

# Single-cell multimodal imaging uncovers energy conversion pathways in biohybrids

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Microbe–semiconductor biohybrids, which integrate microbial enzymatic synthesis with the light-harvesting capabilities of inorganic semiconductors, have emerged as promising solar-to-chemical conversion systems. Improving the electron transport at the nano–bio interface and inside cells is important for boosting conversion efficiencies, yet the underlying mechanism is challenging to study by bulk measurements owing to the heterogeneities of both constituents. Here we develop a generalizable, quantitative multimodal microscopy platform that combines multi-channel optical imaging and photocurrent mapping to probe such biohybrids down to single- to sub-cell/particle levels. We uncover and differentiate the critical roles of different hydrogenases in the lithoautotrophic bacterium *Ralstonia eutropha* for bioplastic formation, discover this bacterium’s surprisingly large nanoampere-level electron-uptake capability, and dissect the cross-membrane electron-transport pathways. This imaging platform, and the associated analytical framework, can uncover electron-transport mechanisms in various types of biohybrid, and potentially offers a means to use and engineer *R. eutropha* for efficient chemical production coupled with photocatalytic materials.

Utilizing solar energy to efficiently synthesize higher-value chemicals from earth-abundant molecules such as CO<sub>2</sub> remains a long-standing challenge. Microbe–semiconductor biohybrids have emerged as promising systems to address this challenge by combining the high light-harvesting efficiencies of inorganic semiconductors with the capacity of microbes to orchestrate complex chemical transformations<sup>1–6</sup>. In the ‘decoupled biohybrids’ (Fig. 1a, top), semiconductor-based photovoltaics power water electrolysis to generate H<sub>2</sub>, which is subsequently fed to lithoautotrophic bacteria (for example, *Ralstonia eutropha* and

*Methanosarcina barkeri*) to fix CO<sub>2</sub> to value-added chemicals (for example, biomass, isopropanol and methane)<sup>7,8</sup>. In ‘integrated biohybrids’ (Fig. 1a, bottom), the microbes (for example, *Sporomusa ovata*, *Moorella thermoacetica* and *Saccharomyces cerevisiae*) are directly interfaced with semiconductor photoelectrodes or photocatalysts, where photogenerated electrons are taken up by the microbes to drive downstream biosynthesis of acetic acid, methane, shikimic acid, polymers and so on<sup>9–12</sup>.

Key to fully exploiting such biohybrid systems is the engineering of semiconductors and bacteria on the basis of mechanistic, quantitative

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guidance for improving energy conversion efficiencies. Although bulk-level studies have provided important insights<sup>7,10,13–16</sup>, their interpretations are complicated by the ubiquitous heterogeneities of microbial cells in gene expression<sup>17</sup> and of semiconductor particles in photocatalytic activity<sup>18,19</sup>. Such heterogeneities demand quantitative measurements at the single- and sub-cell/particle levels.

In this Article we develop a generalizable, multimodal imaging approach, combining multi-channel fluorescence microscopy and photoelectrochemical current mapping, to interrogate the players and energy conversion pathways in microbe–semiconductor biohybrids for CO<sub>2</sub> fixation down to single-molecule, single-cell and single/sub-particle level (Fig. 1b). We focus on the lithoautotrophic Gram-negative bacterium *Ralstonia eutropha* (a.k.a. *Cupriavidus necator*), which was previously used in decoupled biohybrids<sup>7</sup> using H<sub>2</sub> as the sole energy source to fix CO<sub>2</sub> into the bioplastic polyhydroxybutyrate (PHB)<sup>20</sup>. We dissect the different roles of membrane-bound and soluble hydrogenases towards PHB formation in the cell grown under H<sub>2</sub> and CO<sub>2</sub>. We discover a surprisingly large, nanoampere-level electron-uptake capability of *R. eutropha* from semiconductor photoelectrodes with different energy levels. We further show that this large interfacial electron transport is dominantly mediated by species other than H<sub>2</sub>, yet hydrogenases play critical roles in sustaining these photoelectrochemical currents. These findings raise the possibilities of using *R. eutropha* in both decoupled and integrated biohybrids and provide quantitative insights to guide microbial engineering for enhanced energy conversion.

## Results and discussion

### Role of hydrogenases in bioplastic production

*R. eutropha* uses two types of hydrogenase to metabolize H<sub>2</sub> (Supplementary Fig. 1): a membrane-bound hydrogenase (MBH), which comprises HoxGKZ subunits and is known to deliver electrons to the respiratory chain that generates a proton gradient across the membrane for producing ATP, and a cytosolic soluble hydrogenase (SH), which comprises HoxFUYHI subunits and generates NADH for downstream biosynthesis<sup>21–23</sup>. To probe their roles in bioplastic production under lithoautotrophic growth, we examined their cellular concentrations in relation to cellular PHB production at the single-cell level using optical imaging (Fig. 1b).

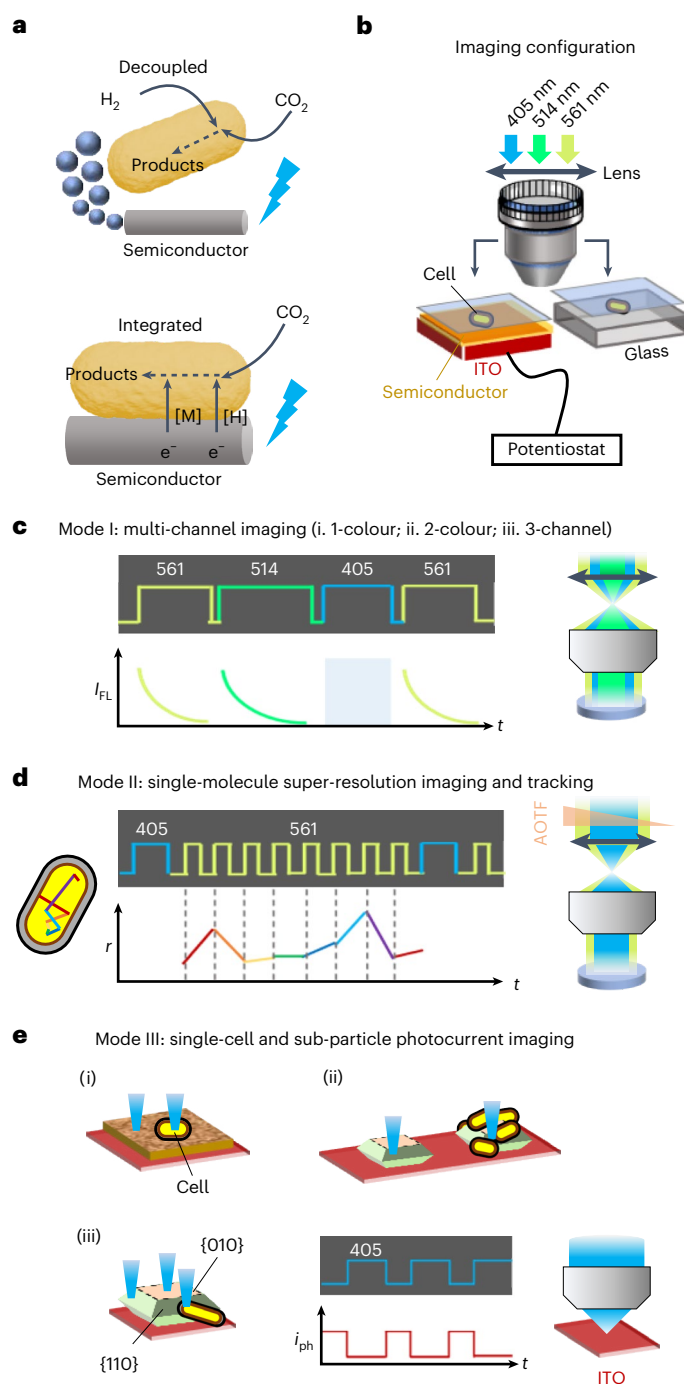
To visualize PHB, we tagged PhaP1, a PHB granule-association protein<sup>24</sup>, or PhaC, a PHB synthase<sup>25</sup>, with the yellow fluorescent protein mVenus (mV) at their respective chromosomal loci, enabling their imaging down to single-molecule sensitivity (Supplementary Sections 1.2–1.4). Both proteins decorate PHB granules<sup>26</sup>, and their expression levels also scale with the amount of PHB in bulk cultures<sup>27</sup>.

