

# Additional file 1

Thiel, K., Patrikainen, P., Nagy, C., Fitzpatrick, D., Pope, N. Aro, E-M., and Kallio, P\*. Redirecting photosynthetic electron flux in the cyanobacterium *Synechocystis* sp. PCC 6803 by the deletion of flavodiiron protein Flv3

\* Corresponding author. Email: [pataka@utu.fi](mailto:pataka@utu.fi)

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**Fig. S7.** Growth curve of *Synechocystis* wild-type and  $\Delta flv3$  strains grown under 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light for 24h and 72h, indicating the sampling points for Fig. S8. Page 8.

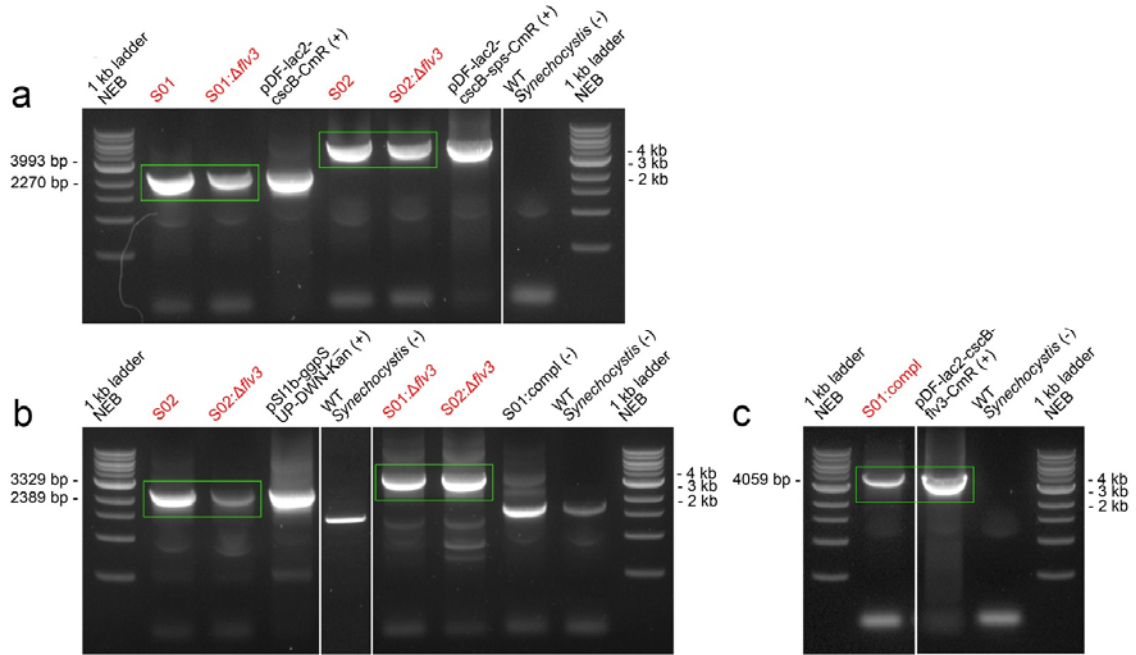
**Fig. S8.** Quantitation of the relative ATP and NADPH content of *Synechocystis* wild-type and  $\Delta flv3$  strains grown under 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light for 24h and 72h. Page 9.

**Table S1.** Summary of the calculated significances for the cellular gas fluxes measured by MIMS (**Figs. 2-5**) for the *Synechocystis* strains generated in this study. Page 10-11.

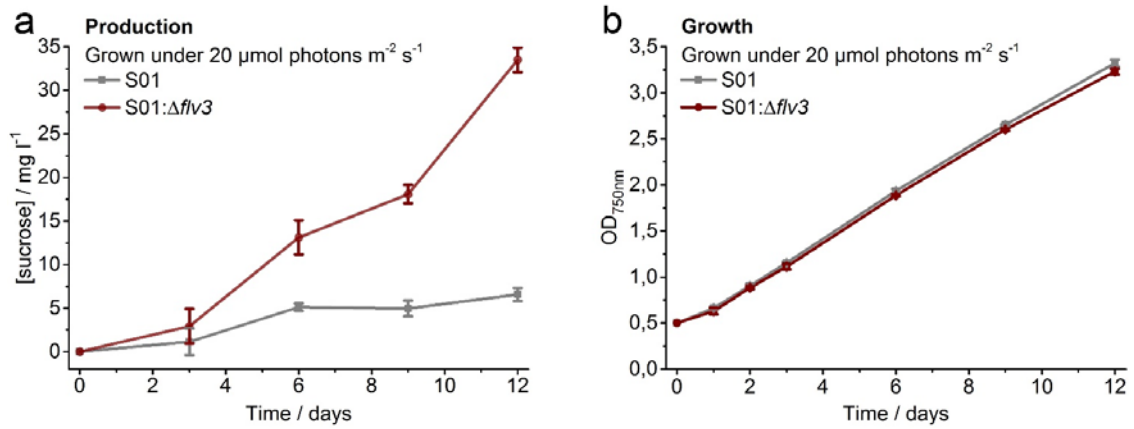
**Table S2.** Simplified list of enzymatic reactions towards sucrose, PHB and glycogen in *Synechocystis* from the common metabolic intermediate glyceraldehyde-3-phosphate, and estimation of relative ATP/NADPH demand between the pathways. Page 12-13.

