

Supporting Information
for
Biofilm Development on Carbon Nanotube/Polymer
Nanocomposites

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MWCNT Oxidation

Pristine MWCNTs were oxidized by adding 1 gram of MWCNTs (Nanocyl NC7000, outer diameter 9.5 nm, 1.5 μ m length, 90% purity) to 0.3 M HNO₃ and refluxing for 5 h at 110 °C. The resulting O-MWCNTs were washed with deionized water continuously until the pH of the filtrate reached 7 and then dried in an oven (Forced Convection Oven, Felisa Ovens, Zapopan, Jalisco, Mexico) overnight at 80 °C.¹ The O-MWCNTs had an oxygen content of 4.1% as determined by X-ray photoelectron spectroscopy (XPS) (PHI 5600 XPS, 58.7 eV pass energy, 0.125 eV/step, Mg K α X-rays) and CasaXPS software (Teignmouth, UK).

Octadecyltrichlorosilane (OTS) Modification of Glass Slides

Reaction of OTS with Glass Slide Surfaces

Glass slides (1 x 25 x 75 mm, FisherBrand Frosted Microscope Slides, Cat #12-550-13) were first soaked in detergent solution (Alconox, Cat #1004) for 30 min and then rinsed with Milli-Q water. Slides were next etched with 4.0 M NaOH for 30 min, rinsed with Milli-Q water, and dried in a uniform stream of nitrogen gas. Subsequently, the clean glass slides were placed in a gravity convection oven (Model 16 GCA, cat.# 31477, Precision) at 85 °C and dried overnight. The next day, slides were immersed in a 0.2% v/v OTS/hexane (95% GR ACS, Cat #HX0302-3, EMD) solution for 30 min and then ultra-sonicated in hexanes followed by ethanol to remove excess OTS and hexanes, respectively. Slides were then dried with a uniform stream of nitrogen, put in the oven at 85 °C for 30 min, and autoclaved.²⁻⁵

Characterization of OTS Modification

XPS analysis of OTS-modified slides revealed a decrease in the Si(2p) signal and an increase in the C(1s) signal compared to a native glass microscope slide, consistent with the expectations of OTS modification. Specifically, we used the equation, $d = \lambda \cos\theta \ln\left(\frac{I_0}{I}\right)$,⁶ where λ is the inelastic mean free path

of the silicon (2p) photoelectron from the literature (2.7 nm),⁷ θ is the take-off angle (54.7°), (I_0/I) represents the fractional change in the Si(2p) signal after OTS modification, and d is the depth of the OTS overlayer. The decrease in Si(2p) signal was indicative of a film approximately 1 nm thick over a large sample area (0.8 x 2 mm). These results indicate that the glass slide was sufficiently coated by an OTS layer to allow the CNT/PCL samples to attach to the underlying support. A lack of CNT/PCL detachment in aqueous media in all aspects of this study provided further evidence that the OTS layer firmly adhered the CNT/PCL to the OTS-modified glass surface.

Preparation of PCL and CNT/PCL Samples

Spray-Coating PCL and O-CNT/PCL Nanocomposites on OTS-modified Glass Slides for Biofilm Studies

To prepare CNT/PNC samples, OTS-modified glass slides were placed onto a hot plate (Fisher Scientific™ Isotemp™ Basic Stirring Hotplates) at 85 °C and sprayed with approximately 4 mL (200 sprays) of CNT/PNC casting solution at a pressure of 30 PSI using a Badger 200 Single Action, Internal Mix Series Air-brush (Badger Air-Brush Co. Franklin Park, IL). Two slides were placed in a row and sprayed together from a distance of approximately 15 cm. After spray-coating, samples were immediately put into separate sterile containers. Spray-coating was performed inside a sterile biosafety cabinet (Labconco Purifier Class II Biosafety Cabinet) as shown in Figure S2.

Slow-drying Thicker CNT/PCL Nanocomposites for Photolysis

To prepare thicker CNT/PCL nanocomposites suitable for photolysis, the 2% w/w O-MWCNT/PCL casting solution was added in 5 mL aliquots to aluminum dishes (44 mm diameter, 12.5 mm height, Fisherbrand) and the CNT/PCL samples were subsequently generated by solvent evaporation overnight. Once the CNT/PCL nanocomposites had dried, they were peeled from the aluminum dishes.

Spray-Coating CNT/PVOH Nanocomposites for SEM

As a comparison to O-MWCNT/PCL nanocomposites, the surface structure of an O-MWCNT/PVOH nanocomposite with the same O-MWCNT loading was imaged using SEM. To prepare these O-MWCNT/PVOH nanocomposites, a 2 mg/mL stock PVOH solution (Sigma Aldrich, $M_w=31,000$ -50,000, 98%-99% hydrolyzed) and a 0.05 mg/mL O-MWCNT stock suspension (NanoLab Inc., PD15L5-20-COOH, Lot. # 06-16-10, outer diameter 15 ± 5 nm, length 5-20 μm from the manufacturer) were combined aseptically to prepare a casting solution containing 2% w/w O-MWCNT/PVOH. A pure PVOH solution was also prepared as a reference. These casting solutions were then shaken vigorously, sonicated for 5 min, and added to spray bottles. Autoclaved glass slide pieces (1 cm^2) were placed onto a hot plate at $150\text{ }^\circ\text{C}$ and sprayed 30 times ($1.07\text{ mL/spray} \pm 0.05\text{ mL/spray}$) from a consistent distance (approximately 25 cm) in 10 s intervals to flash dry the casting solution upon contact. All spray-coating was carried out inside a sterile biosafety cabinet (Labconco Purifier Class II Biosafety Cabinet).

SEM Analysis

For SEM analysis, PCL, CNT/PCL, PVOH, and CNT/PVOH nanocomposites were spray-coated onto 1-cm^2 -sized OTS-modified glass pieces according to the procedures described in Section I of the experimental section and sputter-coated with platinum (Quorum Technologies Polaron SC7640 Auto/Manual High Resolution Sputter Coater, 12 mA/800V plasma current, and ~ 3 min at 0.5 nm/min) to prevent charging under the electron beam. A cold-cathode field emission scanning electron microscope (JEOL 6700F, 10 keV, 8.0 nm working distance, LEI & SEI detectors) with a 1.0 nm resolution at 15 keV was used to image samples. For each sample, replicate images (≥ 5 images) of random areas across the surface were taken. To demonstrate sample-to-sample consistency, 2% w/w O-MWCNT/PCL nanocomposites were spray-coated on different occasions and imaged.

Microbial Frozen Stocks

To prepare the frozen stocks, cells were grown in LB broth (Lennox, Fisher Scientific) overnight at 37 °C to the exponential phase. The cell suspensions were distributed into 1 mL Eppendorf tubes with sterile glycerol (10% v/v final concentration) and stored at -80 °C.

Basal Mineral Media (BMM) Composition

BMM contained 7.18 mM K₂HPO₄, 2.79 mM KH₂PO₄, 0.757 mM (NH₄)₂SO₄, 0.0406 mM MgSO₄*7H₂O, and 0.5 v/v% trace elements solution (3.75 mM H₃BO₃, 0.0605 mM ZnSO₄*7H₂O, 0.0296 mM FeSO₄(NH₄)₂SO₄*6H₂O, 0.034 μM CoSO₄*7H₂O, 26 μM (NH₄)₆Mo₃O₂₄*4H₂O, 32 μM CuSO₄*5H₂O, and 36 μM MnSO₄*4H₂O).

LIVE/DEAD Staining and Biofilm Imaging

LIVE/DEAD Staining Procedure

The LIVE/DEAD biofilm viability kit consists of two nucleic acid molecular probes: green-fluorescent SYTO 9 to stain living cells and red-fluorescent propidium iodide (PI) to stain dead cells. An aqueous stain stock solution was prepared containing 3 μL of 3.34 mol/L SYTO 9 in dimethylsulfoxide (DMSO) and 3 μL of 20 mmol/L propidium iodide (PI) in DMSO for every 1 mL of autoclaved Milli-Q water.⁸ This stock solution was shaken and covered with aluminum foil to minimize light exposure to the photosensitive stains. After an experiment, samples were transferred to a 20 mL depleted media (DM) bath to remove loosely adhered cells by holding the frosted end of the underlying glass slides with forceps since this was an area of the sample that was never imaged. Immediately after removal from DM, 0.5 mL of the aqueous stain stock solution was added gently to the samples and left to sit for 20 min in the dark.

Samples were then washed, stained, washed, and coated in VectaShield in a staggered fashion and immediately brought to the Integrated Imaging Center at JHU for imaging.

Several staining procedures were followed that were utilized in our previous study. These included prevention of sample dry-out during the staining process, washing samples with depleted media to maintain ionic strength and prevent lysis, and complete thawing of the frozen LIVE/DEAD stain stocks to ensure the proper concentrations of stains were used.

Confocal Laser Scanning Microscopy (CLSM) Imaging

CLSM imaging was performed using a Zeiss LSM 510 Multiphoton Confocor 3 laser scanning microscope (Carl Zeiss, Germany) in the Integrated Imaging Center at JHU. Images were obtained using a 40x water immersion objective with a 1.2 numerical aperture (N.A.). A 488 nm Argon laser (30 mW at 5% output) to excite the SYTO 9-stained cells and a 543 nm He/Ne laser (1.2 mW) to excite PI-stained cells were used in dual channel mode. The gains were adjusted using the brightest red and green cells in the range indicator mode: this was possible since the biofilms always contained a small number of living and dead cells. Images were obtained using ZEN software (Carl Zeiss, 2009, Thornwood, NY).

LIVE/DEAD Staining Controls

Background Staining Controls

PCL, 2% w/w O-MWCNT/PCL, and photodegraded 2% w/w O-MWCNT/PCL samples were LIVE/DEAD stained in the absence of *P. aeruginosa*. This allowed the CNT/PNC contribution to the fluorescence background to be assessed. A small amount of background fluorescence was present for all three PCL-containing samples, although the laser gains had to be increased by approximately 10% on these confocal image to obtain a visibly fluorescent image.⁹ Additionally, the background fluorescence faded

during imaging which was not the case for the biofilms imaged in this study. Nevertheless, care was taken to eliminate any contribution from PCL or CNT fluorescence by choosing the lower boundary of the confocal stack based on a strong fluorescent signal and clear evidence of microbial shapes.

OTS Staining Control

OTS-modification of glass slides is often used in studies involving microorganisms and has not been shown to cause cell death.^{10, 11} Nevertheless, to ensure OTS had no cytotoxic effect on *P. aeruginosa*, OTS slides were inoculated under static conditions for 6 h, a sufficient length of time to coat the OTS slides with microorganisms. Samples were LIVE/DEAD stained and imaged in replicate areas according to the protocol used in this study (Figure S16). In line with expectations, results from these studies revealed that minimal cell death occurred on OTS-modified glass surfaces.

Confocal Image Analysis

COMSTAT 2 Analysis of Biofilm Images

Since SYTO 9 stains all biofilm cells while PI only stains dead cells, the green fluorescent (SYTO 9) channel was chosen to determine biomass and biofilm thickness. Since SYTO 9 only weakly fluoresces in dead cells, the green fluorescent signal threshold was increased to 150 for COMSTAT 2 analysis to ensure all biofilm cells were counted. Connected volume filtering was used to exclude planktonic cells in the biofilm analysis.¹²⁻¹⁴

Manual Thickness Measurements of Confocal Images for Comparison to COMSTAT 2 Analysis

To validate the COMSTAT 2 results, the COMSTAT 2 thickness analysis was compared to manual thickness measurements using ImageJ software (NIH, Bethesda, MD) for PCL and 2% w/w O-MWCNT/PCL samples under both DFR and static conditions. Manual thickness measurements were

made in five areas of each biofilm using the side view of each reconstructed XYZ projection. At least 6 replicate side views were measured and averaged for each biofilm regime. The dead layer thicknesses were also measured manually for the red fluorescent microbial layer that formed under drip flow reactor conditions (Figure 2) and for the red-fluorescent microbial layer that formed on the photodegraded CNT/PCL nanocomposites (Figure 5). Only manual thickness measurements were made for the photodegraded CNT/PCL nanocomposites because COMSTAT 2 analysis of the biofilm coating was not possible with the curved configuration of the underlying CNT/PNC (wrapped around a glass slide for photodegradation).

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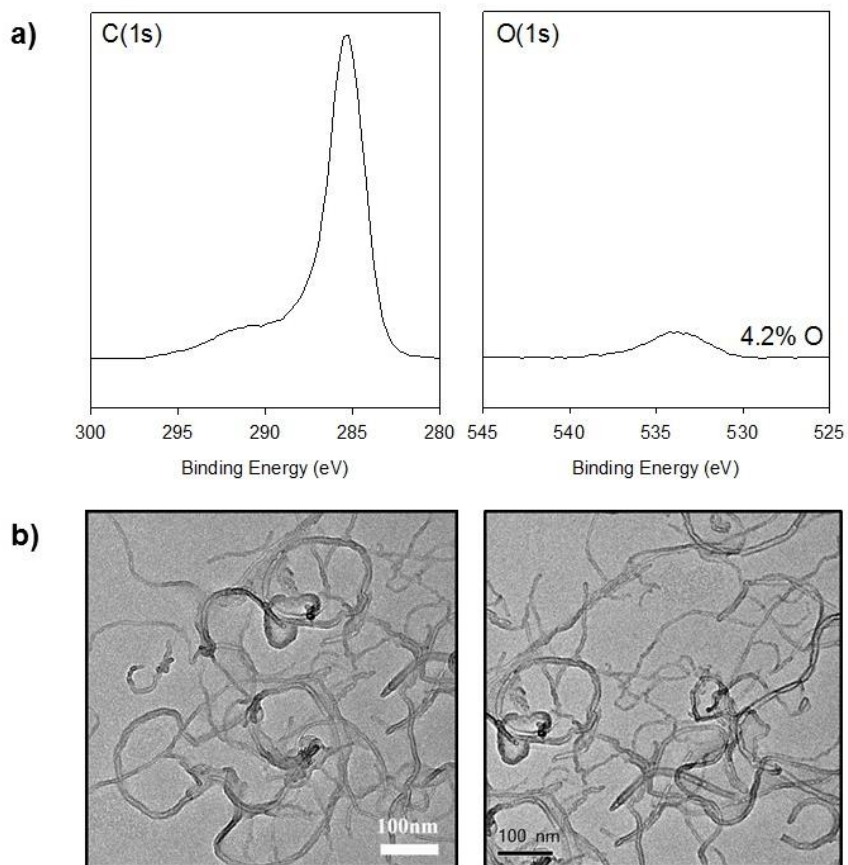


Figure S1: Characterization of O-MWCNTs by a) XPS and b) TEM. Using the TEM images, an O-MWCNT diameter distribution of 9.4 ± 1.2 nm was calculated as the average of 15-20 measurements using DigitalMicrograph software.

