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Live Cell Analysis of Shear Stress on Pseudomonas aeruginosa using the Automated Higher-Throughput Microfluidic System
--Manuscript Draft--

1 **TITLE:**
2 **Live Cell Analysis of Shear Stress on *Pseudomonas aeruginosa* using an Automated Medium-**
3 **Throughput Microfluidic System**
4
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30 **KEYWORDS:**
31 *Pseudomonas aeruginosa,* enhanced green fluorescent protein, microfluidics, shear, biofilm,
32 minimal media, fluorescence microscopy, bright-field microscopy, biofilm coverage, image
33 analysis
34

35 **SUMMARY:**
36 Here, we describe the use of a medium-throughput microfluidic bioreactor coupled with a
37 fluorescent microscope for the analysis of shear stress effects on *Pseudomonas aeruginosa*
38 biofilms expressing green fluorescent proteins, including instrument set up, the determination of
39 biofilm coverage, growth rate, and morphological properties.
40

41 **ABSTRACT:**
42 A medium-throughput microfluidic *in vitro* bioreactor coupled with fluorescence microscopy has
43 been used to study bacterial biofilm growth and morphology, including *Pseudomonas aeruginosa*
44 (*P. aeruginosa*). Here, we will describe how the system can be used to study the growth kinetics

45 and the morphological properties such as the surface roughness and textural entropy of *P.*
46 *aeruginosa* strain PA01 that expresses an enhanced green fluorescent protein (PA01-EGFP). A
47 detailed protocol will describe how to grow and seed PA01-EGFP cultures, how to set up the
48 microscope and autorun, and conduct the image analysis to determine growth rate and
49 morphological properties using a variety of shear forces that are controlled by the microfluidic
50 device. This article will provide a detailed description of a technique to improve the study of
51 PA01-EGFP biofilms which eventually can be applied towards other strains of bacteria, fungi, or
52 algae biofilms using the microfluidic platform.
53
54 **INTRODUCTION:**
55 Here, we will demonstrate a method to measure the effect of shear stress on the formation of
56 fluorescent *Pseudomonas aeruginosa* (*P. aeruginosa*) PA01 biofilms using an automated
57 medium-throughput microfluidic system.
58
59 Biofilms are communities of microorganisms, such as bacteria, organized by an extracellular
60 polymeric substance that are attached to a support, and are typically found at the interface
61 between a liquid and a solid surface[1]. These biofilm communities can be beneficial to the
62 environment, such as improving water quality in water supply lines and in bioremediation of
63 recalcitrant compounds[2,3]. However, biofilms can also be highly harmful to human health with
64 undesirable consequences. For example, medical devices, such as hip and knee implants, are one
65 type of surface where biofilm accumulation has been a challenge and causes severe medical
66 complications[4,5]. Biofilms can also enter natural water systems, such as rivers and lakes, and
67 infiltrate water supply pipes leading to bacteria contamination in drinking water resulting in
68 infections[6–8]. Biofilms formed in marine environments adhere to ships and other man-made
69 substrates and present a major economic and environmental problem as increased friction leads
70 to increase fuel consumption[9,10]. Antimicrobial coatings, such as Tributyltin, have been
71 developed to prevent these problems but are toxic to marine life[11].
72
73 *P. aeruginosa* is a Gram-negative bacterium with high thriving capabilities in a variety of
74 environmental and nutrimental conditions[12]. *P. aeruginosa* is a common cause of community-
75 and hospital-acquired infections and found to be closely associated to injuries, such as severe
76 burns, and immunocompromised hosts, such as in cystic fibrosis (CF)[5,12,13], AIDS, and cancer
77 patients[5,13]. The formation of *P. aeruginosa* biofilms has been most seriously connected to CF,
78 where chronic lung infections are the leading cause of death for this disease[5].
79
80 A reference strain of *P. aeruginosa*, PA01, is used in this report and is genetically modified to
81 express an enhanced green fluorescent protein (PA-EGFP). EGFP represents a mutant form of
82 GFP with greater fluorescence properties which allows for *in situ* biofilm analysis using
83 fluorescence microscopy[14–16]. This type of fluorescence analysis is advantageous for the study of
84 biofilms because GFP does not interfere significantly with cell growth and function[17]. For
85 example, *Escherichia coli* cells that were tagged with GFP grew well and continuously without
86 having suffered any toxic effects in comparison to the control bacteria[17]. Other reports
87 substantiate this claim[18–20]. Furthermore, the use of a fluorescent reporter such as EGFP is quick
88 and simple, yet only live cells will be measured because dead cells quickly cease to fluoresce[21].

89

90 Biofilms can grow under various environmental conditions including those with different flow
91 rates. For example, films can grow in high shear stress, such as in rivers, where the high water
92 flow conditions lead to a greater microbial diversity[22]. Contrarily, standing water in ponds or oral
93 biofilms experience a much lower shear force[23]. In addition to flow rate, there are other factors
94 that influence biofilm adhesion, including surface roughness and hydrophobicity, media
95 composition, and even the bacterial cell surface[1,4,7,24]. Conditions can also cause variation in the
96 spatial structure or morphology of a biofilm. This includes environmental conditions such as shear
97 stress exerted by a moving fluid or gradients in nutrient availability and biological factors such as
98 the species present in the system, motility of cells, and the specific proteins present in the
99 extracellular polymeric substance[25–27]. Under some conditions, the biofilm will be lawn-like
100 (smooth and flat), while under other conditions the biofilm will be rough, fluffy, or even
101 mushroom-like[28]. While the qualitative difference between biofilm lawns and mushroom
102 structures can be clearly seen in microscopic images, understanding the relationship between
103 film structure and biological processes within the film requires systematic and quantitative
104 methods of describing the morphology. Morphological properties suggested for study by
105 researchers include porosity, fractal dimension, diffusional length, microcolony area at the
106 substratum, microcolony volume, roughness coefficient, and textural entropy[29,30].

