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A Microfluidic Gradient Mixer-Flow Chamber as a New Tool to Study Biofilm Development under Defined Solute Gradients

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Abstract

Understanding the dynamics of biofilm development in response to chemical cues and signals is required toward the development of controllable biofilm-mediated bioprocesses. In this study, we report a **new** biofilm growth system that integrates a **microfluidic gradient mixer** with a biofilm growth chamber. The biofilm growth system allows biofilms grow under defined solute gradients and enables non-destructively monitor the biofilm development dynamics in response to the defined gradients. The solute gradients generated in the system were simulated and then validated experimentally. We then demonstrated the applicability of the biofilm growth system in studying biofilm development under defined solute gradients. Specifically, we examined biofilm development of *Shewanella oneidensis* and *Comamonas testosteroni* under a defined calcium and nitrate gradient, respectively. Using two *C. testosteroni* strains (WDL7 and I2), we further demonstrated the applicability of our biofilm growth system to study the development of co-culture biofilms under a defined solute gradient. Our results showed that the biofilm growth system we developed here can be a promising tool to reveal the dynamics of biofilm development in response to chemical cues and signals as well as the inter-organism interactions in co-culture biofilms.

Keywords: Biofilm, solute gradient, microfluidic, *Shewanella*, *Comamonas*

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Introduction

Biofilm as a surface-associated microbial aggregate encased within a matrix of self-produced extracellular polymeric substances (EPS) is the most ubiquitous and resilient form of microbial lifestyle on the earth (Hansen et al., 2007). Microbial biofilms often exhibit strong structural stability and high tolerance to harsh environmental conditions, rendering biofilms an attractive biocatalytic system for various environmental and biotechnological applications (Gross et al., 2007). Although many studies have demonstrated the advantages of biofilm-based bioprocesses over those using planktonic cultures, the control of biofilm-mediated bioprocesses remains a great challenge. This is primarily due to the highly dynamic and heterogeneous nature of the biofilm lifestyle (Battin et al., 2016; Ren et al., 2018).

To develop strategies for the control of biofilm-mediated bioprocesses, it is essential to understand biofilm development under controlled physicochemical conditions. To this end, several experimental biofilm systems that allow non-destructive, direct microscopic observation and image-based quantification of biofilm development have been developed. Three-channel flow cells and flat plate flow cells are two most commonly used biofilm growth chambers, which enable non-destructive, quantitative analysis of biofilm structures (Hall-Stoodley et al., 1999; Tolker-Nielsen et al., 2011). A planar flow cell capable of precisely controlling the flow velocity and shear force for biofilm development was designed by Packman and colleagues (2011). The planar flow cell enabled the elucidation of the influence of flow velocity on biofilm growth and cell-cell interactions in multispecies biofilms (W Zhang et al., 2011). Another design of biofilm growth was also developed to support the control of hydrodynamic conditions, in which bacterial initial attachment in response to fluid drag forces (e.g., shear stresses and pressure differences) was examined (De La Fuente et al., 2007). To overcome corner effects of rectangular growth

chambers, a micro-bioreactor capable of generating co-flowing streams within the microchannel was developed (Aznaveh et al., 2014; Paquet-Mercier et al., 2014). This device was further modified to incorporate in electrochemical techniques for characterizing biofilm development (Zarabadi et al., 2017).

To achieve a better control of solute gradients, different approaches have been developed and tested (Hong et al., 2012; Kim et al., 2010). For instance, hydrogel matrix-enabled generation of solute gradient was achieved based on molecular diffusion and micropipette injection (Velve-Casquillas et al., 2010). A membrane-supported flow chamber was also shown to be able to generate solute gradient through molecular diffusion (Lin et al., 2015). Various microfluidic devices have also introduced to generate complex solute gradients for biological studies (Hol et al., 2014). For example, a double-inlet microfluidic flow cell was reported to be able to generate solute gradients at the microscale to study biofilm development (W Zhang et al., 2013). This device was modified from a “T” mixer, in which the solute gradients was mainly driven by the convection-diffusion of molecules in the two inlet streams (W Zhang et al., 2013). The width of the solute gradient was determined by the flow velocities of the two streams and varied from the inlet to the outlet of the growth chamber (Hsu et al., 2012; Lin et al., 2015; Song et al., 2014; W Zhang et al., 2013). Microfluidic gradient generators consisting of a network of microchannels can be used to generate linear solute gradients (Ahmed, 2010; Hung et al., 2005; Son et al., 2015) but have not been applied to biofilm studies.