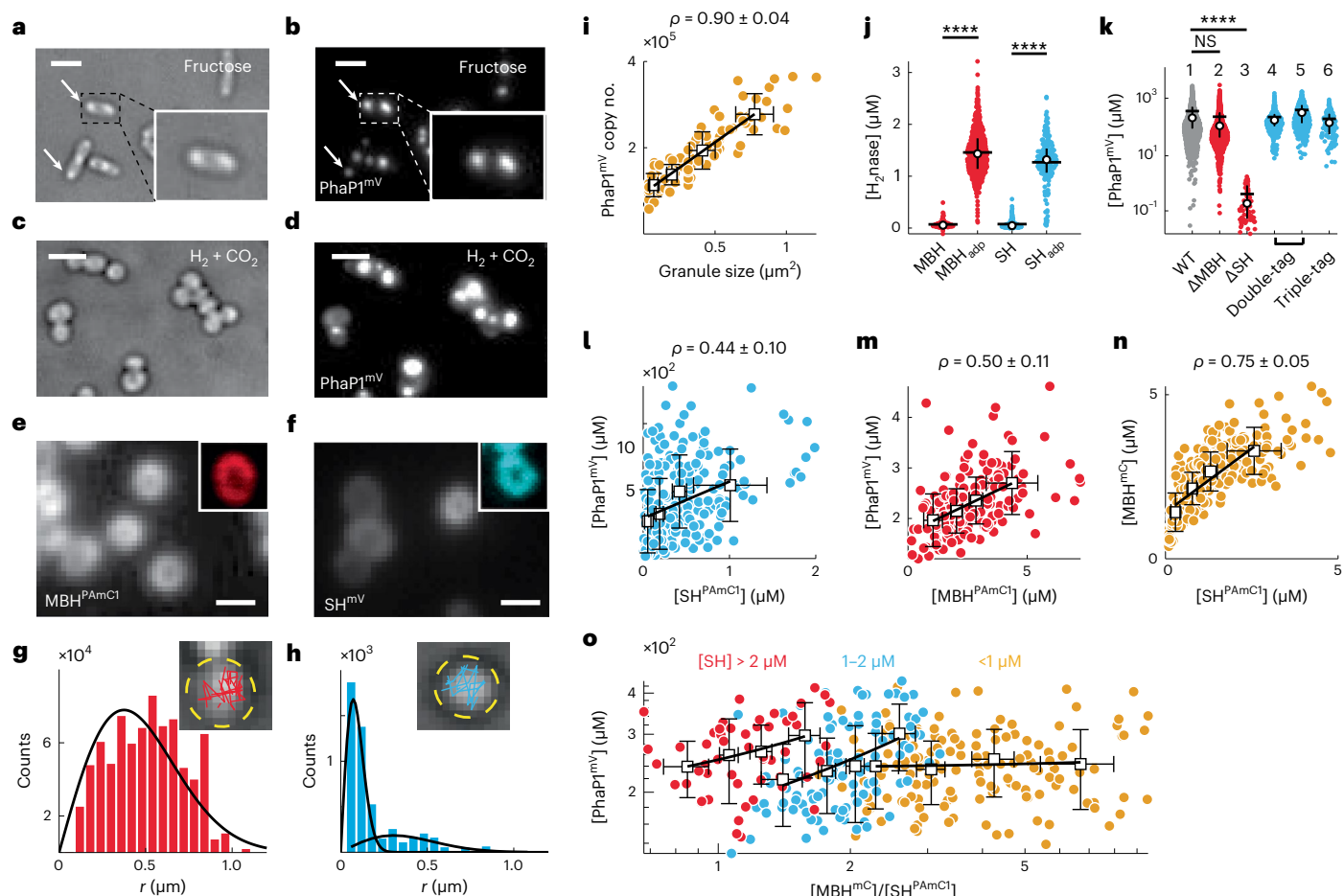
### Fig. 1 | Microbe–semiconductor biohybrids and multimodal imaging platform

**a**, Decoupled and integrated biohybrids for energy conversion. [H]: H<sup>+</sup>/H<sub>2</sub> as the redox mediator, where H<sub>2</sub> can be generated by electrocatalysts or photocatalysts; [M]: other redox mediators. **b**, Multimodal imaging platform (details are provided in Supplementary Sections 1.3–1.5), consisting of a (photoelectrochemical) microfluidic cell on an epifluorescence microscope with multiple, modulated lasers for wide-field and/or focused excitation. Indium tin oxide (ITO) connected to a potentiostat is used as the working electrode for photoelectrochemical current mapping. A 405-nm laser is used for photoactivating PAmC1 and/or exciting the semiconductors, a 514-nm laser for exciting mV and a 561-nm laser for exciting mC and the photoactivated PAmC1. **c**, Imaging mode I: multi-channel wide-field fluorescence imaging to quantify fluorescent-tagged proteins in single cells, showing the timing (*t*) of lasers (top left), the protein fluorescence *I<sub>FL</sub>* (bottom left) and the laser excitation geometry (right). **d**, Mode II: super-resolution single-molecule tracking by stroboscopic fluorescence imaging. Left: schematic position trajectories in a cell. *r*, displacement; AOTF, acoustic-optic tunable filter. **e**, Mode III: focused laser illumination to probe single-spot photoelectrochemical currents on single cells in contact with a semiconductor film (i) or with single semiconductor particles (ii and iii). *i<sub>ph</sub>*, photocurrent.

We first imaged PhaP1<sup>mV</sup> or PhaC<sup>mV</sup> in *R. eutropha* grown with fructose as the carbon source (that is, non-adapted condition). These cells are micrometre-long and rod-shaped, with PHB granules clearly visible in bright-field transmission images (Fig. 2a). Under wide-field fluorescence imaging (Fig. 1c, mode I-i), PhaP1<sup>mV</sup> and PhaC<sup>mV</sup> expectedly colocalize with PHB granules (Fig. 2b and Supplementary Fig. 7b–e), confirming the function and integrity of the tagged proteins (Supplementary Section 2). Furthermore, the PhaP1 and PhaC copy numbers of individual cells, determined from wide-field fluorescence imaging, scale linearly with PHB granule sizes (Fig. 2i and Supplementary Fig. 10g), validating PhaP1/PhaC as quantitative reporters for PHB level. Because of its higher copy numbers and stronger linear correlation with granule sizes than PhaC, we use PhaP1 as the PHB reporter in the following.