**Table S3.** List of plasmids generated and used in this study. Page 14.

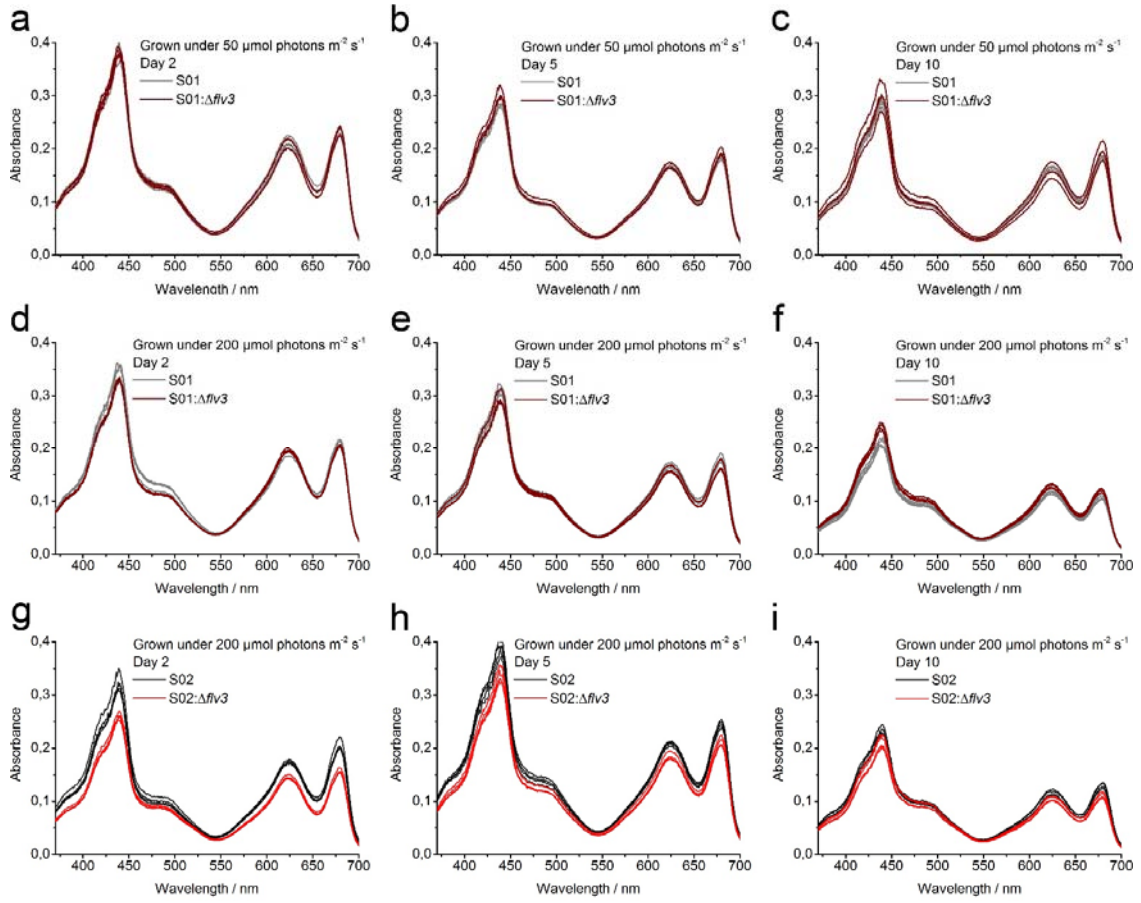
**Table S4.** List of PCR primers used in this study. Page 15.



