Optogenetic control of *B. subtilis* gene expression using the CcaSR system

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Key words

Optogenetics, *Bacillus subtilis*, two-component system, CcaSR, synthetic biology

Abstract

Optogenetics enables precise control of gene expression in a variety of organisms. We recently developed the first system for optogenetic control of transcription in *Bacillus subtilis*. This system is based on CcaSR, a light-responsive two-component regulatory system originally derived from *Synechocystis* PCC 6803. The so-called *B. subtilis* CcaSR v1.0 enables activation of gene expression with green light and deactivation with red. As a result, *B. subtilis* CcaSR v1.0 can be used to program gene expression with high quantitative, spatial, and temporal resolution. The expression levels of the CcaS light sensing histidine kinase and the CcaR response regulator are set by the addition of chemical inducers in *B. subtilis* CcaSR v1.0, enabling adjustment of the basal expression level and optimization of the magnitude of gene expression induction. In

principle, *B. subtilis* CcaSR v1.0 should be compatible with expression of any target gene of interest. Here, we provide growth, strain engineering, and light treatment protocols for working with *B. subtilis* CcaSR.

1. Introduction

CcaSR is a light-sensing two-component system (TCS) that originally evolved in the Gram-negative cyanobacterium *Synechocystis* PCC 6803 ***(1,2)***. CcaSR consists of CcaS, a sensor histidine kinase belonging to the cyanobacteriochrome family, and CcaR, an OmpR/PhoB-family response regulator. In order to sense light, CcaS requires phycocyanobilin (PCB), a linear tetrapyrrole that binds covalently to the GAF domain in CcaS to form a holo-CcaS complex. PCB is synthesized in two metabolic steps from cellular heme pools. Synthesis of PCB requires heme oxygenase 1 (encoded by *ho1*) and phycocyanobilin:ferredoxin oxidoreductase (encoded by *pcyA*) ***(3)***. CcaS is produced in the ground state with low autokinase activity, and upon absorbing a green light photon ($\lambda_{max} = 535$ nm), it transitions to an active state with high autokinase activity. Active CcaS phosphorylates itself, and then transfers this phosphoryl group to CcaR. Phosphorylated (active) CcaR then binds to and induces transcription from the P_{cpcG2} promoter. Under red light, CcaS ($\lambda_{max} = 672$ nm) is thought to dephosphorylate CcaR, leading to reduced transcription ***(2)***.

The ability of the CcaSR system to activate transcription in response to green light and deactivate it in response to red light has made it an attractive target for engineering optogenetic control of gene expression in bacteria. Previously, we ported the system to *E. coli* and improved its performance by optimizing the expression of each of the four component proteins, truncating

the output promoter to decrease leakiness and increase dynamic range, and miniaturizing CcaS to further improve dynamic range ***(4-7)***.

Numerous important physiological pathways such as sporulation, biofilm formation, and competence are regulated by stochastic or time-varying patterns of gene regulator activity in *B. subtilis*. Such processes could be rigorously investigated using artificial, light-driven patterns of gene expression in batch culture or single cells. Thus, optogenetic tools could substantially advance understanding of *B. subtilis* biology.

To control gene expression with light in *B. subtilis*, we ported the so-called CcaSR v3.0 system from *E. coli* into *B. subtilis* and re-optimized its function for this new bacterium ***(8)***. These optimizations included codon optimization of the first fifteen codons of *ho1*, *sfgfp*, and *ccaS* as well as the entire sequence of *pcyA* for expression in *B. subtilis*, engineering a translational fusion of Ho1 and PcyA to increase PCB production, and fusing $P_{cpcG2\text{-}172}$ to the strong constitutive *B. subtilis* promoter P_{veg} (resulting in $P_{cpcG2\text{-}veg}$) to increase target gene expression levels. The resulting system was named *B. subtilis* CcaSR v1.0 . It produces a 70-fold increase in transcription rate of a target gene in response to green versus red light and the change in target protein levels can be observed within minutes ***(8)***.

B. subtilis CcaSR v1.0 comprises three genetic modules: a PCB production module (PPM), a light-sensing module (LSM), and a transcriptional output module (TOM) (**Figure 1)**. The PPM encodes an expression cassette for the *ho1-pcyA* fusion. The LSM encodes the so-called *ccaSmini#10* variant ***(6)*** under the control of the xylose-inducible promoter $P_{xylA(+47)}$, which we engineered for superior xylose response during the construction of *B. subtilis* CcaR v1.0, as well as its cognate repressor *xylR* (see **Note 1**) ***(8,9)***. The TOM encodes *ccaR* under the control of

IPTG-inducible promoter $P_{hy\text{-}spank}$, its cognate repressor gene *lacI*, and the target gene under control of $P_{cpcG2\text{-}veg}$.

