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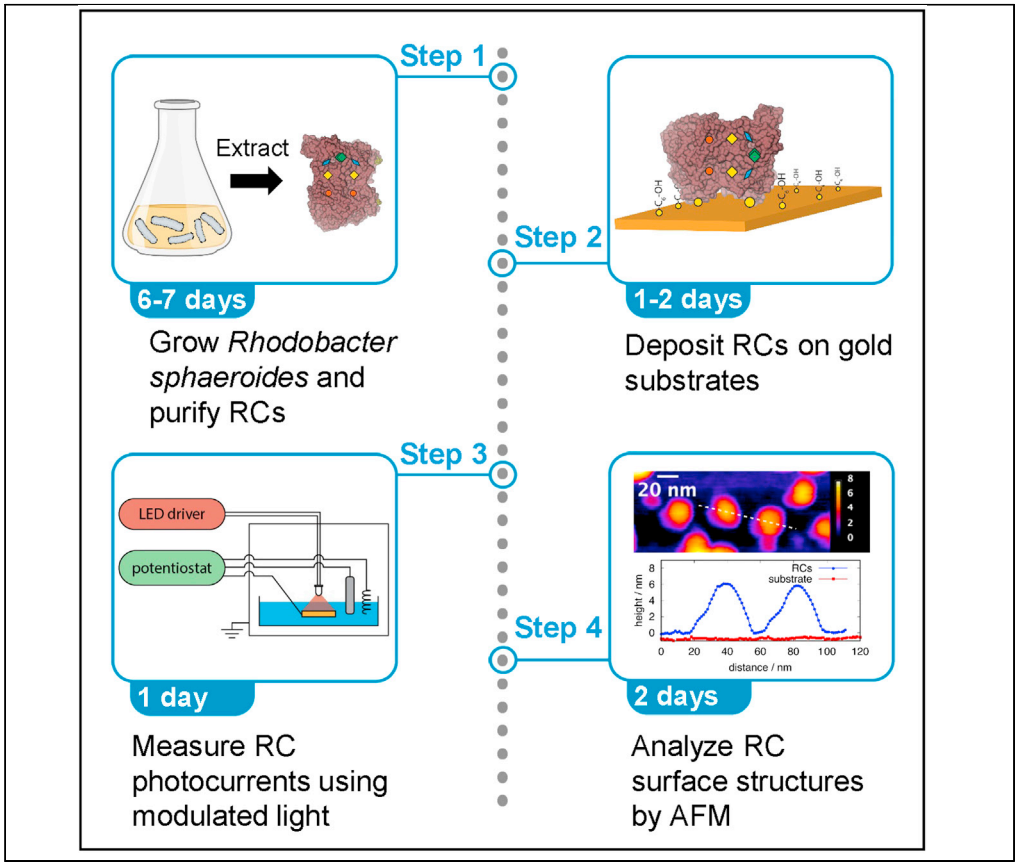
Purification and preparation of *Rhodobacter sphaeroides* reaction centers for photocurrent measurements and atomic force microscopy characterization



The formation of defined surfaces consisting of photosynthetic reaction centers (RCs) in biohybrid solar cells is challenging. Here, we start with the production of engineered RCs for oriented binding. RCs are deposited onto gold electrodes, and 6-mercapto-1-hexanol (MCH) is used to displace multilayers and non-specifically adsorbed RCs. The resulting electrode surfaces are analyzed for photocurrent generation using an intensity-modulated light and lock-in amplifier. Atomic force microscopy (AFM) is used to characterize the surface and the formation of RC structural assemblies.

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Highlights

Express and purify RC membrane proteins from *Rhodobacter sphaeroides*

Deposit RCs and MCH on gold electrodes for formation of structural assemblies

Measure RC photocurrents with an intensity-modulated LED and lock-in detection

Prepare single crystal gold electrodes for AFM of RCs and image processing

Jun et al., STAR Protocols 3, 101044
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Purification and preparation of *Rhodobacter sphaeroides* reaction centers for photocurrent measurements and atomic force microscopy characterization

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SUMMARY

The formation of defined surfaces consisting of photosynthetic reaction centers (RCs) in biohybrid solar cells is challenging. Here, we start with the production of engineered RCs for oriented binding. RCs are deposited onto gold electrodes, and 6-mercapto-1-hexanol (MCH) is used to displace multilayers and non-specifically adsorbed RCs. The resulting electrode surfaces are analyzed for photocurrent generation using an intensity-modulated light and lock-in amplifier. Atomic force microscopy (AFM) is used to characterize the surface and the formation of RC structural assemblies.

For complete details on the use and execution of this profile, please refer to Jun et al. (2021).

BEFORE YOU BEGIN

In this protocol, we begin with the steps required to grow *Rhodobacter sphaeroides* bacteria in order to express and purify engineered reaction center proteins. The photosynthetic proteins are covalently bound to a gold surface and used to harvest light energy, which they then convert into electrons and transfer to the electrode for photocurrent generation.

Replicable and oriented deposition of proteins on a surface is challenging. We use 6-mercapto-1-hexanol (MCH) to competitively displace non-specifically adsorbed RCs and multilayer formations. Varying incubation times result in different structural assemblies, ranging from a sub-monolayer to multilayers. There is flexibility in changing multiple parameters in the optimization process to obtain the desired surface configuration.

Finally, the protein-modified surfaces are characterized using modulated light intensity and lock-in amplifier to detect photocurrent generation, and we detail the steps to create a single crystal gold bead for visualizing protein structural assemblies with AFM.

Prepare media, buffers, and solutions following the recipes in the "materials and equipment" section.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli DH5α	Thermo Fisher Scientific	Cat #: 18265017
Rhodobacter sphaeroides RCx^R	Jun et al. (2014)	N/A
Chemicals, peptides, and recombinant proteins		
Tryptone	Fisher Bioreagents	Cat #: BP1421-500
Yeast extract	Fisher Bioreagents	Cat #: BP1422-500
NaCl	Fisher Bioreagents	Cat #: BP358-212
$MgCl_2$	EMD Millipore Sigma	Cat #: 5980-OP
$CaCl_2$ dihydrate	EMD Millipore Sigma	Cat #: 102382
Tris base	Fisher Bioreagents	Cat #: BP152-1
DNase I	Sigma-Aldrich	Cat #: DN25-1G
N,*N*-Dimethyldodecylamine *N*-oxide solution (LDAO)	Fluka Chemika	Cat #: 40236
Imidazole	EMD Millipore Sigma	Cat #: 4015
Dimethyl sulfoxide (DMSO)	Acros Organics	Cat #: AC610420010
Kanamycin sulfate	Bio Basic	Cat #: KB0286
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	GoldBio	Cat #: I2481C25
Ni^{2+}-NTA agarose resin	Qiagen	Cat #: 30210
100 kDa centrifugal filter unit	Millipore Sigma	Cat #: UFC9100
DEAE-650M anion resin	Toyopearl	Part #: 07473
Hydroquinone	Sigma-Aldrich	Cat #: H9003-100G
6-Mercapto-1-hexanol (MCH)	Sigma-Aldrich	Cat #: 725226-1G
KOH	Fisher Scientific	Cat #: P250-1
NaOH	Electron Microscopy Sciences	Cat #: B10252-34
H_2SO_4 (95%–98%)	Sigma-Aldrich	Cat #: 258105-2.5L-PC
HNO_3 (68%–70%)	Anachemia	Cat #: 62786-460
HCl (36.5%–38%)	Sigma-Aldrich	Cat #: 258148-2.5L-GL
Recombinant DNA		
pIND4-DM	Jun et al. (2021)	N/A
Software and algorithms		
Gwyddion	http://gwyddion.net/	Ver. 2.58
UV Solutions	Hitachi	Ver. 2.1
MATLAB	https://www.mathworks.com/	Ver. 2020
Other		
Ag/AgCl reference electrode	eDAQ	Cat #: ET072-1
SCE reference electrode	CH Instruments	Cat #: CHI150
Potentiostat	Metrohm	Model #: PGStat30
Linear potential sweep generator	Princeton Applied Research	Model #: 175
Lock-in amplifier	Stanford Research	Model #: SRS830
LED driver	ILX Lightwave	Model #: LDX-3207B
810 nm LED	DigiKey	Part #: 1125-1158-ND
860 nm LED	DigiKey	Part #: 475-1200-ND
Butane	Colibri Premium	Cat #: X11230BU
Gold wire	Alfa Aesar	Cat #: 14725-BW
Spectrophotometer	Hitachi	Model #: U-3010
AFM	Agilent Technologies	Model #: 5500; N9610A
AFM cantilevers	Ted Pella	Cat #: TAP150AL
Argon (99.998%)	Praxair	Cat #: Ar 4.8
ddH_2O	Merck Millipore	Cat #: Milli-Q Integral 5

MATERIALS AND EQUIPMENT

LB medium (pH 7.0)		
Reagent	Final concentration	Amount
Tryptone	10 g/L	2.5 g
Yeast extract	5 g/L	1.25 g
NaCl	10 g/L	2.5 g
ddH_2O	n/a	To 250 mL
Total	**n/a**	**250 mL**

Adjust the pH to 7.0 as necessary with HCl or NaOH and autoclave to sterilize. Store at 19°C–21°C for a maximum of 3 months.

RLB medium (pH 7.0)		
Reagent	Final concentration	Amount
Tryptone	10 g/L	40 g
Yeast extract	5 g/L	20 g
NaCl	10 g/L	40 g
1 M $MgCl_2$	810 µM	3.24 mL
1 M $CaCl_2$	510 µM	2.04 mL
ddH_2O	n/a	To 4 L
Total	**n/a**	**4 L**

Adjust the pH to 7.0 as necessary with HCl or NaOH and autoclave to sterilize. Store at 19°C–21°C for a maximum of 3 months.

