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Supporting Information

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Spectroscopic Slicing to Reveal Internal Redox Gradients in Electricity-Producing Biofilms**

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S1: Customized electrochemical flow cell

The experiments were carried out in a custom-made three electrodes electrochemical cell (EC) specially designed to be mounted on the stage of an optical microscope and controlled by an Autolab PGSTAT 101 potentiostat. The three electrodes were an Ag/AgCl-3M NaCl reference electrode (RE), a platinum wire counter electrode (CE) and an ITO working electrode (WE). All potentials provided in the manuscript are referred versus the Ag/AgCl (3 M NaCl) reference electrode (0.209 V vs. SHE). Media is pumped into the chamber through a stainless steel liquid inlet (L in) at a controlled flow. The liquid outlet connection (L out) is located away from the WE to prevent biofilm drying in case of flow interruption. A low gas overpressure allows pushing the liquid out of the cell, maintaining the filling level and ensuring anoxia in the overhead space. To ensure that cells were using the electrode as the electron acceptor, no other acceptor was added to the culture media.

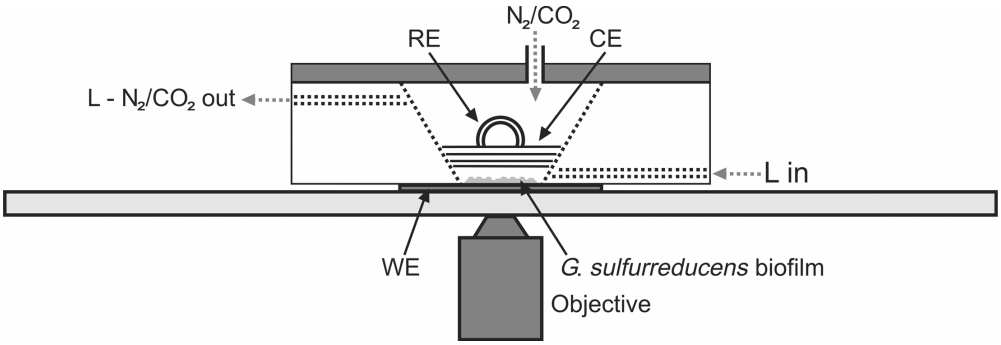

Figure S1. Schematic diagram of the electrochemical flow cell (EC) designed for microscopic observation.

S2a. Biofilm growth - determination of biofilm thickness and surface coverage

Structural information of the bacterial population was obtained from the electrode surface up to the solution by optical slicing of the surface-associated biofilm with a phase contrast Nikon Eclipse T*i*-U inverted microscope using a long working distance objective (ULWD Nikon, 60X, with correction ring) to focus on stepped focal planes. Biofilm thickness was measured as the distance between the electrode surface and the upper biofilm slice in optical sectioning having a covered area >1%. Biofilm coverage was calculated as the percentage of slice area covered by cells at increasing focal distance from the electrode. Digital analysis of the images obtained at each optical slice was performed using the public domain Image J software[1] as shown in Figure S2a. Contrast was enhanced and background subtracted using the rolling ball tool with 1 µm radius (B). The resulting image was binarized and thresholded to show only *in focus* bacteria in each focal plane (C), allowing the calculation of the covered area in every slice image.

