

## **A Synthetic Biology Approach to Engineering Living Photovoltaics**

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## Abstract

The ability to electronically interface living cells with electron accepting scaffolds is crucial for the development of next-generation biophotovoltaic technologies. Although recent studies have focused on engineering synthetic interfaces that can maximize electronic communication between the cell and scaffold, the efficiency of such devices is limited by the low conductivity of the cell membrane. This review provides a materials science perspective on applying a complementary, synthetic biology approach to engineering membrane-electrode interfaces. It focuses on the technical challenges behind the introduction of foreign extracellular electron transfer pathways in bacterial host cells and the past and future efforts to engineer photosynthetic organisms with artificial electron-export capabilities for biophotovoltaic applications. The article highlights advances in engineering protein-based, electron-exporting conduits in a model host organism, *E. coli*, before reviewing state-of-the-art biophotovoltaic technologies that use both unmodified and bioengineered photosynthetic bacteria with improved electron transport capabilities. A thermodynamic analysis is used to propose an energetically feasible pathway for extracellular electron transport in engineered cyanobacteria and identify metabolic bottlenecks amenable to protein engineering techniques. Based on this analysis, an engineered photosynthetic organism expressing a foreign, protein-based electron conduit yields a maximum theoretical solar conversion efficiency of 6-10% without accounting for additional bioengineering optimizations for light-harvesting.

## 1. Introduction

A current focus of synthetic biology aims to solve one of the major challenges society faces today: reducing the dependence on fossil fuels and lowering the carbon footprint of a growing population. One approach to addressing this challenge is to engineer biophotovoltaics; that is, to create biohybrid devices that harness solar energy to produce electricity. Protein engineering approaches have been used to attach light-harvesting proteins to material surfaces<sup>3,4</sup>. However, in addition to inherent protein instabilities, light-harvesting proteins undergo accelerated degradation mechanisms when prolonged illumination leads to the production of damaging reactive oxygen species and photoinhibition<sup>5,6</sup>. Compared to silicon-based photovoltaics and biophotovoltaic devices based on isolated proteins, devices utilizing autonomously replicating and self-repairing microorganisms, henceforth referred to as living photovoltaics, could benefit from lower fabrication and maintenance costs. While no single organism is optimized to both convert solar energy into redox energy (photosynthesis) and produce external electrical current (extracellular electron transfer), these two capabilities are separately found in cyanobacteria and exoelectrogenic bacteria, respectively. Thus, synthetic biologists can embark on combining these capabilities to create living photovoltaics.

In this review, we highlight underlying principles and advancements in using synthetic biology approaches to engineer living photovoltaics, focusing on the ability of vital cells to interact with electrodes at the nanoscale. It begins with an introduction on living photovoltaics, discussing the challenges of charge extraction from living cells and focusing on natural approaches to charge transfer found in exoelectrogens. This is followed by a brief description of synthetic biology and protein engineering that is exemplified by endeavors undertaken to reconstitute an extracellular electron transfer pathway in the model organisms *E. coli*. After a general discussion on interfacing wild-type cyanobacteria with electrodes for light-harvesting applications, we present recent breakthroughs in bioengineered cyanobacteria with exoelectrogenic capabilities. Finally, we propose an alternative approach to bioengineering cyanobacteria and present a theoretical analysis of maximum photosynthetic and biochemical conversion efficiencies.

## 2. Living Photovoltaics Are Limited by Efficient Charge Transfer

The prospect of harvesting electrons from the energy metabolism of microorganisms forms the basis of microbial electrochemical technologies (METs) used for renewable and carbon neutral energy production, wastewater treatment, and biosensing<sup>7</sup>. While most METs incorporate heterotrophic bacteria that convert organic substrates to electrical power, the nearly limitless availability of solar energy has given rise to biophotovoltaics that generate current from oxygenic photosynthetic organisms (or parts thereof) in the absence of organic substrates<sup>8</sup>. Living photovoltaics consist of whole microorganisms, such as microalgae or cyanobacteria, that absorb light to catalyze the water-splitting reaction of oxygenic photosynthesis and transfer high-energy electrons to an anode. These technologies contrast with syntrophic anaerobic photosynthesis, where cell growth is driven by electron transfer to the photosynthetic cell from an electrode or interspecies electron transfer from a heterotrophic partner<sup>9-13</sup>. In living photovoltaics, a current is generated when the electrons pass through an external load to reduce an electron acceptor at the cathode. Most photosynthetic microorganisms also generate current in the dark when the internal carbon storage is oxidized to fuel the respiratory energy metabolism<sup>14,15</sup>.

Due to their relatively simple physiology and low basal energy requirements, cyanobacteria have been favored over more complex eukaryotic micro algae for living photovoltaics<sup>8</sup>. Different unicellular and filamentous strains have been incorporated in various setups. While early studies relied on artificial mediators for electron transfer between the cyanobacteria and electrodes,<sup>14,16,17</sup> more recent work has focused on the sole use of natural transfer mechanisms for electron transfer<sup>15,18–20</sup>. Theoretical calculations estimate the photocurrent generation capacity of cyanobacteria to be 700-7700 mW/m<sup>2</sup><sup>8</sup>, although most living photovoltaics do not exceed a few mW, with the best performing device achieving only around 100 mW in an optimized lab environment<sup>21</sup>.

The primary challenge in using photosynthetic cyanobacteria for living photovoltaics is the inability to effectively move photogenerated charge carriers within the cell to an external electrode<sup>22,23</sup>. This difficulty arises from multiple insulating barriers in the cell; both the plasma membrane that separates the living cell from its non-living environment and the thylakoid membranes that contain the photosynthetic apparatus are largely impermeable to ions and polar molecules, including most cellular redox carriers. Until recently, nearly all approaches to electrically link cells to electrodes have focused on the materials engineering of electrodes or synthetic redox mediators that abrogate these barriers to facilitate charge transfer. While the addition of artificial mediators can support electron transfer from cyanobacterial cells<sup>16,24</sup>, these mediators do not selectively accept electrons from only the charge carriers involved in photosynthesis. Rather, they accept electrons from any of the redox active molecules in the cell with an appropriate midpoint potential. This lack of selectivity is believed to be partially responsible for the cellular toxicity often observed with the use of exogenous mediators. Since costly and potentially toxic exogenous compounds compromise the commercial feasibility of living photovoltaics, recent research has focused on alternative approaches, such as engineering electrodes<sup>25</sup>. For electrodes that do not directly permeate the insulating cell membrane, the electrical and surface characteristics of the anode plays a critical role in extracellular electron transfer<sup>26</sup>. Anodes modified with a flexible redox polymer<sup>27</sup>, porous ceramic materials<sup>28</sup>, indium tin oxide-coated materials<sup>29</sup>, and nanomaterials<sup>30</sup> were shown to enhance electron transfer compared to conventional carbon-based anodes.

