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A novel FbFP-based biosensor toolbox for sensitive *in vivo* determination of intracellular pH

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Keywords: FRET biosensor, flavin-binding fluorescent proteins (FbFP), yellow fluorescent protein (YFP), intracellular pH, bacterial acid stress response

Highlights

- Construction of new genetically encoded biosensors for pH
- The FluBpH toolbox covers a broad, physiologically relevant pH detection range from 4.0 to 9.0.
- Due to its exceptionally low pKa of 5.7, FluBpH 5.7 can be applied in highly acidic compartments and environments
- The FluBpH sensors allow non-invasive analysis of biological processes *in vivo* with high spatiotemporal resolution.

ABSTRACT

The intracellular pH is an important modulator of various bio(techno)logical processes such as enzymatic conversion of metabolites or transport across the cell membrane. Changes of intracellular pH due to altered proton distribution can thus cause dysfunction of cellular processes. Consequently, accurate monitoring of intracellular pH allows elucidating the pH-dependency of (patho)physiological and biotechnological processes. In this context, genetically encoded biosensors represent a powerful tool to determine intracellular pH values non-invasively and with high spatiotemporal resolution.

We have constructed a toolbox of novel genetically encoded FRET-based pH biosensors (named Fluorescence Biosensors for pH or FluBpH) that utilizes the FMN-binding fluorescent protein EcFbFP as donor domain. In contrast to many fluorescent proteins of the GFP family, EcFbFP exhibits a remarkable tolerance towards acidic pH (pK_a ~ 3.2). To cover the broad range of physiologically relevant pH values, three EYFP variants exhibiting pK_a values of 5.7, 6.1 and 7.5 were used as pH-sensing FRET acceptor domains. The resulting biosensors FluBpH 5.7, FluBpH 6.1 and FluBpH 7.5 were calibrated *in vitro* and *in vivo* to accurately evaluate their pH indicator properties. To demonstrate the *in vivo* applicability of FluBpH, changes of intracellular pH were ratiometrically measured in *E. coli* cells during acid stress.

Keywords: - FRET biosensor

- flavin-binding fluorescent proteins (FbFP)

- yellow fluorescent protein (YFP)

- intracellular pH

- bacterial acid stress response

1. Introduction

The intracellular pH is one of the most important factors for the metabolic activity and viability of cells. Changes in the intracellular pH can lead, for instance, to protein misfolding and inhibition of enzymatic activities which can even cause cell death. The physiological intracellular pH of most bacteria is constant and lies within the narrow range of pH 7.4-7.8 (Booth, 1985; Krulwich et al., 2011; McLaggan et al., 1998; Padam et al., 1976; Padan et al., 1981; Slonczewski et al., 1981). As a consequence, neutrophilic bacteria that thrive in environments with neutral pH have developed different mechanisms for maintaining their intracellular pH value, which are well described (Krulwich et al., 2011; Slonczewski et al., 2009).

Despite its strict regulation, intracellular pH values can alter during periods of acid or alkaline stress (De Biase and Lund., 2015; Foster, 2004; Maurer et al., 2005; Zilberstein et al., 1984). This includes, for example, the passage of the acidic barrier of the stomach by some pathogens such as *E. coli* O157: H7 before colonization of the gastrointestinal tract (Arnold and Kaspar, 1995; Benjamin and Datta, 1995; Conner and Kotrola, 1995). Therefore, *E. coli* developed additional strategies to maintain its cytoplasmic pH at a minimum of pH 4.3 in order to survive strong acidification of the environment (Iyer et al., 2002; Richard and Foster, 2004).

In addition, many different pathogenic and non-pathogenic bacteria are perfectly adapted to (transiently or permanently) acidic environments. A well-known example is the biotechnologically relevant organism *Lactococcus lactis* that preferentially grows at environmental pH values of 6.5 (Andersen et al., 2009; Hutkins and Nannen, 1993). During glucose consumption, however, *L. lactis* forms lactic acid in high amounts which results in a moderate acidification of the surrounding environment - a process which is of crucial importance in dairy and milk processing industry (Ghaffar et al., 2014). During this process, the

intracellular pH of *L. lactis* changes from approximately 7.2 to 6.0 (Andersen et al., 2009; Neves et al., 2002). In contrast, the human pathogenic bacterium *Helicobacter pylori* can even survive at a constant low external pH (Stingl et al., 2002) in the human stomach, thereby causing gastric ulcers (Smith, 2003). Furthermore, many different extremophilic bacteria and archaea could be isolated from various highly acidic environments like sulfuric pools, geysers and areas polluted by acid mine drainage (Baker-Austin and Dopson, 2007; Johnson, 1998).

The high diversity of neutrophilic and acidophilic microbes as well as their role in pathophysiology, biotechnology and preservation of the environment clearly illustrates the importance of monitoring intracellular pH values, which can help to get vital insights into general and individual acid stress response strategies. To this end, different fluorescent biosensors have been developed and are currently used for the analysis of intra- and extracellular pH values. These sensors can be divided into two groups: The first group encompasses fluorescent dyes, such as SNARFs, LysoSensors or BCECF (Han and Burgess, 2010; Lin et al., 2001; Rink et al., 1982; Whitaker et al., 1991), which are often used as pH indicators offering pK_a values (i.e. the pH value at which 50% of the fluorescence is still detectable) suitable for detection ranges from pH 3.5 to pH 8. Fluorescent dye-based pH sensors have several drawbacks including cytotoxic effects, limited cellular uptake or leakage of the dye from cells (Han and Burgess, 2010). The second group comprises genetically encoded fluorescent protein (FP)-based pH biosensors, which were developed to overcome some of the problems that are specifically associated with fluorescent dyes. One of the most important advantages of FP sensors is the ability to target them to specific cell types and cellular compartments or structures in a non-invasive manner (Bizzarri et al., 2009; Zhang et al., 2002). These sensors either consist of single FPs or fusions of two FPs as FRET or tandem fusion pairs. Single FP systems such as pHluorin, E^2GFP and pHRed (Bizzarri et al., 2006; Miesenböck et al., 1998; Tantama et al., 2011) allow quantitative pH determinations *via* a pH-dependent ratiometric change of their excitation peaks. Genetically encoded pH sensors that are based on tandem fusion or FRET fusion proteins (e.g. GFpH, YFpH, ClopHensor and pHlameleons (Arosio et al., 2010; Awaji et al., 2001; Esposito et al., 2008)) contain two fluorescent protein domains: The first domain (in case of FRET sensors the FRET donor domain) exhibits a distinct tolerance

towards acidic pH thereby providing a fluorescence signal that is not significantly affected by changes of the pH value in a defined range (Sawano and Miyawaki, 2000). The fluorescence signal of the second domain (which acts as FRET acceptor domain in the corresponding biosensor constructs), in turn, is strongly affected by acidic pH. The resulting change of FRET efficiency thus allows for the ratiometric pH readout *via* the two fluorescence emission intensities when exciting the donor.

