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# Development and Quantitation of *Pseudomonas aeruginosa* Biofilms after *in vitro* Cultivation in Flow-reactors

Yingdan Zhang[1], Jingru Zhao[2], Hang Cheng[1], Jing Wang[1], Liang Yang[1, 3, *] and Haihua Liang[2, *]

[1]School of Medicine, Southern University of Science and Technology, Shenzhen, China; [2]College of Life Sciences, Northwest University, Xi'an, ShaanXi, China; [3]Shenzhen Key Laboratory of Gene Regulation and Systems Biology, Southern University of Science and Technology, Shenzhen, China
*For correspondence: yangl@sustech.edu.cn; lianghh@nwu.edu.cn

**[Abstract]** Characterization of biofilm formation and metabolic activities is critical to investigating biofilm interactions with environmental factors and illustrating biofilm regulatory mechanisms. An appropriate *in vitro* model that mimics biofilm *in vivo* habitats therefore demands accurate quantitation and investigation of biofilm-associated activities. Current methodologies commonly involve static biofilm setups (such as biofilm assays in microplates, bead biofilms, or biofilms on glass-slides) and fluidic flow biofilm systems (such as drip-flow biofilm reactors, 3-channel biofilm reactors, or tubing biofilm reactors). Continuous flow systems take into consideration the contribution of hydrodynamic shear forces, nutrient supply, and physical transport of dispersed cells, which define the habitat for biofilm development in most natural and engineered systems. This protocol describes the assembly of 3 flow-system setups to cultivate *Pseudomonas aeruginosa* PAO1 and *Shewanella oneidensis* MR-1 model biofilms, including the respective quantitation and observation approaches. The standardized flow systems promise productive and reproducible biofilm experimental results, which can be further modified according to specific research projects.

**Keywords:** Biofilm characterization, Fluid-flow biofilm reactors, *Pseudomonas aeruginosa*

**[Background]** Biofilm is the most prevalent growth mode of microbial organisms in nature, industry, and clinical habitats; it is commonly recognized as bacterial communities embedded in the self-generated matrix of extracellular polymeric substances (EPS) (Flemming *et al.*, 2016). The spatial organization of a biofilm aggregate is highly heterogeneous and has diverse metabolic activities, rendering the biofilm robust and tolerant to numerous types of environmental stress (Van Dyck *et al.*, 2021). Hence,biofilms are promising in industry to ferment for nutrient conversion, degrade hazardous compounds for bioremediation, and generate electricity in microbial fuel cells (Coenye and Nelis, 2010). Conversely, biofilms also raise public concerns such as biofouling and biofilm-associated infections. Biofilms are the leading cause of chronic wound infections and infections on biomedical devices, such as cardiac valves, tracheal tubes, and catheters (Del Pozo *et al.*, 2018). An improved understanding and characterization of biofilm formation, dispersion, and activities will shed light on biofilm control strategies.

A typical biofilm life cycle usually involves 5 stages: initial reversible attachment of bacteria, stable and irreversible attachment, maturation, dispersion, and dispersed free-living bacteria (Martin *et al.*,

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2021). The biofilm communities show active social behaviors and interaction with environmental factors, which in return regulate the cellular metabolism of biofilms. Numerous methodologies and devices have been documented for the investigation of biofilm morphology and development *in vitro*, commonly categorized as static biofilm assays and continuous-flow biofilm systems. The static biofilm assays - such as those in microplates and on air-liquid interface coverslips or colony biofilm assays and Kadouri drip-fed biofilm assays - are prevalently applied to the screening of early events in biofilm formation (Merritt *et al*., 2005). The static biofilm assays are high-throughput and easily executed with common laboratory equipment; however, nutrient supply in static biofilm assays is limited with respect to developing mature biofilm communities that mimic nature.

Given the fact that biofilms are frequently observed under fluidic flow conditions in nature and engineered systems, nutrient availability, physical transport, and hydrodynamic shear forces significantly impact biofilm formation and metabolism (Mattei *et al*., 2018). To address the question of how biofilm responds to environmental stresses and communicating signals, it is important to create a standardized and reproducible protocol for cultivating *in vitro* biofilms in flow systems mimicking the *in vivo* fluid habitats (Cowle *et al*., 2020). The development of flow biofilm reactors has been advanced by emerging microfluidics manufacturers and designs, with the most common flow systems including drip-flow systems (Gonzalez *et al*., 2014), tubular reactors (Winn *et al*., 2014), planar flow-cell (Zhang *et al*., 2011), and 3-channel flow-cell (Sternberg and Tolker-Nielsen, 2006; Pamp *et al*., 2009). Further modifications, such as segmented flow cells (Karande *et al*., 2014), gradient-generator flow cells (Zhang *et al*., 2019), and flow-velocity microfluidic flow cells (Liu *et al*., 2019), are specifically modified and designed to meet specific research purposes.

In this protocol, we addressed three commonly applied flow-system setups for different research purposes with *Pseudomonas aeruginosa* PAO1 and *Shewanella oneidensis* MR-1 as model organisms. The tubing biofilm reactor mimics the biofilm habitats in pipelines, tracheal tubes, catheters, *etc*., allowing biofilm harvest with adequate biomass. The 3-channel flow-cell system is designed for the non-invasive spatiotemporal observation of biofilm morphologies with a continuous and steady nutrient supply. The recycling biofilm reactor enables the study of biofilm metabolic responses toward its bioremediation metabolites and/or environmental chemicals (Sternberg and Tolker-Nielsen, 2006; Weiss Nielsen *et al*., 2011; Pamp *et al*., 2019). Overall, the standardized flow systems promise productive and reproducible biofilm observation and quantitation, which can be further modified according to the specific research project.