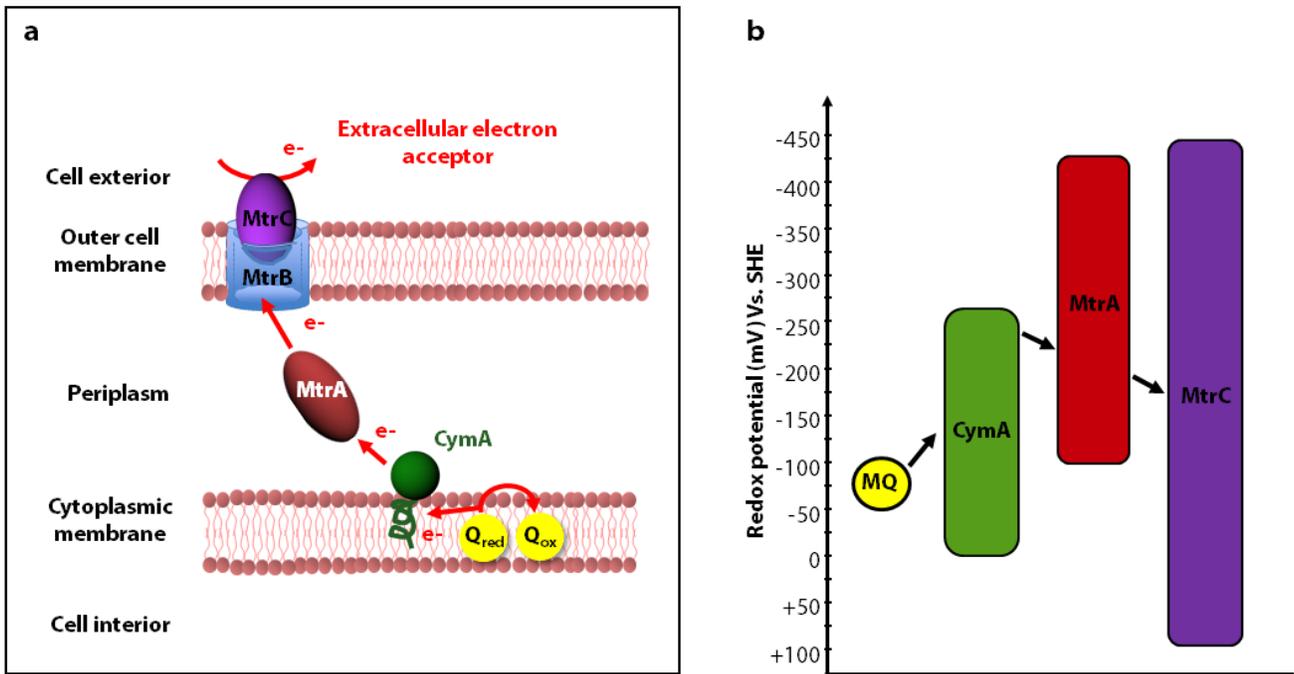
In addition to device optimization, the recent expansion of the synthetic biology toolbox allows scientists to bioengineer pathways for shuttling electrons from the intracellular carbon fixing metabolism to external electrodes for efficient current extraction from cyanobacteria. Using this approach, new proteins that harvest electrons from specific charge carriers can be introduced into the cell and used to deliver electrons to an electrode<sup>31</sup>. The DNA of the organism thus encodes for autonomous current extraction; cyanobacteria engineered in this manner are therefore genetically pre-dispositioned to behave as living photovoltaics. This bioengineering approach to developing living photovoltaics forms the basis of a major new research focus in the area of METs.

### **3. Efficient Charge Transfer in Exoelectrogens**

Certain naturally-occurring microorganisms can transfer electrons to metals and metal oxides located outside of the cell in a process called extracellular electron transfer (excellently reviewed by Shi *et al.*<sup>32</sup>). These microorganisms, called exoelectrogens, have evolved both soluble electron mediators and redox-active protein structures that allow electrons to penetrate the insulating outer membrane. The extracellular electron pathways are used during respiration, a process whereby an organism oxidizes

an electron donor, usually an organic molecule, and passes the extracted electrons through a series of redox reactions to a terminal electron acceptor. The energy released during the biochemical redox reactions is harnessed to generate a proton motive force, which in turn is used to make ATP or power other cellular functions. Under aerobic conditions, the terminal electron acceptor is oxygen, which can diffuse into the cell. However, under anaerobic conditions, exoelectrogens export their electrons to a terminal electron acceptor outside the cell. In nature, the terminal electron acceptor is often an insoluble metal, such as iron oxide. However, electrodes can act as terminal electron acceptors for exoelectrogens in the context of bioelectrochemical applications<sup>33,34</sup>. Two commonly studied, naturally occurring exoelectrogens are *Geobacter sulfurreducens*<sup>33</sup> and *Shewanella oneidensis*<sup>35</sup>. Reviews on synthetic biology approaches for improving current production and other applications in naturally occurring exoelectrogens such as *S. oneidensis* and *G. sulfurreducens* are provided elsewhere in the literature<sup>36</sup>.

Though a variety of natural molecular pathways exist for extracellular electron transport, the majority of these pathways rely on multiheme c-type cytochromes. These proteins bind several closely stacked, iron-containing heme groups that behave as redox-active electron transfer sites, and they are located in the periplasm or are associated with the cytoplasmic or periplasmic membranes<sup>37</sup>. The best characterized conduit is the MtrCAB pathway in *S. oneidensis* MR-1 (Figure 1). A thorough discussion of extracellular electron transfer mechanisms in the MtrCAB pathway in *S. oneidensis* is provided in the literature<sup>34,37–39</sup>. To briefly summarize, the oxidation of nutrients during anaerobic respiration generates reducing equivalents stored in the membrane-confined quinone pool. Quinols donate electrons to the protein CymA, which is located in the inner membrane and has four heme *c* cofactors. CymA then donates these electrons directly or through additional periplasmic cytochromes to MtrA, a soluble protein with ten heme *c* cofactors located in the periplasmic space between the cytoplasmic and outer membrane of the cell. As part of a 1:1:1 MtrCAB complex, MtrA transfers the electrons to MtrC, which also contains ten heme *c* cofactors as well as a lipid tail that anchors it in the outer cell membrane. Although there is no solved crystal structure for the MtrCAB complex, studies have suggested that MtrA has an elongated, wire-like structure that snugly fits into the integral membrane protein MtrB, which likely interacts with the extracellular MtrC protein on the other end<sup>40</sup>. Once reduced, MtrC is able to donate its electrons to a range of extracellular electron acceptors, including electrodes. The membrane-anchored c-type cytochrome OmcA that interacts with MtrC can act as a terminal reductase and facilitate electron transfer, though it is not an essential component for electron transfer by MtrCAB<sup>41,42</sup>. In addition to direct electron transfer, *S. oneidensis* can also release flavins that act as soluble mediators or bound co-factors that shuttle electrons from MtrC to the final electron acceptor<sup>33,38</sup>.



**Figure 1 | Extracellular Electron Transfer Pathway in *S. oneidensis*.** (a) During anaerobic respiration, metabolites, such as sugars, are oxidized to release energy. The quinone pool becomes reduced (Q<sub>red</sub>), and upon quinone oxidation (Q<sub>ox</sub>), electrons are donated to the membrane bound protein, CymA. Electrons are then transferred from CymA to MtrA. MtrA transfers electrons to MtrC, an extracellular protein capable of reducing extracellular electron acceptors through a pore formed by the MtrB porin. (b) The corresponding energy diagram shows comparable redox potentials for the proteins involved in the pathways and no significant change in energy. Such a pathway is capable of electron transfer in both the forward and reverse directions<sup>39</sup>. The redox potentials of the hemes are affected by the surrounding environment<sup>40</sup>, and interactions such as menaquinone (MQ) binding to CymA may change the protein potential in a manner that favors electron transfer<sup>41,42</sup>. Approximate potentials are shown.

Despite being evolutionarily unrelated to *S. oneidensis*, *G. sulfurreducens* and other *Geobacter* species utilize proteins that appear to be functionally analogous to the MtrCAB pathway while sharing the same localization<sup>43</sup>. Electrons from the cytoplasmic quinone pool are transferred to periplasmic cytochromes, through porin-cytochrome trans-outer membrane protein complexes, and finally to insoluble metals outside the cell<sup>44</sup>. *G. sulfurreducens* encodes multiple homologous proteins that can form different porin-cytochrome pathways and are believed to function in a parallel manner<sup>32</sup>. In addition to insoluble metals, some *Geobacter* species can transfer electrons to cells of the same or different species<sup>32,50,51</sup>. For example, *Geobacter* spp. assembles conductive nanowires, appendages that can extend up to 20 microns from the cell surface. These proteinaceous filaments are composed of thousands of pilin subunits that are anchored to the cytoplasmic membrane and span the periplasmic space and outer membrane. The conductivity of *Geobacter* spp nanowires is attributed to closely stacked aromatic amino acids on the filament surface, but the exact nature of the charge transport along individual pili is still a matter of debate<sup>45–48</sup>. The filaments are associated with OmcS, an abundant multi-heme c-type cytochrome that may facilitate the extracellular reduction of metal oxides<sup>49</sup>.

