Supplementary Material

Biosynthesis of Orthogonal Molecules Using Ferredoxin and Ferredoxin-NADP⁺
Reductase Systems Enables Genetically Encoded PhyB Optogenetics

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Illumination Circuits and Software.

To obtain programmable control needed to drive the high-power LEDs used in our experiments, we designed the light control system shown in Figure S5. Using this system, we have precise timing and light-intensity control for 8 experimental boxes that required red and/or far-red illumination. Each black box can house a standard 6-well, 12-well, 24-well, 96-well plate or can be fitted for a single dish with minimum modifications. The system can be replicated for experiments requiring a larger number of boxes or experimental conditions. Far-red and red lights can be controlled independently if placed in the same box. For our experimental setup, boxes contained either far-red 735nm LEDs or red 660nm LEDs. The light control system employs: (a) an Arduino Uno and voltage regulation circuits, managed through a (b) user interface developed in LabVIEW (National Instruments).

The voltage regulation circuit is shown in Figure S6. Coupled with the Arduino signals, this system delivers light pulses with precise timing and intensity control to the experiment boxes. The circuit is built using a LM317T linear voltage regulator (STMicroelectronics), a NPN general-purpose amplifier (2N2222, Fairchild Semiconductors), a resistor and a trimmer potentiometer (Helitrim, model 75PK10K). An external power supply was outfitted for the circuit (Safety Mark, 12V 1.5A Switch-mode power supply). The power supply allows the circuit to vary its current and voltage needs depending upon the intensity chosen by a user using the trimmer potentiometer.

The LabVIEW user interface, available for download at https://github.com/mcatanho/Kyriakakis_et_al_SupplementaryFiles (See Supplementary Note), controls the Arduino and connected circuits. It allows the user to connect to the Arduino effortlessly and to control experimental conditions such as time delay before illumination, a total duration of sample illumination, and pulse frequencies for each individual illumination box. It also contains digital displays of all relevant experimental times (Figure S5).
Kinetic Model Development and Parametrization.

We demonstrate the biochemical interactions among the enzymes shown in Figure 1A in the production of PCB through a kinetic model developed with the PySB framework. The model’s code, equations, and simulation files are available for download at https://github.com/mcatanho/Kyriakakis_et_al_SupplementaryFiles (See Supplementary Note). The quantitative mathematical model was parametrized (Table S5) by experimental data and uses ordinary differential equations to describe the changes in the concentration of the molecular components of the reaction.

For the model, we assume that the production of PCB can be described by the set of sequential steps shown in Table S4, and depicted in Figure 1A. This kinetic model builds upon Tu et al. description of the four electron reduction of biliverdin IX-alpha (BV) to phycocyanobilin (PCB), catalyzed by cyanobacterial phycocyanobilin:ferredoxin oxidoreductase (PcyA). As demonstrated experimentally in this work, the ferredoxin (Fd) and ferredoxin:oxidoreductase (FNR) complex is of paramount importance to the redox metabolism in plants and cyanobacteria, working as an electron transfer complex to reduce or oxidize enzymes in different pathways, further acting to reduce or NADP+ to NADPH or the reverse of this reaction. As described in Figure 1A, the first step in the PCB production pathway involves the formation of the HO1:Heme complex, which receives electron transfers from reduced ferredoxin (Fd\text{red}), producing BV. Following a PcyA:BV complex is formed, which in turn also receives electron transfers from Fd\text{red}, leading to the production of PCB. As the preferred electron donor for HO1 and PcyA, reduced Fd allows for continuous turnover of those enzymes in the PCB production pathway.

The reactions described above to produce PCB are shown in Scheme 1. The model assumes that those molecules are present in vitro at stoichiometry levels compatible with our transient transfection plasmid ratio. For simplicity, the model ignores differences in overall expression and degradation of each enzyme. Our model does not assume degradation of heme or BV, since we assumed there were saturating amounts in the cell medium. We also assume
that the oxidized ferredoxin, a result of the electron transfer to the HO1:Heme and PcyA:BV complexes, is renewed in the NADP+/NADPH pathway catalyzed by FNR. We probed the proposed model directly as proposed in literature \(^{2,7,8}\), and similar pathways published. We complement this work showing the model’s agreement with the hypothesized pathway, confirming that in the presence of heme, Fd and FNR are the rate limiting factors to produce PCB, confirmed experimentally in Figure 1B. We also show in Figure S7A, how PCB’s production dependence on Heme and the NADP/NAPDH pathway, characterized by the presence of Fd and FNR, are interlinked.

**Design and Parametrization of the Mathematical Model.**

Coupled, first order, ordinary differential equations (ODEs), parametrization of the model was performed using previously reported endogenous PCB production curves \(^8\). The reaction schemes below were translated into the PySB rule-based language. Rates were calculated through a parametric sweep method utilizing maximum-likelihood minimization for model-fitting procedures. The rule-based model simulates PCB production, following the reactions described in the **Scheme 1** below.

**Scheme 1**

1. Formation of the Heme and HO1 complex
2. Formation of Fd\(_{\text{red}}\):HO1:Heme complex, electron transfer from Fd\(_{\text{red}}\), producing BV
3. Formation of the BV:PcyA complex
4. Fd\(_{\text{red}}\):PcyA:BV complex formation, and electron transfer from Fd\(_{\text{red}}\), producing PCB
5. FNR-enabled Fd reduction
6. Spontaneous degradation of PCB, as described by Mueller *et al.* \(^8\).
The set of coupled ordinary differential equations obtained from those reactions, following mass-action kinetics\(^9\), is shown in **Scheme 2**.

