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# Flavin Mononucleotide-Based Fluorescent Reporter Proteins Outperform Green Fluorescent Protein-Like Proteins as Quantitative *In Vivo* Real-Time Reporters▽

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**Fluorescent proteins of the green fluorescent protein (GFP) family are commonly used as reporter proteins for quantitative analysis of complex biological processes in living microorganisms. Here we demonstrate that the fluorescence signal intensity of GFP-like proteins is affected under oxygen limitation and therefore does not reflect the amount of reporter protein in *Escherichia coli* batch cultures. Instead, flavin mononucleotide (FMN)-binding fluorescent proteins (FbFPs) are suitable for quantitative real-time *in vivo* assays under these conditions.**

In the last decade, quantitative analysis of cellular processes has enabled detailed insights into the complexity of metabolic and regulatory networks in various microorganisms, especially in the fields of systems and synthetic biology. Within the respective research areas, fluorescent proteins (FPs) are used as versatile *in vivo* reporters to study gene regulation and protein synthesis, folding, localization, and activity (1, 5, 7) in bacteria and yeast. The most widely used FPs are the green fluorescent protein (GFP) and its derivatives. However, their use as *in vivo* reporter proteins is restricted by various environmental factors affecting their signal intensity, including the availability of oxygen during maturation (18, 20). In addition, time-consuming protein folding and chromophore formation cause a time lag between the detectable fluorescence signal and the amount of reporter protein produced at a certain time point. Recently, we have developed an alternative family of fluorescent proteins that binds flavin mononucleotide (FMN) as a chromophore (3, 4). In contrast to all members of the GFP family, these novel FMN-binding fluorescent proteins (FbFPs) exhibit bright cyan-green fluorescence under both aerobic and anaerobic conditions and can be used to label facultative and strict anaerobic bacteria (3, 16) and yeast (19).

Here, we comparatively analyzed whether FbFP and a GFP-derived enhanced yellow fluorescent protein (YFP) can be used as reporters for quantitative real-time analysis of gene expression in living *Escherichia coli* cells. Furthermore, we studied whether the detectable FP fluorescence intensities correlate with the amounts of reporter proteins continuously during cell growth. Therefore, both FPs were expressed in *E. coli* using vector pRhokHi-2 (12), which harbors the *aphII* promoter of the kanamycin resistance gene and allows the constitutive expression of both fluorescence proteins at moderate levels. The pRhokHi-2-YFP expression vector, encoding yellow fluorescent GFP-10C derivative YFP (15) (available from Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), was constructed as described by Katzke et al. (12). Expression vector pRhokHi-2-FbFP, encoding *E. coli* FbFP (EcFbFP) (GenBank accession number ABN71355) (evoglow-Bs2; evocatal GmbH, Düsseldorf, Germany), was generated by PCR amplification of the fluorescence reporter gene, while NdeI and XhoI restriction sites were introduced into its 5′ and 3′ ends, respectively. Subsequently, the reporter gene was cloned into the corresponding sites of the expression vector pRhokHi-2. $P_{aphII}$-dependent FP expression basically avoids protein misfolding caused by high-level gene expression and alterations of FP expression levels due to changes in the inducer-to-cell ratio during cultivation.

In order to continuously monitor FP expression and *in vivo* fluorescence, *E. coli* DH5α (8) strains carrying the FP-encoding expression vector pRhokHi-2-YFP or pRhokHi-2-FbFP were generated. The respective empty vector was used as a negative control. Subsequently, the recombinant *E. coli* strains were cultivated in TB medium (13) under defined conditions (37°C, 700 rpm shaking frequency, 3 mm shaking diameter) in a BioLector microbioreactor system (m2p-labs GmbH, Aachen, Germany) over 16 h. To allow aeration of the cultures, a 48-well microtiter plate (MTP) (Greiner bio-one GmbH, Frickenhausen, Germany) was sealed with air-permeable sealing tape (AB-0718; ABgene, Epsom, United Kingdom). During cultivation, the BioLector provides quantitative online data of (i) biomass