Construction of a strain for optical control of a novel target gene requires first building a custom TOM that places the target gene under the control of $P_{cpcG2\text{-}veg}$, replacing *sfgfp.* We recommend replacing *sfgfp* via Golden Gate assembly, a molecular cloning strategy that uses restriction enzymes and a ligase to scarlessly assemble several DNA fragments ***(10,11)*** (**Section 3.1**). The resulting product constitutes a linear integration module (LIM), which is a double stranded DNA molecule with homology to the genome, enabling integration of DNA into the chromosome through homologous recombination. LIMs can be produced through assembly techniques (e.g. Gibson Assembly, Golden Gate) and/or amplified via PCR. The LIM containing the custom TOM is transformed to wildtype *B. subtilis* (**Sections 3.2-3.3**). After confirming successful integration, the genome of the TOM strain is purified to use as a genome integration module (GIM) (**Section 3.4**). A GIM is a purified genome that contains a single LIM integrated somewhere in the chromosome. It can be transformed to a *B. subtilis* strain in order to integrate the LIM with high efficiency due to the high degree of homology between the GIM and the recipient chromosome at the LIM locus. Both the published LSM (in the *xylA* locus of the *B. subtilis* genome) and the customized TOM (in the *amyE* locus) must be transformed as GIMs to the published base strain containing the PPM in the *thrC* locus (**Sections 3.5 and 3.6**).

To run an optogenetic experiment with this system, we recommend exposing engineered cells growing at exponential phase to light signals in a Light Plate Apparatus (LPA), a commercially-available open source device that can deliver two independent light signals to each well of a 24-well plate ***(12)*** (Sections 3.7-3.9). The cells are exposed to custom light programs, enabling quantitative control of gene expression.

2. Materials

Strains from the original report of *B. subtilis* CcaSR v1.0 ***(8)*** can be obtained from the Bacillus Genetic Stock Center (BGSC). For those bearing a single LIM, the annotated sequence of the LIM is available through GenBank. Strain genotypes, BGSC numbers, and GenBank accession numbers can be found in **Table 1**. When preparing media, filter sterilize all components and combine aseptically. Standard PCR equipment and reagents are required.

2.1 Strains

1. PY79 (wildtype *B. subtilis*, can also be obtained from BGSC).
2. S028.
3. S051.
4. S061.

2.2 Media and Antibiotics

1. Iron chloride solution with citric acid: 50 mM $FeCl_3$ and 100 mM $C_6H_8O_7$ in water. Filter sterilize. Store at 4°C protected from light.
2. T-base: Add 9.91 g ammonium sulfate ($(NH_4)_2SO_4$), 69.68 g potassium phosphate dibasic (K_2HPO_4), 29.94 g potassium phosphate monobasic (KH_2PO_4), and 5.15 g sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) to a sterile bottle. Water up to 500 ml. Mix thoroughly and heat to dissolve. Filter sterilize.
3. M9 media formulated for *B. subtilis* optogenetics: For 1 L, add the following to 700 ml filtered deionized water in a sterile water: 200 ml 5X M9 salts, 20 ml 10% casamino

acids, 20 ml iron chloride solution with citric acid, 8 ml 50% glycerol, 2 ml 50 mM $MnSO_4$, 2 ml 1 M $MgSO_4$., and 1 ml 100 mM $CaCl_2$. Add more water up to 1 L. See **Notes 2-3**.

4. SpC media: 20 ml 10x T-base, 4 ml 10% yeast extract, 3 ml 1.2% w/v $MgSO_4$, 2 ml 50% glucose, 0.5 ml 10% casamino acids, water up to 200 ml.
5. SpII media: 20 ml 10x T-base, 14 ml 1.2% w/v $MgSO_4$, 2 ml 50% glucose, 2 ml 10% yeast extract, 1 ml 100 mM $CaCl_2$, 0.2 ml 10% casamino acids, water up to 200 ml.
6. SpII-EGTA: 1000 µl 10x T-base, 700 µl 1.2% w/v $MgSO_4$, 200 µl 0.1M EGTA, 100 µl 50% glucose, 100 µl 10% yeast extract, 100 µl 1% casamino acids, 50 µl 100 mM $CaCl_2$, water to 10 ml.
7. Concentrated SpII-EGTA: 2500 µl 10x T-base, 1750 µl 1.2% w/v $MgSO_4$, 500 µl 0.1M EGTA, 250 µl 50% glucose, 250 µl 10% yeast extract, 250 µl 1% casamino acids, 125 µl 100 mM $CaCl_2$, water up to 10 ml.
8. D-xylose: 50% w/v in water. See **Note 4.**
9. Isopropyl β-d-1-thiogalactopyranoside (IPTG): 1 M in water. See **Note 5.**
10. Spectinomycin, 100 mg/ml in water.
11. Erythromycin, 0.5 mg/ml in ethanol.
12. Chloramphenicol, 35 mg/ml in ethanol (optional, see **Notes 5-6**).
13. Kanamycin, 50 mg/ml in water (optional, see **Notes 6-7**).
14. Tetracycline, 10 mg/ml in water (optional, see **Note 7**).
15. Starch plates: Combine the following in a sterile 1 L bottle: 25 g LB Miller, 15 g bacteriologial agar, 10 g starch, water up to 1 L. Autoclave the bottle containing the suspension. After autoclaving, shake the bottle to mix the preparation and pour into plates

as normal, 10-15 ml per plate. Allow plates to dry under flame, then store in a bag at 4°C. Pre-warm before use.

16. Gram's iodine, stabilized.
17. 50% glycerol.

2.3 PCR and Golden Gate Supplies

1. BsaI restriction enzyme (NEB).
2. 10x NEB T4 buffer.
3. NEB T4 ligase.
4. DMSO recommended for all PCRs (see **Note 8**).