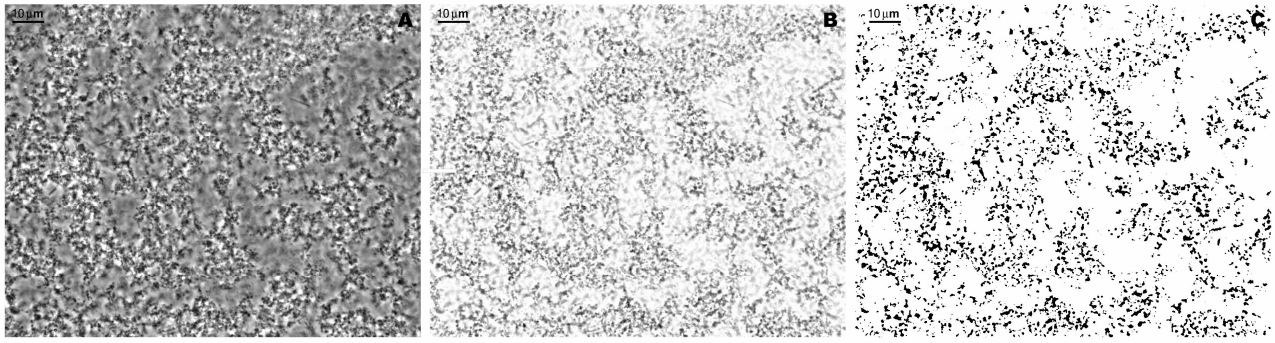

Figure S2a. Steps in the digital image analysis of microscopic images of *Geobacter sulfurreducens* biofilm: (A) original phase contrast image of a biofilm optical slice; (B) image obtained after enhancing contrast and subtracting the background of image in panel A; (C) image obtained after binarization and thresholding of the image in panel B. All steps in image processing were manually applied taking the original image as a reference, to select only *in focus* bacteria. Bars: 10 µm.

[1] C. A. Schneider, W. S. Rasband, K. W. Eliceiri, *Nature Methods* **2012**, *9*, 671

S2b: Biofilm growth - chronoamperometry

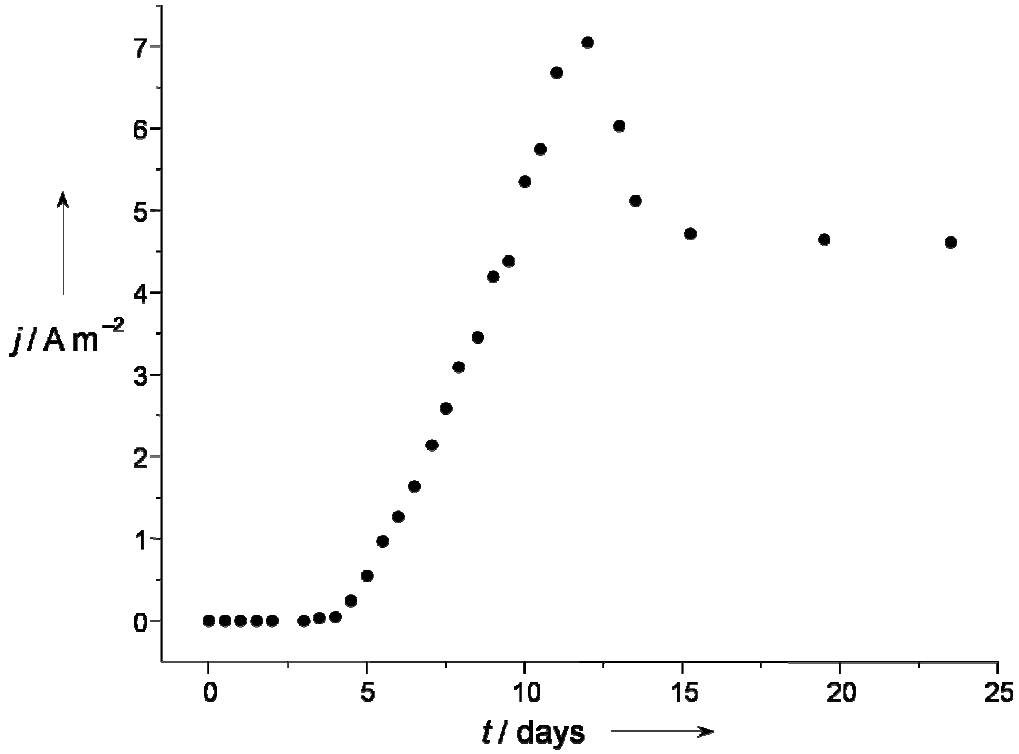

Figure S2b. Typical current evolution plot obtained from a *G. sulfurreducens* biofilm grown at -0.15 V on an ITO electrode in continuous culture using 20mM acetate as electron donor.