## Materials and Reagents

1. Silicon tube (I.D=1 mm, O.D=3 mm, Runze, China)
2. Silicon tube (I.D=3.2 mm, O.D=6.4 mm, Runze, China)
3. Peristaltic pump tube (I.D=1.02 mm, O.D=2.62 mm, Pharmed BPT, Sain-Gobain)
4. Straight connector (Runze, catalog number: DI-016)
5. Straight connector (Runze, catalog number: DI-032)

 

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6. Luer connector (Runze, catalog numbers: RH-G016; RH-M016)
7. Needle (Gauge: 26G, Hypodermic; BD, catalog number: 305111)
8. Syringe (1 ml, BD, Luer-Lock, catalog number: 309628)
9. Silicone glue (Advanced Silicone 2, GE sealant)
10. Parafilm (BEMIS, catalog number: PM996)
11. Metal foil (Maryya, , catalog number: HC081260)
12. 10 µl Inoculation loop (Sangon Biotech, catalog number: F619312)
13. 15 ml Centrifuge tube (Sangon Biotech, catalog number: F602888)
14. Coverslips (Sangon Biotech, catalog number: F518117)
15. Microplates (ThermoFisher, Nunc™, catalog number: 168055)
16. 0.22 µm Syringe filter (Sangon Biotech, catalog number: F513163)
17. 2 ml Microtubes (Maxyclear Snapclock, Axygen, US)
18. LB broth (Sangon Biotech, catalog number: A507002)
19. Calcium chloride (Sangon Biotech, catalog number: A501330)
20. Sodium chloride (Sangon Biotech, catalog number: A100241)
21. QIAamp DNA Micro Kit (QIAGEN, catalog number: 56304)
22. RNeasy Mini Kit (QIAGEN, catalog number: 74104)

## Equipment

1. Peristaltic pump (200 series 16-channel pump, Watson Marlow)
2. Microplate reader (Spark®, Tecan, Switzerland)
3. Flowcytometer (CytoFLEX, Beckman, USA)
4. Probe sonicator (SONICS, model: VCX750)
5. Centrifuge (Eppendorf, model: 5418R)
6. Biosafety Cabinet, BSC (MSC-Advantage™ II, ThermoFisher, USA)
7. Autoclave (Zealway, model: GR-60DA)
8. Confocal Laser Scanning Microscope, CLSM (Zeiss, model: LSM900)

## Software

1. Imaris (Bitplane, Oxford Instrument)
2. ImageJ (https://imagej.net/)
3. Comstat 2 (http://www.comstat.dk/)

## Procedure

A. Tubing biofilm reactors (Figure 1, Figure S1)

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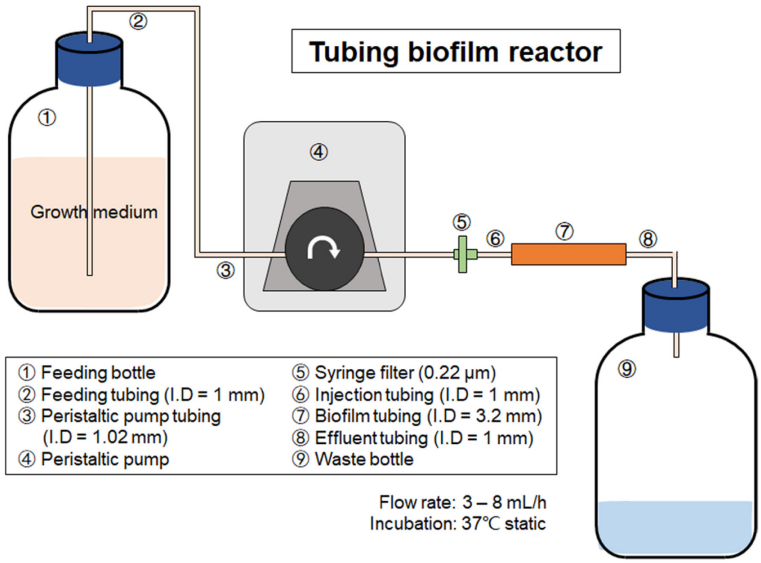


**Figure 1. Illustration of the tubing biofilm reactor**

*Notes:*

1. *Tubing biofilm reactors contain the following parts in sequence: feeding bottle, feeding tubing (I.D=1 mm), peristaltic pump tubing (I.D=1.02 mm), syringe filter (0.2 µm), injection tubing (I.D=1 mm), biofilm tubing (I.D=3.2 mm), waste tubing (I.D=1 mm), and waste bottle.*
2. *All parts of this setup, except ⑤ and ⑦, can be reused for repeated experiments. Please refer to section H for cleanup procedures.*
3. *The tubing biofilm reactors do not allow the spatiotemporal imaging observation of biofilms. They particularly meet the requirement of harvesting a large amount of biomass for -omics analysis.*

1. Connect the feeding tubing to the peristaltic pump tubing with the straight connector. Make sure that the length of the feeding tubing is compatible with the height of the feeding bottle.
2. Connect the other side of the pump tubing to the syringe filter with a female Luer fitting.
3. Connect the other side of the filter to the injection tubing with a male Luer fitting.
4. Connect the injection tubing to the biofilm tubing with a reducing straight connector. The length of the biofilm tubing depends on the experimental design.
5. Connect the biofilm tubing to the waste tubing with a reducing straight connector.
6. Wrap the assembled part (1-5) in metal foil and autoclave to sterilize.
7. Freshly prepare one-tenth LB broth in the feeding bottle and autoclave to sterilize.
8. Autoclave the waste bottle and pour in bleach before use.
9. UV-sterilize pieces of parafilm (10 cm × 10 cm) and metal foil (15 cm × 15 cm) in the biosafety cabinet before use.
10. Place the feeding tubing into the feeding bottle and make sure that the tubing reaches the bottom of the bottle. Place the waste tubing into the waste bottle and make sure that the tubing is close to the bottleneck rather than the bottom. Cover each bottle with sterilized parafilm and

 

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metal foil. This step must be carried out in the biosafety cabinet.

