doi:10.1016/j.jmb.2012.01.001 J. Mol. Biol. (2012) 416, 534–542

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

From Dusk till Dawn: One-Plasmid Systems for Light-Regulated Gene Expression

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Received 25 October 2011; received in revised form 29 December 2011; accepted 1 January 2012
Available online 8 January 2012

Edited by M. Gottesman

Keywords:
gene expression; light-oxygen-voltage; optogenetics; photoreceptor; protein engineering

Signaling photoreceptors mediate diverse organismal adaptations in response to light. As light-gated protein switches, signaling photoreceptors provide the basis for optogenetics, a term that refers to the control of organismal physiology and behavior by light. We establish as novel optogenetic tools the plasmids pDusk and pDawn, which employ blue-light photoreceptors to confer light-repressed or light-induced gene expression in *Escherichia coli* with up to 460-fold induction upon illumination. Key features of these systems are low background activity, high dynamic range, spatial control on the 20-μm scale, independence from exogenous factors, and ease of use. In optogenetic experiments, pDusk and pDawn can be used to specifically perturb individual nodes of signaling networks and interrogate their role. On the preparative scale, pDawn can induce by light the production of recombinant proteins and thus represents a cost-effective and readily automated alternative to conventional induction systems.

Introduction

In nature, sensory, signaling photoreceptors enable organisms to detect light and to adjust their physiology and behavior in response.[1–3] Prominent and well-studied examples include phototropism in higher plants,[4,5] phototactic and photophobic responses in unicellular algae,[6] and entrainment of the circadian clock in *Drosophila*.[7] Sensory photoreceptors fall into six classes with distinct photochemistry: xanthopsins, light-oxygen-voltage proteins, sensors of blue light using flavin adenine dinucleotide, cryptochromes, rhodopsins, and phytochromes.[1–3] At the molecular level, light absorption leads to a change in the biological activity of sensory photoreceptors; thus, *per definitionem* all photoreceptors are light-gated protein switches. This favorable property underpins the novel field of optogenetics.[8,9] Via heterologous expression of specific photoreceptors, desired processes such as enzymatic or transcriptional activity in target cells or organisms can be placed under light control with unprecedented spatiotemporal precision, full reversibility and in a noninvasive manner. To date, optogenetic applications have been almost exclusively based on the use of a few naturally occurring photoreceptors, particularly the light-gated ion channel channelrhodopsin,[6,10] the light-driven

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Abbreviations used: TCS, two-component system; MCS, multiple-cloning site; a.u., arbitrary units; fwhm, full width at half maximum; CAT, chloramphenicol acetyl transferase.

chloride pump halorhodopsin,[11] and light-activated adenyl cyclases.[12–15] Recently, engineered photoreceptors that extend the applicability of optogenetics beyond the limits of natural photoreceptors have been developed.[16]

Of particular interest are photoreceptors that regulate gene expression by light, for example, those from higher plants.[17] Although biotechnological applications of these and related photoreceptors were originally restricted to the native promoters and organisms, several more widely applicable photoreceptor systems for light-regulated gene expression have been engineered in the last decade.[18–23] In particular, we designed the histidine kinase YF1,[21] which employs a light-oxygen-voltage[4,5] blue-light photosensor domain. In the absence of blue light, YF1 phosphorylates its cognate response regulator FixJ, which then drives robust gene expression from the FixK2 promoter. Upon light absorption, net kinase activity of YF1 and consequently gene expression are greatly reduced.[21] However, all current systems for light-regulated gene expression suffer from limitations that may impede their widespread use: dependence on nonnative chromophores that must be supplied exogenously[19] or by introduction of appropriate cofactor-synthesis genes,[20,23] a decrease (rather than an increase) in gene expression by light absorption,[20,21] high background activity,[20,22] low dynamic range,[20,22,23] and limited portability since multiple plasmid components are required.[19–23] We address these limitations in the portable one-plasmid systems pDusk and pDawn, which are based on the YF1/FixJ system and enable light-repressed or light-activated gene expression in *Escherichia coli*. pDusk and pDawn are easy to implement in the laboratory and are suited to both preparative and analytic purposes.