Resuspension buffer (pH 8.0)		
Reagent	Final concentration	Amount
1 M Tris pH 8.0	50 mM	1 mL
1 M NaCl	200 mM	4 mL
1 M $MgCl_2$	0.5 mM	10 µL
DNase I	Approx. 1.25 mg/mL	Approx. 25 mg
ddH_2O	n/a	To 20 mL
Total	**n/a**	**20 mL**

Adjust the pH to 8.0 as necessary with HCl or NaOH and use within a week. Store at 19°C–21°C for a maximum of 1 week.

Wash buffer (pH 8.0)		
Reagent	Final concentration	Amount
1 M Tris pH 8.0	50 mM	100 mL
1 M NaCl	200 mM	400 mL
30% LDAO (w/v)	0.03% (w/v)	2 mL
ddH_2O	n/a	To 2 L
Total	**n/a**	**2 L**

Adjust the pH to 8.0 as necessary with HCl or NaOH and use within a week. Store at 19°C–21°C for a maximum of 1 week.

Alternatives: If a different surfactant is desired, such as *N*-dodecyl β-D-maltoside (DDM) detergent, use at 0.02% (w/v).

Elution buffer (pH 8.0)		
Reagent	Final concentration	Amount
1 M Tris pH 8.0	50 mM	1 mL
1 M NaCl	200 mM	4 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
30% LDAO (w/v)	0.03% (w/v)	20 µL
2 M imidazole	100 mM	1 mL
ddH_2O	n/a	To 20 mL
Total	**n/a**	**20 mL**
Adjust the pH to 8.0 as necessary with HCl or NaOH and use within a week. Store at 19°C–21°C for a maximum of 1 week.		

Alternatives: If a different surfactant is desired, such as *N*-dodecyl β-D-maltoside (DDM) detergent, use at 0.02% (w/v).

Anion loading buffer (pH 8.0)		
Reagent	Final concentration	Amount
1 M Tris pH 8.0	50 mM	2.5 mL
30% LDAO (w/v)	0.03% (w/v)	50 µL
ddH_2O	n/a	To 50 mL
Total	**n/a**	**50 mL**
Adjust the pH to 8.0 as necessary with HCl or NaOH and use within a week. Store at 19°C–21°C for a maximum of 1 week.		

Phosphate buffered saline (pH 7.2)		
Reagent	Final concentration	Amount
NaCl	137 mM	4 g
KCl	2.7 mM	0.1 g
Na_2HPO_4	10 mM	0.71 g
KH_2PO_4	1.8 mM	0.12 g
ddH_2O	n/a	To 500 mL
Total	**n/a**	**500 mL**
Adjust the pH to 7.2 as necessary with HCl or NaOH and use within a week. Store at 19°C–21°C for a maximum of 1 week.		

STEP-BY-STEP METHOD DETAILS

Expression of the "double mutant" (DM) reaction centers (RCs) from *Rhodobacter sphaeroides*

Timing: 4 days

This step begins the process of creating the "Double Mutant" (DM) reaction centers (RCs) for the purpose of the electrochemical experiments. The DM RC is a derivative of the wild type RC, where all native Cys are replaced with Ala or Ser (C(H156)A, C(H234)S, C(L92)S, C(L108)S, C(L247)S) (Mahmoudzadeh et al., 2011); two Cys are introduced in hydrophilic regions to promote binding to a gold surface via a gold-thiol covalent bond (Jun et al., 2021).

Note: This step is based on a previous protocol (Jun et al., 2014).

1. *Day* 1. In a test tube, inoculate 10 mL LB supplemented with kanamycin at a final concentration of 25 µg/mL with a few crystals from a glycerol freezer stock of *R. sphaeroides* cells containing the pIND4-DM plasmid encoding the DM RC. Grow for approximately 24 h at 30°C, shaken or rotated on a wheel.

2. *Day* 2. Add the 10 mL overnight culture to a 1 L Erlenmeyer flask containing 200 mL LB supplemented with kanamycin at a final concentration of 25 μg/mL. Grow for approximately 24 h at 30°C, shaken at 200 RPM.
3. *Day* 3. Split the 200 mL overnight culture into four 50 mL aliquots. Use these aliquots to add to four 2 L Erlenmeyer flasks containing 500 mL RLB supplemented with kanamycin at a final concentration of 25 μg/mL. To each flask, add isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM to induce protein production. Grow for 16 h at 30°C, shaken at 200 RPM.
4. *Day* 4. At the end of the 16 h induction period, optical density (OD_{650}) of the cell culture should be 1.5–2 and orange-red in colour.
   a. Pellet cells by centrifuging at 12000×*g* for 15 min at 4°C in a J-LITE JLA 8.1000 rotor (Beckman Coulter) or equivalent.
   b. Discard the supernatant and weigh the cell pellet (wet mass). Typical yields are 7 g cell pellet per L culture.

Pause Point: If necessary, the pellets can be stored at −20°C for up to 6 months.

Purification of the DM RCs

Timing: 3 days

This step extracts and purifies the DM RCs from the cell pellet. Keep the sample and buffers on ice or 4°C throughout the duration of the purification process.

5. *Day* 1. Resuspend the cell pellet by stirring in the resuspension buffer, using 2 mL of buffer per gram of cell pellet (e.g., resuspend a 7 g pellet with 14 mL buffer).
   a. If the pellet is frozen, add the resuspension buffer to thaw before resuspending by stirring.
6. Lyse the cell suspension using a French pressure cell press chilled to 4°C. Maintain a pressure between 22500 and 25000 PSI.

   Note: Ensure that the lysate is not very viscous. Lysate should flow easily in well-defined drops. A gooey lysate indicates that not enough DNase was added. Add a few more crystals of DNase to the lysate and stir gently (to not cause frothing) for 1–2 min. Ensure that lysate is no longer viscous before proceeding to the next step.

7. Centrifuge the lysate at 10000×*g* for 20 min at 4°C in a JA-20 rotor (Beckman Coulter) or equivalent.

   Note: The pellet contains unwanted whole cells and large cell membrane fragments. The chromatophores, containing the RCs, are found in the supernatant.

8. Pipette the supernatant into a separate beaker. Note the supernatant volume.
9. Using a magnetic stir bar, stir the supernatant in the dark at room temperature (19°C–21°C and add 30% *N*,*N*-dimethyldodecylamine *N*-oxide solution (LDAO) (w/v) dropwise to a final concentration of 1% (w/v). Add 2 M imidazole to a final concentration of 5 mM. Stir for 20–30 min.
10. Centrifuge the solubilized solution at 164000×*g* for 20 min at 4°C in a Type 70 Ti rotor (Beckman Coulter) or equivalent.

    Note: The pellet contains insolubilized cell membrane fragments and precipitated proteins. Solubilized RCs are found in the supernatant.

11. Pipette the supernatant (avoid the loose pellet) into a new beaker.

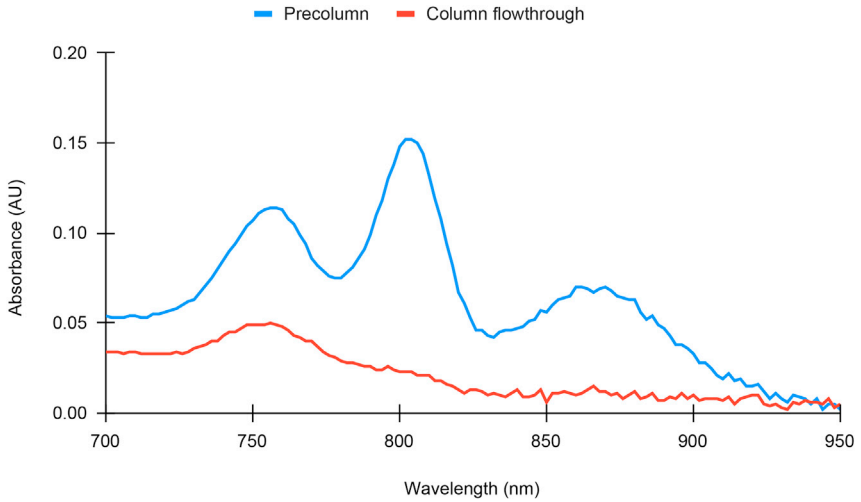

Figure 1. Absorbance spectra of 10-fold dilutions of solubilized RCs prior to incubation with the Ni^{2+}-NTA resin (precolumn sample, blue) and following incubation with the Ni^{2+}-NTA resin (column flowthrough, red)

The decrease in absorbance at 804 nm in the flowthrough compared to the precolumn sample indicates successful binding of the RCs to the resin.

Note: To ensure that RCs are present in the supernatant, dilute (10-fold) the supernatant with the wash buffer and measure the absorbance spectrum (250 nm–1000 nm). Absorbance peaks should be present at 760, 804, and 860 nm (corresponding to the cofactors in the RCs), as shown in Figure 1 (blue trace).

⚠ CRITICAL: The supernatant should not be turbid. Turbidity indicates that proteins are precipitating and will clog the column in subsequent steps. If turbidity is observed, centrifuge again at 164000×*g* for 20 mins at 4°C in a Type 70 Ti rotor (Beckman Coulter) or equivalent. Further addition of LDAO or imidazole is not recommended. Ensure the supernatant is on ice and work quickly.

12. Equilibrate clean Ni^{2+}-NTA agarose resin (approximately 4–5 mL of resin in a gravity column) with 2 column volumes wash buffer. Transfer the resin into a 50 mL conical tube.
13. Add the supernatant to the 50 mL conical tube containing the equilibrated resin. Rock gently overnight (15–18 h) in the dark at 4°C.
14. *Day* 2. Pour the overnight resin suspension onto a gravity column. Allow liquid to flow through into a clean beaker.
    a. Measure the absorbance spectrum of the flowthrough, diluted 10-fold in wash buffer.