Previously described genetically encoded pH sensors employ different variants of the green fluorescent protein (GFP), of which a combination of CFP and YFP is mostly used for FRET fusion proteins (Esposito et al., 2008; Hellwig et al., 2004). While all of the YFP variants share very similar spectral characteristics, several derivatives exhibit significant differences in their pH susceptibility. EYFP, for example, offers a high pH sensitivity with a pK_a of 7.1 (Llopis et al., 1998; Ormö and Cubitt, 1996). Many EYFP variants with additional mutations show different pK_a values: The EYFP-H148G variant, for instance, is very sensitive towards neutral to alkaline pH with a pK_a value of 8.0 (Wachter et al., 1998). Fluorescence of both, EYFP and EYFP-H148G is known to be further impaired by halide ions like chloride (Wachter et al., 2000, 1998; Wachter and Remington, 1999). In contrast, the EYFP variant Citrine is known for its fast maturation, high chloride tolerance and relatively low pK_a of 5.7, which is caused by the Q69M mutation (Griesbeck et al., 2001). The described differences in pH sensitivity are caused by distinct accessibilities of the chromophores to the protons of the solvent (Hanson et al., 2002). The chromophore protonation, in turn, results in a reduction of absorption at 514 nm, which is accompanied by a pH dependent decrease of the fluorescence emission at 527 nm (McAnaney et al., 2005). The commonly employed FRET donor protein CFP, in turn, has a pK_a value of 6.4 (Llopis et al., 1998), whereas the enhanced cyan fluorescent protein (ECFP) is less sensitive to pH with a reported pK_a of 5.6 (Erard et al., 2013). This renders it a suitable donor domain for FRET-bases pH biosensors. However, the sensitivity of biosensors using ECFP significantly decreases below pH 5.5 (Esposito et al., 2008; Hellwig et al., 2004). Additionally, to the best of our knowledge, the so far established single FP- and tandem fusion sensors using GFP derivatives exhibit moderately high pK_a values ranging from 5.8 to 7.3 (Benčina, 2013).

In this report, we present the establishment and detailed characterization of novel genetically encoded FRET-based pH biosensors suitable for detecting pH values below 5.5 by implementing the alternative, highly pH resistant FRET donor domain EcFbFP. The FMN-binding fluorescent proteins (FbFP) represent promising alternative candidates to commonly used CFP variants because of their similar emission wavelength (Drepper et al., 2007; Wingen et al., 2014). Recently, the functionality of EcFbFP together with EYFP as a FRET-based biosensor for molecular oxygen could be demonstrated (Eichhof and Ernst, 2016; Potzkei et al., 2012). This prompted us to investigate the suitability of different FbFP-YFP fusion constructs as pH sensors. The resulting fluorescence biosensors for pH designated as FluBpH 5.7, 6.1 and 7.5 cover a broad, physiologically relevant pH range from 4.0 to 9.0. *In vivo* applicability could successfully be demonstrated by monitoring changes of intracellular pH in living *E. coli* cells under acidic conditions.

2. Materials and methods

2.1. Bacteria and growth conditions

E. coli strain DH5α (Hanahan, 1983) was used for DNA replication and construction of the expression vectors encoding the fluorescent reporter proteins EcFbFP and the different EYFP derivatives as well as the biosensors FluBpH 5.7, 6.1 and 7.5. *E. coli* strain BL21 (DE3) (Novagen, distributed by Merck KGaA, Darmstadt, Germany) was used for the expression of the fluorescent reporter proteins as well as for the biosensors FluBpH 5.7, 6.1 and 7.5. *E. coli* JM109 (DE3) (Promega GmbH, Mannheim, Germany) was used for the acid resistance assay (section 2.6). For protein expression and purification, bacterial cells were grown at 30 °C overnight in autoinduction terrific broth (TB) medium containing 12 g/l hydrolyzed casein, 24 g/l yeast extract, 9.4 g/l K_2HPO_4, 2.2 g/l KH_2PO_4, (pH 7.2), 4 ml/l glycerol, 0.05 % glucose, 0.2 % lactose. The EG minimal medium was used for the acid resistance assay (Lu et al., 2013), containing 73 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$, 0.8 mM $MgSO_4$ and 10 mM citrate supplemented with 0.5% glucose. All media were supplemented with 50 μg/ml kanamycin to maintain the expression vectors.

2.2. DNA constructs and cloning

The construction of the expression vector pRhotHi-2-FluBO and pRhotHi-2-FbFP was described previously (Drepper et al., 2007; Potzkei et al., 2012). To generate the pH sensor FluBpH 7.5, the N-terminal Clontech EYFP domain (S65G/V68L/S72A/T203Y/H231L, Clontech Laboratories, Inc, USA) of FluBO (Potzkei et al., 2012) was mutated to introduce the H148G substitution, described by Wachter and co-workers (Wachter et al., 1998) using QuikChange PCR and the following primers: EYFP-H148G fw: 5` AGTACAACTACAACAGCGGGAAC-3` and EYFP-H148G rev.: 5` TCGGCCATGATATAGACGTTCCCGCT-3`. For FluBpH 6.1 the V68L mutation present in the Clontech EYFP domain of FluBO was reversed, to yield a valine at the respective position, as described for EYFP (Llopis et al., 1998), using the primers: EYFP-L68V fw: 5`-ACCTTCGGCTACGGCGTCCAGTG-3` and EYFP-L68V rev.:

