

Light-Inducible Recombinases for Bacterial Optogenetics

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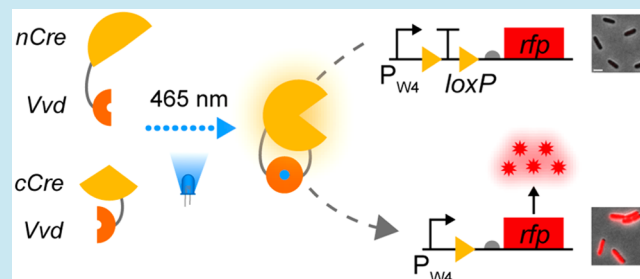


Supporting Information

ABSTRACT: Optogenetic tools can provide direct and programmable control of gene expression. Light-inducible recombinases, in particular, offer a powerful method for achieving precise spatiotemporal control of DNA modification. However, to-date this technology has been largely limited to eukaryotic systems. Here, we develop optogenetic recombinases for *Escherichia coli* that activate in response to blue light. Our approach uses a split recombinase coupled with photodimers, where blue light brings the split protein together to form a functional recombinase. We tested both Cre and FLP recombinases, Vivid and Magnet photodimers, and alternative protein split sites in our analysis.

The optimal configuration, Opto-Cre-Vvd, exhibits strong blue light-responsive excision and low ambient light sensitivity. For this system we characterize the effect of light intensity and the temporal dynamics of light-induced recombination. These tools expand the microbial optogenetic toolbox, offering the potential for precise control of DNA excision with light-inducible recombinases in bacteria.

KEYWORDS: optogenetics, recombinase, photoactivation, inducible recombinase, Cre



Optogenetic tools enable novel applications for synthetic biology.^{1–5} These tools typically use light to control expression of genes, often relying on light-dependent changes in protein state to control protein–protein interactions,^{6,7} promoter systems,^{8,9} and ion channels.¹⁰ Optogenetic systems offer many advantages over traditional chemical approaches for controlling gene expression due to the direct and programmable nature of light as an input. Using light instead of small molecules can give precise spatiotemporal control over regulation, and can circumvent the need to change media or otherwise disrupt the system to add or remove a chemical inducer. As light is easily programmable using electronics, optogenetic tools can also interface with dynamic computer-based control and feedback.^{11,12}

Microbial optogenetic approaches have revealed a myriad of new applications that take advantage of the precise, programmable nature of light. As examples, light has been used to control expression of enzymes involved in biofuel synthesis^{13,14} and to regulate bacterial growth *via* metabolic control.^{15,16} In addition, it has been used to enable light-activated drug release from hydrogels¹⁷ and patterning of *Escherichia coli* onto multiple materials,¹⁸ indicative of the wide ranging potential of optogenetic approaches. At present, the current bacterial optogenetic toolset primarily includes one or two-component systems^{8,19–22} and split proteins.^{7,23,24}

Recombinases are proteins that recognize specific 30–50 base pair (bp) sequences of DNA, and excise the “target” DNA between the sites along with one of the recognition sites. Their ability to manipulate DNA makes them particularly useful for

complex cellular logic circuits and engineering gene circuits with memory.^{25–27} Light-inducible recombinases have been notably useful in mammalian systems^{6,28–32} and yeast.³³ Having recombinases that are inducible at the protein-level can allow specific cells within a population to be targeted for recombination in response to spatial patterning of light, and there is no need to change media or wait for a chemical inducer to diffuse. Recombinase enzymes can be made light sensitive by splitting the gene into N-terminal and C-terminal fragments, and linking a sequence for a light-sensitive photodimer to each fragment. Upon light induction, the photodimer undergoes a conformational change that allows it to dimerize, bringing the two fragments together. This split-protein approach has been shown to work for both chemogenetic and optogenetic split-recombinases in eukaryotic systems.^{6,29} However, although split-recombinases for prokaryotes do exist, inducibility has not been characterized and they can be slow, for example, requiring 24 h for DNA excision.³⁴

Here, we develop and optimize an optogenetic recombinase for *E. coli*. We focus primarily on split Cre linked to Vivid (Vvd) photodimers. Cre is a commonly used tyrosine recombinase from the P1 bacteriophage that excises DNA