This study aimed to develop a biofilm growth system that allows us to investigate biofilm development under spatially defined solute gradients by coupling a microfluidic gradient mixer with a biofilm growth chamber. The system is compatible with confocal laser scanning microscopy (CLSM) and allows non-destructive, continuous monitoring of biofilm development

under defined solute gradients. The solute gradients generated in the biofilm growth chamber were simulated and validated experimentally. By using *Shewanella oneidensis* and *Comamonas testosteroni* as model organisms, we then demonstrated the application of this biofilm growth system in quantifying biofilm development under defined calcium and nitrate gradients.

Materials and Methods

Design and Fabrication of Microfluidic Gradient Mixer-Flow Chamber

The microfluidic gradient mixer-flow chamber was designed by coupling a microfluidic chemical mixer to a biofilm growth chamber (Figure 1). The chemical mixer consists of two inlets, seven mixing levels and nine outputs which connect to the growth chamber. The growth chamber also connects with four cell inoculation inlets/outlets which are independent of the chemical mixer. This device is fabricated by three layers: the top layer, containing a microfluidic chemical mixer; the middle layer, which is a biofilm growth chamber; and the bottom layer, which is a glass slide substratum for biofilm growth and microscopic observation. The top and middle layers were made of polydimethylsiloxane (PDMS). The fabrication protocols were modified based on commonly applied PDMS fabrication procedures (Eddings, 2008; Guo et al., 2014; Sia et al., 2003) with details described in the Supporting Information (SI).

Simulation of Solute Gradients in the Flow Chamber

Simulation of solute gradients was conducted using the COMSOL Multiphysics 4.3b (COMSOL, Inc.) (Hashim, 2012). The details of simulation and mixing efficiency were described in SI. Briefly, the 3D geometry was designed in AutoCAD 2007 (Autodesk) and was imported into COMSOL as *stl* file. The modules of *Laminar Flow* (Equations 1 and 2) and *Transport of*

Diluted Species (Equation 3) in COMSOL were applied to simulate the fluid flow, chemical mixing and diffusion inside the biofilm growth chamber:

$$\frac{\partial \rho}{\partial t} + \rho \cdot \nabla \vec{u} = 0 \quad (\text{Equation 1})$$

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla p + \nabla \cdot \left(\mu (\nabla \vec{u} + (\nabla \vec{u})^T) - \frac{2}{3} \mu (\nabla \cdot \vec{u}) \vec{I} \right) + \vec{F} \quad (\text{Equation 2})$$

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \vec{u} \cdot \nabla c + R \quad (\text{Equation 3})$$

where ρ is the flow density, $\vec{u}$ is the flow velocity, p is the fluid pressure, μ is the fluid dynamic viscosity, $\vec{F}$ is the external force, D is the diffusion coefficient, c is the chemical concentration, and R is the chemical reaction rate.

The chemical concentration at each mixing output in each mixing level of the microfluidic gradient generator can be calculated by the chemical dilution equation shown as **Equation 4** (Lee et al., 2009):

$$C_i = \frac{V_{L,i}}{V_{L,i} + V_{R,i}} C_0 + \frac{V_{L,i}}{V_{L,i} + V_{R,i}} C_{i+1} \quad (\text{Equation 4})$$

where C_0 represents the original chemical concentration at the input, C_i is the chemical concentration at mixing output, V is the flow rate that is tightly linked with the channel length L and flow resistance R .