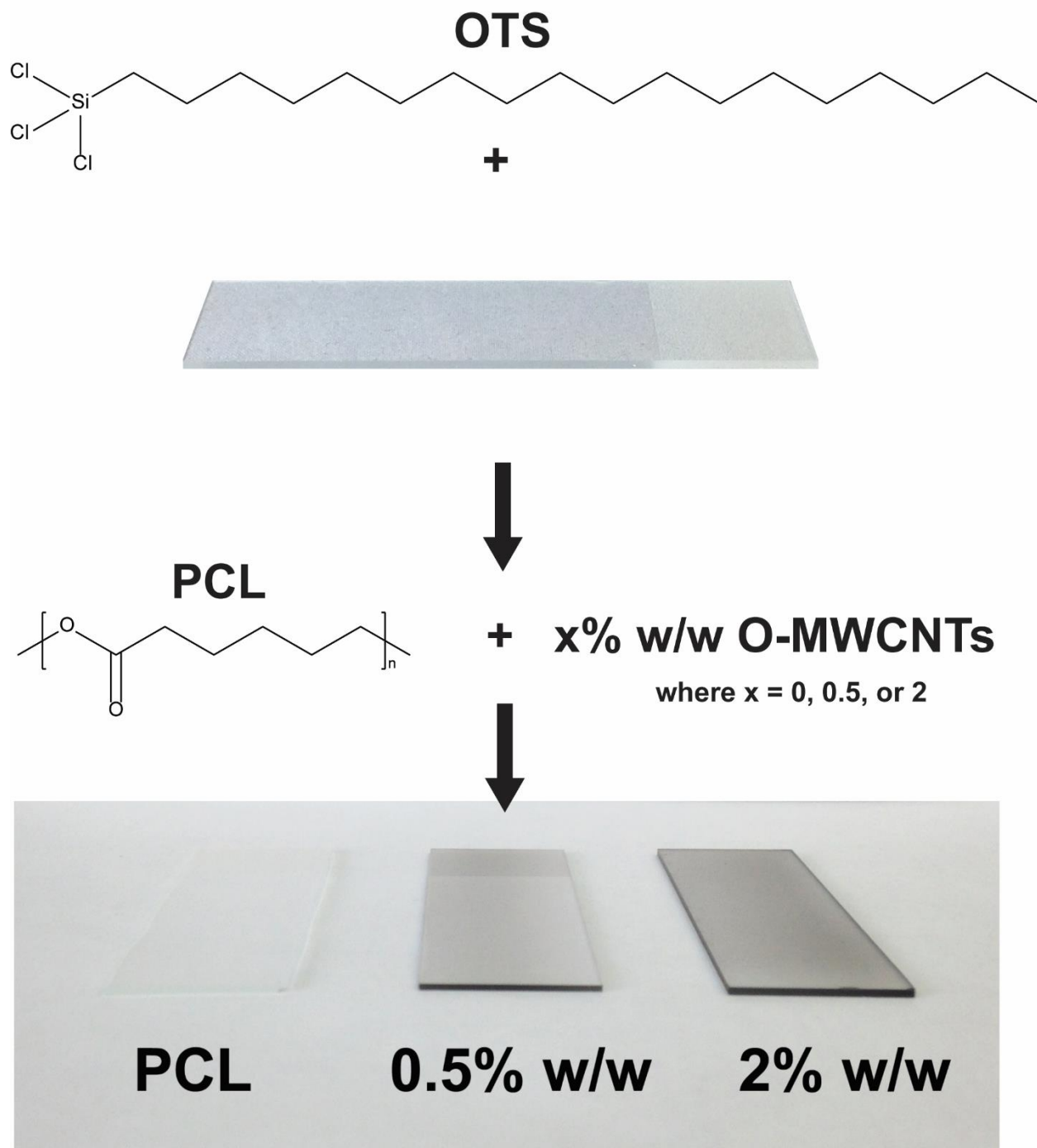


Figure S2: Octadecyltrichlorosilane-modified glass slides spray-coated with PCL, 0.5% w/w O-MWCNT/PCL, and 2% w/w O-MWCNT/PCL casting solutions.

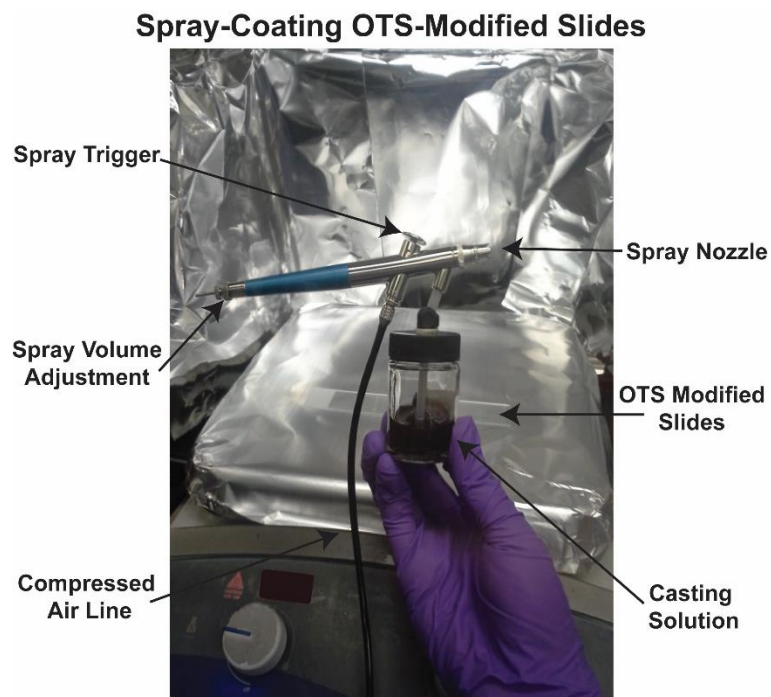


Figure S3: The setup used to spray-coat the CNT/PNC casting solutions onto OTS-modified glass slides.

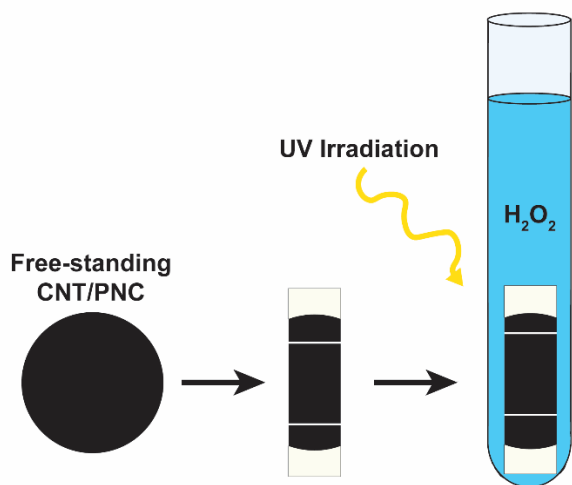


Figure S4: An illustration depicting the simulated weathering of CNT/PNCs. 2% w/w O-MWCNT/PCL nanocomposites were photolyzed in the presence of H_2O_2 to accumulate CNTs at the surface of the nanocomposite prior to microbial exposure.

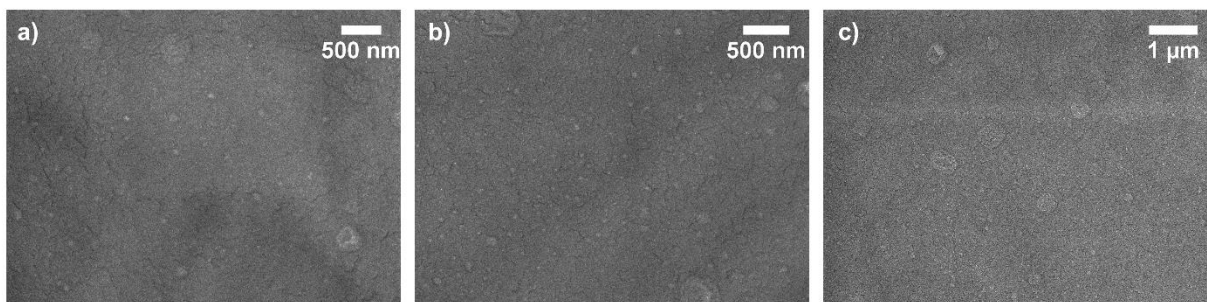


Figure S5: SEM images of PCL samples in replicate areas.

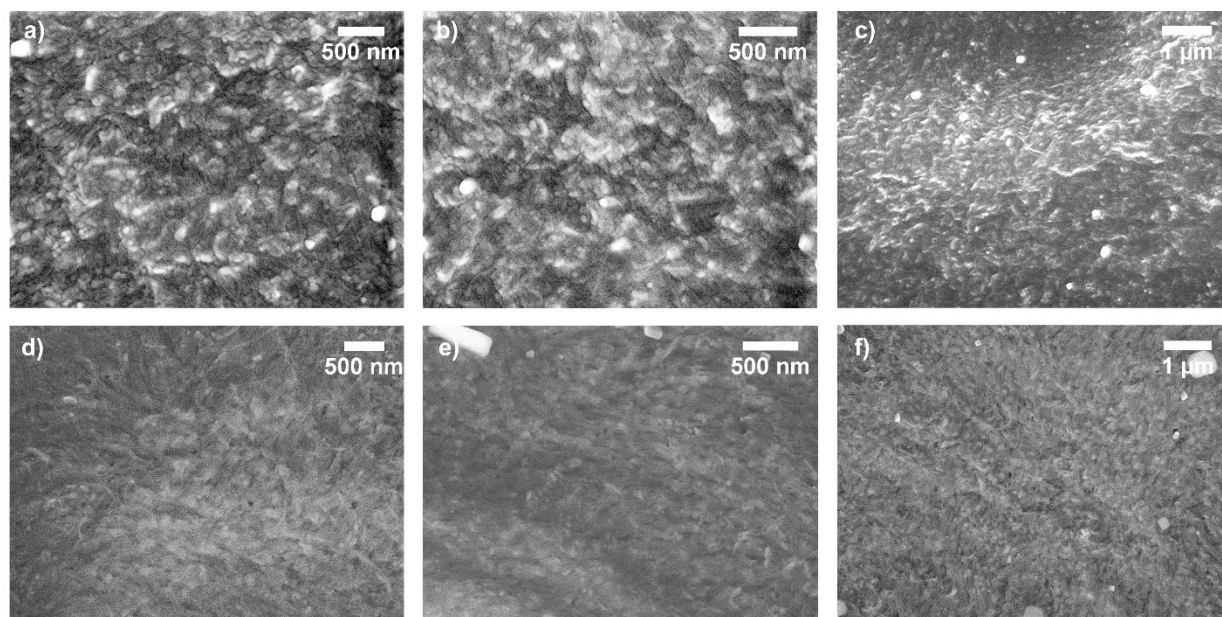


Figure S6: SEM images of 2% w/w O-MWCNT/PCL samples. The top row and second row are SEM images from two separately prepared samples. Each row contains replicate areas from that particular sample.

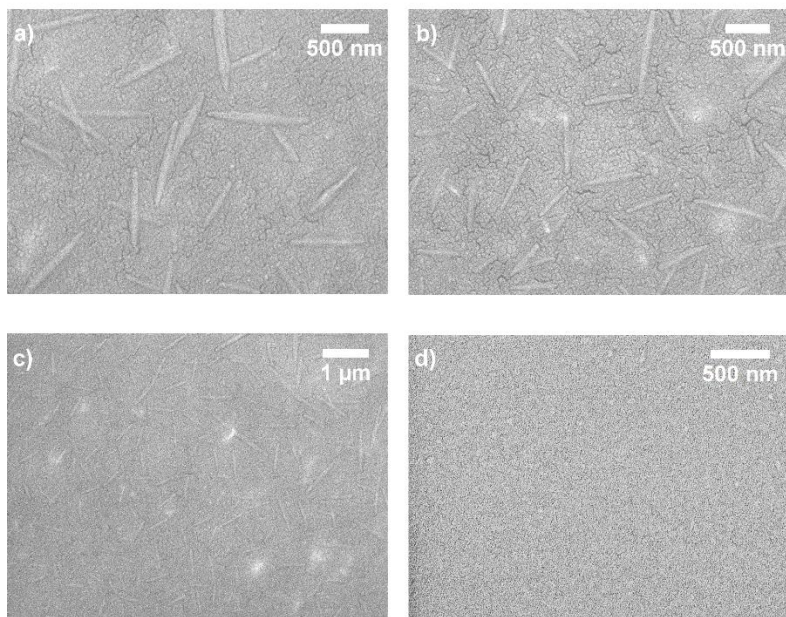


Figure S7: SEM images of 2% w/w O-MWCNT/PVOH (a-c) samples in replicate areas as well as pure PVOH (d) for reference.

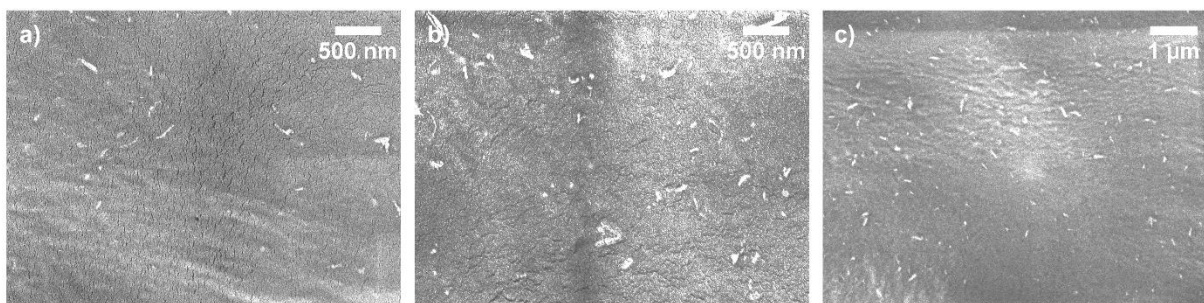


Figure S8: SEM images of 0.5% w/w O-MWCNT/PCL samples in replicate areas.

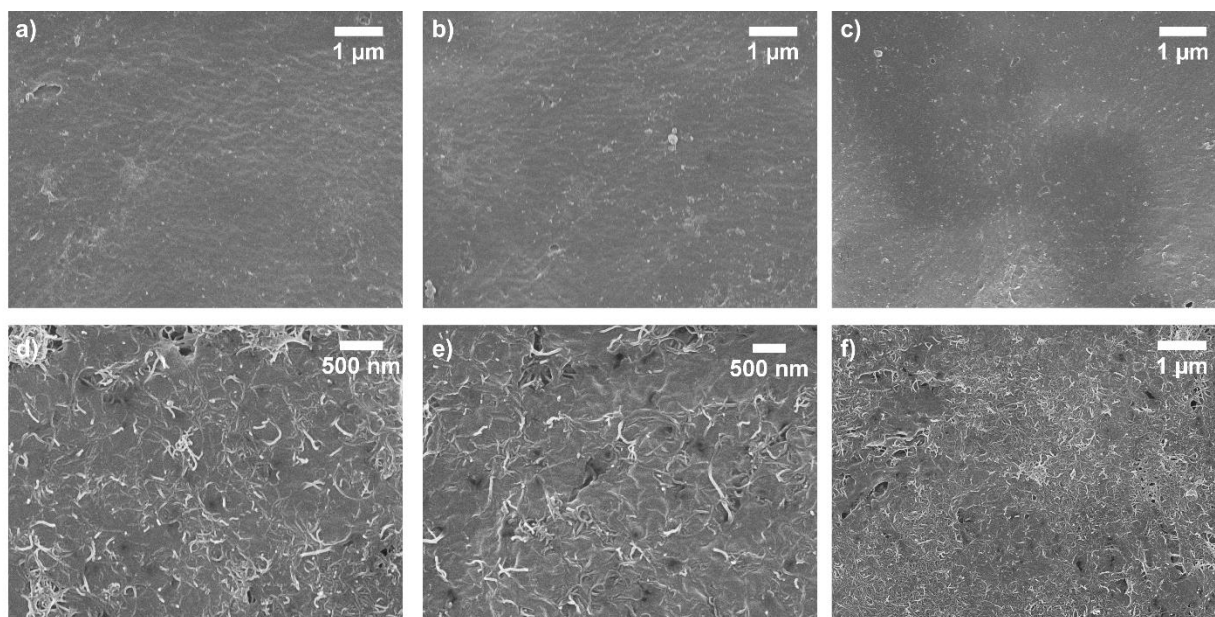


Figure S9: SEM of thick 2% w/w O-MWCNT/PCL nanocomposites (replicate areas) with no underlying support before (a-c) and after (d-f) irradiation in the presence of 0.5 M H_2O_2 .