Results

We previously demonstrated that the engineered light-responsive two-component system (TCS) YF1/FixJ can induce gene expression by up to 70-fold under dark relative to blue-light conditions.[21] However, broader application of the YF1/FixJ TCS has so far been hindered by its implementation on two separate and relatively large plasmids and by its expression from the strong inducible T7-lacO promoter. In addition, gene expression is repressed upon blue-light absorption, while certain biotechnological and optogenetic applications may require that gene expression is increased rather than decreased by light. We sought to address these points and constructed the plasmids pDusk and pDawn that offer light-repressed and light-induced gene expression, respectively. As shown in Fig. 1, all components of the YF1/FixJ TCS were assembled on the single medium-copy plasmid pDusk in which YF1 and FixJ are constitutively expressed from the $LacI^q$ promoter[24] as a bicistronic operon. Target genes can be introduced in a single step via a multiple-cloning site (MCS) downstream of the pFixK2 promoter, and their expression is put under light control. To enable quantitative analysis, we cloned the red-fluorescent reporter protein DsRed Express2 (in the following, simply referred to as DsRed)[25] into the MCS to yield pDusk-DsRed in which expression could be measured by red fluorescence (Supplementary Table 1). *E. coli* cultures carrying pDusk-DsRed were grown for 20 h at 37 °C in the dark or under constant blue light (470 nm) of intensities between 1 and 100 nW cm^{-2} to determine the effect of light dose on expression. DsRed expression decreased with increasing blue-light intensities in a hyperbolic manner by a factor of 12 ± 1 and with a half-maximal light dose (ED_{50}) of 1.7 ± 0.5 nW cm^{-2} (Fig. 2a). Note that this is 5.8-fold less than the induction factor of ~70 we obtained previously for the YF1/FixJ TCS under rather different conditions in which a different reporter gene, a different *E. coli* strain, and strong overexpression conditions were used.[21]

We then set out to invert the signal polarity of light-regulated gene expression; that is, we sought a system in which gene expression is increased by light instead of being repressed by light. Following pioneering work by Elowitz and Leibler,[26] by Gardner *et al.*,[27] and later studies by Tabor *et al.*,[28] we achieved this inversion of signal polarity by insertion of a gene-inversion cassette into pDusk to obtain pDawn (Fig. 1). In pDawn, the YF1/FixJ TCS drives expression of the λ phage repressor cI from

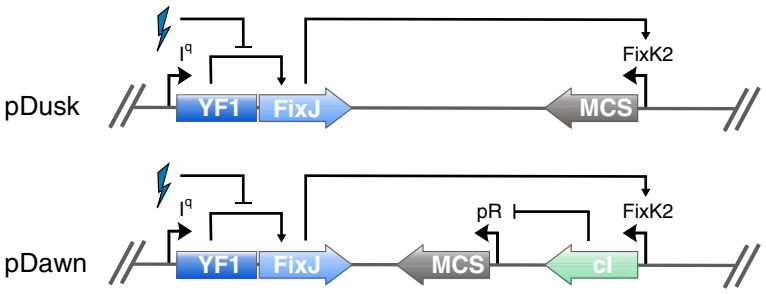

Fig. 1. Plasmids pDusk for light-repressed and pDawn for light-activated gene expression in *E. coli*. In pDusk, the engineered TCS YF1/FixJ drives gene expression from the pFixK2 promoter in a blue-light-repressed fashion. Insertion of the λ phage repressor cI and the λ promoter pR in pDawn inverts signal polarity and renders gene expression light-activated.

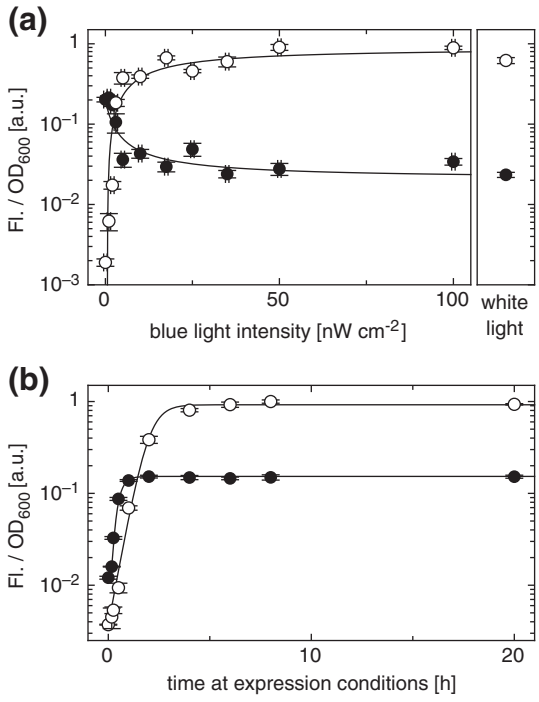

Fig. 2. (a) DsRed expression from pDusk (●) decreases hyperbolically with increasing intensity between 0 and 100 nW cm^{-2} of 470-nm light; DsRed expression from pDawn (○) increases under the same conditions. Illumination with white light leads to similar expression levels as under blue light in pDusk and pDawn (right panel). Data were obtained on multiple days and are averages of 5 replicates ± standard deviation; lines denote fits to hyperbolic functions. (b) DsRed expression from pDusk (●) and pDawn (○) increases hyperbolically with time spent under inducing conditions. Data were obtained in a single experiment and are averages of 3 replicates ± standard deviation; lines denote fits to hyperbolic functions.

the pFixK2 promoter, which, in turn, represses expression from the strong λ promoter pR.[29] Again using DsRed as the reporter, gene expression is greatly enhanced with increasing blue-light intensity by a factor of 460 ± 20 and with an ED_{50} of 12 ± 3 nW cm^{-2}. That is, introduction of the inversion cassette not only inverted but also greatly amplified the effects of blue light. As λ pR is a very strong promoter, expression levels under inducing conditions are higher in pDawn than in pDusk; conversely, expression levels under non-inducing conditions are lower in pDawn than in pDusk since λ cI is a very strong repressor. A conventional white light source can replace the blue light source with similar effects on gene expression from pDusk and pDawn (Fig. 2a); dedicated lamps or lasers are thus not necessary.