We then quantified PHB in *R. eutropha* cells lithoautotrophically grown with H<sub>2</sub>, CO<sub>2</sub> and air (that is, the adapted condition; Methods





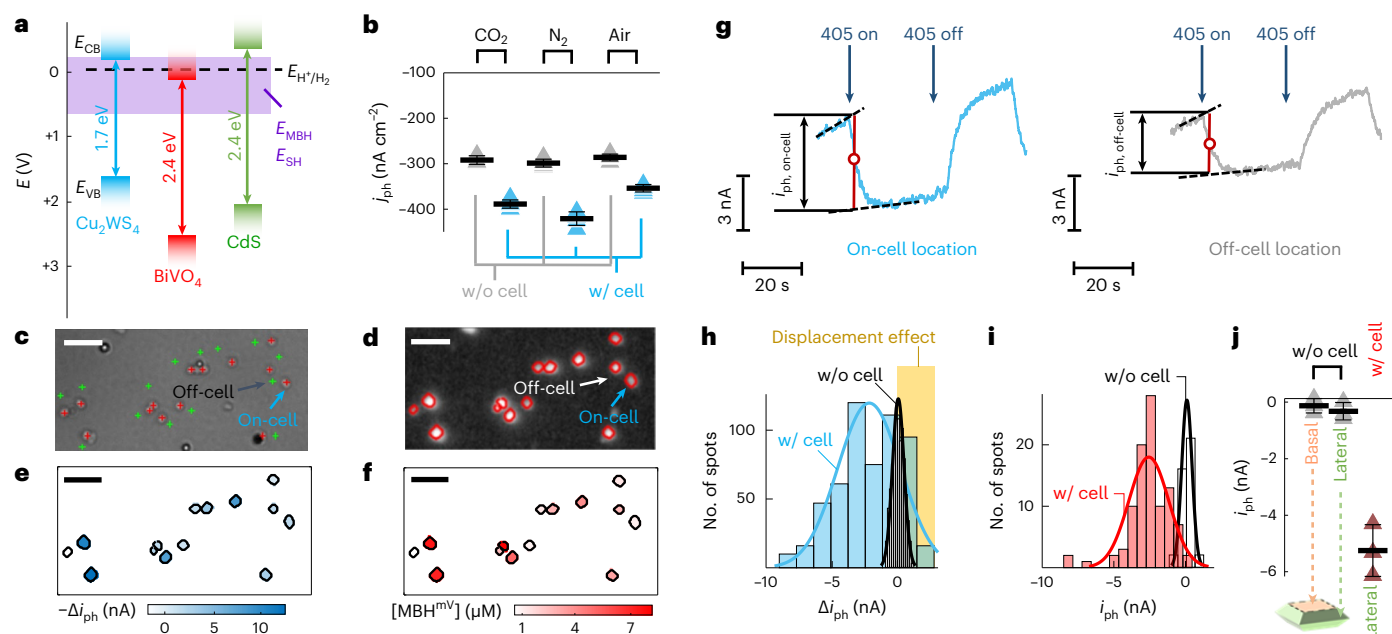
**Fig. 2 | Roles of hydrogenases in bioplastic formation under lithoautotrophic growth.** **a**, Bright-field transmission image of PhaP1<sup>mV</sup> *R. eutropha* cells grown with fructose (that is, non-adapted). PHB granules appear as punctate spots, two of which are marked by arrows. **b**, Wide-field fluorescence image corresponding to **a**, showing the PhaP1<sup>mV</sup> decorating PHB granules. **c,d**, Same as **a,b**, but for PhaP1<sup>mV</sup> *R. eutropha* cells grown with H<sub>2</sub> and CO<sub>2</sub> (that is, adapted). Scale bars, 2  $\mu$ m (**a–d**). **e,f**, Wide-field fluorescence images of adapted MBH<sup>PAmC1</sup> (**e**) and SH<sup>mV</sup> (**f**) cells. Insets: false-coloured confocal fluorescence images. Scale bars, 1  $\mu$ m. **g,h**, Cell-confinement-effect-deconvoluted displacement length  $r$  distributions of MBH<sup>PAmC1</sup><sub>HoxG</sub> (**g**) and SH<sup>mV</sup><sub>HoxY</sub> (**h**) from single-molecule tracking (Supplementary Section 7). Black lines: fitted one (**g**) or two (**h**) Brownian diffusion state displacement distributions. Insets: representative single-molecule tracking trajectories (red/blue lines) overlaid on the transmission image of a cell. Yellow dashed lines: cell contours. **i**, Correlation between PhaP1<sup>mV</sup> copy number and PHB granule size in single non-adapted cells (yellow dots). Black open squares are binned and averaged results. Across individual bins there are approximately equal numbers of data points to be statistically equivalent. Black line: linear fit as a guide

to the eye (same for **i–o**).  $\rho$ , Pearson's correlation coefficient with 95% confidence interval calculated from the original data points.  $n = 95$  cells. **j**, Cellular concentrations of MBH<sup>mV</sup><sub>HoxG</sub> and SH<sup>mV</sup><sub>HoxY</sub> in non-adapted (columns 1 and 3; sample size  $n = 126$  and 589) and adapted cells (columns 2 and 4; sample size  $n = 177$  and 194). Two-sided  $t$ -test, \*\*\*\* $P < 0.0001$ . **k**, Cellular PhaP1<sup>mV</sup> concentrations in different adapted strains. Column 1: PhaP1<sup>mV</sup>, sample size  $n = 2,417$ ; column 2: PhaP1<sup>mV</sup>,  $\Delta$ MBH ( $\Delta$ hoxG),  $n = 1,561$ ; column 3: PhaP1<sup>mV</sup>,  $\Delta$ SH ( $\Delta$ hoxY),  $n = 66$ ; column 4: MBH<sup>PAmC1</sup><sub>HoxG</sub>, PhaP1<sup>mV</sup>,  $n = 513$ ; column 5: SH<sup>PAmC1</sup><sub>HoxY</sub>, PhaP1<sup>mV</sup>,  $n = 579$ ; column 6: MBH<sup>mV</sup><sub>HoxG</sub>, SH<sup>PAmC1</sup><sub>HoxY</sub>, PhaP1<sup>mV</sup>,  $n = 130$ . NS, not significant. Two-sided  $t$ -test, \*\*\*\* $P < 0.0001$ . In **j** and **k**, white circles denote the median, horizontal black lines the mean, vertical black lines the 25% to 75% quantile. **l–n**, Cellular protein concentration correlations in adapted single cells (coloured dots) between PhaP1<sup>mV</sup> and SH<sup>PAmC1</sup><sub>HoxY</sub>,  $n = 243$  cells (**l**), PhaP1<sup>mV</sup> and MBH<sup>PAmC1</sup><sub>HoxG</sub>,  $n = 190$  cells (**m**), MBH<sup>mV</sup><sub>HoxG</sub> and SH<sup>PAmC1</sup><sub>HoxY</sub>,  $n = 313$  cells (**n**). **o**, Cellular PhaP1<sup>mV</sup> concentration versus [MBH<sup>mV</sup><sub>HoxG</sub>]/[SH<sup>PAmC1</sup><sub>HoxY</sub>] in single adapted cells (coloured dots), sorted into three groups of [SH]. Error bars in **i** and **l–o** indicate s.d.

and Supplementary Section 1.2). The cells are short, with PHB granules (although unclear in transmission images) distinctively marked by PhaP1<sup>mV</sup>/PhaC<sup>mV</sup> fluorescence (Fig. 2c,d and Supplementary Fig. 10e,f). Compared with the non-adapted cells, these adapted cells also contain more PHB, which further increases at higher H<sub>2</sub> concentrations (Supplementary Fig. 11a); therefore, this PHB results from lithoautotrophic growth.