2.4 Equipment and Kits

1. Wizard Genomic DNA Purification Kit from Promega.
2. Wizard Gel Purification Kit from Promega.
3. Light Plate Apparatus (LPA). The LPA is a device that can deliver two independent light signals to each well of a 24-well plate ***(12)***. A guide to building, programming, and using the LPA is available in Gerhardt et al (2019) ***(13)***. Alternatively, LPAs can be purchased through Flash Genetics (flashgenetics.com).
4. ArcticWhite opaque 24-well clear-bottom plates (arcticwhiteusa.com).
5. Reflective aluminum foil seals (VWR, Radnor, PA, USA).

3. Methods

3.1 Design and Construction of the Transcriptional Output Module

1. Design and order primers for Golden Gate assembly of the custom TOM, which will place the target gene downstream of $P_{cpcG2\text{-}veg}$ (see **Notes 9-12**). One pair of primers must amplify the gene of interest and introduce recognition sites for the Type II restriction enzyme BsaI, as well as sticky ends with homology to adjacent fragments. Two other pairs will amplify the 5' and 3' fragments of the TOM (flanking the gene of interest). We have provided the primer pairs necessary for PR amplification of the 5' and 3' fragments, as well as primer templates for amplifying your gene of interest--see **Table 2**. These primers introduce the sticky end 5'-TATG-3' upstream of the gene of interest, and 5'-TAAC-3' downstream (see **Note 13**). **Figure 2** provides a reference for the cloning scheme.
2. Perform PCR amplification of the 5' and 3' fragments of the TOM (using S051 as a template), as well as the PCR amplification of your gene of interest. **Table 3** provides recommended PCR reaction conditions (see **Notes 14-16**).
3. Perform agarose gel electrophoresis (recommended conditions: 1% agarose gel, 90 V, ~30-50 min depending on total size of PCR product) on your PCR reactions and identify the appropriately sized band for your product.
4. Perform gel extraction and purification to obtain purified DNA fragments. This avoids off-target recombination in later transformations due to residual genomic DNA.
5. Perform a Golden Gate reaction to link the 5' and 3' portions of the TOM to your target gene. On ice, mix together equimolar quantities of each of the three purified DNA fragments in a PCR tube. Total DNA mass should be around 200 ng, with a volume less than 11.5 µl. Add water up to 11.5 µl. Then add 1.5 µl 10x NEB T4 Buffer, 1 µl BsaI

enzyme, and 1 µl NEB T4 Ligase. Flick or pipette gently to mix. Then run the reaction using the thermocycler conditions provided in **Table 4**. See **Notes 17-18.**

3.2 Preparation of competent *B. subtilis*

1. Streak out the recipient strain (PY79) on an LB agar plate and grow overnight at 37°C.
2. Inoculate a single colony of the recipient strain into 2 ml of SpC media and grow at 37°C, shaking.
3. Grow until early stationary phase (see **Note 19**). This usually takes about 4.5 hours. Early stationary phase can be detected when the OD_{600} value of the culture stops changing significantly over time.
4. Dilute the liquid culture 1:10 into pre-warmed SpII media. 1 ml of diluted SpII culture will yield enough cells for two transformations; scale the volume of SpII media accordingly.
5. Grow for 90 min at 37°C, shaking.
6. Pellet the cells (3 min at 4,000 x g, room temperature) and decant the supernatant--save the supernatant. It contains secreted competence factors. See **Note 15.**
7. Resuspend pellets in a volume of supernatant equal to 10% of the culture volume that was spun down. For example, if 1 ml of culture was spun down, resuspend in 100 µl of supernatant.
8. At this stage, cells are ready for transformation. Either transform immediately or add sterile glycerol to a final concentration of 15% and freeze at -80°C for future use. See **Note 20**.

3.3 Chromosomal Integration of TOM in wildtype PY79

1. Add the following to a 1.5 ml microcentrifuge tube: 50 µl competent cells (PY79), 20 µl *concentrated* SpII-EGTA, and 30 µl Golden Gate reaction (see **Notes 21-23**).
2. Incubate the mixture at 37°C, shaking, for 1 hour (see **Note 24**).
3. Plate the cells on LB with spectinomycin (100 µg/ml) for selection and incubate overnight at 37°C (see **Note 25**).

3.4 Confirmation of TOM integration and sequence

1. Grow colonies from the transformation plate to early exponential phase in LB media.
2. Make temporary freezer stocks
3. Spot plate cultures on pre-warmed starch plates to perform a starch test (see **Notes 26-27**).
4. Grow starch plates overnight at 37°C.
5. The following day, pour Gram's iodine solution (1-2 ml) over the plates such that it forms a thin coat. Allow to incubate at room temperature for 5 min, then pour off the iodine solution. Cells with an intact *amyE* locus are able to degrade starch and will form a red/yellow halo after incubation with iodine; cells with a disrupted locus (implying successful integration) will be unable to form the halo.
6. Once integration is confirmed, grow the cells overnight at 37°C in liquid LB media and amplify the locus via PCR. The following primers amplify the TOM for sequencing (not including the spectinomycin resistance marker): `CCATAAAACTAAAGTAAGTGTAAACCTATTCATTG` (FWD) and `CATAAAGGTCATTGTTGACGCG` (REV). If using NEB Phusion polymerase, use an

annealing temperature of 63°C. Total length is 4.1 kb, plus the length of your gene of interest.