5` TAGCGGGCGAAGCACTGGACGCCGTA-3`. After QuikChange PCR the parental template DNA was hydrolysed with *Dpn*I to select for mutation-containing, recombinant DNA. To generate FluBpH 5.7, the N-terminal Clontech EYFP was exchanged against Citrine (Griesbeck et al., 2001). To this end, the corresponding Citrine-encoding gene was isolated from pREST CFP-mglb-Citrine (Deuschle et al., 2005; Moussa et al., 2014) using *Nde*I and *Sac*I restriction sites and subsequently cloned into pRhotHi-2-FluBO. The new plasmids pRhotHi-2-FluBpH 5.7, pRhotHi-2-FluBpH 6.1 and pRhotHi-2-FluBpH 7.5 were subsequently sequenced (Eurofins MWG Operon, Ebersberg, Germany). For the expression of the three FluBpHs the corresponding genes were subsequently cloned into the *Nde*I and *Xho*I restriction sites of pRhotHi-2 (Katzke et al., 2010).

2.3. Protein purification

The fluorescent proteins as well as the biosensors were purified as His_6-tagged proteins using Ni-NTA metal-ion-exchange-chromatography-superflow-columns (Qiagen, Hilden, Germany), under standard operating conditions as described by the manufacturer. The purified proteins were stored at 4 °C in protein storage buffer containing 10 mM NaCl, 10 mM NaH_2PO_4, pH 8.0.

2.4. Spectral analysis and pH titration

The absorption and fluorescence properties of EcFbFP, the EYFP derivatives and FluBpHs were determined in protein storage buffer, using a UV-2450 absorption spectrophotometer (Shimadzu) and a QuantaMaster 40 fluorescence spectrophotometer (PTI). The absorption spectra were recorded by scanning from λ = 330 - 650 nm; the emission spectra were recorded at excitation wavelengths of λ = 380 nm (FluBpHs), 450 nm (EcFbFP) or 515 nm (EYFPs). The maximum absorption of the purified proteins did not exceed 0.1 in the visible region for all fluorescence measurements.

For *in vitro* pH titrations of FPs three buffers with different chloride concentrations were used: (1) Buffer A was a Na_2HPO_4 / citric acid buffer (pH 4.0 – 9.0) without any chloride ions, prepared as described by McIlvaine (McIlvaine, 1921). (2) Buffer B contained 125 mM KCl, 20 mM NaCl, 0.5

mM $CaCl_2$, 0.5 mM $MgCl_2$, and 25 mM of one of the buffer substances Mes, Mops, Hepes, or Bicine (according to their individual buffering capacities) and pH values were adjusted to 4.0 – 9.0 as described previously using either HCl or NaOH (Llopis et al., 1998). (3) To generate a buffer containing physiological chloride concentrations (in the following designated as physiological buffer), 6 mM NaCl were added to buffer A. The *in vitro* measurements of fluorescence emission of single FPs and fluorescence emission ratios of FluBpHs were performed in a Tecan Infinite M1000 PRO 96 well plate reader. Each well contained a buffer solution with a defined pH value from 4.0 to 9.0 in 0.2 increments and the purified protein of interest with a final absorption of 0.1 at λ = 450 nm for EcFbFP or at λ = 515 nm for the EYFP-derivatives as well as the pH biosensors FluBpH 5.7, 6.1 and 7.5. In order to accurately determine the pH robustness of the EcFbFP fluorescence signal, the pH range was extended to pH 2.6 - 11.5. Titration curves were fitted to a Bolzmann sigmoid curve and the pK_a values were determined as the pH at which half maximal fluorescence intensity was still detectable.

2.5. In vitro *and* in vivo *calibration of the FluBpH pH biosensors*

The biosensors FluBpH 5.7, 6.1 and 7.5 were calibrated *in vitro* in each of the aforementioned buffer systems. For this purpose, the samples were excited at λ = 380 nm and the emission spectra were recorded from λ = 480 to 650 nm. The fluorescence ratio of emission at λ_{max} = 527 and 495 nm was then assigned to the corresponding pH value in the tested buffer system. For the *in vivo* pH calibration, the antibiotic nigericin (Sigma-Aldrich, distributed by Merck KGaA, Darmstadt, Germany) was used to equilibrate the intracellular pH of *E. coli* cells, expressing one of the biosensors, with the external pH of buffer A. The buffer was adjusted to cover a pH range from 4.0 - 9.0 and was supplemented with 150 mM KCl, because nigericin acts as a K^+/H^+ ionophore. For this, *E. coli* BL21 (DE3) cells were harvested, washed with 100 mM Tris/HCl buffer pH 7.5 and resuspended in the buffer A solutions (pH 4.0 - 9.0) at an OD_{580} = 0.5. After this, the *E. coli* cells were treated with 1 mM EDTA and 50 µM nigericin, as described previously (Ahmed and Booth,

1983). Determination of *in vivo* fluorescence ratios of the expressed FluBpHs 5.7, 6.1 and 7.5 were performed as described for the *in vitro* calibration.

2.6. FluBpH-based monitoring of intracellular pH in E. coli *JM109 (DE3) during pH stress*

The acid stress assay was performed as described by Lu *et al.* (Lu et al., 2013) with one minor variation. Overnight cultures of JM109 (DE3) were grown in autoinduction medium for 20 h to express the pH biosensor FluBpH 5.7. The cells were diluted to an OD_{580} = 0.1 in EG medium with a pH value of 7.5 (positive control) and 2.5, supplemented with either 1 mM glutamine or 1 mM glutamate, which are known to activate the acid response system 2 (AR2) (Lu et al., 2013; Richard and Foster, 2004). EG medium pH 2.5 without glutamine or glutamate was chosen as a negative control. In order to exclusively observe AR2-mediated responses, all samples were additionally supplemented with 0.4 % glucose thereby supressing the acid response system 1 (AR1) (Foster, 2004). After incubation at 37 °C for 3 h, samples were taken for fluorescence measurements to determine the intracellular pH value of the *E. coli* cells that survived the acid shock. To exclusively determine intracellular pH values of intact cells, samples with an OD_{580} = 0.1 were taken and washed once with EG medium with the same pH value and supplements. Afterwards the FluBpH 5.7 fluorescence emission spectra were measured as described for the pH titration and the fluorescence ratios (527 nm / 495 nm) were compared to the *in vivo* calibration curves to determine the intracellular pH value during acid response. Additionally, cells were inoculated on LB agar plates, supplemented with 50 µg/ml kanamycin, to determine the survival rates by counting colony forming units (CFUs).