Improvements in the understanding of the electron-transfer pathways in natural exoelectrogens have

fueled significant advancements in introducing naturally occurring conduits in foreign cells. Utilizing these pathways to bypass the insulating membranes of photosynthetic cyanobacteria has the potential to significantly enhance electron transfer in living photovoltaics. The feasibility of this approach has been demonstrated in the model organism *E. coli*. To date, the MtrCAB conduit in *S. oneidensis* remains the only naturally exoelectrogenetic pathway that has been functionally introduced in its entirety to a foreign cell. The next section focuses on the progression towards realizing the expression of the MtrCAB pathway in *E. coli* for the purpose of bioengineering whole cell-electrode interfaces.

#### 4. Overcoming Challenges in the Bioengineering of Extracellular Electron Pathways in *E. coli*

The primary challenge in expressing the MtrCAB pathway in a foreign host lies with the number and variety of post-translational processes required to express the proteins. As with most electron transfer systems, the MtrCAB-pathway comprises several c-type cytochromes with iron-containing heme *c*. In most bacteria, multi-protein cyt *c* maturation systems (Ccm) are required to covalently link the heme-cofactors with recipient apoproteins, and these maturation systems may vary significantly between different species<sup>50</sup>. Moreover, the translocation and secretion systems of the host bacteria must be able to move the heterologous proteins from the cytoplasm into or across the plasma membrane, the periplasmic space, or the outer membrane to establish a complete electron path from the cytoplasm to the extracellular space. If protein expression imposes a heavy metabolic burden or leads to the accumulation of harmful products such as free hemes, the expression must also be tuned to minimize the effects of diminished cell growth and instability of the recombinant DNA, which reduce overall protein yields<sup>51</sup>.

Because of these complications, initial studies focused on expressing only portions of the *S. oneidensis* pathway. In an initial breakthrough study, an MtrA-expressing *E. coli* strain was shown to reduce soluble Fe(III)NTA in its periplasm<sup>52</sup>. Since the native *ccm* operon of *E. coli* is not expressed under aerobic conditions and aerobic growth is preferred for protein expression, this and all subsequent studies had to rely on strains that included a plasmid for the constitutive expression of *E. coli*'s native cyt *c* maturation system under aerobic conditions. Following this work, it was demonstrated that NapC, a homolog of CymA in *E. coli*, could functionally replace CymA as an Fe(III) reductase in *S. oneidensis* and that CymA- and MtrACymA-expressing *E. coli* strains could transfer electrons to membrane-permeable chelated metals in the periplasm<sup>53,54</sup>. Although these strains were able to extract electrons from the cellular metabolism, they were unable to reduce extracellular metals in the absence of synthetic mediators, presumably due to the electrochemical barrier posed by the outer membrane.

Alternative outer membrane proteins involved in extracellular electron transfer have also been expressed in *E. coli*. For example, Palmer, Richardson, and coworkers expressed OmcA, an outer membrane paralog of MtrC found in *S. oneidensis* MR-1, in *E. coli*<sup>55</sup>. Their work showed that OmcA was capable of reducing extracellular insoluble iron oxide only when it is correctly localized to the extracellular face of the outer membrane. This work highlights the added difficulty outer membrane cytochromes pose for heterologous expression; the proteins must be congruent with the host's post-translational secretion systems to be localized correctly, underlining the importance of thorough biochemical characterization of extracellular electron proteins and pathways.

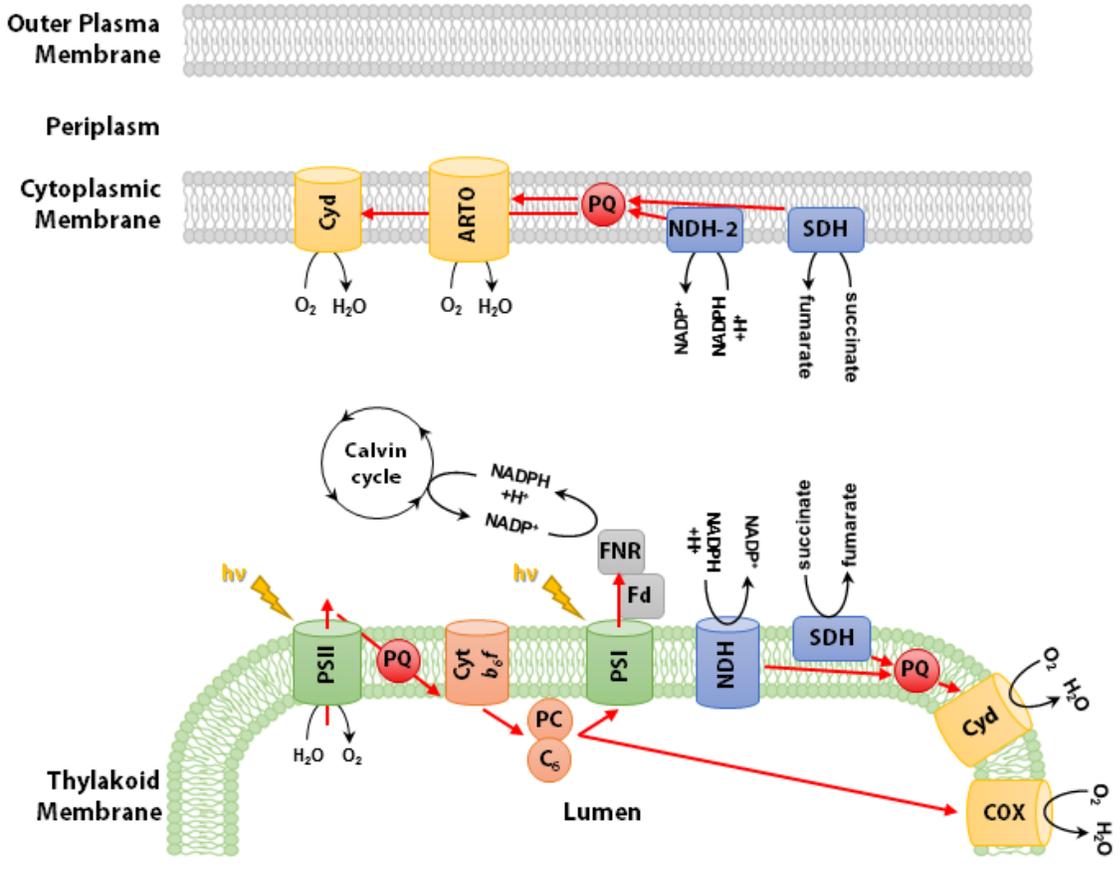
Building upon this study, Ajo-Franklin and co-workers were the first to demonstrate extracellular electron transfer in engineered *E. coli* through the MtrCAB pathway<sup>56</sup>. Although the introduction of this