7. Separate the PCR product using gel electrophoresis as before.
8. Extract and purify the band corresponding to the appropriate size.
9. Submit the purified locus for sequencing (Sanger or amplicon).
10. Following sequence confirmation of the locus, perform a genome extraction of the new strain and store the purified genome at -20°C. We recommend the Wizard Genomic DNA Purification kit from Promega.

3.5 Integration of the LSM on PPM base strain to produce S065

1. Purify the genome of *B. subtilis* strain S061, containing the LSM integrated into the *xylA* locus (see **Notes 28-31**).
2. Prepare competent cells of *B. subtilis* strain S028, containing the PPM in the *thrC* locus.
3. Add the following to a 1.5 ml microcentrifuge tube: 50 µl competent cells, 50 µl (non-concentrated) SpII-EGTA, and 500 ng purified S061 genome.
4. Incubate the mixture at 37°C, shaking, for 2.5 hours (see **Note 24**).
5. Plate the cells on LB + erythromycin (0.5 µg/ml) and incubate overnight at 37°C.
6. Inoculate candidate colonies into LB media and make temporary glycerol stocks.
7. We recommend checking colonies for the presence of both the PPM and the LSM in their respective loci by PCR. Re-sequencing is typically not necessary.

3.6 Integration of the TOM on S065

1. Prepare competent cells of the chassis strain S065.

2. Add the following to a 1.5 ml microcentrifuge tube: 50 µl competent cells, 50 µl (non-concentrated) SpII-EGTA, and 500 ng purified genome containing custom TOM.
3. Incubate the mixture at 37°C, shaking, for 1 hours (see **Note 24**).
4. Plate the cells on LB + spectinomycin (0.5 µg/ml) and incubate overnight at 37°C.
5. Inoculate candidate colonies into LB media and make temporary glycerol stocks.
6. Confirm the successful integration of the TOM using the starch test as described in section 3.4. This strain will be referred to as the "experimental strain".

3.7 Preparation of glycerol aliquots

1. Streak out the experimental strain on LB agar plates and grow overnight at 37°C for preparation of glycerol aliquots (see **Notes 32-33**).
2. Prepare M9 media and add inducers (IPTG and xylose) in the same concentration that you plan to use for the experiment. See Section 3.9 for notes on choosing these concentrations.
3. Pick a colony, inoculate in 2 ml M9 media and grow until early exponential phase (OD_{600} ~0.05). This typically requires 45-90 min.
4. Combine 1.4 ml culture with 600 µl of 50% glycerol as if making a freezer stock. Mix thoroughly. This will provide enough volume for 20 glycerol aliquots (50 µl each).
5. Measure the OD_{600} of the mixture. Use as a blank a mixture of 700 µl M9 media and 300 µl glycerol (50%). Record this OD.
6. Dispense 50 µl of the mixture into each of 20 PCR tubes. Place the tubes in a secondary container such as a 50 ml Falcon tube and label with the strain, date, and OD.
7. Place the tubes at -80°C. Alternatively, aliquots can be flash-frozen using liquid nitrogen.

3.8 Designing a light program

1. Use Iris to design your desired light program for the LPA ***(13)*** (see **Notes 34-40**).
2. Download the light program file onto an SD card containing the necessary dc and gcal files (see **Note 41**).

3.9 Running an experiment

1. Prepare the necessary quantity of M9 media. You will need 500 µl per well, along with a pilot tube of media (2 ml is usually sufficient) for OD_{600} measurements at the end of the experiment if applicable.
2. Add inducers (IPTG and xylose) to the media based on required dynamic range and basal expression level (see **Notes 42-45)**. Mix thoroughly. We recommend final inducer concentrations of 17.0 µM IPTG, 0.025% w/v xylose.
3. Pre-warm media to 37°C.
4. Separate the media into tubes for each strain being used for your experiment, as well as one pilot tube (uninoculated).
5. Thaw a single-use frozen aliquot for each strain and sub-inoculate into the pre-warmed media. We recommend employing a starting OD_{600} between 10^{-5} and 10^{-4}. Discard any remaining unused volume from the single-use aliquot.
6. Distribute media into wells of the optogenetic 24-well plate (ArticWhite opaque 24-well clear-bottom plates). We recommend 500 µl per well.
7. Seal the plate with a reflective seal aluminum foil seal and secure to the LPA device.

8. Activate the light program and allow cells to incubate at 37°C, shaking, while exposed to light signals. We recommend an experimental duration of 8-10 hours.
9. Remove plates from the incubator and remove the seal (see **Note 46**).
10. Perform endpoint assays on the cultures. Possible assays include measurement of optical density, microscopy, flow cytometry or FACS, Western blotting, or RNA-seq (see **Note 47**).