3. Results and discussion

3.1. Characterization of the pH stability of EcFbFP and EYFP variants

EYFP variants are known to be susceptible to acidic pH (Bizzarri et al., 2006; Griesbeck et al., 2001; Llopis et al., 1998), rendering them suitable for use as pH-sensing acceptor domains within a FRET-based biosensor design. In contrast, the spectral and photophysical properties of FbFPs have only been characterized at neutral pH (Drepper et al., 2013, 2007; Wingen et al., 2014). To assess the pH tolerance of EcFbFP and thus its applicability as an alternative FRET donor domain for novel pH biosensors, we first purified the flavoprotein and analysed its fluorescence over a broad pH range (Fig 1). Remarkably, EcFbFP exhibits a pronounced pH stability resulting in an almost constant fluorescence signal within a pH range from 4.0 to 9.0. To determine the pK_a value of EcFbFP, an extended stability test was conducted covering a broadened pH range from 2.6 to 11.5 (supplementary Fig. S1). As reflected by the change of the EcFbFP fluorescence spectrum (supplementary Fig. S1A), the fluorescence intensity is significantly affected by the release of the chromophore from the protein (λ_{max} of free FMN = 520 nm) which occurs upon EcFbFP denaturation at pH values lower than 3.2. In the pH range of 4.0 to 3.2, EcFbFP is probably partially unfolded, accompanied by a significant decrease of fluorescence at λ_{max} of 495 nm, resulting in a very low pK_a of ~3.2.

The previously described FRET-based pH biosensor pHlameleon utilizes ECFP as donor domain, which is characterized by a pK_a value of 5.6 (Erard et al., 2013). Other examples are the FRET-based pH biosensors GFpH and YFpH (Awaji et al., 2001). These two pH sensors utilize the recombinant green fluorescent protein GFPuv as pH tolerant donor domain with a pK_a value of ~4.6 - 5.4 (Yang et al., 1996). In comparison, the fluorescence signal of EcFbFP is remarkably stable over a very wide pH range from 3.4 to 10.8. We therefore concluded that its pronounced pH tolerance qualifies EcFbFP as an alternative FRET donor domain that is well suited for creating new pH biosensors in conjunction with YFP.

In order to evaluate the YFP variants as potential FRET acceptor domains that can be used for monitoring intra- end extracellular pH over the entire physiological range, we conducted the same experiments with Citrine, EYFP and EYFP-H148G, which were reported to exhibit pK_a values of 5.7, 7.1 and 8.0, respectively (Griesbeck et al., 2001; Llopis et al., 1998; Wachter et al., 1998). As expected, all three EYFP variants showed a distinct pH sensitivity of their fluorescence signals (Fig. 1).

Fig. 1. Normalized fluorescence intensities of EcFbFP, Citrine, EYFP and EYFP-H148G at different pH-values. The fluorescent proteins were diluted in physiological buffer (supplemented with 6 mM NaCl) adjusted to pH values from 4.0 to 9.0 by combining defined amounts of citric acid and Na_2HPO_4. Fluorescence spectra were recorded and normalized to their maximum fluorescence intensity in the corresponding buffer at pH 9.0. All measurements were performed at 22 °C. All measurements where performed in triplicates, showing the average value with standard deviations.

The loss of fluorescence is a well-known phenomenon of different EYFP variants and can be attributed to the protonation of the chromophore (Elsliger et al., 1999; McAnaney et al., 2005; Seward et al., 2013). However, in contrast to the published data, we determined pK_a values of 5.7, 6.1 and 7.5, for Citrine, EYFP and EYFP-H148G, respectively. These tests were performed in a Na_2HPO_4 / citric acid buffer, containing 6 mM NaCl, which corresponds to the physiological chloride concentration found in *E. coli* (Mukhtarov et al., 2013) and most mammalian cells (Arosio and Ratto, 2014). While the pK_a value for Citrine is in agreement with the published value of 5.7 (Griesbeck et al., 2001), our results differ from the published values for EYFP (pK_a = 7.1, Llopis et al., 1998) and EYFP-H148G (pK_a = 8.0, Wachter et al., 1998). This observation might be explained by the different buffer system used in previous studies, which contained 125 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Llopis et al., 1998; Wachter et al., 1998). Since both fluorescent proteins are known to be influenced by halide ions (Jayaraman et al., 2000; Wachter et al., 2000; Wachter and Remington, 1999), we repeated the pH titration experiments using the respective buffer

system (designated as buffer B in the above mentioned publications; Fig. S2B). In addition, we used the Na_2HPO_4 / citric acid buffer without NaCl (Fig. S2A), designated here as buffer A (see experimental section for details) as a negative control. As expected, the pK_a of Citrine was not affected by increased chloride concentrations, which is in agreement with literature (Griesbeck et al., 2001). In contrast, the pK_a values of EYFP and EYFP-H148G were significantly lower in the absence of chloride ions (buffer A - EYFP: 5.9, EYFP-H148G: 7.3) whereas high chloride concentrations resulted in a significant increase of pK_a values (buffer B - EYFP: 7.1, EYFP-H148G: 7.8) compared to the physiological buffer, which can be attributed to the chloride sensitivity of these two YFP variants (Wachter and Remington, 1999).