107

108 Bioreactors are used in the study of biofilms to mimic real life conditions[31]. Drip flow reactors
109 (DFR) represent a low-shear environment where nutrients in media flow slowly across cells that
110 are attaching to a surface over time to form a biofilm with a high cell density[32]. CDC reactors are
111 bioreactors that create a high shear stress fluid environment by control of a stirring rod that is
112 continuously rotating within the media filled tank[33]. These types of bioreactors are simple to set
113 up, but they are limited in scope because of the relatively low sample size, the high consumption
114 of media, large amounts of biohazardous waste produced from media drip flows ranging from
115 125 µL/minute for drip flow reactors to more than 1 mL/min for CDC reactors, and the need to
116 autoclave large amounts of glassware and waste media[34]. Biofilms do not grow evenly across the
117 surface in a drip flow reactor because the low shear of media causes trailing along the larger
118 conglomerates of *P. aeruginosa* bacteria therefore, the biofilm growth is not very smooth and
119 the uneven samples cannot be easily analyzed with fluorescence microscopy[35,36] .

120

121 Some common bioreactor limitations are overcome by using a medium-throughput microfluidic
122 bioreactor, where only milliliters of media are required, and the reaction plates are small and
123 readily disposable after autoclaving[37]. Furthermore, depending on the number of wells, many
124 replications can be performed in just one reactor run, which provides sufficient amount of data
125 to conduct meaningful statistical analysis. In **Figure 1**, the different components of the
126 microfluidic-microscopy system that allow for controlled conditions, including temperature and
127 flow rate[38–40], are shown. The bioreactor is coupled with fluorescence microscopy to visualize the
128 fluorescence of the EGFP tag in PA01 under applied low through high shear conditions that will
129 mimic more realistic scenarios that are encountered in the environment or in the biomedical
130 field.

131

132 [Place Figure 1 here]

133
134 An excerpt of the microfluidic plate is shown in **Figure 2**. The most commonly used plates consist
135 of 48 wells. One experiment requires one inlet and one outlet wells, a total of 2 wells. This allows
136 for 24 simultaneous experiments that can be performed with various experimental conditions,
137 such as bacterial strains, antimicrobial treatments, and media varied from channel to channel
138 and controlled shear flow for each column of six channels. The experimental temperature is also
139 controlled with one temperature setting throughout the plate. The microfluidic channels show
140 that each channel has a serpentine region to provide sufficient back pressure and controlled
141 shear.
142
143 [Place Figure 2 here]
144
145 A step by step guide is provided to allow users of microfluidic bioreactors that are coupled with
146 fluorescence microscopy to conduct novel biofilm experiments using different shear
147 environments. This method will allow for the expansion of experiments involving other
148 microorganisms besides bacteria, such as fungi and algae, that have medical and environmental
149 applications[41–43]. The detailed approach describes how to culture PA01-EGFP, inoculate a 48-well
150 plate and set up the microfluidic device and software, set up the fluorescent microscope, and
151 demonstrate the software analysis to obtain the biofilm coverage, growth rate, and
152 morphological properties such as surface roughness.
153
154 **PROTOCOL:**
155
156 1. **Media Preparation**
157
158 1.1. Prepare minimal media (MM) with 0.25% glucose. To make 1 L of MM with 0.25% glucose,
159 add 200 mL of sterile M9 salt solution, 2 mL of sterile 1 M $MgSO_4$, 100 µL of sterile 1 M $CaCl_2$, and
160 12.5 mL of sterile 20% (w/v) glucose to water (dH_2O) at a final volume of 1 L.
161
162 1.2. Transfer required media to a sterile bottle using sterile techniques. Prepare the amount
163 required depending on the number of channels being used. Typically, each channel requires 200
164 µL for priming, 300 µL for seeding and 1300 µL for the 24 h experiment.
165
166 1.3. Place the bottle in an incubator or water bath set at the experimental temperature. The
167 media should be at the experiment temperature before use to avoid bubbles forming in the
168 microchannels.
169
170 2. **Preparation of an Overnight and Experimental Culture of PA01-EGFP.**
171
172 2.1. Place 15 mL of experimental media into a sterile sidearm flask and inoculate with one or two
173 colonies from an agar plate streaked with PA01-EGFP. Expand the culture for 12-16 h on an
174 incubator/shaker table at 37 °C and 180-220 rpm.
175
176 2.2. Measure the OD_{600} of the overnight culture. When the OD_{600} is greater than 0.80, dilute the

177 overnight culture to the final OD of 0.8 using fresh MM. Place the experimental culture in an
178 incubator or water bath at 37 °C until needed for seeding the microfluidic channels. Check the
179 OD_{600} again immediately before seeding to ensure that it has not changed significantly from the
180 target OD_{600}, 0.8 in this case.
181
182 3. **Equipment Startup**
183
184 3.1. Set up the system station according to the user guide, similar to the setup in **Figure 1**. To
185 avoid an error in the connection of the instrument to the software, turn on the instrument in the
186 following order:
187 PC Workstation
188 Fluorescence Module. Make sure the fluorescence shutter is ON (blue light by shutter button on)
189 Hardware Controllers
190 Imaging system Controller (see **Table of Materials**)
191 CCD Camera
192 Imaging Station (Microscope)
193
194 NOTE: The temperature of the heated plate should be adjusted to the desired experimental
195 temperature.
196
197 3.2. Start the control application and enter the plate number located on the label on the side of
198 the plate.
199
200 NOTE: After control application starts there will be two separate application windows, one for
201 the software that controls the microscope/imaging software and one for the control module that
202 controls the pump and the well-plate interface. This is shown in **Figure S1**, where the "Multi
203 Dimensional Acquisition" menu is open on the top and the Control Module is set for an autorun
204 on the bottom.
205
206 4. **Priming and Seeding the Microfluidic Plate**
207
208 NOTE: The priming, seeding, bacterial attachment and growth are illustrated in **Figure 3.**
209
210 4.1. Remove the 48-well microfluidic plate from the packaging making sure to not touch the glass
211 surface at the bottom of the plate. Clean the glass slide at the bottom of the plate well with a
212 lens tissue, a lint free cloth, or a low-lint wipe.
213
214 4.2. To prime the microfluidic channels, pipette 200 µL of 37 °C MM into the output well, being
215 careful to avoid bubbles. Place the plate into the plate stage and wipe the interface with ethanol,
216 allowing to dry, before sealing it onto the plate stage.
217
218 4.3. In **Manual Mode** on the Control Module, set **Fluid** as LB at 37 °C and **Max Shear** at 5.00
219 dyne/cm^2. Click the output wells to activate flow from the output to the input well, priming the
220 channels. After the 5 min priming, pause the flow to prepare for seeding. Carefully remove the

221 plate from the stage and pipette residual media from the output but not removing any of the
222 media from the inner circle that leads to the microfluidic channels.