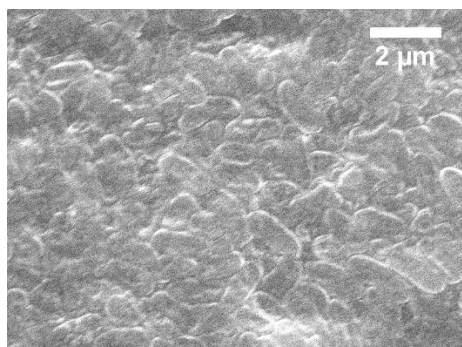


Figure S10: SEM image of a 2% w/w O-MWCNT/PCL nanocomposite surface after an attempt to remove attached biofilms with sodium polyphosphate. Despite treatment, biofilms still covered the underlying material.

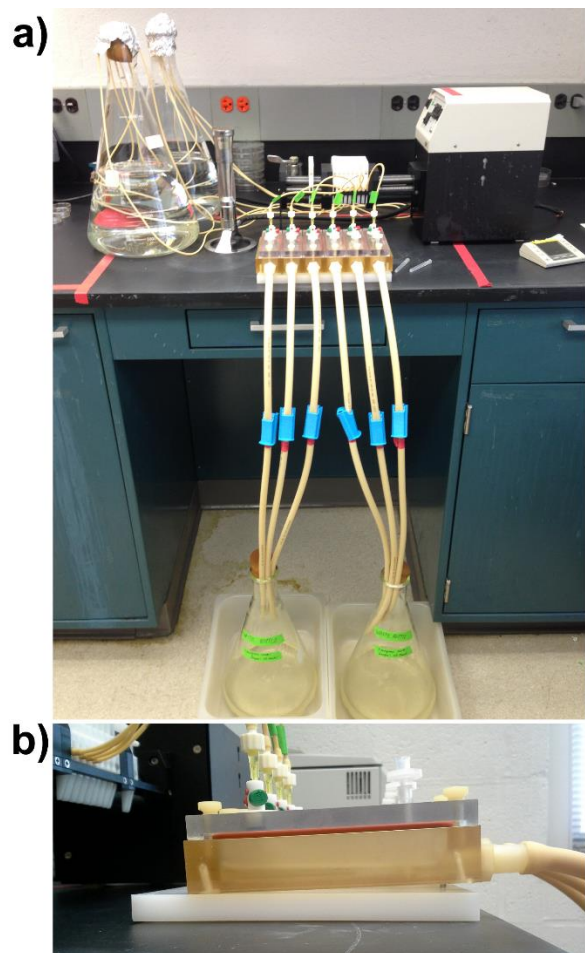


Figure S11: Drip flow reactor setup (a) with a side view of the negative angle used to fully cover the hydrophobic slides (b).

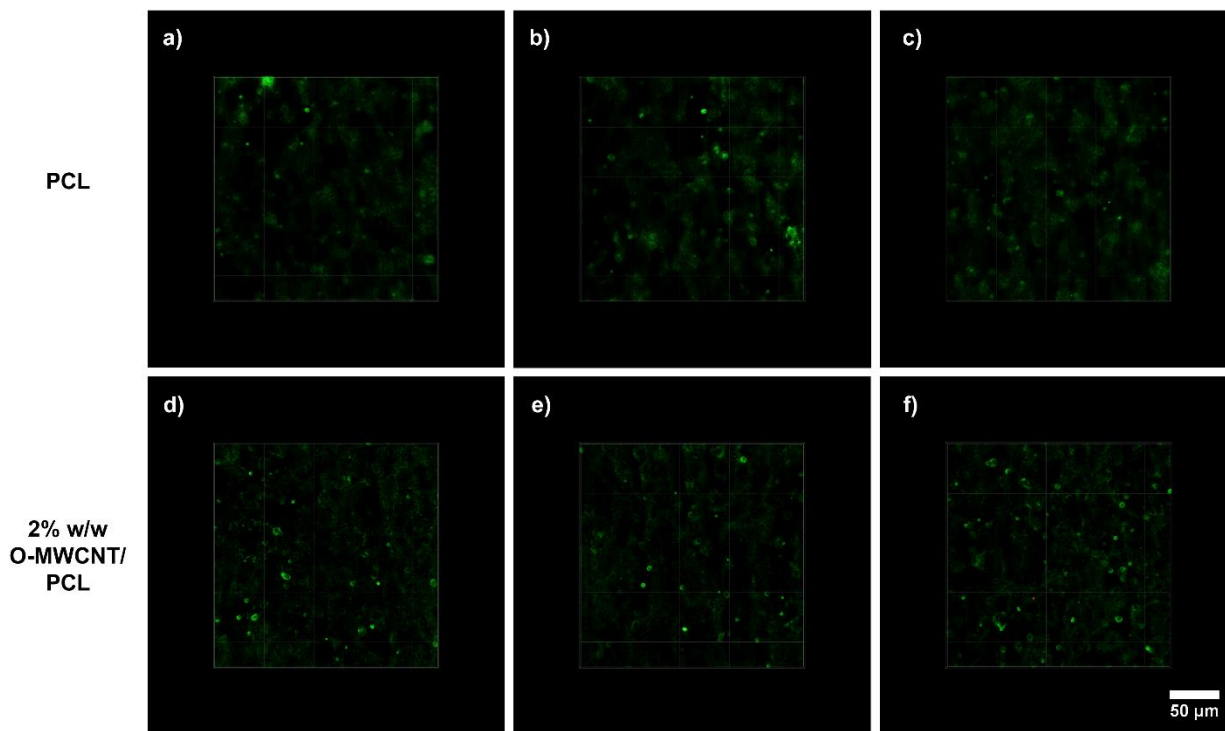


Figure S12: Abiotic background fluorescence controls of LIVE/DEAD stained PCL and 2% w/w O-MWCNT/PCL nanocomposite surfaces. Gains were adjusted approximately 10% higher than those used for biofilm-containing samples to make the background staining slightly more visible in the images.

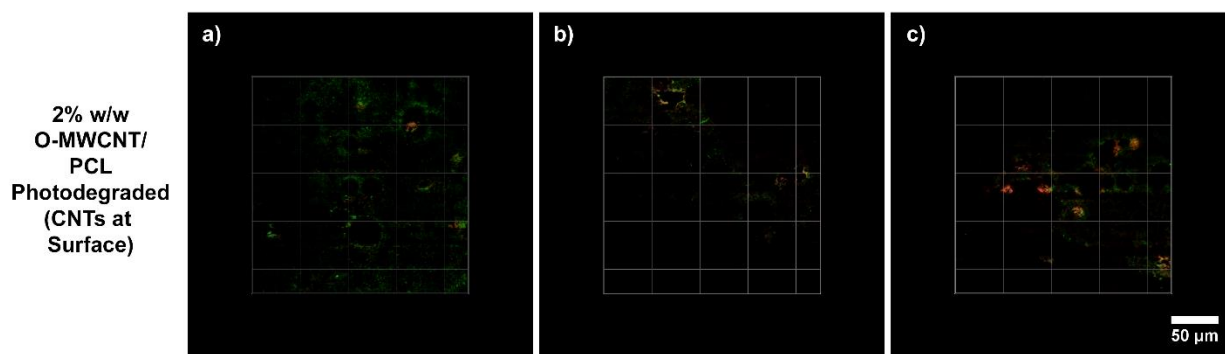


Figure S13: Abiotic background fluorescence controls of LIVE/DEAD stained 2% w/w O-MWCNT/PCL nanocomposite surfaces that had been photodegraded. Gains were adjusted approximately 10% higher than those used for biofilm-containing samples to make the background staining slightly more visible in the images.

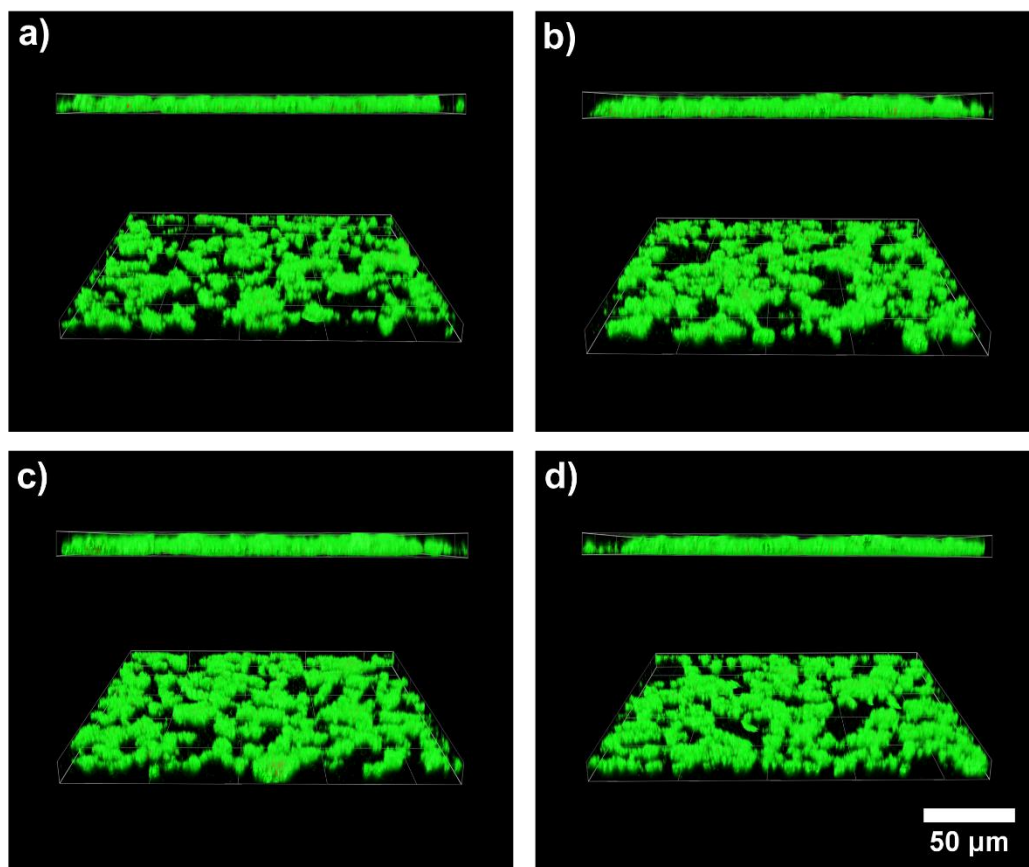


Figure S14: CLSM images of LIVE/DEAD stained biofilms on glass slides grown under static conditions for 2 weeks (similar to Regime 3 in Figure 4). Replicate areas of the same sample are shown.

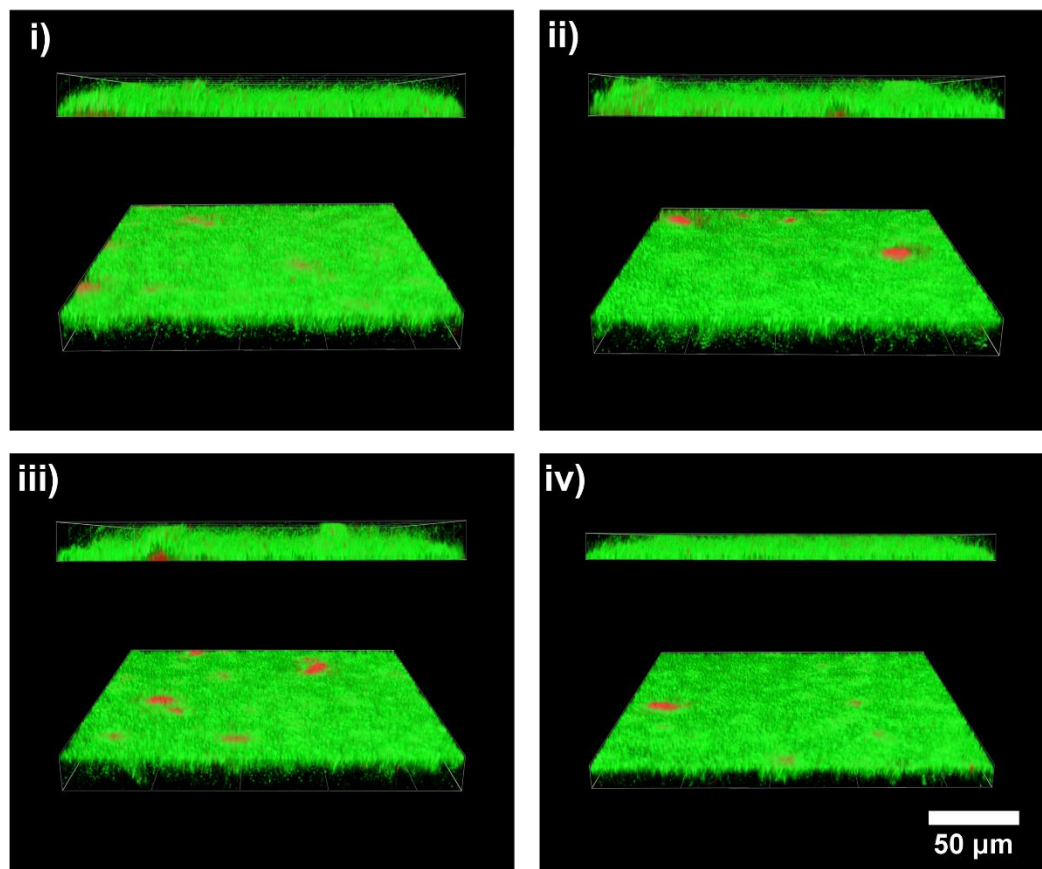


Figure S15: CLSM images of LIVE/DEAD stained biofilms on glass slides grown under DFR conditions for 72-96 h (similar to regime 4 in Figure 2). Replicate areas of the same sample are shown.