Note: Compare this spectrum of the flowthrough to the spectrum of the starting material supernatant. The 804 nm peak should be much smaller in the flowthrough, indicating that RCs have bound to the resin (Figure 1).

Note: The binding of the RCs, which are dark purple in colour, should cause the Ni^{2+}-NTA agarose resin to also turn dark purple. (Figure 2).

15. Add wash buffer continuously to the column.
    a. Measure the absorbance spectrum of the flowthrough, undiluted, after approximately 5 column volumes of wash buffer, then every 50–100 mL of wash buffer.
    b. Monitor the absorbance at 280 nm. Wash until this value plateaus to <0.05 AU, then allow the wash buffer to drain to the surface of the resin (Figure 3).

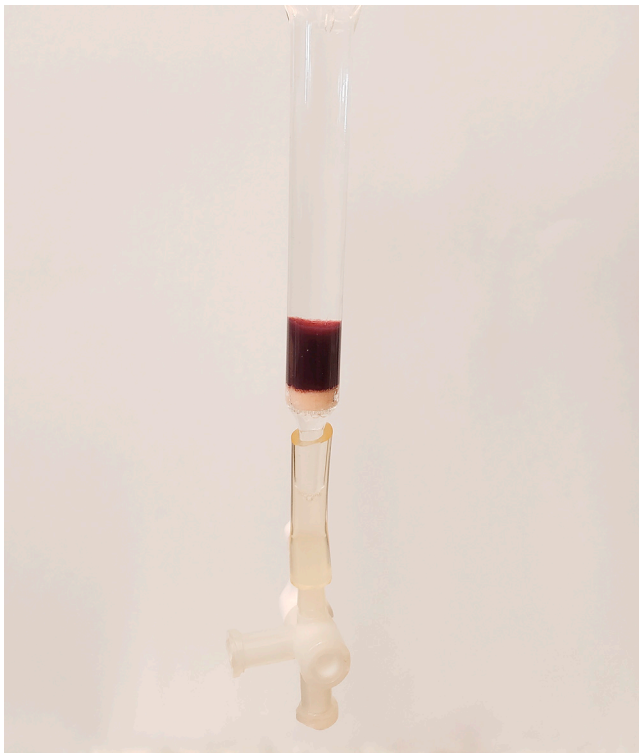

Figure 2. The Ni^{2+}-NTA agarose resin appears dark purple in colour after the binding of RCs

Note: The absorbance at 280 nm indicates the presence of proteins, due to aromatic amino acids (Trp, Tyr, Phe).

16. Add 5 column volumes of elution buffer to the column, then allow the buffer to flow slowly.
    a. Monitor the colour of the eluent. Once a pale purple colour is visible (i.e., A_{804} >0.015 AU), start collecting the sample. Collect until the flowthrough is no longer coloured.
    b. Measure the absorbance spectrum of the elution, diluted 20- or 50-fold in elution buffer. A clean protein will have an A_{280}:A_{804} ratio of <1.4.

⚠ CRITICAL: If the A_{280}:A_{804} ratio is <1.4, proceed to step 17. If the ratio is >1.4, proceed to steps 18–24, where the RCs can be cleaned up further using anion exchange chromatography.

Note: If storing RCs overnight, since there may not be enough time to complete step 17 in a single day, dilute 10-fold in wash buffer as they are less stable at the high imidazole concentrations found in the elution buffer.

17. Buffer exchange the RCs into PBS pH 7.2 and 0.03% LDAO using a 15 mL 100 kDa NMWL centrifugal filter unit.
    a. Add the eluent (from step 16) to a 100 kDa NMWL centrifugal filter unit. Centrifuge at 5000×*g* for 25 min at a time at 4°C in a JS-5.3 rotor (Beckman Coulter) or equivalent, until the volume of the retentate is less than 0.5 mL, then discard the flowthrough.
    b. Add PBS to the retentate to a final volume of 15 mL. Centrifuge at 5000×*g* for 25 min at 4°C in a JS-5.3 rotor (Beckman Coulter) or equivalent, or until the volume of the retentate is less than 0.5 mL, then discard the flowthrough.
    c. Repeat the previous step.

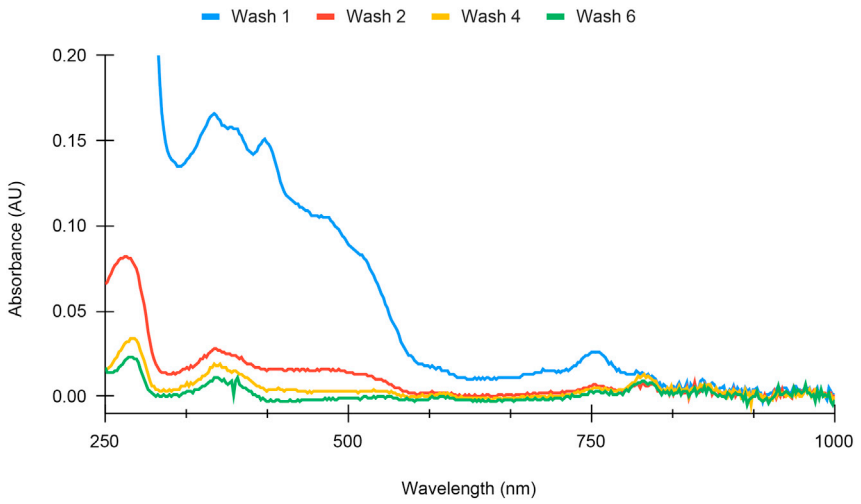

Figure 3. Absorbance spectra of column washes during purification by Ni^{2+}-NTA affinity chromatography

Spectra are measured approximately every 5–10 column volumes and shows a decrease in absorbance at 280 nm with continued washing.

Note: This step exchanges the RCs in Tris to PBS in preparation for electrochemistry.

Pause Point: Store the RCs in PBS and 0.03% LDAO in the dark at 4°C for up to a week or −80°C for up to 2 months. If stored at −80°C, avoid multiple freeze-thaw cycles as this will damage the protein.

18. *Day* 3. If necessary (i.e., A_{280}:A_{804} ratio is >1.4), an anion exchange chromatography step yields higher purity RCs (steps 18–24). Buffer exchange the RCs into the anion loading buffer using a 15 mL 100 kDa NMWL centrifugal filter unit.
    a. Add the eluent (from step 16) to a 100 kDa NMWL centrifugal filter unit. Centrifuge at 5000×*g* for 25 min at a time at 4°C in a JS-5.3 rotor (Beckman Coulter) or equivalent, until the volume of the retentate is less than 0.5 mL, then discard the flowthrough.
    b. Add anion loading buffer to the retentate to a final volume of 15 mL. Centrifuge at 5000×*g* for 25 min at 4°C in a JS-5.3 rotor (Beckman Coulter) or equivalent, or until the volume of the retentate is less than 0.5 mL, then discard the flowthrough.
    c. Repeat the previous step.

Note: This step removes the NaCl in the elution buffer in preparation for loading onto an anion exchange chromatography column.

19. Equilibrate the anion exchange resin with approximately 5 column volumes of anion loading buffer, then allow the buffer to drain to the surface of the resin.
20. Gently add the RCs to the column, ensuring the resin bed is not disturbed. Let the sample flow through.

⚠ CRITICAL: Make sure that the RCs are in a buffer that does not contain NaCl.

21. Add 5 column volumes of anion loading buffer and collect the flowthrough in approximately 5 mL aliquots.
    a. Measure the absorbance spectrum of each flowthrough aliquot. Monitor the absorbance at 804 nm to determine if RCs are bound or eluting. RCs can be considered to be eluting if A_{804} is over 0.015 AU.
    b. Add more buffer until the RCs have stopped eluting, then proceed to the next step.

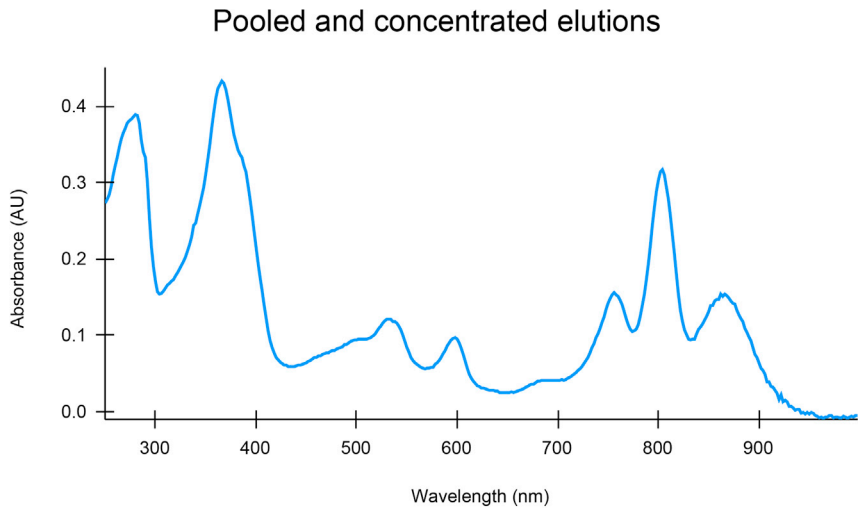

Figure 4. Absorbance spectrum of pooled and concentrated elution fractions after purification by anion exchange chromatography

Fractions collected from the column are analyzed for the presence of RCs (A_{804} >0.015 AU) and for protein purity (A_{280}:A_{804} <1.4).

22. Repeat the previous step at increasing concentrations of NaCl: 5 mM, 10 mM, 30 mM, 60 mM, and 80 mM.
23. Pool the fractions of RCs with sufficient purity (i.e., A_{280}:A_{804} ratio of <1.4, Figure 4).
24. Proceed to step 17 to buffer exchange the RCs into PBS pH 7.2.