3.2. Construction and in vitro *characterization of fluorescence-based biosensors for pH determination (FluBpH)*

In a previous study, we could demonstrate that EcFbFP and YFP can basically be fused to generate a functional FRET system. Spectral analysis of the resulting fluorescence biosensor for molecular oxygen, FluBO, revealed that the FRET donor domain EcFbFP can specifically be excited at λ = 380 nm (Potzkei et al., 2012). After the characterization of the pH-dependent fluorescence response of EcFbFP and the selected YFP variants we further investigated in this study if similar fusion proteins can be used to generate novel biosensors that allow for precise pH readout in a ratiometric manner, over the entire physiologically relevant range (Fig. 2).

Fig. 2. Schematic representation of the FRET-based fluorescence biosensor for pH (FluBpH). The fusion protein consists of EcFbFP as donor domain (green circle) and Citrine, EYFP or EYFP-H148G as respective acceptor domains (yellow barrel), connected by a small peptide linker. Specific excitation of EcFbFP (λ_{Ex} = 380 nm) results in an exclusive excitation of EcFbFP as shown previously (Potzkei et al. 2012), so that efficient FRET (green arrow) should result in a low donor (λ_{max} = 495 nm) and a high acceptor (λ_{max} = 527 nm) fluorescence under neutral pH conditions. Acidification, in turn, should lead to a stepwise decrease of the acceptor fluorescence accompanied by an increase of donor fluorescence.

We therefore constructed three EcFbFP-YFP fusion proteins harbouring EcFbFP as pH-tolerant FRET donor domain and one of the EYFP variants as appropriate FRET acceptor domains. The resulting pH biosensors were designated as FluBpH, extended by the pK_a value of the respective EYFP variant determined in the physiological buffer: FluBpH 5.7 (Citrine), FluBpH 6.1 (EYFP) and FluBpH 7.5 (EYFP-H148G). All three biosensor variants were expressed in *E. coli* BL21 (DE3) and subsequently purified for *in vitro* characterization. To calibrate the pH sensors, we performed pH titrations in the same buffer systems (physiological buffer, buffer A and B) and recorded the fluorescence spectra (exemplarily shown for FluBpH 5.7 in supplementary figure S1C), to determine the emission ratio of the FRET acceptor (YFP variants, 527 nm) and FRET donor (EcFbFP, 495 nm) domains upon specific excitation of EcFbFP (λ_{ex}=380 nm). As shown in Fig. 3, the shapes of the resulting curves closely resemble those of the individual YFP variants in the respective buffer systems, which was expected considering the pH stability of EcFbFP.

Fig. 3. *In vitro* calibration curves of pH biosensors FluBpH 5.7, 6.1 and 7.5. The fluorescence ratios of FluBpH 5.7, 6.1 and 7.5 are plotted as a function of pH in a range of pH 4.0 to 9.0 in three different buffers (see section 2.4). **A:** The physiological buffer contains 6 mM NaCl **B:** The chloride-free buffer A. **C:** Buffer B contains 147 mM chloride. Error bars indicate the standard deviation of three independent measurements.

According to the sigmoidal calibration curves, FluBpH 5.7 is suitable for pH detection in a range of approximately 4.2 to 7.0 with a very low pK_a of 5.7 in all three tested buffer systems (Tab. 1), demonstrating its high tolerance towards chloride ions. For FluBpH 6.1 and 7.5, in contrast, the valid pH detection range depends on the predominant chloride concentration as already described for the respective EYFP variants (Jayaraman et al., 2000; Wachter et al., 2000; Wachter and Remington, 1999): In the chloride free buffer A, FluBpH 6.1 is suitable for measurements from pH 5.0 to 7.0, with a pK_a of 5.9 (Fig. 3B, Tab. 1). However, even a low NaCl concentration of 6 mM (physiological buffer, Fig. 3A) leads to shifted calibration curve with a pK_a of 6.1. This effect is

even more pronounced in buffer B, which contains high chloride concentrations resulting in a pK_a of 7.1 (Tab. 1). In case of FluBpH 7.5, a similar effect could be observed, but in this case the chloride-mediated pK_a shift is less pronounced, resulting in a pK_a of 7.3 under chloride free conditions (buffer A), 7.5 in the physiological buffer and 7.8 in buffer B (Tab. 1).

Table 1

Properties of FluBpH biosensors determined *in vitro*.

Name of the biosensor	Acceptor domain	pK_a			pH detection range (physiological buffer)
		Physiological buffer (6 mM [Cl^-])	Buffer A (0 mM [Cl^-])	Buffer B (147 mM [Cl^-])	
FluBpH 5.7	Citrine	5.7 ± 0.06	5.7 ± 0.07	5.7 ± 0.02	~ 4.2 – 7.0
FluBpH 6.1	EYFP	6.1 ± 0.03	5.9 ± 0.02	7.1 ± 0.05	~ 5.0 – 7.4
FluBpH 7.5	EYFP H148G	7.5 ± 0.03	7.3 ± 0.04	7.8 ± 0.2	~ 7.0 – 9.0

The pK_a value of FluBpH 5.7 that contains Citrine is constant in all three buffers, while pK_a values of the FluBpHs with the EYFP variants EYFP 6.1 and EYFP H148G are affected by the chloride concentration. All values are mean values of three independent measurements including corresponding standard deviations.

Overall, the pH-mediated change of FluBpH fluorescence ratios exhibited remarkably high dynamic ranges (Fig. 3). In case of FluBpH 5.7, the fluorescence ratio increased from approximately 1.0 at pH 4.0 to 6.3 at pH 9.0, in all tested buffers. A similar change of fluorescence ratios could be observed for FluBpH 6.1 in a pH range of 5.0 to 9.0 with a maximal factor of 4.7 in physiological buffer and buffer A and a factor of 4.5 in buffer B. FluBpH 7.5, however, offers a comparatively low dynamic range with a change of its fluorescence ratio from 0.8 (pH 5.5) to 1.7 (pH 9.0) as determined in all three buffers. Nevertheless, this sensor allows for ratiometric pH determination between pH 7.0 and 9.0, with sufficient sensitivity. Since FluBpH 7.5 only differs from FluBpH 6.1 by two amino acid substitutions, the observed differences in the dynamic range might be explained

by a reduced molecular extinction coefficient and/or a lower fluorescence quantum yield of the EYFP-H148G acceptor domain.