In another experiment, we assessed whether intermediate expression levels can be achieved in pDusk and pDawn by variation of the duration of blue-light illumination. To this end, we grew cultures carrying pDusk-DsRed or pDawn-DsRed for varying times under inducing conditions, that is, dark for pDusk or 100 nW cm^{-2} blue light for pDawn, and then transferred them to non-inducing conditions, that is, 100 nW cm^{-2} blue light for pDusk or dark for pDawn for a total of 20 h. To allow DsRed maturation, we then incubated cultures under non-inducing conditions prior to fluorescence measurements (Fig. 2b). The fluorescence data indicate that expression from pDusk and pDawn saturates as a function of time spent under inducing conditions with half-maximal times (t_{50}) of 0.51 ± 0.02 h and 2.3 ± 0.1 h, respectively. Thus, expression levels controlled by pDusk or pDawn can be continuously adjusted by either light intensity (Fig. 2a) or duration of illumination (Fig. 2b).

The above measurements report on the ensemble average of fluorescence and thus do not identify whether cell populations carrying pDusk or pDawn respond homogeneously to illumination and if not, the nature of any heterogeneity. To address this point, we used flow cytometry to assess at the single-cell level the performance of pDusk and pDawn. Consistent with the ensemble measurements, the mean fluorescence for pDusk-DsRed clones is 19 times higher in the dark [mean, 1.1×10^4 arbitrary units (a.u.); full width at half maximum (fwhm), 1.2×10^3 a.u.] than under 100 nW cm^{-2} blue light (mean, 5.8×10^2 a.u.; fwhm, 1.1×10^2 a.u.) (Fig. 3a); mean fluorescence for pDawn-DsRed is 460 times higher under 100 nW cm^{-2} blue light (mean, 7.0×10^4 a.u.; fwhm, 6.7×10^3 a.u.) than in the dark (mean, 1.5×10^2 a.u.; fwhm, 4.3×10^1 a.u.) (Fig. 3b). Notably, blue light switches both pDusk and pDawn in an all-or-none fashion with little or no overlap in the extent of single-cell fluorescence in the dark and under 100 nW cm^{-2} blue light. In particular, our data reveal that there are essentially no unresponsive cells; at the population level, light switching of gene expression thus occurs in a strictly concerted manner. At a non-saturating blue-light intensity of 2 nW cm^{-2}, the distributions uniformly shift to intermediate fluorescence values for both pDusk (mean, 2.8×10^3 a.u.; fwhm, 3.7×10^2 a.u.) and pDawn (mean, 3.2×10^4 a.u.; fwhm, 2.8×10^3 a.u.).

In subsequent experiments, we focused on pDawn because of its high induction ratio and favorable signal polarity. For practical applications of pDawn, it is important to know how fast the system reacts to illumination. To analyze the response dynamics of gene expression, we grew cultures harboring pDawn-DsRed in the dark to an OD_{600} (optical density at 600 nm) of ~0.4 before transferring them to inducing conditions (100 nW cm^{-2} blue light). At different times, samples were taken, cell division and protein expression were stopped by antibiotic addition, and DsRed was allowed to mature.

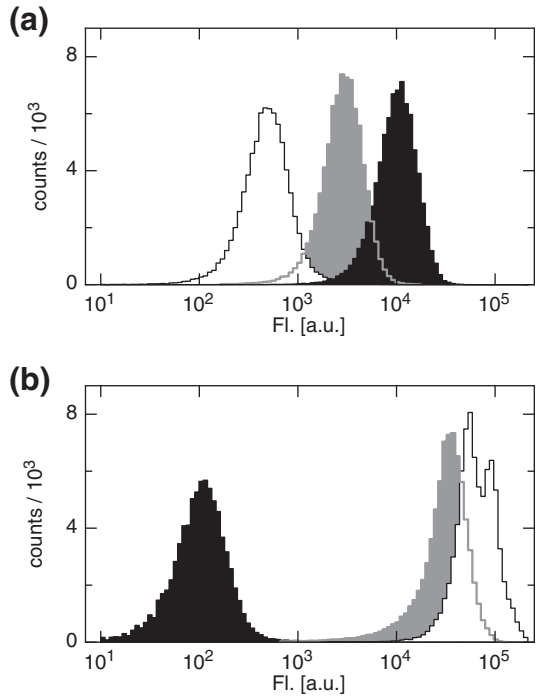

Fig. 3. (a) Flow cytometry of *E. coli* cells harboring pDusk-DsRed grown in the dark (black) or under 2 nW cm^{-2} (gray) or 100 nW cm^{-2} blue light (white). A total of 10^5 cells were analyzed for each population; data were obtained on multiple days. (b) Flow cytometry of *E. coli* cells harboring pDawn-DsRed grown in the dark (black) or under 2 nW cm^{-2} (gray) or 100 nW cm^{-2} blue light (white). A total of 10^5 cells were analyzed for each population; data were obtained on multiple days.