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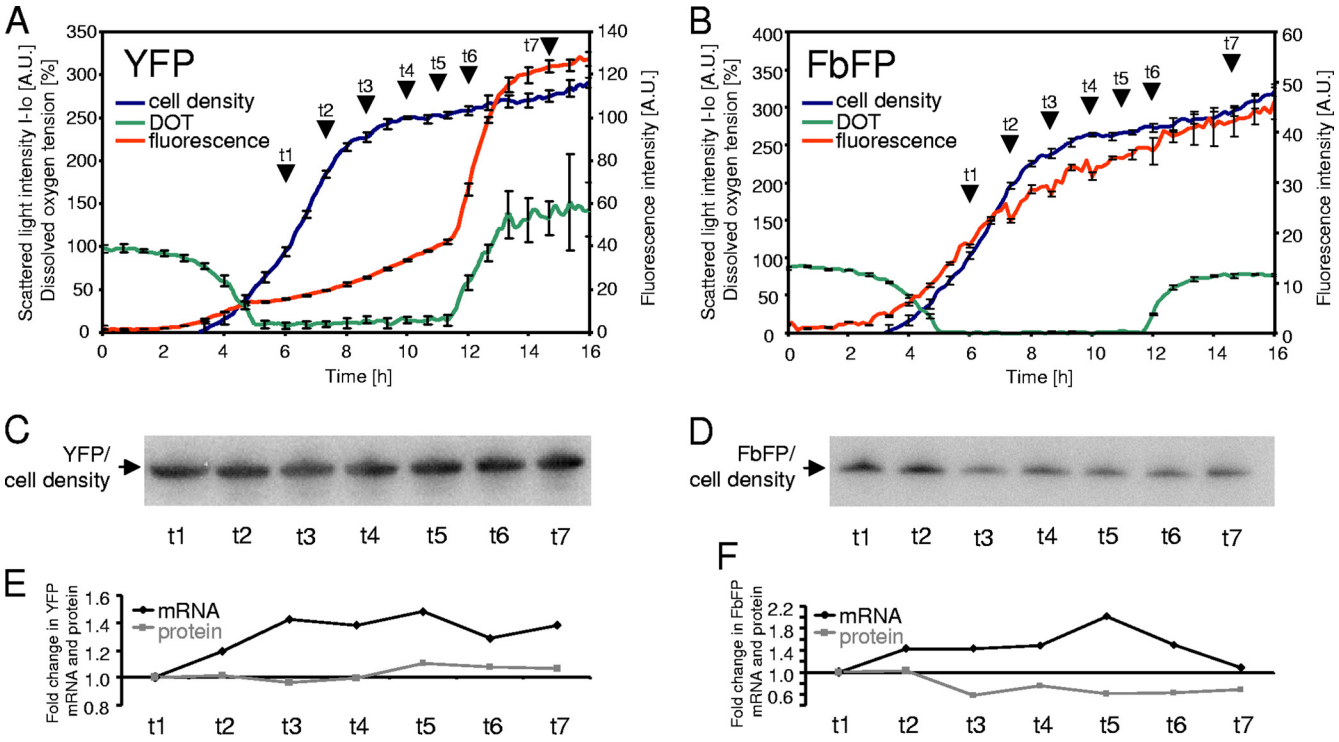


FIG. 1. Comparative analysis of YFP and FbFP expression and *in vivo* fluorescence in *E. coli*. *E. coli* DH5α carrying expression plasmid pRhokHi-2 encoding YFP (A) or FbFP (B) was batch cultured using a BioLector microbioreactor system. The development of biomass (cell density), dissolved oxygen tension (DOT), and FP-mediated fluorescence were permanently online monitored in each well of the MTP. The growth of cultures was monitored with scattered light (I-Io) at an excitation wavelength of 620 nm. Fluorescence emission of FPs was recorded at 540 nm (YFP) and 492 nm (FbFP). Data represent averages from four cultivations (means ± standard deviations). A.U., arbitrary units. Accumulation of YFP (C) and FbFP (D) in *E. coli* was analyzed at the indicated time points (t1 to t7, marked in panels A and B with black triangles) by immunodetection using specific antisera. All cell extracts were adjusted to the same cell density (corresponding to an $OD_{600}$ of 10). Accumulation of transcripts of YFP (E) and FbFP (F) was analyzed by RT-PCR using the same cell extracts as for panels C and D. Values are averages from three independent measurements.

via measuring of scattered light at an excitation wavelength of 620 nm, (ii) dissolved oxygen tension (DOT) using an immobilized oxygen-sensitive fluorescent dye (m2p-labs GmbH, Aachen, Germany), and (iii) FP-mediated fluorescence in a continuously shaken MTP, as described previously (11, 13, 17). YFP fluorescence was measured at an excitation wavelength of 510 nm and an emission wavelength of 540 nm, whereas FbFP fluorescence was monitored at 492 nm upon excitation at 460 nm. Background fluorescence of the control strain *E. coli* DH5α (pRhokHi-2) was used to normalize the fluorescence signals during cultivation of the YFP and FbFP expression strains (Fig. 1A and B).