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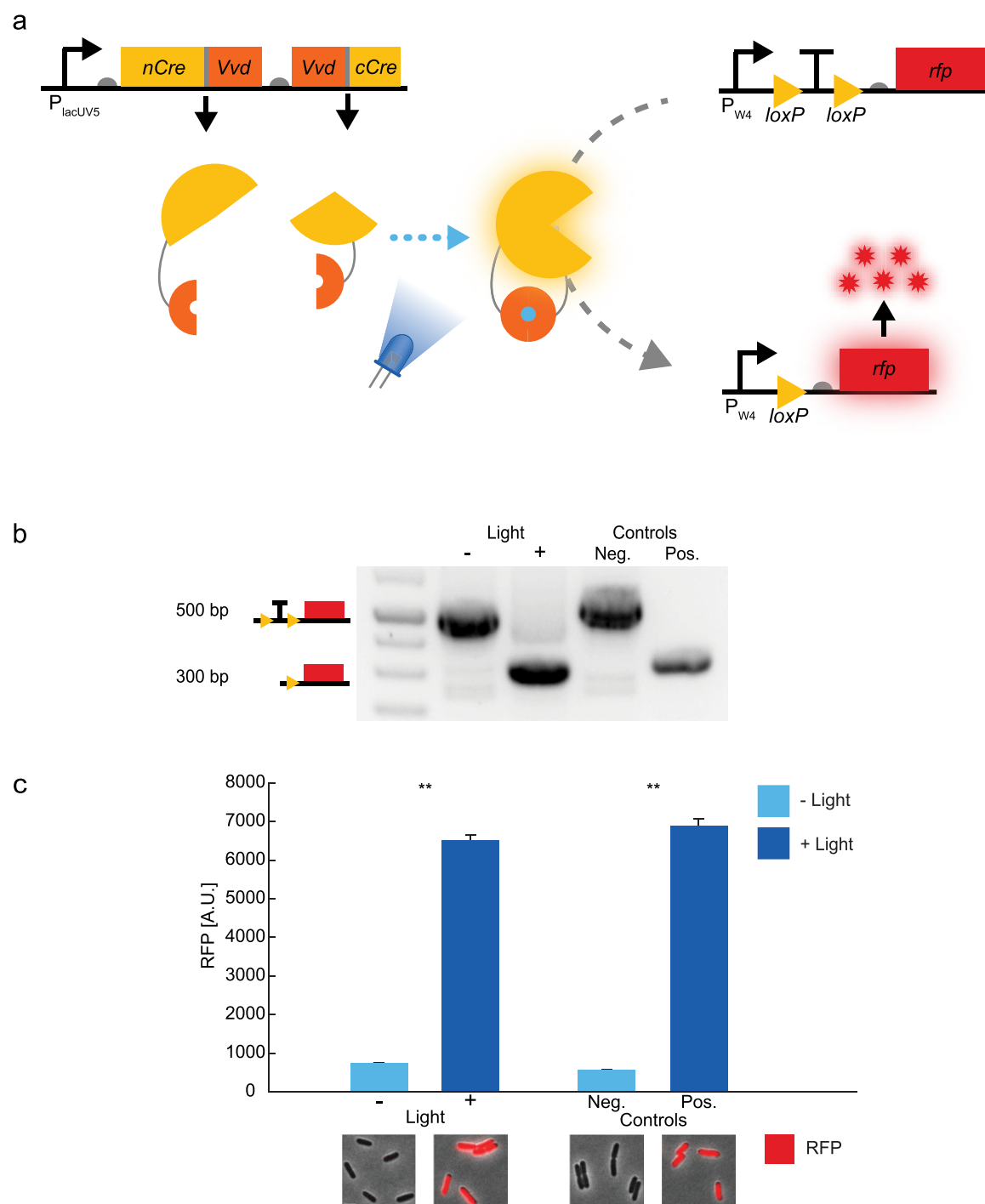


Figure 1. Light-inducible recombination in *E. coli*. (a) Split Cre fragments are linked to Vvd photodimers and expressed under the control of an IPTG-inducible promoter (P_{lacUV5}). When exposed to blue light, Vvd dimerizes, forming functional Cre protein. Cre can then act on the reporter plasmid, excising the *loxP*-flanked transcription terminator and allowing expression of RFP. RFP is under the control of a constitutive promoter (P_{W4}). (b) Gel electrophoresis images showing DNA excision. PCR of the reporter region containing *loxP*-flanked terminator shows a 500 bp band when full terminator is intact, and 300 bp band after recombination. Negative control contains cells with the reporter plasmid alone (terminator upstream of *rfp*); positive control contains cells with recombinase and a pre-cut reporter plasmid (no terminator upstream of *rfp*). (c) Single-cell fluorescence microscopy showing RFP expression for cells with and without light exposure. Insets below show representative cell images (scale bar = 2 μ m). Error bars show standard error around the mean ($n \approx 300$ cells per sample). In addition, we tested for statistical significance between conditions with and without light exposure using a two-tailed Welch's *t*-test by using individual microscopy images as replicates, $**P < 0.005$.

flanked by *loxP* sites.³⁵ Vvd is derived from the fungus *Neurospora crassa*, and homodimerizes under blue light and separates in the dark.^{36,37} In developing our light-inducible recombinase we also explored Flp recombinase and Magnet photodimers,³⁸ as well as multiple protein split sites within

each recombinase. Here, we introduce an optimized design, which we denote Opto-Cre-Vvd, which excises target DNA completely in 2 h. We also characterize sensitivity to ambient light exposure, the impact of light intensity, and the response time of the system.