**Scheme 2**

\[
\frac{d[Heme](t)}{dt} = -k_1[Heme][H01] + k_2[H01:Heme]
\]

\[
\frac{d[H01](t)}{dt} = -k_1[Heme][H01] + k_2[H01:Heme] + k_6[H01:BV]
\]
\[\frac{d[Fd_{\text{red}}]}{dt}(t) = -k_9[PcyA:BV][Fd_{\text{red}}] + k_{10}[Fd_{\text{red}}:PcyA:BV] - k_3[Fd_{\text{red}}][H01:Heme] + k_13[Fd_{\text{oxy}}] + k_4[Fd_{\text{red}}:H01:Heme]\]  

(3)

\[\frac{d[Fd_{\text{oxy}}]}{dt}(t) = k_{11}[Fd_{\text{red}}:PcyA:BV] - k_{13}[Fd_{\text{red}}] + k_5[Fd_{\text{red}}:H01:Heme]\]  

(4)

\[\frac{d[PcyA]}{dt}(t) = k_8[PcyA:BV] + k_{12}[PcyA:PCB] - k_7[PcyA][BV]\]  

(5)

\[\frac{d[Heme:HO1]}{dt}(t) = k_1[Heme][H01] - k_3[Fd_{\text{red}}][H01:Heme] - k_2[H01:Heme] + k_4[Fd_{\text{red}}:H01:Heme]\]  

(5)

\[\frac{d[Fd_{\text{red}}:H01:Heme]}{dt}(t) = k_3[Fd_{\text{red}}][H01:Heme] - (k_4 + k_3)[Fd_{\text{red}}:H01:Heme]\]  

(6)

\[\frac{d[H01:BV]}{dt}(t) = k_5[Fd_{\text{red}}:H01:Heme] - k_6[H01:BV]\]  

(7)

\[\frac{d[BV]}{dt}(t) = -k_7[PcyA][BV] + k_6[H01:BV] + k_8[PcyA:BV]\]  

(8)

\[\frac{d[PcyA:BV]}{dt}(t) = -k_9[PcyA:BV][Fd_{\text{red}}] - k_8[PcyA:BV] + k_{10}[Fd_{\text{red}}:BV:PcyA] + k_7[BV][PcyA]\]  

(9)

\[\frac{d[Fd_{\text{red}}:PcyA:BV]}{dt}(t) = k_9[Fd_{\text{red}}][PcyA:BV] - (k_{10} + k_{11})[Fd_{\text{red}}:PcyA:BV]\]  

(10)

\[\frac{d[PcyA:PCB]}{dt}(t) = -k_{12}[PcyA:PCB] + k_{11}[Fd_{\text{red}}:PcyA:BV]\]  

(11)

\[\frac{d[PCB]}{dt}(t) = k_{12} * [PcyA:PCB] - k_{\text{degPCB}} * [PCB]\]  

(12)

### 1.1.1. Fitting the Model to Experimental Data.

The model’s unknown parameters were determined by a maximum likelihood approach fitted to the data shown in Muller et al.\(^8\). Units are defined in S.I. units with concentrations as the number.
of molecules for species (\#molecules, or \(c\)), and parameters as bimolecular rate constants in \(\text{#molecules/s}^{-1}\) (or \(c/s^{-1}\)).

**Sum-of-Squares and Parameter Estimation**

We assume that the system of ordinary differential equations (ODE) shown in Scheme 2 can be represented as a dynamical system given by an \(N\)-dimensional state variable \(x(t) \in \mathbb{R}^N\), at time \(t \in I = [t_0, t_f]\), which is the unique and differentiable solution for the initial value problem given by:

\[
\dot{x}(t) = f(x(t), t, \theta) \quad x(t_0) = x_0
\]

As such, the ODE depends on certain parameters \(\theta \in \mathbb{R}^{np}\). Also, let \(Y_i\) denote the data of measurement \(i = 1, \ldots, n\), where \(n\) represents the total amount of data. Moreover, the data \(Y_i\) satisfies \(Y_i = g(t_i, \theta) + \sigma_i \epsilon_i\), for some function \(g : \mathbb{R}^d \rightarrow \mathbb{R}^{obs}\), and \(d \geq obs, \sigma_i > 0\) and \(\epsilon_i\) are independent and standard Gaussian distributed random variables. The function \(g(\cdot)\) is continuously differentiable. To estimate the parameters \(\theta\), given the initial conditions, utilizing the principle of maximum-likelihood to yield a cost function to be minimized gives us:

\[
\mathcal{L}(\theta) = \sum_{i=1}^{n} \frac{(Y_i - g(x(t_i; \theta), \theta))^2}{2\sigma_i^2}
\]

We perform a direct minimization of \(\mathcal{L}\) with respect to \(\theta\) to obtain the parameters shown in Table S5, and used throughout the experiments described next.