11. Place the pump tubing onto the peristaltic pump and start the pump at max speed to purge the system with growth medium. Make sure that the system is free of bubbles before setting the flow rate back to normal.
12. Shift the tubing biofilm reactor with the peristaltic pump into a temperature-controlled incubator. Make sure that the biofilm tubing is at a similar level as the bottleneck of the waste bottle to avoid any backflow.

B. Three-Channel flow-cell biofilm reactor (Figure 2, Figure S2)

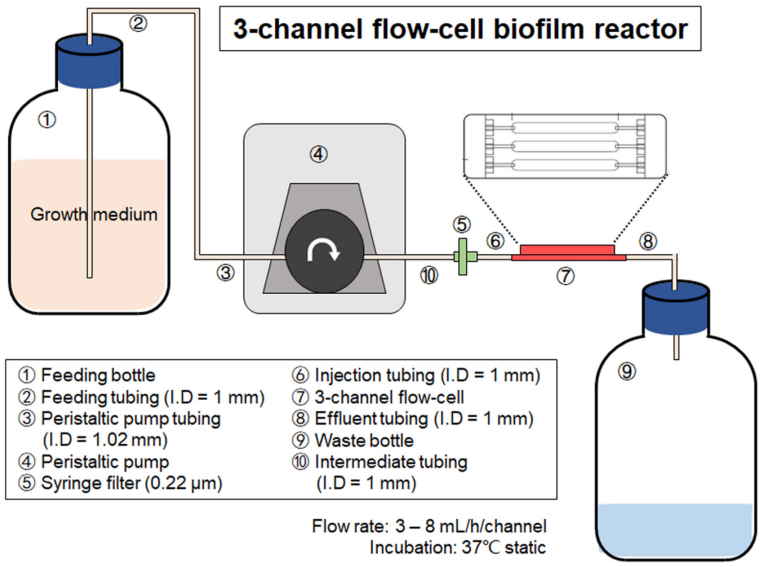


**Figure 2. Illustration of the 3-channel flow-cell biofilm reactor**

*Notes:*

1. *The 3-channel flow-cell system contains the following parts in sequence: feeding bottle, feeding tubing (I.D=1 mm), peristaltic pump tubing (I.D=1.02 mm), intermediate tubing (I.D=1 mm), syringe filter (0.2 μm), injection tubing (I.D=1 mm), biofilm tubing (I.D=3.2 mm), waste tubing (I.D=1 mm), and waste bottle.*
2. *All parts of this setup, except ⑤, can be reused for repeat experiments. Part ⑦ can be reused by removing the coverslip and cleaning with 70% ethanol. For the reuse of other parts, refer to section H for the cleanup procedures.*
3. *The 3-channel biofilm system is most commonly applied to study the biofilm formation and activities of* P. aeruginosa, Staphylococcus aureus, *etc. This setup is slightly modified from the traditional version; the bubble trap is replaced by a syringe filter and the air-connecting tubing in the feeding bottle is removed. According to the authors' experience, the bubble trap is the major cause of leaking, and removal of the air-connecting tubing does not impact the oxygen level in the media. This device is feasible for the non-invasive spatiotemporal observation of biofilm morphology and community compositions, although biofilm harvest is not*

 

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*recommended.*

1. Wash the 3-channel flow cell with ethanol and dry it in the fume hood. Make sure that the surface of the flow cell is clean and dry upon assembly.
2. Place a thin layer of silicon glue on the walls of the 3-channel flow-cell and place a coverslip on top. Carefully press the coverslip toward the base of the flow-cell until it is fully sealed without any bubbles in the sealing area. Leave the sealed flow-cell at room temperature overnight to fully dry the glue.
3. Connect the feeding tubing to the peristaltic pump tubing with a straight connector.
4. Connect the other side of the pump tubing to an intermediate tubing with a straight connector. Make sure that the length of intermediate tubing allows the flow-cell unit to be moved to the stage of the confocal microscope.
5. Connect the other side of the intermediate tubing to the syringe filter with a female Luer fitting.
6. Connect the other side of the filter to the injection tubing with a male Luer fitting.
7. Connect the injection tubing to one channel of the 3-channel flow-cell.
8. Connect the waste tubing to the other side of the flow-cell channel.
9. Assemble the tubing with the feeding bottle, waste bottle, and peristaltic pump using the same procedures as described for the tubing biofilm reactors.