Note: RC concentrations can be calculated by using the Beer-Lambert law, given the extinction coefficient ($\epsilon_{804\ nm}$ = 288 mM^{-1}-cm^{-1}) for the RC (Straley et al., 1973) and the absorbance value at 804 nm corresponding to the accessory bacteriochlorophylls. For example, if A_{804} = 0.288, then the [RC] = 0.288 / 288 mM^{-1}-cm^{-1} / 1 cm = 1 μM.

⚠ CRITICAL: Monitor for changes in the absorbance spectrum of the RC, which may indicate loss of structural integrity. For example, a decrease in the 804 nm peak and a concomitant increase in the 760 nm peak indicates that the RCs are degrading (Hughes et al., 2006).

Preparation of gold flag electrodes for RC and 6-mercapto-1-hexanol (MCH) deposition

Timing: 30–45 min

In this step, gold flag electrodes are cleaned electrochemically by cyclic voltammetry (CV) using a potentiostat.

25. Rinse a glass electrochemical cell with ddH_2O followed by 100 mM KOH. Fill the cell with 100 mM KOH such that the gold electrode is fully immersed.

Note: Ensure the electrochemical cell has been cleaned previously in a hot acid bath (1:1 ratio (v/v) of H_2SO_4:HNO_3) and rinsed thoroughly in pure water (>18 MΩ-cm).

26. Gently bubble the electrolyte with argon gas to remove dissolved oxygen.
27. Once degassed, connect the gold electrode to the working electrode lead from the potentiostat. Similarly, connect a flamed annealed platinum electrode and an Ag/AgCl reference electrode rinsed in ddH_2O to the counter and reference electrode leads, respectively. Ensure that the electrodes are fully submerged in the electrolyte.

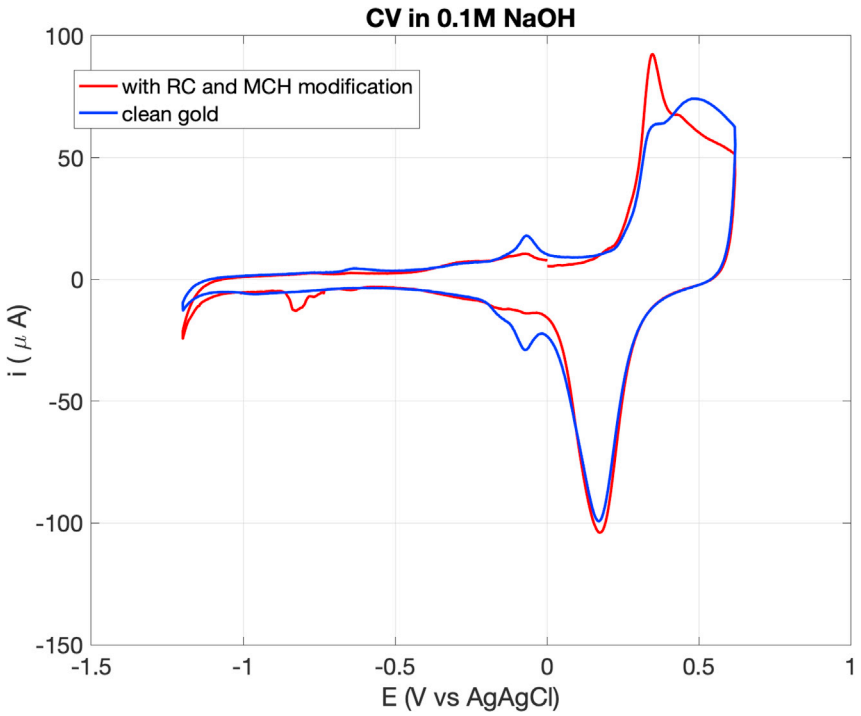

Figure 5. CVs of gold flag electrodes are measured in 0.1 M KOH at 25 mV/s of a cleaned gold electrode (blue) and an RC- and MCH-covered electrode (orange)

The well-defined oxidation peak for the cleaned gold electrode at +0.3 V is an indication that the surface is free from contaminants.

28. Set up a CV with the following parameters:
    a. Scan rate: 25 mV/s
    b. Lower vertex: −1.25 V vs. Ag/AgCl
    c. Upper vertex: +0.55 V vs. Ag/AgCl
    d. Cycles: 15

Note: Cleaning is considered complete once the CV has reached an equilibrium and displays features that are consistent with gold (Figure 5).

29. Rinse the gold electrode with ddH_2O and remove from the holder and using tweezers to hold the gold stem, flame anneal (heat in a butane flame until the gold electrode is dark orange in colour).

⚠ CRITICAL: Careful that the gold does not overheat and melt.

Pause Point: The cleaned electrode can be stored in a clean and sealed container, in a position such that the surface is not in contact with the sides.

Deposition of RC and MCH on gold electrodes

Timing: 1 h to 2 days

In this step, RCs purified from previous steps are deposited onto gold electrodes. This procedure applies to both the gold flag electrodes for photocurrent measurements and single crystal gold bead electrodes for AFM imaging. MCH is used to displace multilayers and non-specifically

adsorbed RCs. Varying the MCH incubation time period controls the formation of different RC structural assemblies.

30. Fully submerge a cleaned gold electrode in 100 μL of an RC solution in a 1.5 mL microcentrifuge tube of the desired concentration (e.g., 0.5–10 μM). Incubate for the desired period of time (e.g., 10 min to 24 h) in the dark at 4°C.
31. Rinse the electrode to remove excess RCs by gently dipping in PBS.

⚠ CRITICAL: Be cautious to not aggressively agitate or contact the sides of the container while rinsing.

32. Fully submerge an RC-deposited gold electrode in 100 μL of a 100 μM MCH solution in a 1.5 mL microcentrifuge tube. Incubate for the desired period of time (e.g., 1–24 h) in the dark at 4°C.

⚠ CRITICAL: MCH will adsorb to many things. Take caution to avoid contaminating other experiments.

33. Rinse the electrode by gently dipping in PBS. The electrode should be used immediately.

⚠ CRITICAL: As formation of well-defined structural assemblies of RCs is the purpose of the experiment, the variables here control the structures. For example, the incubation time and concentration of RCs control how much protein material is initially bound to the electrode surface. The incubation time and concentration of MCH control how much protein material is displaced – non-specifically bound proteins are removed preferentially over those bound covalently.

Measuring photocurrents from RCs

Timing: 6–8 h

In this step, the gold flag electrodes with RCs and MCH deposited on the surface are tested to measure the generation of photocurrents under an intensity-modulated illumination from an LED light source.

Note: This step is based on a previous protocol (Jun et al., 2019).

34. Set up an electrochemical cell with 2 mL of PBS in a cuvette (plastic or glass). Insert the gold working electrode (from the previous step), platinum counter electrode, and Ag/AgCl reference electrode (Figure 6). Connect the electrodes to a potentiostat. See Figure 7 for an example of the equipment setup.
    a. Add an aliquot of 1 M hydroquinone (in DMSO), the sacrificial electron donor, to achieve the desired final concentration (e.g., 1–20 mM).

35. Run a CV as follows:
    a. Scan rate: 2 mV/s

    Note: Not all potentiostats can scan at such low rates. We find that faster CV scan rates do not allow the use of low pass filters on the lock-in amplifier, resulting in large noise in the photocurrent signals.

    b. Upper voltage: 0.30 V vs. Ag/AgCl
    c. Lower voltage: −0.10 V vs. Ag/AgCl
    d. 2 scans

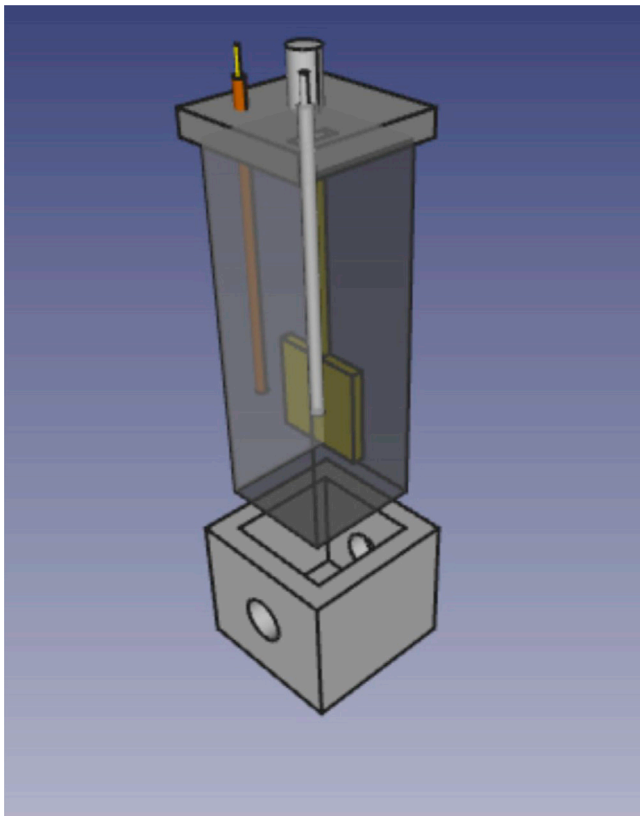

Figure 6. A schematic of the spectroelectrochemical cell

The cell is constructed in a plastic cuvette with the gold flag electrode oriented perpendicular to the LED positions, which are indicated by the holes in the bottom piece into which the cuvette is placed during measurements.

Note: Ensure that the currents measured are small (i.e., <10 $\mu A/cm^2$). This indicates that the MCH layer is effectively blocking faradaic reactions that occur on bare gold or through the MCH layer. While larger faradaic currents can be tolerated, we obtain a better signal-to-noise ratio of the photocurrents by minimizing the faradaic reactions at the electrode surface.

Note: The photocurrents are measured at the same time as the faradaic current. The lock-in amplifier extracts the photocurrents from the total current (faradaic and photocurrent).