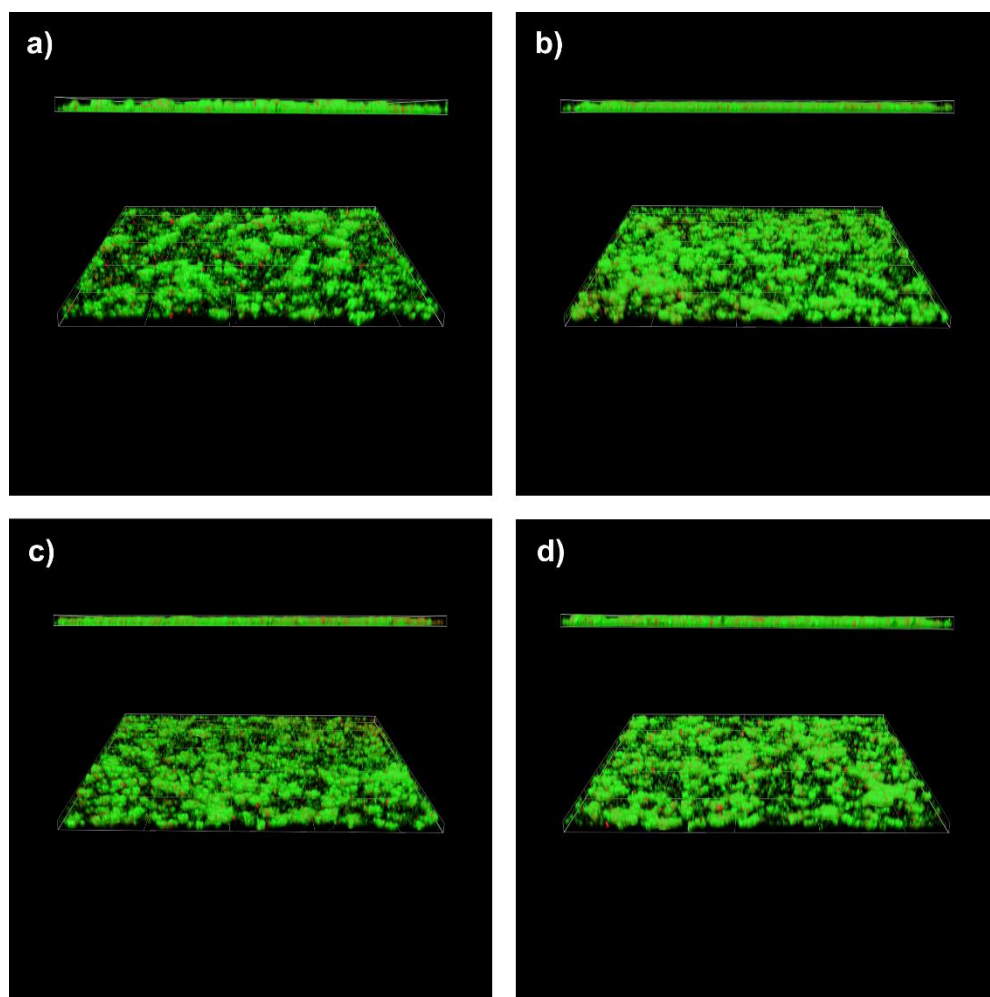
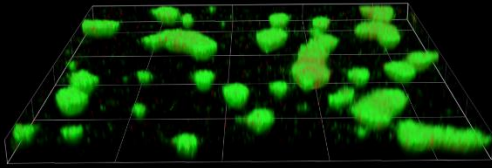


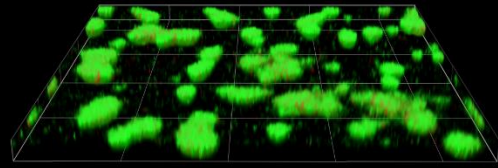
Figure S16: CLSM images of LIVE/DEAD stained biofilms grown on OTS-modified glass slides under static conditions for 6 h. Minimal cell death occurred as indicated by the large number of green-fluorescent cells. Replicate areas of the same sample are shown.

i)

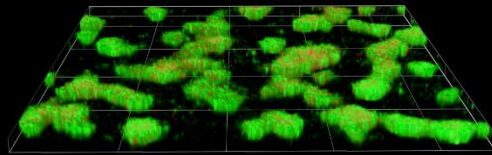


PCL Regime 1

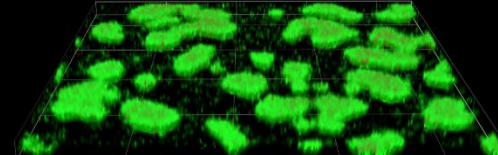
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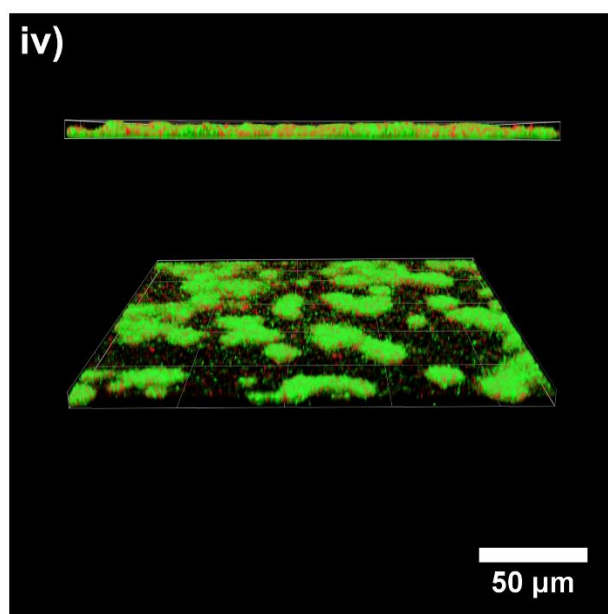
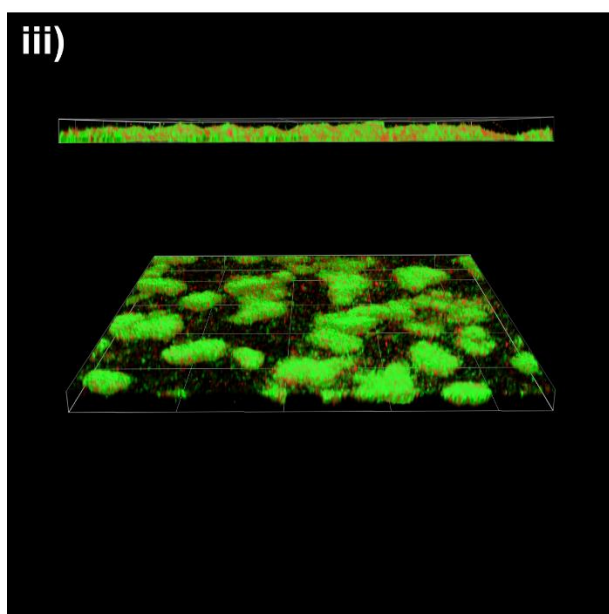
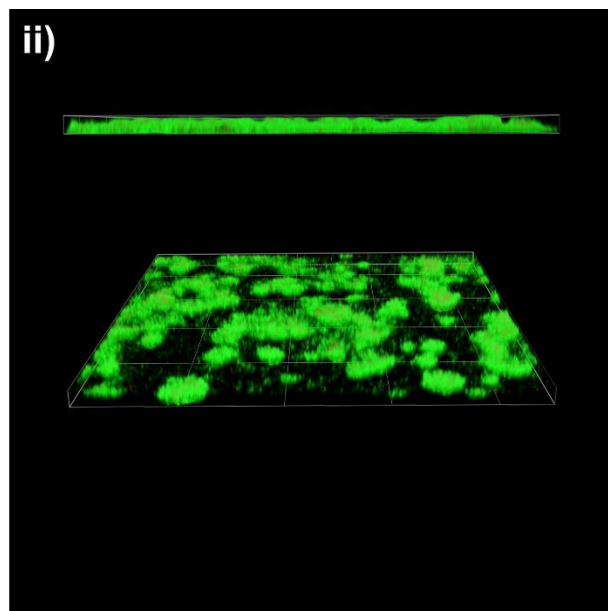
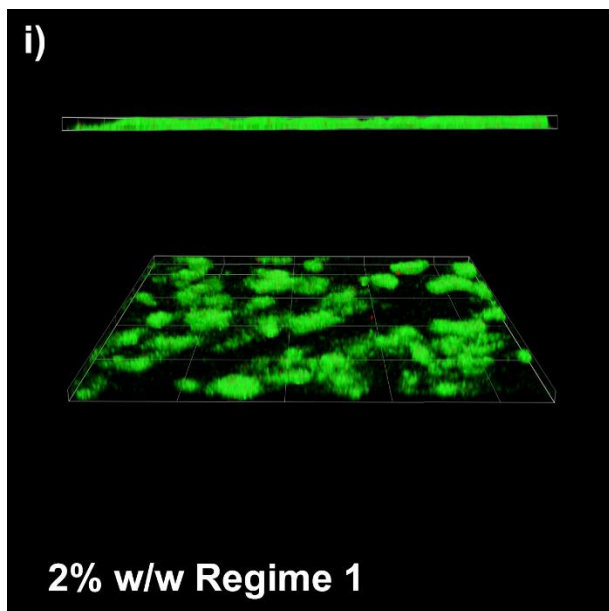
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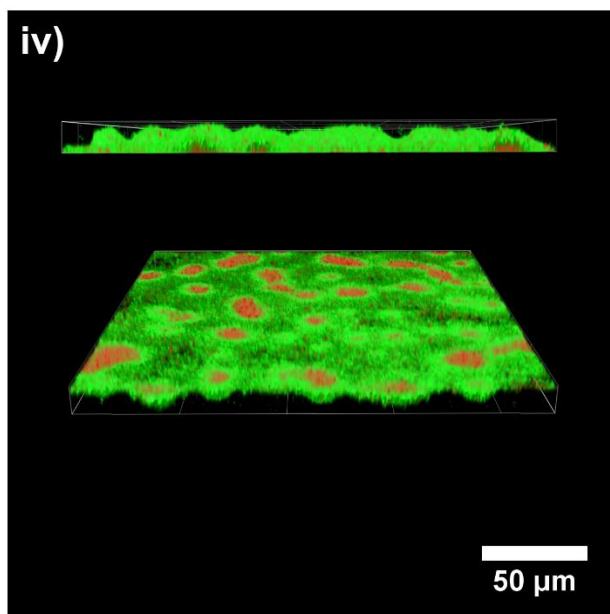
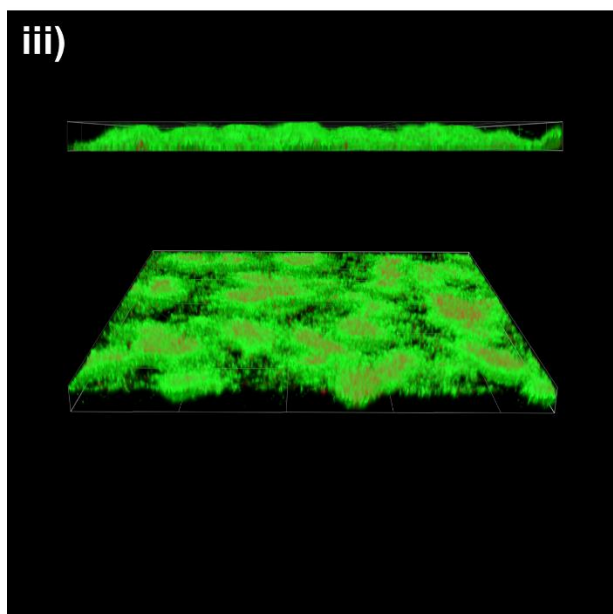
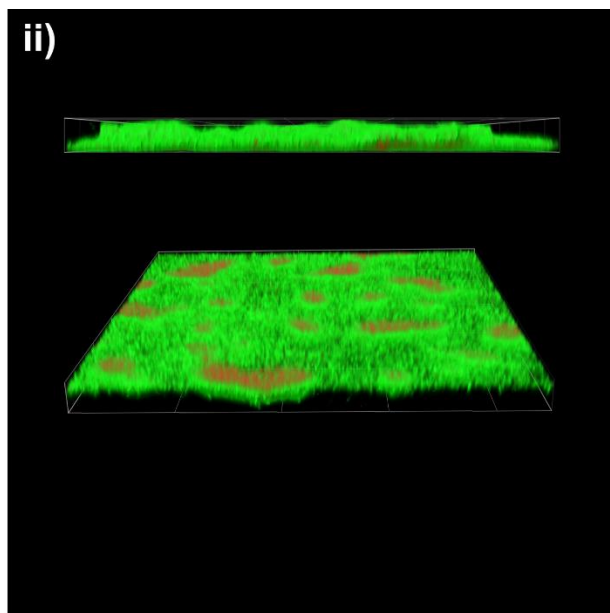
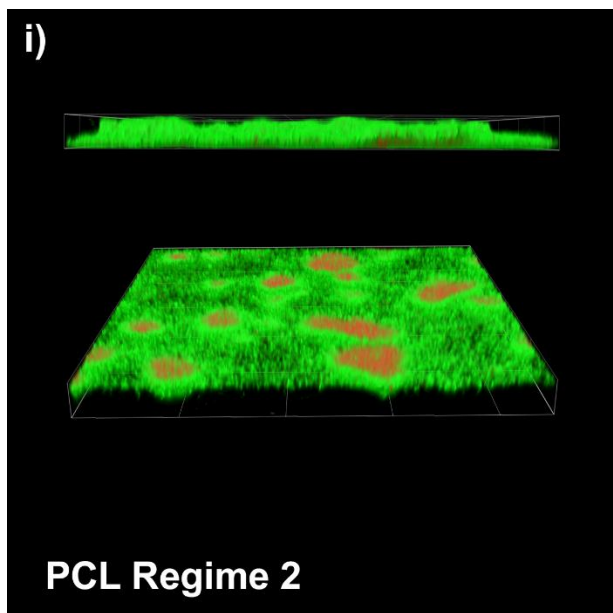