S2c: Biofilm growth - dependence of current production on biofilm thickness

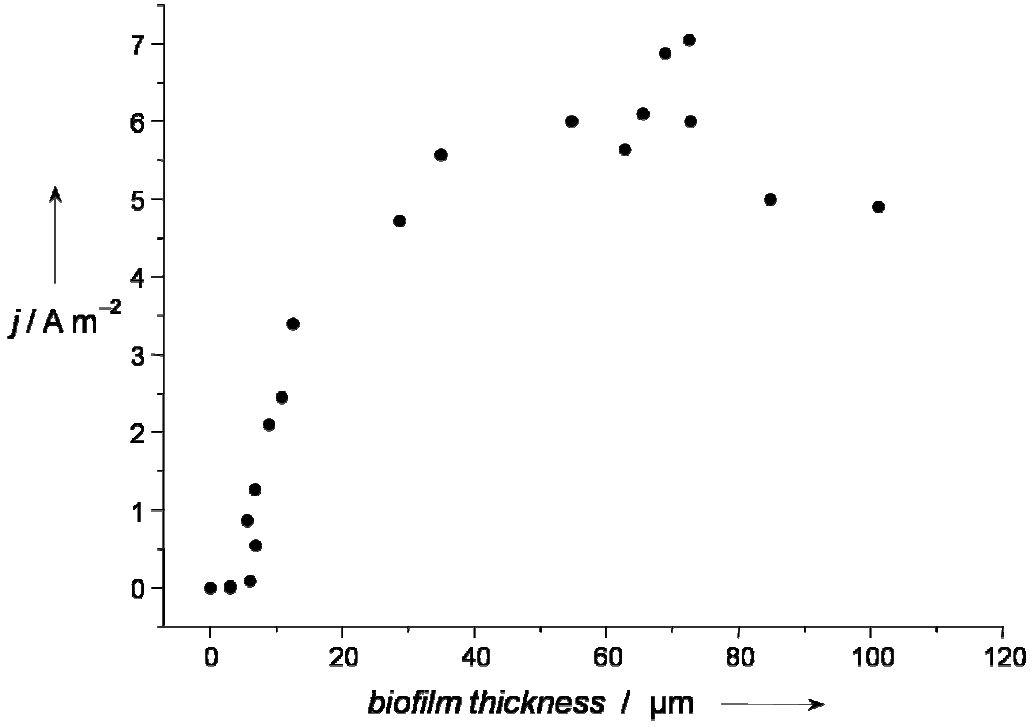

Figure S2c: Dependence of current output on the thickness of a biofilm of *G. sulfurreducens* growing on an ITO electrode polarized at -0.15 V. Biofilm thickness was measured as the distance between focal planes centred at the electrode surface and that at the upper biofilm slice having a covered area >1% in optical sectioning, as described in the experimental section.

S3a: Confocal Raman microscopy - dependence of the Raman spectral profile with the applied potential