Within the first 4 h of cultivation, where cells switched from the log phase to the early exponential growth phase, YFP and FbFP fluorescence as well as cell density increased simultaneously. Remarkably, development of YFP *in vivo* fluorescence (Fig. 1A) stagnated after 5 h of cultivation, although exponential growth of the expression strain was not affected. More surprisingly, after 11.5 h of cell growth, YFP fluorescence rapidly increased about 2.5-fold within the next 2 h. At this point, the culture had clearly reached the stationary growth phase. In contrast, fluorescence of FbFP increased continuously until the *E. coli* culture reached the stationary growth phase (Fig. 1B).

Analysis of dissolved oxygen tension during *E. coli* cultivation demonstrated that the high respiratory activity of the bacterial cells during the logarithmic and the early stationary growth phases led to a limitation of oxygen in both expression cultures (Fig. 1A and B). Obviously, the delayed YFP fluorescence development corresponded to the phase of low oxygen tension. Hence, we analyzed whether $O_2$ deprivation during logarithmic cell growth leads to either low YFP expression levels or inefficient chromophore maturation. To this end, we determined the amounts of accumulated transcript and protein at various representative time points during cellular growth for both YFP and FbFP (t1 to t7) (Fig. 1C to F). For the analysis of transcript and protein levels, eight 500-μl TB medium cultures were inoculated in parallel with aliquots of precultures to yield an initial cell density of 0.1 (optical density at 600 nm [$OD_{600}$]). Sampling was conducted at the given time points (t1 to t7) without interruption of the shaking movement. Subsequently, the $OD_{600}$ of the samples was determined using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA), and cells were harvested by centrifugation (14,000 rpm, 4°C, 10 min). Total RNA was isolated from aliquots of cell cultures by using an RNeasy mini kit (Qiagen, Hilden, Germany). One hundred fifty nanograms of total RNA was used for reverse transcription and subsequent real-time PCR (RT-PCR) with a QuantiTect SYBR green one-step RT-PCR kit (Qiagen, Hilden, Germany). Reverse transcription and real-

time PCR were performed using a Mastercycler epgradient S with a realplex[4] module (Eppendorf, Hamburg, Germany). For specific detection of FP transcripts, the following primer pairs were used: *YFP*-f primer, 5′-AGAAGAACGGCATCAAGGT GAACT-3′, and *YFP*-r primer, 5′-GGACTGGTAGCTCAGG TAGTGGTTG-3′; *fbfp*-f primer, 5′-TGCAGATCCAGAACT ACAAGAAGGAC-3′, and *fbfp*-r primer, 5′-TATTCCTTCT GCTTGGTGATGTCG-3′; and *aphII*-f primer, 5′-CTATCAG GACATAGCGTTGGCTACC-3′, and *aphII*-r primer, 5′-GA ACTCGTCAAGAAGGCGATAGAAG-3′. The transcript obtained from the constitutively expressed *aphII* gene was used as an internal standard and reference. For data analysis, threshold cycle ($\Delta C_T$) values of YFP and *aphII* as well as FbFP and *aphII* transcripts were calculated from a minimum of triplicate measurements. Levels of YFP and FbFP mRNA were determined by calculating $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for the respective transcripts. The $\Delta C_T$ value calculated for transcripts accumulated at t1 was referred to as the reference value for each time point (t2 to t7). Protein extracts corresponding to a final cell density of 1 ($OD_{600}$) were separated by SDS-PAGE (NuPAGE, 4 to 12% Bis-Tris gel, 1 mm; Invitrogen, Paisley, United Kingdom) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratory, München, Germany) by Western blotting (NuPAGE; Invitrogen) using standard methods. YFP and FbFP proteins were visualized using the respective antibodies (rabbit anti-GFP [BD Biosciences, Erembodegem, Belgium] and YtvA-specific antiserum). The overall changes in FP protein levels were quantified by charge-coupled-device (CCD) camera-based analysis of chemiluminescence signals obtained from YFP and FbFP immunodetection (Stella imaging system analyzed with AIDA advanced image data analyzer; Raytest, Straubenhardt, Germany). As expected, quantitative RT-PCR and Western blot analyses clearly demonstrated that both reporter genes were constitutively expressed in *E. coli* (Fig. 1C to F). The change in FP transcript and protein levels never exceeded a factor of 2 over the complete time of cultivation. These results indicated that the delay in YFP fluorescence signal development that occurred *in vivo* during the early logarithmic growth phase as well as its rapid increase during the stationary growth phase was caused predominantly by a reversible inhibition of chromophore maturation under oxygen-limited conditions.