224 4.4. To seed the experimental channels, first pipette 300 μL of MM into the input well followed
225 by pipetting 300 μL of the bacterial culture into the output well into the output well. Place the
226 plate back into the plate stage making sure to wipe the interface before placing it on the plate.

228 4.5. On the Control Module, focus on a single channel using the live camera feed after placing
229 the plate stage onto the microscope stage. While visually monitoring by the live feed, resume
230 flow at 1.00-2.00 dyne/cm^2 for approximately 2-4 s to allow cells to enter the experimental
231 channel but not in the serpentine channels. Leave the plate on the temperature controlled stage
232 for 1 h to allow for cell attachment. During the 1 h incubation, the Control Module and Montage
233 software can be set up for the autorun (Step 5).

235 NOTE: The amount of time needed for seeding will vary with media and organism, so it should be
236 monitored closely by the live feed until optimized and used as a general time frame. Seeding
237 throughout the plate may vary which would require more time of applied flow to certain columns
238 of channels for complete seeding.

240 4.6. After the attachment period, gently remove the plate from the stage and pipette the bacteria
241 from the output first avoiding disturbing the channel. With a new pipette tip, remove the media
242 from the input wells.

244 [Place Figure 3 here]

246 5. **Setting the Software**

248 5.1. In the software, open "**Multiple Dimensional Acquisition**" to control the microscope image
249 acquisition. Under the "**Main**" menu, select "**Timelapse**", "**Multiple Stage Positions**", and
250 "**Multiple Wavelengths**" (**Figure S1A**).

252 5.1.1. Setting up the **Saving** settings, create a simple **Base Name**, making sure that **Increment**
253 **base name if file exists** is checked. Click **Select Directory** to select the folder in which all files will
254 be saved. Include essential details of the experiment in the **Description**.

256 5.1.2. Under the **Timelapse** tab, adjust the **Duration** of the experimental time to 24 h. To control
257 how often images are acquired, set the **Time Interval** so images are acquired every 5 min
258 throughout the experiment. The **Number of Time Points** automatically adjusts with the set
259 parameters.

261 5.1.3. Set stage positions using the live camera feed in the bright field microscope, allowing for
262 correct placement and focus. Starting with the 10X objective, focus on the center of the channel,
263 located above or below the channel numbers engraved on the plate. Switch to the 20X objective,
264 finding the optimal viewing area and focal plane within the channel. Add the position to the list

265   or replace the preset channel position with the new settings.

266

267   5.1.4. Under the **Wavelengths** menu, set the number of wavelengths to 3. For wavelength 1
268   (W1), select the FITC 100% CAM with a 10 ms exposure time. For wavelength 2 (W2), select the
269   Brightfield 50% CAM 50% VIS with a minimal exposure time of 3 ms. For wavelength 3 (W3), set
270   as the **All Closed** filter so no light remains on the last channel between acquisition times.

271

272   5.2. When setting up the Control Module, check to make sure that the manual run has been
273   stopped.

274

275   5.2.1. In the **Edit Autorun** menu under the **Protocol Setup** tab, set a new protocol for 24 h of
276   duration time, flowing in the forward direction at the desired shear rate. Shear rate is varied to
277   study the effect of biofilm growth with shear rate. Click **Add** and **Save As** the protocol.

278

279   5.2.2. Under the **Sequence Setup** tab, create a new sequence by selecting **LB@37degrees** as the
280   default fluid for all channels. Under **Step Iteration** 1, for channels 1-12, select the protocol with
281   the desired shear rate and **enable** all channels. For channels 13-24, select the protocol with the
282   second desired shear rate and **enable** all channels. Select **Apply** and **Save As** the sequence.

283

284   5.2.3. In the **Autorun** menu, select the saved sequence to be used for the autorun.

285

286   6. **Timed Biofilm Growth Experiment Setup**

287

288   6.1. Pipette a maximum of 1300 µL of sterile MM into the input well of the microfluidic plate.
289   Place the plate back onto the plate stage and wipe the interface with ethanol, allowing ethanol
290   to dry completely, before sealing the plate. Recheck the saved stage positions after placing the
291   plate back onto the plate stage to ensure the saved positions are still centered within the viewing
292   channels.

293

294   6.2. Placing the plate stage onto the microscope stage, and be sure that the protocol(s) and
295   sequence(s) are set up correctly. Select **Start** to start the autorun quickly followed by clicking
296   **Acquire** to start the microscope image collection.

297

298   6.3. Adjust the focus and placement of all stage positions between image acquirements. Select
299   **Pause** and, using the live image mode in the bright field wavelength, select **Go To** to view each
300   stage positions. Set new settings by selecting **Set to Current**. Click **Resume** before the next
301   scheduled acquisition time.

302

303   7. **Review and Analyze Image Sequences after the Timed Biofilm Growth Experiment**

304

305   7.1. To review each channel following the 24 h autorun, in the software, open **Review**
306   **Multidimensional Data**, located under **Analysis Tools**. Click on **Select Base File | Select Directory**
307   to navigate to the folder with image sequences for the experiment of interest. In the list of **Data**
308   **Sets** in the folder that appears, select the base name of the data of interest.