4. Notes

1. $P_{xylA(+47)}$ is an engineered form of P_{xylA} from plasmid pAX01, modified to eliminate disruptive elements ***(8,9)***.
2. Glycerol is used as the carbon source because glucose interferes with $P_{xylA(+47)}$.
3. A large batch of M9 media can be prepared ahead of time, separated into aliquots for different experiments, and stored at room temperature protected from light. However, if using this approach, the iron chloride solution and the calcium chloride solution must be withheld from the batch media preparation and added fresh before the experiment.
4. We recommend making large batches of 1 M IPTG and 50% w/v xylose, dividing into small aliquots (5-200 µl), and storing at -20°C.
5. The PPM bears a chloramphenicol resistance marker. A chloramphenicol selection is not necessary for this protocol, but you may wish to confirm chloramphenicol resistance in your strains. Carry out chloramphenicol selections at 5 µg/ml.
6. Chloramphenicol and Kanamycin tend to be weak/unreliable selective markers in *B. subtilis*. We recommend using others when possible, or re-confirming antibiotic resistance after the original selection if using these two markers.

7. Additional modules, such as fluorescent reporters or genomic knockouts, may also be integrated into this strain. We recommend integrating additional modules into the *lacA* locus if necessary. Tetracycline and/or kanamycin can be used as selective markers at concentrations of 10 µg/ml and 5 µg/ml, respectively.
8. DMSO can be added to a final concentration of 3% v/v to disrupt secondary structure of template DNA during PCRs.
9. To enable strong expression of most any gene of interest, we also include the transcriptional insulator RiboJ and the strong RBS MF001 upstream of the target gene.
10. The published TOM, available in the genome of strain S051, uses *sfGFP* as the target gene. To replace it, we will construct a new linear integration module (LIM) with a new target gene, but maintaining homology for the *amyE* locus.
11. LIMs are double stranded DNA molecules containing the DNA payload to be integrated, flanked by homology arms that enable integration into genomic DNA via homologous recombination ***(14).*** Transformation of *in vitro*-assembled linear DNA fragments with sufficient homology to the genome (~2 kb on either flank) will result in genomic integration by homologous recombination.
12. Note that these sticky ends assume that your gene of interest begins with an ATG start codon and ends with a TAA stop codon; they can be modified if either of these is not true.
13. Boiled cell culture can be used as template (2 µl/50 µl reaction).
14. **Table 3** provides the recommended annealing temperature and extension time if using NEB Phusion polymerase. Adjust these parameters if using a different polymerase.

15. We recommend the use of GC buffer for PCR reactions using *B. subtilis* genomic DNA as a template.
16. Note that the temperature of the restriction digestion step (Step 1) may need to be adjusted if using an enzyme other than BsaI.
17. Keep completed Golden Gate reactions at 4°C until transformation.
18. We will leverage the natural competence of *B. subtilis*, which arises under conditions of nutrient deprivation
19. Avoid pelleting cells at low temperatures (4°C or less), as *B. subtilis* cells will lyse.
20. We recommend using fresh competent cells when possible, and only using fresh competent cells when transforming Golden Gate reactions.
21. The custom TOM is first integrated into the genome of wildtype cells in order to enable identification of constructs with the correct sequence, without the presence of the full system, which may exert a mutagenic pressure on the TOM construct. Additionally, the genomes of the strain containing only the TOM may be purified and used for easy integration of the TOM on other strains.
22. If you have less than 30 µl Golden Gate reaction, complete with water.
23. 400 ng of DNA in 30 µl of Golden Gate reaction is usually recommended; 200 ng is sufficient if the resistance marker is spectinomycin (as it is for the TOM).
24. When the resistance marker is spectinomycin, 1 hour is sufficient. Otherwise, 2.5 hours is recommended.
25. When transforming DNA to *B. subtilis* recipient strains already bearing selective markers in other locii, only the antibiotic corresponding to the selective marker in the donor DNA

should be used for selection. Including additional selective markers is unnecessary and places excessive stress on cells, sometimes reducing transformation efficiency.

26. Alternatively, PCR amplification of the *amyE* locus can be used to confirm integration; make sure to design the PCR reaction such that at least one primer is outside the linear fragment which was transformed.
27. We recommend including wildtype cells (PY79) as a negative control and the published TOM strain S051 as a positive control for this test. These strains can be struck out on LB agar and grown overnight along with the transformation plate, then grown to exponential phase and spot platted onto the starch plates along with the new strains being tested.
28. A strain containing both the PPM and LSM forms the chassis for the engineered system. We refer to this strain as S065. S065 can be constructed by transforming the purified genome of S061 to competent cells of S028.
29. Note that S065 only needs to be constructed once, and can be used as a recipient strain for any custom TOM.
30. When transforming a GIM, always ensure both the GIM strain and recipient strain are the same base strain (e.g. PY79) of *B. subtilis* to avoid an uncertain genotype.
31. Also, we recommend transforming one GIM containing a single new integration module at a time.
32. Generating precise and reproducible experimental results from optogenetic experiments requires consistent patterns of cell growth on different days. To achieve this goal, we begin experiments from single-use aliquots of cells frozen during exponential growth at a specified optical density at 600 nm (OD_{600}). We have found that this approach enables reproducible growth on different days, as well as minimizes lag phase.

