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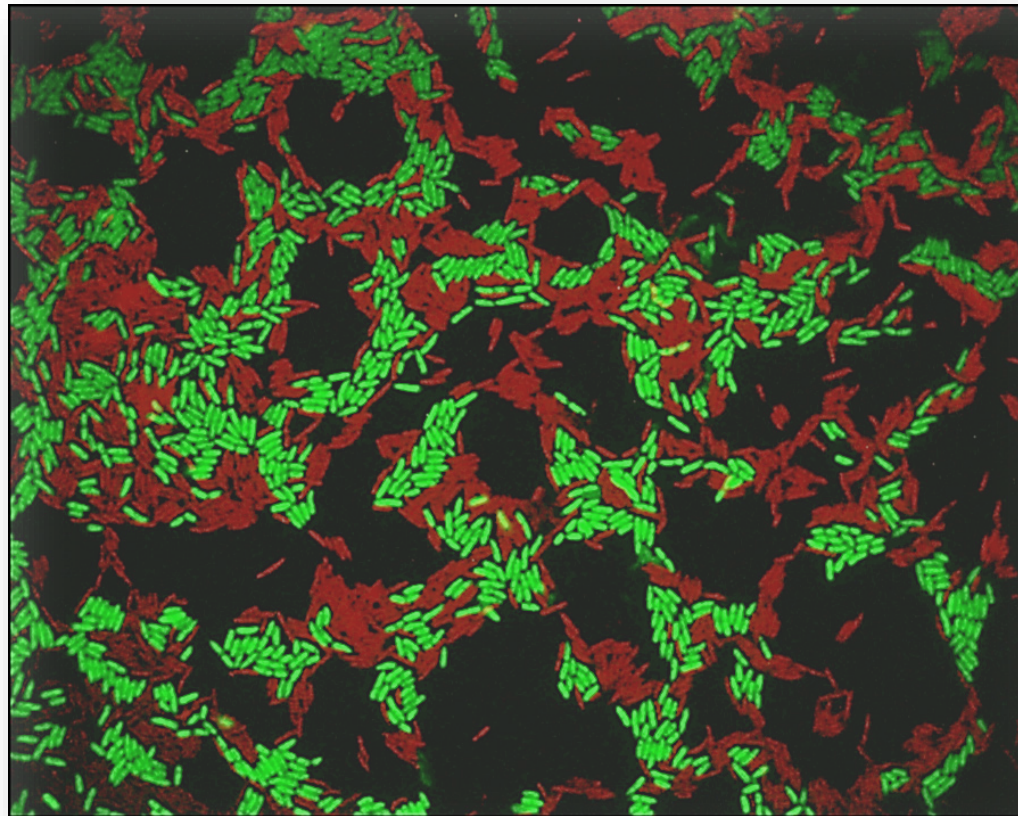
Environmental Quality and Installations

Method for Localizing and Differentiating Bacteria within Biofilms Grown on Indium Tin Oxide

Spatial Distribution of Exoelectrogenic Bacteria within Intact ITO Biofilms via FISH

Clint M. Arnett and Justin D.T. Lange

November 2017



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Method for Localizing and Differentiating Bacteria within Biofilms Grown on Indium Tin Oxide

Spatial Distribution of Exoelectrogenic Bacteria within Intact ITO Biofilms via FISH

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Abstract

With a limited supply of fossil fuel, there has been great interest in the development of new technologies that can take advantage of renewable fuel sources or convert energy stored in waste to usable energy. One such class of technologies are microbial fuel cells (MFCs), which can convert various carbohydrate rich sources as well as wastewater into electricity via biological catalysts. However, electrical current generation in these microbial driven systems is typically low making these technologies unsuitable for widespread use. In order for MFCs to become a viable alternative energy source, methods are needed to better understand the relationship between microbes and electron transfer. This work outlines a method for spatially differentiating exoelectrogenic bacteria within intact biofilms grown on a conductive surface. The technique involves the rapid generation of biofilms by using a drip flow bioreactor (DFR) on indium tin oxide (ITO)-coated slides, in situ fixation of bacteria within the biofilms on the ITO surface, and determining species differentiation and location by probing with fluorescence in situ hybridization (FISH). This method was shown to effectively distinguish two exoelectrogens within biofilms on a conductive surface, which could serve as a novel means to study MFCs in greater detail.

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Preface

This study was conducted for the U.S. Army Engineer Research and Development Center Environmental Quality/Installations (ERDC-EQ/I) Basic Research Program under Project 404938, “Microbe-surface Interactions in Biofouling and Corrosion.” The technical monitor was Dr. Elizabeth Ferguson, Lead Technical Director for ERDC-EQ/I.

The work was performed by the Environmental Processes Branch (CNE) of the Installations Division (CN), U.S. Army Engineer Research and Development Center, Construction Engineering Research Laboratory (ERDC-CERL). At the time of publication, Mr. H. Garth Anderson was Chief, CEERD-CNE; Mr. Donald K. Hicks was Acting Chief, CEERD-CN; and Dr. Elizabeth Ferguson, CEERD-EM-J was the Technical Director for ERDC-EQ/I. The Interim Deputy Director of ERDC-CERL was Ms. Michelle J. Hanson, and the Interim Director was Dr. Kirankumar V. Topudurti.

This research was supported in part by appointments at the Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE), through a cooperative agreement between the U.S. Department of Energy and the U.S. Army ERDC-CERL. We wish to thank Justin R. Smith for his assistance in the initial drip flow reactor (DFR) setup and imaging hybridizations via inverted microscopy.

COL Bryan S. Green was the Commander of ERDC, and Dr. David W. Pittman was the Director.

Abbreviations

ATCC	American Type Culture Collection
BTU	British thermal units
CCD	Charge coupled device
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DFR	Drip flow bioreactor
DoD	Department of Defense
EO	Executive Order
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
ITO	Indium tin oxide
MFC	Microbial fuel cell
PFA	Paraformaldehyde
QE	Quantum efficiencies
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TSB	Tryptic soy broth
UHP	Ultra-high purity

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1 Introduction

1.1 Background

There is a finite amount of fossil fuel remaining in the world, and at currently predicted rates of consumption, it is estimated that strategic reserves could be depleted within 50 years if alternative energy sources are not sought (Berg and Korte 2008). The U.S. military is the single largest consumer of fossil fuels in the world, using 340,000 barrels of oil per day, which equates to roughly 1000 trillion British Thermal Units (BTUs) of energy per fiscal year (Resilience 2007). This large consumption is concerning, because the Department of Defense (DoD) obtains a significant amount of its oil from foreign sources, which are potentially hostile. In order to promote sustainability, the federal government was mandated under Executive Order (EO) 13693 to reduce consumption of traditional fossil fuels (EO 13693 2015). This EO is a multifaceted directive to reduce dependency on foreign oil, lessen the nation's carbon footprint, and spawn new sustainable technologies that reduce operational costs.

Over the last decade, bioelectrochemical systems and technologies have been rapidly emerging as a feasible means to generate electrical energy from renewable and waste products. Such technologies have the potential to lessen dependency on foreign fossil fuel sources and convert waste to a useful source of clean energy. In bioelectrochemical systems, bound energy in the form of various organic matter is released by using microorganisms as catalysts. This release creates oxidation redox potentials at the anode and a reduction reaction at the cathode. This process generates a differential potential between the anode and the cathode, which allows electron flow and produces an electrical current that can be harnessed for use (Bajracharya et al. 2016). Many of these systems can be driven by the colonization of electro-active bacteria on the anode surface in the form of a biofilm, and the biofilm can convert organic substrates into electrons while oxygen is reduced at the cathode to complete the redox reaction (Muskateem 2015). Not only are such systems useful for releasing chemical energy bound in organic molecules, but these same systems can also be used for production of sustainable chemicals and used in applications such as biosensors, bioremediation, and wastewater treatment (Logan et al. 2006). Any technologies that have the potential to lessen the logistical

load and provide energy from waste are of great interest to the U.S. Army and the DoD as a whole (Holcomb 2011).

One of the most studied types of microbial-driven electrochemical systems is microbial fuel cells (MFC), which produce electrical current from the oxidation of organic matter. The anode materials used in MFC are typically carbonaceous-based materials due to their low cost, ease of fabrication, and resistance to corrosion (Wei et al. 2011). However, these materials suffer from low electrical conductivity (Mustakeem 2015) and can also have a negative effect on bacterial attachment to the anode surface (Pec et al. 2010). Other substrates such as various rare metals have been demonstrated as effective anode materials; however, the cost associated with these materials precludes their use for large-scale applications; thus, economically, rare metals can only be used on the nano- to micro-scale for applications such as sensors (Choi et al. 2011). Stainless steel exhibits high conductivity and is inexpensive, but it corrodes easily in MFC systems (Dumas et al. 2007). Furthermore, all of these electrode materials are not transparent, which limits optical methods that can be used to study these systems to better improve efficiencies.

A possible alternative electrode material is indium tin oxide (ITO), which is used extensively in the electronics industry as a relatively inexpensive, transparent, and conductive material. The relative ease at which ITO can be deposited and etched on glass surfaces can provide a vast assortment of surface area modifications that can rival carbon cloth electrodes. Additionally, recent studies have demonstrated the adhesion of electrochemically active bacteria to ITO-coated surfaces as well as demonstrated efficient electron transfer to produce an electrical current (Schmidt et al. 2017; Viridis et al. 2016). The reasons that ITO is used so frequently in industrial applications are the same reasons it's an attractive material for MFCs—low resistance and transparency. These are two key characteristics that can also be an important means to study electron transfer mechanisms in complex electrode biofilms. Studies have shown that diverse microbial communities are the key to increasing MFC efficiencies (Logan 2009); however, limitations exist on how these microbial communities can be studied on electrode surfaces. Using a transparent conducting surface such as ITO presents the unique opportunity to examine intact biofilm assemblages by using a variety of optical techniques such as fluorescence in situ hybridization (FISH).