309

310 7.2. Once the data of interest is selected, click **View** to review this data. Select a wavelength to
311 review that data under **Wavelength** window (**Figure S2**).

312

313 7.3. Review the image sequence for each channel by selecting the channel number under **Stage**
314 **Position** and using the video controls to analyze the data for the 289 time points (assuming that
315 images are obtained every 5 min over a 24 h time frame). Take note of useable growth data.

316

317 NOTE: In each channel, watch for the development of air bubbles and clogs that will disturb the
318 biofilm growth within the channel, affecting the data. However, if these occur later in the run,
319 data prior to these circumstances may be found useful.

320

321 7.4. After reviewing and selecting the desired data for analysis, create stacks of the images for
322 each channel. Under the **File** menu, select **Open Special | Build Stack | Numbered Names**. In the
323 **Build Stack** window, select the **Select First Image** button then select the first image for the stack
324 in the file folder; select the **Select Last Image** button and then select the file corresponding to
325 the last image in the stack. Click on **OK** to open the stack with all the images of the channel in
326 chronological order. Stacks can be saved under the **File** menu, **Save As** as tiff files.

327

328 NOTE: Do not create stacks with the thumb images. These files are not very useful and can be
329 deleted to save room on the hard drive. Also, image files are saved as the following:

330

331 BASE NAME_WAVELENGTH_sCHANNEL#_tIMAGE#.

332

333 For example, 07062018_W1FITC 100_s12_t112 is for the channel 12, time point 112, image in
334 the first wavelength-FITC 100% camera data with the base name "07062018".

335

336 7.5. Finally, select **Save As** from the **File** drop-down menu to save the sequence as a tiff stack.
337 Stacks can also be overlaid and saved as a movie. This is addressed in step 9.

338

339 7.6. Before quantifying the % surface coverage, calibrate the image distances. Under **Analysis**
340 **Tools**, click on **Calibrate Distances**. Select the appropriate calibration measurement and click
341 **Apply to All Open Images**.

342

343 7.7. While the stack is on the first image of the sequence, click on the **Threshold** button. Use
344 **Auto Threshold for Light Objects** to threshold for fluorescent signal (FITC wavelength) and **Auto**
345 **Threshold for Dark Objects** to threshold in bright field. Adjust threshold to the coverage that
346 represents the coverage of the image.

347

348 7.7.1. For fluorescent thresholds, utilize a channel with non-fluorescent cells to establish any
349 background signal and set the minimum threshold value to exclude signal detected in channels
350 containing these non-fluorescent cells to ensure the signal measurement does not overestimate
351 the area of fluorescence. This may be different from run to run so it is a good idea to use at least
352 one, if not more, channel(s) for non-fluorescent controls.

353

354 7.7.2. For bright field thresholds, if all cells are not covered by the orange threshold signature,
355 adjust the maximum threshold value either via the sliding toolbar or using the **Threshold Image**
356 (found under the **Analysis Tools**) so that all cells are covered but any background is excluded.

357

358 NOTE: While these threshold values ideally would be used for all images within a stack and for all
359 channels, threshold values selected for one image/channel may not be appropriate for other
360 images or channels, therefore the user may need to adjust the threshold range periodically. For
361 this reason, it is a good idea to always record the maximum and minimum threshold values used
362 should the data need to be reviewed later.

363

364 7.8. To quantify the coverage, under **Analysis Tools**, click **Show Region Statistics**. Make sure that
365 **Use Threshold** is checked, the **Entire Image** is selected, and under **Obtained Data**, make sure the
366 desired measurements/settings are selected (threshold area, average, standard deviation, min,
367 max, and % threshold area). Click on **Open Log** and make sure DDE file is selected before clicking
368 **OK**. Select **Microsoft Excel** and click **OK**. In the spreadsheet that will open, click **Log Data** to
369 automatically record the measurements of the image being analyzed.

370

371 7.9. Leaving this spreadsheet open, use the same sheet to collect all image analysis data for a
372 single stack. In the stack, go to the fourth image and threshold to the optimal settings. Click on
373 **Log Data** in the **Show Region Statistics** screen and the values are logged into the spreadsheet.
374 Repeat this for analysis of images every 15 min.

375

376 8. **Other Analysis Including Morphology and Surface Coverage Measure Using Python scripts**
377 **from the Biofilm Morphology Suite**

378

379 8.1. Install a distribution of Python 3.6 that includes standard scientific modules by using a
380 standard   scientific   Python   distribution   such   as   Anaconda,   available   at
381 https://www.anaconda.com/download.

382

383 8.2. Obtain the Biofilm Morphology Suite from GitHub in a browser by navigating to
384 https://github.com/cdwentworth/Biofilm-Morphology-Suite.git, then select **Clone or download**
385 (green button) and select **Download Zip**. Unzip the file and move the code folders to a working
386 directory.

387

388 8.3. Measure percent thresholded coverage by copying the image stack into the "**Coverage**"
389 folder. Use a terminal window to navigate to that folder and then execute the script with the
390 command

391

392 python bfCoverage.py *tiffStackName.tif*

393

394 from the command line interface, where *tiffStackName.tif* is the name of the file containing the
395 tiff stack of images. A text file containing the coverage measurements at each time point will be
396 created with the name *tiffStackName.txt* and a graphics file with a plot of coverage as a function

397   of time will be created with the name *tiffStackName.png*.

398

399   8.4. Use the same procedure specified in step 8.3 to measure the other morphological
400   characteristics: biofilm accumulation, roughness coefficient, and textural entropy. Use the
401   bfAcc.py script for the accumulation measurement, the roughCoef.py script for the roughness
402   coefficient measurement, and the TEvsTime.py script for the textural entropy measurement.

403

404   9. **Other Software Applications – Making Overlays and Movies**

405

406   9.1. If multiple wavelengths are used, create overlay stacks including these wavelengths. Open
407   all stacks of interest and calibrate the distance as done in step 7.6.