**Implementation of Experiments.**

Our model was used to gain insight into the dependencies of this pathway and to further validate our experimental results. HO1 and PcyA were assumed to be at equimolar amounts and Fd at
1/10\(^{th}\) of that molar concentration. Unless stated otherwise, the following initial conditions were used. If not listed, the initial concentrations were set to zero at \(t=0\).

\[
\begin{align*}
[Heme](0) &= 100 \\
[HO1](0) &= 10 \\
[Fd_{\text{red,oxi}}](0) &= 5 \\
[PcyA](0) &= 10 \\
\end{align*}
\]

**Experiment 1: Fd and Heme dependence**

We determined experimentally the rate limiting factors are Fd, followed by Fd+FNRI and finally heme. To model this experimental result, we performed a sweep over initial concentrations of Fd ([Fd](0)), heme ([Heme](0)), and rate of renewal of Fd by FNRI (\(k_{13}\)). The result of those sweeps are shown in **Figure S7A** (Heme concentration vs. Fd renewal) and **Figure S7B** (Heme concentration vs. Fd concentration). The resulting graphs show the dependency of PCB production on those molecules, and how the initial condition of each affects the rate of production of PCB.

**Experiment 2: 2E vs 4E.**

Our experimental results show that PCB is only produced to high levels under the presence of Fd, PcyA, and HO1. To model this experimental result, we modified the following parameters to simulate the lack of compatible Fd, namely a “two enzyme” (2E) case, that limits the production of PCB versus the output of the pathway when all four enzymes (4E) are present. For the 2E case, we set [Fd_{\text{red,oxi}}](0) to zero (**Figure S8A**).

**Experiment 3: Species Specificity as Demonstrated by Different Binding Coefficients.**

To demonstrate how the species specificity between Fd and HO1/PcyA plays a pivotal role in the amount of PCB produced, we performed a decreasing sweep through the parameters \(k_3\) and \(k_9\),
which control binding of HO1 and PcyA to Fd respectively. The sweeps were started at the
parameter’s value as described in Table S5 to 1e-3 c/s⁻¹. The resulting graph is shown in Figure
S8B.

Experiment 4: Variable Levels of Heme.
In this experiment, we performed a sweep over a range of Heme concentrations, from 100, 10, 5,
1 and 0.1 c. This experiment, similar to Figure S7, shows the heme dependency of PCB
production. The respective graph is shown in Figure S8C.
SUPPLEMENTARY TABLES

Table S1. Similarity Tables for Ferredoxin and Ferredoxin-dependent Bilin Reductases.

(A) The similarity of ferredoxin-dependent bilin reductases and similarity of Fds. (B) The similarity of ferredoxins with eukaryotic sequences containing signal sequences. (C) The similarity of ferredoxins with eukaryotic sequences with signal sequences removed. Sequence alignments were done using UniProt (http://www.uniprot.org/).

Fd types: ■ Cyanobacterial; ■ Chloroplastic; ■ Mitochondrial.
Species: ■ Cyanobacterial, ■ Arabidopsis, ■ Yeast; ■ Human.
Table S2. Plasmids Used in This Study.

Genes for enzymes were synthesized by Genscript and Integrated DNA Technologies. Plasmids and sequences will be made available on Addgene or upon request.

| Plasmid Number | Description                                      | Source          | Addgene Plasmid ID |
|----------------|--------------------------------------------------|-----------------|--------------------|
| pMZ-802        | FLuc under control of pTet (tetO13-CMVmin-FLuc-pA) | Müller et al.   | N/A                |
| pPKm-102       | pcDNA3 - mOrange                                 | This study       | 90493              |
| pPKm-105       | pcDNA3 - PhyB NT - GBD,                          | This study       | 104853             |
| pPKm-112       | pcDNA3 - MTAD - PIF3,                            | This study       | 90494              |
| pPKm-113       | pcDNA3 - MTAD - PIF6,                            | This study       | 90495              |
| pPKm-118       | pcDNA3 - 5X UAS - pFR Luciferase                 | This study       | 90491              |
| pPKm-145       | Empty plasmid, pSIN-EF1-alpha-IRES-puro         | This study       | 90505              |
| pPKm-163       | pcDNA3 - PIF3 - GBD,                             | This study       | 104854             |
| pPKm-195       | pcDNA3 - PhyB NT - MTAD                          | This study       | 90496              |
| pPKm-196       | pcDNA3 - PIF6-DBD                                | This study       | 90511              |
| pPKm-202       | pcDNA3 – CMVmin 5X UAS - pFR - Luciferase        | This study       | 90492              |
| pPKm-226       | pcDNA3 - PIF3 – VPR                              | This study       | 90497              |
| pPKm-227       | pcDNA3 - VPR - PIF3                              | This study       | 90498              |
| pPKm-230 | pSIN - EF1-alpha - PIF3 - MTAD - IRES - PhyB - GBD | This study | 90499 |
| pPKm-231 | pSIN - EF1-alpha - MTS - tFd - P2A - MTS - tFNR, encoding for mitochondrial-tagged *Thermosynechococcus elongatus* Ferredoxin (Fd) and Ferredoxin-NADP(+) oxi0doreductase (FNR) | This study | 90500 |
| pPKm-232 | pSIN - EF1-alpha - MTS tHO1 - P2A - MTS - tPCYA, encoding for mitochondrial-tagged *Thermosynechococcus elongatus* Heme Oxygenase-1 (HO1) and phycocyanobilin:ferredoxin oxidoreductase (PcyA) | This study | 90501 |
| pPKm-233 | pSIN - EF1-alpha - sFD - P2A - MTS - sFNR, encoding for *Synechococcus sp.* Ferredoxin (Fd) and Ferredoxin-oxidoreductase (FNR) | This study | 90508 |
| pPKm-234 | pSIN - EF1-alpha - MTS sHO1 - P2A - MTS - sPCYA, encoding for mitochondrial-tagged *Synechococcus sp.* Heme Oxygenase (HO1) and phycocyanobilin:ferredoxin oxidoreductase (PcyA), | This study | 90507 |
| pPKm-235 | pSIN - EF-1alpha - MTS sHO1 - P2A - MTS - sPCYA, | This study | 90509 |
| pPKm-240 | pSIN - EF1-alpha cyto-sHO1-P2A – cyto-sPcyA, encoding for cytoplasmic-tagged *Synechococcus sp* HO1 and PcyA | This study | 90510 |
|----------|-------------------------------------------------------------------------------------------------|----------|------|
| pPKm-241 | pSIN - EF1-alpha - cyto-sFd - P2A - cyto-sFNR, vector encoding for cytoplasmic-tagged *Synechococcus sp* Fd and FNR | This study | 104855 |
| pPKm-234 | pSIN - EF1-alpha - mOrange-P2A-mitosfGFP, mOrange and mitochondrial-tagged sfGFP | This study | 90506 |
| pPKm-244 | pSIN – EF1-alpha - MTS - tHO1 - P2A - MTS - tPCYA - IRES - MTS - tFD - P2A - MTS - tFNR | This study | 90502 |
| pPKm-245 | pSIN - EF1-alpha - MTS - tHO1 - P2A - MTS - tPCYA - P2A - MTS - tFD - P2A - MTS - tFNR | This study | 90503 |
| pPKm-248 | pSIN - EF1-alpha - MTS - tPCYA - IRES - MTS - tHO1 - | This study | 90504 |
| Vector | Description | Source | Part Number |
|--------|-------------|--------|-------------|
| pPKm-292 | pcDNA3 – GAL4_DNA BD - MTAD | This study | 105816 |
| pPKm-293 | pcDNA3 – TET DNA BD - MTAD | This study | 105817 |
| pPKm-300 | pSIN - EF1-alpha - MTS - tFd, encoding for mitochondrial-tagged *Thermosynechococcus elongatus* Ferredoxin (Fd) | This study | 104626 |
| pRL-TK | Control reporter for constitutive expression of wildtype Renilla luciferase (Rluc) under pRL-TK | Promega | E2241 |
Table S3. Transfection and Illumination Details for each Figure.