RESULTS

To make Cre light sensitive, we split it into N-terminal (nCre) and C-terminal (cCre) fragments, with Vvd photodimers attached to the internal end of each fragment (Figure 1a). When exposed to blue light, Vvd changes conformation to allow dimerization, bringing the Cre fragments together.

Cre excises DNA fragments between *loxP* sites that are oriented in the same direction. We used this to develop a reporter for the efficiency of our recombinase constructs. We placed a transcription terminator flanked by *loxP* sites between the gene for red fluorescent protein (*rfp*) and a constitutive promoter. In the absence of Cre, the terminator prevents transcription of *rfp*. Functional Cre excises the terminator, leading to RFP production (Figure 1a). To perform these tests, we used a light plate apparatus (LPA).³⁹ We exposed samples to 465 nm blue light using LEDs for 1 h and then took samples for polymerase chain reaction (PCR) immediately following light exposure. To verify that the recombinase was excising the target DNA properly, we first used PCR to check the length of the plasmid region containing the *loxP*-flanked terminator with and without exposure to light. We used a forward primer upstream of the promoter, and a reverse primer in the *rfp* gene to amplify the region, which is approximately 500 bp if the terminator and both *loxP* sites are intact, and 300 bp when the terminator is removed by recombination (Figure 1b). As a negative control, we used cells with the reporter but no recombinase. As a positive control, we used a strain containing recombinase and the reporter plasmid with the terminator excised. In addition, using microscopy we confirmed that after exposure to blue light, cultures showed a clear increase in RFP (Figure 1c). For the microscopy experiments we refreshed cultures overnight to allow full RFP expression and maturation after recombination.

When developing the photoactivatable split recombinase, we considered several variants on the design, including different recombinase enzymes, alternative photodimers, and multiple protein split site locations. First, we tested two widely used recombinases, Cre and Flp (Figure 2a). Flp is a tyrosine recombinase originally native to *Saccharomyces cerevisiae*,⁴⁰ and like Cre has been used as a split photoactivatable recombinase in mammalian systems.^{28,29} We tested each recombinase using previously established split sites (Cre 43,⁶ Flp 374²⁹) with two photodimer options, Vvd and Magnets. In contrast to the blue light-sensitive homodimer Vvd, Magnets are engineered heterodimer Vvd variants with separate positively charged and negatively charged dimer interfaces.³⁸ We worked with these photodimers due to their efficiency in mammalian split-recombinases,^{6,28,29,41} and established function in other bacterial split-protein systems.^{7,23} We found that Cre, especially when paired with Vvd, showed substantially improved activation relative to Flp when exposed to blue light (Figure 2a).

Focusing on Cre recombinase with the Vvd photodimer, we next tested several variants where the protein was split at different locations (Figure 2b). We considered sites reported in the literature, structurally predicted sites, and algorithmically derived sites (Table 1). Literature derived sites included Cre 43,⁶ and Cre 213, 240, 254.²⁹ We also used the SPELL algorithm to determine novel potential split sites.⁴² We ran SPELL on the Cre structure PDB 3MGV,⁴³ which led to predictions for Cre 46 and Cre 137. We also found structurally informed sites for Cre by analyzing the PDB structure 3MGV