Fluorescence indicative of DsRed expression increased with a lag time of 2.0 ± 0.1 h after light exposure (Fig. 4). While this lag time largely precludes the study of kinetic processes on shorter timescales, pDawn is suitable for effecting pronounced changes in gene expression on the timescale of a few hours, even at low light intensities (Fig. 2a).

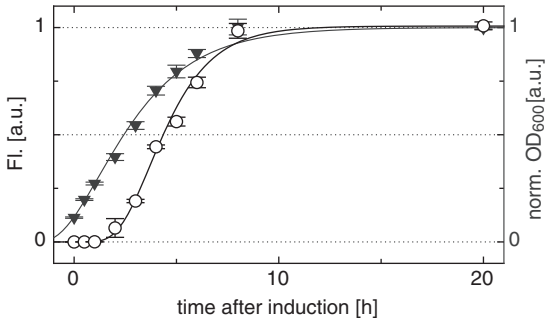

Fig. 4. Delay between cell growth (▼) and onset of protein expression from pDawn-DsRed upon blue-light induction as monitored by fluorescence (○). After incubation under 100 nW cm^{-2} blue light for the indicated times, cell division and protein translation were inhibited by antibiotic addition. Fluorescence was measured for DsRed maturation. Data were obtained in a single experiment and are averages of 3 replicates ± standard deviation; lines denote fits to logistic functions.

Due to its low background activity and high induction ratio, pDawn is well suited to stringent control by light of the expression of arbitrary target genes, which we demonstrate for the antibiotic resistance marker chloramphenicol acetyl transferase (CAT) (Fig. 5). Under dark, non-inducing conditions, growth of *E. coli* cultures harboring pDawn-CAT is delayed or prevented altogether in the presence of 40–150 μg mL^{-1} chloramphenicol. Incubation in constant blue light (100 nW cm^{-2}) induces CAT expression and facilitates growth even in the presence of high concentrations of the antibiotic.

Encouraged by the large light effect obtained for pDawn-DsRed, we investigated whether pDawn can be used on a preparative scale for expression of

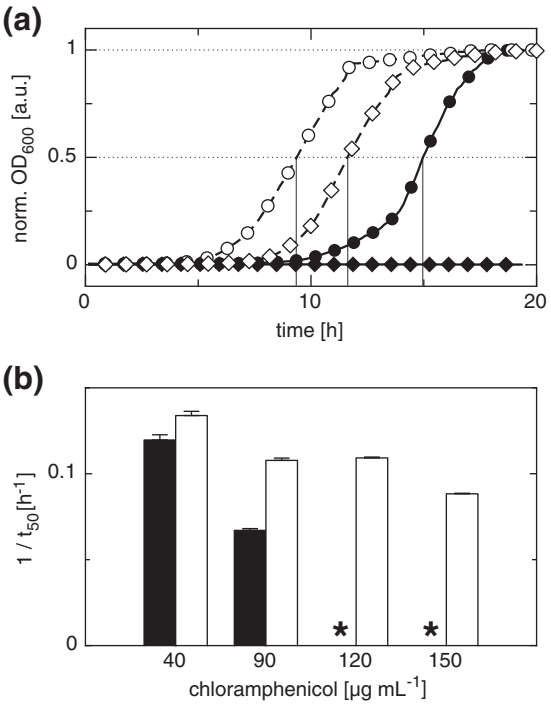

Fig. 5. (a) Growth of *E. coli* cultures bearing pDawn-CAT is delayed at 90 μg mL^{-1} chloramphenicol (●) or prevented altogether at 150 μg mL^{-1} (♦). Light-induced expression of CAT enhances growth in the presence of 90 μg mL^{-1} (○) and 150 μg mL^{-1} (◊) of the antibiotic. Data were obtained on multiple days and are averages of 5 replicates ± standard deviation; lines denote fits to logistic functions. Vertical lines indicate t_{50} values for the growth curves. (b) Times of half-maximal growth (t_{50}) in pDawn-CAT cultures at varying concentrations of chloramphenicol in the dark (black) or under constant blue light (white). Asterisks denote no detectable growth over a 20-h incubation period.

recombinant proteins. For this purpose, 500-mL cultures of *E. coli* carrying either pDawn-DsRed or pDawn-CAT were grown in the dark to an OD_{600} of ~0.4 at which point expression was induced by constant blue light (100 nW cm^{-2}) for ~18 h. After cell harvest, DsRed and CAT were purified by metal-ion affinity chromatography with yields of 40 and 20 mg and purities of 75±15% and ≥95%, respectively (Table 1). For comparison, we also expressed DsRed and CAT from the T7-lacO promoter of the widely used pET vector system[30] following induction with isopropyl-β,D-1-thiogalactopyranoside (IPTG), which produced similar yields and purities. In pET-28c, the onset of protein expression after induction (lag, 1.8±0.1 h) was comparable although the basal activity was 1.7-fold higher and the induction ratio of 160±20 was lower than that in pDawn (Supplementary Fig. 1; Table 1). However, introduction of the pLysS plasmid[30] improves the induction ratio of the pET system to 1100±300 by repressing basal and maximal T7 promoter activity 12-fold and 1.8-fold, respectively.