36. Apply a 13 Hz sine wave to the LEDs (to illuminate the front and back of the gold flag electrode).
    a. Ensure that the amplitude of the sine wave (center-to-peak) does not exceed the offset current to make sure that the sine wave applied to the LEDs is not clipped. For example, in this work, the LEDs had a 40-mA offset and a 70-mA peak-to-peak current.

Note: The sine wave generated by the lock-in amplifier is used as the input to the LED driver and subsequently converted to a current (100 mA/V, e.g., 70 mA peak-to-peak current). This signal is added to the constant current set on the LED driver (e.g., 40 mA offset).

37. Adjust the amplification settings on the lock-in amplifier to increase the signal to a measurable level at the lock-in amplifier outputs. This is a heuristic approach to find the right settings. Set the lock-in amplifier filters at 24 dB roll-off and time constant of 1 or 3 s. Ensure the lock-in is measuring the amplitude (if using a dual-phase lock-in amplifier like the SR830, a non-zero amplitude should be accompanied by a relatively steady phase signal,).

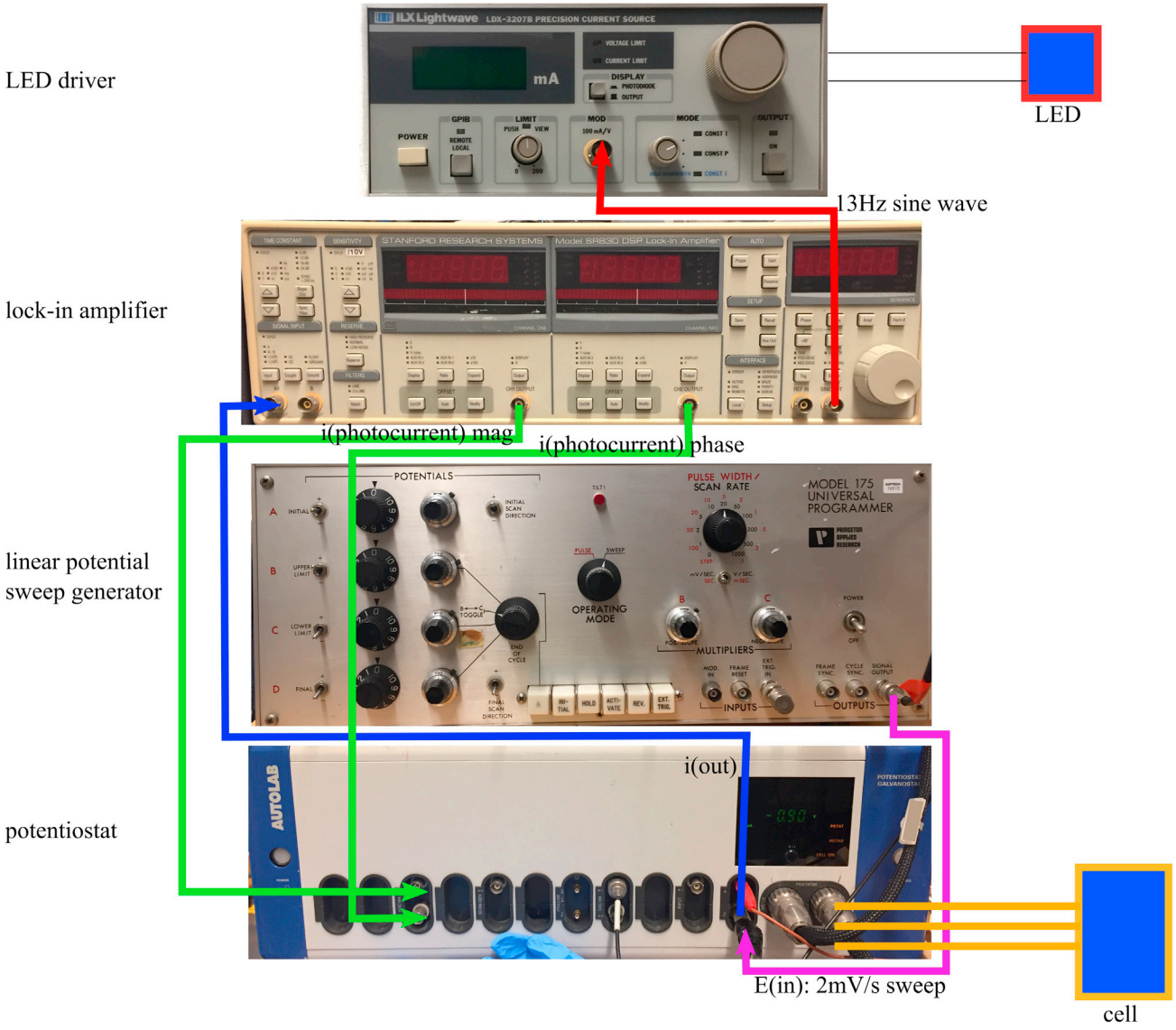

Figure 7. Example of the equipment setup for photocurrent measurements using a modulated light intensity LED, lock-in amplifier, linear potential sweep generator, and potentiostat

a. The demodulated signal amplitude output from the lock-in amplifier is connected to the auxiliary input on the potentiostat.

Note: Ensure that the total current and the amplitude of the photocurrent are measured at the same time.

Note: The total current from the potentiostat is output as a voltage (converted at a value determined by the settings on the potentiostat, e.g., 1 mA/V) and passed into the input of the lock-in amplifier. Adjust the conversion to ensure the input to the lock-in amplifier is not too large, otherwise an input overload will occur.

b. The photocurrent magnitude is determined by using the potentiostat setting for the current (e.g., 1 mA/V) and the amplification setting of the lock-in amplifier (e.g., 10 μV/V). For example, using the previously mentioned settings, if the photocurrent amplitude from the

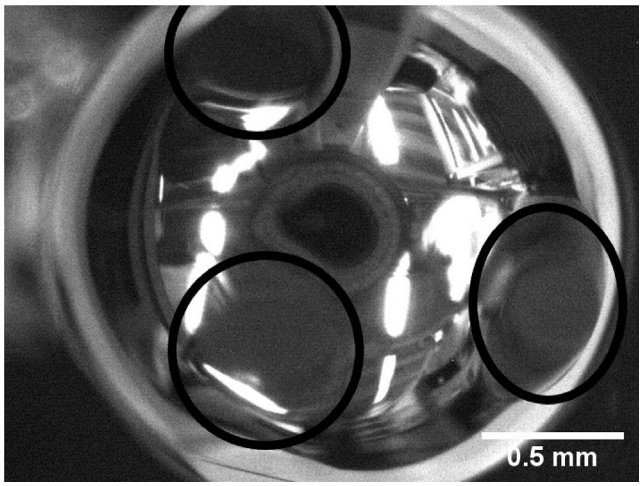

Figure 8. Optical microscope image of the whole gold bead electrode without white light illumination
The oval matte regions represent the 111 surfaces (black circles). The scale bar is 0.5 mm in length.

lock-in amplifier is 2 V, then this would correspond to a photocurrent of 2 V × 10 μV/V × 1 mA/V = 20 nA.

⚠ CRITICAL: Care must be taken to ensure the setting are accurate, as some instruments output a 10 V full scale signal for the setting on the front panel. Make sure to read the manual to be sure of the conversion factor.

38. Begin the photocurrent measurements at 0 V vs. Ag/AgCl. After a stable photocurrent signal is obtained (approximately 15 s at 0 V vs. Ag/AgCl), scan the potential slowly to +0.3 V vs. Ag/AgCl and then to −0.1 V vs. Ag/AgCl (repeatedly) at 2 mV/s. Record the potential, total current, and amplitude from the lock-in-amplifier every 0.1 s.
39. DC photocurrents are measured at the open circuit potential (OCP) of the electrochemical cell. Monitor the potential in the dark (LEDs off) until stabilized (approximately 2 min).
    a. Apply the OCP and continuously measure the current. Monitor the change in the current as the LEDs are cycled on and off (approximately 10–30 s) using a constant current (i.e., sine wave is not used here).

Note: Since the photocurrents are very small (on the order of nA), the OCP should be adjusted and optimized to ensure the smallest current possible, which would be achieved once the potential is set to a value where no net current flows (i.e., the net rate of HQ redox is zero).

Preparation of a single crystal gold electrode for AFM imaging

Timing: 2–3 h

In this step, a single crystal gold electrode is prepared and cleaned.

40. Cut approximately 1.5 cm of gold wire (1 mm in diameter) and secure to a holder using additional gold wire and glass tubing.
41. Rinse the gold wire with ddH_2O thoroughly. Immerse the wire in a solution of aqua regia (1:3 ratio (v/v) of HNO_3:HCl) for 10 min. Rinse with ddH_2O again.
42. Flame the gold wire until the tip of the wire melts. Stop melting when the bead climbs slightly up the wire.

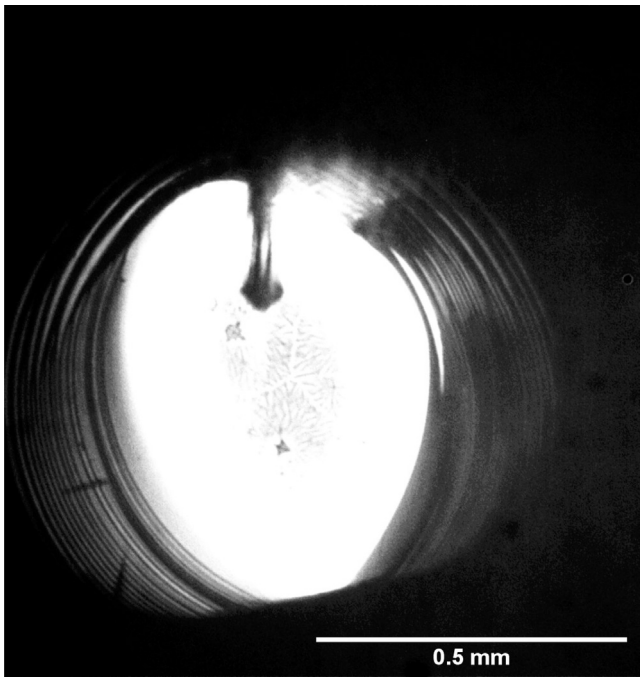

Figure 9. Optical microscope image of the 111 facet on the gold bead surface illuminated with white light
The oval spot represents the 111 surface with an AFM cantilever hovering above the surface. The scale bar is 0.5 mm in length.