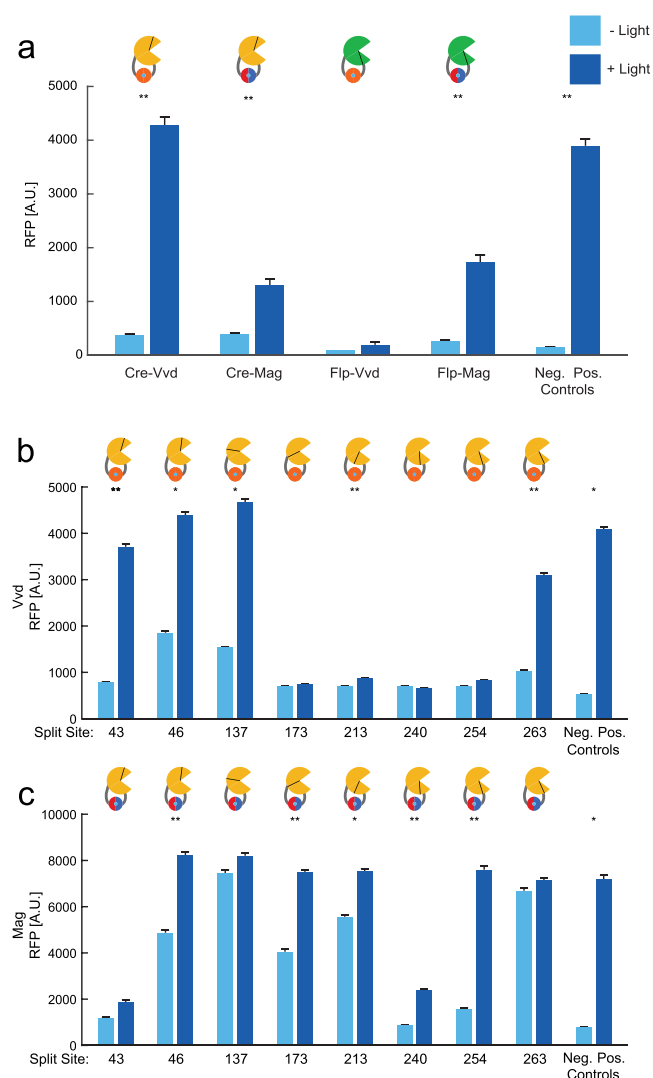


Figure 2. Comparison of optogenetic recombinase protein variants. (a) RFP reporter output with and without light exposure for Cre and Flp recombinases, each with Vvd and Magnet photodimers. Split sites used are Cre with nCre length of 43 AA, Flp with nFlp length of 374 AA. (b) Assay of split sites tested for Cre-Vvd and (c) Cre-Mag. Numbers shown for split sites on the x-axis are the length of nCre. All figure data were obtained using fluorescence microscopy. In all cases, 100 μ M IPTG was used for induction and samples were exposed to 1 h of light at 120 μ W/cm². Error bars show standard error around the mean ($n \approx 750$ cells per sample). Statistical significance comparing conditions with and without light use a two-tailed Welch's *t*-test using microscopy images as replicates. * $P < 0.05$, ** $P < 0.005$.

in PyMol.⁴⁴ Using this crystal-derived structure, we assessed B-factor to select flexible regions within the protein,⁴⁵ and chose split sites between flexible amino acids such as glycine and serine.⁴⁶ This method led us to Cre 173 and 263.

We observed split site-dependent variation in both the level of activation with light and in recombination in the absence of light. Some sites showed almost no activation with light (173, 213, 240, 254). Others sites showed high activation with light exposure, but also increased activation in the absence of light (46, 137). From this screen, we found Cre-Vvd 43 to be our best candidate, as it showed a good fold change in RFP expression in response to light and minimal activation without light. We also tested each split site using the Magnet photodimers (Figure 2c), and observed varied activation at

Table 1. Cre Recombinase Split Sites Used in This Study, Given as Lengths of the nCre Fragment,^a the Amino Acids on Either Side of the Split, and the Source of the Split^b

nCre length (AAs)	amino acids	source
43	NN	Kawano 2016 ⁶ /Weinberg 2019 ²⁹
46	KW	SPELL
137	DR	SPELL
173	DG	PyMOL
213	GV	Weinberg 2019 ²⁹
240	LS	Weinberg 2019 ²⁹
254	RL	Weinberg 2019 ²⁹
263	SG	PyMOL

^aFrom methionine at position 1 to the split site. ^bNote that we use truncated Cre from Kawano (2016),⁶ which starts at AA 18 of native Cre.

different split sites. Of the Cre-Mag variants, Cre-Mag 254 was the most promising candidate, however it showed a growth defect compared to Cre-Vvd 43, so we focused our efforts on Cre-Vvd 43 (Figure S1). Although there were commonalities, not all split sites behaved consistently with both types of photodimers. Overall, we found that Magnets were more likely to strongly activate, but also had much higher activation without light than Vvd. This may be due in part to Vvd's ability to form homodimers, as "incorrect" dimer pairs containing two nCre or two cCre fragments could help to lower formation of Cre in the absence of light. It is also notable that the dark-state expression seen here is higher than in the original mammalian PA-Cre.⁶ Tests of five literature derived split sites for Flp-Mag showed similar split site-dependent results, but were ultimately inferior to the Cre variants (Figure S2). Due to its significant activation and high fold change we chose to use Cre-Vvd 43, which we denote Opto-Cre-Vvd, for further characterization.

An important practical experimental consideration for light inducible recombinases is their sensitivity to ambient light. Therefore, we next tested how Opto-Cre-Vvd performed with 5 min of ambient light exposure. We chose this duration to mirror conditions that might be experienced in a setting where plates are temporarily removed from darkness, such as would be necessary to transfer cultures from growth conditions to flow cytometry or microscopy assays. We found that Opto-Cre-Vvd showed minimal sensitivity to short duration exposure to ambient light (Figure 3a).