A specific advantage of light-induced systems over chemically induced systems is the superior degree of spatial control they afford.[20,22,23] We grew bacteria harboring pDawn-DsRed on an agar slab and used an epi-fluorescence microscope to expose a limited area (radius ~12 μm) to blue light for 100 ms every 10 min. Within 3 h, DsRed expression could be detected within the illuminated area; after 5 h, DsRed expression within the illuminated area was up-regulated by a factor of 55±10 relative to the surroundings (Fig. 6), thus establishing the utility of pDawn for spatially resolved studies of bacterial communities on the micrometer length scale. Similarly, DsRed expression from pDusk-DsRed could be down-regulated in illuminated areas by a factor of 15±5 (not shown). Although direct comparison to related bacterial gene-expression systems is difficult due to use of different reporter genes and lack of quantitative data,[20,23] the contrast ratios we obtain with pDusk and (especially) pDawn appear to be considerably higher than in these systems.

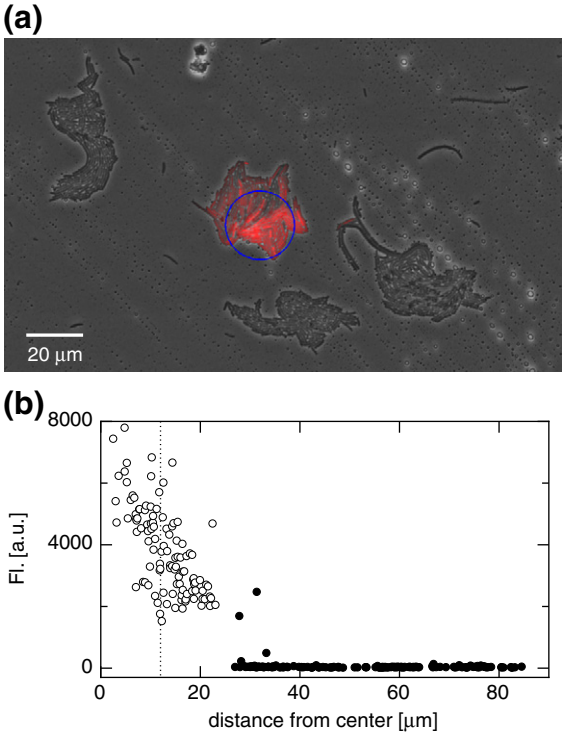

Fig. 6. (a) Overlay of phase-contrast and DsRed fluorescence microscopy images shows spatial control over blue-light-induced gene expression from pDawn-DsRed. The blue circle denotes the area in which blue-light pulses of 100-ms duration were applied every 10 min during a 5-h incubation period at 37 °C. Each of the four visible micro-colonies derives from single cells. (b) Mean fluorescence of single cells within the illuminated micro-colony (center, ○) and within non-illuminated micro-colonies (●) as a function of their distance from the center of the illuminated area. The vertical broken line denotes the radius of the illuminated area.

Table 1. Protein expression from pDawn and pET-28c

Protein	CAT		DsRed			
Plasmid	Yield (mg)[a]	Purity (%)[b]	Yield (mg)[a]	Purity (%)[b]	Induction ratio[c]	Induction delay (h)[d]
pDawn	19	≥95	40	75±15	460±20	2.0±0.1
pET-28c	32	≥95	20	75±15	160±20 (1100±300)[e]	1.8±0.1

[a] From 500 mL of culture.
[b] After affinity purification, as estimated by gel electrophoresis.
[c] Ratio between inducing and non-inducing conditions, measured by DsRed fluorescence per OD_{600}.
[d] Delay between induction and protein expression.
[e] Data obtained in the presence of the pLysS plasmid.

