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An evaluation of genetically encoded FRET-based biosensors for quantitative metabolite analyses *in vivo*

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ABSTRACT

A broad range of genetically-encoded fluorescence biosensors has been developed, allowing the detection of signaling intermediates and metabolites in real time. Many of these biosensors are based on Foerster Resonance Energy Transfer (FRET). The two biosensors of the well-known "Venus-flytrap" type exemplarily studied in this work are composed of a central sugar binding protein flanked by two green fluorescent protein derivatives, namely ECFP as well as Citrine and EYFP, respectively. In order to evaluate FRET-based biosensors as an *in vivo* tool for quantitative metabolite analyses, we have thoroughly studied the effects of pH, buffer salts, ionic strength, temperature and several intracellular metabolites on the signal intensity of both biosensors and both fluorescence proteins. Almost all micro-environmental variations led to considerably different FRET signals, because either the fluorescent proteins or the metabolite binding domains were affected by the tested parameters. This resulted not only in altered FRET ratios between the apo state and the saturated state but also in significant shifts of the apparent binding constant. This underlines the necessity of careful controls in order to allow reliable quantitative measurements *in vivo*.
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1. Introduction

Due to the rapid signal response time FRET-based technologies (Förster, 1948) are highly promising for non-invasive intracellular analyses. Respective genetically encoded biosensors consist essentially of two fluorescent proteins (FPs), which may react on specific environmental changes. Most biosensors contain FPs derived from the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* and related FPs. As GFP and many of its variants were shown to be highly sensitive toward various environmental parameters, among which the influences of pH and halide ions were most intensively studied (Martinière et al., 2013; Newman et al., 2011), less sensitive variants have been generated (Rizzo et al., 2004; Zhang et al., 2003). One of the frequently used less pH- and halide sensitive GFP-variant is Citrine, which additionally shows a high quantum yield (Griesbeck et al., 2001). The broad range of available GFP-based biosensors has recently been extensively reviewed (Newman et al., 2011; Okumoto et al., 2012). Besides, in recent years further FPs, such as the novel class of cyan-green fluorescent flavoproteins were identified as useful FRET partners for GFP-derived FPs *e.g.* for the construction of oxygen-independent *in vivo* bioreporters (Drepper et al., 2013 and references therein) and as oxygen biosensors (Potzkei et al., 2012).

For the quantitative analysis of intracellular metabolites, genetically encoded biosensors of the Venus-flytrap type are frequently used. Such multi-domain biosensors FP_D:BD:FP_A consist of a ligand binding domain (BD) between two usually GFP-based fluorophores acting as FRET donor (FP_D) and acceptor (FP_A) domain, respectively. As a consequence of FRET (energy transfer: $FP_D \rightarrow FP_A$) the emission intensity of the FRET-donor is reduced, whereas the emission of the FRET-acceptor increases. In case of a ligand-binding event to the central BD the FRET-efficiency should change significantly to visualize the presence of bound ligands with a high signal-to-noise ratio (Okumoto et al., 2012). During the last decade the application of many genetically encoded biosensors has successfully been demonstrated following signaling dynamics in living cells (Deuschle et al., 2005; Palmer et al., 2006; Palmer and Tsien, 2006; San Martin et al., 2013, 2014; Spiering et al., 2013; Takanaga et al., 2008).

Abbreviations: AxP, AMP ADP ATP; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; FP, fluorescent protein.

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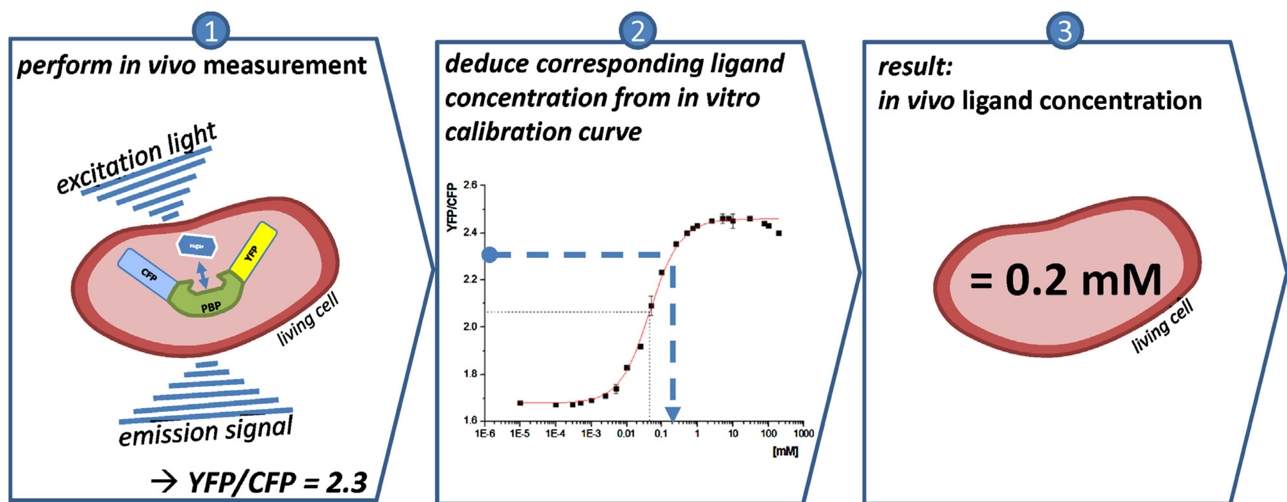

Fig. 1. Flowchart showing the application of genetically encoded FRET biosensors. Step 1 illustrates a common *in vivo* measurement with a cell expressing genetically encoded biosensors. After excitation of the donor fluorophore a FRET-ratio (YFP/CFP) is calculated from the fluorescence emission of the donor- and acceptor fluorophores. Subsequently, the corresponding ligand concentration is deduced from a previously recorded *in vitro*-titration curve (Step 2). As a result a putative *in vivo*-concentration of the investigated ligand (metabolite) is deduced (Step 3).