FISH is a molecular method that uses fluorescent probes to target specific deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences within an organism. The technique is based on the hybridization of complementary sequences between DNA or RNA targets and DNA with a fluorescently conjugated probe. Once in the cell, the fluorophore nucleotide hybridizes its complementary sequence via hydrogen bonds and forms a stable, double stranded, nucleotide tagged with probe. The fluorophore can then be easily detected with various optical methods such as fluorescent microscopy.

Due to the sensitivity and specificity of FISH, the technique has been used successfully to map genes and determine function in many different prokaryotic and eukaryotic organisms (Bottari et al. 2006; Volpi and Bridger 2008; Hu et al. 2014). Additionally, FISH has been used to identify bacteria in a variety of complex samples; however, the bacteria being probed are generally separated from other exogenous materials present, to allow effective hybridization (Bottari et al. 2006). Using FISH within biofilms has proved difficult due to the pronounced extracellular polymeric substance (EPS) surrounding the bacteria. Although EPS is composed primarily of water, substantial amounts of various polysaccharides, proteins, and even extracellular DNA are also present (Flemming et al. 2007). These exogenous molecules can interfere with hybridizations; thus, EPS is typically removed prior to analysis to increase selectivity and sensitivity. This need for EPS removal has limited the use of FISH to characterize intact biofilms because spatial distribution of the microorganisms is lost when the EPS is removed.

In order to better understand multi-species colonization of MFC, methods need to be developed to detect specific microorganisms within intact biofilms on the electrode surfaces. The work reported here describes a method to fix and hybridize bacterial cells within biofilms grown on ITO-coated silica, which can serve as the basis for studying complex microbial assemblages on MFC electrodes.

1.2 Objectives

The objectives of this study were to demonstrate a proof of concept for the in situ fixation and hybridization of bacteria within biofilms grown on an ITO surface. This research provides the foundation for a relatively simple methodology to spatially resolve bacterial species within intact biofilms on a conductive surface.

1.3 Approach

To achieve the overall objective of this research, three major tasks were performed. The first task was to evaluate the chemical fixation process and oligonucleotide probe specificity with planktonic cultures of *Pseudomonas putida* F1 and *Shewanella oneidensis* MR-1. Varied hybridization stringencies, based on formamide concentrations, were evaluated on gelatin-coated glass slides and observed by using inverted microscopy. After the initial probe evaluation, the experiments were repeated on ITO-coated slides (no embedding material) to determine if cells could adhere to the surface and if any imaging interference would be encountered from the ITO. The second task involved rapidly establishing robust biofilms on the ITO surface. For this purpose a 4-chamber drip flow bioreactor (DFR) was evaluated for its ability to form a loosely bound biofilm in less than 48 hr. The third and final task was to determine if bacteria embedded within EPS deposited on the ITO using a DFR could be both fixed and hybridized without disturbing the dimensionality of the biofilm. Both pure culture and co-culture biofilms were grown and examined by using epifluorescence microscopy to differentiate and spatially resolve the two species within the EPS.

A complete discussion of materials and methods is contained in Chapter 2.

2 Materials and Methods

2.1 *P. putida* F1 and *S. oneidensis* MR-1 culture preparation

Cultures of *Pseudomonas putida* strain F1¹ and *Shewanella oneidensis* strain MR-1² were used for all experiments in this report. Stock cultures of *P. putida* F1 were propagated in nutrient broth No. 3,³ and those of *S. oneidensis* MR-1 were propagated in Bacto™ tryptic soy broth⁴ (TSB). Both were grown overnight at 30°C and 200 rpm under aerobic conditions in an incubator/shaker to approximately mid-log phase—that being an OD₆₀₀ of 0.6 or roughly 4.8 x 10⁷ cells/mL for *P. putida* F1 and an OD₆₀₀ of 0.7 or roughly 5.6 x 10⁷ cells/mL for *S. oneidensis* MR-1. Stock cultures were then archived in 10% glycerol (v/v) at -80°C until future use. Sterile controls were made by autoclaving the stock cultures for 15 min at 120°C.

2.2 Chemicals and reagents

All chemicals and reagents used were of the highest purity available and were purchased from major distributors, unless otherwise specified.

2.3 16S rRNA probes

Sequences that targeted unique regions of the 16S ribosomal RNA (rRNA) of species belonging to the genus *Pseudomonas* and *Shewanella* were determined by using probeBase⁵ (Greuter et al. 2016). The sequences chosen were 5'-GATCCGGACTACGATCGGTTT-3' (PSE1284) and 5'-AGCTAATCCCACCTAGGTWCATC-3' (SHEW227) for *P. putida* F1 and *S. oneidensis* MR-1, respectively. The *P. putida* F1 oligo was tagged with Alex Fluor® 488 at the 5' end, and the *S. oneidensis* MR-1 oligo was tagged with Alex Fluor 594 at the 3' end.⁶ Both custom oligonucleotides were synthesized by Integrated DNA Technologies,⁷ using standard commercial techniques. Table 1 shows the characteristics of each oligo, including accession

¹ ATCC® 700007™ obtained from the American Type Culture Collection (ATCC) of Manassas, VA.

² ATCC® 700550™ obtained from the ATCC.

³ Sigma-Aldrich, St. Louis, MO

⁴ Becton, Dickinson, and Company, Sparks, MD.

⁵ <http://www.probebase.net>

⁶ Alex Fluor product obtained from Molecular Probes, Eugene, OR.

⁷ Located in Coralville, IA.

numbers. These probes were used in all experiments performed in this study.

Table 1. Properties of fluorescent labeled oligonucleotides used to target unique regions of the 16S rRNA of *P. putida* F1 and *S. oneidensis* MR-1.

Specificity (genus)	probeBase name	Sequence (5' to 3')	Accession number	Target	Position (bp)	Formamide (%)	Tm (°C)
<i>Pseudomonas</i>	PSE1284	GATCCGGACTACGATCGGTTT	pB-02540	16S rRNA	1284-1304	30	54
<i>Shewanella</i>	SHEW227	AGCTAATCCACCTAGGTWCATC	pB-01191	16S rRNA	227-249	40	55

2.4 Probing 16S rRNA of planktonic cultures on gelatin-coated glass

Fixation of the bacteria was based on the method described by Amann et al. (1990), with modification. *P. putida* F1 was grown to mid-log on nutrient broth, and *S. oneidensis* was grown to mid-log on TSB at 30°C and shaking at 200 rpm. Each culture was harvested by centrifugation (10 min at 4000 x g) and the supernatant decanted. The cell pellets were resuspended with sterile (0.2 µm filtered) phosphate-buffered saline solution (pH 7.4) consisting of 145 mM NaCl, 1.4 mM NaH₂PO₄ and 8 mM Na₂HPO₄ (pH 7.4). The cells were fixed with a 4% paraformaldehyde (PFA) solution prepared by adding 2 g PFA to 50 mL phosphate buffered saline solution consisting of 130 mM NaCl and 10 mM Na₂HPO₄. The mixture was heated to 60°C for 1 hr, then 1 M NaOH was added dropwise until clear. The pH was adjusted to 7.0 with 2 M HCl, and the solution was filter sterilized (0.2 µm) and placed on ice. The PFA solution was poured into a sterile 160 ml serum bottle, capped with sterile butyl rubber stopper, and the head space was exchanged aseptically 3 times with ultra-high purity (UHP) nitrogen to a final headspace pressure of 10 psi. The resulting solution was stable for up to 6 months, when stored in the dark at room temperature.

The PFA solution was added to the cell suspension to a final concentration of 1% and incubated at 4°C for 18 hr. Cells were harvested by centrifugation (10 min at 4000 x g), the supernatant was decanted, and the cell pellets were resuspended in phosphate-buffered saline solution (145 mM NaCl, 1.4 mM NaH₂PO₄, pH 7.4) two times. After the second buffer wash, the pellets were resuspended in absolute ethanol and stored at -20°C until hybridized.

Microscope slides (25 x 75 x 1 mm)⁸ were treated by placing them in a 1 M HCl solution and heating to 60°C for 8 hr. The slides were removed from the acid bath, cooled to room temperature, rinsed with diH₂O and then washed with 95% ethanol, and left to air dry. A solution of 0.01% CrK(SO₄)₂ and 0.1% gelatin was heated to 65°C for 10 min, after which the acid-washed slides were dipped into the solution 5 times for 5 sec each time, and then were left to air dry while resting vertically. After drying, the gelatin-coated slides were stored in a dust-free environment at room temperature until use.