**Figure 1B**
HEK293 cells were transfected 24 hours after plating. Calculations are for each well. Transfected in a 6 well plate. Cells were harvested 44 hours post-transfection followed by Immunoprecipitation and Zn-PAGE as described in methods.

|                  | Plasmid | DNA mass (ng) | DNA Ratio |
|------------------|---------|---------------|-----------|
| NE control       | pPKm-105| 125           | 1/20      |
|                  | pPKm-102| 125           | 1/20      |
|                  | pPKm-145| 1125          | 18/20     |
| M2-sPcyA         | pPKm-105| 125           | 1/20      |
|                  | pPKm-243| 125           | 1/20      |
|                  | pPKm-234| 1125          | 9/20      |
|                  | pPKm-234| 1125          | 9/20      |
| M4-sPcyA         | pPKm-105| 125           | 1/20      |
|                  | pPKm-243| 125           | 1/20      |
|                  | pPKm-234| 1125          | 9/20      |
|                  | pPKm-233| 1125          | 9/20      |
| M2-tPcyA         | pPKm-105| 125           | 1/20      |
|                  | pPKm-243| 125           | 1/20      |
| M4-iPcyA | Plasmid | DNA mass (ng) | DNA Ratio |
|----------|---------|--------------|-----------|
| pPKm-105 | 125     | 1/20         |
| pPKm-243 | 125     | 1/20         |
| pPKm-232 | 1125    | 9/20         |
| pPKm-231 | 1125    | 9/20         |

| M2-Hy2   | Plasmid | DNA mass (ng) | DNA Ratio |
|----------|---------|--------------|-----------|
| pPKm-105 | 125     | 1/20         |
| pPKm-243 | 125     | 1/20         |
| pPKm-235 | 1125    | 9/20         |
| pPKm-145 | 1125    | 9/20         |

| M4-Hy2   | Plasmid | DNA mass (ng) | DNA Ratio |
|----------|---------|--------------|-----------|
| pPKm-105 | 125     | 1/20         |
| pPKm-243 | 125     | 1/20         |
| pPKm-235 | 1125    | 9/20         |
| pPKm-233 | 1125    | 9/20         |

HEK293 cells were transfected 24 hours after plating. Calculations are for each well. Transfected two of each in a 6 well plate, one with and one without heme. 10μM (Frontier scientific) was added 18 hours and 43 hours post-transfection. Cells were harvested 44 hours post transfection followed by Immunoprecipitation and Zn-PAGE as described in methods.
| NE control | Plasmid | DNA mass (ng) | DNA Ratio |
|------------|---------|---------------|-----------|
|            | pPKm-105 | 125           | 1/20      |
|            | pPKm-243 | 125           | 1/20      |
|            | pPKm-145 | 1125          | 18/20     |

| C2         | Plasmid | DNA mass (ng) | DNA Ratio |
|------------|---------|---------------|-----------|
|            | pPKm-105 | 125           | 1/20      |
|            | pPKm-243 | 125           | 1/20      |
|            | pPKm-240 | 1125          | 9/20      |
|            | pPKm-145 | 1125          | 9/20      |