The applicability of an FP as an *in vivo* reporter is dictated by its individual robustness within a respective test system. Thus, an ideal *in vivo* fluorescence reporter must exhibit a constant fluorescence-to-protein ratio that is independent from growth parameters and growth phases. In this regard, our comparative *in vivo* fluorescence measurements in *E. coli* revealed that FbFP but not YFP can be used as a reporter protein for accurate quantitative analyses in living cells. These findings became even more obvious when fluorescence intensities of FbFP and YFP were normalized against cell densities. Figure 2 shows that normalized FbFP fluorescence was almost constant over the entire cultivation time. In addition, the increased fluorescence intensity observed within the first 7 h of cell growth correlated with higher FbFP accumulation (Fig. 1D and F, t1 and t2). In contrast, under equivalent experimental conditions, normalized YFP fluorescence intensity showed dramatic changes

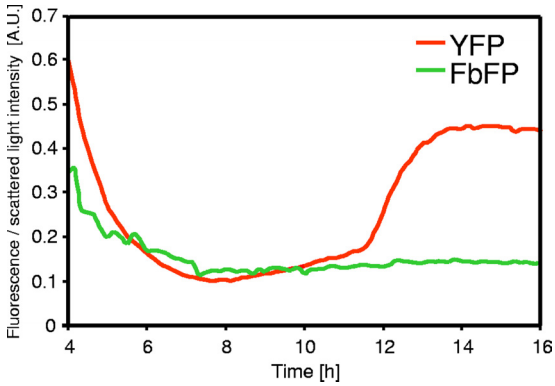


FIG. 2. Fluorescence characteristics of YFP and FbFP during cell growth. Fluorescence intensities derived from online measurements during *E. coli* cultivation (Fig. 1A and B) were normalized against the respective cell densities (scattered light intensity I-Io). A.U., arbitrary units.

over the complete period of cell growth. After a decrease of more than 6-fold during the first 8 h of cell growth, YFP fluorescence subsequently increased 2.5-fold during the late stationary growth phase. Thus, YFP accumulated at constant levels in all phases of bacterial cell growth (Fig. 1C and E), but these levels were not reflected by the fluorescence signals determined during growth. The results demonstrate that changes in normalized YFP fluorescence are caused by an impaired development of YFP signals under the respective environmental or metabolic conditions. Due to this inconsistency in YFP fluorescence intensity, quantification of the fluorescence signal is not sufficient to reliably analyze the expression of the respective reporter gene *in vivo*.

We further analyzed YFP and FbFP fluorescence in the respective *E. coli* DH5α expression strains batch cultured in Luria-Bertani (LB) medium (13) at 37°C in a 48-well MTP by using the BioLector to determine potential changes in YFP fluorescence during cell growth caused by oxygen limitation. As described previously, the cultivation of *E. coli* in unbuffered complex LB medium leads to a fast exhaustion of carbon sources and a rapid basification of the culture medium, resulting in an early inhibition of cell growth (14). As shown in Fig. 3A and B, these limiting growth conditions also caused lower respiratory activity of the *E. coli* cells, as reflected by a minimal dissolved oxygen tension of approximately 50% at the end of the logarithmic growth phase. Presumably, the development of YFP fluorescence (Fig. 3A) is not affected by oxygen depletion, as observed in TB medium (Fig. 1A). In contrast to FbFP-mediated fluorescence, which increased parallel to the cell density, analysis of YFP-mediated fluorescence revealed a remarkably delayed development of the reporter signal (Fig. 3A and B). As a direct consequence, the ratio between YFP fluorescence and cell density was, again, not constant during cell cultivation but showed a 2-fold increase in fluorescence after the bacteria reached the stationary growth phase (Fig. 3C). In contrast, FbFP-dependent fluorescence normalized against cell density exhibited an almost linear behavior over 16 h of cultivation (Fig. 3C). The maturation of GFP-like proteins is a process that includes time-consuming protein folding (2, 6) and chromophore formation (9, 10) and may thus significantly