Experimental Validation of the Solute Gradients

The simulation results of the solute gradients were validated by experimental results using bovine serum albumin (BSA), CaCl_2 , and KNO_3 as the model chemicals. The model chemicals were dissolved in sterilized MiliQ water with concentrations at 2 mg/L, 0.68 mM, and 4 mM for BSA, CaCl_2 and KNO_3 , respectively. The model chemical solution was introduced into the

microfluidic gradient mixer through inlet 1, with water being introduced through inlet 2. The flow rate of both inlets was maintained at 3 mL/h using syringe pumps (NE-1000, New Era Pump Systems Inc., NY, USA). A duration of 30 min was set for equilibrium after the chamber was fully-filled, followed by sample collection from each outlets of the chamber. BSA was quantified using the BCA assay following the protocol described elsewhere (Y Zhang et al., 2014). Ca^{2+} concentration was quantified using Ion Chromatography (Rieman et al., 2013). Nitrate concentration was determined by using the HACH kits with a HACH DR 3900 photometer (HACH, Loveland, Co) based on manufacturer's instructions (Wu et al., 2015). Comparison of the experimental validation and simulation was evaluated by the goodness of fit as shown in Equation 5:

$$R^2 = 1 - \frac{\sum_{i=1}^9 (C_{s(i)} - C_{e(i)})^2}{\sum_{i=1}^9 (C_{s(i)} - C_{s\text{ ave}})^2} \quad (\text{Equation 5})$$

Where R^2 is the goodness of fit, i represents the i^{th} effluent position, C_s is the simulated concentration and C_e is the experimental concentration.

Bacterial Strains and Growth Conditions

S. oneidensis MR-1 tagged with a green fluorescent protein (MR-1-gfp) (Y Zhang et al., 2014), *C. testosteroni* WDL7 wild-type (WDL7-wt), and *C. testosteroni* I2 tagged with a red fluorescent protein (I2-rfp) (Boon et al., 2002) were used. The modified MM1 medium and M9 medium were used for the growth of *S. oneidensis* and *C. testosteroni*, respectively (Ding et al., 2014; Wu et al., 2015).

Biofilm Development of *S. oneidensis* under a Defined Ca^{2+} Gradient

MM1 media with and without 0.68 mM CaCl_2 were supplied at a flow rate of 3 mL/h to the fluidic system through medium inlets 1 and 2, respectively using syringe pumps. The inlet media flows were stopped after the whole chamber was fully filled, and *S. oneidensis* MR-1-gfp inocula were introduced into the biofilm growth chamber through the cell inoculation inlets. The inocula were prepared by diluting an overnight culture of MR-1-gfp using fresh MM1 medium without CaCl_2 to a final OD_{600} of 0.1. After inoculation of 5 mL inocula into the chamber, all flows stopped for 2 h, allowing the bacteria to attach onto the chamber. The media flow resumed at a rate of 3 mL/h for each inlet.

Biofilm Development of *C. testosteroni* under a Defined Nitrate Gradient

C. testosteroni WDL7-wt and I2-rfp single-species biofilms were cultivated in the gradient-generator-flow chamber following the similar protocol. Briefly, modified M9 minimal medium with and without 4 mM KNO_3 was delivered through medium inlets 1 and 2, respectively, at a flow rate of 3 mL/h for biofilm growth. Inocula of WDL7-wt / I2-rfp by diluting the overnight culture with fresh M9 medium without nitrate to a final OD_{600} of 0.1 were injected into the growth chamber. All flows were stopped for 2 hours to facilitate bacterial initial attachment before each inlet medium flow resumed at a rate of 3 mL/h.

Similarly, the same protocol for chemical gradient generation and biofilm development was applied to develop WDL7 and I2 co-culture biofilms. The overnight cultures of WDL7 and I2 were both adjusted to a density of OD_{600} at 1, which were mixed at a volume ratio of 1:1. The mixture was further adjusted to a density of OD_{600} at 0.1 with fresh M9 medium for inoculation in the growth chamber.