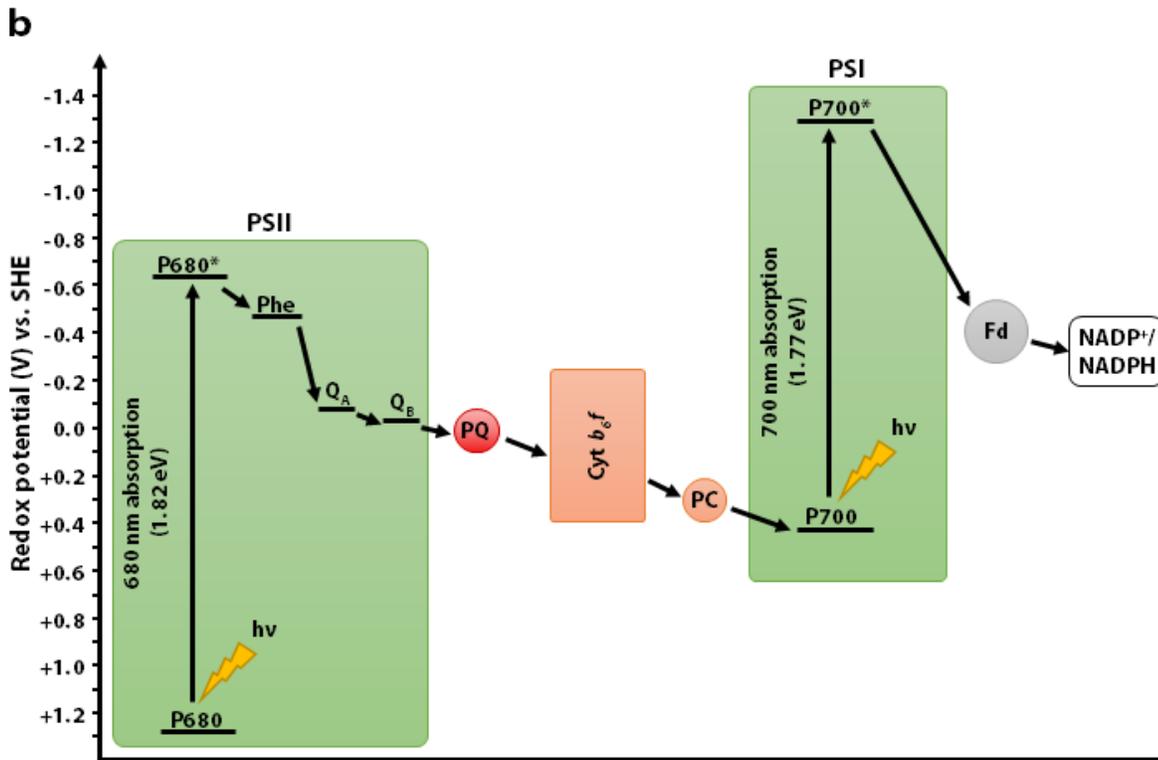
pathway was shown to boost extracellular electron transfer rates on a per cell basis, the engineered strain grew more slowly than its parent strain under aerobic conditions<sup>57</sup> and was unable to maintain biomass under iron-reducing conditions<sup>58</sup>. The authors speculated that high expression levels resulted in perturbations to cell metabolism. Consequently, the impact on cell growth and survival could be minimized and improved electron transfer was achieved by systematically tuning both MtrCAB and Ccm expression to moderate levels<sup>57</sup>. Further improvement was achieved by co-expressing CymA to circumvent the unnatural interaction between MtrA and NapC, an interaction that serves as a bottleneck in shuttling electrons from the quinone pool to the MtrCAB complex in *E. coli*. The resulting *cymA-mtrCAB E. coli* strain reduced both solid Fe<sub>2</sub>O<sub>3</sub><sup>65</sup> and an electrode<sup>66</sup> at a significantly faster rate than the strain expressing only MtrCAB. Moreover, cyclic voltammetry of these strains showed that re-reduction of MtrCAB was faster with co-expression of CymA, confirming the importance of appropriate protein-protein interactions<sup>58</sup>.

Since the first introduction of *mtrA* in *E. coli* in 2003, it has taken over a decade's worth of research to engineer a strain capable of expressing the entire CymA-MtrCAB pathway to an extent that the pathway achieves several of its native functions in a non-native host<sup>36</sup>. However, analysis of this extensive body of work, which mainly focused on *E. coli*, establishes several design rules for introducing extracellular electron transfer pathways in other hosts. Three specific important design rules are: i) the host must be able to recognize motifs in the heterologous genes that signal post-translational modifications (i.e. secretion, cofactor insertion, localization) and appropriately express the machinery required for those modifications<sup>55</sup>; ii) favorable protein-protein interactions are necessary for efficient and molecularly-defined electron transfer<sup>58</sup>; and iii) low expression levels are needed to make these highly post-translationally modified proteins without pleiotropic consequences to the host<sup>54,56,57</sup>. Beyond these design rules, the last decade of work – and more generally the study of exoelectrogens and bioelectronics - has shown that a molecular-level understanding of how these engineered hybrid systems behave is only possible by multi-faceted biochemical, spectroscopic, electrochemical, and metabolic characterizations. This complete characterization is needed to rule out other mechanisms that may contribute to increased current, such as increased cell permeability, increased production of soluble mediators, etc.

Moving forward, the community can look to use these design rules to (more rapidly) engineer extracellular electron transfer in new hosts to enable new applications. One particular host of interest to the energy community is cyanobacteria, the only prokaryotic organisms capable of oxygenic photosynthesis (Figure 2). This platform, together with a genetically engineered conduit for extracting the separated charge, offers the opportunity to couple light-driven water splitting to current production.

**a**





**Figure 2 | Water-splitting Through Photosynthesis. (a)** Light is absorbed by photosystem II (PSII), resulting in the generation of an electron-proton pair. The hole is used by surrounding oxygen-evolving complexes for oxygen generation, whereas the electron is subsequently transferred to photosystem I (PSI), where light is used to excite the electron to a higher energy state. The excited electron is ultimately used in NADPH production. Abbreviations: succinate dehydrogenase (SDH), NAD(P)H dehydrogenase NDH, NDH-2), plastoquinone (PQ), cytochrome *b<sub>6</sub>f* complex (Cyt *b<sub>6</sub>f*), plastocyanin (PC), cytochrome *c<sub>6</sub>* (*C<sub>6</sub>*), ferredoxin (Fd), ferredoxin-NADP<sup>+</sup> reductase FNR, cytochrome-*c* oxidase (COX), alternative respiratory terminal oxidase (ARTO), bd-quinol oxidase (Cyd). **(b)** A comparison of the redox potentials of the proteins involved in photosynthesis illustrates that low energy (more positive redox) interactions largely occur prior to PSI re-excitation. Approximate potentials are shown.

## 5. Wildtype Cyanobacteria in Photovoltaic Devices

The bottleneck in living photovoltaics that use photosynthetic organisms is the transfer of electrons from the thylakoid membranes across the cytoplasmic and periplasmic membranes. This limitation has been identified from multiple, experimental studies which, when taken together, identify membrane transport as the rate-limiting step: (1) rapid, increased photocurrent from isolated photosynthetic membrane fractions containing fewer photosynthetic complexes compared to whole-cell measurements<sup>22</sup>, (2) high currents obtained by inserting a nanoelectrode into the photosynthetic membranes of a chloroplast<sup>60</sup>, (3) improved electron transfer by engineering the electrode surfaces for improved interaction with the cell membrane<sup>13,24,61</sup>, (4) enhanced photocurrent extraction in the presence of membrane-permeable mediators<sup>11,15,62</sup>, and (5) comparative analysis calculating a lower charge extraction rate than predicted from oxygen evolution rates<sup>63</sup>.