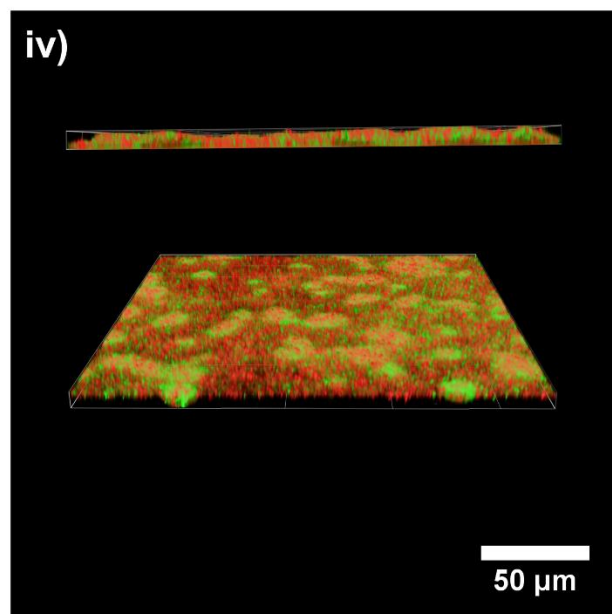
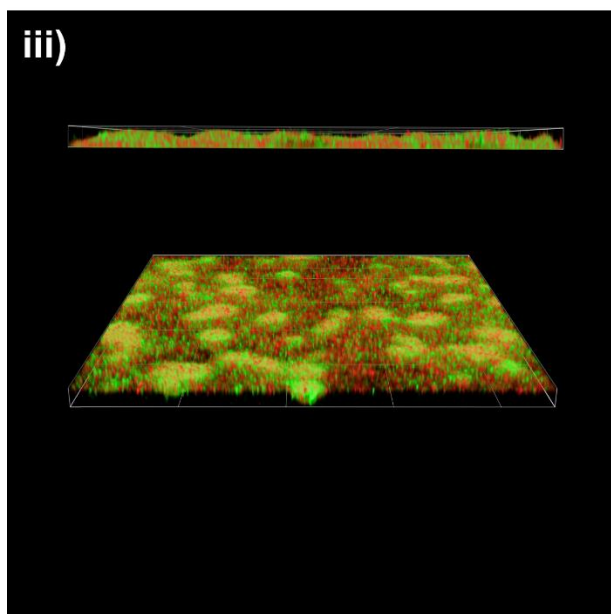
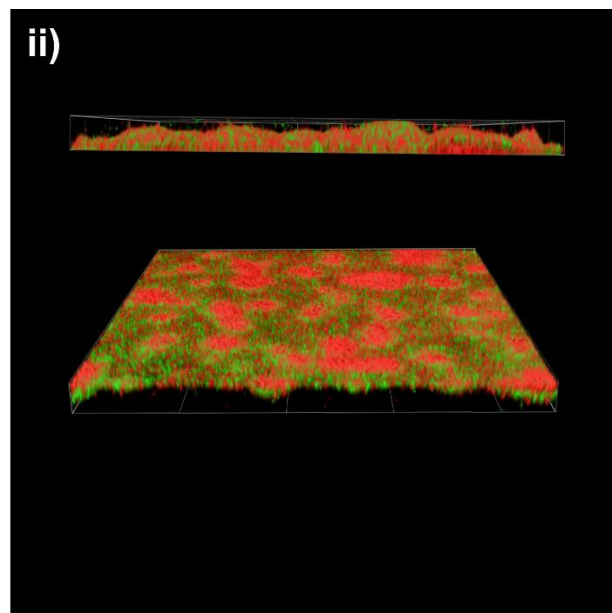
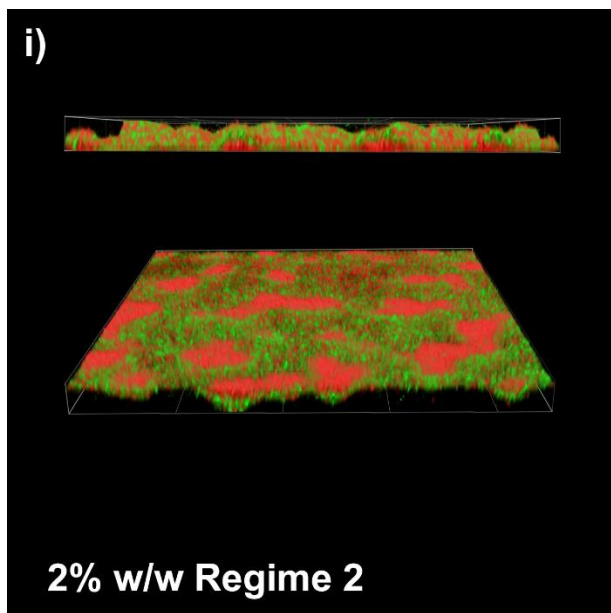
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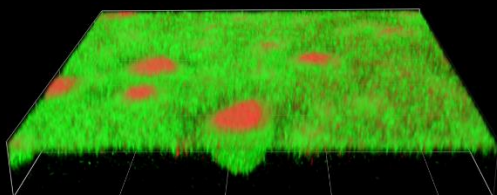
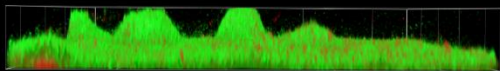
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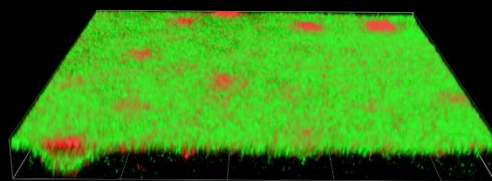
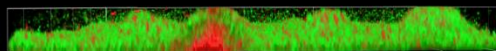


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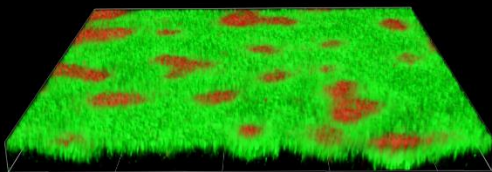


PCL Regime 3

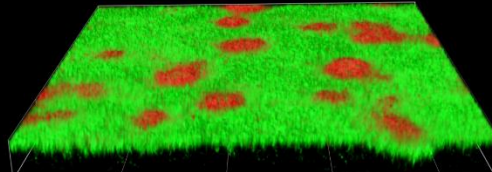
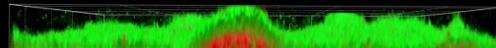
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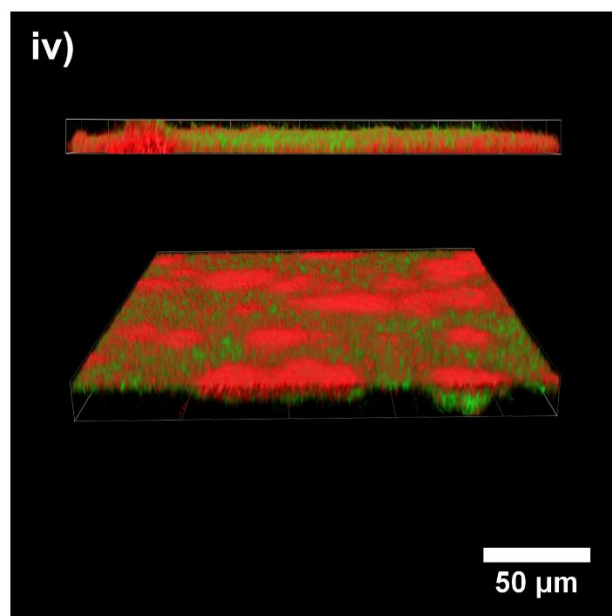
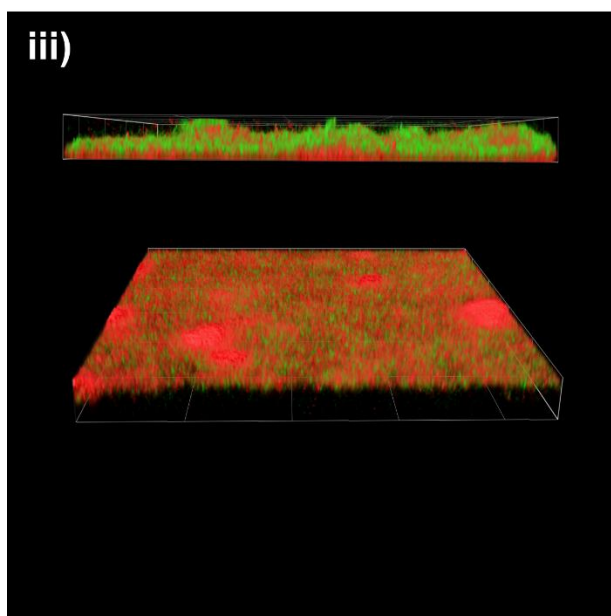
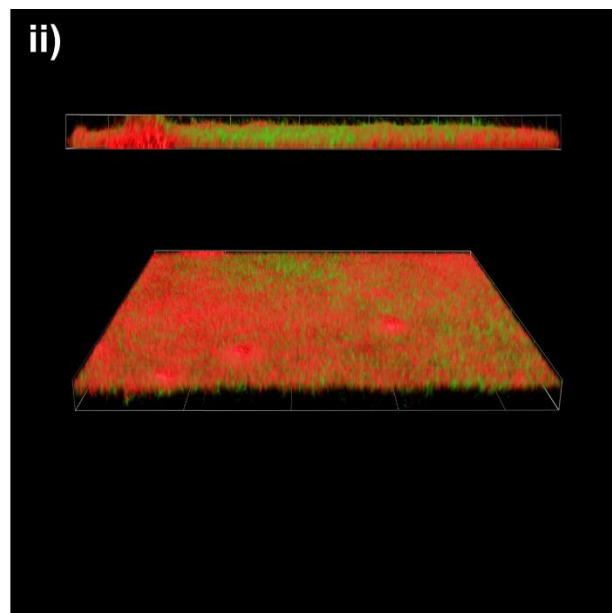
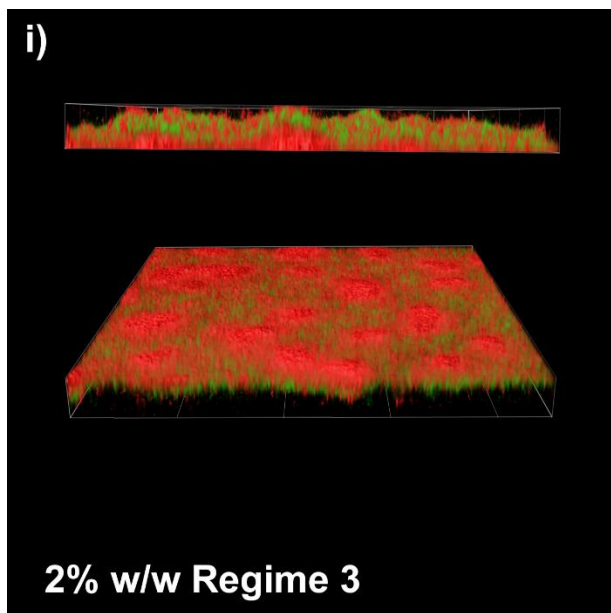
iii)

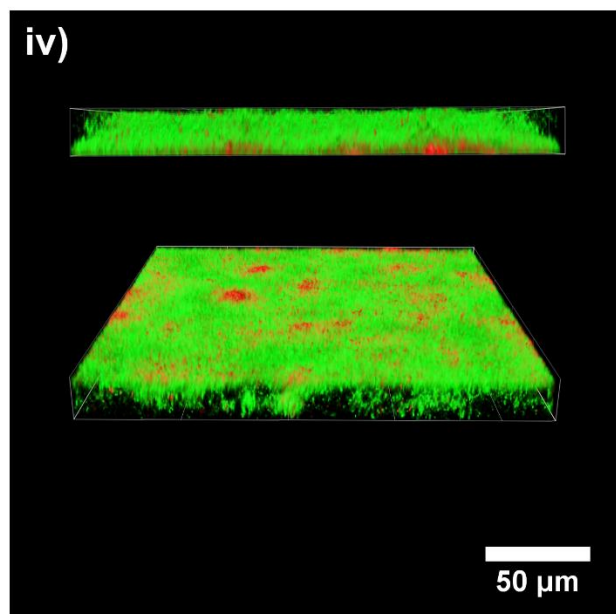
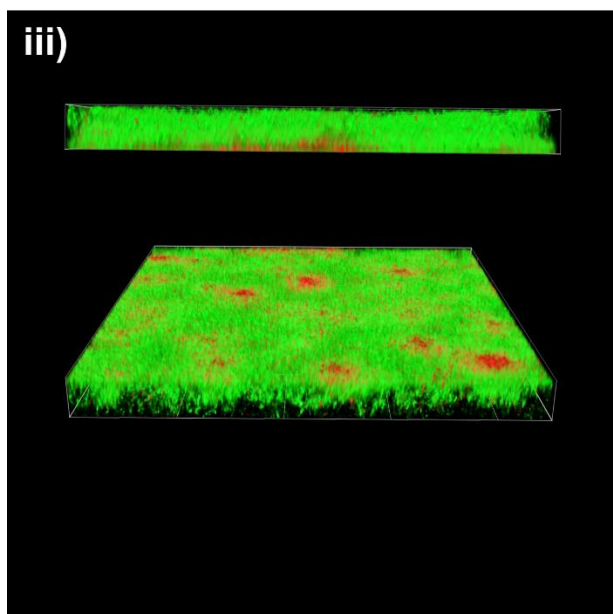
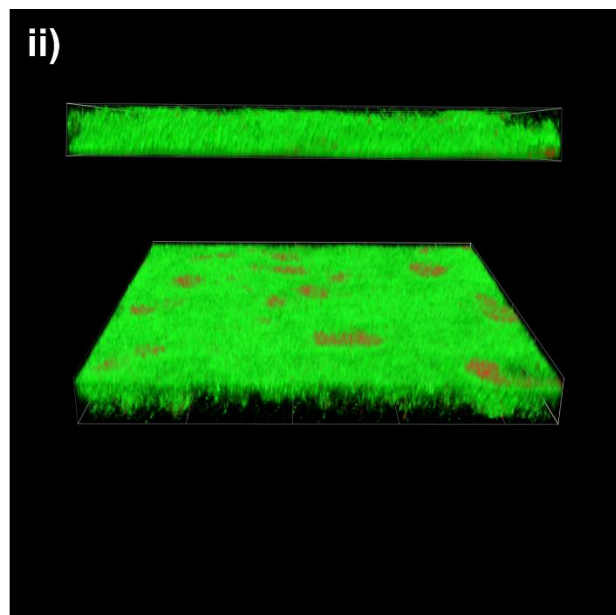
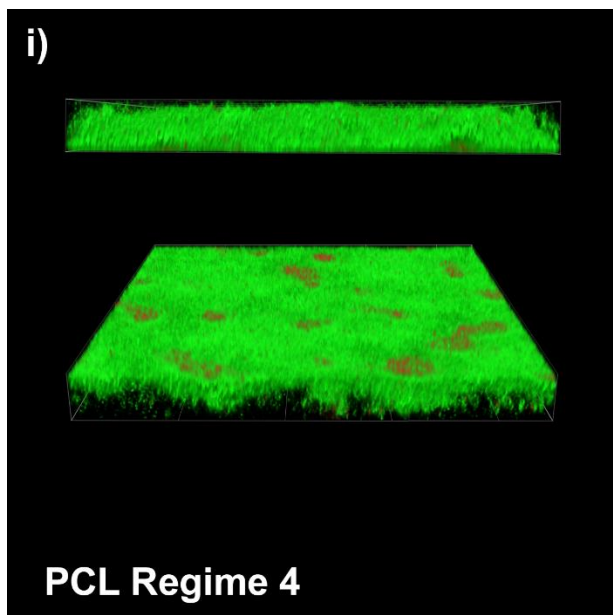


iv)