33. Alternatively, exponential phase precultures can be diluted to a starting OD_{600} between 10^{-5} and 10^{-4} and used to start the experiment.
34. LPA devices generate custom light signals according to a Light Program File (.lpf) placed on an SD card. Gerhardt et al (2019) describes how to use the Iris software to program basic to advanced light signals ***(13)***.
35. We recommend employing a total experiment duration of 8-10 hours, with 3-5 hours of red light pre-conditioning at 20 µmol m^{-2} s^{-1}. This preconditioning step allows the cells to grow and adjust to experimental conditions before the CcaSR system is activated by green light.
36. When determining how many wells to use for each experimental condition, consider the number of replicates necessary to achieve the desired statistical power. Also consider the required amount of material for downstream analysis, remembering that the experimental time course should conclude before the cells enter stationary phase, as the function of the CcaSR system is likely to change during stationary phase growth.
37. Be sure to set aside wells for appropriate controls (e.g. cells lacking fluorescent reporters to be used as measures of autofluorescence).
38. If light exposures of various durations are required, consider using a staggered-start approach to ensure all samples will finish simultaneously, enabling easier downstream assays ***(15).***
39. The maximum recommended green light intensity is 50 µmol m^{-2} s^{-1}.
40. When programming light signals, consider that patterns of target gene expression will not resemble the dynamical light signals. Green and red light intensity control the fraction of active sensor kinase in the cell, which in turn influences the amount of target gene

transcription according to a Hill function. If simple behaviors (e.g. “turn on expression at t = 6 hours” or “slowly increase expression over the course of 2 hours”) are required, light programs can be easily designed to achieve these behaviors. However, if more complex and/or precise patterns of gene expression are required, a mathematical model of how light signals determine gene expression is necessary. A simple ODE model that replicates light response dynamics is provided by Castillo-Hair et al (2019) ***(8,16)***.

41. We recommend inserting the card into the LPA device and starting the device to ensure the file exhibits the anticipated behavior.
42. The dynamic range of the CcaSR system will be determined by the level of steady-state expression of the CcaS and CcaR proteins within the cell. For example, a higher steady-state level of CcaR is predicted to increase the rate of basal expression of the target gene due to increased auto-dimerization and binding to $P_{cpcG2\text{-}veg}$. Chemical inducer levels should be selected to achieve the desired expression range and basal expression level (i.e. leaky expression of the target gene in red light).
43. If using our M9 media formulation, the following inducer concentrations can be used: 17.0 μM IPTG, 0.025% w/v xylose. These inducer concentrations enable a 13.5x fold change in steady state expression of *sfGFP* from saturating red light to saturating green light, with a basal expression of 1.9×10^3 MEFL under saturating red light--see calibration data in **Figure 3**.
44. If a larger fold change is desired, inducer concentrations of 10.0 μM IPTG and 0.010% xylose (w/v) can be employed with reduced iron (III) citrate concentration (reduce from 50 mM to 1 mM) to achieve a 70x fold change with similar basal expression. This is the inducer scheme used in Castillo-Hair et al (2019) ***(8)***, and inducer calibration data is

available in the supplementary material. Note that reducing iron (III) citrate concentration increases the rate of mutational inactivation of the PPM, preventing the use of glycerol aliquots. Sub-inoculation of an exponentially growing culture must be used instead.

45. The basal expression level and dynamic range generated by the provided inducer combinations will not be suitable for all applications. For example, if a toxic gene is being expressed, basal expression levels will likely be too high, even under saturating red light, and inducer concentrations will need to be adjusted. If the target gene product is easy to measure (e.g. a fluorescent protein), various combinations of inducers can be tried, with each combination being exposed to saturating green light and saturating red light, as in **Figure 3**. This approach enables determination of inducer levels that offer desirable dynamic range and basal expression levels. Alternatively or in addition, genetic design tools, such as alteration of the ribosome binding site preceding the target gene, as well as the addition of protein degradation tags, can be employed ***(17)***.
46. Because the CcaSR light response is fast (i.e. seconds to minutes), cell phenotype could change rapidly upon exposure to ambient light after removal from the LPA device. Therefore, we recommend keeping samples covered as long as possible and performing assays immediately upon removal.
47. Perturbations to *B. subtilis* gene regulatory networks may produce altered phenotypes, such as increased rates of sporulation, chaining, or biofilm matrix production. Ensure that assays employed are robust to such variation.

5. References

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6. Figure Captions

Figure 1: CcaSRv1.0 strain overview. The complete system includes three linear integration modules in separate genomic loci.

Figure 2: Cloning scheme for CcaSRv1.0 with a new target gene. (a) Three PCR reactions are required to generate the fragments necessary for the Golden Gate reaction. (b) A Golden Gate reaction is employed to ligate three fragments according to sticky ends introduced by the primers. (c) The final custom LIM generated by the Golden Gate reaction.

Figure 3: Inducer concentration calibration heatmap. Data represents fold change in sfGFP expression levels grown under red and green light, as measured by the ratio of fluorescence levels obtained under the two conditions. Data was obtained from single replicates.

7. Table Captions

Caption for Table 2: **Bold** text indicates Golden Gate sticky ends. Underlined text indicates the BsaI recognition sequence.