The application of such ratiometric sensors for quantitative metabolite analysis in living cells often relies on an *in vitro* calibration by measuring a ligand-titration curve in an aqueous buffer with arbitrary pH, buffer salt, and ionic strength. From such titration curves the affinity (K_d-value) of the sensor is deduced from a semi-logarithmic plot of the fluorescence intensity ratio (*e.g.* YFP/CFP) of both chromophores over a logarithmic concentration scale (Fig. 1). The central inflection point of the resulting S-curve is subsequently used to deduce the K_d-value. In order to analyze *in vivo* data, the respective YFP/CFP fluorescence intensity ratio is compared with the respective value of the calibration curve to determine the metabolite concentration *in vivo*.

Although this method is generally accepted, it has some limitations. First of all the K_d-value can only be estimated from the titration curve, which resembles typical substrate concentration-dependent hyperbolic Michaelis–Menten curves known from velocity studies with enzymes. In both cases a strong signal change is observed in a narrow concentration range. In order to deduce the K_d-value from such curves, the data set must be fitted with a respective (ideal) function. However, a usual dataset shows more or less deviations from this ideal function, especially under saturation conditions. In the semi logarithmic plot (Fig. 1) K_d is the central inflection point and strongly dependent on the number of data points, their errors and the fit quality to the ideal model. Second, and this is the topic of the present manuscript, the fluorescence intensity ratio and the apparent K_d can dramatically be influenced by environmental factors, such as pH, temperature, ions and cellular metabolites, which can hardly be simulated in *in vivo* calibrations.

Several factors are known which affect the FRET-efficiency, such as the distance between the donor and acceptor fluorophores, the overlap of the donor emission and the acceptor absorption spectra, and the orientation of the dipoles of both fluorophores (Dale et al., 1979; Okumoto et al., 2012; Stryer and Haugland, 1967).

The goal of this study was to evaluate the reliability of such FRET-based sensor systems for an *in vivo*-application, focusing on factors which are most likely different between *in vitro* calibration and *in vivo* application. For this purpose, we selected two well established biosensors for glucose and maltose, respectively (Fehr et al., 2002; Takanaga et al., 2008). These biosensors use very common FRET-pairs namely the enhanced cyan fluorescent protein (ECFP) as FRET donor and enhanced yellow fluorescent protein (EYFP) and Citrine, respectively, as FRET acceptors. Their central ligand binding domain consists of well characterized periplasmic binding proteins from *E. coli* with known crystal structures (PDB: 2FVY, 1ANF). Both sugar biosensors were broadly used for quantitative metabolite analyses in prokaryotes, yeasts, plants, and mammalian cells showing an excellent signal-to-noise ratio (Bermejo et al., 2011; Fehr et al., 2002; Hou et al., 2011; Liemburg-Apers et al., 2011; Takanaga et al., 2008).

We characterized both sensors thoroughly to explore the influence of micro-environmental effects, such as pH, buffer salts, buffer concentration, ions, and cellular metabolites on the biosensor and also on the single FPs. Our data show that the tested biosensors are very sensitive toward most of the studied parameters, demonstrating that care should be taken during the calibration of such systems in order to deduce quantitative data.

2. Material and methods

2.1. Sensor preparation

The glucose sensor pRSET FLII[12]Pglu600μ (Deuschle et al., 2005; Fehr et al., 2005) and the maltose sensor pRSET FLIPmal-25μ (Fehr et al., 2002) were from addgene (http://www.addgene.org). The protein sequences of both sensors are shown in Fig. S15. Characteristic mutations relative to GFP (UniProt accession no. P42212) in the respective FPs were:

ECFP: F64L, S65T, Y66W, N146I, M153T, V163A (Kremers et al., 2006, Clontech)

EYFP: S65G, V68L, S72A, T203Y (variant 10c in (Ormö et al., 1996), Clontech)

Citrine: S65G, V68L, Q69M, S72A, T203Y (Griesbeck et al., 2001)

E. coli BL21 (DE3) GOLD cells (Stratagene) were transformed with those vectors and positive colonies were selected on agar plates through ampicillin resistance. BL21 (DE3) Gold cells were grown in 2 L shaking flasks with baffles using 400 mL lysogeny broth medium (LB) containing 100 μg mL^{-1} ampicillin. Cultivation and sensor expression was carried out for 48 h at 28 °C in the dark, without IPTG-induction. Cells were harvested by centrifugation, resuspended in 20 mM MOPS buffer (pH 7.3), and disrupted by ultrasonication at 4 °C using an UP200s (Hielscher, Teltow, Germany). The sensor proteins carried an N-terminal

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