There were 10 µL of fixed cells spotted onto the gelatin-coated slides and allowed to dry at room temperature. The cells were then dehydrated with 50%, 80%, and 100% ethanol for 3 min each and left to air dry. After drying, the cultures were hybridized with the fluorescently labeled probes, based on the method described by Manz et al. (1992). Custom oligonucleotides targeting each bacterial species were mixed to a final concentration of 50 ng DNA mL⁻¹ in molecular graded water. This stock solution was used in a ratio of 1 volume to 9 volume of hybridization buffer. The hybridization buffer consisted of the following final concentrations: 900 mM NaCl, 20 mM Tris-HCl, 30%-40% formamide, 0.01% sodium dodecyl sulfate (SDS), and molecular grade water up to 2 mL. Note that SDS is added last to prevent precipitation. The 10 µL of the hybridization buffer containing probe(s) was spotted onto the fixed cultures and evenly distributed by gently spreading the mixture to entirely cover the cells. Hybridizations were performed in standard 50 mL polyethylene tubes with screw caps. Blotting paper was cut to 25 x 75 mm, placed into the tube under the slide, and saturated with the remaining hybridization buffer. Hybridization vessels were incubated horizontally at 46°C for 90 min. Wash buffer was prepared in 50 mL volumes for each hybridized slide by adding the following final concentrations: 56–112 mM NaCl, 20 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.01% SDS. This solution was heated to 48°C in a water bath before use. Hybridizations were gently rinsed dropwise with 1 mL of the wash buffer, then completely submerged in the buffer and incubated at 48°C for 25 min at a 45° angle. After incubation, the hybridized samples were gently washed with deionized water and allowed to air dry prior to imaging. Separate hybridization vessels were used for each individual condition tested. The probes for each species were eval-

⁸ Fisherfinest Premium Microscope Slides, Thermo Fisher Scientific, Asheville, NC

uated at the optimal formamide concentration (refer to Table 1), and imaged by using inverted microscopy. After the initial evaluation each species was hybridized individually and in co-culture with 35% formamide and again imaged by using inverted microscopy.

2.5 Inverted fluorescence microscopy of 16S rRNA probed planktonic cultures on gelatin-coated glass

Initial evaluations of the 16S rRNA molecular probes were performed by using an inverted microscope⁹ and image analysis software.¹⁰ The microscope was equipped with a 100x oil immersion objective and a charge coupled device (CCD) camera.¹¹ Cells fixed and hybridized on the gelatin-coated slides were inverted, spotted with a drop of immersion oil,¹² and imaged. Differential interference contrast (DIC) microscopy was used to image the bacteria without fluorescence. Excitation and emission were accomplished by using filter cubes established to detect Alexa Fluor® 488 and Alexa Fluor® 594, which had excitation and emission profiles of 490/525 and 590/617 nm, respectively. A filter that was sensitive to fluorescein isothiocyanate (FITC)¹³ was used for Alexa Fluor 488, and another filter cube¹⁴ (Texas red) was used for Alexa Fluor 594. Excitation for the FITC cube set was 470–495 nm and the emission was 510–550 nm. Excitation for the Texas red cube set was 545–580 nm and the emission was 610 nm. Exposure time of the samples to light was kept to a minimum to prevent photobleaching of the fluorescent dyes. Additionally, FITC and Texas red intensities (%), brightness, and contrast were kept consistent throughout all inverted microscope imaging sessions as a means to normalize fluorescence output relative to each sample examined. Image overlays were constructed by using image processing software.¹⁵

⁹ Olympus IX 81, Olympus America, Center Valley PA.

¹⁰ MetaMorph Microscopy Automation and Image Analysis Software, Molecular Devices, Sunnyvale, CA.

¹¹ Image EM, Hamamatsu, Bridgewater, NY.

¹² Olympus Type-F immersion oil

¹³ Olympus U-MNIBA3

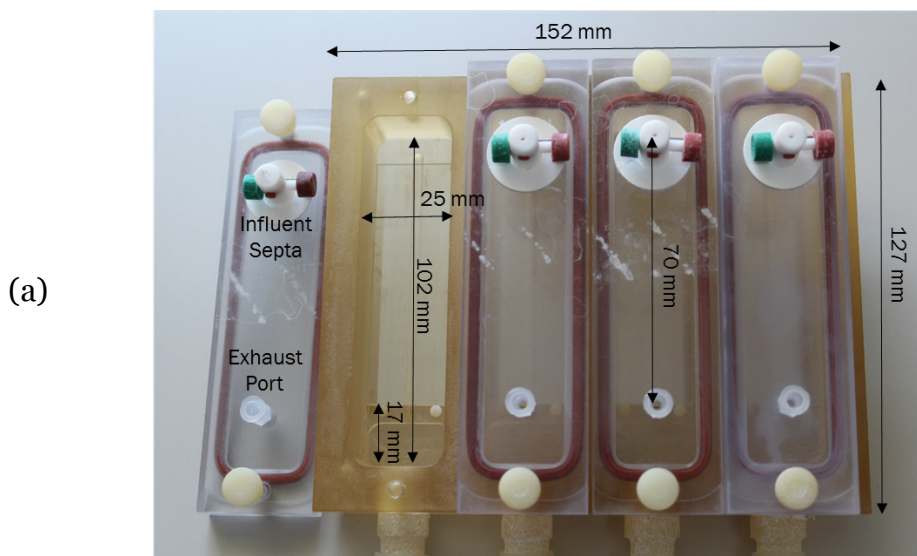
¹⁴ Olympus U-MWIY2

¹⁵ ImageJ software, developed by W. Rasband, National Institutes of Health (<https://imagej.nih.gov/ij/>).

2.6 Biofilm growth on ITO-coated glass

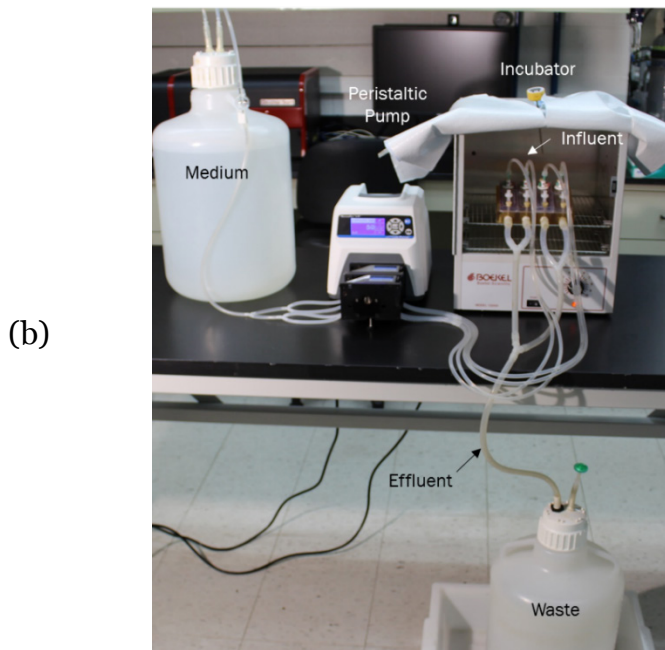
Four-chamber DFRs (DFR 110-4) were purchased.¹⁶ The reactors' bases were constructed from polysulfone, and the chambers were machined to accommodate standard 25 x 75 mm substrates (Figure 1). Pure culture biofilms of both species, as well as a co-culture, were established on translucent, 25 x 75 mm, non-polarized, ITO-coated glass microscopy slides.¹⁷ Slides were cleaned with 100% ethanol to remove any contaminating materials from the surface, allowed to air dry, and then placed in the DFRs. Slides, DFRs, and all associated tubing and materials were sterilized by autoclaving at 120°C for 20 min.

Figure 1. DFR system used to rapidly form biofilms on ITO-coated glass substrates; (a) bioreactor with ITO-coated slides, and (b) complete drip flow bioreactor system.



¹⁶ Biosurface Technologies, Bozeman, MT.

¹⁷ SPI Supplies, West Chester, PA.



To establish biofilms on the ITO-coated glass surfaces, *P. putida F1* and *S. oneidensis* MR-1 were allowed to grow statically at 30°C for 6 hr in a logarithmic growth phase at an angle of zero. Five mL of *P. putida F1* and *S. oneidensis* MR-1 glycerol stocks were used to inoculate 25 mL of 3 g/L TSB per chamber. In order to keep cell densities equal, reactor chambers containing co-cultures were inoculated with 2.5 mL of each strain. After 6 hr of static incubation, the drip angle of the bioreactors was increased to 5°, and a continuous flow of nutrients (TSB, 270 mg/L) was supplied at 50 mL hr⁻¹ via a peristaltic pump through a glass flow break. The DFRs were operated at 30°C for 48 hr under low-fluid shear conditions to promote rapid growth near the air-liquid interface.

After 48 hr incubation, the slides were removed from the DFR and the cells were fixed and hybridized similar to the protocol described in section 2.4, with the exception that the cells were not harvested but rather, they were fixed directly within the intact biofilm on the slide surface. In addition, samples were not dried at any stage of the fixation or hybridization steps. Briefly, immediately after removal from the DFR, the biofilms were partially dehydrated by gently overlaying the entire surface with 50%, 80%, and 100% ethanol. Prior to fixing, the absolute ethanol was allowed to volatilize without letting the biofilms completely dry. Then the slides were entirely covered with fixative buffer (200-400 µL) and allowed to incubate for 18 hr at 4°C. In order to completely cover the slides with probe

and hybridization buffer, volumes were increased to 200 μL and 500 μL , respectively. After fixing and hybridizing, the biofilms were gently rinsed with dH_2O and immediately imaged by using epifluorescence microscopy.