8. Tables

Table 1: BGSC strains

Name	**Description**	**BGSC code**	**GenBank Accession Number**
S028	*thrC*::PPM v0.3	1A1606	MK995048
S051	*amyE*::TOM v0.2	1A1608	MK995068

S061	*xylA*::LSM v0.3a	1A1607	MK995072
S063	CcaSR v1.0 (*thrC*::PPMv0.3; *amyE*::TOMv0.2; *xylA*::LSMv0.3a)	1A1609	N/A

Table 2: Recommended primers for PCR

Primer Name	**Sequence**	**Reaction Partner**
prTOM_5_FWD	GTTCAGCTCAGTGATACCTGC	prTOM_5_REV
prTOM_5_REV	TTAGGTCTCG***CATA***GTAGTTCCTCCTTATGTAAGCTT	prTOM_5_FWD
prTOM_3_FWD	TTAGGTCTCG***TAAC***CAGGCATCAAATAAAACGAAAGG	prTOM_3_REV
prTOM_3_REV	GCGAAGCCGGTAAAGAAGAAG	prTOM_3_FWD
prTOM_GOI_FWD (custom)	TTAGGTCTCG***TATG***_____ [insert sequence to amplify gene of interest (GOI), 5' end]	prTOM_GOI_REV
prTOM_GOI_REV (custom)	TTAGGTCTCG***GTTA***_____ [insert sequence to amplify gene of interest, 3' end reverse complement]	prTOM_GOI_FWD

Table 3: Recommended PCR conditions

Fragment	**Forward Primer**	**Reverse Primer**	**Annealing Temperature * [°C]**	**Length** [kb]	**Extension Time***	**Template**
5’	prTOM_5_FWD	pr_TOM_5_REV	63	3.8	170 s	S051**
3’	prTOM_3_FWD	prTOM_3_REV	63	4.6	210 s	S051**
GOI	prTOM_GOI_FWD	prTOM_GOI_REV	–	–		–

Table 4: Golden Gate Thermocycler Program

Step	**Name**	**Temperature [°C]**	**Time [min]**
1	Restriction	37	3
2	Ligation	16	4
3	Go to 1, 24 times		
4	Final restriction	50	5
5	Enzyme inactivation	80	5
6	Hold	4	Forever

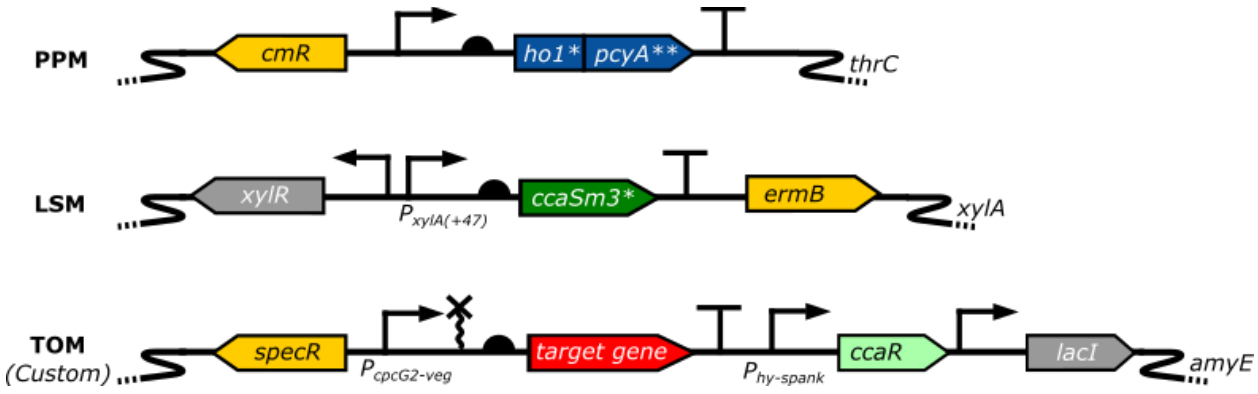
PPM
cmR
ho1*
pcyA**
thrC
LSM
xylR
$P_{xylA(+47)}$
ccaSm3*
ermB
xylA
TOM
(Custom)
specR
$P_{cpcG2\text{-}veg}$
target gene
$P_{hy\text{-}spank}$
ccaR
lacI
amyE

Figure 1

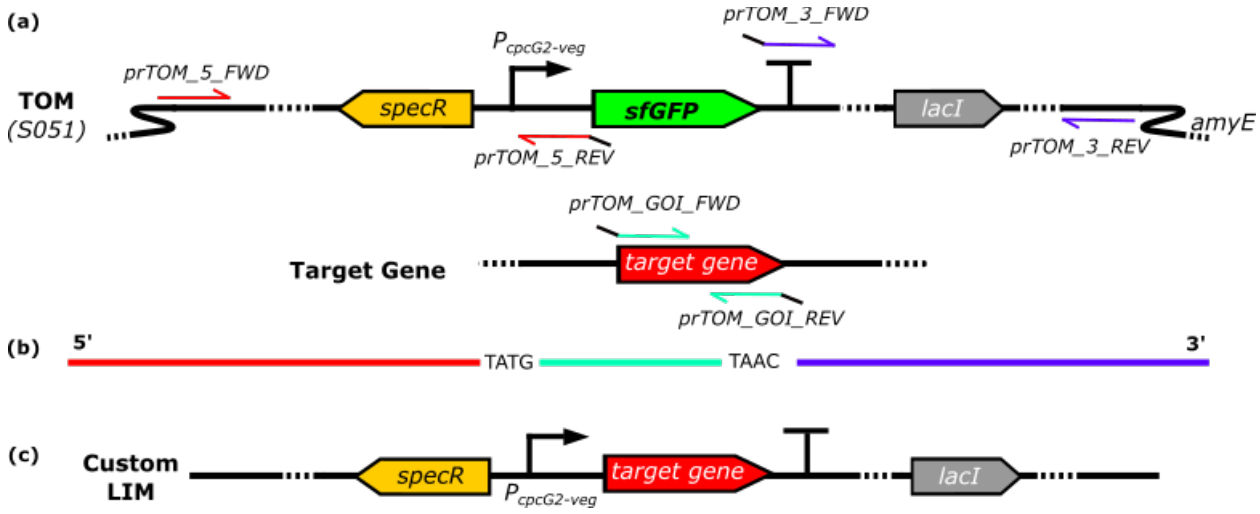
(a)
TOM
(S051)
prTOM_5_FWD
specR
P cpcG2-veg
sfGFP
prTOM_3_FWD
lacI
amyE
prTOM_5_REV
prTOM_3_REV
Target Gene
prTOM_GOI_FWD
target gene
prTOM_GOI_REV
(b)
5'
TATG
TAAC
3'
(c)
Custom
LIM
specR
P cpcG2-veg
target gene
lacI