CLSM Image Acquisition and Processing

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3 149 Biofilm development in the growth chamber was monitored using a confocal laser scanning
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5 150 microscope (CLSM, ZEISS LSM780, Oberkochen, Germany) following the modified method
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8 151 described previously (Wu et al., 2015). Briefly, nucleic acid specific fluorescent dyes, SYTO9
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10 152 (stains all cells) and propidium iodide (PI, stains membrane compromised cells) (ThermoFisher
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12 153 Scientific, Massachusetts, US), were used whenever necessary. The CLSM images were taken at
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14 154 different time points at specific locations with an acquisition of $425\ \mu\text{m} \times 425\ \mu\text{m} \times 100\ \mu\text{m}$
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16 155 (length $\times$ width $\times$ depth). And 5 CLSM images were taken at adjacent locations as replicates for
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18 156 further statistical analysis. The CLSM tile-image was set with a size of $425\ \mu\text{m} \times 20\ \text{mm} \times 100$
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20 157 μm with a scaling of $0.42\ \mu\text{m} \times 0.42\ \mu\text{m}$. Images were further processed by image processing
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22 158 and analysing software IMARIS (Bitplane, Belfast, UK) and Comstat2 with 5 replicates for each
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24 159 (Heydorn et al., 2000).

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29 160 **Results and Discussion**

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32 161 **Design of the Microfluidic Gradient Mixer-Flow Chamber**

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35 162 The microfluidic gradient mixer-flow chamber was designed by combining a microfluidic
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37 163 gradient mixer with a biofilm cultivation chamber, aiming to generate defined solute gradients
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39 164 for biofilm studies (Figure 1). The microfluidic gradient mixer consisted of two medium inlets,
40
41 165 seven levels of mixing cascade and nine outlets, which can generate nine chemical gradient
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43 166 concentrations for the downstream biofilm chamber. The growth chamber was designed as 35
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45 167 $\text{mm} \times 20\ \text{mm} \times 2\ \text{mm}$, which connected to four inoculation inlets / outlets as well as nine
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47 168 sampling outlets. To better illustrate the positions within the chamber, x-axis was defined as the
48
49 169 direction normal to the flow and y-axis was the direction in parallel to the flow (Figure 2A).

The chemical concentration of each output stream in the i^{th} level is able to be estimated by **Eq.4**, although the diffusion of particles also took place between each mixed stream. The mixing efficiency was also estimated based on the simulation results at the mixing section (**Figure S2**). In the growth chamber, chemical mixing was shown as molecule convection-diffusion process which was able to be estimated by **Eq.3**. In principle, the convection only occurred along y-axis and diffusion was minimal for consideration except along x-axis, which was estimated as minimal for our preset experimental conditions (Walker et al., 2005). This two-inlets-gradient generator utilizing a cascaded-mixing overcomes the disadvantages present in simple “T” mixers and fluid-filled reservoirs which rely on convection-diffusion and / or diffusion over the short distance. In the “T” mixers, the chemical gradient generation is mainly driven by chemical convection-diffusion (**Eq.3**) which is highly time and length dependent (Walker et al., 2005). In addition, this biofilm growth system was designed to minimize the physical environmental variances for biofilm development by maintaining the laminar flow condition in the growth chamber (velocity distribution shown in **Figure S1**). The Reynolds number within the chamber was less than 10 when the inlet flow rate was lower than 10 mL/h, which was minimal compared to the critical Reynolds number at 2300 for transition and turbulence (Ismagilov et al., 2000; Stroock et al., 2002). Therefore, solute gradient in the flow chamber along the x-axis can be considered as the sole environmental variance for biofilm development within this gradient generator-flow chamber system.