C. Recycling flow-cell system (Figure 3)

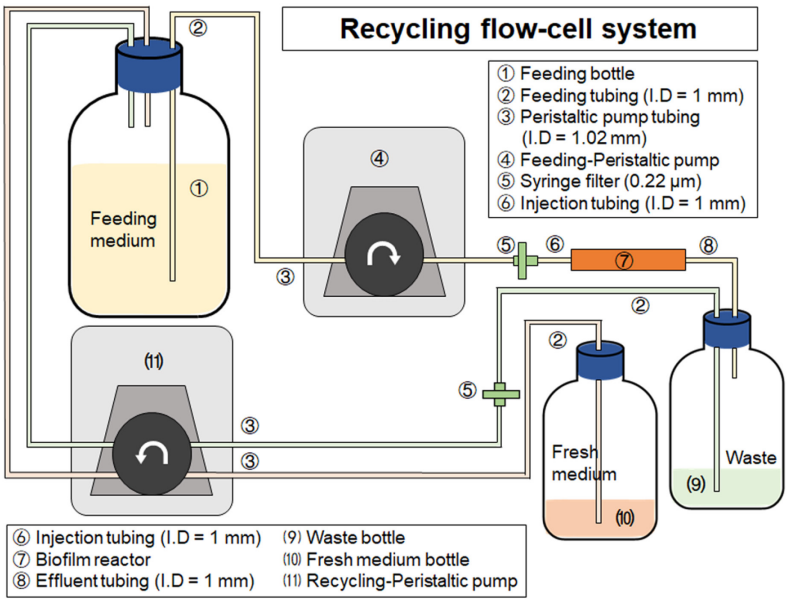


**Figure 3. Illustration of the recycling flow-cell system**

*Notes:*

1. *The recycling flow-cell system contains the following parts in sequence: feeding bottle, feeding tubing (I.D=1 mm), peristaltic pump tubing (I.D=1.02 mm), flow-cell reactor, waste tubing (I.D=1*

 

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*mm), waste bottle with recycling tubing, and fresh medium bottle with supply tubing.*

2. *All parts of this setup, except ⑤ and ⑦, can be reused for repeat experiments. Please refer to section H for cleanup procedures.*
3. *The recycling flow-cell system is commonly applied to study the bioremediation of toxicants and environmental compounds with a view to mimicking the wastewater treatment plants. Part ⑦ is not limited to tubing and/or 3-channel chambers. The feeding ratio of fresh medium to waste medium should be modified by each project.*

1. Assemble the feeding parts by following the same procedures 1-5 in the section of “Assembly of the tubing reactor.”
2. Assemble the recycling waste parts: Connect the feeding tubing to a syringe filter with a female Luer fitting; connect the pump tubing to the other side of the syringe filter with a male Luer fitting; connect the other side of the pump tubing to another feeding tubing (recycling feeding tubing) with a straight connector.
3. Assemble the recycling fresh medium parts: Connect the feeding tubing to the pump tubing with a straight connector; connect the other side of the pump tubing to another feeding tubing (recycling feeding tubing) with a straight connector.
4. Wrap each part individually in metal foil and autoclave to sterilize.
5. Freshly prepare one-tenth LB broth in both the feeding and fresh-medium bottle.
6. Autoclave the waste bottle.
7. UV-sterilize pieces of parafilm (10 cm × 10 cm) and metal foil (15 cm × 15 cm) in the biosafety cabinet before use.
8. Place the three feeding tubings into the feeding bottle, waste bottle, and fresh-medium bottle, respectively. Make sure that the tubing reaches the bottom of each bottle. Place the waste tubing into the waste bottle and make sure that the tubing is close to the bottleneck rather than the bottom. Place the end of the recycling feeding tubing from both the waste and fresh-medium parts into the feeding bottle. Make sure that the tubings are close to the bottleneck rather than the bottom. Cover each bottle with sterilized parafilm and metal foil. This step must be carried out in the biosafety cabinet.
9. Place the feeding pump tubing onto the feeding peristaltic pump and place the two recycling and feeding tubings onto the recycling peristaltic pump.
10. Adjust the rotation speed of the feeding pump to be twice that of the recycling pump.
11. Start the feeding pump first at the highest speed to purge the system with growth medium. Make sure that the system is free of bubbles before setting the flow rate back to normal.
12. Shift the whole system into a temperature-controlled incubator and start both pumps.

D. Biofilm inoculation and cultivation (Figure S3)

1. Prepare the inoculum: cultivate bacteria overnight at the appropriate temperature and dilute to the desired cell density with growth medium. Typically, 1 × $10^8$ cells/ml for *Pseudomonas*

 

*aeruginosa* can promise a robust biofilm.

2. Load the bacterial inoculum into a syringe with a 26G needle. Make sure that the volume of the inoculum fills the biofilm reactor.
3. Stop the peristaltic pump and clamp off the injection tubing before inoculation.
4. Sterilize the insertion site on the biofilm tubing with 75% ethanol.
5. Load the bacterial suspension into each biofilm reactor.
6. Clamp off the effluent tubing.
7. Remove the needle and seal the hole immediately with silicone glue.
8. Allow attachment for 2 h without flow in the incubator. Make sure that the inner surface of the biofilm reactor faces down for initial attachment.
9. Turn over the biofilm reactor so that the inner surface of biofilm attachment faces up.
10. Start the flow (3-8 ml/h/channel) and remove the clamps.
11. Cultivate the biofilm in the incubator for the desired duration before harvest.

E. Biofilm treatment

1. Direct perfusion
   a. Prepare the $CaCl_2$ solution at the desired concentrations in LB broth (0 mM, 0.34 mM, and 0.68 mM) and load into a 1-ml syringe with a 26G needle.
   b. Prepare the control solution containing LB broth and the chemical vector ($H_2O$ in this case) and load into a 1-ml syringe with a 26G needle.
   c. Stop the peristaltic pump and clamp off the injection tubing before perfusion.
   d. Sterilize the insertion site on the biofilm tubing with 75% ethanol.
   e. Inject and load the chemical solution into the biofilm tubing.
   f. Remove the needle and seal the hole immediately with silicone glue.
   g. Allow static treatment for the desired duration.
   h. Start the flow (3-8 ml/h/channel) and remove the clamp, or harvest the biofilms directly.
2. Continuous loading
   a. Prepare a new bottle of sterile medium containing $CaCl_2$ (0.68 mM) and a bottle of sterile medium containing the vector for the drug solution ($H_2O$).
   b. Sterilize pieces of parafilm and metal foil.
   c. Stop the peristaltic pump and clamp off the feeding tubing.
   d. Shift the feeding tubing into the new feeding bottle containing calcium chloride/vector.
   e. Cover the feeding bottle with parafilm and metal foil.
   f. Remove the clamps and start the pump; make sure that there are no bubbles inside the system.