To visualize cellular MBH and SH, we tagged their respective subunits HoxG and HoxY with mV or the photoactivatable red fluorescent protein PAmCherry1 (PAmC1), where PAmC1 also enables single-molecule tracking and super-resolution imaging (Fig. 1d, mode II; note that different fluorescent tags show similar results, Supplementary Section 3). Confocal and wide-field fluorescence imaging both show that MBH<sup>PAmC1</sup><sub>HoxG</sub> is membrane-localized, as expected (Fig. 2e).

Single-molecule tracking of MBH<sup>PAmC1</sup><sub>HoxG</sub> resolves a single diffusion state with a diffusion constant of  $1.67 \pm 0.07 \mu\text{m}^2 \text{s}^{-1}$  (Fig. 2g), typical of mobile membrane proteins<sup>28,29</sup>. The H<sub>2</sub>-oxidizing capability of MBH<sup>PAmC1</sup><sub>HoxG</sub> is also indistinguishable from the non-tagged version (Supplementary Fig. 8). These findings support the tagged MBH being intact and functional in the cell. Surprisingly, SH<sup>mV</sup><sub>HoxY</sub> (functionality confirmed in Supplementary Fig. 8g and Supplementary Table 3), besides being in the cytosol, also shows cell envelope localization (Fig. 2f), which we attribute to SH's interaction with inner-membrane or membrane-bound proteins, or its partial secretion to the periplasm, as some hydrogenases can be secreted out of cells<sup>30</sup> (Fig. 4). Moreover, single-molecule tracking of SH<sup>PAmC1</sup><sub>HoxY</sub> identified two diffusive states with diffusion constants of  $0.10 \pm 0.01$  and  $1.3 \pm 0.2 \mu\text{m}^2 \text{s}^{-1}$  (Fig. 2h), consistent with SH's multiple locations. Furthermore, both MBH and



**Fig. 3 | Electron uptake of *R. eutropha* from photoelectrodes in integrated biohybrids.** **a**, Energy levels of the conduction band (CB) and valence band (VB) edges of  $\text{Cu}_2\text{WS}_4$ ,  $\text{BiVO}_4$  and  $\text{CdS}$  and the redox potential ranges (purple shade) of MBH (0.2 to 0.7 V) and SH ( $-0.02$  to  $0.1$  V)<sup>23,38</sup>, all referenced to the RHE ( $\text{H}^+/\text{H}_2$ ). **b**, Photoelectrochemical current densities at 0.2 V of a bulk  $\text{Cu}_2\text{WS}_4$  thin-film electrode with and without deposited multi-layer adapted *R. eutropha* cells in minimal medium under different gas environments (individual values are indicated by triangles). Horizontal black lines denote the mean. Error bars indicate s.d. from five on/off cycles. **c**, Transmission image of adapted *R. eutropha* cells encoding MBH<sup>mV</sup><sub>HoxG</sub> on a  $\text{Cu}_2\text{WS}_4$  film. Single-spot photocurrents were measured with a focused 405-nm laser illuminating at on-cell (red crosses) and nearby off-cell (green crosses) locations. **d**, Fluorescence image corresponding to **c** (intensity is given in log scale to show contrast). Red lines show the cell contours. **e**, Colour-coded map of single-cell photocathodic current enhancement at 0.2 V, showing a correlation with local MBH concentration in **f**. **f**, Colour-coded map of MBH<sup>mV</sup><sub>HoxG</sub> local concentration at the laser focus in each cell. **g**, Representative local photocurrent–time ( $i$ – $t$ ) trajectories at 0.2 V at an

on-cell location and its corresponding off-cell location under 405-nm light chopping, as denoted by arrows in **c** and **d**. Scale bars in **c**–**f**, 5  $\mu\text{m}$ . Black dashed lines represent linear fitting of current before and after turning on light; the red lines with circle mark the time of the steepest change in current determined from the corresponding  $\Delta i/\Delta t$  trajectory (Supplementary Information Section 1.5). **h**, Histograms of single-cell  $\Delta i_{\text{ph}}$  (blue) obtained on  $\text{Cu}_2\text{WS}_4$  ( $n = 511$  cells) and a control  $\text{Cu}_2\text{WS}_4$  sample that contains no cells (black,  $n = 891$  spots) at 0–0.3 V. For the control,  $\Delta i_{\text{ph}}$  is between adjacent spots (Supplementary Information Section 1.5). The yellow shading denotes the cells whose intrinsic photocurrent is small and their overall  $\Delta i_{\text{ph}}$  is positive due to dominance by the cell's displacement of water from the  $\text{Cu}_2\text{WS}_4$  surface. **i**, Histograms of  $i_{\text{ph}}$  obtained on single  $\text{BiVO}_4$  particles with (red,  $n = 98$  spots) and without (black,  $n = 41$  spots) adapted *R. eutropha* at 0–0.3 V. Solid lines in **h**–**i** are Gaussian fits as guides to the eye. **j**, Comparison of  $i_{\text{ph}}$  obtained on the cell-interfacing lateral facet, and on the cell-free lateral and basal facets of single  $\text{BiVO}_4$  particles at 0.3 V (individual values are shown as triangles). Horizontal black lines denote the mean. Error bars indicate s.d. of measurements on three different particles contacting different cells.

SH cellular concentrations are upregulated by >20-fold upon adaptation (Fig. 2j), corroborating their importance in  $\text{H}_2$  metabolism<sup>31</sup>.

Yet, we find that MBH and SH play different roles regarding PHB formation. Upon deleting MBH (that is,  $\Delta\text{hoxG}$ ), the PhaP1<sup>mV</sup> level stays unchanged, whereas deleting SH (that is,  $\Delta\text{hoxY}$ ) decreases the PhaP1<sup>mV</sup> level by  $\sim 10^3$  times (Fig. 2k, column 2 versus 1; 3 versus 1). Therefore, SH is critical for oxidizing  $\text{H}_2$  to supply reducing equivalents (that is, NADH) towards PHB synthesis from  $\text{CO}_2$  fixation (Fig. 4, steps (i) and (v)), while MBH is nonessential (but exhibits facilitating roles, as discussed in the following).

Genetic deletion does not inform, however, whether and how PHB production scales with SH level. To probe this, we tagged both SH<sub>HoxY</sub> and PhaP1 with PAmC1 and mV, respectively, and quantified them via two-colour imaging in the same cell (Fig. 1c, mode I-ii). This double tagging does not affect PHB synthesis (Fig. 2k, column 5 versus 1), concurrently confirming that tagging HoxY does not affect its function. At the single-cell level, PhaP1 and SH levels show a clear positive correlation (Fig. 2l), consistent with the critical role of SH in PHB production and, more importantly, showing that more SH facilitates more PHB synthesis with  $\text{H}_2$  and  $\text{CO}_2$  lithoautotrophic growth.