In contrast to metal-reducing bacteria such as *S. oneidensis* and *G. sulfurreducens*, extracellular electron

transfer would be disadvantageous for carbon fixing cyanobacteria during normal photoautotrophic growth as it would diminish their ability to reduce CO<sub>2</sub>. However, when exposed to high-light or carbon limiting conditions, cyanobacteria may benefit from an extracellular electron sink to avoid over-reduction of the photosynthetic electron transport chain. The existence of efficient extracellular electron transport pathways in cyanobacteria that are comparable to the pathways in metal-respiring exoelectrogens remains largely unfounded; the absence of specific redox peaks in cyclic voltammetry measurements of cyanobacterial cultures<sup>64</sup> and the low currents produced in living photovoltaic systems<sup>65</sup> contradict the existence of such dedicated systems. Despite this, recent research endeavors have nonetheless succeeded in developing mediatorless, living photovoltaics that rely on elusive, electron-transfer mechanisms that are inherent to cyanobacteria<sup>8,66</sup>, though the exact molecular mechanisms behind this electrogenic activity are unknown. Some studies have explored the possibility of electron transfer through naturally occurring nanowires that were formed by *Synechocystis* and *Microcystis aeruginosa* PCC 7806 under CO<sub>2</sub>-limiting conditions<sup>67,68</sup>. However, conflicting data on the size of these structures introduce some uncertainty regarding the exact nature of the electron transfer mechanism. While Sure *et al.* suggested nanowires with a diameter of 4.5-7 nm to be Type IV pili, the structures observed by Gorby *et al.* were measured to be 100+ nm in diameter and were hypothesized to be membrane extensions, bundles of thin pili, or other hitherto unrecognized cell appendages<sup>69</sup>. To date, a thorough analysis characterizing the electron transfer capabilities of cyanobacterial nanowires *in vivo* remains lacking. In addition to nanowires, other potential electron transfer pathways may include ferric reductases in the cytoplasmic membrane, naturally produced mediators like flavins or quinones, or excreted oxidizable substrates<sup>8</sup>. The production of H<sub>2</sub> by cyanobacteria has also been discussed as a possible source for anodic current production, though this mechanism seems unlikely given that hydrogenases are inactivated by oxygen evolution during photosynthesis in unicellular cyanobacteria<sup>70</sup>.

Despite being conceptually simple, research on mediatorless, single-strain living photovoltaics has only been pursued for the past 7 years; in contrast, the general field of electricity generation from microorganisms dates back over 100 years<sup>71</sup>. Table 1 summarizes mediatorless, living photovoltaics that have been developed using different single cyanobacteria species. As shown in the table, living photovoltaics were characterized under a wide range of conditions and configurations, varying in strain type, illumination flux and spectra, solar cell configuration, electrode type, calculation of active electrode area, normalization of chlorophyll concentration, light and dark incubation times, and electrical load. Earlier studies have also explored the use of genera-specific electrochemical measurements<sup>18</sup>, and a few studies have characterized cellular biophotovoltaic devices using single-wavelength illumination conditions<sup>72-75</sup>. Because standardized conditions have yet to be adapted by the community, a comparative analysis of the device performances remains limited in scope and interpretation. Nonetheless, even the highest power output of 105 mW/m<sup>2</sup> recently reported by Bombelli and co-workers<sup>21</sup> corresponds to an overall device efficiency of less than 1% , highlighting the need to optimize current devices.

**Table 1 | Summary of Biophotovoltaic Literature.** This table summarizes relevant literature on living photovoltaics using single-strain cyanobacteria in the absence of exogenous mediators under white light illumination.

polyA = polyaniline; CP = carbon paint; CC = carbon cloth; CB = carbon base; PTFE = polytetrafluoroethylene; polyP = polypyrrole; ITO = indium tin oxide-coated polyethylene terephthalate; PANI = polyaniline; CNT = carbon nanotube

[a-d] Power output was calculated from the normalized value reported, multiplied by the chlorophyll concentration after 4 days of growth.

[e] Power output was calculated from the maximum value of 7.5 W/m<sup>3</sup> reported in the literature, multiplied by the volume of the anodic chamber (60 mL)

and divided by the electrode area (0.0015 m<sup>2</sup>).

[f] Original article erroneously suggests illumination conditions of 3000 mol photons m<sup>-2</sup> s<sup>-1</sup> which has been corrected to μmol photons m<sup>-2</sup> s<sup>-1</sup> in the table.

Study Reference	Strain	White Light Illumination Conditions (W/m <sup>2</sup> )	Anode / Cathode (Working / Counter / Reference)	Max. Power (mW/m <sup>2</sup> )
61	<i>Synechocystis sp. PCC 6803</i>	~100 lux (6,500 K)	polyA-coated CP / Pt-CC-CB-PTFE / Ag/AgCl	0.95
61	<i>Synechocystis sp. PCC 6803</i>	~100 lux (6,500 K)	polyP-coated CP / Pt-CC-CB-PTFE / Ag/AgCl	1.3
76	<i>Spirulina platensis</i>	30 μmol photons m <sup>-2</sup> s <sup>-1</sup>	Pt / Pt	6.5
15	<i>Synechocystis sp. PCC 6803</i>	10 W/m <sup>2</sup>	ITO / Pt-coated glass	0.114
15	<i>Synechococcus sp. WH 5701</i>	10 W/m <sup>2</sup>	ITO / Pt-coated glass	10
29	<i>Pseudanabaena limnetica (Oscillatoria limnetica)</i>	~8 W/m <sup>2</sup>	ITO / Pt-coated glass	~0.02 [a]
29	<i>Pseudanabaena limnetica (Oscillatoria limnetica)</i>	~8 W/m <sup>2</sup>	stainless steel / Pt-coated carbon paper	~0.006 [b]
29	<i>Pseudanabaena limnetica (Oscillatoria limnetica)</i>	~8 W/m <sup>2</sup>	PANI-coated glass / Pt-coated carbon paper	~0.003 [c]
29	<i>Pseudanabaena limnetica (Oscillatoria limnetica)</i>	~8 W/m <sup>2</sup>	carbon paper / Pt-coated carbon paper	~0.002 [d]
77	<i>Synechocystis sp. PCC 6803</i>	10,000 lux	carbon fiber / carbon fiber	0.3 [e]
19	<i>Spirulina platensis</i>	3000 μmol photons m <sup>-2</sup> s <sup>-1</sup> [f]	Au mesh / carbon cloth	10
20	<i>Nostoc sp. ATCC 27893 (Anabaena PCC 7120)</i>	760 W/m <sup>2</sup> (3,100K)	CNT-modified carbon paper / laccase-CNT-modified carbon paper	35
21	<i>Synechocystis sp. PCC 6803</i>	42 W/m <sup>2</sup>	InSnBi / Pt	105

## 6. Engineering of Electron Pathways in Cyanobacteria for Energy Applications

The wild-type cyanobacteria used in the living photovoltaic devices shown in Table 1 have undergone billions of years of evolution to yield photosynthetic microorganisms with robust mechanisms of dynamic repair and adaptability. These microorganisms have been specifically engineered by nature for enhanced survival rather than maximal light harvesting, which is detrimental to photoautotrophic health. In fact, photosynthetic cells actively dissipate up to 80% of the light they have captured to prevent the formation of damaging reactive oxygen species under intense illumination conditions<sup>78,79</sup>. Considering that only 45% of the solar spectrum is accessible to photosynthetic cells<sup>80</sup>, nature has largely engineered cyanobacteria to harness what is optimally required for survival. This conservative light-harvesting design is at odds with solar cell design principles that maximize light absorption, charge separation, and charge transfer across the entire solar spectrum. As such, synthetic biology can be used to re-purpose natural light-harvesting complexes for more efficient solar cell applications.

Different approaches can be used to engineer cyanobacteria for improved photovoltaic behavior<sup>81</sup>. One approach is to increase total light absorption by broadening the absorption spectrum of the cell. This can be achieved by engineering the photosystems (PSII and PSI) to broaden the light absorption range

into non-overlapping regions of the solar spectrum as well as tuning the arrangement and peak absorption of the surrounding light-harvesting complexes<sup>81,82</sup>. However, given that photoautotrophs readily dissipate absorbed light that is in excess of the optimal energy requirements for survival, a logical approach to engineering biological devices would be to first engineer a pathway for dissipating excess energy through improved exoelectrogenic activity prior to broadening the absorption spectrum of the cell.