2.7 Epifluorescence microscopy of hybridized biofilms on ITO-coated glass

Bacteria fixed and hybridized within intact biofilms were imaged with a Nikon Eclipse Ci epifluorescence microscope equipped with a DS-Fi2 digital camera and DS-L3 camera control unit.¹⁸ Filter cubes used were Nikon ET/m CH/TR for Alex Fluor 594, and the C-FL YFP was used for Alex Fluor 488. VectaShield[®] mounting medium was used per the manufacturer's specification in an effort to prevent photobleaching of the fluorescent dyes on glass slides.¹⁹ Image overlays were created using Adobe[®] Photoshop[®].²⁰

¹⁸ Nikon Instruments Inc., Melville, NY.

¹⁹ Vector Laboratories, Burlingame, CA.

²⁰ Adobe Systems Inc., San Jose, CA.

3 Results/Discussion

3.1 Selection of model microorganisms, *P. putida* F1 and *S. oneidensis* MR-1

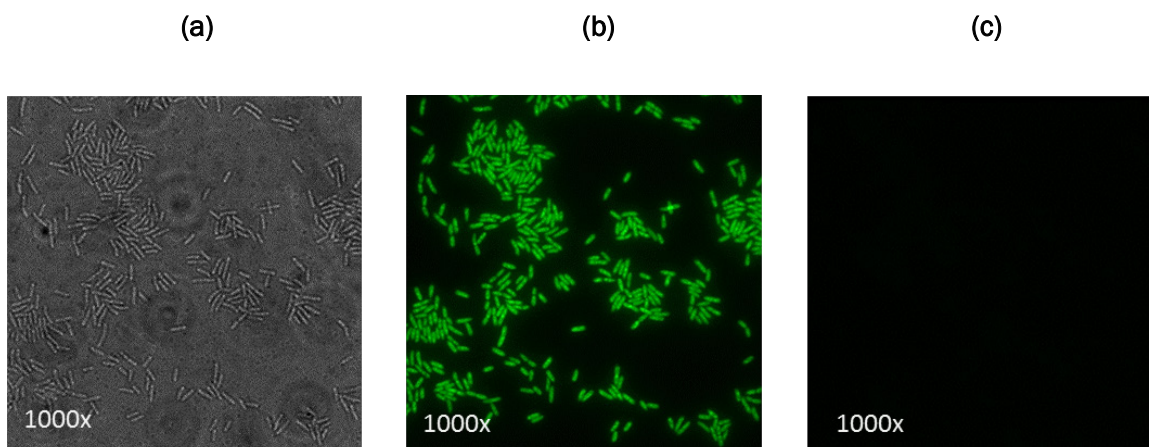
P. putida F1 and *S. oneidensis* MR-1 were chosen as model organisms for the described methods development due to their ability to rapidly colonize and produce robust biofilms. Each genera consist of species that are known to form biofilms on smooth surfaces such as glass, which make them excellent candidates for attachment and growth on ITO (Waters et al. 2008; Wang et al. 2011; Mbaye et al. 2013). Both organisms are commercially available, lack pathogenicity, and molecular probes that target unique regions of the 16S rRNA are well established (Greuter et al. 2016). In addition, bacteria belonging to the genera *Pseudomonas* and *Shewanella* are known exoelectrogens capable of transferring electrons through their cell membranes to external environments (Logan 2009; El-Naggar et al. 2010). This ability to shuttle electrons to an electrode has made members of these genera the focus of many MFC studies (Logan and Regan 2006; Watson and Logan 2010; Friman et al. 2012; Majumder et al. 2014; Kouzuma et al. 2015). Furthermore, ITO-coated glass slides were chosen as a growth surface to represent a transparent, conductive material with prospective applications in MFC construction.

3.2 Planktonic cultures hybridized on gelatin-coated glass slides

To evaluate the 16S rRNA probes selected for this study, planktonic cultures of *P. putida* F1 and *S. oneidensis* MR-1 were grown to mid-log, spotted onto gelatin-coated glass slides, fixed, hybridized with the recommended formamide concentrations, and imaged using an inverted microscope. Gelatin-coated glass was used in the initial probe evaluations to facilitate the attachment and retention of cells to the slide surface while being hybridized and imaged. The optimized formamide concentration for PSE1284 hybridization is reported to be 30%, which was used for the initial evaluations (Greuter et al. 2016). The probe targets the conserved region of the 16S rRNA at positions 1284-1304 bp of Pseudomonads (Gunasekera et al. 2003). Figure 2 shows hybridized and non-hybridized (no probe) *P. putida* F1 using an oil-immersion objective at a 1000x total magnification. Embedded *P. putida* F1 were shown to readily hybridize at a 30% formamide stringency when grown and fixed planktonically. All cells observed demonstrated fluorescence when viewed with FITC settings

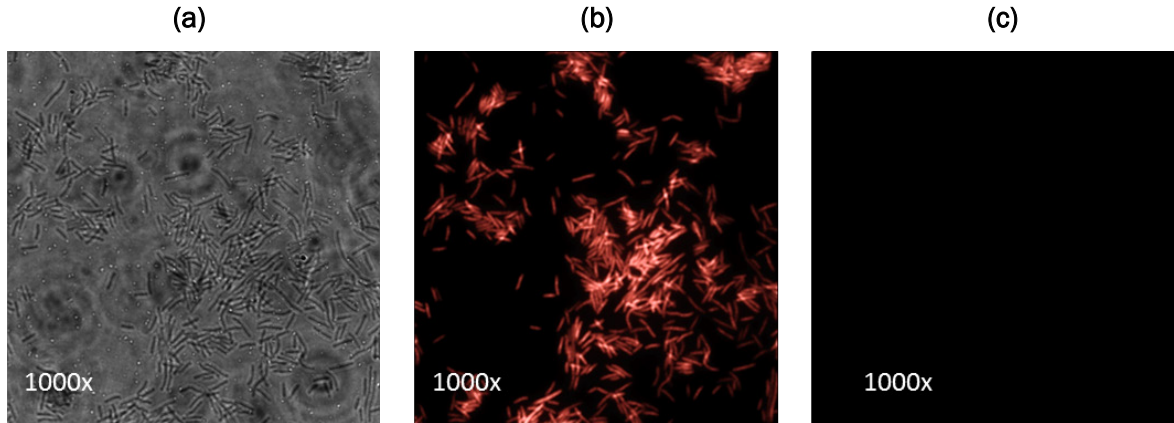
and no cells showed any fluorescence in the non-hybridized controls or when the excitation and emission profile was switched from 490/525 to 590/617 nm (Texas red; data not shown). Additionally, little to no background fluorescence was observed due to residual PSE1284 probe within the gelatin matrix.

Figure 2. Planktonic *P. putida* F1 hybridized with PSE1284 and 30% formamide on gelatin-coated glass slides; (a) DIC image of hybridized culture, (b) hybridized cells viewed with FITC fluorescence, and (c) non-hybridized cells viewed with FITC fluorescence.



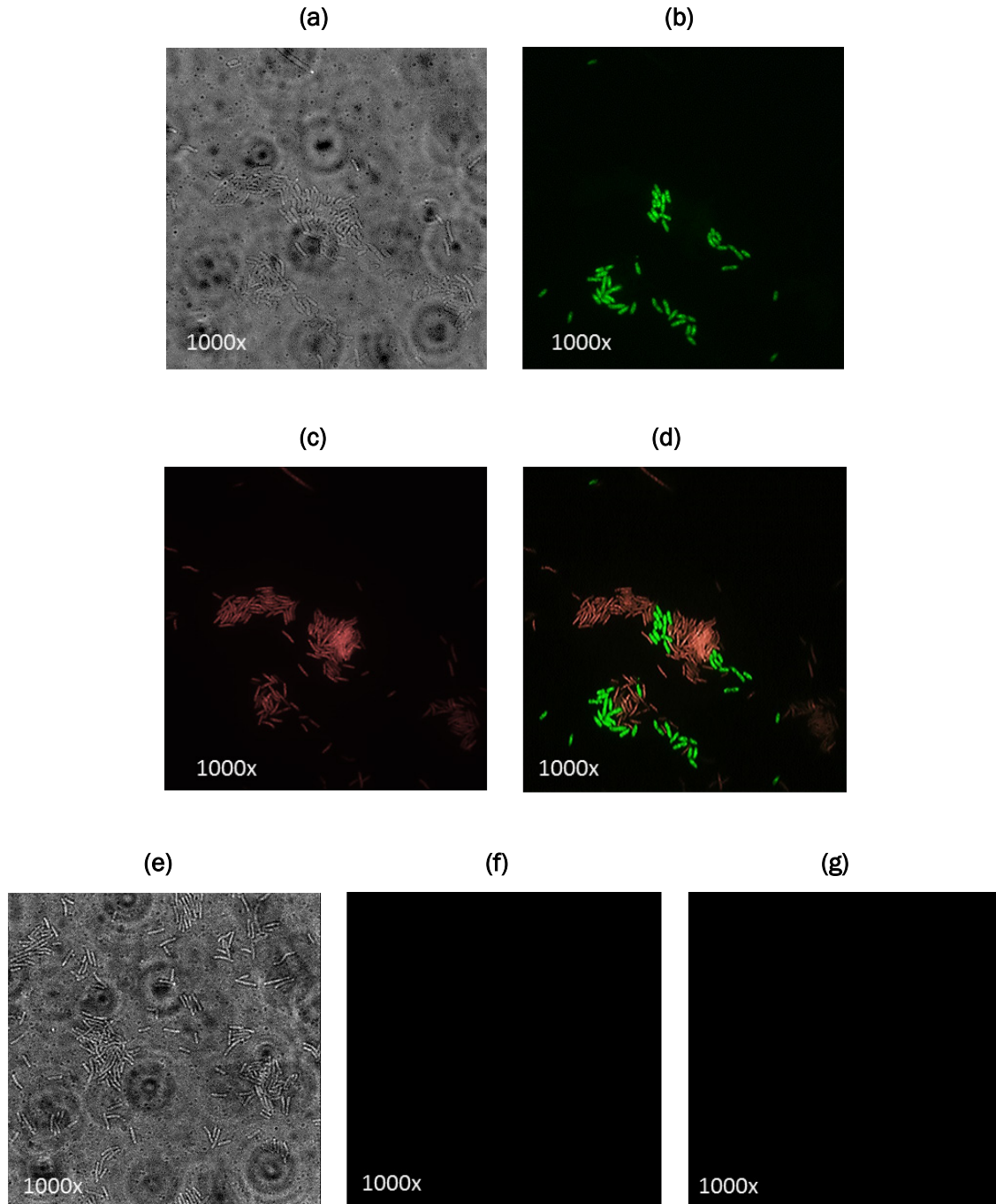
Hybridized and non-hybridized planktonically grown *S. oneidensis* MR-1 on gelatin-coated slides are shown in Figure 3. The optimal formamide concentration for SHEW227 is listed to be 40% by probeBase and this concentration was used for the preliminary hybridizations. The probe targets positions 227-249 of the 16S rRNA of species belonging to the genus *Shewanella* (Greuter et al. 2016; Hugget et al. 2008). *S. oneidensis* MR-1 cells were shown to hybridize at the 40% formamide concentration. All cells viewed exhibited fluorescence when viewed with the Texas red setting (590/617 nm) and no cells showed fluorescence in the non-hybridized controls (no probe). Cells did not fluoresce when the excitation and emission was changed to the FITC setting (490/525 nm), and no apparent background fluorescence was observed due to unbound SHEW227 probe.