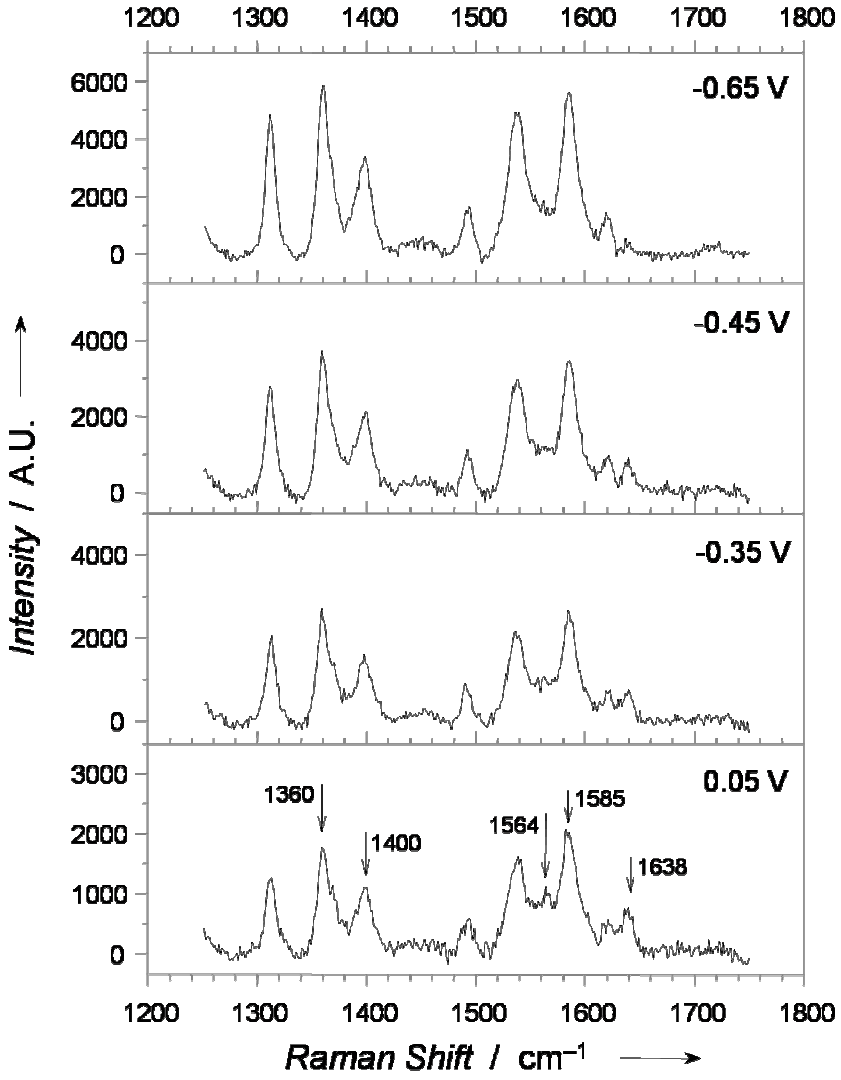

Figure S3a. Raman spectrum of fully developed *G. sulfurreducens* biofilm at the electrode interface level under different applied potentials. Arrows indicate peaks ascribed to resonant excitation of heme groups of c-type cytochromes.