| C4         | Plasmid | DNA mass (ng) | DNA Ratio |
|------------|---------|---------------|-----------|
|            | pPKm-105 | 125           | 1/20      |
|            | pPKm-243 | 125           | 1/20      |
|            | pPKm-240 | 1125          | 9/20      |
|            | pPKm-241 | 1125          | 9/20      |

| M2         | Plasmid | DNA mass (ng) | DNA Ratio |
|------------|---------|---------------|-----------|
|            | pPKm-105 | 125           | 1/20      |
|            | pPKm-243 | 125           | 1/20      |
|            | pPKm-234 | 1125          | 9/20      |
|            | pPKm-145 | 1125          | 9/20      |

| M4         | Plasmid | DNA mass (ng) | DNA Ratio |
|------------|---------|---------------|-----------|
|            | pPKm-105 | 125           | 1/20      |
HEK293 cells were transfected 24 hours after plating. Calculations are for each well in a 6-well plate. Cells were harvested 44 hours post transfection followed by Immunoprecipitation and Zn-PAGE as described in methods.

| Plasmid   | DNA mass (ng) | DNA ratio |
|-----------|---------------|-----------|
| pPKm-105  | 125           | 1/20      |
| pPKm-243  | 125           | 1/20      |
| pPKm-232  | 1125          | 9/20      |
| pPKm-145  | 1125          | 9/20      |
| pPKm-231  | 1125          | 9/20      |
| pPKm-300  | 1125          | 9/20      |
### NE

| Plasmid | DNA mass (ng) | DNA ratio |
|---------|---------------|-----------|
| pPKm-105 | 125           | 1/20      |
| pPKm-243 | 125           | 1/20      |
| pPKm-145 | 2250          | 18/20     |

### Figure 3D

HEK293 cells were transfected 24h after plating, followed by a medium change 24h after transfection. For illumination, 1μmol/m2/s 1-minute pulses of red light were delivered for 24h, starting 12h after the medium change. Cells were kept in darkness before and after illumination. Lysis was performed 72h after transfection, and samples stored in -20C until assayed.

### 9HP:9EV (1:1 ratio HP:EV)

| Plasmid | DNA mass (ng) | DNA ratio |
|---------|---------------|-----------|
| pPKm-102 | 425.0         | 25.5/30   |
| pPKm-105 | 16.7          | 1/30      |
| pPKm-112 | 16.7          | 1/30      |
| pPKm-232 | 16.7          | 1/30      |
| pPKm-202 | 16.7          | 1/30      |
| pRL-TK   | 8.3           | 0.5/30    |

### 9HP:9FF (1:1 ratio HP:FF)

| Plasmid | DNA mass (ng) | DNA ratio |
|---------|---------------|-----------|
| pPKm-102 | 408.3         | 24.5/30   |
| pPKm-105 | 16.7          | 1/30      |
| pPKm-112 | 16.7          | 1/30      |
| pPKm-232 | 16.7          | 1/30      |
| pPKm-231 | 16.7          | 1/30      |
| pPKm-202 | 16.7          | 1/30      |
| Plasmid | DNA mass (ng) | DNA ratio |
|---------|--------------|-----------|
| pRL-TK  | 8.3          | 0.5/30    |

### 17HP:1EV (17:1 ratio HP:EV)

| Plasmid | DNA mass (ng) | DNA ratio |
|---------|--------------|-----------|
| pPKm-102 | 158.3       | 9.5/30    |
| pPKm-105 | 16.7        | 1/30      |
| pPKm-112 | 16.7        | 1/30      |
| pPKm-232 | 283.3       | 17/30     |
| pPKm-202 | 16.7        | 1/30      |
| pRL-TK   | 8.3         | 0.5/30    |

### 17HP:1FF (17:1 ratio HP:FF)

| Plasmid | DNA mass (ng) | DNA ratio |
|---------|--------------|-----------|
| pPKm-102 | 141.7       | 8.5/30    |
| pPKm-105 | 16.7        | 1/30      |
| pPKm-112 | 16.7        | 1/30      |
| pPKm-232 | 283.3       | 17/30     |
| pPKm-231 | 16.7        | 1/30      |
| pPKm-202 | 16.7        | 1/30      |
| pRL-TK   | 8.3         | 0.5/30    |

**Figure 3F**

HEK293 Cells were transfected 24h after plating, followed by a medium change 24h after transfection. For illumination, 1 μmol/m²/s 1-minute pulses of red light were delivered for 24h, starting 12h after the medium change. Cells were kept in darkness before and after illumination. Cell lysis was performed 72h after transfection, and samples stored in -20°C until assayed.
| Plasmid   | DNA mass (ng) | DNA ratio |
|-----------|---------------|-----------|
| pPKm-102  | 10            | 1/50      |
| pPKm-230  | 225           | 22.5/50   |
| pPKm-245  | 225           | 22.5/50   |
| pPKm-202  | 20            | 2/50      |
| pRL-TK    | 20            | 2/50      |

**Figure 4,** Cells were transfected 24h after plating, followed by a medium change 24h after transfection. In Figure 4D, red light at 1μmol/m²/s, 0.1μmol/m²/s, 0.01μmol/m²/s and 0.001 μmol/m²/s were delivered for a total of 24 hours.