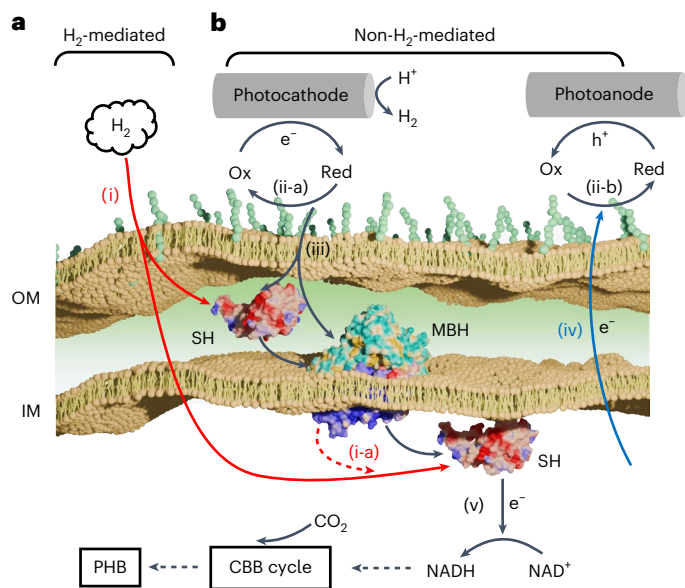
In parallel, we tagged and imaged both MBH<sub>HoxG</sub> and PhaP1 in the same cell. Overall, the PhaP1 level stays the same, regardless of MBH tagging (Fig. 2k, column 4 versus 1). At the single-cell level, PhaP1 and

MBH levels also show a positive correlation (Fig. 2m), even though MBH is nonessential for PHB production. We attribute this correlation to the correlated MBH and SH expression (imaged by doubly tagging them; Fig. 2n), as both genes are regulated by the same regulatory hydrogenase (Supplementary Fig. 1c)<sup>21,32</sup>.

We wondered whether MBH's coexistence with SH could facilitate bioplastic formation. We therefore tagged PhaP1, MBH and SH with mV, mCherry (that is, mC) and PAmC1, respectively, and quantified them in the same cell. Here we combine wavelength and time separation to achieve three-channel imaging (Fig. 1c, mode I-iii): mV and mC are separated in wavelength, while PAmC1 and mC are separated in time utilizing PAmC1's photoactivation. Interestingly, in cells with an SH level of  $<1 \mu\text{M}$ , PhaP1 level is independent of the cellular concentration ratio  $[\text{MBH}]/[\text{SH}]$  (Fig. 2o, yellow dots). However, when the SH level is higher, the PhaP1 level clearly increases with  $[\text{MBH}]/[\text{SH}]$  (Fig. 2o, blue/red dots), and this increase is not due to higher  $[\text{SH}]$  within each group (Supplementary Fig. 19). Therefore, although MBH is nonessential, it facilitates PHB production with SH present (Fig. 4, step i-a), and more so with higher SH levels.

### Single-cell electron-uptake capability

Although *R. eutropha* was only employed in decoupled biohybrids (Fig. 1a, top)<sup>7</sup>, other lithoautotrophic bacteria have been interfaced directly with semiconductors to form integrated biohybrids for



**Fig. 4 | Schematics of dominant electron-transport pathways for bioplastic formation in biohybrid systems.**

**a**, Under lithoautotrophic growth with H<sub>2</sub> + CO<sub>2</sub> (that is, decoupled biohybrids), H<sub>2</sub> gas enters a cell and is oxidized by SH (i), which provides electrons to generate NADH towards CO<sub>2</sub> fixation for PHB synthesis through Calvin–Benson–Bassham (CBB) cycle (v); here, MBH has a facilitating role (i–a). **b**, At the microbe–semiconductor interface (that is, integrated biohybrids), electron uptake from the photocathode takes place dominantly via redox mediators (for example, possible flavin derivatives, secreted SH; ii–a) other than H<sub>2</sub> and further passes to MBH and SH as required (iii), towards eventual CO<sub>2</sub> fixation to PHB. Electron outflow to the photoanode is via redox mediators (ii–b), which may have overlapping species with ii–a (for example, possible flavin derivatives and so on), and does not require either SH or MBH (iv). OM, outer membrane; IM, inner membrane; Ox/Red, oxidized/reduced forms of redox mediators.

solar-to-fuel conversions (Fig. 1a, bottom)<sup>9–11</sup>. We therefore examined whether *R. eutropha* can accept electrons from photoelectrodes.

First, we interfaced *R. eutropha* with Cu<sub>2</sub>WS<sub>4</sub>, a photocathode material with visible absorption and a conduction band edge ( $E_{CB}$ ) appropriate for reducing protons, MBH, SH and other common cellular redox proteins such as NADH dehydrogenase and cytochrome *c* (Fig. 3a)<sup>23,33</sup>. Consistently, under 405-nm light excitation and an applied potential of 0.2 V (versus reversible hydrogen electrode (RHE) for all potentials cited here), Cu<sub>2</sub>WS<sub>4</sub> thin-film photoelectrodes alone show clear steady-state photocathodic currents from proton reduction in minimum medium (Fig. 3b, grey symbols), which is stable for >3 h (Supplementary Fig. 20). This photocathodic behaviour is independent of whether the environmental gas is N<sub>2</sub>, air or CO<sub>2</sub>, indicating indiscernible O<sub>2</sub> or CO<sub>2</sub> reduction on Cu<sub>2</sub>WS<sub>4</sub> under our conditions (Fig. 3b). Strikingly, upon depositing layers of adapted *R. eutropha* cells, steady-state photocathodic currents are enhanced (Fig. 3b, blue symbols), indicating that *R. eutropha* can take up electrons from Cu<sub>2</sub>WS<sub>4</sub>, either directly or via certain redox mediators. Such cell-induced photocathodic current enhancement exists across N<sub>2</sub>, air or CO<sub>2</sub> environments (Fig. 3b), which is understandable, because the photocurrent (here, electron uptake) dominantly reflects upstream electron transfer near the cell–semiconductor interface rather than downstream cellular processes that involve electron movements (for example, respiration or CO<sub>2</sub> fixation). Control experiments on dead cells show no such photocurrent enhancement, indicating that this enhancement is specific to live cells (Supplementary Section 9).

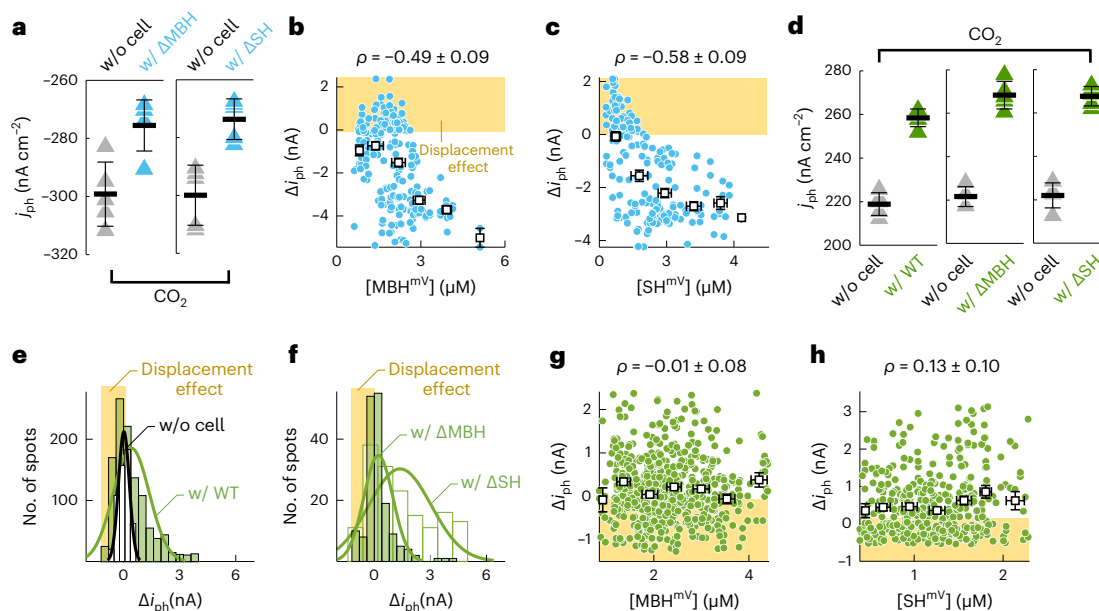
Although our bulk measurements demonstrate the ability of *R. eutropha* to take up electrons, the multi-layer cell deposition makes