Next, we tested experimental parameters for Opto-Cre-Vvd, including the light intensity used for induction, the timing of light exposure, and concentration of IPTG for recombinase induction. When optimizing split sites and photodimer variants, we used a blue light intensity that corresponded to the maximum value accessible with the LEDs used in the LPA (120 $\mu\text{W}/\text{cm}^2$) to minimize excision times (Figure 3b). Using microscopy, we observed no discernible differences in cell morphology with and without light exposure in these conditions, suggesting that phototoxicity effects were minimal with this exposure level (Figure S1a). We also confirmed that our constructs and IPTG induction levels did not have adverse effects on cell growth in bulk cultures (Figure S1b). Overall, we found that even at much lower light intensities, we observed complete excision when samples were exposed to blue light for a longer time (Figure 3b). Cultures exposed to lower intensities of light showed partial excision after 1 h, while cultures exposed to high intensity light showed near-complete excision. When we exposed cultures to constant blue light for 4

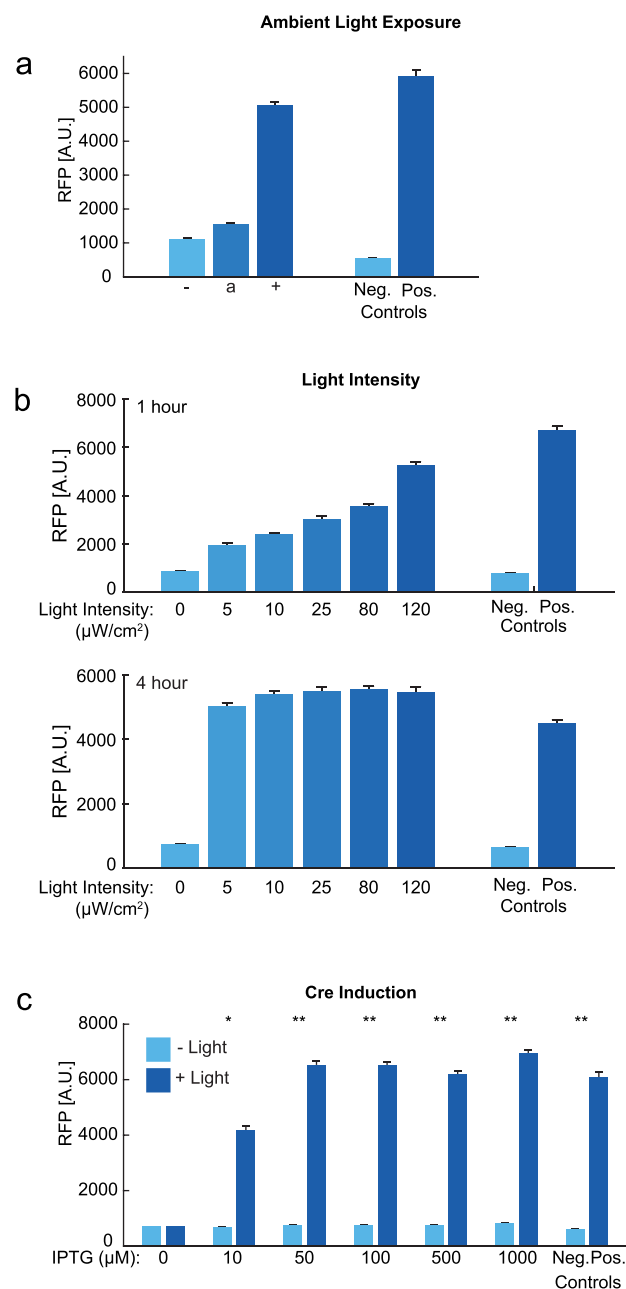


Figure 3. Characterization of Opto-Cre-Vvd. (a) RFP reporter output for Opto-Cre-Vvd without light (−), with a 5 min exposure to ambient light (a), and with full exposure to blue light (+). (b) Effect of blue light intensity on Opto-Cre-Vvd activation when exposed for 1 or 4 h. (c) Effect of IPTG induction levels for Opto-Cre-Vvd. Statistical significance comparing conditions with and without light use a two-tailed Welch's *t*-test using microscopy images as replicates. **P* < 0.05, ***P* < 0.005. In all cases, error bars show standard error around the mean from microscopy data (*n* ≈ 350 cells per sample).

h, we found that all intensities of light yielded comparable, high levels of excision (Figure 3b). In addition, we found Opto-Cre-Vvd to have consistent, low basal levels of activation without light between experiments conducted on different days (Figure S3).