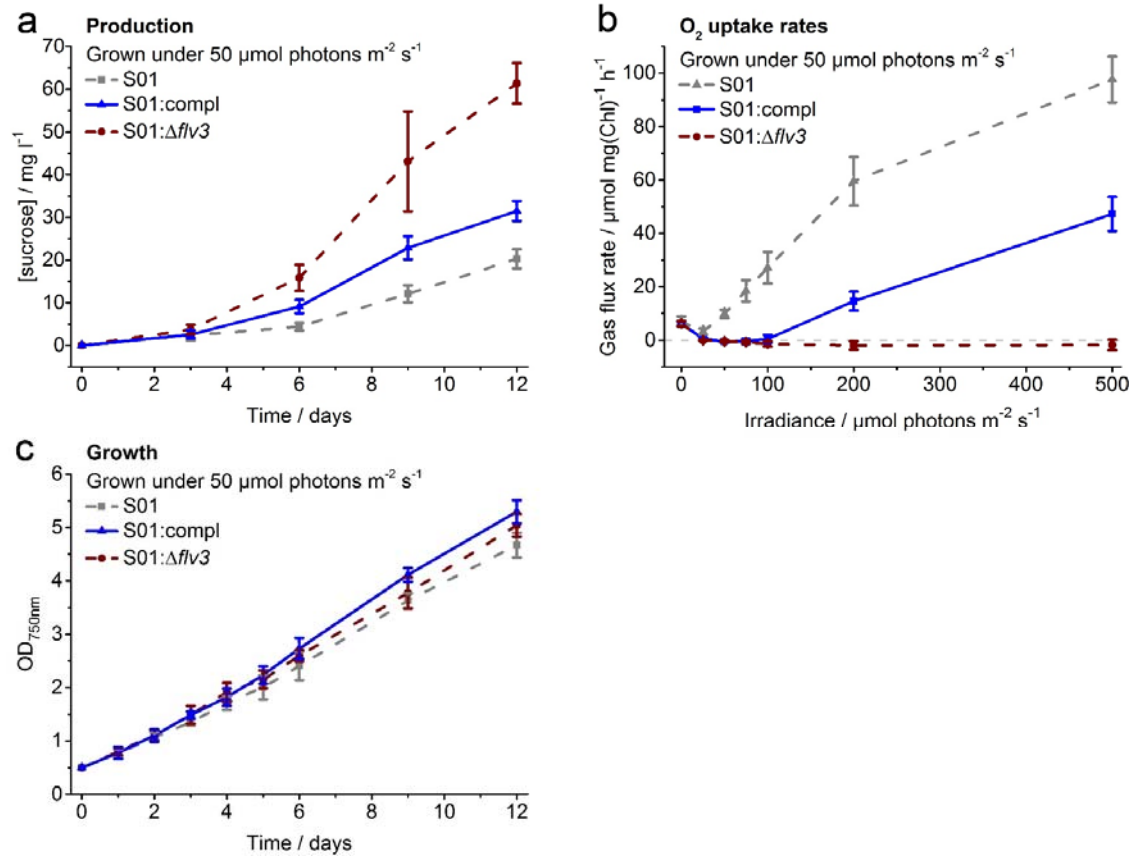
**Fig. S1.** Colony PCR verification of the *Synechocystis* strains generated in this study (red font; see Table 1) as visualized by agarose gel electrophoresis. **(a)** The presence of the expression cassette for CscB (S01; S01:Δflv3) and CscB+SPS (S02; S02:Δflv3) was confirmed using the corresponding plasmids as positive controls (pDF-lac2-cscB-CmR; pDF-lac2-cscB-sps-CmR), and WT *Synechocystis* as a negative control (primers in pDF-lac2\_FOR and pDF-lac2\_REV; see Table S4). **(b)** The deletion of *ggpS* (S02; S02:Δflv3) was confirmed using the plasmid pSI1b-ggpS\_UP-DWN-Kan as a positive control, and WT *Synechocystis* as a negative control (primers ggpS\_UP\_FOR and ggpS\_DWN\_REV; see Table S4). The deletion of *flv3* (S01:Δflv3; S02:Δflv3) was confirmed using the Flv3 complementation strain (S01:compl), and WT *Synechocystis* controls (primers flv3\_FOR and flv3\_REV; see Table S4). **(c)** The presence of CscB+Flv3 overexpression plasmid (S01:compl lane 2) was confirmed by using pDF-lac2-cscB-flv3-CmR as a positive control and wild-type *Synechocystis* as a negative control (primers pDF-lac2\_FOR and pDF-lac2\_REV; see Table S4). The expected sizes for the PCR products are shown on the left, and the correct bands have been highlighted in green.