S3b: Confocal Raman microscopy - spectral reconstruction

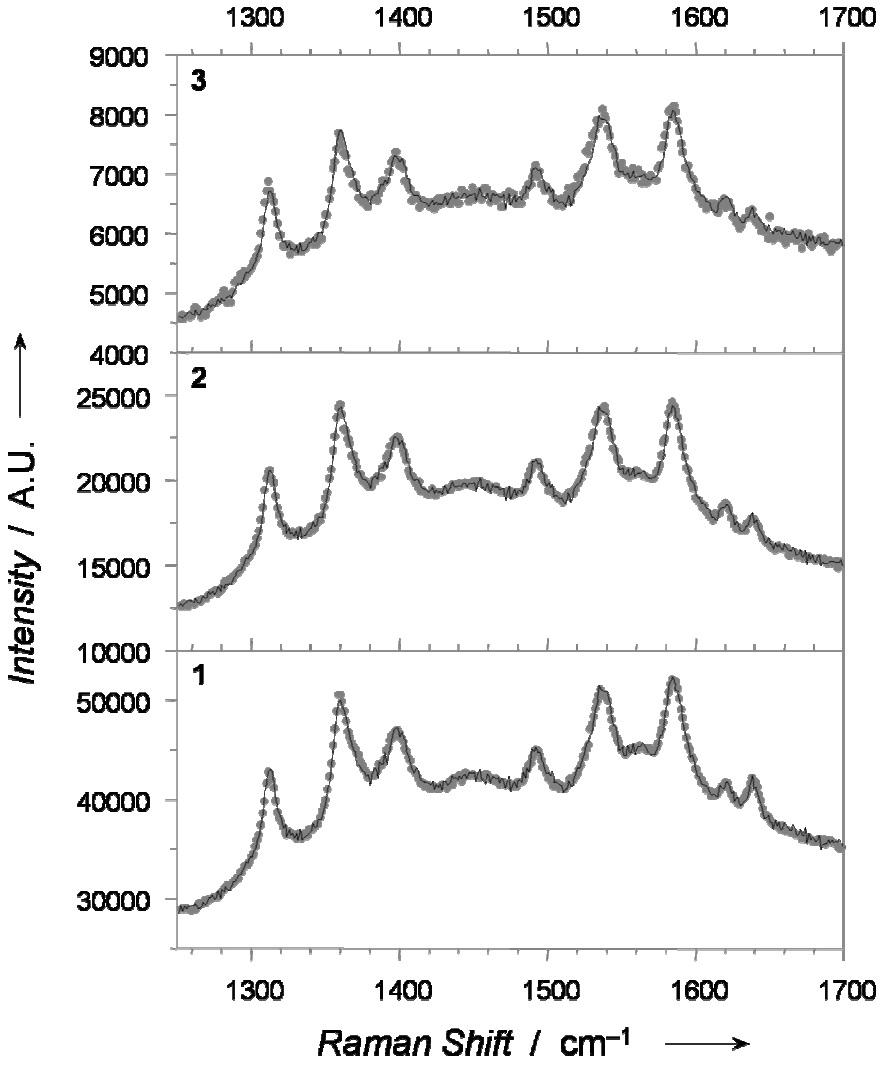

Figure S3b. Example of the spectral reconstruction generated by the fitting procedure for three local Raman spectra taken at 10 (1), 30 (2) and 50 (3) µm depth, in the experiment performed at -0.40 V. The acquired Raman spectrum (●) is linearly expanded as a combination of: i) totally oxidized spectrum; ii) totally reduced spectrum; iii) a five degree polynomial that accounts for changes in baseline. The fitting procedure finds by least squares an optimum set of parameters of the linear expansion, generating the reconstructed spectrum shown in each case as (—). The first two coefficients of the linear expansion are directly related with by-weight contributions of reduced and oxidized forms.

S3c: Confocal Raman microscopy - dependence of the molar fraction of reduced and oxidized cytochromes and current production with the applied potential