3.3. In vivo *calibration of FluBpHs*

The most important prerequisite for the accurate determination of intracellular pH values is a precise calibration of the applied sensor with respect to the cellular system of interest. Therefore, we conducted an *in vivo* calibration of the FluBpH sensors using *E. coli* cells as an appropriate test system. To manipulate the intracellular pH for *in vivo* calibration within intact cells, the cytoplasmic membrane of live *E. coli* cells was permeabilized for protons using the K^+/H^+ ionophore nigericin (Pressman, 1976; Reed, 1979). When working with Gram-negative bacteria like *E. coli*, it is also necessary to treat the cells with EDTA, in order to facilitate the passage of nigericin through the outer membrane (Ahmed and Booth, 1983). In a first experiment, we therefore harvested *E. coli* cells expressing FluBpH 5.7 and resuspended them in a buffer with a pH value ranging from 4.0 to 8.0 in 0.2 increments. To analyse the ratiometric response of nigericin-mediated acidification of the *E. coli* cytoplasm, we subsequently analysed FluBpH-mediated fluorescence in aliquots of the same cell cultures after treatment with (i) nigericin and EDTA, (ii) with nigericin only or (iii) with EDTA only. The resulting fluorescence ratios of FluBpH 5.7 plotted against the extracellular pH are depicted in Fig. 4A.

Fig. 4. *In vivo* calibration of FluBpH 5.7 in *E. coli* cells using the K^+/H^+ ionophore nigericin. **A**: Fluorescence ratio of *E. coli* cells expressing FluBpH 5.7 as a function of external pH after addition of EDTA and nigericin (E+ N+, blue), nigericin (E- N+, red), EDTA (E+ N-, green) or without nigericin and EDTA (E- N-, black). **B**: Comparison of the *in vitro* and *in vivo* calibration curves of FluBpH 5.7 derived from data shown in Fig 3A and 4A. Both curves show good correlations, demonstrating the applicability of FluBpH 5.7 under *in vivo* conditions. The data represents the average of three independent measurements with the corresponding standard deviation.

As expected, addition of EDTA and nigericin in combination with different extracellular pH values (pH 4.0 – 8.0) resulted in a pronounced change of the fluorescence ratio between donor and acceptor domain of the intracellularly located FluBpH 5.7 (Fig. 4A, blue graph). Most remarkably, under these conditions, the *in vivo* and *in vitro* calibration curves (physiological buffer) exhibit an almost identical sigmoidal shape with highly comparable fluorescence ratios at the corresponding pH values (Fig. 4B). This data clearly indicates that due to the activity of the ionophore a complete equilibrium of protons could be achieved between the interior and exterior of the bacterial cell. More importantly, the pH-dependent change of FluBpH 5.7 fluorescence emission is virtually not affected by components of the cytoplasm. Similar data could be obtained for *in vivo* calibration of FluBpH 6.1 (calibration range: pH 4.0 – 8.0, supplementary Fig. S3) and FluBpH 7.5 (calibration range: pH 5.5 – 9.0, supplementary Fig. S4). In contrast, by solely adding nigericin (Fig. 4A, S3A and S4A; red graphs) or EDTA (green curves), the intracellular pH is only slightly affected by acidic and alkaline pH values as reflected by a minor change of the fluorescence ratio of FluBpHs, which corroborates the synergistic effects of nigericin and EDTA. In summary, the results of the *in vivo* calibration experiments clearly prove the applicability of the FluBpH toolbox for *in vivo* monitoring of pH over the full physiological range. It should be noted however, that halide ion concentrations as well as other cellular and environmental factors can affect the fluorescence signal of the biosensors (Moussa et al., 2014), which can vary significantly with respect to the biological systems of interest. Therefore, we strongly recommend performing an *in vivo* calibration of the FluBpH variants in the respective cellular background as described here.

3.4 Determination of intracellular pH in E. coli *during acid stress response*

After demonstrating the *in vivo* applicability of the new pH biosensors, we first analysed the effect of increasing acidification of the cell exterior on cytoplasmic pH under similar conditions as applied for the FluBpH *in vivo* calibration. In contrast to previous experiments, we now omitted nigericin and EDTA as components capable of artificially affecting the proton transfer across the

cytoplasmic membrane. As shown in Fig. 4A, under these conditions the fluorescence ratio of FluBpH 5.7 (black curve) is nearly constant at an extracellular pH of 5.5 to 8.0 (i.e. the pH range allowing unrestricted growth of *E. coli*, Krulwich et al., 2011; Slonczewski et al., 2009) with a deduced intracellular pH of 6.7 – 7.0. This observation resembles previously published data (Padam et al., 1976; Slonczewski et al., 2009; Wilks and Slonczewski, 2007). In these studies, the authors determined intracellular pH values of 7.4 - 7.8 in *E. coli* cells at neutral extracellular pH using either radiolabelled, membrane-permeant probes, phosphorus NMR spectroscopy and pH sensitive GFP derivatives (Krulwich et al., 2011; Padam et al., 1976; Slonczewski et al., 1981; Wilks and Slonczewski, 2007). However, due to the low pK_a value of FluBpH 5.7, this biosensor is not well suited for monitoring changes of intracellular pH in the neutral to basic range, a fact that might be responsible for the deviation of results originating from the different pH detection techniques. Therefore, we subsequently applied the biosensors FluBpH 6.1 and 7.5 for *in vivo* detection of intracellular pH at the respective extracellular conditions (pH 6.5 – 8.0). Here, ratiometric pH detection revealed intracellular pH values ranging from 7.2 to 7.6 (FluBpH 6.1, Fig. S3A) and 7.0 to 7.6 (FluBpH 7.5, Fig S4A), respectively, which corroborates the previously published values (Padam et al., 1976; Slonczewski et al., 2009; Wilks and Slonczewski, 2007).