Figure 3. Planktonic *S. oneidensis* MR-1 hybridized with SHEW227 at 40% formamide on gelatin-coated glass slides; (a) DIC image of hybridized culture, (b) hybridized cells viewed with Texas red fluorescence, and (c) non-hybridized cells viewed with Texas red fluorescence.



Next, hybridization of PSE1284 and SHEW227 were evaluated at a mean formamide concentration of 35% and imaged as described above. Based on visual observations, hybridization performed with 35% formamide with both probes showed no loss in stringency or fluorescent emission with each bacterium (data not shown). Subsequently, a mixture of *P. putida* F1 and *S. oneidensis* MR-1 (50:50) was examined to determine probe specificity towards targeted 16S rRNA of each bacterium, using 35% formamide. Probing the co-culture with PSE1284 and SHEW227 easily differentiated *P. putida* F1 and *S. oneidensis* MR-1 from each other (Figure 4). When excitation and emission wavelengths were switched from FITC and Texas red (490/525 to 590/617 nm), both species were easily identifiable at various areas of the slide under their respective fluorescent settings. As with hybridization performed with optimal formamide concentrations, no fluorescence was observed in the non-hybridized controls (no probe) and little to no background fluorescence was observed.

Figure 4. Mixture of planktonic *P. putida* F1 and *S. oneidensis* MR-1 hybridized with PSE1284 and SHEW227 at 35% formamide on gelatin-coated glass slides; (a) DIC image of hybridized co-culture, (b) hybridized cells viewed with FITC fluorescence, (c) hybridized cells viewed with Texas red fluorescence, (d) overlay of images b and c, (e) DIC image of non-hybridized co-culture, and (f) non-hybridized cells viewed with FITC fluorescence, and (g) non-hybridized cells viewed with Texas red fluorescence.



Preliminary probing with planktonic cells on gelatin-coated glass clearly demonstrated the specificity of each probe towards their target rRNA sequence. Furthermore, it was demonstrated that each probe can be used with less than optimal formamide stringencies and still be specific to each of the target species.

Hybridization stringency is defined as the extent at which hydrogen bonding between mismatch nucleic acids can be formed. Under high-stringency conditions hybridization of complementary sequences must match exactly; however, under low-stringency conditions hybridization of mismatched bases is permitted. The degree of stringency can be controlled by salt concentration and hybridization temperature, as well as formamide concentration. Increased hybridization stringencies can be achieved by increasing incubation temperatures, lowering salt concentrations, and/or decreasing formamide concentrations. Decreasing formamide concentration of a hybridization reaction effectively increases the melt temperature of nucleic acids by disrupting hydrogen bonding. Although we chose to only alter formamide concentration in this study, in order to expand this technique to more diverse and or unknown microbial cultures, combinations of salt and formamide concentrations as well as incubation temperature will need to be carefully considered (Yilmaz et al. 20012). In addition, probe labeling also needs to be evaluated to ensure minimal overlap between excitation and emission wavelengths of selected fluorophores. Alexa Fluor 488 and 594 were chosen for their minimal overlap in wavelengths and compatibility to FITC and Texas red filter sets. Sensitivity and selectivity of the designed probe is contingent on these factors and are the focus of future studies.

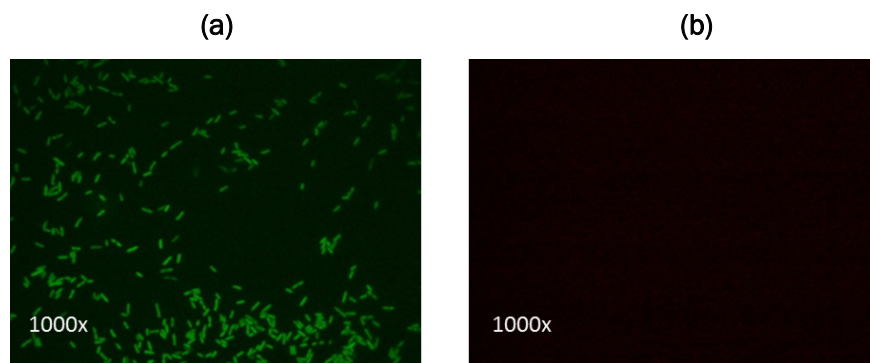
Due to distorted resolution of the inverted microscope, presumably caused by ocular contamination (see DIC images above), all subsequent imaging of experiments performed with ITO-coated glass was performed on a Nikon Eclipse Ci epifluorescence microscope equipped with a DS-Fi2 digital camera and ET/m CH/TR and YFP filter cubes.

3.3 Planktonic cultures hybridized on ITO-coated slides

After the successful differentiation of planktonically grown *P. putida* F1 and *S. oneidensis* MR-1 on gelatin-coated glass, each experiment was repeated on ITO-coated glass using 35% formamide. Planktonic *P. putida* F1 was hybridized directly on the ITO without the addition of embedding material (gelatin) on the surface of the slides. Figure 5 shows *P. putida* F1

probed with PSE1284 and imaged by epifluorescence with a FITC filter cube. All hybridized cells exhibited fluorescence and none of the cells showed any fluorescence when the filter was changed to Texas red.

Figure 5. Planktonic *P. putida* F1 hybridized with 35% formamide on ITO-coated glass slides; (a) hybridized cells with FITC epifluorescence, and (b) hybridized cells with Texas red epifluorescence.



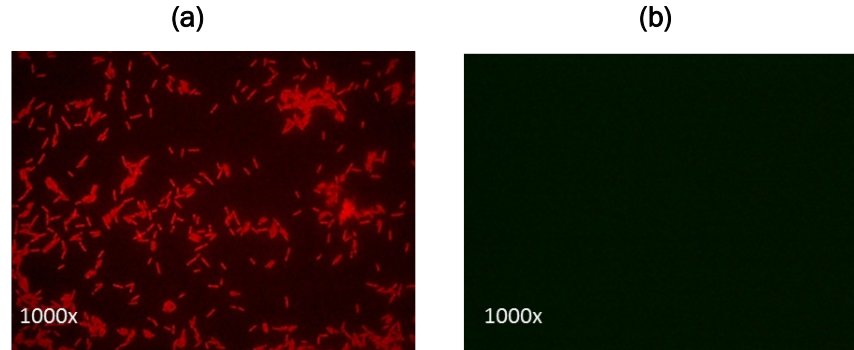
An attempt to prevent photobleaching (to preserve extended viewing and storage) was made by overlaying the cells with an antifade mounting media²¹ per the manufacturer's specifications. However, background fluorescence became problematic, particularly when viewing cells under the Texas red setting. Others have suggested that VectaShield may be incompatible with Texas red, but FITC is generally regarded as compatible (Max Plank Institute for Medical Research 2017). To determine if the ITO surface was causing the increased background fluorescence, planktonically grown bacteria were hybridized on glass slides and mounted with VectaShield. Cells on uncoated glass did not exhibit the same background fluorescence, indicating a compatibility issue between the mounting medium and the ITO coatings. For this reason, the use of an antifade preservative was ceased and the length of the epifluorescent microscopy sessions were kept to a minimum to prevent photobleaching of the hybridized cells.

Planktonically grown *S. oneidensis* MR-1 were then fixed directly on the ITO surface (i.e., no embedding agent) and hybridized with 35% formamide. In the presence of SHEW227 probe, planktonic *S. oneidensis* MR-1

²¹ VectaShield® Antifade Mounting Media, Vector Laboratories, Burlingame, CA.

readily fluoresced when examined under the Texas red setting, and no fluorescence was observed when viewed with FITC epifluorescence (Figure 6).