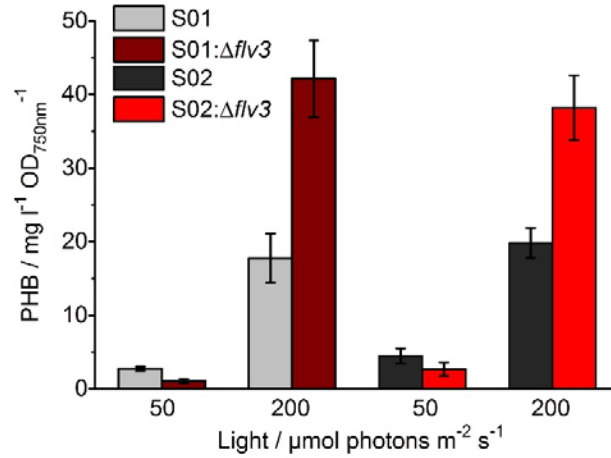
**Fig. S2.** Characterization of engineered *Synechocystis* strains grown under continuous light of 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The strains S01 (over-expression of sucrose permease CscB) and S01:Δflv3 (over-expression of CscB and inactivation of flavodiiron protein Flv3) were evaluated in respect to (a) sucrose production ( $\text{mg l}^{-1}$ ) and (b) growth ( $\text{OD}_{750\text{nm}}$ ). The control strain S01 is shown in grey and the strain S01:Δflv3 in brown. The strains were cultivated in 1 %  $\text{CO}_2$  in the presence of 400 mM supplemented NaCl. In each case, the average and standard deviation were calculated from three to four independent experiments.



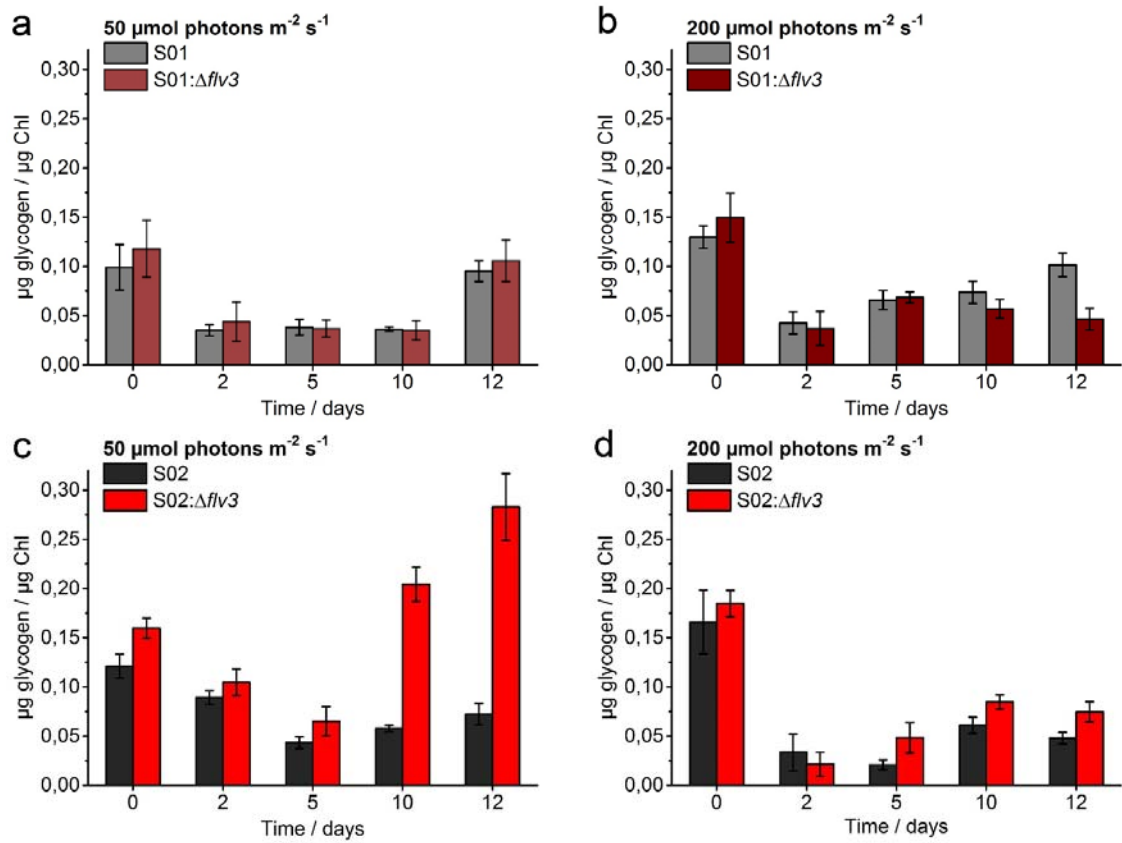
**Fig. S3.** Spectrophotometric analysis of pigments present in engineered *Synechocystis* strains (Table 1) grown under different light conditions. Absorption spectra (370 nm – 700 nm) of the strains S01 (over-expression of sucrose permease CscB) (grey) and S01:Δ*flv3* (over-expression of CscB and inactivation of flavodiiron protein Flv3) (brown) grown for (a, d) two days (b, e) five days and (c, f) 10 days under continuous growth light (50 and 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , respectively). (g, h, i) Corresponding absorption spectra of the strains S02 (over-expression of CscB and sucrose phosphate synthase SPS, and inactivation of glucosylglycerolphosphate synthase GGPS) (black) and S02:Δ*flv3* (over-expression of CscB and SPS, and inactivation of GGPS and Flv3) (red) grown under continuous growth light of 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The strains were cultivated in 1 %  $\text{CO}_2$  in the presence of 400 mM NaCl. The graphs present four independent experiments and have been normalized to 750 nm.