Figure 2

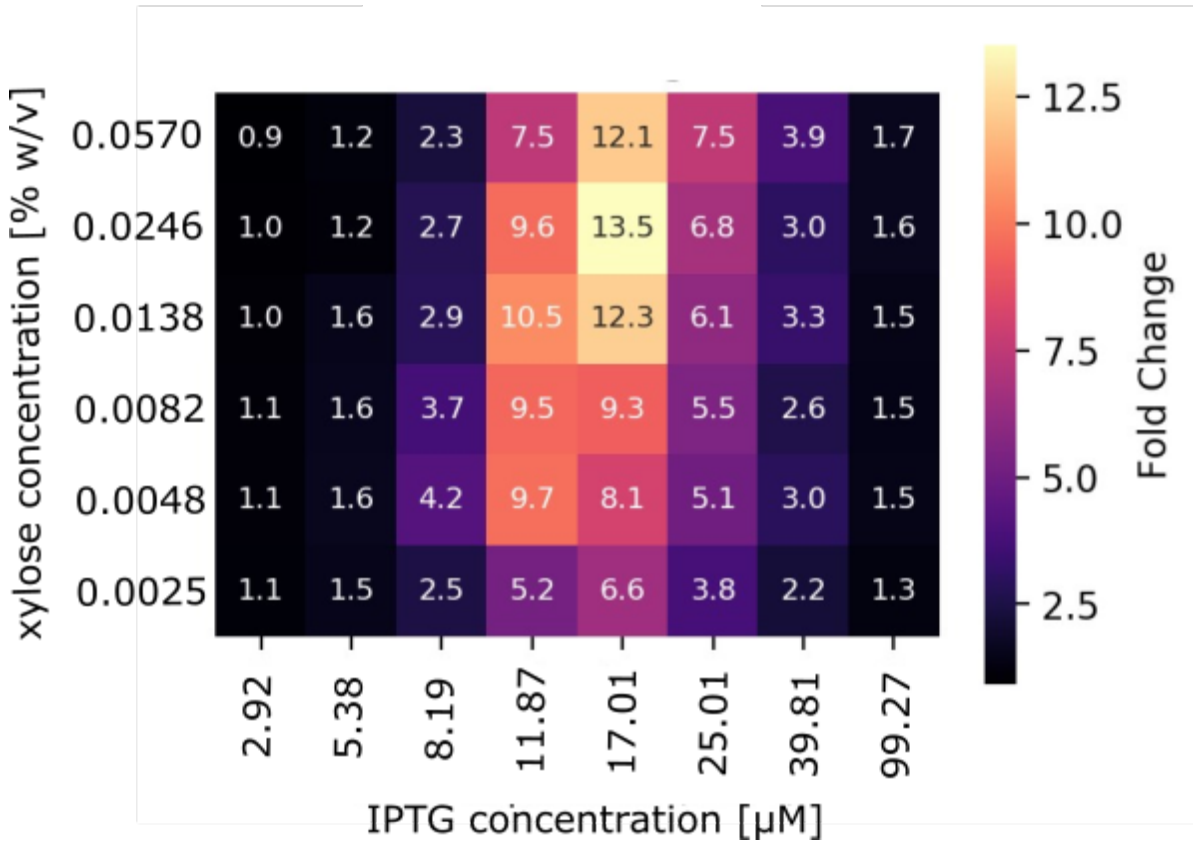
xylose concentration [% w/v]
0.0570
0.0246
0.0138
0.0082
0.0048
0.0025
0.9 1.2 2.3 7.5 12.1 7.5 3.9 1.7
1.0 1.2 2.7 9.6 13.5 6.8 3.0 1.6
1.0 1.6 2.9 10.5 12.3 6.1 3.3 1.5
1.1 1.6 3.7 9.5 9.3 5.5 2.6 1.5
1.1 1.6 4.2 9.7 8.1 5.1 3.0 1.5
1.1 1.5 2.5 5.2 6.6 3.8 2.2 1.3
2.92
5.38
8.19
11.87
17.01
25.01
39.81
99.27
IPTG concentration [μM]
12.5
10.0
7.5
5.0
2.5
Fold Change

Figure 3