189 Solute Gradients in the Microfluidic Gradient Generator-Flow Chamber

Simulation results of the gradients in the microfluidic gradient generator-flow chamber are shown in **Figure 2**. The gradient was generated along the x-axis from $x = 0$ mm to $x = 20$ mm, whereas no significant gradient was observed along the y-axis from $y = 0$ mm to $y = 35$ mm

(Figure 2B). Moreover, different diffusion coefficients (D_c) of the solutes within a wide range from 10^{-5} to 10^{-13} m²/s showed no apparent impacts on the gradient generated in the chamber effluent side where $y = 35$ mm (Figure 2C). These simulated results suggested the applicability of the biofilm growth system to generate stable gradients for various commonly used chemicals ranging from macromolecules to low-molecular weight organic compounds and inorganic ions. To validate the simulated results, we used BSA, nitrate, and CaCl₂ as model chemicals to quantify the chemical gradients at the sampling outlets (equal to positions at $y = 35$ mm) of the growth chamber. The gradients generated in our experiments agreed well with the simulated results, with a correlation coefficient (R^2) of 0.90, 0.90, 0.76 for BSA, CaCl₂ and nitrate, respectively (Figure 2D). According to the experimental validation, the highest solute concentration in the sampling outlet was at a similar level with the highest inlet concentration, suggesting a minimal adsorption (< 3%) of the solute onto the chamber.

205 Development of *S. oneidensis* Biofilms under a Defined Ca²⁺ Gradient

We grew *S. oneidensis* MR-1-gfp biofilms under a defined gradient of CaCl₂ in MM1 medium in the microfluidic chamber. The structural heterogeneity of the biofilms growing in the upstream may influence the solute gradient profiles at the downstream in the growth chamber. Hence, we examined the biofilm development by taking CLSM images at locations close to the inlet position ($y = 3.5$ mm; $x = 0$ to 20 mm) to minimize the influence of biofilm growth at the upstream. In addition, the depth of the growth chamber (2 mm) is more than 10× of the biofilm thickness (up to 120 μm) so that the influence of biofilm growth on the overall gradient pattern in the growth chamber is also minimal. The chemical concentrations at the inlet positions were estimated from simulation and validation results.

215 The representative images were taken on day 3, 5, and 7 of biofilm cultivation, respectively
216 (Figure 3A). Quantitative analyses of the biofilm biovolume, surface coverage and roughness
217 were carried out based on the tile image of the 7-day biofilms (Figure 3B, C; Figure S3). The
218 biovolume showed an increasing trend from $(5.7 \pm 4.3) \times 10^5 \mu\text{m}^3$ to $(7.6 \pm 3.5) \times 10^6 \mu\text{m}^3$ with
219 the increased Ca^{2+} concentration from 0 to 0.68 mM (Figure 3B).

220 Intriguingly, surface coverage and roughness (represented as the standard deviation of average
221 biofilm thickness) (DiCesare et al., 2012; Heydorn et al., 2000; Shen et al., 2018) of the biofilms
222 were relatively constant with the increase of Ca^{2+} from 0 to 0.5 mM in the medium. However,
223 when Ca^{2+} concentration reached 0.5 mM and higher, the biofilm coverage on the surface
224 increased dramatically, reaching almost 100%, whereas the roughness decreased to 0.1 (Figure
225 3C). Low surface coverages with a high roughness of the biofilms developed at locations where
226 calcium concentration was lower than 0.5 mM suggested a high dependence of Ca^{2+} for the
227 biofilm development of *S. oneidensis*. The biofilm of MR-1-gfp developed to relative uniform
228 3D structures covering the whole bottom surface instead of as separated microcolonies where
229 calcium supply was high (> 0.5 mM).