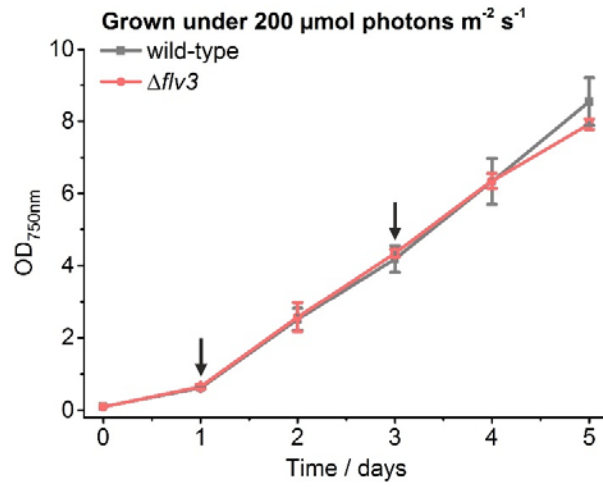
**Fig. S4.** Partial complementation of Flv3 inactivation in engineered sucrose-producing *Synechocystis*  $\Delta flv3$  strain grown under continuous light of  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The strain S01:compl (over-expression of sucrose permease CscB and flavodiiron protein Flv3 in the  $\Delta flv3$  background) (blue line) was evaluated in respect to (a) sucrose production ( $\text{mg l}^{-1}$ ), (b) O<sub>2</sub> uptake measured on cultivation day 5 and (c) growth (OD<sub>750nm</sub>). The strains S01 (over-expression of CscB) and S01:Δflv3 (over-expression of CscB and inactivation of flavodiiron protein Flv3) shown in grey and red dashed lines, respectively, have been included for comparison (see Fig. 2). The strains were cultivated in 1 % CO<sub>2</sub> in the presence of 400 mM supplemented NaCl. In each case, the average and standard deviation were calculated from three to four independent experiments.



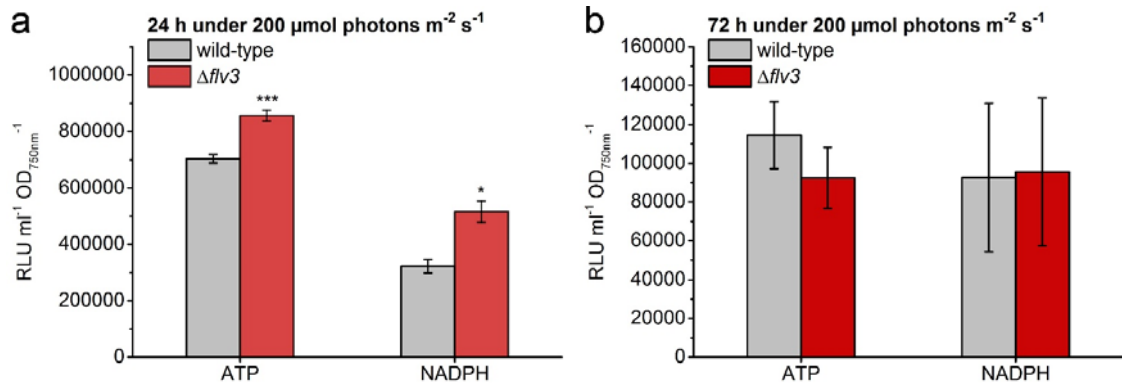
**Fig. S5.** Quantitative analysis of polyhydroxybutyrate (PHB) content in the engineered *Synechocystis* strains (Table 1) grown under different light conditions. The amount of PHB was measured from the strains S01 (over-expression of sucrose permease CscB) (grey), S01:Δflv3 (over-expression of CscB and inactivation of flavodiiron protein Flv3) (brown), S02 (over-expression of CscB and sucrose phosphate synthase SPS, and inactivation of glucosylglycerolphosphate synthase GGPS) (black) and S02:Δflv3 (over-expression of CscB and SPS, and inactivation of GGPS and Flv3) (red) grown under continuous 50 or 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light for 12 days (1 %  $\text{CO}_2$  in the presence of 400 mM NaCl). PHB was broken down to 3-hydroxybutyrate and the concentration was determined colorimetrically using a commercial D-3-Hydroxybutyric Acid Assay Kit (Megazyme, US). In each case, the average and standard deviation were calculated from four independent cultivations.