Note: When the wire begins to melt, it turns from a bright yellow colour to bright orange. Stop melting when a very small bead can be observed moving up the wire.

43. Rinse the gold wire with ddH_2O thoroughly. Immerse the wire in a solution of aqua regia for 10 min. Rinse with ddH_2O again.
44. Repeat the previous two steps until the bead reaches approximately 2–3 mm in diameter.
45. After immersion in aqua regia, the etching pattern is examined under a microscope to look for a highly symmetrical pattern that corresponds to the crystallography. Once the surface of the bead has a symmetric etch pattern and does not have any grain boundaries (these show up clearly as dark lines in the microscope images of the electrode), melt the electrode for the final time to smooth out the surface.

Alternative: Check for the presence of a large flat reflective region on the surface – this is the 111 facet. Repeat the melting and cooling until this region is large enough to place the AFM tip on it (approximately 0.1 mm in diameter). For AFM measurements, the need for a single crystal bead electrode is not critical, just the presence of large 111 facets.

⚠ CRITICAL: Once the highly symmetric pattern is achieved on the surface without evidence of grain boundaries, when remelting the electrode, do not let the molten gold go past the top of the bead (i.e., stop melting before it reaches the stem). If the flame is not withdrawn in time, there is a high chance the single crystal pattern will not be retained. Such an electrode will most likely not regain its single crystal character.

46. Assemble a glass electrochemical cell for cleaning the electrode surface using a 0.1 M solution of NaOH and purged with argon. Connect the single crystal gold electrode to the working electrode lead from the potentiostat. Similarly, connect a flamed annealed platinum electrode and rinsed saturated calomel reference electrode to the counter and reference electrode leads, respectively.

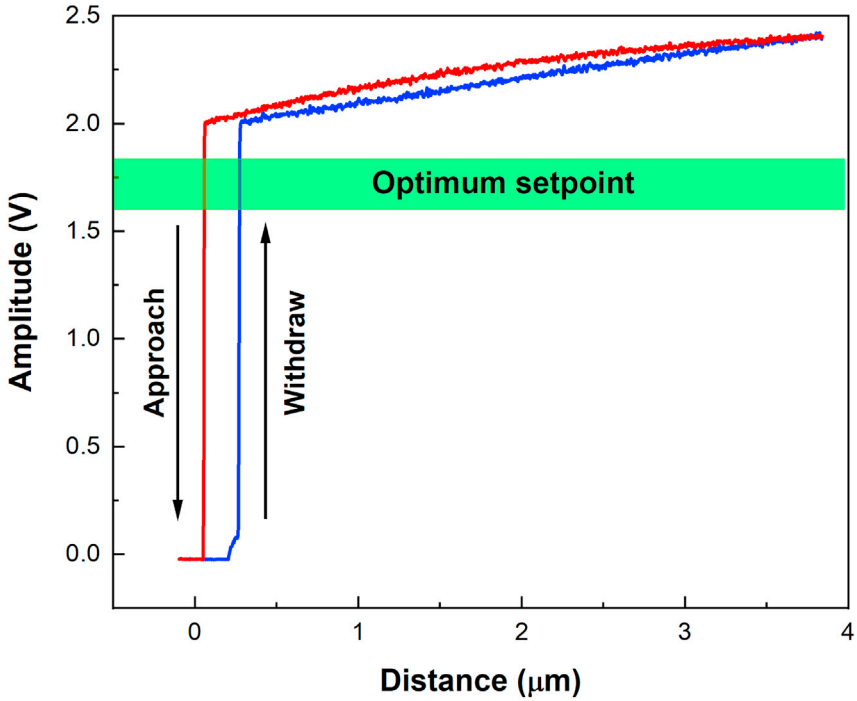

Figure 10. The approach curve represents the forces acting on the AFM tip

The red and blue traces represent the approach of the tip to the surface and withdrawal, respectively. The green box illustrates the optimal range for the setpoint to be selected before the scan begins.

Note: Ensure the electrochemical cell has been cleaned previously in a hot acid bath of H_2SO_4 and HNO_3.

47. Set up a CV with the following parameters:
    a. Scan rate: 50 mV/s
    b. Lower vertex: −1.4 V vs. SCE
    c. Upper vertex: +0.6 V vs. SCE
    d. Cycles: 30

Note: Cleaning is considered complete once the CV has reached an equilibrium and displays features that are consistent with gold (Figure 5).

48. Rinse the gold electrode with ddH_2O and flame anneal.

⚠ CRITICAL: Careful that the gold does not overheat and melt.

49. The clean single crystal gold bead electrode can now be used for the deposition of RCs as previously described in the section deposition of RC and MCH on gold electrodes for future AFM imaging.

Pause Point: The cleaned electrode can be stored in a clean, sealed container such that the surface is not in contact with the sides.

AFM imaging of a single crystal gold electrode

Timing: 1–2 h (setup: steps 50–56); 8–24 h (imaging: step 57)

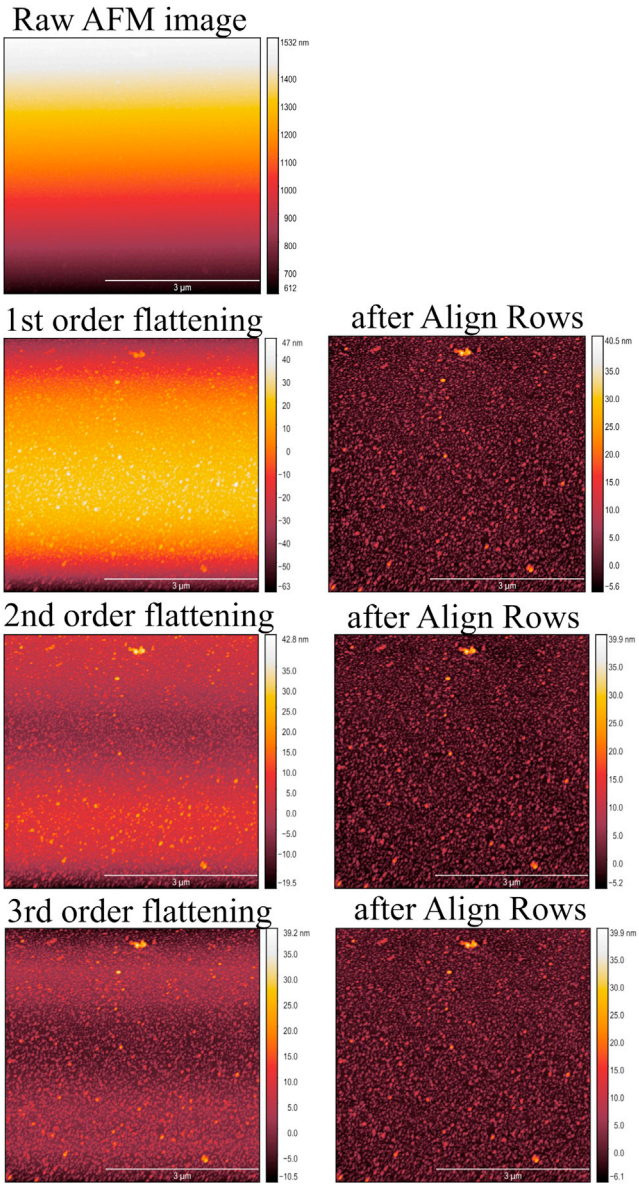

Figure 11. Example of the AFM flattening procedure
The scale bar is 3 μm in length.

This step outlines the procedure to image a 111 facet on a single crystal gold bead electrode.

50. Rinse the single crystal gold bead electrode with PBS for 10 s. Rinse with ddH_2O for 30 s to remove residual salt. Dry under a stream of argon.
51. Mount the electrode on the AFM stage and transfer to an optical microscope to coarsely align the 111 facet horizontally (i.e., parallel to AFM stage that has been pre-aligned to be perpendicular to laser beam).

Note: The 111 facet is a darker circle or an oval spot on the electrode (Figure 8). It can be distinguished from the rest of the surface as it appears more matte. The rest of electrode surface is lustrous like a mirror, whereas the 111 facet is not, since it tends to reflect light in a

Table 1. Summary of RMS roughness and angle of inclination (θ) parameters for images before and after polynomial subtraction and row alignment

Flattening method	Raw data	1st order flatten	Row alignment	2nd order flatten	Row alignment	3rd order	Row alignment
RC-covered surface							
RMS roughness (nm)	251.9	20.9	3.481	5.62	3.469	4.04	3.465
Angle of inclination (θ)	9.54°	0.24°	0.23°	0.22°	0.23°	0.22°	0.23°
Flat gold surface (MCH-covered)							
RMS roughness (nm)	21.49	1.176	0.766	0.511	0.502	0.445	0.438
Angle of inclination (θ)	0.42°	0.01°	0.01°	0.01°	0°	0°	0°

direction away from the viewer and so appearing as though it was matte when viewed under the microscope and illuminated from the sides of the electrode.

Note: Do not illuminate the electrode with white light directly, as the reflection from the 111 facet will make it hard to visualize.

52. Illuminate the electrode surface in the AFM holder with the output from a columnated laser diode. The resulting reflection off the 111 facet is directed onto the surface of paper and will be visible as an intense circular reflection pattern.

Note: Take care to wear appropriate laser safety glasses, as the reflection from the bead can be difficult to control.