In our design, the split Cre fragments are under the control of a lacUV5 promoter to prevent excision of the target DNA prior to induction and subsequent light exposure. We found that inducing with IPTG concentrations above 50 μM for 2 h

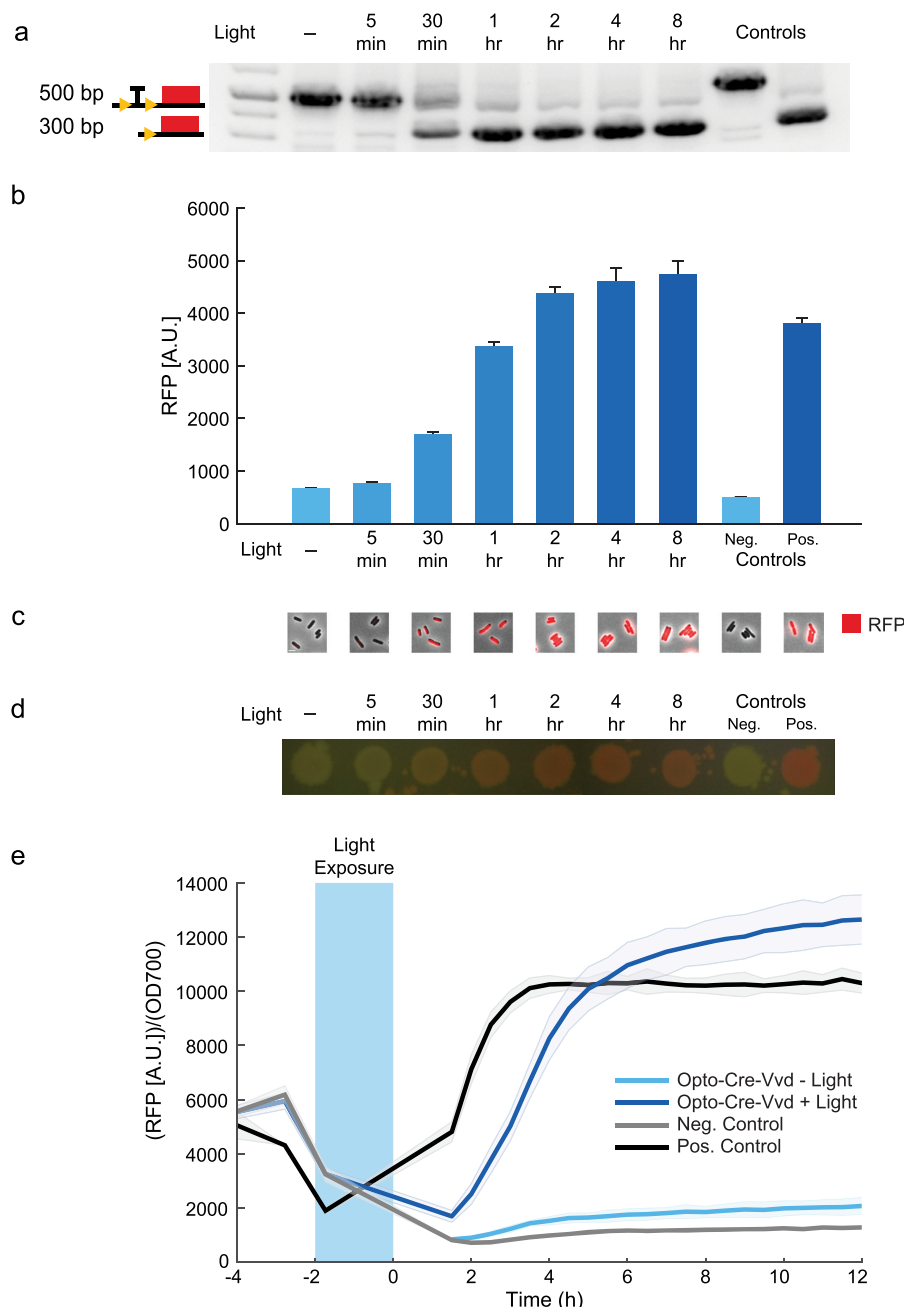


Figure 4. Light exposure duration necessary to induce excision by Opto-Cre-Vvd. (a) DNA gel image showing reporter bands with and without transcription terminator excision, (b) single-cell fluorescence microscopy averages of RFP values, (c) representative microscopy images (scale bar = 2 μ m), and (d) samples of culture spotted on agar plates of Opto-Cre-Vvd exposed to different durations of blue light. Error bars show standard error around the mean ($n \approx 750$ cells per sample). (e) Opto-Cre-Vvd activation in real-time, with the blue bar indicating light exposure. Shaded error bars represent standard deviation around the mean from plate reader data ($n = 3$ wells).

prior to light exposure was sufficient to induce Cre for light activation (Figure 3c). We used 100 μ M IPTG as a standard value, which remains solidly above the induction threshold for our experiments.