F. Biofilm harvest

1. Biofilm harvest from the tubing reactor:
   a. Stop the flow and clamp off both the injection and waste tubings.

b. Take the section including the injection tubing, biofilm tubing, and waste tubing off the system into the biosafety cabinet.
c. Place the biofilm tubing horizontally and carefully remove the injection and waste tubings.
d. Let the liquid contents of the biofilm tubing drip into a sterile centrifuge tube.
e. Cut the biofilm tubing from the inlet to the outlet and scrape out the biomass using an inoculation loop. Collect all biomass into the centrifuge tube used above.
f. Adjust the volume of each harvest sample to the same level.
g. Water-sonicate the harvested biofilms at room temperature for 5 min to dissociate the bacterial aggregates. Repeat this step if large aggregates are present in the samples.
h. Vortex the samples before aliquoting for different quantitation methods.

2. Biofilm harvest from the microfluidic devices/3-channel flow-cells

*Note: Most microfluidic devices are sealed with silica glue, plasma bonding, and heat bonding, which are not easy to disassemble for biofilm harvest.*

a. Stop the flow and clamp off both the injection and waste tubings.
b. Shift the devices carefully into the biosafety cabiniet.
c. Replace both the injection and waste tubings with short silica tubing without losing the biofilm contents.
d. Allow the liquid contents to drip into a sterilized centrifuge tube from one side of the device.
e. Connect the other side of the device to a syringe containing PBS buffer.
f. Purge the channel forward and reverse with buffer.
    i. Adjust the volume of each harvested sample to the same level.
    ii. Water-sonicate the harvested biofilms at room temperature for 5 min to dissociate the bacterial aggregates. Repeat this step if large aggregates are present in the samples.
g. Vortex the samples before aliquoting for different quantitation methods.

G. Biofilm observation and quantitation

1. Relative density quantitation: load 100 µl dispersed biofilm suspension into a 96-well microplate with at least 5 replicates for each sample. Read the $OD_{600}$ using a microplate reader. The relative density represents the relative biomass formed in each sample.
2. CFU counting: prepare a serial dilution ($10^1$, $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ ×) of the dispersed biofilm suspension with LB broth. Drop 10 µl diluted suspension onto LB agar plates with at least 5 replicates for each dilution (Herigstad *et al*., 2001). Cultivate in a 37°C incubator for 16 h and subsequently count the colonies.

   *Note: This method usually aims to selectively cultivate bacteria with specific metabolic activities in the biofilm communities.*
3. Flow cytometry analysis (Desai *et al*., 2019): prepare a 1,000× dilution of the dispersed biofilms in 0.9% NaCl buffer. Count and analyze the treated and control biofilms following the manufacturer's protocol.

*Note: This method usually targets the relative quantitation of bacteria with different fluorescent tags.*

4. Omics study (Tan *et al*., 2014; Ding *et al*., 2019): Isolate and purify DNA/RNA/protein from the dispersed biofilm suspension following the manufacturer's instructions for the isolation kit.
   a. Total genome isolation: centrifuge 1 ml dispersed biofilm suspension at 8,000 × *g* for 3 min and remove the supernatant. Isolate total DNA from the bacterial pellets using a DNA isolation kit (QIAamp DNA micro kit).
   b. Total RNA isolation: immediately centrifuge 1 ml dispersed biofilm suspension at 8,000 × *g* for 1 min; carefully wash the cell pellets twice with NaCl. Isolate the total RNA following the manufacturer's instructions for the kit (RNeasy Mini Kit).
      *Note: Snap-freeze the bacterial pellets using liquid nitrogen if the sample handling procedures exceed 10 min.*
   c. Total protein isolation: aliquot 1 ml dispersed biofilm suspension in 2-ml microtubes and place in an ice-water bath. Lyse the bacteria using a probe sonicator at 250 W for 30 min with 4-s intervals per 2-s sonications. Centrifuge the total lysates at 20,000 × *g* for 1 h to collect the total dissolved protein in the supernatant.
5. Biofilm observation and quantitation by confocal laser scanning microscopy (CLSM).
   *Note: The biofilm reactor is only compatible with CLSM systems when its attachment surface is transparent and has a thickness less than 0.5 mm.*
   a. Stop the pump and clamp off both the injection and effluent tubings of the flow-cell.
   b. Shift the whole system with the pump into the CLSM facility room using a trolly.
   c. Place the flow-cell onto the CLSM sample stage with the coverslip facing down (for reversed microscope only).
   d. Start the pump and remove the clamps.
   e. Image the 3D structures, tracing the movement of particles/bacteria, *etc*.
   f. Image analysis approaches:
      Software: Imaris, Comstat2, ImageJ, *etc*.
      Calculation: biovolume of total and each aggregate, relative fluorescence intensity of total and each aggregate, surface coverage, roughness, number of aggregates, *etc*.

H. Clean-up and reuse of tubings
1. Remove parts ⑤ and ⑦ from each system and reconnect the tubing with straight connectors.
2. Fill the feeding bottle with 70% ethanol and flush the tubing system at 1 ml/min for 1 h.
3. Purge the tubing system with air by placing the feeding tubing in the air with the peristaltic pump working at max speed for 10 min.
4. Repeat steps 2-3 three times.
5. Fill the feeding bottle with $H_2O$ and flush the tubing system at 1 ml/min for 1 h.

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6. Purge the tubing system with air by placing the feeding tubing in air with the peristaltic pump working at max speed for 10 min.
7. Repeat steps 5-6 three times.
8. Disconnect each part of the system, dry all parts, and wrap-up all parts in metal foil.