**Figure 5** Similarly, in Figure 4E, continuous illumination for 24h was delivered to the cells, in the intensities listed above. For Figure 4F, red light at 0.1 and 1μmol/m²/s was continuously delivered or shone for 1-minute pulses every
4 minutes, 9 minutes or 29 minutes, starting 12h after medium change for a total of 24h. For Figures 4G, red light at the intensity of 1μmol/m²/s was delivered to the cells every 30 minutes, every hour, every 2 hours, every 4 hours, 6 hours, 8 hours or every 12 hours. For Figure 5B, cells were kept in darkness, illuminated with far-red light, red light for 24 hours, or with 12 hours or red light followed by darkness or far-red light. For Figure 5C, cells were illuminated with red light at 1 μmol/m²/s and given a 1 min red light pulse every 5 minutes for 24 hours. In all cases, cells were kept in darkness before and after illumination. Far-red samples were kept under constant illumination starting at medium change. Cell lysis was performed 72h after transfection, and samples stored in -20C until assayed.

| All conditions | Plasmid  | DNA mass (ng) | DNA ratio |
|----------------|----------|---------------|-----------|
|                | pPKm-102 | 10            | 1/50      |
|                | pPKm-230 | 225           | 22.5/50   |
|                | pPKm-248 | 225           | 22.5/50   |
|                | pPKm-202 | 20            | 2/50      |
|                | pRL-TK   | 20            | 2/50      |
HEK293 Cells were transfected 24h after plating on polylysine-coated coverslips. 43 hours later media was changed with media+5µM PCB (Frontier Scientific P14137) added to the NE+PCB control. One hour later cells were rinsed in PBS and fixed in 4%Paraformaldehyde for 10 minutes. Next cells were incubated in permeabilization buffer (5% BSA + 0.3% TritonX-100 in PBS) for 30min, followed by primary antibodies overnight at 4°C in antibody buffer (2% BSA + 0.2% TritonX-100 in PBS; anti-flag mouse monoclonal 1:1000 (Sigma F3165) anti-HA rabbit polyclonal 1:500 (Santa Cruz Y-11)); Next coverslips were rinsed twice and washed three time in PBS and then incubated in antibody buffer with goat anti-mouse AlexaFluor 488 1:1000 (Thermo-Fisher A11001) goat anti-rabbit AlexaFluor 568 1:1000 (Thermo-Fisher A11011)). Coverslips were then mounted with Fluoromount-G (SouthernBiotech 0100-20). Images were taken using a DeltaVision RT Deconvolution Microscope.

| NE control | Plasmid    | DNA mass (ng) | DNA Ratio |
|------------|------------|---------------|-----------|
| pPKm-105   | 100        | 4/20          |
| pPKm-145   | 400        | 16/20         |

| C2         | Plasmid    | DNA mass (ng) | DNA Ratio |
|------------|------------|---------------|-----------|
| pPKm-105   | 100        | 4/20          |
| pPKm-240   | 375        | 15/20         |
| pPKm-145   | 25         | 1/20          |

| C4         | Plasmid    | DNA mass (ng) | DNA Ratio |
|------------|------------|---------------|-----------|
| pPKm-105   | 100        | 4/20          |
HEK293 cells were transfected 24 hours after plating. For this experiment, 15uM of PCB (Frontier Scientific) was added 47h after transfection. Light at 10 μmol/m²/s was delivered 1h after PCB was added, and cells were illuminated for 1 minute every 4 minutes for 24 hours. 24h after illumination, cells were lysed and stored at -20C until assay.

### Table: DNA Mass and Ratio

| Plasmid     | DNA mass (ng) | DNA Ratio |
|-------------|---------------|-----------|
| pPKm-105    | 100           | 4/20      |
| pPKm-234    | 375           | 15/20     |
| pPKm-233    | 25            | 1/20      |

**M2**

| Plasmid     | DNA mass (ng) | DNA Ratio |
|-------------|---------------|-----------|
| pPKm-240    | 375           | 15/20     |
| pPKm-241    | 25            | 1/20      |

**M4**

| Plasmid     | DNA mass (ng) | DNA Ratio |
|-------------|---------------|-----------|
| pPKm-105    | 100           | 4/20      |
| pPKm-234    | 375           | 15/20     |
| pPKm-233    | 25            | 1/20      |

**Figure S2C**

### Table: DNA Mass and Ratio

| Plasmid     | DNA mass (ng) | DNA Ratio |
|-------------|---------------|-----------|
| pPKm-102    | 1579          | 12/19     |
| pPKm-196    | 263           | 2/19      |
| pPKm-195    | 263           | 2/19      |
| pPKm-118    | 263           | 2/19      |
| pRL-TK      | 132           | 1/19      |

**P6-DBD**
| Plasmid   | DNA mass (ng) | DNA Ratio |
|-----------|---------------|-----------|
| pPKm-102  | 1579          | 12/19     |
| pPKm-163  | 263           | 2/19      |
| pPKm-195  | 263           | 2/19      |
| pPKm-118  | 263           | 2/19      |
| pRL-TK    | 132           | 1/19      |