We were also interested in exploring the duration of light exposure that cells need to induce full RFP expression (Figure 4). To test this, we exposed separate cultures to light for 5 min, 30 min, 1, 2, 4, or 8 h. Cultures exposed to light for less than 8 h were kept in the dark following light exposure for the remainder of the time. At the end of the 8 h period, we refreshed all cultures and grew them for 2 h without light to allow for protein maturation and then assessed transcription

terminator excision both genotypically and phenotypically. Genotypic excision was assessed by PCR and gel electrophoresis (Figure 4a). Phenotypic results were assessed by microscopy (Figure 4b,c) and spotting on agar (Figure 4d). We observed general agreement between all characterization methods. Opto-Cre-Vvd shows substantial RFP expression with 1 h of blue light exposure, and RFP values comparable to the positive control, indicative of near-complete activation, by 2 h. Live time course results, which include the time for protein maturation, indicate RFP expression within 2 h after exposure to blue light, and expression comparable to the positive control by 4 h post-exposure (Figure 4e).

■ DISCUSSION

We have developed, optimized, and characterized a light-inducible recombinase for *E. coli*. We found that both Cre and Flp associated with either Vvd or Magnet photodimers have the potential for photoactivatable recombination. However, split site location and the recombinase-photodimer pairing impact efficacy. Our most promising candidate, Opto-Cre-Vvd, exhibits blue light-dependent excision and low sensitivity to ambient light. We also found that Opto-Cre-Vvd shows activation at both low and high light intensities, but at different time scales. The construct can cut completely within 2 h, which is comparable to the time frame observed for mammalian photoactivatable Cre⁶ and existing Magnet-based split proteins in *E. coli*.⁷

A well-characterized recombinase tailored to *E. coli* is a powerful new tool for bacterial optogenetics. Moving forward, this offers expanded potential for interfacing engineered cells with computational control *via* light. Applications include the ability to target subpopulations of cells, real-time genetic modifications, or experiments where small molecule inducers are impractical due to crosstalk. For example, this could enable studies on population dynamics through controlled spatial development of subpopulations, establishing interfaces between neighboring cells. A future version of this system may also be useful for changing genetic state during biomolecule production, such as in metabolic engineering applications, as light is an inexpensive inducer. Cre could also be used to activate or inactivate multiple genes using constructs similar to a dual-fluorescent assay.³⁴ Along these lines, future extensions to this system may involve development of orthogonal light-inducible recombinases for bacteria. Alternate photodimer systems that are sensitive to other wavelengths of light could be used to multiplex the approach.⁴⁷ It would also be interesting to develop an in-depth understanding of the differences between Cre and Flp, to perform detailed characterization at the molecular level, or to engineer variants with alternative optogenetic tools such as CRY2/CIB^{30,31} or iLID.⁴⁸ As an immediate application, Opto-Cre-Vvd can be used as-is to perform gene knock outs in real time and is compatible with plate-based or microscopy platforms. These light-inducible recombinases expand the optogenetic methods available for bacteria and have great potential for the design of novel synthetic circuits.

■ METHODS

Strains and Plasmids. Expression studies use *E. coli* strain MG1655. All recombinase constructs use a plasmid with a high-copy ColE1 origin and an ampicillin resistance cassette where the recombinase genes are under the control of an IPTG-inducible lacUV5 promoter to prevent activation from light exposure prior to experiments. The construct is derived from the pBbE5a BioBrick plasmid.⁴⁹ All reporter constructs use a medium-copy p15A origin plasmid with a kanamycin resistance cassette and the gene for red fluorescent protein (mRFP1)⁵⁰ under the control of a constitutive, medium-strength promoter (denoted P_{W4}), which is modified from the phage T7 A1 promoter: TTATCAAAAAGAGTATTGCA-TTAAAGTCTAACCTATAGGAATCTTACAGCCATCG-AGAGGGACACGGCGAA (underline indicates mutations from original T7 A1 promoter).⁵¹ Plasmids were constructed using the Gibson assembly method.⁵² Primer data can be found in Table S1.

Original Cre and Magnet heterodimer plasmids are from Weinberg (2019).²⁹ The original Flp gene sequence was derived from the pCP20 plasmid from Datsenko and Wanner (2000).⁵³ We obtained the Vivid homodimers from AddGene plasmid #58689 (mV-NcVV-LOV_231) deposited by Harald Janovjak.³⁷ We express Cre split with a photodimer pair as an operon (Figure 1a). The N-terminal fragment of Cre (nCre) is followed by a 10 AA glycine-serine linker and a photodimer. A separate RBS is used to express the second photodimer linked by a 10 AA glycine-serine linker to the C-terminal fragment of Cre (cCre).