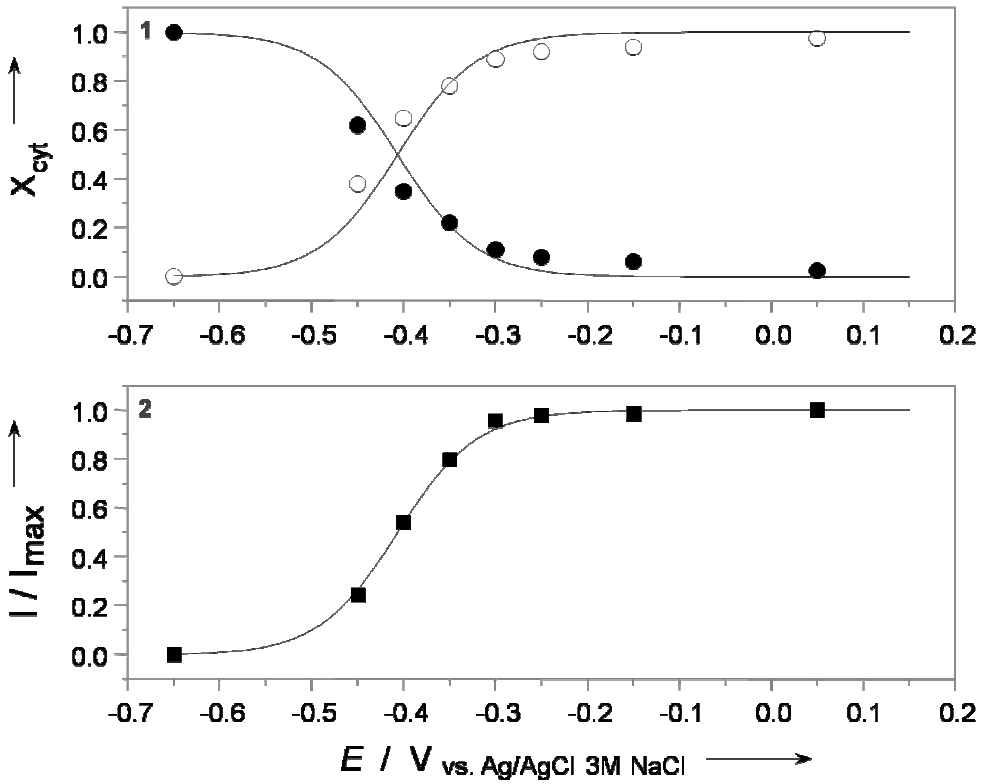

Figure S3c. Dependence of 1) the fraction of reduced (●) and oxidized (○) cytochromes at the biofilm/electrode interface level and 2) the current output with the applied potential (■). Imax: 150µA

S3d: Confocal Raman microscopy – data reproducibility

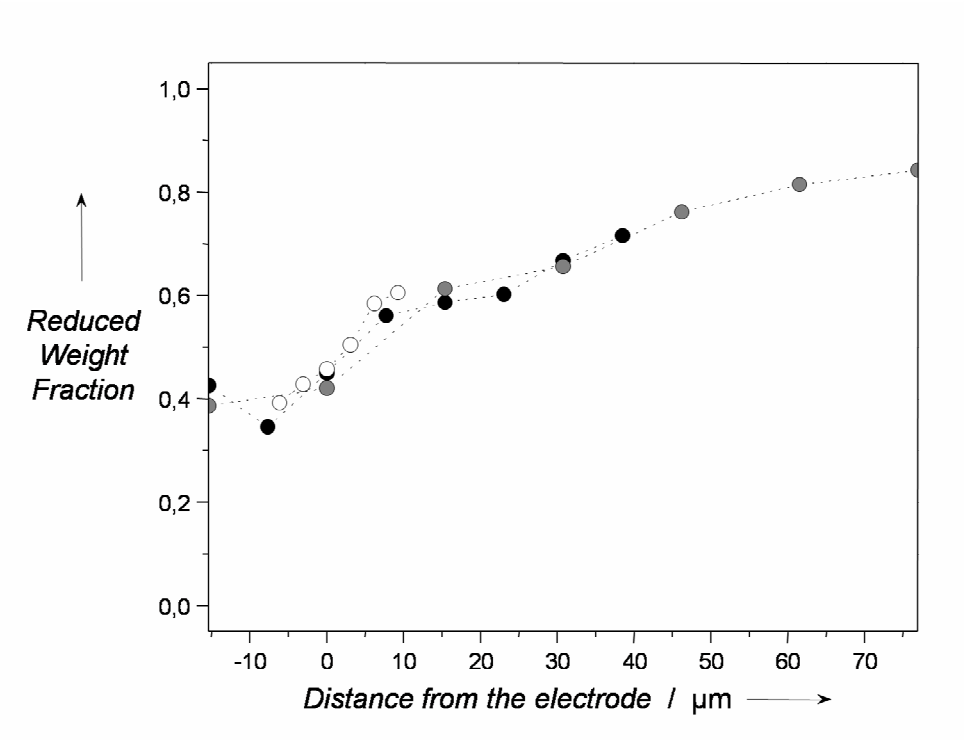

Figure S3d. Example of data reproducibility typically obtained in confocal Raman experiments. Data of the biofilm shown in Figure 2 taken in nearly the same sample spot at -0.40 V under different acquisition conditions: steps of 2 µm, 10% laser power, 24 s by acquisition (○); steps of 10 µm, 50% laser power , 9 s by acquisition (●); steps of 5 µm, 50% laser power, 48 s by acquisition (●).