One possibility is to genetically engineer cyanobacteria in a manner analogous to that described above for *E. coli* by introducing electron exporting protein pathways such as the MtrCAB pathway into cyanobacteria. As described above, engineering such pathways in *E. coli* has been a lengthy endeavor, pushing the metabolic limits of heterologous protein expression. Translocating this pathway to less well-characterized organisms such as cyanobacteria poses additional expression challenges. Whereas multiple foreign, heme-containing proteins had been expressed in *E. coli* years before MtrA expression was first attempted<sup>83,84</sup>, biologists have only recently begun to develop a comprehensive understanding of the proteins involved in the Ccm pathway in cyanobacteria<sup>50,85</sup>. Though cyanobacteria may benefit from the inherent ability to mature c-type cytochromes under aerobic growth conditions<sup>86</sup>, to the best of our knowledge, there is only one successful demonstration of a foreign heme-containing cytochrome expressed in cyanobacteria<sup>87</sup>. Therefore, challenges in extending this platform to cyanobacteria may include developing a system for the expression of specialized Ccm maturation pathways in addition to the heterologous cytochromes and iteratively optimizing expression conditions as done over the past decade with *E. coli*, albeit now in a host with a growth rate that can be over 30 times slower.

Although the field eagerly awaits further advancements in heme-containing protein expression in cyanobacteria, researchers have started to engineer cyanobacteria for improved extracellular electron transfer mechanisms through alternative approaches. One such study deleted terminal oxidases to enhance extracellular electron transfer<sup>88</sup>. During aerobic respiration, the cell metabolizes sugar through oxidation, producing low energy electrons. These electrons are passed along a series of oxidases until they arrive at the terminal oxidase, which transfers electrons to oxygen, the final electron acceptor. Under intense illumination, terminal oxidases prevent the over-reduction of the photosynthetic electron transport chain, thereby decreasing oxidative stress. By deleting the terminal oxidases, the authors rationalized that the electrons otherwise "wasted" on oxygen can be redirected through alternative pathways capable of power generation. The authors were able to redirect metabolic electron flux in manner that increases power output, though the exact mechanism for this extracellular transport is unknown.

In 2016, Sekar *et al.*<sup>87</sup> engineered the cyanobacterium *Synechococcus elongatus* PCC 7942 for extracellular electron transfer by heterologously expressing OmcS, an outer membrane protein involved in extracellular Fe(III) oxide reduction by *G. sulfurreducens*<sup>89</sup>. This work is believed to be the first and only demonstration of heterologous expression of a foreign cytochrome *c* in cyanobacteria. Although extracellular electron transport in *G. sulfurreducens* through the OmcS pathway is poorly understood, some studies suggest that OmcS is a heme-containing protein localized along the pili that serves a functionally similar role as MtrC in *S. oneidensis*<sup>49,90</sup>. As a heme-containing protein, OmcS expression in cyanobacteria faces challenges similar to MtrCAB expression in *E. coli*, though it benefits from several technical advantages: (1) the expression of only one (OmcS) instead of three (MtrA, MtrB, MtrC) proteins, (2) the absence of integral membrane proteins such as MtrB, which introduce additional complications in post-translational modifications, protein folding, and membrane-targeting, (3) protein

localization to just one sub-cellular compartment instead of two to three compartments (periplasm for MtrA, outer membrane for MtrB and MtrC, and inner membrane for CymA), and (4) the expression of a six-heme protein (OmcS) instead of two ten-heme proteins (MtrC, MtrA). These advantages allow for a streamlined genetic engineering approach with just a single gene needed to express OmcS, collectively decreasing the overall demand for the metabolic resources required for protein expression and post-translational modifications. The expression of OmcS in *S. elongatus* PCC 7942 was verified by heme staining in the soluble protein fraction of *S. elongatus*, which the authors attributed to the loose association of the protein to the periplasmic membrane.

The OmcS-engineered strain demonstrated improved light-dependent ferricyanide reduction compared to wild-type strains<sup>87</sup>. Open-circuit potential and amperometry measurements of the bioengineered and wild-type *S. elongatus* PCC 7942 strains were performed using a carbon nanotube-modified carbon paper working electrode. This study showed a similar decrease in open-circuit voltage for the engineered strains compared to the wild-type strain under both illuminated and dark conditions, which suggests large contributions from respiration in addition to contributions from photosynthesis. Amperometry measurements confirmed an approximately ninefold increase in photo-current for the engineered cells compared to the wild-type cells.

One explanation offered by Sekar *et al.* for the increased current is that OmcS shuttles electrons extracted from the plastoquinone pool and/or from plastocyanin to the electrode. In the former case, the authors hypothesize that, under excess illumination conditions, OmcS can extract photosynthetic electrons from the over-reduced plastoquinone pool to dissipate reducing equivalents similar to terminal oxidases<sup>88</sup>. Previous measurements have shown that OmcS has a midpoint redox potential of -212 mV (vs. SHE), with complete reduction at c.a. -375 mV and complete oxidation at c.a. -50 mV<sup>90</sup>. Thus, reduction of OmcS by the plastoquinone pool (c.a. +80 mV)<sup>91</sup> is significantly less energetically favorable than reduction of cytochrome  $b_6f$  (-130 - +355 mV)<sup>92</sup> by the plastoquinone pool. Similarly, in the second proposed pathway, the reduction of OmcS by plastocyanin (+370 mV)<sup>93</sup>/cytochrome  $c_6$  (c.a. +320 mV)<sup>94</sup> is even more energetically unfavorable. Thus, additional factors, such as the coupling of OmcS with the membrane-bound electron transport chains by specific mediators, could be necessary to make either of these pathways favorable. Current-voltage (I-V), power density, and additional biochemical characterizations of the engineered and wild-type devices are needed to elucidate the mechanism, and these measurements are the focus of ongoing research<sup>87</sup>. These studies will significantly advance the fundamental metabolic understanding that is needed to engineer living photovoltaics in cyanobacteria.

## 7. Theoretical Analysis of Bio-engineered Cyanobacteria Devices

A thermodynamic analysis of different engineering approaches can be used to calculate maximum theoretical efficiencies and identify favorable approaches for engineering devices. In this analysis, we model a bioengineered system that utilizes the MtrCAB pathway for charge extraction (Figure 3). This specific pathway is chosen because (1) it is the only defined exoelectrogenic pathway that has been fully and functionally reconstituted in a foreign host cell, ensuring that all the proteins involved in this specific pathway have been identified, and (2) redox potentials reported in the literature allow us to calculate approximate efficiencies.

Photosynthetic systems have very efficient strategies for charge separation, allowing photosynthetic