53. By moving the bead using tweezers, align the reflected beam with the path of the incident light, resulting in a 0° angle between the incident and reflected beams. This ensures that the 111 facet of the electrode is aligned horizontally for optimal AFM imaging conditions. The electrode is now finely aligned with the AFM sample holder. This procedure does not eliminate sample tilt as the AFM tip/cantilever may not always be parallel to the AFM sample holder.

Note: The alignment does not have to be exactly 0°. Some tolerance is acceptable as the tilt can be corrected during image processing.

54. Transfer the finely aligned electrode on the AFM sample holder to the AFM microscope. The 111 facet is found as a bright oval spot, when illuminated with white light in the AFM (Figure 9).

Note: The best spot for imaging is the center of the 111 facet, as it has the flattest surface. Avoid imaging on the edge of the facet as multiple atom high steps are present.

Note: The suggested cantilever for imaging is one with a nominal resonance frequency of 150 kHz and a spring constant of 5 N-m.

55. Approach the tip to the surface as usual. Perform a force curve measurement using tapping mode to ensure good contact with the surface and to determine the surface quality. The force curve should consist of clean approach and withdraw lines with relatively small hysteresis (i.e., no cusps or steps on the vertical part) (Figure 10).

Note: Cusps on the vertical part, may indicate adherence of contaminants to the tip from the surface.

56. Manually set the optimum setpoint at approximately 80%–90% of the value between surface and the approach cusp (Figure 10, green box).
57. Scan at a frequency of 0.14 lines/sec, sampling 4096 lines per image.

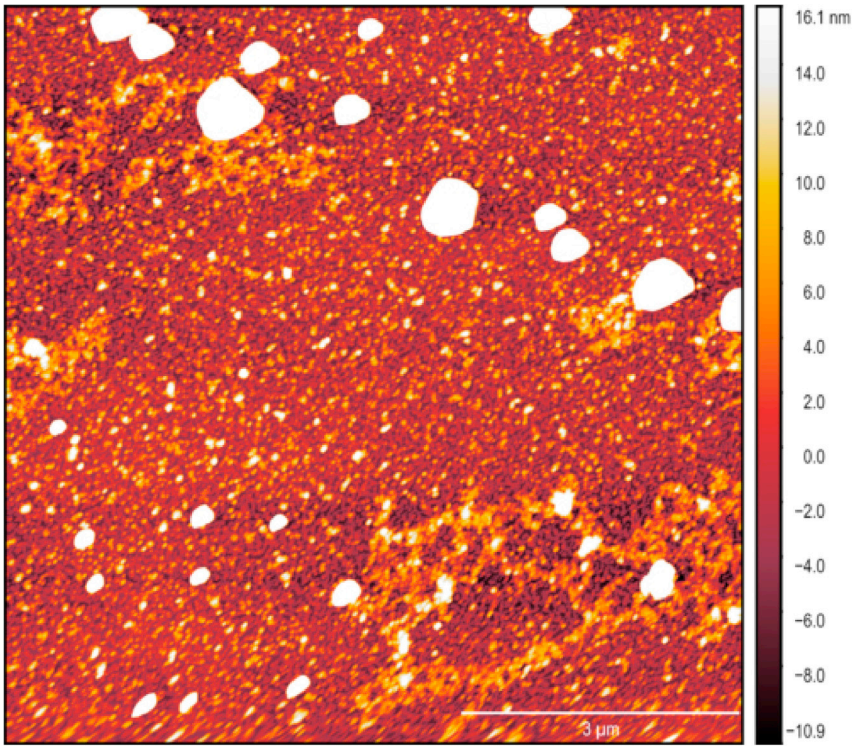

Figure 12. Example of an AFM image of the 111 facet modified with RCs and MCH
Tall, white features are from salt crystals due to the evaporation of the electrolyte. The scale bar is 3 µm in length.

a. Repeat as necessary. Scans can be completed overnight.

Processing of a raw AFM image

Timing: 1 h

This step outlines the procedure to process AFM images using Gwyddion, an open-source software available for all major operating systems. The following steps correct for the background and tilt of the AFM images taken from the 111 facet.

58. Even after aligning the surface using steps 52 and 53, the AFM image will still be tilted. Removing this planar background or tilt from the image is performed using the "Remove Polynomial Background" method.
    a. Optimal results are obtained by trial-and-error, but some simple guidelines are provided to ensure that the flattening does not introduce artifacts. Correction for the background is accomplished by fitting a polynomial to the whole image in both the X and Y dimensions. The choice of the polynomial is never usually above 3rd order. As shown in Figure 11, 1st order correction removes the tilt, but low frequency waves are still present. This can be corrected using the "Align Rows" option to align the rows of lines that are out of the average plane (Figure 11). The RMS roughness can be monitored, using the "Statistical Quantities", method to evaluate the flattening process. As shown in Figure 11 and Table 1, the RMS height stays constant after 2nd order background correction and performing "Align Rows" function. This characteristic is a reasonable metric to limit background correction to where it no longer affects the RMS roughness values (shown in Table 1).

59. Perform a particle analysis (size, height) after obtaining a flattened image.

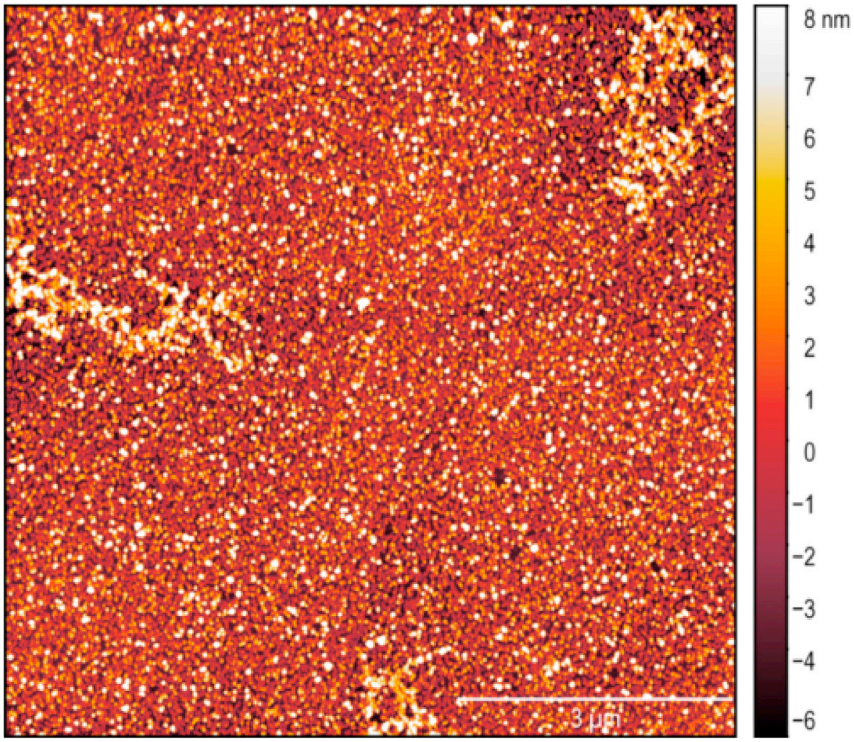

Figure 13. Example of an AFM image of the 111 facet modified with RCs and MCH without salt crystals
The scale bar is 3 µm in length.

a. Set a threshold of 1 nm and create a mask. Erode the mask twice and dilate once to facilitate watershed separation of the particles. Watershed the resulting image to delineate or separate the particles. Convolution with the tip limits the accuracy of particle footprint estimates; therefore, accurate watershed is considered to be more important than accurate particle footprint determination.

Note: The mask is used to analyse the AFM image by restricting the particles to be >3 pixels in area and exclude any particles on the edge of the image.

b. Create a histogram of the maximum height and the distribution of the footprint area for each particle.

EXPECTED OUTCOMES

After purification of the DM RC, the UV-vis absorbance spectrum of the protein should be similar to that in Figure 4. In particular, the A_{280}:A_{804} ratio should be less than 1.4, indicating that the preparation is of high purity and suitable for subsequent electrochemistry experiments.

Examples of expected outcomes and further details of photocurrent measurements, modelling to the Marcus-Hush-Chidsey theory, statistical analysis, and AFM images can be found in Jun et al. (2019) and Jun et al. (2021).

AFM imaging gives an indication of surface coverage with RCs but can also show unwanted features, such as contamination. For example, Figure 12 shows an AFM image where salt precipitated on the surface after the electrolyte evaporated; in comparison, Figure 13 is a scan where no salt is present, and RCs cover the surface. The salt features are large with heights of several hundred nm (Figure 14,

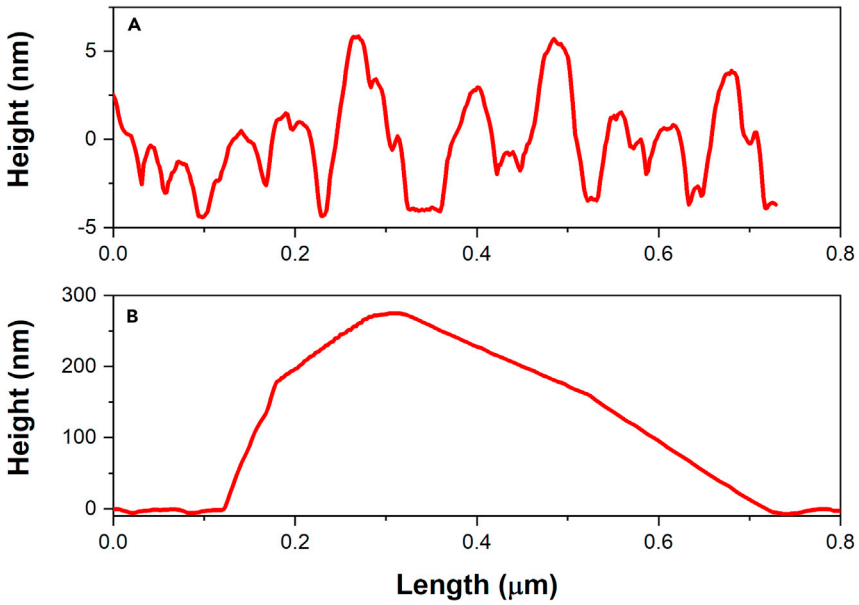

Figure 14. Line scans extracted from Figure 12

(A) Line scan without a salt crystal.