## Data analysis

A. Relative density quantitation

1. Raw data (Table 1)

**Table 1. Demo results for the $OD_{600}$ readings of dispersed biofilms of PAO1 wildtype and mutants**

| Replicates | Blank | PAO1 wild-type | PAO1 mutant1 | PAO1 mutant2 | PAO1 mutant3 | PAO1 mutant4 |
|---|---|---|---|---|---|---|
| 1 | 0.66 | 2.28 | 1.51 | 1.68 | 2.28 | 2.41 |
| 2 | 0.70 | 2.04 | 1.73 | 1.89 | 1.96 | 2.15 |
| 3 | 0.50 | 1.99 | 1.55 | 1.77 | 2.17 | 2.31 |
| 4 | 0.34 | 2.01 | 1.90 | 1.80 | 2.21 | 2.41 |

2. Calculate the average value and standard deviation (Table 2)

**Table 2. Demo results analysis for the $OD_{600}$ readings of dispersed biofilms of PAO1 wildtype and mutants**

| | Blank | PAO1 wild-type | PAO1 mutant1 | PAO1 mutant2 | PAO1 mutant3 | PAO1 mutant4 |
|---|---|---|---|---|---|---|
| Average | 0.55 | 2.08 | 1.67 | 1.79 | 2.16 | 2.32 |
| Stdev | 0.17 | 0.13 | 0.18 | 0.09 | 0.14 | 0.12 |

3. Plot the results (Figure 4)

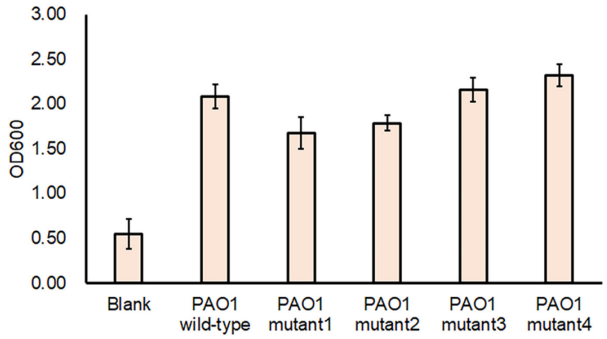


**Figure 4. Demo results showing the relative density of dispersed biofilms of PAO1**

 

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**wildtype and mutants**

B. CFU counting results for the dispersed biofilms (Figure 5)

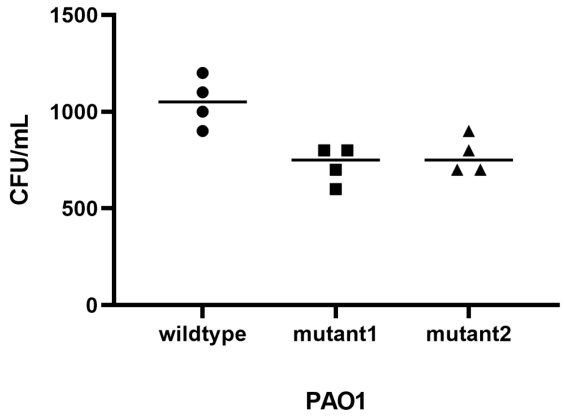


**Figure 5. Demo results for the CFU counting of dispersed biofilms**

C. Flow cytometry analysis (Figure 6)

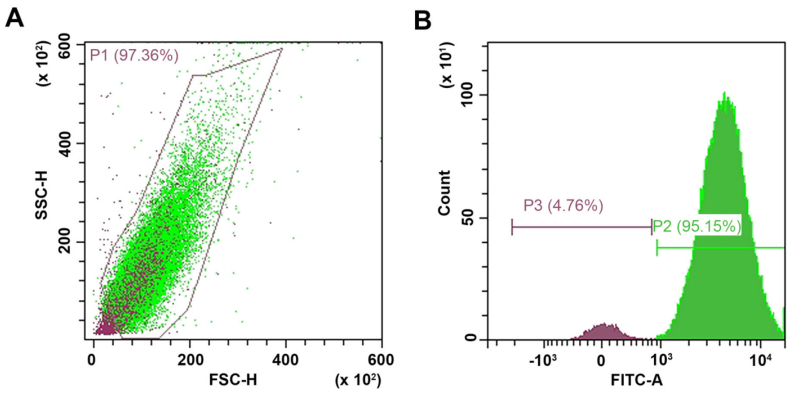


**Figure 6. Demo illustration of the flow cytometry results for dispersed PAO1-GFP-tagged biofilms**

D. Transcriptomics analysis of the biofilms (Figure 7)

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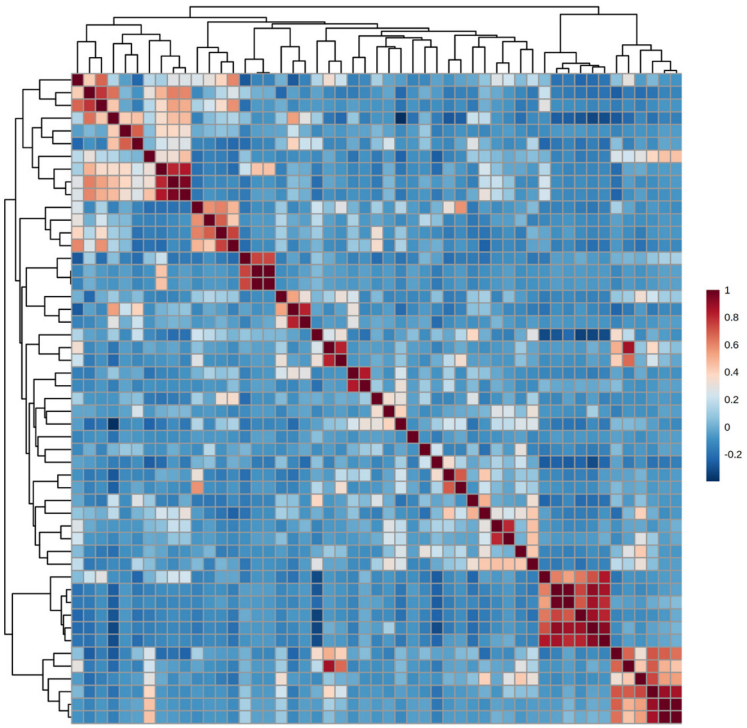