230 These results are consistent with previous studies in which Ca^{2+} was shown to be a critical factor
231 that enhanced biofilm development of *S. oneidensis* (Theunissen et al., 2010). Ca^{2+}
232 supplemented in growth medium has been revealed to enhance biofilm formation by affecting
233 cell attachment, motility, and biofilm structures (Cruz et al., 2012). In many bacterial species,
234 Ca^{2+} regulates extracellular polymeric substance production during biofilm development
235 (Patrauchan et al., 2005; Sarkisova et al., 2005). Meanwhile, Ca^{2+} -binding proteins play
236 important role in bacterial surface adhesion as attachment factors (Porsch et al., 2013). In *S.*
237 *oneidensis*, Ca^{2+} -binding proteins have been reported to be involved in initial attachment, where

the Ca^{2+} stimulates auto-aggregation of MR-1 cells (Satchell, 2011; Sivakumar et al., 2015; Theunissen et al., 2010).

Development of *C. testosteroni* Biofilms under a Defined Nitrate Gradient

To demonstrate the applicability of our biofilm growth system in comparing responses of different strains to the same solute gradient, we grew *C. testosteroni* strain WDL7 and strain I2-rfp biofilms under a defined gradient of NO_3^- from 0 to 4 mM. Representative CLSM images at various NO_3^- concentrations are shown in **Figure 4A**. Quantitative analysis was carried out based on the tile images, and representative biovolumes corresponding to four x positions were plotted together with estimated NO_3^- concentration (**Figure 4B**).

The biofilm biovolumes of both strains WDL7 and I2 showed a positive correlation with the NO_3^- in the medium. At positions with NO_3^- concentration higher than 3 mM, the biovolumes of WDL7 biofilms were significantly higher than those of I2 biofilms, e.g., $(3.02 \pm 0.71) \times 10^6 \mu\text{m}^3$ (WDL7) vs $(3.39 \pm 0.38) \times 10^5 \mu\text{m}^3$ (I2) in the presence of 4 mM NO_3^- . In contrast, at positions where NO_3^- concentration was no more than 1 mM, comparable biofilm biovolumes were observed for both WDL7 and I2. Genomic analysis suggested that both WDL7 and I2 are capable of reducing nitrate, whereas only I2 is able to utilize NO_3^- as an energy source under anoxic conditions (Wu et al., 2015). Our observation that NO_3^- promotes I2 biofilm development is consistent with previous work showing that NO_3^- enhances I2 biofilm stability (Wu et al., 2015).

Interestingly, WDL7 is not capable of using NO_3^- as an energy source for biofilm growth, the biofilm development of WDL7 was also found to be enhanced at higher NO_3^- concentrations. It is possible that NO_3^- serves as a biofilm formation-promoting factor in WDL7, referencing to the

260 NO_3^- sensing and responding system in *P. aeruginosa* where NO_3^- affects motility and virulence
261 of the cells (Van Alst et al., 2007).

262 Our observations and quantitative comparisons elucidated different responses of biofilm
263 development by different organisms to the same solute gradient. Our results demonstrated the
264 applicability of the biofilm growth system developed here to the comparative analysis of biofilm
265 development of different organisms under same solute gradients.

266 **Development of *C. testosteroni* WDL7 and I2 Co-culture Biofilms under a Defined Nitrate** 267 **Gradient**

268 We further demonstrate the applicability of our biofilm growth system to study the development
269 co-culture biofilms under a defined solute gradient. Specifically, we grew *C. testosteroni* strains
270 WDL7 and I2-rfp co-culture biofilms in the gradient generator flow chamber in which NO_3^-
271 concentrations ranged from 0 to 4 mM. The biofilm was monitored by CLSM imaging at $y = 3.5$
272 mm along x direction at early maturation by staining both strains with SYTO9 (Ex/Em at
273 495/535 nm). Representative CLSM images of the co-culture biofilms are shown in **Figure 5A**.
274 The presence of NO_3^- is not essential for the development of the co-culture biofilm, while the
275 total biofilm biovolume exhibited a positive correlation with NO_3^- concentration. Although the
276 two organisms were inoculated at a ratio of 1:1, WDL7 appeared to be dominant (~80% of total
277 biovolume) in the co-culture biofilms (**Figure 5B**). **The enhanced biofilm biovolume by**
278 **increased nitrate concentration is mainly contributed by WDL7 instead of I2.**

279 Non-destructive monitoring and quantitative analyses of the development of co-culture biofilms
280 under a defined gradient enabled by the biofilm growth system we developed here provide a new
281 approach to investigate inter-organism interactions in co-culture biofilms.