Figure 6. Planktonic *S. oneidensis* MR-1 hybridized with 35% formamide on ITO-coated glass slides; (a) hybridized cells with Texas red epifluorescence, and (b) hybridized cells with FITC epifluorescence.



To determine the selectivity of each probe towards its intended target, each species was planktonically grown, fixed, and mixed in a 1:1 ratio. The cell mixture was hybridized simultaneously with PSE1284 and SHEW227 at 35% formamide. Figure 7 shows epifluorescence of the *P. putida* F1 and *S. oneidensis* MR-1 mixed culture, after probe hybridization. Each probe clearly demonstrated selectivity for its respective bacterial target on the ITO surface. Roughly half the cells imaged were selective for PSE1284 under the FITC setting, and the other half were selective for SHEW227 with Texas red. When overlaid, the species differentiation and distribution was clearly observed on the ITO-coated glass. No fluorescence was observed in the planktonically grown mixed culture without PSE1284 and SHEW227 under the FITC or Texas red settings on the ITO surface (Figure 8).

Figure 7. Planktonic *P. putida* F1 and *S. oneidensis* MR-1 hybridized with 35% formamide on ITO-coated glass slides; (a) image of hybridized cells without fluorescence, (b) hybridized cells with FITC epifluorescence, (c) hybridized cells with Texas red epifluorescence, and (d) overlay of images b and c.

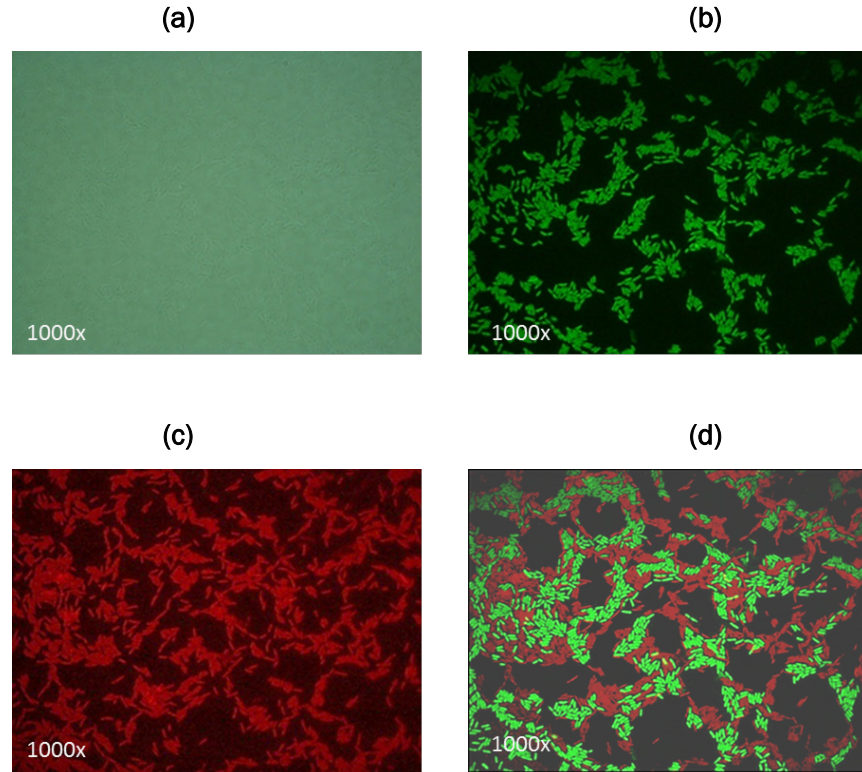
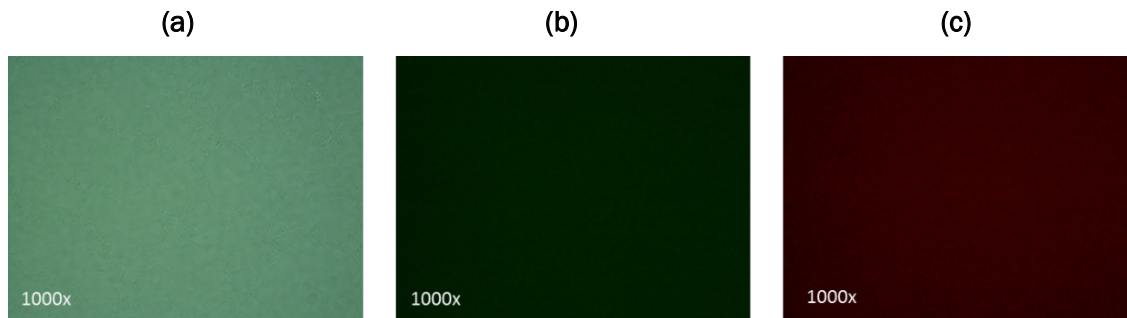


Figure 8. Planktonic *P. putida* F1 and *S. oneidensis* MR-1 no 16S rRNA probe added to hybridizations on ITO-coated glass slides; (a) image of non-hybridized co-culture without fluorescence, (b) non-hybridized cells with FITC epifluorescence, and (c) non-hybridized cells with Texas red epifluorescence.



These experiments demonstrated that the ITO glass surface did not appear to have any undesirable effect on binding the cells to the slides, nor was

there any noticeable negative effect on epifluorescence when viewed with FITC and Texas red settings. In contrast, ITO may have actually improved FISH sensitivity. A technical report published by Roper Scientific (1999) suggests that ITO enhances quantum efficiencies (QE), which make it very useful for the detection of various fluorescent proteins and fluorophores by improving sensitivity and dynamic range while minimizing light dosage. This enhanced QE may have been the cause of the autofluorescence and elevated background observed when using VectaShield® mounting medium. However, the use of antifade preservatives may not be necessary, due to the potential for ITO to increase sensitivities to fluorescent proteins and fluorophores at decreased exposure times and intensities. Nevertheless, fluorescent probes should be chosen carefully to ensure compatibility with the ITO surface.

3.4 Bacteria fixed and hybridized within intact ITO biofilms

After successfully demonstrating probe specificity, and that ITO had no apparent negative effects on cell adhesion and imaging, biofilms production was evaluated on the ITO-coated slides. Then, an attempt to fix and hybridize cells within the EPS of the intact biofilms was made to provide spatial distribution of the species within the biofilm.

Typically, biofilm formation on surfaces can take several days to weeks contingent on hydrodynamics and available nutrients (Stoodley et al. 1999; Allen et al. 2002; Oliveira et al. 2007). To accelerate the production of robust biofilms on ITO-coated slides, a DFR was chosen for growth of *P. putida* F1 and *S. oneidensis* MR-1 in pure cultures and in co-cultures. The DFR used was a plug flow reactor with laminar liquid flow that resulted in biofilm formation close to the liquid air interface (ASTM International 2013). Under continuous flow operation, the angle of the reactor was set at 5° to achieve very low fluid shear conditions, which had a residence time of less than 2 min. Influent was delivered through polytetrafluoroethylene septa ports at the top of the reactor, and air/gas was vented at the bottom through 0.2 µm syringe filters. Effluent exited the base at the vented end of the reactor through a 1.3 cm exit port to waste.

The continual flow of nutrients and elimination of waste was shown to promote rapid biofilm formation on the ITO slides. The combination of low shear conditions, continuous flow of nutrients, and waste removal resulted in the production of repeatable biofilms in less than 24 hr. Biofilm formation on the slides was clearly visible by the naked eye after as little as

6 hr of static incubation (data not shown). After 24 hr of drip-flow incubation, very pronounced EPS were observed in the chambers inoculated with *P. putida* F1 and the co-culture (Figure 9). Less growth was observed in the chamber inoculated with only *S. oneidensis* MR-1; nonetheless, it was still visible with the naked eye and no growth was observed in the sterile control. There was clearly a synergistic effect when the bacteria were grown together as a co-culture. Pseudomonads are known as primary biofilm colonizers and likely helped to facilitate the proliferation of *S. oneidensis* MR-1 (Dang and Lovell, 2000; Bernborn et al. 2013).

After 48 hr of incubation, the biofilms appeared slightly more substantial when visually observed, but the variance was marginal (Figure 10). This indicated that a mature stable biofilm was established within 24 hr by using a DFR. Although adequate biofilm formation was produced in as little as 24 hr of drip flow operation, the biofilms were allowed to further mature for an additional day before performing in situ fixations to minimize sloughing from the slide surfaces. An expanded view of the co-culture is shown in Figure 11, which clearly shows a pronounced biofilm after 48 hr of dripping TSB over the ITO surface.

Figure 9. Bacterial biofilm formation on ITO-coated glass substrates after 24 hr incubation; (a) sterile control, (b) *P. putida* F1, (c) *S. oneidensis* MR-1, and (d) co-culture with *P. putida* F1 and *S. oneidensis* MR-1.

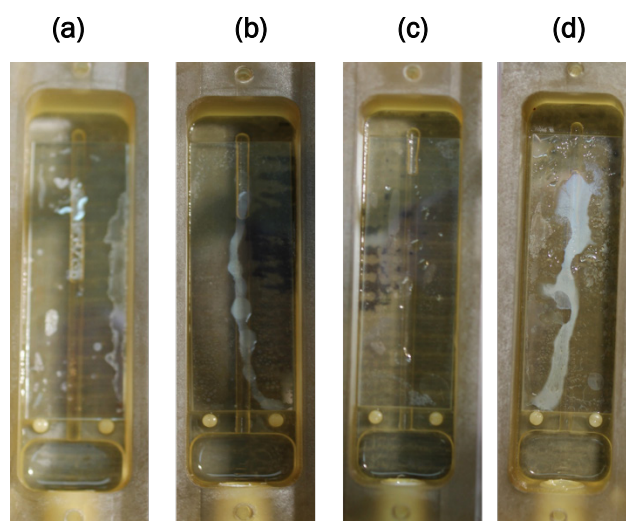


Figure 10. Bacterial biofilm formation on ITO-coated glass substrates after 48 hr incubation; (a) sterile control, (b) *P. putida* F1, (c) *S. oneidensis* MR-1, and (d) co-culture with *P. putida* F1 and *S. oneidensis* MR-1.