408

409   9.1.1. Under **Analysis Tools**, select **Overlay Images**. Set the sources as the stacks of interest. In
410   the FITC stack, click on the rainbow circle and select green color filter to be added in order to
411   highlight this wavelength.

412

413   9.1.2. Use **bal** to adjust the overlay so that the FITC stack is apparent in the images. After all
414   adjustments have been made, select **All Planes** for both stacks and click **Apply**. Save the overlays
415   in the same way as stacks described in step 7.5 or as a movie described in step 9.2.

416

417   9.2. To save stacks or overlays as a timelapse movie, under **MM Standard** and **Stack**, click on
418   **Make Movie**. Select the source as the stack or overlay that is desired. Click **Save** and save as a
419   **Microsoft Video 1** with a quality set between 70-80.

420

421   **REPRESENTATIVE RESULTS:**
422   **Figure 4a** demonstrates the percent thresholded area over time from a 24 h run at shear flows
423   of 0.2, 0.5, and 1.0 dyne/cm^2. The biofilm coverage or percent threshold surface area (C [%]) was
424   different for all three shear settings. The biofilm coverage was fastest at 1.0 dyne/cm^2 shear,
425   where the threshold area increased from 2-5% to 100% after 200 min of growth and reached a
426   stationary phase after 400 min. At 0.5 dyne/cm^2, the biofilm coverage was delayed and began to
427   increase at 400 min reaching 100% coverage after 800 min. The lowest shear at 0.2 dyne/cm^2
428   clearly demonstrated the slowest increase in biofilm coverage, where percent coverage started
429   to increase at 500 min but never reached beyond the 65% threshold area. These results indicated
430   shear had a direct influence in biofilm surface coverage. The higher shear seemed to be a more
431   optimal condition for biofilm growth, possibly due to the fact that the media provided the
432   bacteria with more nutrients so that the biofilm can proliferate quicker.

433

434   [Place Figure 4 here]

435

436   **Figure 4b** is consistent with the findings shown in **Figure 4a**. The biofilm accumulation (N[rel])
437   was measured based on the assumption that the GFP signal at a point in an image is proportional
438   to the live cell density at that position. It demonstrates that total relative measurement biofilm
439   accumulation increased as a function of time using 0.2, 0.5, and 1.0 dyne/cm^2 over a 24 h time
440   period, and the growth rate declined from highest shear stress to lowest shear stress. There is a

441 clear time period giving exponential growth from which a quantitative growth rate can be
442 calculated.
443
444 **Figure 4c** demonstrates the roughness coefficient (R_a) at shear flows of 0.2, 0.5, and 1.0
445 dyne/cm^2. Surface roughness, quantified by the roughness coefficient, measures the variance in
446 the thickness profile of the film. The formal definition is
447

448
$$R = \frac{1}{N} \sum_{i=1}^{N} \frac{|T_a - T_i|}{T_a}$$

449
450 where T_i is the i-th thickness measurement, T_a is the average thickness, and N is the number of
451 thickness measurement[30]. The procedure described in this investigation measures the thickness
452 associated with living cells. A flat arrangement of cells will yield a roughness coefficient of zero
453 while significant variations of thickness from the average will yield a roughness coefficient
454 greater than one. Similar to the shear influence on the growth rate and the percent threshold
455 coverage of the film, the biofilms exhibited different topographies over time. Overall, R_a
456 decreased over time for all shear conditions indicating that all surfaces became smoother.
457 However, in comparison to the lowest shear of 0.2 dyne/cm, the higher shear settings of 0.5 and
458 1.0 dyne/cm^2 resulted in a smoother surface over time indicating that a faster shear flow
459 contributed to smoother and more even surface, and the highest shear of 1.0 dyne/cm^2 reaching
460 below 0.2 R_a.
461
462 The smoothness, regularity, or coarseness of a surface can also be expressed in textural entropy
463 (TE). TE is a property used in image analysis to measure the degree of randomness in a two-
464 dimensional image. Its calculation is based on the gray level co-occurrence matrix, defined by
465 Haralick *et al*., which looks at whether pixel values at one location are correlated with pixel values
466 at another location[44]. A high degree of correlation will lead to low entropy. **Figure 4d** depicts the
467 TE at shear flows of 0.2, 0.5, and 1.0 dyne/cm^2. The TE increased over time for all shear conditions
468 but the highest shear stress at 1.0 dyne/cm reached the maximum TE (1.0) earlier than the lower
469 shear stresses at 900 min. The lowest shear of 0.2 dyne/cm^2 had the lowest TE (0.8) reaching its
470 maximum after 1000 min. However, the intermediate shear stress of 0.5 dyne/cm^2 reached its
471 maximum TE (1.2) much later than the high or low shear stress conditions.
472
473 The roughness coefficient and TE measure different features. While a flat film would have a low
474 roughness coefficient and low entropy, a film with significant variation in thickness would have a
475 high roughness coefficient but could still be low entropy if the variation is sinusoidal rather than
476 random. In this case, R_a decreased with increased shear stress and time while the TE trends
477 cannot be directly related to shear stress applied to biofilm formation over time.
478
479 **FIGURE AND TABLE LEGENDS:**
480 **Figure 1: Individual components of the Microfluidic System.** The individual components are
481 listed from left to right: 1. CCD Camera, 2. High Resolution Inverted Microscope with Automated
482 Stage, Automated Fluorescence Module, and Autofocus Module, 3. Plate Stage, 4: Imaging

483 system Interface, 5: Manual Microscope Stage Control, 6: Vapor Trap, 7: Imaging system
484 Controller (including Temperature Controller), 8: Hardware Controllers, 9: Fluorescence
485 Controller, 10: Uninterruptible Power Supply, 11: External Hard Drive for Image Storage, 12: PC
486 Workstation.

488 **Figure 2: Visualization of the microfluidic channels and viewing window.** Two inlet and outlet
489 wells with the microfluidic channels connecting them are highlighted with red and green dyes.
490 The dye makes visible a serpentine region in each channel which creates sufficient back pressure
491 and controlled shear during fluid flow. Each viewing channel (within the red circle) can be imaged
492 with desired wavelengths. Shown are bright field (top) and fluorescent (bottom) microscopy
493 images of a single channel with a PA01-EGFP biofilm using a 20X objective. Scale bar = 80 μm.