Discussion

The portable one-plasmid systems pDusk and pDawn offer efficient light-regulated gene expression in *E. coli*. Both plasmids can easily be implemented in the laboratory without any need for specialized equipment, and a single cloning step suffices to put expression of target proteins under the control of light. The underlying photoreceptor YF1 autonomously incorporates its ubiquitously available flavin-mononucleotide chromophore; thus, addition of auxiliary enzymes or cofactors is not required. As the performance of the light-inducible pDawn rivals that of the widely used pET system, pDawn can be used on a preparative scale for production of recombinant proteins. In

comparison to induction by chemical means, for example, by IPTG, light induction is noninvasive, which reduces the risk of contamination, and cost-effective. Moreover, pDawn readily lends itself to automation: protein expression could be initiated at certain set points and adjusted by variation of time and intensity of illumination. In this manner, production yield and purity could be optimized, which is crucial in many areas including biotechnology and structural biology.[31] Considering all these properties, we believe that pDawn will complement and, perhaps in certain cases, supersede conventional systems for induction of protein expression.

pDusk and pDawn could become important tools in optogenetics research where they facilitate applications that demand high spatial resolution. As evidenced by significantly higher dynamic range and lower background expression, pDawn outperforms related systems such as the red- and green-light-sensitive gene circuits developed for "bacterial photography".[20,23] In addition, these plasmids do not require any additional components such as genes that encode enzymes for chromophore synthesis[20,23] or incorporation. They are thus well suited to the study of metabolic and signaling networks. pDawn could be used to accurately perturb arbitrary nodes of such networks via light-induced expression, and the response could be studied at the systems level. Since pDusk and pDawn can be switched by light in a concerted manner and afford spatial control on the 20-μm scale, they are also well suited to the study of spatial and temporal aspects of the behavior of microbial communities. However, gene expression is an inherently slow process, and our plasmids only allow light-induced perturbations on the timescale of hours. For higher temporal resolution, signaling networks may be perturbed at the level of the gene products, for example, via light-activated protein dimerization.[22,32,33]

The present study adds to a growing number of examples that demonstrate the potential of engineered photoreceptors for optogenetics.[16] However, it is noteworthy that despite their indisputable potential, no single engineered photoreceptor[16,19–23,32–36] has found broad deployment in optogenetics to date. Arguably, the main factors that have impeded their wider adoption are high background activity, low dynamic range, and difficulty in implementation. The properties of even the most impressive representatives such as the blue-light-regulated GTPase PA-Rac1[35] fall short of those of certain natural, evolutionarily highly optimized photoreceptors. A key challenge for designers of effective, engineered photoreceptors is therefore to produce photoreceptors that display the dynamic range and low background activity necessary for robust application *in vivo*. Along with recent reports,[37,38] our present work demonstrates one way to accomplish this goal. Incorporation of both natural and engineered photoreceptors into synthetic genetic circuits can potentiate the degree and the specificity of light control over physiology and behavior at the level of the whole organism.

Materials and Methods

Construction of pDusk and pDawn

The plasmids pDusk and pDawn derive their backbone from the widely used pET system to ensure compatibility with existing protocols and promote ease of use.[30] The plasmid pDusk was derived from the pET-28c vector (Novagen, Madison, WI, USA) by excising the T7-lacO promoter region with BglII and XbaI and replacing it with the promoter region of the *Bradyrhizobium japonicum* FixK2 protein (locus bll2757), namely, nucleotides −247 to −1 relative to the translation start site. Two MfeI sites were introduced by site-directed mutagenesis immediately upstream and downstream, respectively, of the LacI open reading frame, to enable replacement of LacI by the YF1/FixJ operon.[21] The YF1/FixJ operon replicates the gene structure of the parent *B. japonicum* FixL/FixJ operon (bll2760 and bll2759): the genes encoding YF1 and FixJ are separated by three nucleotides and are expressed bicistronically. For further optimization, the fluorescent reporter gene DsRed Express2 (GenBank FJ226077)[25] was cloned under the control of the pFixK2 promoter using NdeI and HindIII. (For simplicity, we refer to the reporter gene as DsRed henceforth.) To improve expression levels of YF1/FixJ and hence of DsRed, we introduced the I^q mutation by site-directed mutagenesis into the LacI promoter, which increases gene expression of YF1/FixJ about 10-fold.[24] Further improvement of DsRed expression was achieved by adjusting the relative expression levels of YF1 and FixJ via introduction of a ribosome-binding site between YF1 and FixJ. At this stage, we identified a spontaneous mutation, denoted R1, which led to substantially higher levels of DsRed expression. DNA sequencing revealed a two-nucleotide deletion at the 3′ end of the YF1 gene. Subsequently, DsRed was excised and the original MCS was restored. Removal of surplus restriction sites within the pFixK2 promoter, the YF1 gene, and the FixJ gene by site-directed mutagenesis yielded the plasmid pDusk.

The plasmid pDawn was directly derived from pDusk by inserting a gene-inversion cassette via the XbaI site. The gene-inversion cassette was chemically synthesized (Mr. Gene, Regensburg, Germany) and corresponds to the BioBrick BBa_Q04510, which derives from the work of Elowitz and Leibler[26] and Tabor *et al.*[28] Briefly, insertion of the gene-inversion cassette puts the λ repressor cI under control of the pFixK2 promoter and places the λ promoter pR immediately upstream of the MCS.[29] The λ repressor cI carries a C-terminal LVA tag, which greatly decreases its intracellular lifetime, to improve the response dynamics of the inverted system.[39]

Nucleotide sequences for pDusk and pDawn have been deposited in the GenBank database under accession numbers JN579120 and JN579121.