**Figure 7. Demo illustration of the transcriptomics analysis of biofilms: correlation across the significantly dysregulated genes**

E. Biofilm observation and quantitation by CLSM

1. *S. oneidensis* MR1-GFP was cultivated in a 3-channel flow-cell for 7 days. Biofilm morphology was observed by CLSM on days 3, 5 and 7. Representative images are shown in Figure 8.

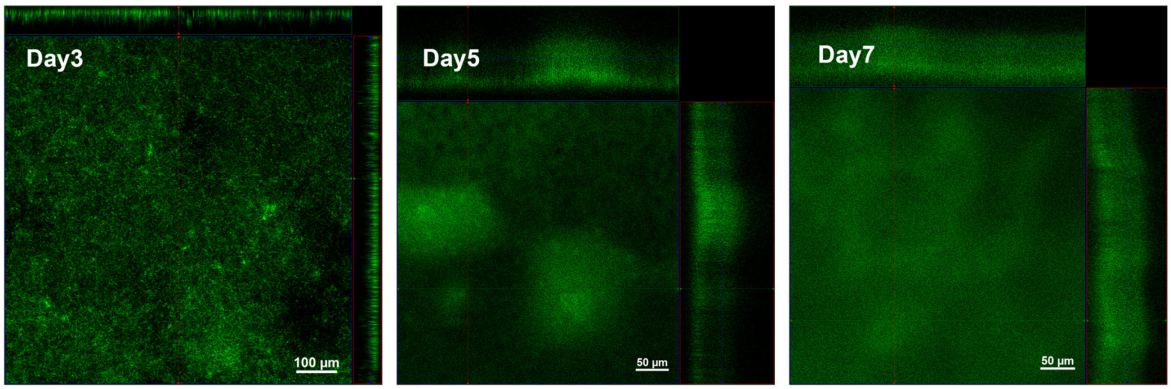


**Figure 8. Ortho images of *S. oneidensis* MR1-GFP biofilms during cultivation.** The biofilm was developed from a thin layer of single microcolonies on day 3 to thick and robust flat biofilms on day 7.

2. The *S. oneidensis* MR1-GFP biofilms were cultivated in microfluidic systems for three days with four different calcium concentrations. Subsequently, biofilm formation was evaluated by CLSM imaging for each concentration, followed by image analysis. As shown in Figure 9, biofilm

 

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formation is positively correlated with calcium supply, with increased biovolume and surface coverage.

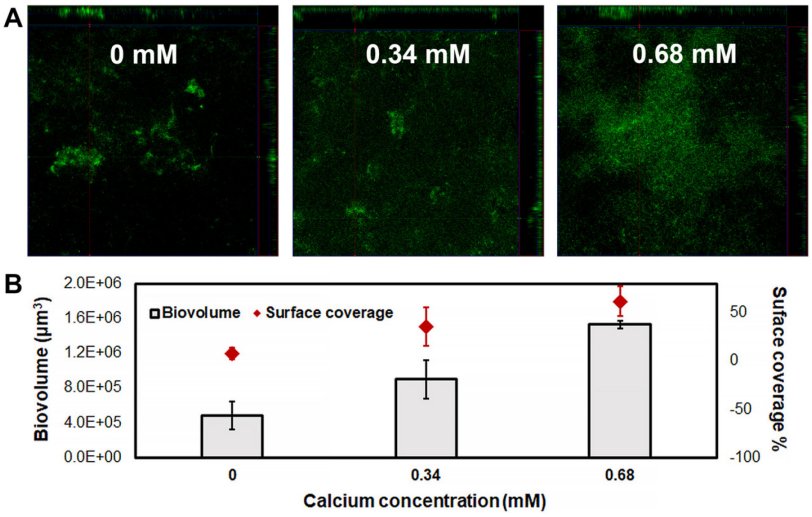


**Figure 9. CLSM images and analysis of sample biofilms cultivated in microfluidic devices with different calcium supplys. A.** CLSM imaging. Green: SYTO9 stain for all biomass. **B.** Biovolume and surface coverage were calculated from the CLSM images.

a. Biovolume analysis of biofilms based on CLSM imaging results
   i. Open the Imaris and create a new “arena” following the manufacturer’s instructions; create respective subfolders (*i.e.*, “Treated,” “Control”).
   ii. Upload CLSM images into the subfolders. For example, load “PAO1-wild type.czi” into the subfolder “Control.”
   iii. In the analysis panel, create a surface with an adjusted threshold. Make sure that the surface covers most of the fluorescence signals.
   iv. Calculate the volume and surface coverage in the mounted “surface.”
   v. Export the volume information into Excel.
   *Note: Each piece of volume information in the list represents the calculated biovolume of an individual microcolony. The sum of the volume list is the total volume of the image.*
b. Surface coverage and roughness analysis based on CLSM imaging results
   i. Make sure that the file format of the images is compatible with Comstat2.
   ii. Start Comstat2 and add folder destinations to upload the image files.
   iii. Choose the target image file and adjust to the proper threshold.
   iv. Choose the target parameters and start the analysis according to the manufacturer’s instructions.
c. Morphology observation based on CLSM imaging results
   i. Open the CLSM images in Zen.