**Fig. S6.** Quantitative analysis of glycogen content at different time-points (0-12 days) in engineered *Synechocystis* strains (Table 1) grown under different light conditions. The amount of glycogen was measured from strains S01 (over-expression of sucrose permease CscB), S01: $\Delta\text{flv3}$  (over-expression of CscB and inactivation of flavodiiron protein Flv3), S02 (over-expression of CscB and sucrose phosphate synthase SPS, and inactivation of glucosylglycerolphosphate synthase GGPS) and S02: $\Delta\text{flv3}$  (over-expression of CscB and SPS, and inactivation of GGPS and Flv3) grown under continuous growth light of (a, c) 50 and (b, d) 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (1 %  $\text{CO}_2$  in the presence of 400 mM NaCl). In a and b, the control strain S01 is shown in grey and the strain S01: $\Delta\text{flv3}$  in brown and, in c and d, the control strain S02 is shown in black and the strain S02: $\Delta\text{flv3}$  in red. Glycogen was broken to glucose and analyzed using a commercial Sucrose/D-Glucose Assay Kit (Megazyme, US). In each case, the average and standard deviation were calculated from four independent cultivations, and normalized to chlorophyll *a* content.



**Fig. S7.** Growth (OD<sub>750nm</sub>) of *Synechocystis* wild-type and  $\Delta flv3$  strains cultivated under 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  continuous light. The samples for ATP and NADPH analysis (see Fig. S8) were taken on days 1 and 3, as indicated by black arrows. The average and standard deviation for each time-point were calculated from four independent cultures.



**Fig. S8:** ATP and NADPH content of *Synechocystis* wild-type and  $\Delta flv3$  strains after cultivation under continuous 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light for (a) 24h and for (b) 72h. The values are presented in Relative Luminescence Units (RLU) per ml of culture per OD<sub>750nm</sub>, the averages and standard deviations representing three independent cultures with three technical replicates each (n = 9). The statistical difference (*t*-test) between the strains is denoted as three (p < 0.01) or one (p < 0.05) asterisk above the  $\Delta flv3$  error bar.

**Table S1.** Summary of the calculated significances for the cellular gas fluxes measured by MIMS (see Figs. 2-5) for the *Synechocystis* strains generated in this study. The significances represent pairwise *t*-test comparisons of each of the measurement points between a specified  $\Delta flv3$  strain and the corresponding control strain using four independent replicates ( $n = 4$ ). Statistical difference is shown in green background, and the direction of the change is indicated by + (increased value in  $\Delta flv3$  strain in comparison to the control) and - (reduced value in  $\Delta flv3$  strain in comparison to the control). Three signs correspond to  $p < 0.01$ , two signs  $p < 0.02$  and one sign  $p < 0.05$ . Results found not to be statistically significant are presented in grey background.