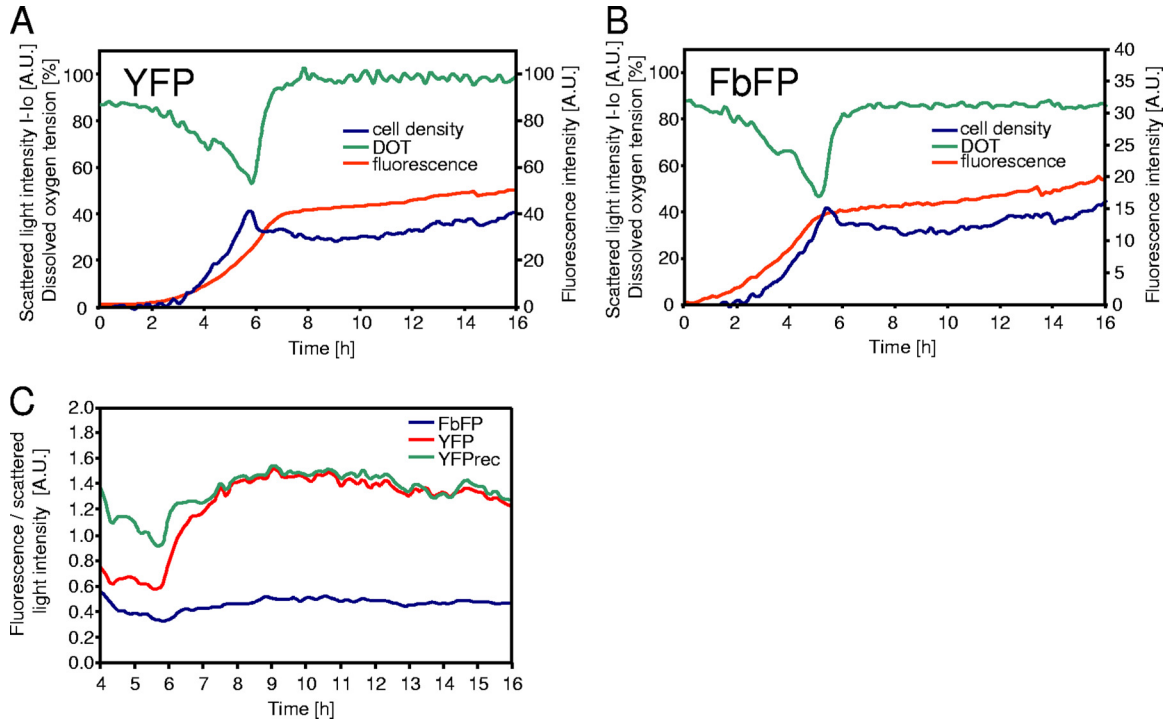


FIG. 3. Comparative expression and *in vivo* fluorescence of YFP (A) and FbFP (B) in *E. coli* DH5α cultured in LB medium. Development of biomass (cell density), dissolved oxygen tension (DOT), and FP-mediated autofluorescence were online monitored in each well of the MTP. Background fluorescence from *E. coli* DH5α (pRhokHi-2) was subtracted. Values are means from four cultivations. (C) Fluorescence characteristics of YFP and FbFP during cell growth in LB medium. Fluorescence intensities derived from online measurements during *E. coli* cultivation (A and B) were normalized against the respective cell densities (scattered light intensity I-Io). In addition, YFP fluorescence intensity was recalculated ($YFP_{rec}$) assuming a maturation half time of 1 h. A.U., arbitrary units.

delay the development of fluorescence signals. Calculation of the YFP fluorescence signals while assuming an average YFP maturation time of 1 h, as indicated by the respective online fluorescence measurements (Fig. 3A), revealed that fluctuations of normalized YFP fluorescence observed in the logarithmic growth phase were partially reduced (Fig. 3C, $YFP_{rec}$). Therefore, these results further support our initial assumption that the use of GFP derivatives as *in vivo* real-time reporters is also limited by time-demanding maturation.

In this article, we describe the performances of two different fluorescent proteins when used as real-time *in vivo* reporters in a microbioreactor system. We demonstrated that FbFP, in contrast to YFP, is insensitive toward variable levels of dissolved oxygen during cell cultivation and that its fluorescence signal develops immediately after gene expression. Fluorescence signals generated by FbFP rather than GFP-related FPs thus reflect *in vivo* gene expression and protein production levels. Their unique properties make FbFPs superior tools for systems and synthetic biology as well as for various biotechnological approaches.

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