50 μ m





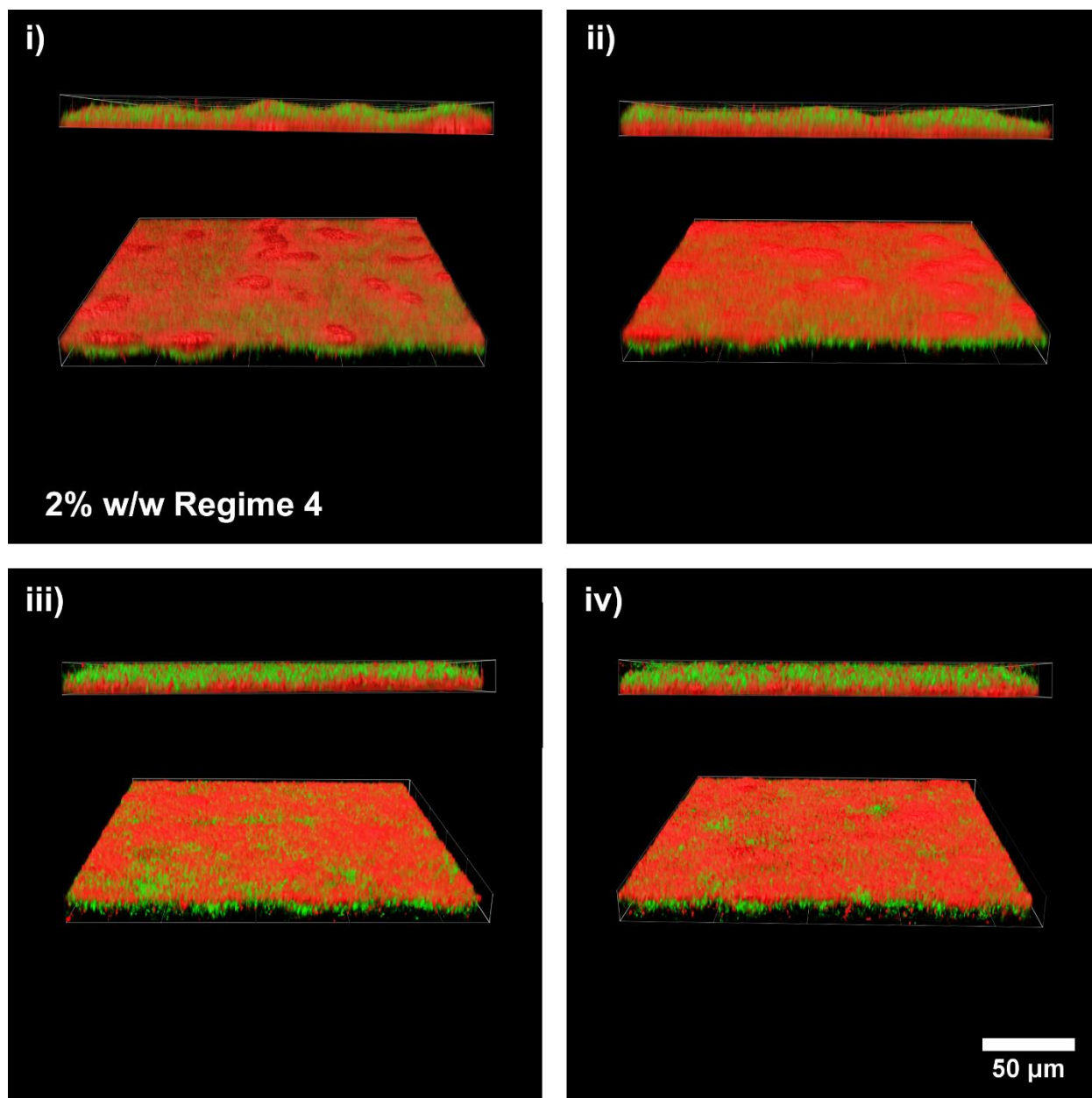


Figure S17: CLSM images of biofilms on PCL and 2% w/w O-MWCNT/PCL at Regimes 1-4 grown in the drip flow reactor. The top row of each 4-panel set represents replicate images from one sample while the bottom row represents replicate images from a second sample inoculated on a different occasion. The biofilms grown on PCL in each regime correspond to the 2% w/w biofilms by row; these biofilms on PCL were grown at the same time and under the exact same conditions as those on the 2% w/w.

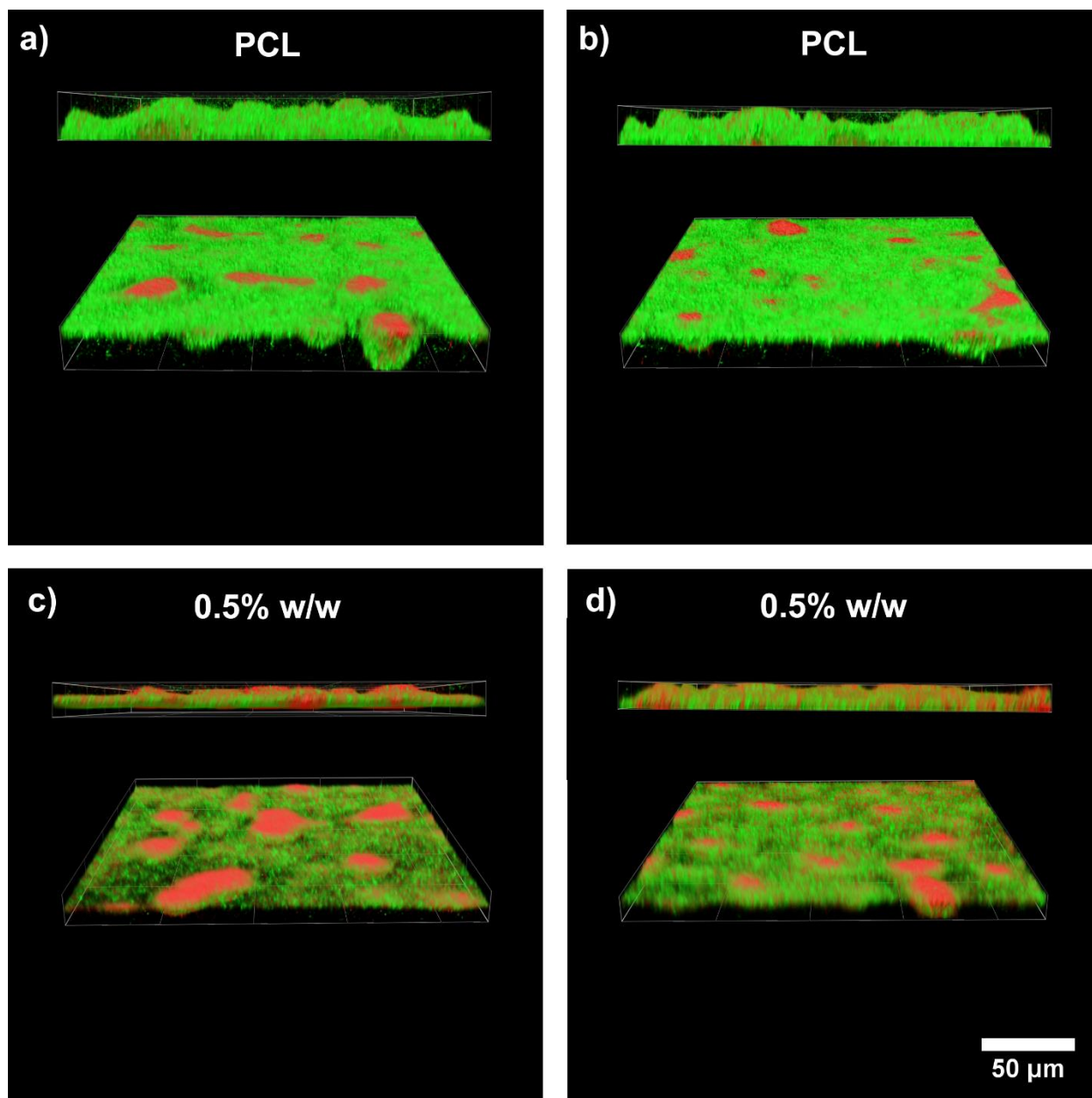
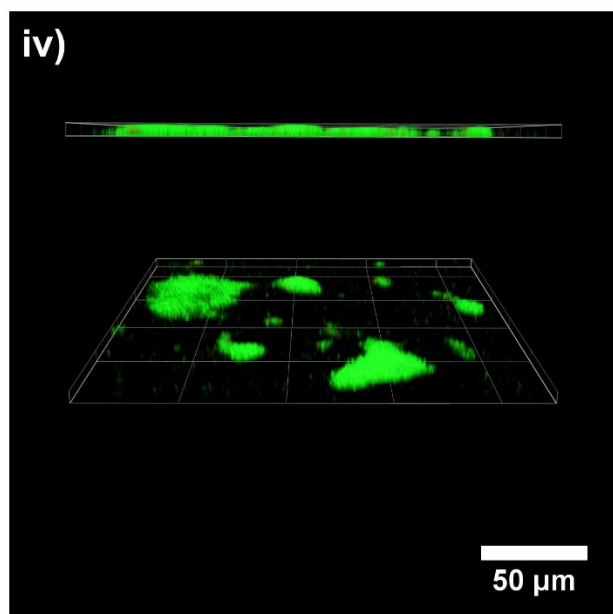
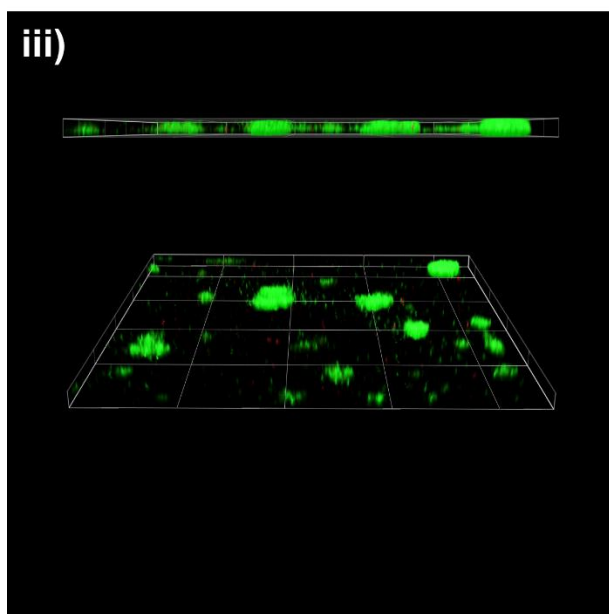
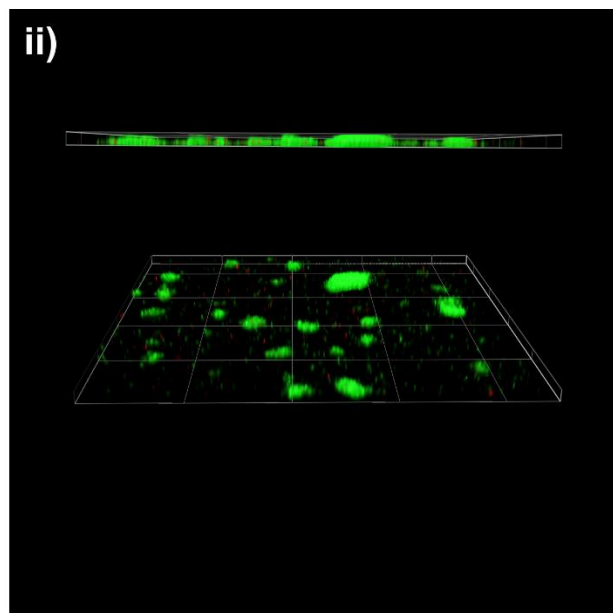
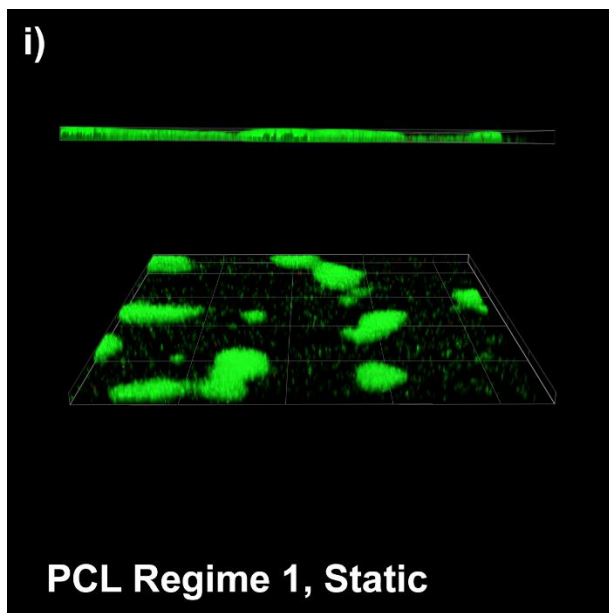
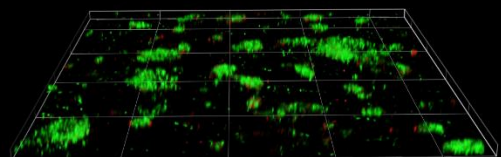
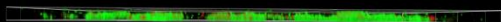


Figure S18: CLSM images of biofilms on PCL and 0.5% w/w O-MWCNT/PCL grown in the drip flow reactor to Regime 4 ($22 \pm 3 \mu\text{m}$ thickness for PCL). The top row represents duplicate images (different sample areas) of biofilms grown on PCL (a and b) at the same time and under the same conditions as the duplicate images (different sample areas) of biofilms grown on 0.5% w/w in the bottom row (c and d).

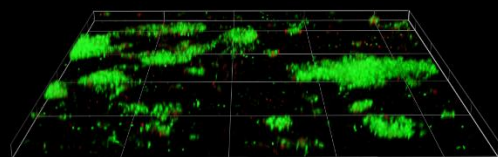


i)

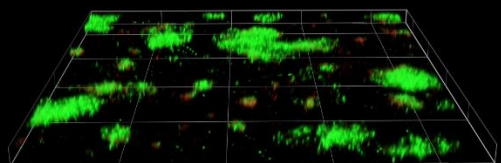


2% w/w Regime 1, Static

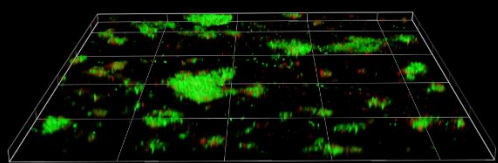
ii)