| Strains<br>/ Figure ref.                      | Growth light intensity<br>/ $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ |                                | Irradiance in MIMS /<br>$\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ |     |     |     |     |     |     |
|-----------------------------------------------|---------------------------------------------------------------------------|--------------------------------|-----------------------------------------------------------------------|-----|-----|-----|-----|-----|-----|
|                                               |                                                                           |                                | 0                                                                     | 25  | 50  | 75  | 100 | 200 | 500 |
| S01 and<br>S01: $\Delta flv3$<br>(see Fig. 2) | 50                                                                        | Gross O <sub>2</sub> evolution |                                                                       | #   | #   | #   | #   | --- | --- |
|                                               |                                                                           | O <sub>2</sub> uptake          |                                                                       | --- | --- | --- | --- | --- | --- |
|                                               |                                                                           | Total C uptake                 |                                                                       | #   | #   | #   | ++  | +++ | +++ |
|                                               |                                                                           | Net O <sub>2</sub> evolution   |                                                                       | #   | #   | #   | +++ | +++ | +++ |
| S01 and<br>S01: $\Delta flv3$<br>(see Fig. 3) | 200                                                                       | Gross O <sub>2</sub> evolution |                                                                       | +   |     | -   | -   | -   | --  |
|                                               |                                                                           | O <sub>2</sub> uptake          |                                                                       | +++ |     |     |     | --- | --- |
|                                               |                                                                           | Total C uptake                 |                                                                       | --- | --- | --- |     |     | +++ |
|                                               |                                                                           | Net O <sub>2</sub> evolution   |                                                                       |     | -   | -   |     |     | +   |
| S02 and<br>S02: $\Delta flv3$<br>(see Fig. 4) | 200                                                                       | Gross O <sub>2</sub> evolution |                                                                       | --- | --- | --- | --- | --- | --- |
|                                               |                                                                           | O <sub>2</sub> uptake          |                                                                       | +++ | +++ |     | --- | --- | --- |
|                                               |                                                                           | Total C uptake                 |                                                                       | --- | --- | --- |     | +++ | +++ |
|                                               |                                                                           | Net O <sub>2</sub> evolution   |                                                                       | --- | --- | --- | --  | +   | +++ |

|                                              |   |   |    |   |                                   |                                                                                    |
|----------------------------------------------|---|---|----|---|-----------------------------------|------------------------------------------------------------------------------------|
| S02 and<br>S02:Δ <i>flv3</i><br>(see Fig. 5) | # | # | 50 | # | Gross O <sub>2</sub><br>evolution |  |
|                                              |   |   |    |   | O <sub>2</sub> uptake             |                                                                                    |
|                                              |   |   |    |   | Total C<br>uptake                 |                                                                                    |
|                                              | # |   |    |   | Net O <sub>2</sub>                |                                                                                    |
|                                              | # |   |    |   | evolution                         |                                                                                    |

**Table S2:** Simplified list of enzymatic reactions towards sucrose, PHB and glycogen in *Synechocystis* from the common metabolic intermediate glyceraldehyde-3-phosphate, and estimation of relative ATP/NADPH demand between the pathways.

| Enzyme<br>[EC ID]                                                       | ATP/NADPH<br>produced or used | Reactions /<br>target molecule | Net          |
|-------------------------------------------------------------------------|-------------------------------|--------------------------------|--------------|
| <b>Polyhydroxybutyrate; PHB</b>                                         |                               |                                |              |
| Glyceraldehyde-3-phosphate dehydrogenase<br>[EC 1.2.1.59]               | 0                             | 2                              | 0            |
| Phosphoglycerate kinase<br>[EC 2.7.2.3]                                 | + 1 ATP                       | 2                              | + 2 ATP      |
| Phosphoglycerate mutase<br>[EC 5.4.2.1]                                 | 0                             | 2                              | 0            |
| Enolase<br>[EC 4.2.1.11]                                                | 0                             | 2                              | 0            |
| Pyruvate kinase<br>[EC 2.7.1.40]                                        | + 1 ATP                       | 2                              | + 2 ATP      |
| Pyruvate dehydrogenase<br>[EC 1.2.4.1 / 1.8.1.4 / 2.3.1.12]             | 0                             | 2                              | 0            |
| $\beta$ -ketothiolase<br>[EC 2.3.1.9]                                   | 0                             | 1                              | 0            |
| Acetoacetyl CoA reductase<br>[EC 1.1.1.36]                              | - 1 NADPH                     | 1                              | - 1<br>NADPH |
| PHB synthetase<br>[EC 2.3.1.-]                                          | 0                             | x                              | 0            |
| <b>Total: + 4 ATP, - 1 NADPH</b>                                        |                               |                                |              |
| <b>Glycogen</b>                                                         |                               |                                |              |
| D-Fructose 1-phosphate D-glyceraldehyde-3-phosphate-lyase [EC 4.1.2.13] | 0                             | 1                              | 0            |
| Fructose-bisphosphatase<br>[EC 3.1.3.11]                                | 0                             | 1                              | 0            |
| Glucose-6-phosphate isomerase<br>[EC 5.3.1.9]                           | 0                             | 1                              | 0            |
| Phosphoglucomutase<br>[EC 5.4.2.2]                                      | 0                             | 1                              | 0            |
| Glucose-1-phosphate adenylyltransferase<br>[EC 2.7.7.27]                | - 1 ATP                       | 1                              | - 1 ATP      |
| Glycogen synthase<br>[EC 2.4.1.21]                                      | 0                             | x                              | 0            |
| <b>Total: -1 ATP</b>                                                    |                               |                                |              |