495 **Figure 3: Experimental overview of priming, seeding, and attachment of PA01-EGFP in the**
496 **microfluidic channels.** The priming, seeding, and attachment is outlined. The first step of priming
497 requires fresh media introduced to the output. The seeding entails equal volumes of media and
498 bacterial culture in the input and output wells, respectively. The culture should not pass the
499 viewing segment of the experimental channel (red line) to avoid clogging the serpentine
500 channels. After the incubation period is over, fresh media continuously flows from the input, to
501 the viewing chamber and into the outlet. This initiates the attachment and growth of the bacterial
502 biofilm.

504 **Figure 4: Percent threshold area, total biofilm accumulation, roughness coefficient, and**
505 **textural entropy.** a) Percent threshold area (C [%]) over time using 0.2, 0.5, and 1.0 dyne/cm^2
506 over a 24 h time period. Data was obtained from one channel for each shear condition. b) Total
507 biofilm accumulation (relative measure) as a function of time using 0.2, 0.5, and 1.0 dyne/cm^2
508 over a 24 h time period. The black lines are least-squares fits to an exponential model. Data was
509 obtained from one channel for each shear condition. c) Roughness coefficient of PA01-EGFP using
510 shear stress values of 0.2, 0.5, and 1.0 dyne/cm^2 monitored over 24 h. Data was obtained from
511 one channel for each shear condition. d) Textural entropy of PA01-EGFP using shear stress values
512 of 0.2, 0.5, and 1.0 dyne/cm^2. Data was obtained from one channel for each shear condition
513 (Valquier-Flynn, H., Sutlief, A.L., Wentworth, C.D., 2018).

515 **Figure S1: Image capture of the control software windows.** Software window with **Multi**
516 **Dimensional Acquisition** menu open (Top). Control Module set for an AutoRun (Bottom).

518 **Figure S2: Image capture of the software for reviewing data.** Application window after selecting
519 the Data Set of interest using the **Review Multi Dimensional Data** tool from the **Analysis Tools**
520 menu.

522 **DISCUSSION:**
523 The microfluidic system and image analysis procedure discussed here focuses on the execution
524 of microfluidic biofilm experiments to determine morphological properties that do not require
525 the full three-dimensional information typically found from confocal microscope studies. These
526 included microcolony substratum coverage (percent coverage), surface roughness measured by

527 the roughness coefficient, and textural entropy. A method for estimating total relative biofilm
528 cell accumulation is also presented from which growth rates in log phase can be calculated.
529
530 There are several small but significant steps that should be highlighted in this method. Wiping
531 the interface with alcohol helps avoid contamination of other bacteria in going from experiment
532 to experiment but also from well to well in one experiment. Priming and seeding are also very
533 important because priming allows the user to determine which channels are allowing the media
534 to flow through the channels without any disturbances or clogging. Channels should also not be
535 disturbed (*i.e.* they should be constantly full of media) after priming to increase the chances of a
536 successful experiment with no air bubbles or clogging. The seeding step can be varied according
537 the type of bacteria and so should be optimized for cell attachment. For example, if cells do not
538 seem to attach, surface modifications may have to occur on the microfluidic plate prior to seeding
539 or longer incubations times may be needed. It is also critical to make sure that the microscope is
540 correctly set up to obtain an in-focus image and should be monitored periodically throughout the
541 experiment to assure that quality images are obtained. If the focus is off, the microscope can and
542 should be adjusted as the experiment continues. During the image acquisition time, the last
543 wavelength should be set to **All Closed** in order to avoid the exposure of filters and lighting to
544 only one channel during the waiting time that occurs in between the image acquisitions. Also,
545 the image analysis that determines the % surface coverage was designed in house because the
546 Montage software manual did not explicitly describe the procedure. Furthermore, in order to
547 expand on the image analysis and determine other characteristics such as surface roughness,
548 *etc.*, open source Python-based code[45] was developed in house and shared on the gitHub
549 repository. There are also limitations on how much data can be stored and managed on the local
550 hard drive, so an external hard drive or online data sharing is needed such as CyVerse[46].
551
552 Conventional bioreactors, such as the CDC reactor and the drip flow reactor[34], require a lot
553 media, provide fewer sample sizes, and demand a high amount of sterilization of equipment. In
554 contrast, the advantages of this higher throughput platform include the ability to control shear,
555 flow rates, and the assumption that the *in vitro* experiments closely resemble *in vivo* conditions.
556 Disadvantages of the system include the multiple accessories and software that require a
557 meticulous setup that must be performed in the correct order of events. Furthermore, the
558 manual that is provided for the equipment does not fully explain each step of the experiments,
559 and the software commands, and consequently, many mistakes occur during the experiments,
560 including clogging of channels, lack of growth or attachment, or the lack high quality microscopy
561 images or movies. The instrument itself and consumables, such as the microfluidic plates, are
562 also relatively expensive with a price tag of over $200 per plate and are not reusable. Thus, while
563 the technique lends powerful results, the technical expertise required for its use is relatively high
564 and requires repeated training by experts in the field. This report attempts to resolve this issue
565 by providing a guide to new users of these bioreactors to study biofilms characteristics.
566
567 The microfluidic system, which is able to perform cellular analysis, has gained considerable
568 attention for various scientific modalities, such as in microbiology, immunology, hematology,
569 oncology, and stem cell research. More specifically, the technology has resulted in many
570 publications describing topics that are highly relevant to medical applications[37,47], including

571   microbial oral adhesion[48], determining the effects of biosurfactants on *Pseudomonas aeruginosa*
572   and *Staphylococcus aureus*[49,50], host pathogen interactions in *E. coli*[51], *Streptococcus*
573   adherence[52], and treatment of cystic fibrosis[53]. Given the fact that this microfluidic system is very
574   versatile, it is anticipated that more and more systems will be distributed throughout the world.
575