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Conclusions

We report a new biofilm growth system in which controllable and defined solute gradients can be generated. We demonstrate the applicability of the biofilm growth system in studying biofilm development under defined solute gradients. Our results showed that the biofilm growth system we developed here can be a promising tool to reveal the dynamics of biofilm development in response to chemical cues and signals as well as the inter-organism interactions in co-culture biofilms.

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The authors declare no competing financial interest.

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Figure Captions

Figure 1. A. The microfluidic gradient mixer flow cell contains two major parts: the gradient mixer with two medium inlets and the growth chamber with four inoculation inlets/outlets. **B.** Design and specifications of the gradient mixer flow cell: the gradient mixer contains seven chemical mixing levels and dimensions of the device are shown. **C.** This device is fabricated in three layers: the bottom is a microscope cover glass for direct microscopic observation; the middle layer is a 2 mm PDMS sheet of open biofilm chamber and bacterial inoculation and effluent channels; and the top layer is the chemical mixer made of PDMS.

Figure 2. A. Schematic illustration of the positions in the chamber. The x-axis is defined normal to the fluid flow and the y-axis is defined parallel to the fluid flow. **B.** Simulated chemical gradients along the x-axis at various y positions in the chamber. **C.** Simulated gradients of chemicals with different diffusion coefficients (D_c) along the x-axis at $y = 35$ mm. **D.** Experimental validation results (shown as data points) and comparison with simulation result (line).

Figure 3. A. Representative CLSM images of *S. oneidensis* MR1-gfp biofilms at different x positions (y position was kept at $y = 3.5$ mm) taken on day 3, day 5 and day 7. The CaCl_2 concentration for each x position is predicted according to simulation results. **B.** Biofilm biovolume at day 7 were calculated based on tile images by Imaris, which were plotted along x-axis together with predicted calcium concentrations. **C.** Roughness and surface coverages at day 7 were calculated which were plotted along calcium concentrations. Surface coverage was present in percentage; and roughness was determined by the standard deviation of average thickness of the biofilms.

Figure 4. A. Representative CLSM images of *C. testosteroni* WDL7 and I2 monoculture biofilms under different nitrate concentrations. Images were taken at early maturation stage (day 4) by bacterial SYTO9 staining along x-axis where y = 3.5 mm. **B.** Quantitative analysis of CLSM tile images for WDL7 and I2 monoculture biofilms at selected positions were plotted along x-axis together with predicted nitrate concentration. (* $p \leq 0.05$)

Figure 5. A. Representative CLSM images of *C. testosteroni* WDL7 and I2-rfp dual-organism biofilms under nitrate concentration gradients in medium (all images were kept at y = 3.5 mm); all cells were stained by SYTO9 and were observed at 495/535 nm (in green); I2-rfp cells were observed directly at 561/593 nm (in red). All scale bars are 50.0 μm . **B.** Biofilm biovolume of WDL7 and I2-rfp in biofilms were calculated based on the CLSM images which were plotted along x-axis together with predicted nitrate concentration. (* $p \leq 0.05$)

Figure 3

67x124mm (300 x 300 DPI)

Figure 4

84x81mm (300 x 300 DPI)

Figure 5

84x65mm (300 x 300 DPI)

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Supporting Information

A Microfluidic Gradient Mixer-Flow Chamber as a New Tool to Study Biofilm Development under Defined Solute Gradients

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1 Microfluidic gradient mixer flow cell fabrication