Upon stronger acidification of the cellular environment (pH 4.0 – 5.5) the fluorescence ratio of FluBpH 5.7 further decreases. According to the *in vitro* calibration curve, the cytoplasmic pH finally dropped to a value of ~ 6.2 at an external pH of 4.0. However, bacteria like *E. coli* and *H. pylori* are known to survive even harsher pH values of 2 to 3 (Foster, 2004), for instance when passing or colonizing the human stomach. The acid resistance mechanisms of *E. coli* are well described in previous studies (Lu et al., 2013; Richard and Foster, 2004). Amongst others, the decarboxylase/antiporter-dependent acid response system 2 (AR2) is known to stabilize the internal pH at 4.3 as determined by measuring the distribution of radiolabelled salicylic acid across the membrane (Richard and Foster, 2004). This is achieved by the decarboxylation of glutamine/glutamate – a catalytic process which consumes protons (Castanie-Cornet et al., 1999;

Foster, 2004; Lin et al., 1996, 1995; Richard and Foster, 2004). However, to the best of our knowledge, this phenomenon has not been analysed so far by applying FP-based sensors or fluorescent dyes. Therefore, we used FluBpH 5.7 to monitor this important biological process, thereby evaluating the biosensor's *in vivo* applicability at very low pH. After standard cultivation, *E. coli* JM109 (DE3) cells expressing FluBpH 5.7 were transferred into EG medium, adjusted to pH 2.5. In order to activate AR2, two aliquots were subsequently supplemented with either 1mM glutamine or 1mM glutamate. As control experiments, we used cell samples of the same batch that were set at pH values of 2.5 and 7.5, respectively, without adding those supplements. After adaptation to acid stress (incubation for 3 h at 37 °C) we measured the FluBpH-mediated fluorescence ratio of intact cells and also assayed the *E. coli* survival rate by determining the corresponding colony forming units. As shown in Table 2, activation of AR2 by glutamate or glutamine is sufficient to ensure the survival of a small amount of *E. coli* cells during acid stress at pH 2.5. Remarkably, these cells are able to stabilize their internal pH at ~4.3 ± 0.2, according to the fluorescence ratio of FluBpH 5.7.

Table 2

FluBpH 5.7-based analysis of cytosolic pH during acid stress in *E. coli*.

Growth medium and supplements	survival rate (%)	fluorescence ratio of FluBpH 5.7 (527nm / 495 nm)	intracellular pH value
EG medium pH 7.5	100	5.3 ± 0.09	6.8 ± 0.09[a]
EG medium pH 2.5	0	n.d	n.d
EG medium 1 mM Glu pH 2.5	1.4 ± 0.5	1.3 ± 0.12	4.3 ± 0.12
EG medium 1 mM Gln pH 2.5	1.3 ± 0.7	1.4 ± 0.17	4.3 ± 0.17

The survival rate of *E. coli* JM109 (DE3) cells in EG medium (pH 7.5) was defined as 100 %. Incubation in EG medium (pH 2.5) without the addition of glutamine or glutamate resulted in cell lysis and thus no clear fluorescence spectra could be measured (n.d.: not detectable). Addition of either 1 mM glutamine or glutamate resulted in a low percentage of surviving cells (Glu: 1.4 % ± 0.5; Gln: 1.3% ± 0.7). FluBpH 5.7-dependent fluorescence ratios of these cells indicated an intracellular pH of 4.3 in each case. [a]This pH value of the negative control at pH 7.5 is lower than expected, presumably because FluBpH 5.7 is not well suited for measurements around pH 7, as discussed above. All determined values are average values with the corresponding standard deviation of three independent measurements.

This value exactly matches the one previously reported using radiolabelled salicylic acid (Richard and Foster, 2004). Additionally, cells unable to activate any of the acid stress responses completely died off, which was probably accompanied by full cell lysis as suggested by the absence of any detectable FluBpH fluorescence signals after the acid treatment. These observations clearly illustrate that the non-invasive FRET-based pH biosensor FluBpH can be applied for *in vivo* monitoring of intracellular pH during the acid stress response in bacteria. Furthermore, the biosensor toolbox covers the broad physiological pH range found in the cytoplasm of acidophilic, neutrophilic and alkaliphilic bacteria and archaea, which can range from 4 to 9 (Slonczewski et al., 2009). The pH

detection limits known from established FRET-based pH biosensors (Awaji et al., 2001; Esposito et al., 2008) could thus be significantly expanded.

4. Conclusions

In this study, we developed a novel FRET-based pH biosensor toolbox consisting of three EcFbFP-EYFP fusion proteins termed FluBpH 5.7, 6.1 and 7.5, which allows the precise determination of pH values inside living cells. The biosensor proteins contain EYFP variants as pH sensor domains that offer different pK_a values, so that the entire physiological pH range, including very acidic compartments and environments, can principally be analysed. In particular, the exceptionally low pK_a of 5.7, the high dynamic range of the ratiometric fluorescence readout and the independence of chloride ions make FluBpH 5.7 the most sensitive and robust member of the FluBpH toolbox. FluBpH 5.7 therefore provides a new and non-invasive way to study bio(techno)logical, pathophysiological and biomedical processes *in vivo* that depend on or are affected by low pH.

Conflict of interest

The authors declare no financial or commercial conflict of interest

Author Contributions

Conceived and designed the experiments: CR, JP, TG, TD. Performed the experiments: CR and MW. Analysed the data: CR and JP. Contributed reagents/materials/analysis tools: TG, KEJ. Wrote the paper: CR, MW, TG, TD. All authors have approved the final article.