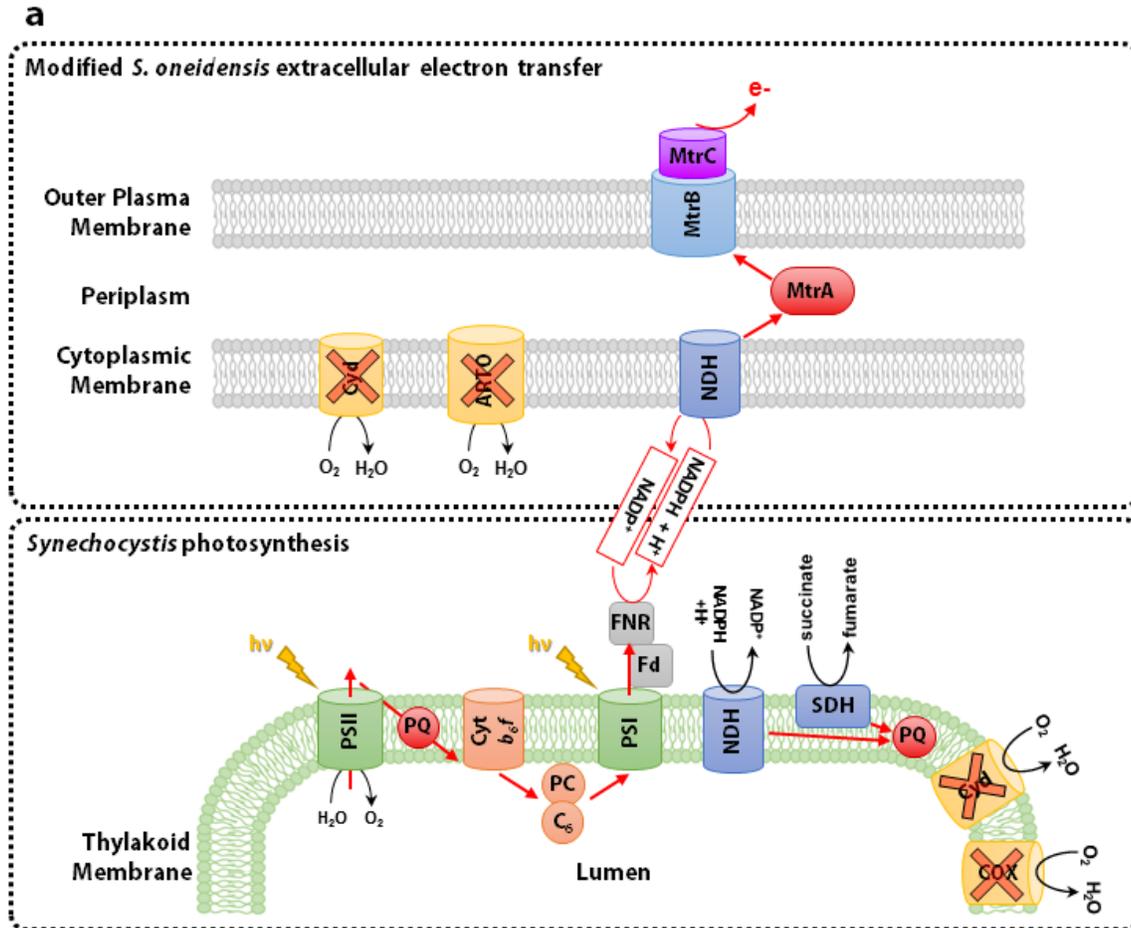
organisms to operate at nearly 100% quantum efficiency under optimal conditions<sup>95</sup>. In other words, nearly every photon that is absorbed yields a separated electron-proton pair. However, since light-harvesting pigments absorb light that is largely limited to the visible region of the solar spectrum, photosynthetic organisms can only access approximately 45% of the solar spectrum<sup>80</sup>. Assuming that 100% of the photons with energies larger than the PSII band gap (680 nm, 1.8 eV) are absorbed at sea level (ASTM), the effective upper limit becomes 37%<sup>96</sup>. The Shockley-Queisser limit of a single-junction, 1.8 eV band-gap photovoltaic under AM 1.5 illumination conditions, which accounts for thermal dissipation of photons with energies above the bandgap energies, is approximately 24%.

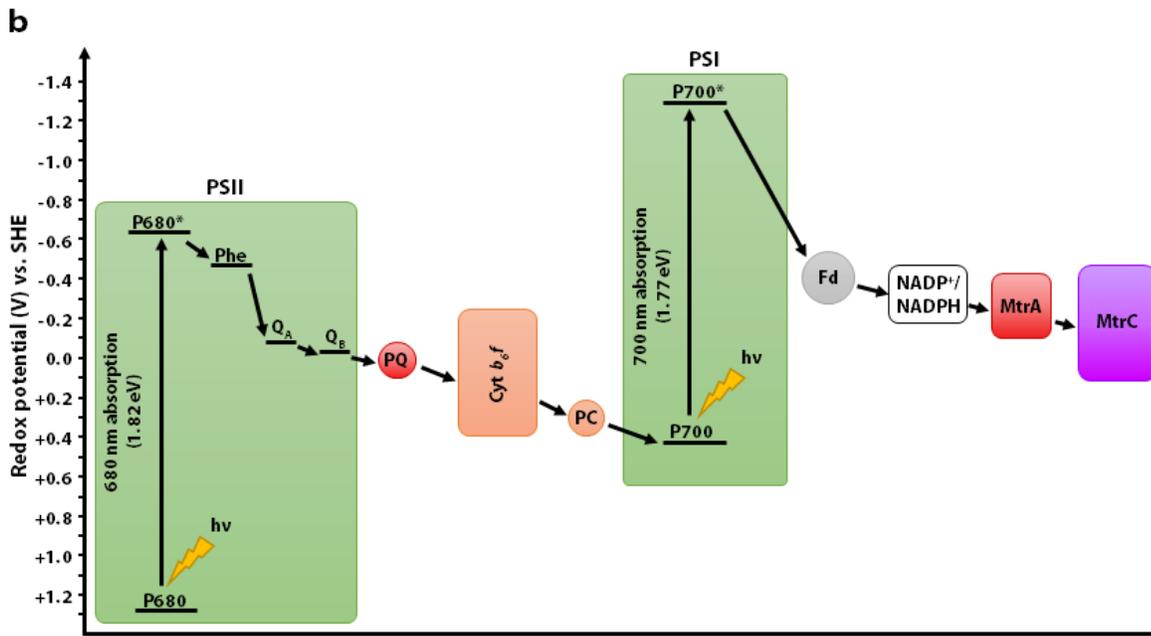
Unlike protein-based biophotovoltaics, which can directly interface PSII to an electrode<sup>97</sup>, the whole-cell devices discussed herein only extract electrons after a series of electrochemical reactions that convert the separated charge into biochemical fuel, and this electrochemical conversion contributes to additional energy losses. Approximately 1.23 eV is required to split water, and this highly endergonic reaction is the most thermodynamically challenging reaction known to occur in living systems. As such, water splitting occurs in the oxygen-evolving complex through oxidation by the active site, P680, whose cation radical is the strongest biological oxidizing agent known. As shown in Figure 2, the subsequent electron acceptors have redox potentials that are largely above 0 mV. In contrast, the multi-heme proteins involved in extracellular electron transfer shown in Figure 1 have redox potentials that are largely below 0 mV, suggesting more favorable electron extraction following re-excitation at PSI. Following PSI excitation, the electron is used to produce NADPH, a mobile carrier of reducing equivalents, involved in carbon fixation and sugar production during the Calvin Cycle. Engineering an extracellular electron pathway that extracts charge after NADPH production (1) does not directly compete with NADPH production by re-directing electrons otherwise used for NADPH synthesis and (2) circumvents challenges with otherwise having to localize proteins in the thylakoid membrane to intercept electrons during the series of charge-transfer reactions<sup>60</sup>. In contrast, NADPH readily transverses the cytoplasm, directly accessing the cytoplasmic membrane.

In *S. oneidensis*, electrons are transferred from the cytoplasm to menaquinone to CymA before MtrA reduction. In the proposed mechanism shown in Figure 3, direct electron transfer from NADPH to MtrA in cyanobacteria would result in minimal energy transfer losses. Electron transfer from NADPH to MtrA is an energetically feasible reaction (Figure 3b), though it requires the expression of an unnatural protein specifically engineered for this function (see discussion in the Conclusions and Outlook section).

Based on this proposed scheme, theoretical maximum efficiencies were calculated for the conversion of light to electric energy via the extracellular transfer of photosynthetic electrons by the MtrCAB pathway and by the outer membrane cytochrome OmcS. This was done for a closed biophotovoltaic system reducing oxygen at the cathode working under optimal conditions and assuming that (1) all photochemical active photons are evenly absorbed by both photosystems, (2) the electrons follow the Z-schema of linear electron transport, (3) the only mediator between photosynthesis and MtrCAB or OmcS-mediated electron transfer is NADPH, and (4) no losses occur due to carbon fixation. For the reduction of one molecule of NADPH, four photons with an average energy of 205 kJ mol<sup>-1</sup><sup>98</sup> need to be absorbed, requiring a total of 820 kJ mol<sup>-1</sup>. For the final protein-based electron mediator OmcS, the values were based on the midpoint potential of -212 mV<sup>90</sup>. Since the decaheme MtrC, shows a broad redox-potential range spanning approximately +100 to -400 mV, the change in free energy for the electron transfer to oxygen ( $E_0' = 816$  mV) was calculated assuming a minimal value of +100 mV and a maximal value of -320 mV corresponding to the potential for NADPH oxidation. Based on these values,

the standard Gibbs free energy change ( $\Delta G^\circ$ ) was found to range from 129 kJ to 219 kJ for the MtrCAB pathway and 198 kJ for OmcS, yielding a maximum conversion efficiency of 15.7-26.7% of the absorbed energy to available (electrical) energy. This translates to a conversion efficiency of roughly 6-10% for MtrCAB and 9% for OmcS relative to the total solar irradiation.