2 Device fabrication was modified from commonly used methods, and three general steps were
 3 taken to create the whole chamber (Eddings, Johnson, & Gale, 2008; Guo et al., 2014; Sia &
 4 Whitesides, 2003). The first step was to fabricate the molds of top and middle layers. The mold
 5 of gradient generator was fabricated through photolithography approach, and the aluminum
 6 mold for flow cell chamber was fabricated by machining. The second step was to cast the two
 7 PDMS layers against the molds. Briefly, PDMS with base to reagent ratio of 10 to 1 was mixed
 8 thoroughly and vacuumed for 10 min for primary degassing before being poured into the molds.
 9 Then the molds with PDMS were vacuumed for 30 min to fully degas and placed under 70°C
 10 for 2 h to solidify. Last step was to bond the three layers together with plasma treatment. Before
 11 the bonding, necessary cleaning and hole punching was done. Cleaning was done in a
 12 sequential way by washing initially with isopropanol, followed with acetone, and finally with
 13 distilled water. The washed layers were dried by nitrogen blow and placed under 70°C for 30
 14 min. After 1 min high level plasma treatment and aligned bonding, the whole chamber was
 15 placed under 100°C for at least 1 h to achieve a strong and permanent bonding. The chamber
 16 was then ready for the following experiments.

17 Simulation of Solute Gradients in the Flow Chamber

18 The COMSOL model and geometry files were attached. The assumptions of the simulation
 19 model were listed as below:

- 20 (1) The simulation was done using stationary conditions, i.e., we only simulated the
 21 equilibrium stage of the fluid dynamics and chemical mixing behaviors.

$$22 \quad \frac{\partial \rho}{\partial t} = 0, \frac{\partial \vec{u}}{\partial t} = 0, \text{ and } \frac{\partial c}{\partial t} = 0.$$

(2) The chemical mixing was simulated based on diluted solutes, i.e., the dissolved chemicals has negligible influences on the fluid properties (e.g., viscosity and flow patterns).

(3) Larminar flow was considered in the simulation instead of turbulent

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla p + \nabla \cdot \left(\mu (\nabla \vec{u} + (\nabla \vec{u})^T) - \frac{2}{3} \mu (\nabla \cdot \vec{u}) \vec{I} \right) + \vec{F}$$

(4) The outlet pressures were considered as the same as the local atmospheric pressure.

Besides, constant chemical concentrations were used for inlets, and no constraints were added to the outlets. The inflow velocity of each inlet was assumed as uniform at 37.5 m/h (3 ml/h). The boundary surfaces except the inlets and outlets, were considered as stationary with no slip for fluid dynamics and no flux for chemical mixing.

The Z structure was coupled to enhance the solute mixing in the gradient generator. The mixing efficiency at a certain cross-section of the mixing channel was defined by (Soleymani, Kolehmainen, & Turunen, 2008):

$$\alpha = 1 - \sqrt{\frac{\sigma^2}{\sigma_{max}^2}} \quad \text{with} \quad \sigma^2 = \frac{1}{n} \sum_{i=1}^n (C_i - \bar{C})^2$$

where σ^2 is the ratio of variance of concentration, σ_{max}^2 is the maximum variance of the mixture, $\bar{C}$ is the mean value of the concentration field, C_i is the concentration for the i^{th} cell in the cross sectional area of interest, and n is the number of grid points inside the cross-section.

Herein, the perfect mixing shall have a α at 1, whereas no mixing occurred when α at 0.

The COMSOL model package is available via
<https://www.dropbox.com/s/mjna9pd4ij8stxq/COMSOL.rar?dl=0>

Figure S1. Velocity distribution in the microfluidic gradient mixer flow cell (unit: m/s). Input velocity for each inlet is 37.5 m/h (3 ml/h).

Figure S2. Mixing efficiency of the microfluidic gradient mixer. Perfect mixing: $\alpha = 1$; no mixing: $\alpha = 0$. (* position with max σ^2)

Figure S3. CLSM Tile image (top view) of the *S. oneidensis* biofilms at day 7 with CaCl_2 gradient in the microfluidic gradient mixer-flow cell system.

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