| <b>Sucrose</b>                                                          |   |   |   |
|-------------------------------------------------------------------------|---|---|---|
| D-Fructose 1-phosphate D-glyceraldehyde-3-phosphate-lyase [EC 4.1.2.13] | 0 | 2 | 0 |
| Fructose-bisphosphatase [EC 3.1.3.11]                                   | 0 | 2 | 0 |
| Glucose-6-phosphate isomerase [EC 5.3.1.9]                              | 0 | 1 | 0 |
| Phosphoglucomutase [EC 5.4.2.2]                                         | 0 | 1 | 0 |
| Glucose-1-phosphate uridylyltransferase [EC 2.7.7.9]                    | 0 | 1 | 0 |
| Sucrose phosphate synthase [EC 2.4.1.14]                                | 0 | 1 | 0 |
| Sucrose-phosphate phosphatase [EC 3.1.3.24]                             | 0 | 1 | 0 |
| Sucrose permease [EC 2.7.1.69]                                          | 0 | 1 | 0 |
| <b>Total: No net change</b>                                             |   |   |   |

**Table S3.** Plasmids generated and used in this study.

| <b>Plasmid name</b>    | <b>Use or function</b>                                              | <b>References</b> |
|------------------------|---------------------------------------------------------------------|-------------------|
| pDF-lac2               | <i>E. coli</i> / <i>Synechocystis</i> shuttle vector                | (1)               |
| pDF-lac2-cscB-CmR      | Expression of CscB                                                  | This study        |
| pDF-lac2-cscB-flv3-CmR | Expression of CscB and Flv3                                         | This study        |
| pDF-lac2-cscB-sps-CmR  | Expression of CscB and SPS                                          | This study        |
| pSI1b_ggpS_UP_DWN_Kan  | Disruption of <i>Synechocystis</i> <i>ggpS</i> with Km <sup>R</sup> | This study        |
| pNiv(S3)               | Assembly vector carrying RBS S3                                     | (1)               |

**Table S4.** PCR primers used in this study for amplifying the homologous recombination regions from the genomic DNA of *Synechocystis*, for amplifying the kanamycin resistance cassette from pCOLADuet-1 and for colony PCR verification of the generated strains. Restriction sites used for subsequent cloning steps are underlined, extra overhangs are in lowercase and sequences complementary to the amplified region are in uppercase.

| Primer       | Sequence (5'>3')                  | Restriction sites |
|--------------|-----------------------------------|-------------------|
| ggpS UP FOR  | tatCATATGATGAATTCATCCCTTGTGATCC   | NdeI              |
| ggpS UP REV  | tatCCCGGGTCGTGGACCCAAAACAATG      | XmaI              |
| ggpS DWN FOR | tatCCTAGGAACAAGATTTAGGCGGTAAGAGG  | AvrII             |
| ggpS DWN REV | tatAAGCTTGGGTAGTTCCACCGCACA       | HindIII           |
| KmR FOR      | tatGTCGACGCGCTAGCATGCCTATTTGT     | SalI              |
| KmR REV      | tatCCATGGCTGAGCAATAACTAGCATAACCCC | NcoI              |
| pDF-lac2 FOR | GTTGACTTGTGAGCGGATAACAATGATACTTA  |                   |
| pDF-lac2 REV | CCGCTTCTGCGTTCTGATTTAATCTG        |                   |
| flv3 FOR     | CATATGTTCACTACCCCCCTCCCC          |                   |
| flv3 REV     | CTCGAGTTAGTAATAATTGCCGAC          |                   |

## References

1. K. Thiel *et al.*, Translation efficiency of heterologous proteins is significantly affected by the genetic context of RBS sequences in engineered cyanobacterium *Synechocystis* sp. PCC 6803. *Microb Cell Fact* **17**, 34 (2018).
2. C. J. Joshi, C. A. M. Peebles, A. Prasad, Modeling and analysis of flux distribution and bioproduct formation in *Synechocystis* sp. PCC 6803 using a new genome-scale metabolic reconstruction. *Algal Research* **27**, 295-310 (2017).

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