576   Some specific protocol steps should be carefully considered. Media can be diluted to 50% with
577   dH_2O to help prevent bubbles and clogging but was not required in this case. The specific value
578   of OD_{600} used for seeding must be determined using trial runs of a growth experiment to see
579   what works best for the particular set of conditions used. Bubbles in the wells prior to sealing can
580   lead to bubbles in the microfluidic channels and should be removed by either popped or sucked
581   out with a pipette tip. It is important to keep bacteria out of the small serpentine channels. By
582   having equal volumes of media in the input and output during the seeding process, flow due to
583   pressure from the liquid volume will be controlled so flow is only due to the applied pressure
584   from the system. The calibration distances should be set up during installation by the company
585   representative. These settings are specific for each camera.
586

587   There are several challenges that occur when finding the most representative threshold for an
588   image. Setting maximum threshold values can be difficult if the average pixel intensity across
589   regions of the background are not consistent caused by either selecting a stage position that is
590   not at the center of the channel or from debris on the plate. Under the **MM Standard**, click on
591   **Process**, and then select **Background and Shading Correction** tool to correction for these
592   inconsistencies. However, this tool is generally only helpful if the user has taken images of the
593   channels before seeding that they can use as reference images. Or, if background/shading
594   reference images are unavailable, the user will need to use their judgment to set a threshold
595   value that covers the most cell area without including background for the entire image.
596   Alternatively, select representative areas to measure that exclude regions of inconsistency by
597   click **Rectangular Region**, **Ellipse Region**, or **Trace Region** to select a region and select **Active**
598   **Region** rather than **Entire Image** on the **Show Region Statistics** window (under **Analysis Tools**).
599   If a representative region is utilized for thresholding the bright field image, the same region
600   should be used for measurement of the corresponding FITC image. It is helpful to record the
601   specific spatial statistics (**Left**, **Top**, **Width**, **Height**, **Area**, **Perimeter**) associated with that
602   representative region so the same region will be found and measured on the corresponding FITC
603   image.
604

605   To prevent a buildup of data on the hard drive that will cause the computer to slow down, an
606   external hard drive can be purchased for data storage. Another option for data storage and
607   facilitating data sharing is the CyVerse bioinformatics platform. Create an account on the CyVerse
608   system by going to http://www.cyverse.org/ Once logged in, launch the Discovery Environment
609   then select "Log into CyVerse". Select "**Data**" and navigate to your the folder. If the image stack
610   is on the local computer then select "**Upload**" then "**Simple Upload from Desktop**". Find the
611   image stack file and select for upload. The file or a folder can be shared with collaborators if they
612   have a CyVerse account and are granted permission. Sharing of the data folder to the general
613   public requires that metadata be added for each file using CyVerse approved standards. This
614   procedure will not be discussed here because this is not within the scope of this work.

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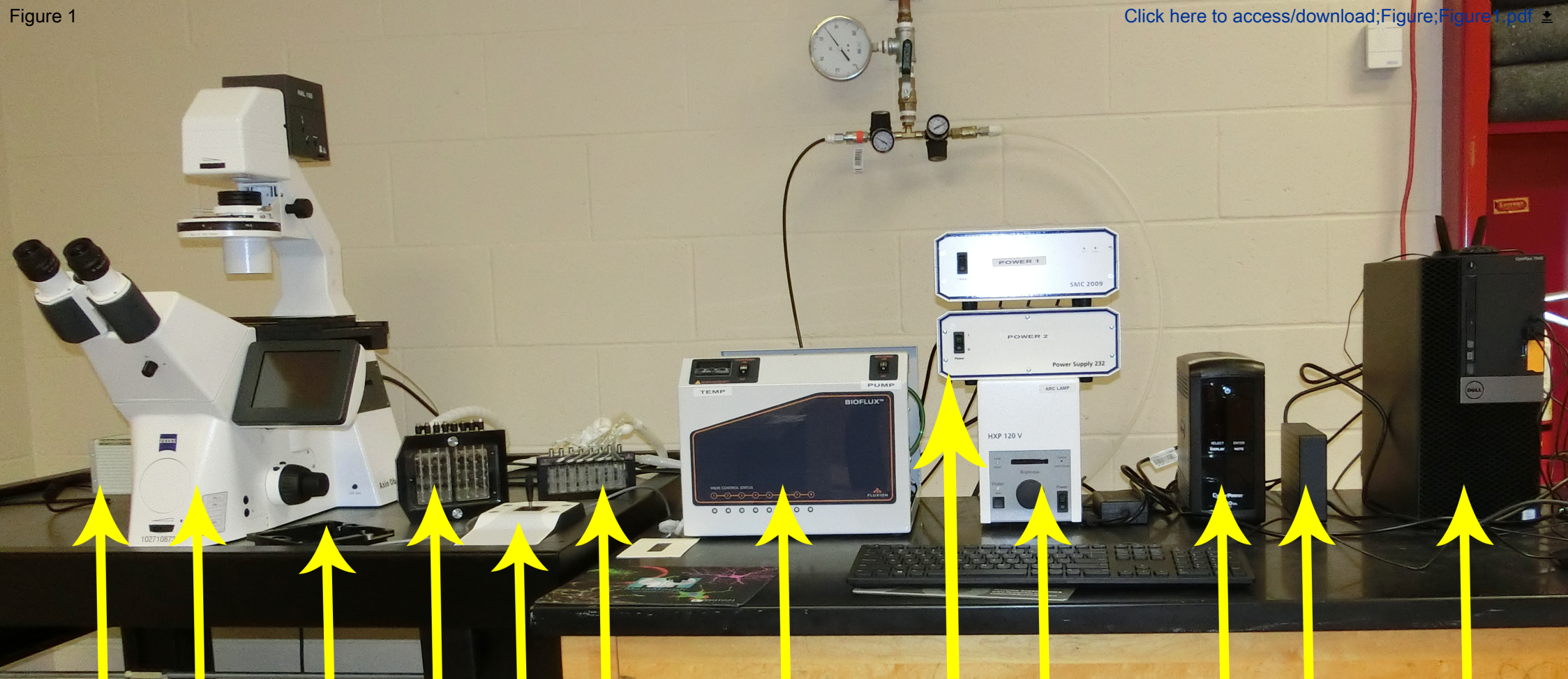
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773