**Figure 3 | Coupling the MtrCAB Pathway to Photosynthesis in Cyanobacteria. (a)** The introduction of the MtrCAB pathway in cyanobacteria offers one approach to extracting photo-generated charge from water splitting during photosynthesis. The pathway includes the reduction of MtrA from NADPH produced during photosynthesis. **(b)** A comparison of the redox potentials of the proteins involved in the photosynthetic and MtrCAB pathways suggests a thermodynamically feasible pathway using NADPH. The NADPH to MtrA electron transfer may require the expression of a chimeric protein that has been engineered to facilitate the transfer of electrons to a periplasmic MtrA instead of the membrane-bound plastoquinone pool. Approximate potentials are shown.

## 8. Conclusions and Outlook

This review highlights a specific research trajectory in the living photovoltaics field that exploits parallel, convergent efforts in materials science and biological engineering towards realizing living photovoltaics with optimized electron transfer behavior. Traditionally, membrane-electrode interactions have been tuned by modifying the electrode composition and surface, as well as screening various combinations of mediators to facilitate electron transfer. Recent studies have focused on a complementary synthetic biology approach that requires the expression of redox-active foreign proteins that span the outer membrane of the cell. The primary advantage of using such bioengineered strains for energy applications is that electron transfer is autonomously and molecularly encoded by the cell's genome; electron transfer can be achieved in the absence of mediators, which contribute to increased device cost and instability, and unlike mediators, electrons can be selectively and specifically withdrawn from different intracellular redox pools through the use of molecular recognition. Direct electron transfer circumvents energy losses incurred as a result of multiple, consecutive electron transfer mechanisms, decreasing the overpotential of the cell.

*E. coli* serves as a model host organism for demonstrating proof-of-concept approaches to increase whole-cell, extracellular electron transfer using synthetic biology techniques. Although multiple naturally exoelectronic pathways have been identified in species such as *S. oneidensis* and *G.*

*sulfurreducens*, only one pathway, the MtrCAB conduit from *S. oneidensis*, has been functionally expressed in its entirety in *E. coli*. Though the expression of MtrA, MtrB, and MtrC are minimally required for extracellular transfer<sup>52,56</sup>, the co-expression of CymA was found to improve overall extracellular electron transfer<sup>54,59</sup>. Unfortunately, the current co-expression of CymA and MtrCAB is a significant challenge to the cell, resulting in cells that are poorly suited for the introduction of additional heterologous genetic circuitry. One possible approach to addressing this limitation is to apply site directed mutagenesis or direct evolution to engineer MtrA for improved interaction with the endogenous *E. coli* proteins involved in the electron transfer pathway. In theory, the expression of an engineered MtrA protein should improve the overall electron transfer at the pathway bottleneck without increasing the resources required for protein expression compared to the wild-type pathway.

The next logical step in applying this platform to biophotovoltaic devices is to express such a conduit in photosynthetic cells such as cyanobacteria. Unfortunately, the synthetic biology tools available for cyanobacteria are limited compared to the tools available for *E. coli*, and co-expression of MtrA, MtrB, and MtrC has not yet been achieved in cyanobacteria. However, a recent study has demonstrated for the first time the expression of a single, foreign, heme-containing protein, OmcS, in cyanobacteria that has been shown to improve extracellular electron transfer<sup>87</sup>. While the comparable maximum theoretical efficiencies calculated for the MtrCAB (10%) and OmcS (9%) pathways do not significantly favor one pathway from a thermodynamic perspective, the technical feasibilities of the different pathways largely lie with challenges in protein expression and metabolic understanding of the systems. For instance, the OmcS pathway benefits from a simpler expression system that may be less metabolically burdensome, whereas the MtrCAB pathway offers an identifiable charge transfer pathway that can be more clearly defined.

Well-defined pathways allow biologists to engineer the metabolism of the cell in a rational manner with the goal of optimizing electron transfer. Analysis of extracellular transfer pathways involving photosynthetically derived electrons reveals that one possible pathway includes electron extraction from NADPH, which is indirectly reduced by electrons extracted from light-induced water splitting. This proposed pathway requires a cytoplasmic membrane protein capable of oxidizing NADPH and reducing a suitable electron acceptor like MtrA. Such a protein does not naturally exist in cyanobacteria and requires protein engineering efforts to enable this reaction. One approach would be to create protein-fusion chimera consisting of the NADPH oxidizing domains of NADPH dehydrogenase or oxidase and the MtrA-reducing domain of CymA.

In addition, protein engineering may also be used to enhance light-harvesting to improve efficiencies<sup>81</sup> in living photovoltaics. Since survival serves as the evolutionary selection pressure, photosynthetic organisms have evolved to access only a fraction of the solar spectrum and dissipate excess energy to minimize protein photodamage. Previous studies have engineered light-sensitive proteins to absorb light at different wavelengths<sup>99</sup>, effectively increasing the range of wavelengths that can be absorbed by the solar spectrum. Increasing light absorption by expressing additional pigments<sup>100</sup> or truncating the light harvesting antennae size to minimize wasteful energy dissipation and increase light penetration in dense cultures<sup>80</sup> have been proposed as effective approaches for enhancing device efficiencies. In combination with biological limitations, device configurations may also limit overall efficiencies. The absorption of light by water or the complete or partial absorption of light by overlaying electrodes may diminish device efficiencies, though the extent of impairment depends on the specific configuration<sup>108</sup>. For example, one device configuration immobilizes light-harvesting bacteria on the

surface of a transparent electrode that is placed at the top of the device, allowing the bacteria to absorb light before it penetrates the aqueous compartment below<sup>15</sup>. A similar configuration has been adapted for dye-sensitized solar cells (DSSCs), for example<sup>109</sup>. Another device configuration used on the industrial scale for algal biofuel production consists of small diameter tubes that maximize the surface area to volume ratio of the reactor to minimize the penetration depth of light and enhance bacterial light absorption<sup>110</sup>.

In summary, the ability to introduce multiple heme-containing proteins to new cells has enabled a new generation of bioengineering technologies for energy applications. Although the latest bioengineered strains demonstrate efficiencies far below theoretical limits, these strains have yet to benefit from protein engineering approaches that have been historically used to tune unnatural chimeric systems. Synthetic biology offers a broad set of tools that, when combined with traditional materials engineering approaches, unlocks unprecedented possibilities for living photovoltaics.

## Acknowledgements

The authors thank Frances H. Arnold for manuscript revisions and suggestions. The authors also thank Jackson K. B. Cahn, Devin L. Trudeau, Thomas Heel, and Sabine Brinkmann-Chen for in-depth discussions on protein engineering techniques and the underlying theory. A.A.B. is thankful for support from the Ruth L. Kirschstein NRSA postdoctoral fellowship from the National Institute of Health (1F32GM112450-01). C.W. is supported by the Swiss Government Excellent Scholarship Grant (2015.0466). The authors thank the Dow Chemical Company-Resnick Institute Bridge/CI2 Innovation Program and the Swiss National Science (SNSF) Advanced Professorship (AP) Energy Grant for funding support.

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