**Figure S3C**

HEK293 cells were transfected 24h after plating, followed by a medium change 24h after transfection. For this experiment, 15uM of PCB (Frontier Scientific) was added 47h after transfection. Light at 1 μmol/m²/s in 1-minute pulses of red light was delivered 1h after PCB was added. Cells were kept in darkness before and after illumination. Lysis was performed 72h after transfection, and samples stored in -20C until assayed.
| P3-MTAD | Plasmid | DNA mass (ng) | DNA ratio |
|---------|---------|---------------|-----------|
| pPKm-102 | 325 | 33/50 |
| pPKm-105 | 50 | 5/50 |
| pPKm-112 | 50 | 5/50 |
| pPKm-118 | 50 | 5/50 |
| pRL-TK | 25 | 2/50 |

| P3-VPR | Plasmid | DNA mass (ng) | DNA ratio |
|--------|---------|---------------|-----------|
| pPKm-102 | 325 | 33/50 |
| pPKm-105 | 50 | 5/50 |
| pPKm-226 | 50 | 5/50 |
| pPKm-118 | 50 | 5/50 |
| pRL-TK | 25 | 2/50 |

| VPR-P3 | Plasmid | DNA mass (ng) | DNA ratio |
|--------|---------|---------------|-----------|
| pPKm-102 | 325 | 33/50 |
| pPKm-105 | 50 | 5/50 |
| pPKm-227 | 50 | 5/50 |
| pPKm-118 | 50 | 5/50 |
| pRL-TK | 25 | 2/50 |

HEK293 cells were transfected 24h after plating, followed by a medium change 24h after transfection. Cells were lysed 72h after transfection, and samples stored in -20C until assayed.
| Renilla                        | Plasmid    | DNA mass (ng) | DNA ratio |
|-------------------------------|------------|---------------|-----------|
|                               | pPKm-102   | 480           | 48/50     |
|                               | pRL-TK     | 20            | 2/50      |

| TET-UAS-CMVmin                | Plasmid    | DNA mass (ng) | DNA ratio |
|-------------------------------|------------|---------------|-----------|
|                               | pPKm-102   | 430           | 43/50     |
|                               | pMZ-802    | 50            | 5/50      |
|                               | pRL-TK     | 20            | 2/50      |

| G4-UAS-Flucmin                | Plasmid    | DNA mass (ng) | DNA ratio |
|-------------------------------|------------|---------------|-----------|
|                               | pPKm-102   | 430           | 43/50     |
|                               | pPKm-118   | 50            | 5/50      |
|                               | pRL-TK     | 20            | 2/50      |

| G4-UAS-CMVmin                | Plasmid    | DNA mass (ng) | DNA ratio |
|-------------------------------|------------|---------------|-----------|
|                               | pPKm-102   | 430           | 43/50     |
|                               | pPKm-202   | 50            | 5/50      |
|                               | pRL-TK     | 20            | 2/50      |

**Figure S4B** HEK293 cells were transfected 24h after plating, followed by a medium change 24h after transfection. Cells were lysed 72h after transfection, and samples stored in -20C until assayed.
| Plasmid   | DNA mass (ng) | DNA ratio |
|-----------|---------------|-----------|
| pPKm-102  | 380           | 38/50     |
| pPKm-293  | 50            | 5/50      |
| pMZ-802   | 50            | 5/50      |
| pRL-TK    | 20            | 2/50      |

| Plasmid   | DNA mass (ng) | DNA ratio |
|-----------|---------------|-----------|
| pPKm-102  | 380           | 38/50     |
| pPKm-292  | 50            | 5/50      |
| pPKm-202  | 50            | 5/50      |
| pRL-TK    | 20            | 2/50      |
Table S4. Parameters for the Model.

| Parameter | Value     | Description                                           |
|-----------|-----------|-------------------------------------------------------|
| k1        | 0.1228    | HO1 and heme binding rate                            |
| k2        | 1e-12     | HO1 and heme unbinding rate                          |
| k3        | 0.5687    | HO1:Heme and Fd$_\text{red}$ binding rate            |
| k4        | 1e-12     | HO1:Heme:Fd$_\text{red}$ unbinding rate              |
| k5        | 0.2285    | Fd$_\text{red}$:HO1:Heme unbinding, forming HO1:BV and Fd$_\text{oxy}$ |
| k6        | 0.4750    | HO1 unbinding from BV, releasing BV                   |
| k7        | 0.1825    | Rate of BV and PcyA binding, forming PcyA:BV         |
| k8        | 1e-12     | PcyA:BV unbinding rate                               |
| k9        | 0.2500    | PcyA:BV and Fd$_\text{red}$ binding rate, forming Fd$_\text{red}$:PcyA:BV |
| k10       | 1e-12     | Unbinding rate of Fd$_\text{red}$:PcyA:BV            |
| k11       | 0.1220    | Fd$_\text{red}$:PcyA:BV unbinding, forming PcyA:PCB and Fd$_\text{oxy}$ |
| k12       | 0.2667    | Unbinding of PcyA:PCB, producing PCB                 |
| k13       | 0.2250    | Reduction of Fd$_\text{oxy}$, forming Fd$_\text{red}$ |
| k$_{\text{deg,PCB}}$ | 0.1567 | Degradation of PCB                                   |
| Heme, at t=0 | 100       | Initial concentration of Heme                         |
| HO-1, at t=0 | 10        | Initial concentration of HO-1                        |
| PcyA, at t=0 | 10        | Initial concentration of PcyA                         |
| Fd$_\text{red,oxi}$, at t=0 | 5        | Initial concentration of Fd (red and oxi)            |

C: arbitrary unit of concentration.