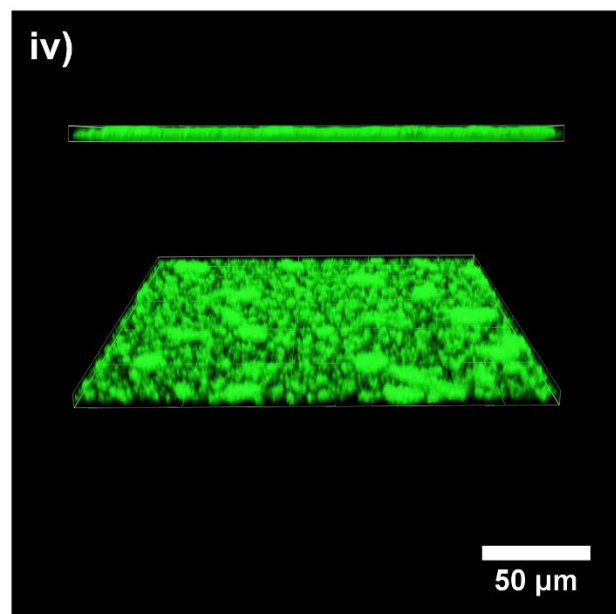
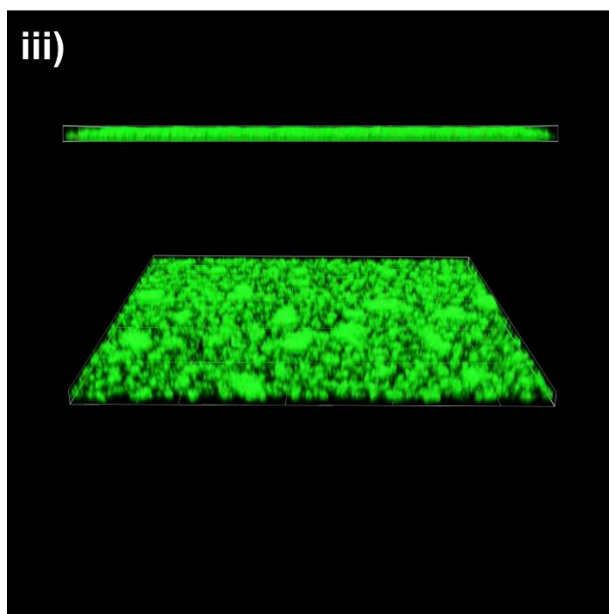
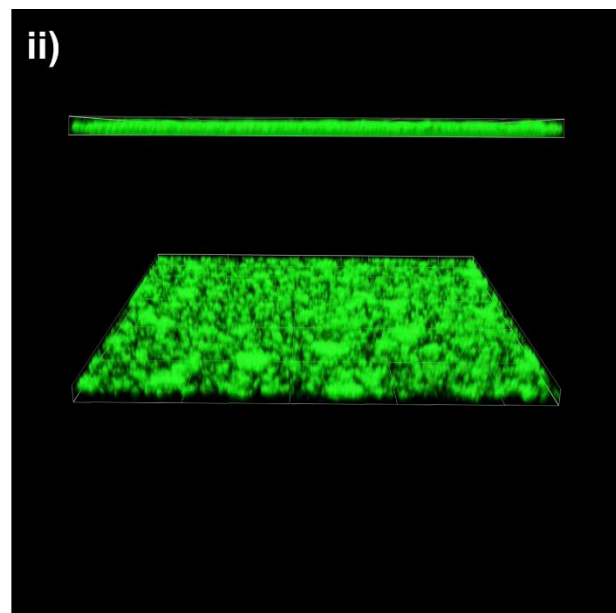
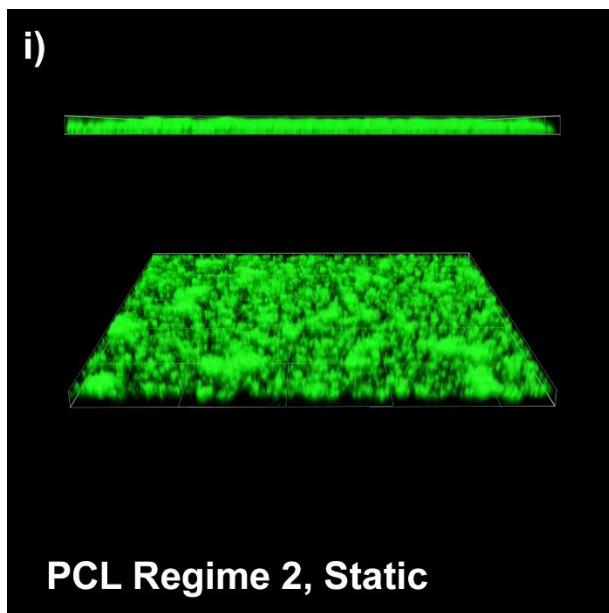
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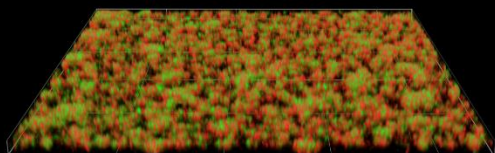
iv)



50 μ m

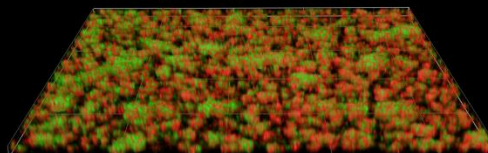


i)

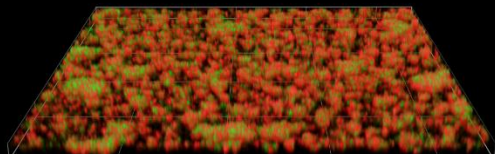


2% w/w Regime 2, Static

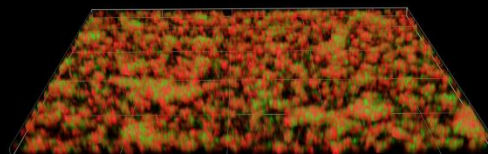
ii)



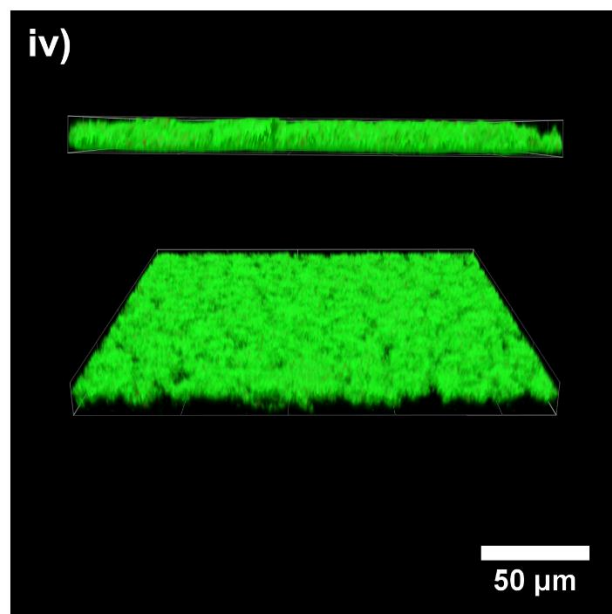
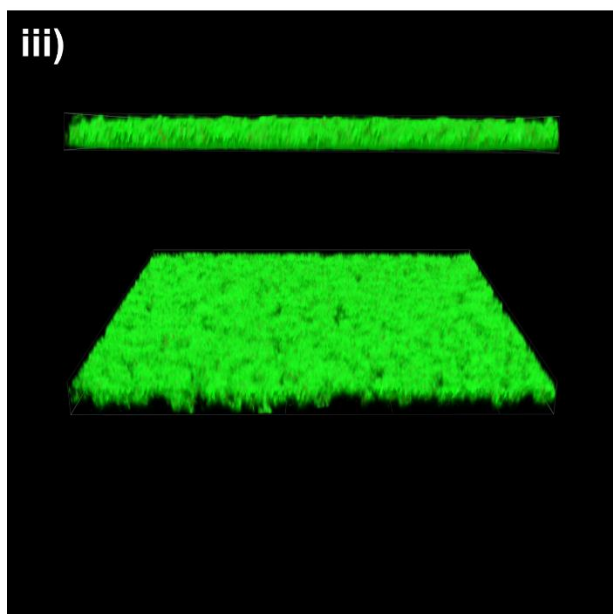
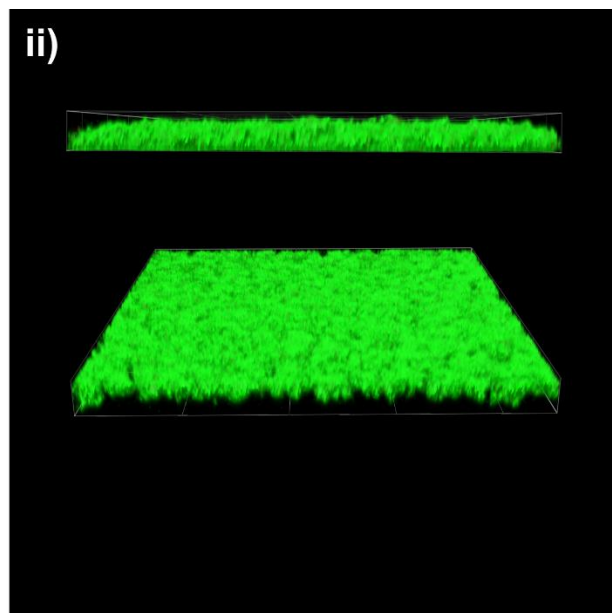
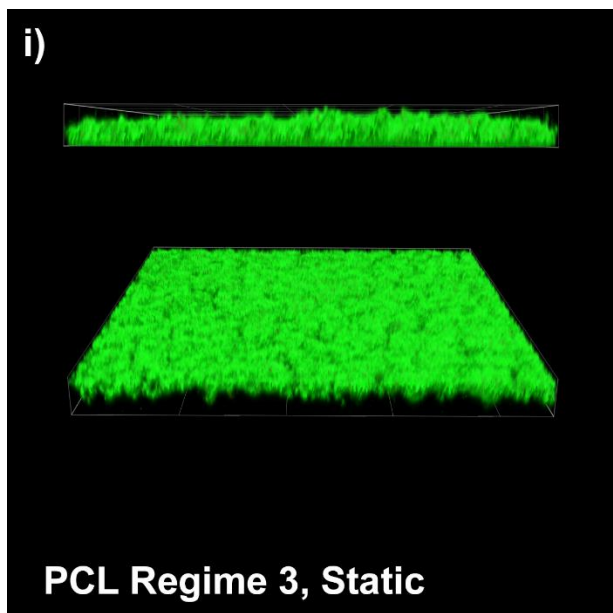
iii)



iv)



50 μm



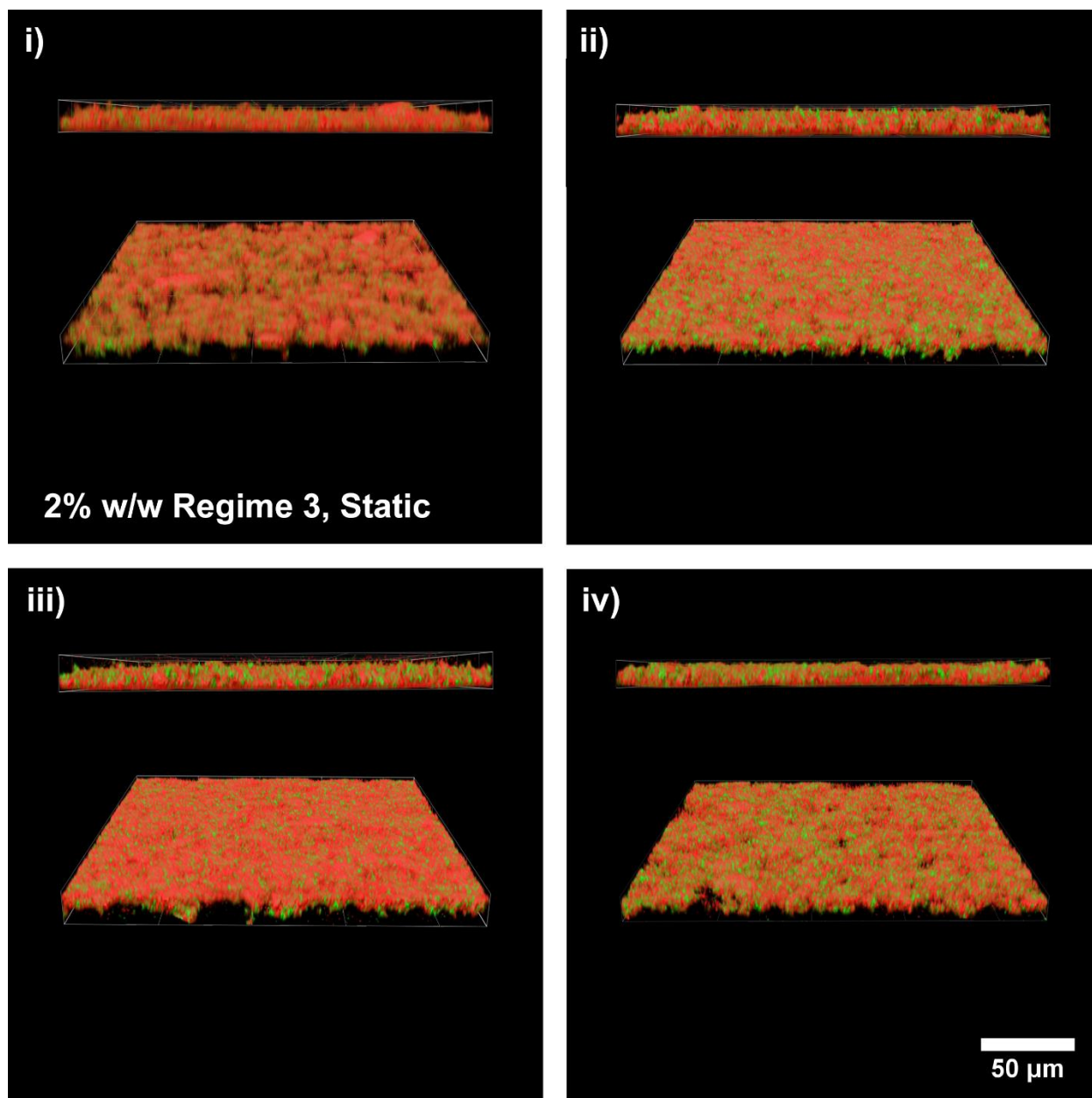


Figure S19: CLSM images of LIVE/DEAD stained biofilms on PCL and 2% w/w O-MWCNT/PCL at Regimes 1-3 grown under static conditions. The biofilms grown on PCL in each regime correspond to the 2% w/w biofilms below for each 4-panel set; these biofilms on PCL were grown at the same time and under the exact same conditions as those on the 2% w/w. Each 4-panel set contains images from different areas of one sample.