 

ii. In the ORTHO panel, select the focusing point with the most representative X, Y, and Z views.
iii. Adjust the threshold of the image.
*Note: The threshold must be set the same for the same batch of experimental images to avoid imaging bias.*
iv. Generate a new image from the current view.
v. In the new image panel, add a scale bar and adjust its front and line size.
vi. In the Analyzing panel, choose Export with the target file type and location; export the Ortho image.

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## Competing interests

The authors declare no competing financial interests.

## References

1. Coenye, T. and Nelis, H. J. (2010). *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Methods* 83(2): 89-105.
2. Cowle, M. W., Webster, G., Babatunde, A. O., Bockelmann-Evans, B. N. and Weightman, A. J. (2020). Impact of flow hydrodynamics and pipe material properties on biofilm development within drinking water systems. *Environ Technol* 41(28): 3732-3744.
3. Del Pozo, J. L. (2018). Biofilm-related disease. *Expert Rev Anti Infect Ther* 16(1): 51-65.
4. Desai, S., Sanghrajka, K. and Gajjar, D. (2019). High adhesion and increased cell death contribute to strong biofilm formation in *Klebsiella pneumoniae*. Pathogens (Basel, Switzerland) 8(4): 277.
5. Ding, W., Zhang, W., Alikunhi, N. M., Batang, Z., Pei, B., Wang, R., Chen, L., Al-Suwailem, A. and Qian, P. Y. (2019). Metagenomic Analysis of Zinc Surface-Associated Marine Biofilms. *Microb Ecol* 77(2): 406-416.
6. Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P. and Rice, S.A., Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 14(9): 563-575.
7. Herigstad, B., Hamilton, M. and Heersink, J. (2001). How to optimize the drop plate method for enumerating bacteria. *J Microbiol Methods* 44(2): 121-129.

 

8. Gonzalez, A.M., Corpus, E., Pozos-Guillen, A., Silva-Herzog, D., Aragon-Piña, A. and Cohenca, N. (2014). Continuous drip flow system to develop biofilm of *E. faecalis* under anaerobic conditions. *ScientificWorldJournal* 2014:706189-706189.
9. Karande, R., Halan, B., Schmid, A. and Buehler, K. (2014). Segmented flow is controlling growth of catalytic biofilms in continuous multiphase microreactors. *Biotechnol Bioeng* 111(9): 1831-1840.
10. Liu, N., Skauge, T., Landa-Marbán, D., Hovland, B., Thorbjørnsen, B., Radu, F.A., Vik, B.F., Baumann, T. and Bødtker, G. (2019). Microfluidic study of effects of flow velocity and nutrient concentration on biofilm accumulation and adhesive strength in the flowing and no-flowing microchannels. *J Ind Microbiol Biotechnol* 46(6): 855-868.
11. Martin, I., Waters, V. and H. Grasemann (2021). Approaches to targeting bacterial biofilms in cystic fibrosis airways. *Int J Mol Sci* 22(4): 2155.
12. Merritt, J.H., Kadouri, D.E. and O'Toole, G.A.(2005). Growing and analyzing static biofilms. *Curr Protoc Microbiol* Chapter 1: Unit-1B.1.
13. Mattei, M.R., Frunzo, L., D'Acunto, B., Pechaud, Y., Pirozzi,F. and Esposito, G., (2018). Continuum and discrete approach in modeling biofilm development and structure: a review. *J Math Biol* 76(4): 945-1003.
14. Pamp, S. J., Sternberg, C. and Tolker-Nielsen, T. (2009). Insight into the microbial multicellular lifestyle via flow-cell technology and confocal microscopy. *Cytometry A* 75A(2): 90-103.
15. Sternberg, C. and Tolker-Nielsen. T. (2006). Growing and analyzing biofilms in flow cells. *Curr Protoc Microbiol* Chapter 1: Unit 1B 2.
16. Tan, S.Y., Liu, Y., Chua, S.L., Vejborg. R.M., Jakobsen, T.H., Chew, S.C., Li, Y., Nielsen, T.E., Tolker-Nielsen, T., Yang, L. and Givskov, M. (2014). Comparative systems biology analysis to study the mode of action of the isothiocyanate compound Iberin on Pseudomonas aeruginosa. *Antimicrob Agents Chemother* 58(11): 6648-6659.
17. Van Dyck, K., Pinto, R.M., Pully, D. and Van Dijck, P. (2021). Microbial interkingdom biofilms and the quest for novel therapeutic strategies. *Microorganisms* 9(2): 412.
18. Weiss Nielsen, M., Sternberg, C., Molin, S. and Regenberg, B. (2011). Pseudomonas aeruginosa and Saccharomyces cerevisiae biofilm in flow cells. *J Vis Exp* (47): 2383.
19. Winn, M., Casey, E., Habimana, O. and Murphy, C.D. (2014). Characteristics of Streptomyces griseus biofilms in continuous flow tubular reactors. *FEMS Microbiol Lett* 352(2): 157-164.
20. Zhang, W., Sileika, T.S., Chen, C., Liu, Y., Lee, J. and Packman, A.I. (2011). A novel planar flow cell for studies of biofilm heterogeneity and flow-biofilm interactions. *Biotechnol Bioeng* 108(11): 2571-2582.
21. Zhang, Y., Li, C., Wu, Y., Zhang, Y., Zhou, Z. and Cao, B. (2019). A microfluidic gradient mixer-flow chamber as a new tool to study biofilm development under defined solute gradients. *Biotechnol Bioeng* 116(1): 54-64.

 