Unless indicated otherwise, all other concentrations were considered to be zero.
**Figure S1.** Imaging endogenously produced PCB in mammalian cells. HEK293 cells were transfected with PhyB alone (NE), PhyB+5μM PCB (NE+PCB), cytoplasmic sho1+spcyA (C2), cytoplasmic sho1+spcyA+spetF+spetH (C4), mitochondrial sho1+spcyA (M2), or mitochondrial sho1+spcyA+spetF+spetH (M4). DAPI DNA stain was imaged using the DAPI channel (purple). PhyB tagged with HA was imaged using anti-HA (green), PcyA tagged with FLAG was imaged using anti-FLAG (red). PCB was imaged using the Cy-5 channel (blue). All images were taken under the same exposure and contrast settings using a 60X (1.40NA) objective.

IRES = Internal Ribosome Entry Site, NLS = Nuclear Localization Sequence, MTS = Mitochondria Targeting Sequence, P2A = 2A self-cleaving peptide, DBD = DNA Binding Domain, R/FR = Red light/Far-red light.
Figure S2: Optimizing PhyB and PIF light switches for mammalian cells. (A) HEK293 cells were transfected, illuminated and incubated in 15µM PCB as shown in the timeline. (B) Plasmid maps of the different PhyB and PIF designs. (C) Comparison of gene activation using several variations of PhyB-PIF light switchable promoters. The negative control consists of UAS luciferase plasmid alone. In the first two experimental conditions, PIF6 or PIF3 are fused to the DBD (P6-DBD and P3-DBD, respectively) and PhyB is fused to the AD (PhyB-AD). The second two experimental conditions contain PhyB fused to the DBD along with PIF6 and PIF3 fused to the AD (P6-AD and PIF3-AD, respectively). Fold gene expression was calculated comparing cells in red light to cells in darkness, after normalizing to a Renilla control (n=6).

DBD = DNA Binding Domain, AD = Activation Domain

(Order bars = s.d. (***) = p<0.001, Statistics were calculated using one-way ANOVA with Bonferroni post-test using GraphPad Prism 5.01).
**Figure S3:** Optimizing PhyB and PIF gene switch. (A) Timeline for experiments where HEK293 cells were transfected and illuminated for 24 hours. (B) Plasmid maps for constructs with MTAD and VPR activation domains fused to the C-terminal or N-terminal of PIF3. (C) Comparison of MTAD and VPR fusions with PIF3 effects of luciferase gene activation. Fold gene expression was calculated comparing cells incubated in red light to cells incubated in far-red light, after normalizing to a Renilla control (n=3).

DBD = DNA Binding Domain, AD = Activation Domain, MTAD = Minimal Trans-Activation Domain, VPR = VP64+P65+RTA, R/FR = Red light/Far-red light

( Error bars = s.d. (***) = p<0.001, Statistics were calculated using one-way ANOVA with Bonferroni post-test using GraphPad Prism 5.01.)
Figure S4: Comparing reporter constructs. (A) Leakiness analysis comparing different reporter vectors. HEK293 cells were transfected using the reporter vector along with Renilla (pRL-TK) alone or with Renilla+filler DNA (pRL-TK +pPKm-102) plasmids. Leaky luciferase values were compared to Renilla alone (n=5) (B) Activation level comparison of Gal4 UAS and TET UAS reporters. HEK293 cells transfected with pPKm-202 or pMZ-802 along with pPKm-292 or pPKm-293 respectively (n=3).

G-UAS = Gal4 UAS, TET-UAS = TET UAS, GDBD = Gal4 DNA Binding Domain, TETDBD = TET DNA Binding Domain.

(Error bars = s.d. (*** = p<0.001, Statistics were calculated using one-way ANOVA with Bonferroni post-test using GraphPad Prism 5.01).
**Figure S5:** Illumination setup consists of black boxes with LED arrays controlled via an Arduino-driven circuitry and a LabVIEW user interface. The system is easily expandable to allow for the control of up to 12 boxes simultaneously. Each box can be activated at different time intervals and at different frequencies.
Figure S6: Circuit Design for LED illumination. Electronic schematic of the circuit used to control the LEDs for each box, coupled with an Arduino UNO. The circuit requires a 9 Volt voltage source and uses simple components. A trimmer potential allows for intensity and brightness control of the LEDs. This circuit can control 6 high power LEDs in series.
Figure S7: Kinetic model results. By varying the initial Heme concentrations and the rate of renewal of Fd$_{ox}$ to Fd$_{red}$, we show the dependence on these parameters in the PCB pathway.
Figure S8: Kinetic model results. (A) We simulate the presence and absence of the FD: FNR complex, demonstrating more robust production of PCB with the 4 enzymes. (B) Decreasing sweep through the parameters $k_3$ and $k_9$, which control binding of HO1 and PcyA to Fd respectively. This graph shows that
with decreasing species specificity, a decrease in PCB production is observed. (C) Varying initial concentrations of heme, demonstrating PCB dependence to Heme levels.
Supplementary Note. Model codes and Illumination user Interface.

1. Full working model (in python 2.7), plus scripts to generate Supplementary Figures S7 and S8. In order to execute the code, it is necessary to install the python package PySB (http://pysb.org/) and dependencies. All files should be placed in the same folder for execution.

2. LabVIEW Visual Interface Note that this requires a working LabVIEW installation in your computer. If you need an executable (.exe) file, please contact the article's corresponding authors.

File:

Kyriakakis_et_al_LabView_Software.vi
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