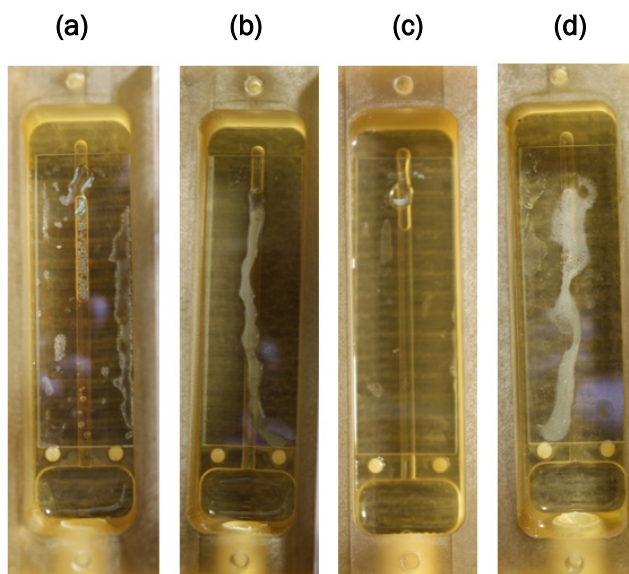
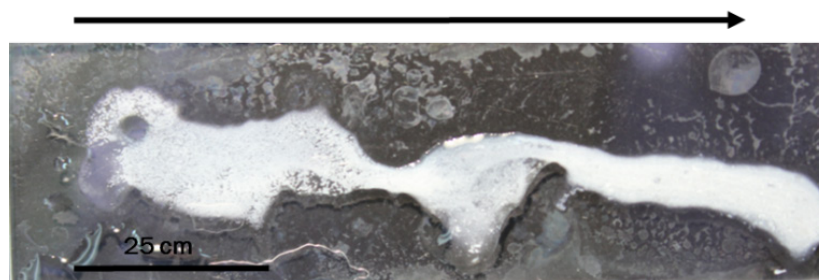


Figure 11. Expanded view of co-culture with *P. putida* F1 and *S. oneidensis* MR-1 after 48 hr incubation. Arrow depicts the flow of nutrients toward waste.



Low shear conditions generally produce biofilms that are very loosely attached to the surface, which is similar to what would be expected on an electrode within a MFC (Goeres et al. 2009). This biofilm attachment was critical, because the fixing and hybridization involve various steps that require complete coverage and washing of the biofilms. It was imperative that the biofilms remain intact for the technique to be used to localize bacteria within the undisturbed biofilms grown on the ITO surfaces. Throughout all experimentation, no apparent migration or loss of biofilm was observed. However, it should be noted that great care was taken to ensure

the biofilms remained intact on the smooth surfaces. Additionally, the biofilms grown were not necessarily representative of an actual MFC biofilm but were chosen purely for methods development that can be extrapolated to other exoelectrogenic species of interest. For this study, the objective was to rapidly produce single and multispecies bacterial biofilms that can be used to evaluate fixation and hybridization parameters within EPS. Ultimately, the goal was to determine if *P. putida* F1 and *S. oneidensis* MR-1 could adhere and produce a biofilm on the surface of ITO-coated glass, and to determine if FISH could be performed without disturbing the structure of the biofilm to reveal spatial distributions between the different species of bacteria.

Slides were removed after 48 hr of continual drip flow, and the cells were fixed and hybridized similar to the planktonic cultures, with the exception that the cells were not harvested but rather, they were fixed and hybridized directly within the intact biofilm on the slide surface. At no point throughout the FISH process were samples allowed to dry. Figure 12 shows a pure culture biofilm of *P. putida* F1 after fixing and hybridizing directly within the EPS with PSE1284. The resolution of the cells was not as clear when compared to planktonic *P. putida* F1, but cells could clearly be observed with FITC epifluorescence. No fluorescence was detected when settings were changed to Texas red. Similar results were seen with pure culture biofilms of *S. oneidensis* MR-1 (Figure 13). Hybridized cells were visibly present when viewed with Texas red epifluorescence, and no fluorescence was detected with FITC epifluorescence. Both pure culture biofilms of *P. putida* F1 and *S. oneidensis* MR-1 demonstrated a decrease in resolution due to being encapsulated in the thick EPS; nevertheless, the results clearly demonstrate the ability to both fix and hybridize both species with fluorophore labeled 16S rRNA probes, directly within the intact biofilms on the ITO surface.

Figure 12. Biofilm *P. putida* F1 hybridized with 35% formamide on ITO-coated glass slides; (a) image of hybridized cells within ESP without fluorescence, (b) hybridized cells within EPS with FITC epifluorescence, and (c) hybridized cells within EPS with Texas red epifluorescence.

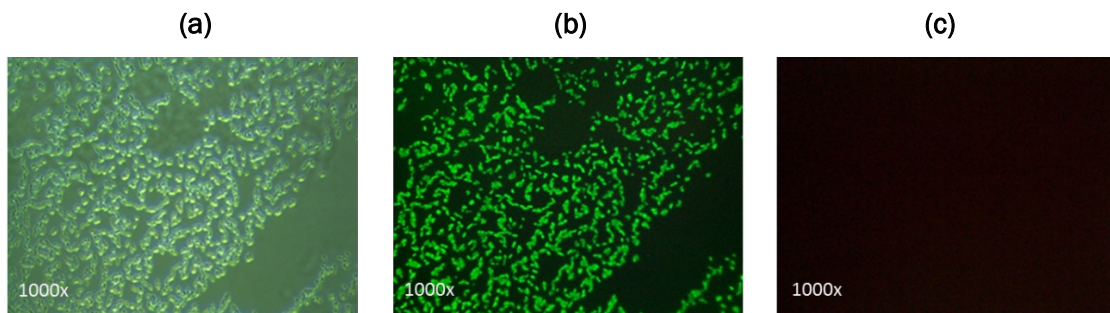
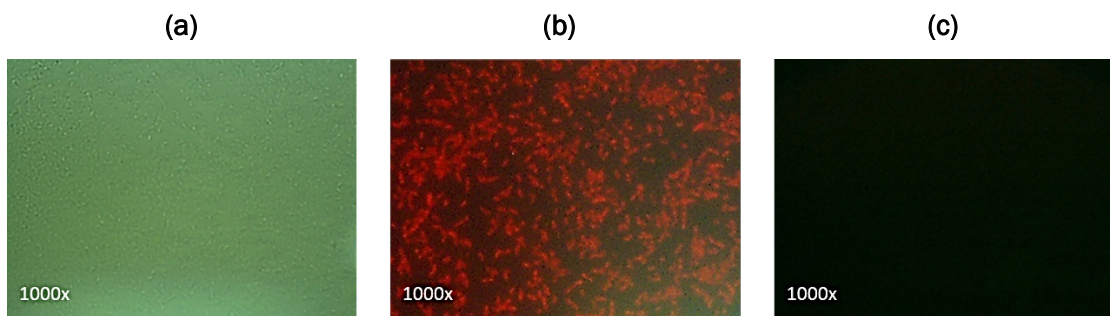


Figure 13. Biofilm embedded *S. oneidensis* MR-1 hybridized with 35% formamide on ITO-coated glass slides; (a) image of hybridized cells within EPS without fluorescence, (b) hybridized cells within EPS with Texas red epifluorescence, and (c) hybridized cells within EPS with FITC epifluorescence.



Co-culture biofilms grown with a mixture of *P. putida* F1 and *S. oneidensis* MR-1 are shown in Figure 14 and Figure 15. As with the planktonic mixed cultures, differentiation and spatial relationships between the species were observed. No fluorescence was observed with either the FITC or Texas red epifluorescence settings with non-hybridized co-culture samples (Figure 16). However, as with the pure culture biofilms, resolution was decreased due to the EPS. The thicker the biofilm, the more difficult imaging became.

Figure 14. Thick biofilm embedded *P. putida* F1 and *S. oneidensis* MR-1 hybridized with 35% formamide on ITO-coated glass slides; (a) image of 16S rRNA hybridized biofilm without fluorescence, (b) hybridized cells within EPS with FITC epifluorescence, (c) hybridized cells within EPS with Texas red epifluorescence, and (d) overlay of images b and c.