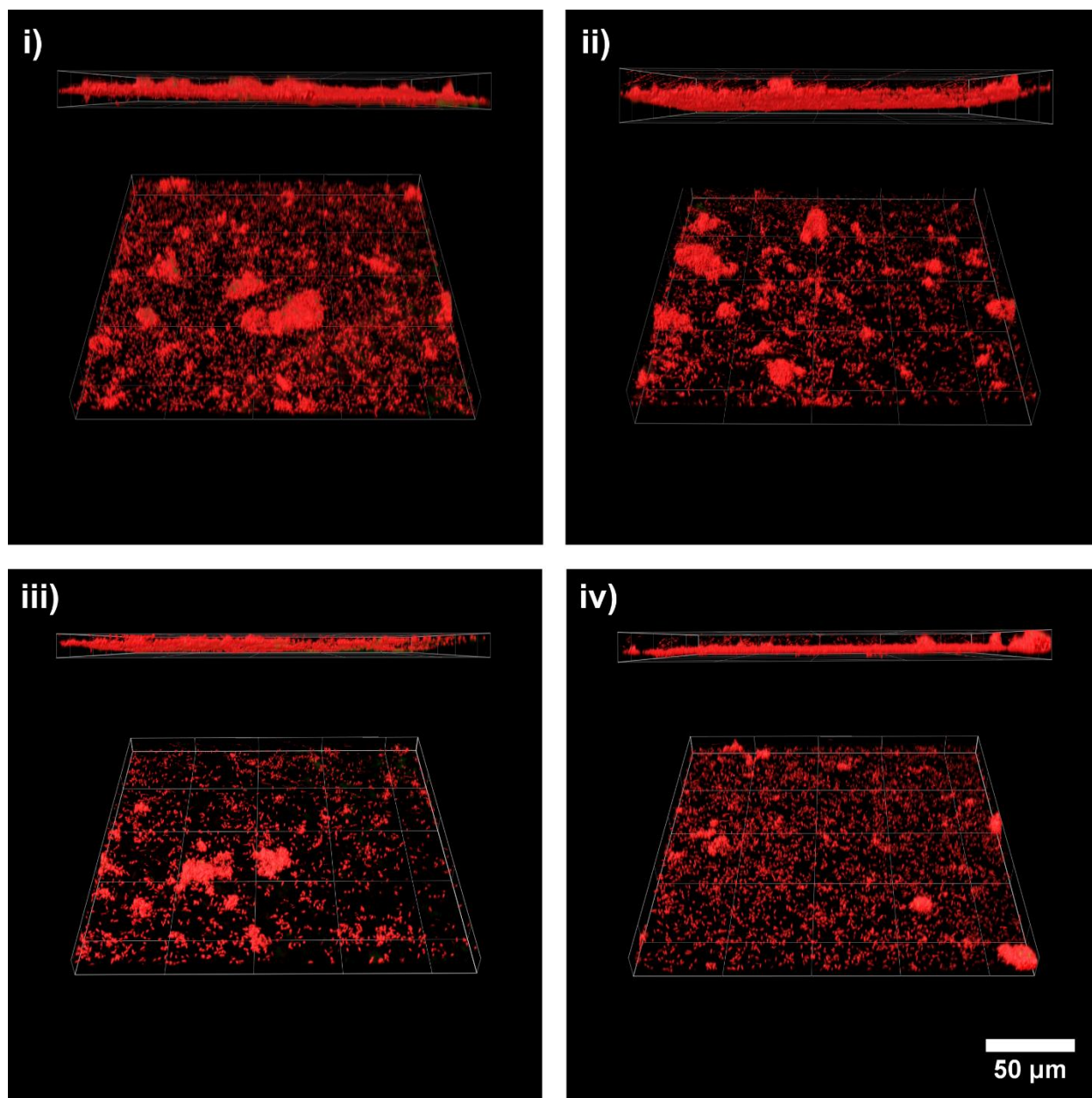


Figure S20: Replicate CLSM images of LIVE/DEAD stained *P. aeruginosa* exposed for 1 h to photodegraded 2% w/w O-MWCNT/PCL nanocomposites. Replicates are from different areas of the same sample.

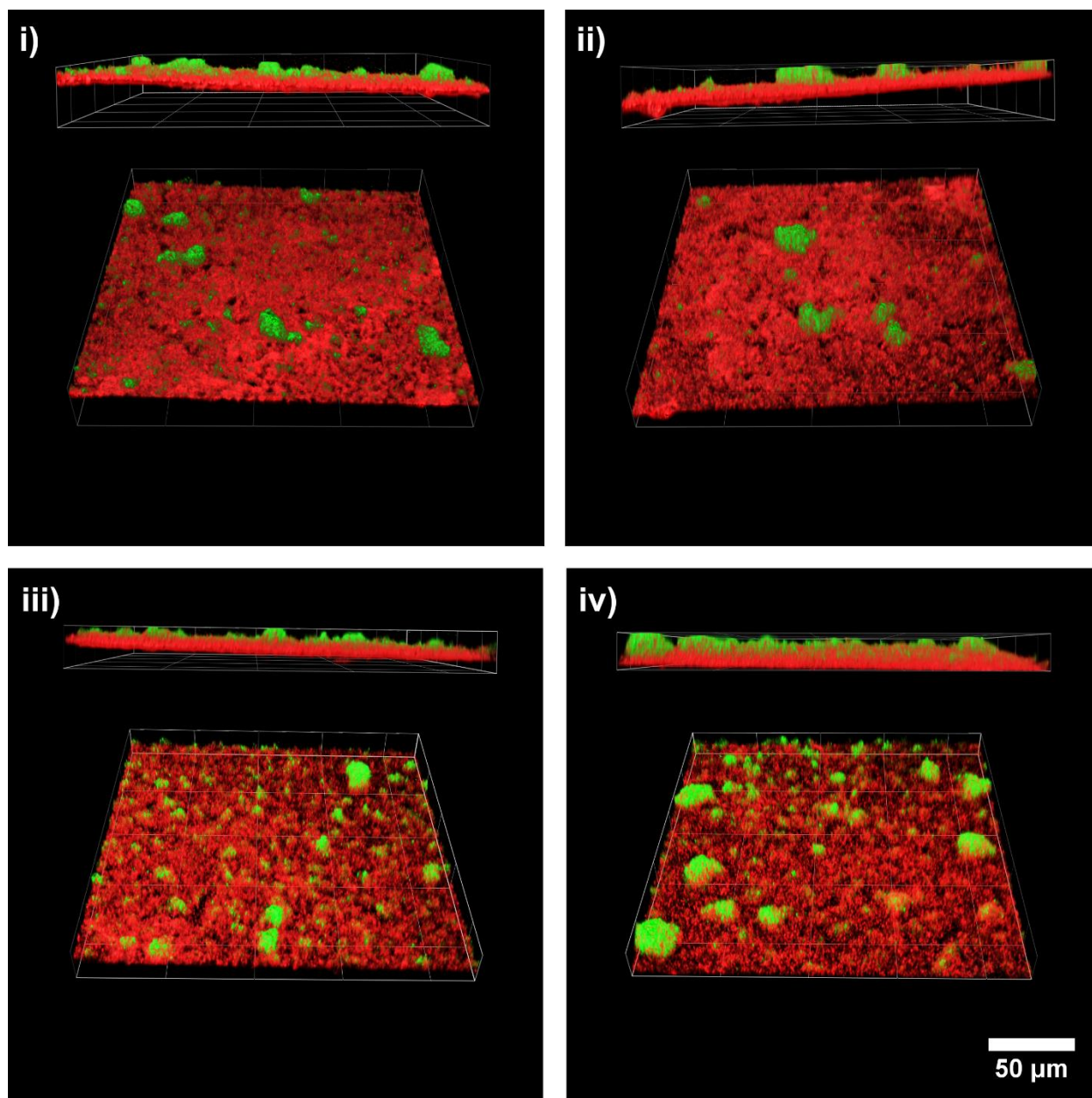


Figure S21: CLSM images of LIVE/DEAD stained *P. aeruginosa* exposed for 2 weeks to photodegraded 2% w/w O-MWCNT/PCL nanocomposites with the acetate food source replenished twice. Replicate images are from different areas of the same sample.

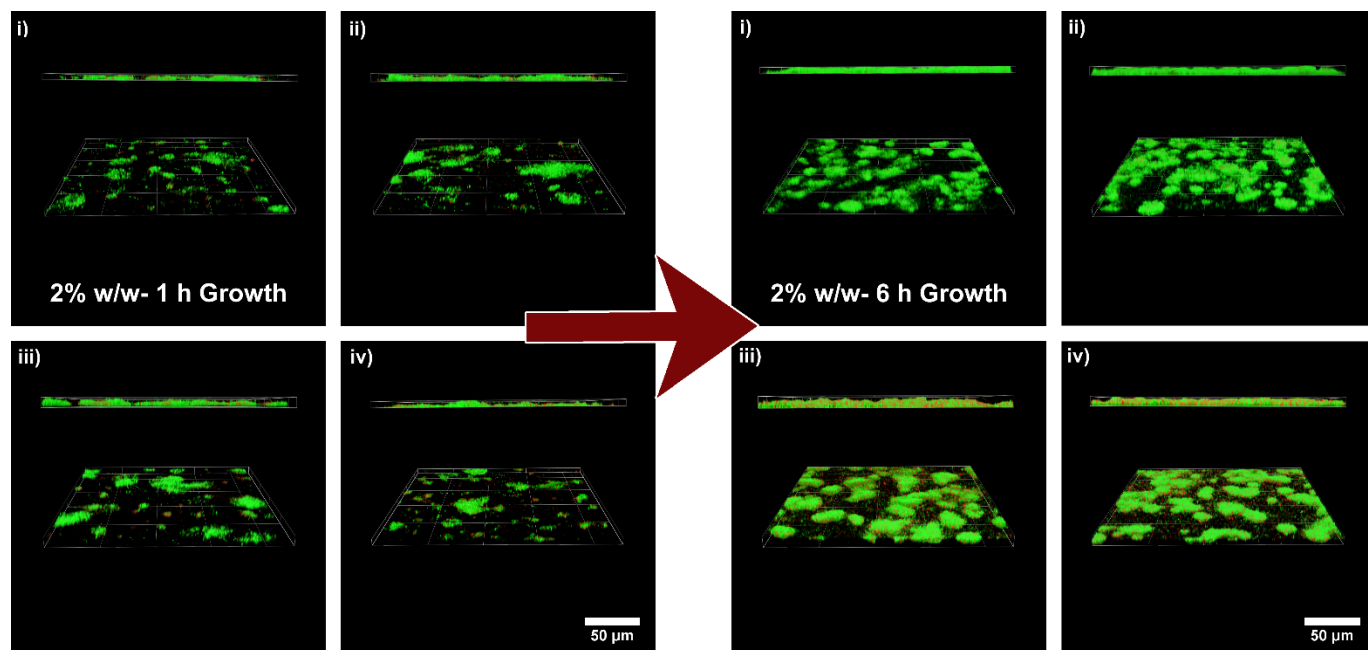


Figure S22: Replicate CLSM images of LIVE/DEAD stained *P. aeruginosa* after 1 h of initial attachment (left) and 6 h of static growth on different areas of 2% w/w O-MWCNT/PCL nanocomposite samples. The progression from 1 to 6 h shows the initially benign nature of the CNT/PCL nanocomposite containing a buried CNT network.

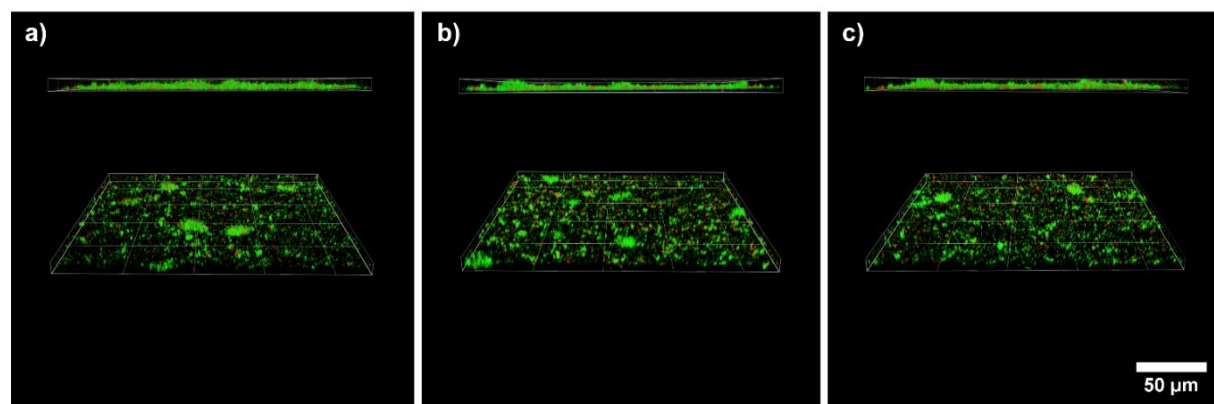


Figure S23: Replicate CLSM images of LIVE/DEAD stained *P. aeruginosa* after 1 h of initial attachment on different areas of a 0.5% w/w O-MWCNT/PCL nanocomposite sample.

2% w/w O-MWCNT/PCL, Static Conditions

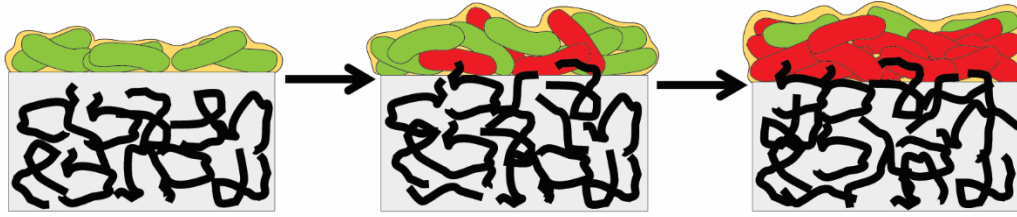


Figure S24: Illustration of biofilm development on 2% w/w O-MWCNT/PCL transformed as a result of polymer biodegradation under static conditions.

a)

Drip Flow Reactor Conditions (2% w/w vs PCL)													
Manual Thickness Measurement					COMSTAT Thickness Measurement				COMSTAT Biomass Measurement				
Regime	PCL Thickness	Std. Dev	2% w/w Thickness	Std. Dev.	PCL Thickness	Std. Dev	2% w/w Thickness	Std. Dev.	PCL Biomass	Std. Dev	2% w/w Biomass	Std. Dev.	
1	8.5	0.6	7.0	0.6	8.2	1.3	6.5	1.5	3.7	1.0	3.3	1.0	
2	12.2	1.9	9.6	1.2	13.2	2.2	10.3	2.3	8.7	1.7	5.6	1.2	
3	17.2	2.5	15.9	1.5	19.4	5.0	18.5	3.6	9.9	1.3	9.1	1.1	
4	20.4	1.8	14.9	1.1	21.8	4.6	15.3	3.1	15.5	4.6	11.2	1.2	

b)

Static Conditions (2% w/w vs PCL)													
Manual Thickness Measurement					COMSTAT Thickness Measurement				COMSTAT Biomass Measurement				
Regime	PCL Thickness	Std. Dev	2% w/w Thickness	Std. Dev.	PCL Thickness	Std. Dev	2% w/w Thickness	Std. Dev.	PCL Biomass	Std. Dev	2% w/w Biomass	Std. Dev.	
1	7.1	0.6	4.7	0.7	4.8	0.6	5.0	1.4	0.9	0.4	0.8	0.3	
2	7.5	0.3	7.1	0.5	6.2	0.6	5.9	1.0	6.8	0.5	4.6	0.7	
3	15.0	0.6	13.0	1.1	16.2	1.3	12.5	1.9	13.8	0.5	8.4	0.9	

c)

Drip Flow Reactor Conditions (0.5% w/w vs PCL)									
COMSTAT Thickness Measurement					COMSTAT Biomass Measurement				
Regime	PCL Thickness	Std. Dev	0.5% w/w Thickness	Std. Dev.	PCL Biomass	Std. Dev	0.5% w/w Biomass	Std. Dev.	
4	21.8	3.5	14.7	1.3	12.1	1.7	8.5	1.7	

d)

Dead Layer Thickness (Drip Flow Reactor- 2% w/w)			Dead Layer Thickness (2% w/w Photodegraded)		
Manual Thickness Measurement			Manual Thickness Measurement		
Regime	Thickness	Std. Dev		Thickness	Std. Dev
3	7.1	0.8		1 hour	5.4
4	6.7	0.6		2 weeks	4.8

Table S1: Comparison of manual and COMSTAT 2 biofilm thickness measurements as well as biomass volume measurements on PCL and 2% w/w O-MWCNT/PCL under DFR (a) and static conditions (b). COMSTAT 2 biofilm thickness and biomass volume measurements on 0.5% w/w O-MWCNT/PCL under DFR conditions (c) and dead layer analysis of biofilms grown on 2% w/w O-MWCNT/PCL nanocomposites in regime 3 and 4 (DFR) as well as on 2% w/w photodegraded O-MWCNT/PCL (static). Each measurement represents the average and standard deviation of at least six samples.