it challenging to quantify a single cell's electron-uptake capability, a key performance metric for microbe–semiconductor biohybrids. To quantify this, we integrated photoelectrochemical mapping into our multimodal imaging platform (Fig. 1e, mode III–i). We sparsely dispersed adapted *R. eutropha* cells on a Cu<sub>2</sub>WS<sub>4</sub> film on a transparent conductive indium tin oxide (ITO) substrate in a photoelectrochemical microfluidic cell (Supplementary Figs. 4 and 5h). With this arrangement, individual cells are easily identifiable in wide-field transmission and/or fluorescence images (Fig. 3c,d). A focused 405-nm laser (~380 nm in diameter) excites charge carriers locally in the semiconductor film and probes a single cell's current versus time (*i*–*t*) response under light chopping at various applied potentials. This laser illumination does not cause a noticeable change in a cell's photoelectrochemical behaviour or morphology (Supplementary Section 10). A typical single-cell *i*–*t* response shows a greater photocathodic current ( $i_{ph}$ ) than that measured at an adjacent location on the semiconductor film (Fig. 3g, left versus right, Fig. 3c and Supplementary Fig. 21); the difference,  $\Delta i_{ph}$ , directly yields an individual cell's photocurrent contribution, a single-cell-level photoelectrochemical measurement.

We measured  $\Delta i_{ph}$  for many cells. In the narrow range of 0–0.3 V,  $\Delta i_{ph}$  has no discernible potential dependence (Supplementary Fig. 23a). The majority (~77%) of the cells show photocathodic current enhancement (that is,  $\Delta i_{ph} < 0$ ; Fig. 3h, blue), consistent with bulk measurements (Fig. 3b). The remaining minor population (~23%) have  $\Delta i_{ph} > 0$ , and we attribute this behaviour to the cell's displacement of water from the Cu<sub>2</sub>WS<sub>4</sub> surface, leading to suppressed proton reduction that dominates over these cells' photocathodic currents (Supplementary Section 8). Importantly, the single-cell photocathodic currents  $\Delta i_{ph}$  average at  $-2.2 \pm 0.2$  nA, which, surprisingly, is  $\sim 10^3$  times larger than the picoampere-level current that cellular hydrogenases are capable of sustaining via H<sub>2</sub>-mediated electron transport (Supplementary Section 11.1).  $\Delta i_{ph}$  due to semiconductor film heterogeneity is only  $0.07 \pm 0.03$  nA (Fig. 3h, black). This large single-cell photocurrent enhancement suggests that either the turnover rates of the hydrogenases were underestimated, or other non-H<sub>2</sub>-mediated electron-transport pathways dominate the cell's photocathodic current here.

To probe the mechanisms underlying these large single-cell photocurrents, we eliminated the H<sub>2</sub>-mediated electron transport by attaching cells to BiVO<sub>4</sub> with  $E_{CB}$  below the proton reduction potential (Fig. 3a)<sup>34</sup>. Using a focused 405-nm laser, we measured local photocurrents on the lateral facets of individual micrometre-sized BiVO<sub>4</sub> particles (Fig. 1e, mode III–ii; Supplementary Fig. 6a). As expected, BiVO<sub>4</sub> particles alone show no photocathodic current at 0.2 V (Fig. 3i, black; Supplementary Fig. 25). Remarkably, BiVO<sub>4</sub> particles surrounded by adapted *R. eutropha* cells exhibit clear photocathodic currents at  $-2.6 \pm 0.3$  nA (Fig. 3i, red; Supplementary Fig. 26). Therefore, non-H<sub>2</sub>-mediated electron-transport pathways indeed exist in *R. eutropha*, and probably dominate the nanoampere-level single-cell photocathodic current on Cu<sub>2</sub>WS<sub>4</sub> (Fig. 4, step ii–a).

To remove complications from particle-to-particle and cell-to-cell variations in our BiVO<sub>4</sub>/cell measurements, we further implemented single-cell, single-particle photocurrent measurement by interfacing larger-sized non-adapted *R. eutropha* cells with BiVO<sub>4</sub> particles with facets larger than the focused laser (Fig. 1e, mode III–iii). The single-cell/single-particle interface is easily identifiable (Supplementary Figs. 5f and 6b), and the focused laser can be placed at different sub-BiVO<sub>4</sub>-particle locations. When the laser focus is on a particle's lateral facet that contacts a cell, a photocathodic current ( $-5.3 \pm 0.9$  nA) is clear (Fig. 3j). In contrast, no appreciable photocathodic current is observed when the laser is at another cell-free lateral or a basal facet of the same particle (Fig. 3j). These single-cell/single-particle results corroborate the existence of non-H<sub>2</sub>-mediated semiconductor-to-cell electron-transport pathways.



**Fig. 5 | Players in electron transport across the microbe–semiconductor interface.** **a**, Photoelectrochemical current densities at 0.2 V of bulk  $\text{Cu}_2\text{WS}_4$  thin-film photocathodes with and without deposited multi-layer adapted *R. eutropha*  $\Delta\text{MBH}$  (that is,  $\Delta\text{hoxG}$ ) or  $\Delta\text{SH}$  (that is,  $\Delta\text{hoxY}$ ) cells in minimum medium under a  $\text{CO}_2$  environment (individual values are shown as triangles). Horizontal black lines denote the mean. Error bars indicate s.d. of five on/off cycles. **b,c**, Correlations of single-cell  $\Delta i_{\text{ph}}$  obtained on a  $\text{Cu}_2\text{WS}_4$  photocathode at 0–0.3 V with local MBH (**b**) or SH (**c**) concentration at the laser focus of the same cell. Blue dots, individual adapted cells. Open squares, binned and averaged results. Yellow shaded areas indicate cells with lower hydrogenase levels that are dominated by the displacement effect, similar to Fig. 3b (same for **e–h**).

$x$  error bars indicate s.d.  $y$  error bars indicate s.e.m.  $n = 230$  cells (**b**), 194 cells (**c**). **d**, Photoelectrochemical current densities at 1.1 V of bulk CdS thin-film photoelectrodes with and without deposited multi-layer adapted *R. eutropha* WT or single-deletion cells in minimum medium under a  $\text{CO}_2$  environment (individual values are shown as triangles). Horizontal black lines denote the mean. Error bars indicate s.d. of five on/off cycles. **e**, Histograms of single-cell  $\Delta i_{\text{ph}}$  of WT adapted *R. eutropha* on CdS thin-film photoelectrodes (green) and a control CdS photoelectrode without cells (black) at 0.7–1.0 V ( $\Delta i_{\text{ph}}$  has no discernible potential dependence in this range; Supplementary Fig. 23b). Solid lines are Gaussian fits as guides to the eye. **f**, As in **e** but using adapted  $\Delta\text{MBH}$  and  $\Delta\text{SH}$  strains. **g,h**, As in **b,c**, but on CdS photoanodes at 0.7–1.0 V. Source data

## Interfacial electron-transport pathways

Hydrogenases, besides metabolizing  $\text{H}_2$ , have been proposed to possibly participate in electron transport involving other redox mediators<sup>10</sup>. Our discovery of non- $\text{H}_2$ -mediated electron-transport pathways between *R. eutropha* and semiconductors prompted us to examine the role of hydrogenases in such pathways. We first examined the bulk photoelectrochemical properties of adapted  $\Delta\text{MBH}$  and  $\Delta\text{SH}$  strains on thin-film  $\text{Cu}_2\text{WS}_4$  photocathodes. Remarkably, neither deletion strain shows the photocathodic current enhancement present in the wild-type (WT) strain (Fig. 5a versus Fig. 3b), indicating that both MBH and SH play critical roles in sustaining electron uptake across the microbe–semiconductor interface (Fig. 4, step iii). For MBH, this role is consistent with it being an inner-membrane protein. For SH, we attribute this role at least in part to it being recruited to the membrane and/or secreted into the periplasm (Figs. 2f and 4).