Cell growth and fluorescence measurements

Unless stated otherwise, all experiments were carried out in the *E. coli* BL21 strain CmpX13,[40] in LB medium plus 50 μg mL^{-1} kanamycin, at 225 rpm and 37 °C in Innova 44R incubator shakers (New Brunswick Eppendorf, Hamburg, Germany). Blue light was applied via a custom-built array of 10×16 light-emitting diodes (470 nm, WEHBL01-DM, Winger Electronics GmbH & Co. KG, Dessau, Germany). Light intensities were measured with a power meter (model 842-PE, Newport, Darmstadt, Germany) using a silicon photodetector (model 918D-UV-OD3, Newport). White light was applied via the built-in photosynthetic light bank of the incubator shaker (GRO-LUX F15T8/GRO/AQ/RP, OSRAM Sylvania, Westfield, IN, 15 W, ~3700 lx). The intensity of the white light in the blue spectral region at 450±10 nm, obtained by inserting an interference filter (FB450-10, Thorlabs GmbH, Dachau, Germany), was measured at 13 nW cm^{-2}. Dark conditions were achieved by covering the windows of the incubator shaker with aluminum foil.

To determine the dependence of gene expression on light intensity in pDusk-DsRed and pDawn-DsRed (cf., Fig. 2a), we grew 5-mL starter cultures to an OD_{600} of ~0.4 under non-inducing conditions (i.e., at 100 nW cm^{-2}, 470-nm light for pDusk-DsRed or in the dark for pDawn-DsRed). Fifty microliters of these cultures was used to inoculate 5-mL cultures, which were then grown for 17 h under varying lighting conditions. Control experiments showed that under these conditions, cultures have reached the stationary phase and DsRed has fully matured.[25] Samples were then diluted 50-fold into LB, and cell growth was arrested by addition of 3.3 mg mL^{-1} chloramphenicol and 0.4 mg mL^{-1} tetracyclin. OD_{600} and DsRed fluorescence were measured with a Tecan M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) in 96-well μClear plates (Greiner BioOne, Frickenhausen, Germany). For fluorescence measurements, excitation and emission wavelengths of 554±9 nm and 591±20 nm were used, respectively. Each data point represents the average of 5 replicates±standard deviation.

To examine the influence of illumination time on gene expression (cf., Fig. 2b), we first grew 200-mL cultures of pDusk-DsRed and pDawn-DsRed under non-inducing conditions (cf., above) to an OD_{600} of ~0.4 and then transferred them to inducing conditions. Five-milliliter aliquots were taken after 0 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, or 20 h; returned to non-inducing conditions; and grown for a total of 20 h. OD_{600} and DsRed fluorescence were determined as above; data points are averages of 3 replicates±standard deviation. The data were fitted using the Levenberg–Marquardt nonlinear least-squares algorithm of proFit (QuantumSoft, Uetikon am See, Switzerland) to determine half-maximal light doses (ED_{50}) and illumination times (t_{50}). Reported confidence intervals are ±asymptotic standard errors.

To determine the induction kinetics of gene expression in pDawn-DsRed (cf., Fig. 4), we grew 100-mL cultures in triplicate in the dark to an OD_{600} of ~0.4 and then transferred them to constant blue light (470 nm, 100 nW cm^{-2}) for 20 h. Samples were taken after 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, or 20 h, and cell growth was stopped by antibiotic addition (cf., above). Prior to measurement of fluorescence and optical density, each sample was kept on ice for ≥2 h to allow for full maturation of DsRed.[25] Data points are averages of 3 replicates±standard deviation. Time courses of OD_{600} and DsRed fluorescence were fitted to generalized logistic functions:

$$f(t) = A + \frac{C}{\left\{1 + \left(2^T - 1\right)\exp\left[-B(t - t_{50})\right]\right\}^{1/T}}$$

where t_{50} denotes the time at which growth has occurred to half-maximal extent; that is, $f(t_{50})=A+C/2$. The induction lag time was calculated as the difference between the values of t_{50} obtained for the time courses of OD_{600} and DsRed fluorescence. In the case of pET-28c-DsRed, induction was started by addition of 1 mM IPTG; measurements and analysis were carried out in corresponding fashion.

Flow cytometry

Cultures of pDusk-DsRed and pDawn-DsRed were grown in the dark or under constant blue light (470 nm, 2 and 100 nW cm^{-2}, respectively) for 20 h. Per construct (pDusk or pDawn) and condition (dark, 2 or 100 nW cm^{-2} blue light), 10^5 cells were sorted on a BD FACS Aria II cell sorter (BD, Franklin Lakes, NJ, USA) based on their DsRed fluorescence (excitation, 561 nm; emission, 585±15 nm). Sensitivity was left unchanged between measurements to allow direct comparison between constructs and conditions. Signals arising from viable cells were discriminated from those originating from aggregates and debris on the basis of forward and sideward scattering signals using FlowJo (Tree Star Inc., Ashland, OR, USA). Data were binned and analyzed using proFit. Fwhm was determined by fitting single-cell fluorescence distributions to log-normal functions.