Plasmids from this study and their sequences are available on AddGene (https://www.addgene.org/Mary_Dunlop/).

Cre Split Site Selection. Split sites for Cre were selected using three methods: chosen from the literature, using the first two optimal choices from the SPELL algorithm (<https://dokhlab.med.psu.edu/spell/>)⁴² based on the PDB structure 3MGV,⁴³ or by using PyMOL (<https://pymol.org/2/>) on 3MGV and selecting two sites around glycine and serine AAs in regions with high B-factor values. Further information and source of each split site for Cre can be found in Table 1. Primers used to make each split are listed in Table S1. Split Cre variants were made by amplifying from plasmids containing Cre without a photodimer from the location of the split site with overhangs for the linker sites. In parallel, we amplified the photodimer and linker inserts and combined *via* Gibson assembly.⁵² Split sites for Flp were chosen from the literature and cloned in a similar fashion; their information can be found in Table S2.

Light Exposure Assays. Strains were grown overnight from a single colony in LB medium containing 100 μ g/mL carbenicillin and 30 μ g/mL kanamycin for plasmid maintenance. The next day, cultures were refreshed 1:100 in selective LB and induced for 2 h with 100 μ M IPTG unless otherwise noted. Blue light exposure was performed using a LPA,³⁹ with two 465 nm wavelength LEDs per well (Thorlabs LED465E), outputting a total of 120 μ W/cm². Unless otherwise noted, cultures were exposed to blue light for 1 h. After exposure, samples were prepared for analysis by PCR to check for target excision by gel electrophoresis, and refreshed in selective LB medium without IPTG overnight. In light intensity experiments, cultures were exposed to light for 4 h total, with intermediate samples taken at 1 h for characterization.

All liquid cultures through the experiment were grown at 37 °C with 220 rpm shaking. Note that agar plates with the Flp recombinase and reporter were also kept at 37 °C at all times, as we observed substantial activation even without light when stored at 4 °C. After transformation, all cultures were kept in the dark throughout the entire experiment with the exception of blue or ambient light exposure periods. For ambient light exposure, samples were exposed to lab lighting in a shaking incubator for 5 min after induction, and then kept in the dark for the remainder of the experiment.

Recombinase Efficiency Characterization. Target DNA excision *via* the recombinase was measured genetically by amplifying the promoter region of the reporter using PCR. We used a forward primer ~200 bp upstream of the first *loxP* site (ATCTTCCCCATCGGTGATGTCG) and a reverse primer ~100 bp downstream of the second *loxP* site (GACGACCTTCACCTT) to check for differences in band length before and after recombination.

Plate reader data were collected on a BioTek Synergy H1 with OD absorbance read at 700 nm to avoid overlap with the RFP spectra, and fluorescence read with excitation at 584 nm and emission at 610 nm.

In addition to the PCR-based measurements, efficiency was also measured visually by imaging plated samples with a mobile phone camera using standard settings (Samsung Galaxy Note 8) on a Blue LED transilluminator through the attached orange filter (IO Rodeo).

Microscopy and Image Analysis. Post light-exposure samples were refreshed overnight in LB medium with 100 $\mu\text{g}/\text{mL}$ carbenicillin and 30 $\mu\text{g}/\text{mL}$ kanamycin for plasmid maintenance to allow full RFP expression and maturation. Before imaging, samples were refreshed for 2 h in 1:100 in MGC medium (M9 salts supplemented with 2 mM MgSO_4 , 0.2% glycerol, 0.01% casamino acids, 0.15 $\mu\text{g}/\text{mL}$ biotin, and 1.5 μM thiamine). Samples were then placed on 1.5% low melting agarose pads made with MGC medium. Cells were imaged at 100x using a Nikon Ti-E microscope. Images were segmented and analyzed using the SuperSegger software⁵⁴ and custom Matlab analysis scripts. Statistical significance (P value) was assessed using a two-tailed Welch's t -test, treating each microscopy image as a sample.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.9b00395>.

Supplementary figures and supplementary tables (PDF)

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Author Contributions

MBS and MJD conceived and designed the experiments. MBS performed the experiments and analyzed the data. WWW provided insight on experimental design and optimization. MBS and MJD wrote the manuscript with input from WWW.

Notes

The authors declare no competing financial interest. Plasmids from this study and their sequences are available on AddGene (https://www.addgene.org/Mary_Dunlop/).

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■ ABBREVIATIONS

Vvd, Vivid; bp, base pair; nCre, N-terminal fragment of Cre recombinase; cCre, C-terminal fragment of Cre recombinase; RFP, red fluorescent protein; PCR, polymerase chain reaction; AA, amino acid; LPA, light plate apparatus; SPELL, split protein reassembly by ligand or light; OD, optical density

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