To quantify the contribution of MBH and SH to the cell-induced photocathodic enhancement ( $\Delta i_{\text{ph}}$ ), we examined single-cell-level correlations between a cell's hydrogenase levels and  $\Delta i_{\text{ph}}$  by combining wide-field fluorescence imaging and single-spot photocurrent measurements for adapted cells on  $\text{Cu}_2\text{WS}_4$  (Fig. 1c,e, model I and III-i). A clear correlation exists between  $\Delta i_{\text{ph}}$  and cellular hydrogenase levels; that is, the magnitude of  $\Delta i_{\text{ph}}$  increases with increasing local cellular MBH and SH concentrations (Figs. 3e,f and 5b,c). Therefore, higher hydrogenase levels are associated with a stronger electron-uptake capability in cells, corroborating the critical role of both MBH and SH in sustaining semiconductor-to-microbe electron transport.

However, because neither MBH nor SH is normally on the outer membrane, they are not expected to be in direct contact with the photoelectrode to accept photogenerated electrons. We hypothesized that there must be redox mediators at the *R. eutropha* surface (for example,

outer membrane and/or extracellular matrix) to shuttle electrons from the photoelectrodes to the hydrogenases. These mediators should include but not be dominated by  $\text{H}^+/\text{H}_2$  based on our results above. To probe potential cell-surface redox mediators, we performed bulk cyclic voltammetry on the supernatant of the adapted cell cultures and identified a catalytic species (possibly secreted SH) as well as two other redox species that could be flavin derivatives (Supplementary Section 12.1). Importantly, to sustain steady-state semiconductor-to-microbe currents, these mediators must be redox-reversible and hence should also be able to mediate anodic, microbe-to-semiconductor electron transport (Fig. 4, step ii-b).

Therefore, to test our hypothesis and probe such cell-surface redox mediators, we interfaced *R. eutropha* with CdS, a typical photoanode material excitable by 405-nm light (Fig. 3a)<sup>35</sup>. On bulk thin-film CdS photoelectrodes with layers of adapted WT cells, cell-induced photoanodic current enhancement is indeed observed (Fig. 5d, left), confirming the reversibility of such redox mediators. Interestingly, for the  $\Delta\text{MBH}$  and  $\Delta\text{SH}$  strains, this cell-induced photoanodic current enhancement persists (Fig. 5d, middle and right), indicating that both types of hydrogenase are not important for electron outflow (Fig. 4, step iv).

We also performed single-cell measurements on CdS photoanodes to eliminate cell-density heterogeneities in the bulk measurements. Most WT cells exhibit pronounced photoanodic current enhancement ( $\Delta i_{\text{ph}}$  of a few nanoamperes; Fig. 5e, green), consistent with bulk measurements (Fig. 5d). A minor population shows photoanodic current suppression ( $\Delta i_{\text{ph}} < 0$ ; Fig. 5e), again attributable to the water displacement effect that is only apparent for single-cell-level measurements and is applicable to both photocathodic and photoanodic behaviours.  $\Delta i_{\text{ph}}$  due to CdS film heterogeneity is small, at  $0.02 \pm 0.03$  nA (Fig. 5e, black).

The  $\Delta\text{MBH}$  and  $\Delta\text{SH}$  strains show comparable  $\Delta i_{\text{ph}}$  values (Fig. 5f versus 5e), consistent with the bulk-level comparison across different strains (Fig. 5d). Moreover, single-cell  $\Delta i_{\text{ph}}$  does not show notable correlation with either MBH or SH local concentration (Fig. 5g,h). These results corroborate that MBH and SH do not play important roles in electron outflow. Altogether, at the microbe–semiconductor interface, the cell-surface non- $\text{H}_2$ -based redox mediators are important for both electron outflow and electron uptake (Fig. 4, steps ii-a and ii-b), whereas MBH and SH are important only for electron uptake (Fig. 4, step iii).

## Conclusions

Microbe–semiconductor biohybrids hold promise for efficient solar-to-chemical conversion. We have demonstrated that *R. eutropha* can (1) metabolize  $\text{H}_2$  for bioplastic synthesis from  $\text{CO}_2$  in decoupled biohybrids, with MBH and SH assuming different roles, and (2) take up electrons efficiently from photoelectrodes via non- $\text{H}_2$ -mediated pathways, with both hydrogenases being crucial. This raises the possibility of using *R. eutropha* in both decoupled and integrated microbe–semiconductor biohybrids, or even synergistically combined, to harvest sunlight to synthesize value-added chemicals. The large electron-uptake capability of *R. eutropha* highlights the potential to leverage synthetic biology to direct more electrons towards bioplastic production in the cell, for example by engineering the protein expression, linkage and kinetics of electron-transport pathways and carbon-fixation cycles. Overall, our non-invasive multimodal imaging approach uncovers valuable mechanistic information down to single-cell resolution, and can identify species critical for electron transport and assess system performance, with enhanced capabilities if further integrated with other tools like NanoSIMS<sup>36,37</sup> and proteomics. The knowledge can also guide the engineering and optimization of diverse biohybrid systems for many types of process, including  $\text{CO}_2$  fixation,  $\text{N}_2$  fixation and pollutant degradation.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41557-023-01285-z>.

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## Methods

### Strain construction, growth condition and semiconductor material synthesis

All genetic engineering, including fluorescent protein tagging and gene knockout, was done using the biparental conjugation method<sup>39</sup>. Briefly, for tagging, the conjugation plasmid was constructed by Gibson assembly of HR1, HR2 and the fluorescent protein gene into pRE118 vector, where HR1 and HR2 are the 1,000-bp regions flanking upstream and downstream of the stop codon of the target gene (*HoxG*, *HoxY* or *PhaPI*), respectively. For gene knockout, only HR1 and HR2 were cloned into the conjugation vector, where HR1 and HR2 are regions flanking upstream and downstream of the entire target gene. The conjugation plasmid was transformed into donor *Escherichia coli* strain WM3064, which was then conjugated with *R. eutropha*. Colonies with correct double crossover were screened by PCR and sequence-confirmed. *R. eutropha* was routinely grown in tryptic soy broth (TSB) overnight at 30 °C, then diluted in minimum medium supplemented with 1% wt/vol fructose for two days. These cells are termed non-adapted cells throughout the Article. For adaptation to the lithoautotrophic condition, non-adapted cells were diluted to an optical density of 0.2 in minimum medium and further grown under H<sub>2</sub>:CO<sub>2</sub> (4:1, total 9% or 30%, balanced with air) for four days<sup>40</sup>. These cells are termed adapted cells. Details are provided in Supplementary Section 1.2.