Chloramphenicol survival assay

The resistance marker CAT was cloned into pDawn using NdeI and HindIII to yield pDawn-CAT. A 5-mL starter culture of pDawn-CAT was grown in the dark (i.e., under non-inducing conditions) to an OD_{600} of ~0.4 and used to inoculate 200-μL cultures containing 40, 90, 120, or 150 μg mL^{-1} chloramphenicol. Cultures were grown in 96-well μClear plates at 37 °C in a Tecan M200 plate reader (4 mm orbital shaking, 37 rpm), and OD_{600} was measured in 10-min intervals. For measurements under light conditions, cultures were illuminated for 30 s (470 nm, 100 nW cm^{-2}) every 10 min. Growth kinetics were fitted to generalized logistic functions using proFit as detailed above; t_{50} values (Fig. 5) represent averages of 5 replicates±standard deviation.

Epi-fluorescence microscopy

Cells carrying pDawn-DsRed were grown overnight (~14 h) in the dark in TB medium plus 50 μg mL^{-1} kanamycin at 37 °C. After dilution to an OD_{600} of ~0.05 and growth for 2 h, 1 μL of the cell suspension was put on an agar slab made of TB and grown on top of a 100× lens of an inverted epi-fluorescence microscope (Nikon TiE, Nikon, Japan) in an incubated chamber at 37 °C. Using an

automated time-lapse protocol, cells were illuminated by blue light every 10 min for 100 ms. Blue light at 436 ± 10 nm was obtained by passing light from a xenon lamp (LambdaXL, Sutter, Novato, USA) through a CFP excitation filter and focused to an area of ~12 μm radius. Every half hour, a 3 × 3 field of fluorescence images of DsRed expression was recorded through appropriate filters (excitation, 560 ± 20 nm; emission, 630 ± 37 nm). DsRed fluorescence develops to its highest level within the illuminated area. During cell division, some cells are displaced from the center of illumination; intermediate DsRed fluorescence levels are observed in these cells. For quantitative analysis (Fig. 6b), the fluorescence images were corrected for inhomogeneous illumination, which amounted to less than 15% within each image. Using phase-contrast microscope images for segmentation, we calculated mean DsRed fluorescence levels per cell. Background fluorescence as determined in cells lacking the pDawn-DsRed plasmid was subtracted. Dynamic range is calculated as the ratio between the mean DsRed fluorescence of cells within the illuminated micro-colony in the center of the image and the mean DsRed fluorescence of cells within micro-colonies outside the illuminated area.

Protein purification

Purification of DsRed and CAT followed a standard purification protocol.[41] Briefly, 500-mL cultures of pDawn-DsRed and pDawn-CAT were grown in 4-L baffled Erlenmeyer flasks at 37 °C to an OD_{600} of ~0.4, and expression was induced by application of 470-nm light (100 nW cm^{-2}); temperature was lowered to 16 °C. After overnight growth (~18 h), cells were harvested and lysed by sonication, and the cleared lysate was purified by metal-ion affinity chromatography using HisPur Cobalt resin (Thermo Scientific, Rockford, IL, USA). His_6-tagged DsRed or CAT was eluted with 200 mM imidazole, and samples were analyzed via polyacrylamide gel electrophoresis. Fractions of high protein concentration and purity were pooled and dialyzed against 10 mM Tris/HCl (pH 8.0) and 10 mM NaCl. Protein concentrations were determined by absorption spectroscopy with extinction coefficients of 35,600 M^{-1} cm^{-1} at 591 nm for DsRed and 43,100 M^{-1} cm^{-1} at 280 nm for CAT. Protein purity was estimated by gel electrophoresis. In control experiments, CAT and DsRed were expressed from the pET-28c plasmid, and gene expression was induced by addition of 1 mM IPTG instead of blue light. All other steps were as described for the pDawn system.

Acknowledgements

We thank Drs. Rebecca Ayers, Sean Crosson, and members of the Möglich laboratory for discussion; Drs. Sean Crosson, Rita Strack, and Robert Keenan for materials; the workshop at Humboldt-Universität zu Berlin for support; and Ms. Christiane Müller for assistance with flow cytometry. Research was supported by National Institutes of Health grant GM036452 (to K.M.) and a Sofja Kovalevskaya Award (to A.M.), which is sponsored by the Alexander von Humboldt Foundation and funded by the German Federal Ministry for Education and Research.

Author Contributions. A.M. and K.M. conceived the project; A.M. designed pDusk and pDawn; R.O. and A.M. planned, performed and evaluated experiments; R.V. and A.E. contributed fluorescence-microscopy data; R.O., K.M. and A.M. wrote the manuscript.

Supplementary Data

Supplementary data to this article can be found online at doi:10.1016/j.jmb.2012.01.001

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