactamase) was firstly inserted into a GFP surface loop. Using the random mutation of insertional fusion protein, entirely new molecular-recognition sites for detection of β-lactamase-inhibitory protein (BLIP) were formed on GFP (Doi and Yanagawa, 1999). As a result the novel allosteric protein-ligand system undergoing fluorescence changes upon binding of target molecules were obtained. Upon conformational changes after protein-ligand binding, fluorescence is increased, and the surface loop bearing receptor is fairly closed in a space to the fluorophore. Using this approach, a double mutant was found that was shown to detect BLIP *in vitro* with micromolar affinity. In principle, all these methods could be used to generate a sensor 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 and coworkers (2006) studied the GFP stability in chlorinated water for injection (WFI) and chlorinated buffered solutions at various pH ranges, to evaluate the exposure time required for chlorine to decrease 90% of GFP

fluorescence intensity (Mazzola et al., 2006). It was shown that GFP fluorescence decreased abruptly when contacting the chlorine in concentrations greater than 150 ppm. The recovery of GFP fluorescence due to renaturation was observed between 30 and 100 ppm chlorine. It was concluded that due to that properties GFP may serve as a suitable fluorescent recognition molecule for biosensor applicable for monitoring of disinfection effectiveness. Further Arosio *et al*. (2010) develop a ratiometric biosensor based on a highly chloride-sensitive *A. victoria* GFP spectral variant (E^2GFP) (Arosio et al., 2010). It was shown that GFP-based ion biosensor is well suited 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.

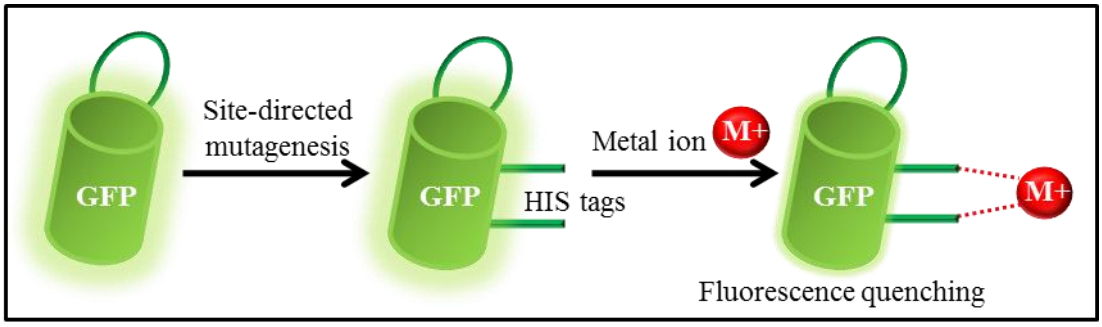

Fig. 4. General scheme of GFP-based metal ions biosensor. Due to site-directed mutagenesis affinity of GFP towards metal ions may be elevated. Due to a binding, metal ion is to close enough to quench the GFP chromophore by energy transfer.

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 (Richmond et al., 2000). Under the influence of Cu^{2+}, Ni^{2+}, or Co^{2+}, the fluorescence of mutants were 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 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 has 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 (Tansila et al., 2007). The channel allows passaging 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 promise a great potential in 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 (summarized in Tab. 2) is based on living organisms' response 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 an inserting bacteria into a microwell array formed at the end of an imaging fiber bundle (Kuang et al., 2004). Inside biosensor, *Escherichia coli* cells carrying arecA fusion with GFP were used as recognition agent for genotoxin detection. Promising paramaters 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 lifetime of two weeks show that this array could be employed for high-throughput drug screening, where only small quantities of an analyte are present.

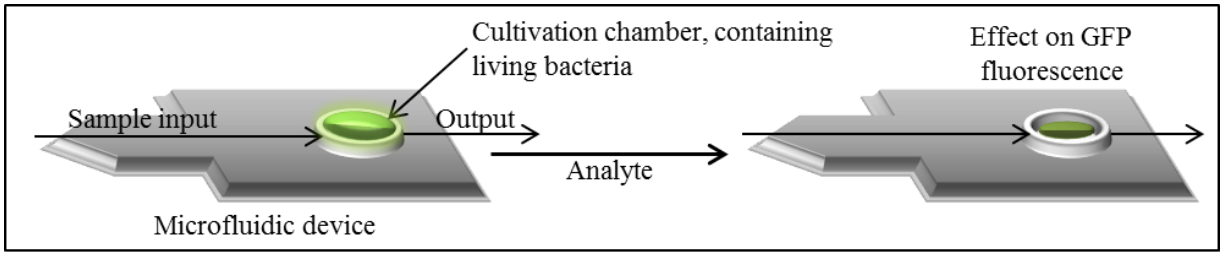

Fig. 5. Overall scheme of microfluidic device, where living organisms in the form of bacteria or yeasts are placed in high-density cultivation chamber. Under an effect of analyte, bacteria may either overexpress or downregulate their GFP formation.

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

diagnostics and antibiotic drug screening, as well as bacterial cell-based biosensor development.

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

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	$0.1–7\times10^{-6}$ 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	$4.88\times10^{-6} – 33.33\times10^{-6}$ 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 over-expression of GFP	Homogenous	(Kuang et al., 2004)
Tetracycline, erythromycin	Bacterial cell-based assay	×	Decrease of fluorescence due to alteration in growth rates and bacterial morphology	Homogenous	(Sun et al., 2011)
Methyl-methanesulfonate,	Bacterial cell-based assay	×	Fluorescence increase, under effect of analyte	Homogenous	(Garcia-Alonso et al., 2009)

Conclusions

The unique properties of GFP, such as great stability and possibility to be readily permutated or mutated, are making GFP as very promising biomolecule in the field of biosensors development. Signal of GFP-based biosensor may be provided *via* quenching caused by protein unfolding under the influence of certain ions, pH changes or *via* FRET performance. Big advantage is the possibility of GFP to be immobilized and even dried while retaining structure and thus biosensor function. Moreover, to become widely useful, green fluorescent proteins should be produced with low costs and low demands on storage prior to use. Although few attempts have been made to develop the field on GFP-based *in vitro* biosensors, more work is clearly needed to exploit the full potential of these powerful molecules. This will involve not only refinements of current devices, but also development of new approaches that combine high temporal and spatial resolution with the increased sensitivity. Arrays based on GFP may become available for household, serving as a rapid and cheap diagnostic tool, as well as a biosensor applicable for screening, providing rapid and accurate information about environmental contamination in the future.

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