All three semiconductor particles (CdS, Cu<sub>2</sub>WS<sub>4</sub> and BiVO<sub>4</sub>) were synthesized in house using hydrothermal methods. The CdS nanoparticles are nanorods with width of ~50 nm and length of ~1 μm. The Cu<sub>2</sub>WS<sub>4</sub> and BiVO<sub>4</sub> particles have truncated bipyramid shapes with sizes varying between 1 μm and 10 μm, respectively. Details are provided in Supplementary Section 1.1 and scanning electron microscopy (SEM) characterization is provided in Supplementary Fig. 5.

### Optical imaging and photoelectrochemical measurements

For the imaging of bacteria without semiconductor, cells were dispersed and immobilized between a glass coverslip and an agarose pad<sup>28</sup>. The sample edges were sealed with epoxy to prevent gel drying. Single-molecule tracking and single-cell protein quantification were performed with a ×60 oil immersion objective (Olympus PlanApo N ×60 oil 1.45 TIRFM UIS 2) on an Olympus IX71 inverted microscope, and the images were captured with an electron-multiplying charge-coupled device (EMCCD) camera (Andor Technology, DU-897E-CSO-#BV). The total magnification was ×115, which leads to a pixel size of 135.4 nm (ref. 41). The circularly polarized continuous-wave lasers were aligned coaxially and inclined at approximately 60° from the optical axis of the objective to decrease background from the medium. mCherry was excited by 561 nm, mVenus by 514 nm, and PAMCherry1 was first activated by 405 nm and then excited by 561 nm. The emission light was collected by the EMCCD camera after passing through appropriate bandpass filters. Single-molecule tracking was done in stroboscopic mode, with a 4-ms laser pulse controlled by an acousto-optic tunable filter and a time lag for each camera frame of 60 ms. The short laser pulse minimized motion blur from the fast movement of proteins. For protein quantification of multiple tagged strains, different proteins were imaged sequentially in the order (1) mCherry, (2) mVenus and (3) PAMCherry1. Details are provided in Supplementary Section 1.3 and Fig. 1b.

For bulk-level photoelectrochemical measurements on annealed semiconductor thin-film electrodes, a three-electrode set-up was used. A Pt wire electrode (BASi, MW-4139) and a Ag/AgCl electrode (BASi, MW-2030) were used as counterelectrode and reference electrode, respectively. For no-cell control experiments, the annealed electrodes were mounted in a quartz cell filled with minimum medium and held at a constant potential. The 405-nm laser was kept on for a duration of 15 s and off for a duration of 10 s while the photocurrent was recorded. A large amount of adapted *R. eutropha* cells were then deposited on the same semiconductor electrode and immobilized with an agarose pad

and a coverslip. The photocurrent was measured again in the presence of cells under exactly the same condition.

For multimodal imaging of single cells coupled with semiconductor films (Cu<sub>2</sub>WS<sub>4</sub> and CdS), a three-electrode photoelectrochemical microfluidic cell was constructed. Semiconductor-deposited ITO was attached to two glass slides and *R. eutropha* cells were dispersed on the semiconductor film surface. The cells were sandwiched between the ITO electrode and another microscope slide by an agarose pad. A Pt wire counterelectrode and a Ag/AgCl reference electrode were assembled into the microfluidic structure, which was then sealed with epoxy to prevent leaking. Minimal medium was introduced into the device by a syringe. The sample was imaged on an inverted Olympus IX71 microscope equipped with a ×60 water immersion objective (Olympus, UPLSAPO60XW) to image through the agarose pad. Cells in the field of view were first imaged and bleached by wide-field laser excitation for protein quantification. Then, a focused 405-nm laser beam was directed to each single cell for the measurement of photocurrent at that location. Photocurrent from a nearby off-cell location was also measured as a control. In the case of BiVO<sub>4</sub>, a single nanoparticle instead of nanoparticle film was used. Focused 405 nm light was directed onto the basal or lateral facet of the BiVO<sub>4</sub>. For details see Supplementary Section 1.5 and Supplementary Figs. 4 and 6.

### Analysis of images and photocurrents

Single-cell protein quantification was calculated as the whole-cell fluorescence intensity divided by the single fluorescent protein intensity. The fluorescence bleaching curve was fitted to a double exponential decay function, where one component corresponds to fluorescent protein bleaching and the other to cell autofluorescence. Off-cell background and cell autofluorescence were subtracted. Details are provided in Supplementary Section 1.4. Protein dynamic information is extracted by fitting the displacement distribution from single-molecule tracking to a multi-state probability distribution function, and the cell-confinement effect is resolved through an inverse transformation algorithm. Details are provided in Supplementary Section 7.

To quantify the photocurrent, currents for the two 3-s durations on either side of the time of turning illumination on, ( $t_{\text{on}} - 4$  s,  $t_{\text{on}} - 1$  s) and ( $t_{\text{on}} + 1$  s,  $t_{\text{on}} + 4$  s), were fitted with two different linear functions, and the difference between these two linear functions at  $t_{\text{on}}$  was taken as the photocurrent (that is,  $i_{\text{ph}}$ ) for this light on/off cycle. Details are provided in Supplementary Section 1.5.

### Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

### Data availability

All data are available in the main text or the Supplementary Information. Raw data supporting the findings of this study are available upon reasonable request. Source data are provided with this paper.

### Code availability

MATLAB codes for data analysis and simulations supporting the findings of this study are provided with this paper.

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## Author contributions

B.F. constructed strains. X.M. synthesized semiconductor materials. B.F. and X.M. designed experiments, performed measurements, wrote codes and analysed data. Y.P., Z.Z. and T.Y. contributed to strain construction, materials synthesis and/or imaging experiments, where Y.P. and Z.Z. contributed equally. W.J. and D.H.F. contributed

to cell culturing. W.L. helped with instrument set-up. B.P., F.S. and B.B. contributed to genetic engineering. M.S. and T.H. contributed to discussions on materials synthesis. P.C. conceived and directed the research. B.F., X.M. and P.C. wrote the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41557-023-01285-z>.

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**Peer review information** *Nature Chemistry* thanks the anonymous reviewers for their contribution to the peer review of this work.

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### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

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| <input type="checkbox"/>            | <input checked="" type="checkbox"/> | For null hypothesis testing, the test statistic (e.g. $F$ , $t$ , $r$ ) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted<br><i>Give <math>P</math> values as exact values whenever suitable.</i>                            |
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*Our web collection on [statistics for biologists](#) contains articles on many of the points above.*

### Software and code

Policy information about [availability of computer code](#)

**Data collection** Super-resolution/wide-field fluorescence and transmission images were collected with Andor iQ2 software; photocurrent data was collected with software CH1200A Electrochemical Analyzer; confocal images were collected with ZEN software. All the above information are described in the main text or SI.

**Data analysis** Customized MATLAB\_R2021a codes were used for data analysis, which are provided by the authors.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

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All data are available in the main text or the supplementary materials. Raw data supporting the findings of this study are available upon reasonable request.

## Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender

N/A

Population characteristics

N/A

Recruitment

N/A

Ethics oversight

N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For single cell measurement, enough data (> 60 cells for each condition) were compiled to ensure statistical significance. We find that such sample size is sufficient to form a smooth distribution and represent data trend.

Data exclusions

When outliers exist, data outside 95 percentile were excluded, which is sufficient to exclude extreme outliers in our case.

Replication

Replications were successful and data from same conditions were combined together. Each condition includes at least three repetitions, which are comparable with each other.

Randomization

Not relevant as no allocation was performed.

Blinding

Not relevant as no grouping was performed.

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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### Methods

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