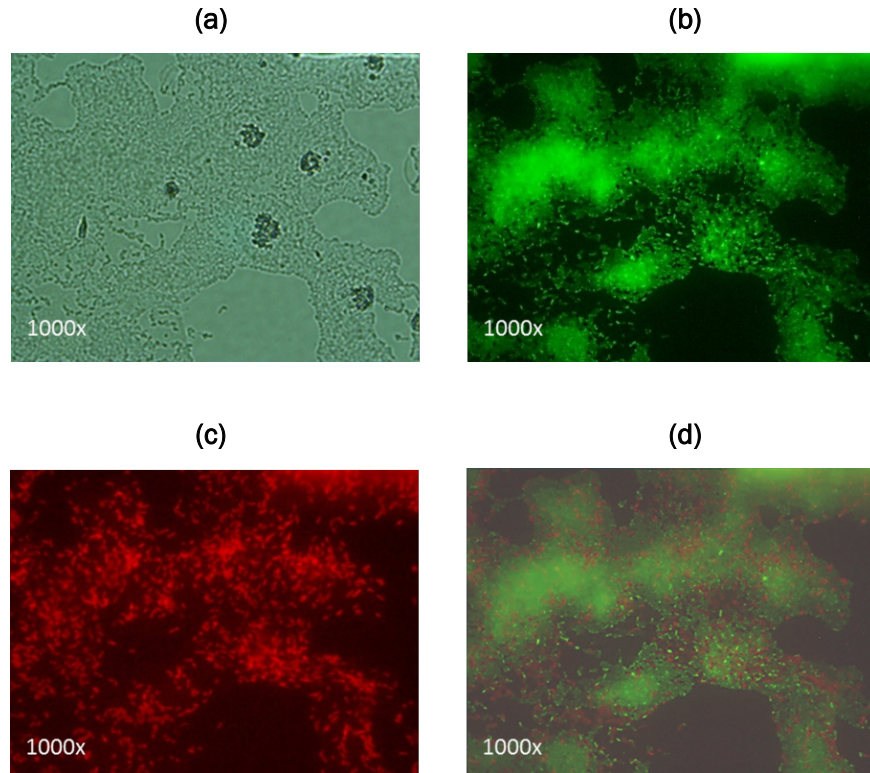
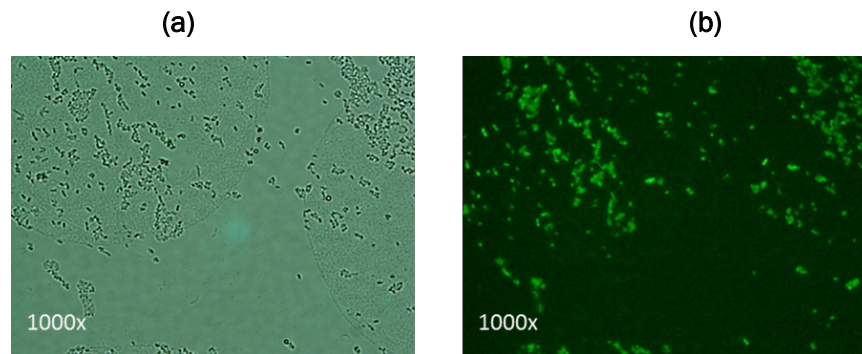


Figure 15. Thin biofilm embedded *P. putida* F1 and *S. oneidensis* MR-1 hybridized with 35% formamide on ITO-coated glass slides; (a) image of 16S rRNA hybridized biofilm without fluorescence, (b) hybridized cells within EPS with FITC epifluorescence, (c) hybridized cells within EPS with Texas red epifluorescence, and (d) overlay of images b and c.



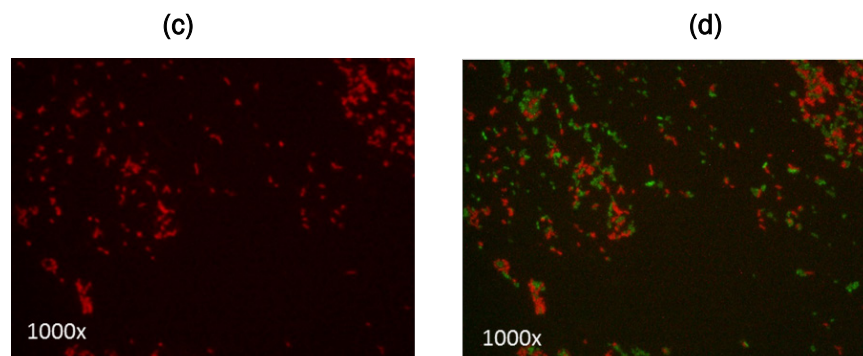
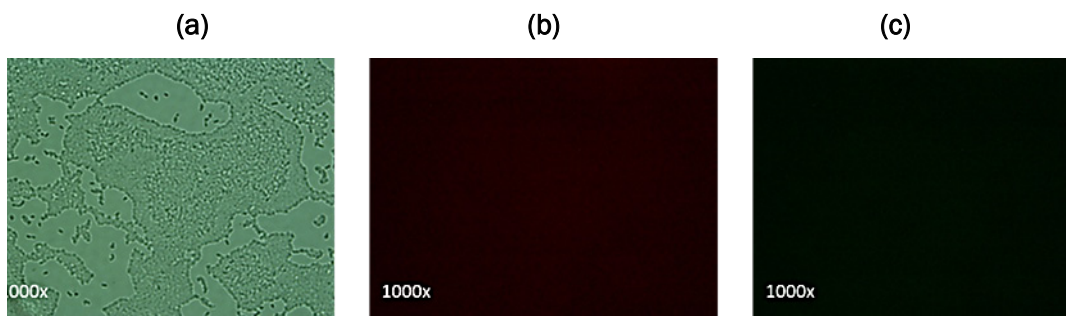


Figure 16. Biofilm embedded *P. putida* F1 and *S. oneidensis* MR-1 no 16S rRNA probe added to hybridizations on ITO-coated glass slides; (a) image of non-hybridized co-culture without fluorescence, (b) non-hybridized cells within EPS with FITC epifluorescence, and (c) non-hybridized cells within EPS with Texas red epifluorescence.



Thickness of the co-culture biofilms were estimated to be greater than 1 mm in those areas receiving nutrients, which resulted in many vertical focal points throughout the EPS. As one focal plane came into view, others became intermixed. Areas with high cell concentrations exhibited increased fluorescent output, which made differentiating individual cells difficult, but did give an indication of areas of predominate biomass for each of the bacteria. *P. putida* F1 was the dominate organism within thick areas of the biofilms (Figure 15), and the distribution between *P. putida* F1 and *S. oneidensis* MR-1 was found to be more even in thin areas of the biofilm receiving less nutrients (Figure 16). Although not available for this study, confocal z-axis microscopy could resolve issues observed here with 2-dimensional imaging. Z-stacking would allow the collection of optical sections through the plane of focus in the z dimension of the EPS to give a

more comprehensive and better resolved image of the biofilm. This capability would provide a 3-dimensional image of the biofilm with precise locations of the individual bacteria being probed in the x, y, and z axis of the EPS.

Recent studies have demonstrated the importance of the structure of EPS and the ability for microorganisms to transfer electrons extracellularly (Xiao et al. 2017). Redox active species were found to be involved in electron transport through the EPS to other microorganisms and to external electron acceptors. The spatial relationship between bacteria and the electron acceptors can influence electron transfer greatly, and electrochemically active elements within EPS likely help to facilitate the process. The method described here for localizing bacterial within intact biofilms has the potential to serve as a powerful tool to study the electron transfer process and better understand complex bioelectrochemical systems.

4 Conclusions

The DFR and methods used in these experiments were capable of establishing pure culture and co-culture biofilms on ITO surfaces within 6 hr of incubation, and each were able to further establish a thick layer of EPS within 24 hr of incubation, under low shear, drip flow conditions. The goal was to rapidly produce a loosely adhered biofilm, which would be indicative of a MFC electrode surface, for testing FISH methodologies within intact biofilms. The 16S rRNA Alexa Fluor labeled probes, PSE1284 and SHEW227, were both found to be very effective at hybridizing *P. putida* F1 and *S. oneidensis* MR-1 grown and fixed planktonically as well as hybridizing both species embedded in thick EPS. Slides coated with ITO had no apparent effect on bacterial attachment and no adverse effects on biofilm production. Contingent on fluorophore selection, ITO may in fact enhance fluorescent reporting, making it an ideal substrate for electrode FISH studies. Both species were readily differentiated and localized within the intact biofilms. In addition, a small degree of depth could also be ascertained, although resolution decreased within thick areas of the biofilm. Employing confocal z-axis microscopy in conjunction with FISH could eliminate this issue and thus serve as a powerful tool to probe deeply into complex EPS. Using this imaging technique in conjunction with microbial biofilm voltammetry can create a better understanding of the spatial distribution of electroactive microorganisms and their effects on extracellular electron transfer through the EPS onto electrodes. In turn, this understanding can help to engineer more efficient MFCs.

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14. ABSTRACT <p>With a limited supply of fossil fuel, there has been great interest in the development of new technologies that can take advantage of renewable fuel sources or convert energy stored in waste to usable energy. One such class of technologies are microbial fuel cells (MFCs), which can convert various carbohydrate rich sources as well as wastewater into electricity via biological catalysts. However, electrical current generation in these microbial driven systems is typically low making these technologies unsuitable for widespread use. In order for MFCs to become a viable alternative energy source, methods are needed to better understand the relationship between microbes and electron transfer. This work out-lines a method for spatially differentiating exoelectrogenic bacteria with-in intact biofilms grown on a conductive surface. The technique involves the rapid generation of biofilms by using a drip flow bioreactor (DFR) on indium tin oxide (ITO)-coated slides, in situ fixation of bacteria within the biofilms on the ITO surface, and determining species differentiation and location by probing with fluorescence in situ hybridization (FISH). This method was shown to effectively distinguish two exoelectrogens within biofilms on a conductive surface, which could serve as a novel means to study MFCs in greater detail.</p>					
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