(B) Line scan going through a salt crystal.

panel B). The salt is easily distinguishable from RC structures – much smaller and approximately 5 nm in height (Figure 14, panel A) and similar to known dimensions (Jones, 2009).

QUANTIFICATION AND STATISTICAL ANALYSIS

Fitting photocurrents to the Marcus-Hush-Chidsey electron transfer theory

The currents from the positive-going and negative-going potential scans are averaged and subsequently used for fitting. The potential data are smoothed with a moving average of 5 datapoints. The photocurrent data are smoothed with a moving average of 50 datapoints (2.5 datapoints per mV, over 20 mV). The photocurrents and faradaic currents are both fit to the Marcus-Hush-Chidsey theory using MATLAB (fitnlm function).

The faradaic currents (both oxidation and reduction) are used in the fitting procedure with three parameters: reorganization energy (`re`, in eV), equilibrium potential (`eo`, in eV) and a scaling factor (`sf`). The first two parameters must be input into the MATLAB formulas as eV, but can be easily converted to V.

After ensuring the `re` is positive, the theoretical current is calculated using the following code in MATLAB (based on Equation 18 in Zeng et al. (2014), where x is a vector that contains the potential data:

```
result = sf*sqrt(pi*re).*tanh((x-eo)./2).*erfc((re-
sqrt(1+sqrt(re)+(x-eo).^2))./(2*sqrt(re)));
```

This code is used by fitnlm to calculate the current for each potential, which is stored as a vector in `result`. The fitting routine minimizes the difference between the data and the fitted values by changing the values of the three parameters (`sf`, `re`, `eo`).

As oxidation currents are only observed in the measured photocurrents, the code used in fitting the data is chosen to only calculate the oxidation current. The code used is based on Equation 17 in Zeng et al. (2014):

```
result = sf*sqrt(pi*re).*(1./(1+exp(-1*(x-eo)))).*erfc((re-
sqrt(1+sqrt(re)+(x-eo).^2))./(2*sqrt(re)));
```

The data used in the fitting of the photocurrents are limited to values of >1 nA. The starting parameter values for the single component fits are:

```
re = 19.45 eV (0.5 V), eo = 7.78 eV (0.2 V) and sf = 50.0.
```

For the two component fits, the starting values for the parameters are:

```
(re = +0.1 V, eo = -0.07 V, sf = 5),  #component 1, convert to eV
(re = +0.5 V, eo = -0.2 V, sf = 5)  #component 2, convert to eV
```

The initial values do not impact the fitted results except for very noisy or low photocurrent data, but convergence is more reliable if the parameters are estimated starting from the one component fitting results. The use of a second component is statistically validated using an F-test (details elaborated in Jun et al. (2021)), which compares the reduced model (one component with three parameters) against the full model (two components with six parameters).

LIMITATIONS

A large faradaic current may result in very noisy photocurrent results, as the current-follower setting on the potentiostat will have to be increased (e.g., from 1 to 10 mA/V) and consequently limits the lock-in amplifier performance.

The use of large time constants for photocurrent measurement may be needed depending on the noise level; this will result in the need for slow potential sweep rates.

The PAR175 linear scan generator is no longer available commercially. Many potentiostats will use a digital-stepped potential to achieve slow scan rates, which will introduce noise in the photocurrent measurement. Another approach to achieving low-noise slow-potential sweep signals is to attenuate a digitally-stepped potential-sweep signal (16-bit DAC) that is generated to have potentials that are 10-fold larger than the range desired (e.g., −3 V to +4 V) followed by an attenuation of 10. Discrete potential steps can be used as well, though the lock-in amplifier may lose the signal when the step change in potential occurs and reacquisition of a stable signal may take tens of seconds or longer for small signals.

The frequency used in the photocurrent measurements is determined by trial-and-error. If the frequencies are too high, slow processes are not accurately measured, whereas at low frequencies lock-in amplifiers have difficulty isolating the signal from the environmental noise. Optimizing the frequency used to drive the LEDs can result in higher quality measurements.

RCs are not stable over a long period of time and may aggregate due to entropic gains or disulphide bonding. The structural stability of the RCs can be monitored by UV-vis absorbance spectroscopy for any characteristic degradation signals (Hughes et al., 2006). Formation of monolayers of RCs may depend on the age of the solution and the aggregation state. Denatured RCs on the surface are difficult to displace completely or reliably using MCH.

TROUBLESHOOTING

Problem 1

Cells not growing to the proper density in time (step 4).

Potential solution

Ensure that cell culture makes up 10%–25% of the nominal flask volume and that the flasks are loosely capped. Flasks can also be shaken at a higher RPM or grown in Fernbach flasks to increase aeration.

Problem 2

Cells appear orange-brown (late stationary or dead culture) rather than orange-red following overnight induction with IPTG (step 4).

Potential solution

Grow culture to stationary phase (roughly 16 h of growth) first, then induce with IPTG for 6–8 h (i.e., not overnight).

Problem 3

RCs are not binding to Ni^{2+}-NTA agarose resin (step 14).

Potential solution

Ensure that RCs are present in the supernatant by measuring the absorbance of a 10-fold dilution. RCs in the lysate should appear as a small peak around 804 nm. Additionally, ensure that the beads are equilibrated in wash buffer prior to use and incubate the supernatant with the beads for a longer period of time.

Problem 4

Protein elution from the Ni^{2+}-NTA column is very impure (i.e., A_{280}:A_{804} >4) (step 16).

Potential solution

Decrease the incubation time of the solubilized RCs in the supernatant with the Ni^{2+}-NTA resin. Additionally, lower the column flow rate during the wash step (to roughly 1–1.5 drops per second), to increase contact time of the buffer interacting with the resin to wash away impurities.

Problem 5

No elution fraction collected from the anion column contains proteins of sufficient purity (step 23).

Potential solution

Collect smaller volume elution fractions (2–3 mL fractions rather than 5 mL) and flow more buffer through the column before increasing the salt concentration.

Problem 6

Photocurrents are noisy or the lock-in amplifier is having difficulty finding a signal (step 37).

Potential solution

Photocurrents are typically 1000–10,000-fold smaller than the faradaic currents, so large faradaic currents will present a challenge to measuring photocurrents.

Improve the MCH coating of the electrode surface to minimize the faradaic reactions from hydroquinone.

Ensure the gold flag electrode is aligned properly, such that the LEDs completely illuminate the surface, and prevent the counter electrode or reference electrode from interfering.

Check for the proper current-follower setting on the potentiostat.

Make sure the reference electrode is reliable and of high quality. Problems in the measurements are usually traced back to a bad reference electrode or faulty connections.

The RCs may have denatured or not be functioning correctly. Structural composition of the RCs can be measured by UV-vis absorbance spectroscopy. Functionality of the RCs can be tested as described in Jun et al. (2018) and Jun et al. (2020). Prepare a new, fresh batch of protein.

More optimal frequencies can be selected to drive the LEDs, as power supply line frequencies may differ depending on geographical regions and countries.

Problem 7

The AFM image shows steps instead of a flat surface (step 54).

Potential solution

The tip is most likely on the edge or just outside of flat 111 facet where multiple atom high steps are present. Reposition the tip such that it is as close to the center of the 111 facet as possible.

Problem 8

The approach curve has cusps for AFM (step 55).

Potential solution

The presence of cusps indicates that the tip is interacting with a substance on the surface. Most commonly, the tip may be interacting with water and so the electrode can be dried with a stream of argon for 0.5–1 min; therefore, imaging under humid conditions is not recommended. If the problem persists, the tip may be contaminated and requires replacement, or the tip is interacting with the electrode surface too strongly and requires remaking of the RC-modified surface.

Problem 9

The AFM image shows an abundance of random peaks that are a few hundred nm in height and appear to be disjointed and discontinuous (i.e., noise) (step 56).

Potential solution

The setpoint is set too high or too low; adjust the setpoint to the optimal value. Additionally, ensure that the laser is in the center of the photodiode. This may also be due to a tip contamination issue in which a new tip would be required.

Problem 10

Salt crystals are present in the AFM image of either MCH-coated gold or RC-coated gold (step 57).

Potential solution

Rinse the electrode thoroughly with ddH_2O for 1–2 min. An example of AFM image that contains salt crystals is shown in Figure 12 and the same surface after rinsing with ddH_2O is shown in Figure 13. Representative line scans are also shown in Figures 14A and 14B for surfaces without and with salt crystals respectively. If the problem persists, sonicating the electrode in ddH_2O for 1–2 min may help, but should be used as the option of last resort, as the treatment is very aggressive.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dan Bizzotto (bizzotto@chem.ubc.ca).

Materials availability

There are restrictions to the availability of the DM RC due to the viability of supply and long-term storage. The *Rhodobacter sphaeroides* strain containing the plasmid expressing the DM RC may be available upon request.

Data and code availability

This study did not generate datasets. A copy of the MATLAB code used for fitting the data will be provided upon demand.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.J. and D.B.; methodology, D.J. and D.B.; investigation, S.Z., A.G., and D.J.; writing – original draft, D.J., S.Z., and D.B.; writing – review & editing, D.J., J.T.B., and D.B.; funding acquisition, J.T.B. and D.B.; resources, D.B., D.J., A.M., and J.T.B.; supervision, D.J., J.T.B., and D.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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