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Fig 1

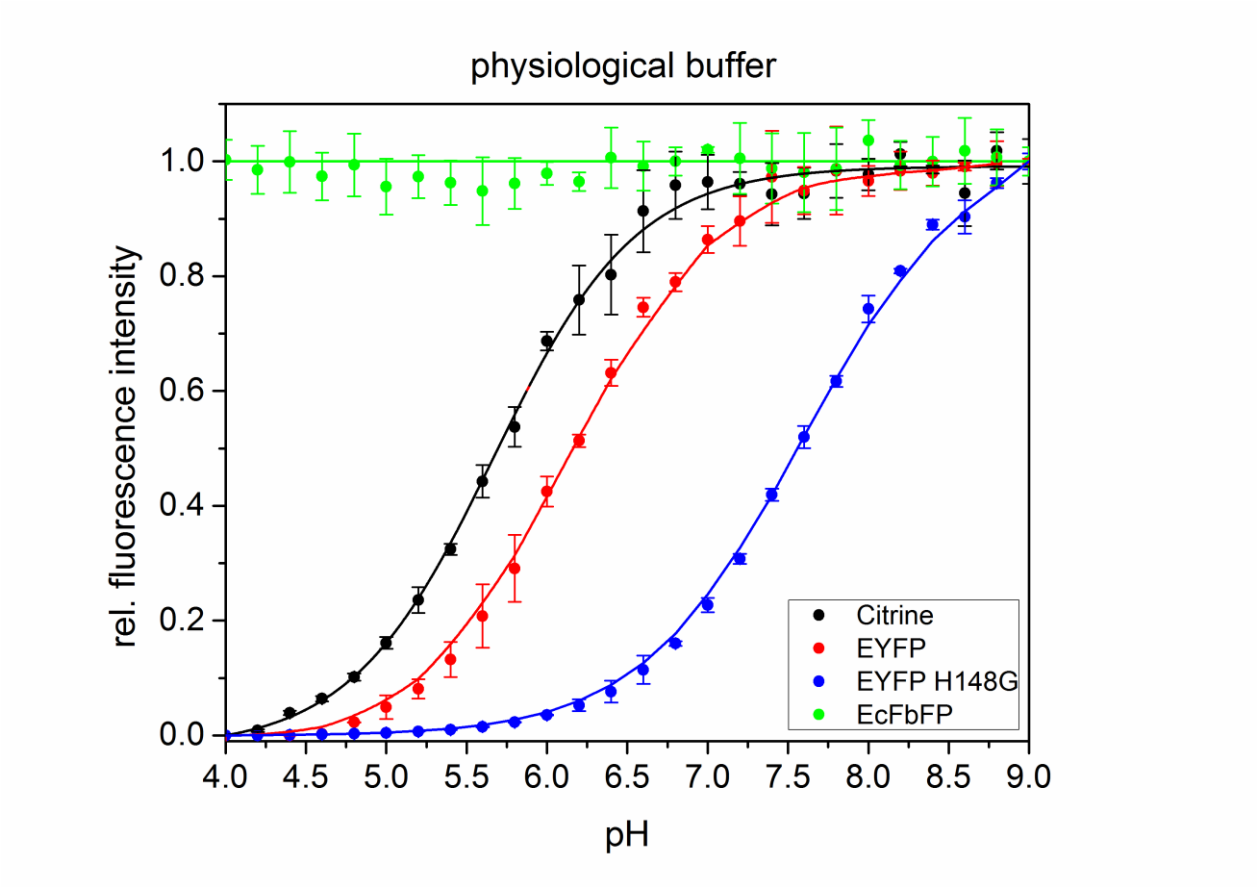

Fig 2

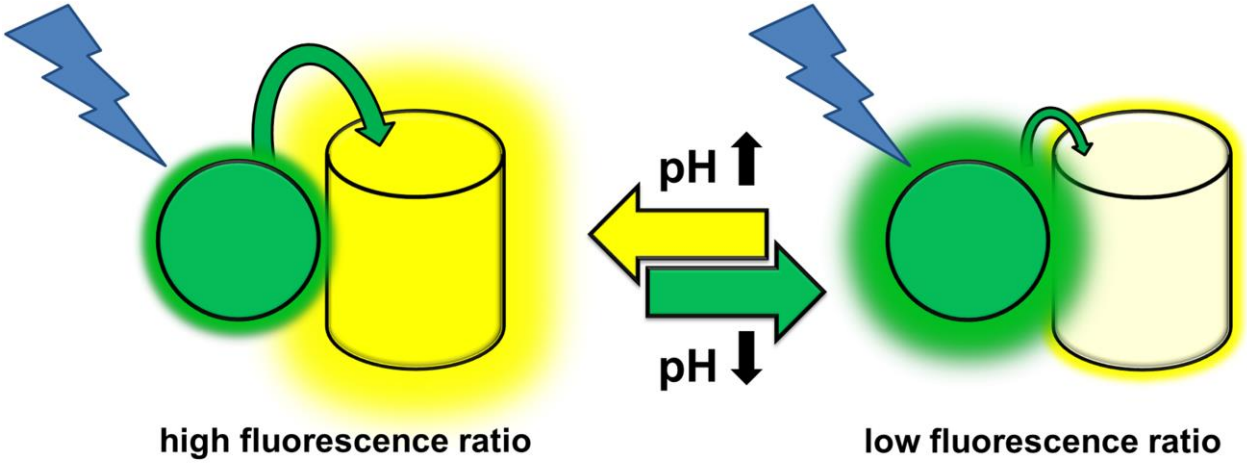

Fig 3

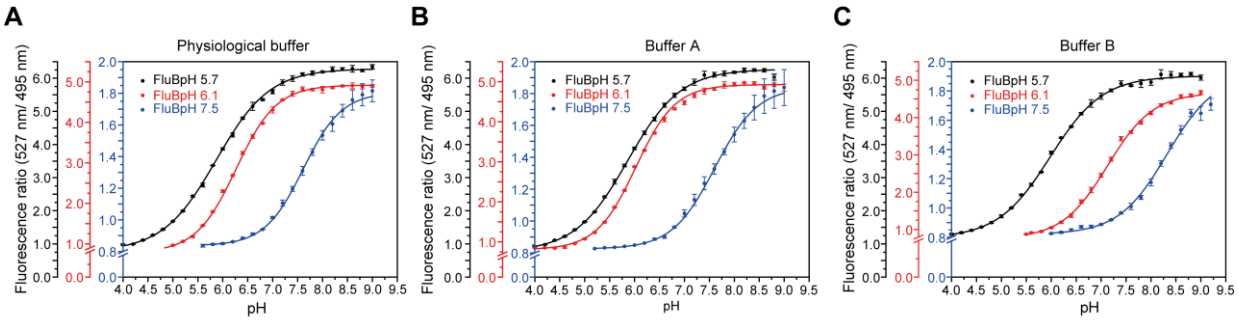

Fig 4