Figure 1

Figure 2

Bright Field Microscopy Image

Inlet Well

Outlet Well

Two Viewing Channels

Inlet Well

Outlet Well

Fluorescence Microscopy Image

Figure 3

Click here to access/download;Figure;Figure3_edited.pdf ⬇
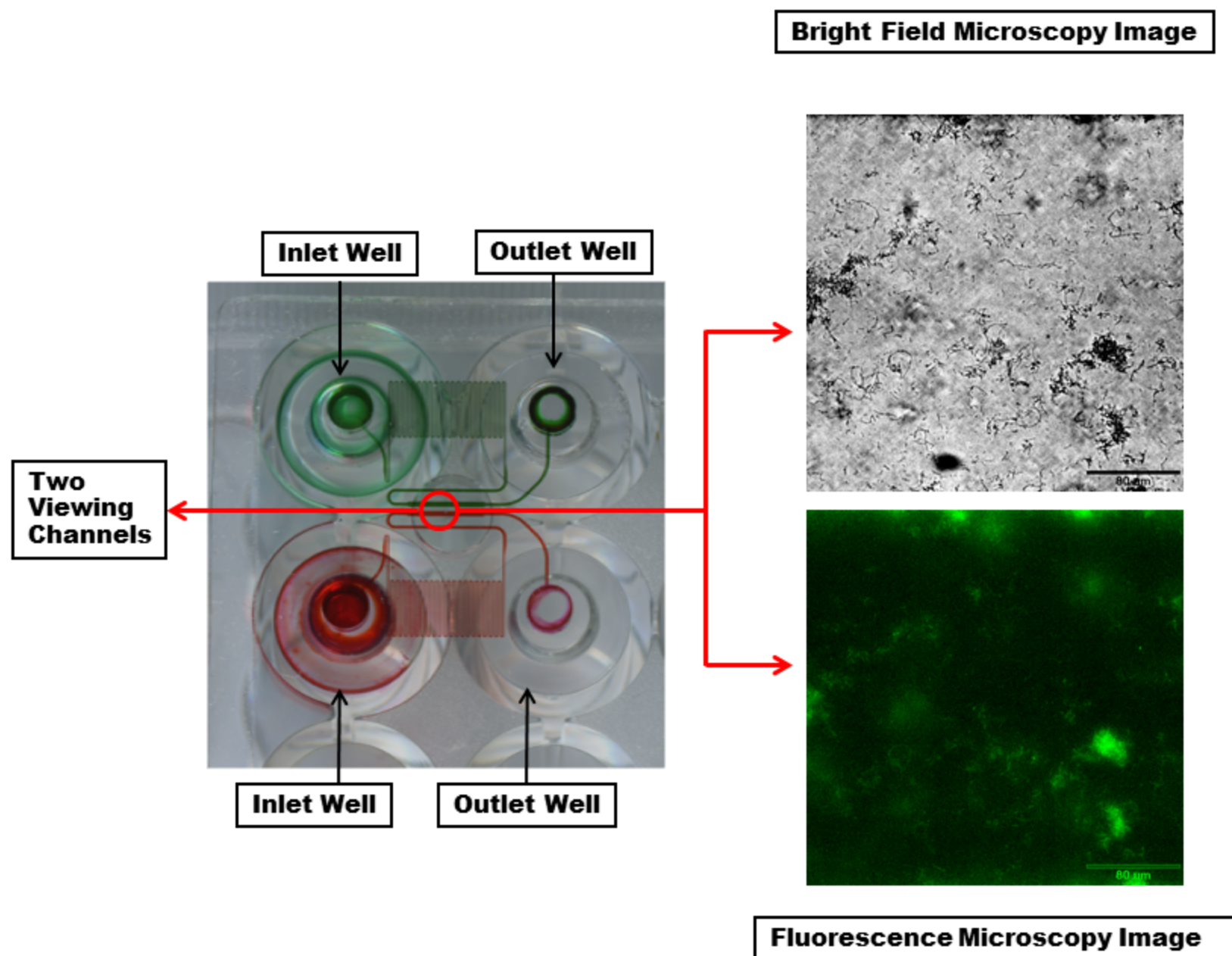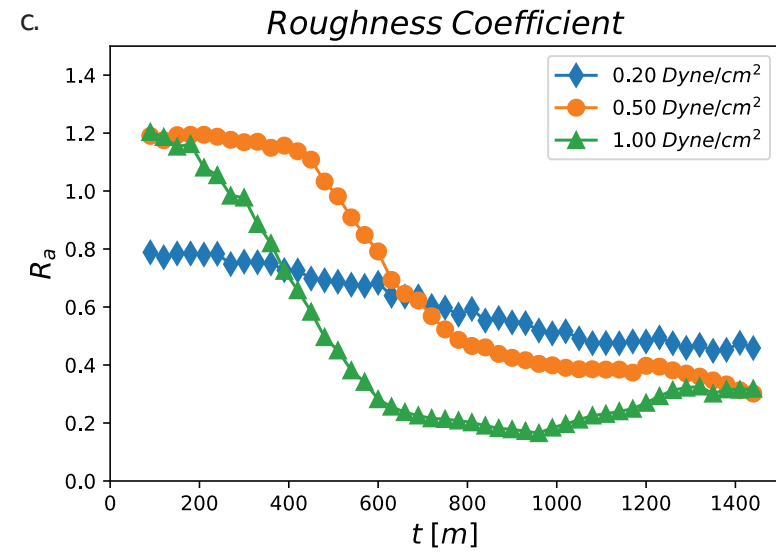

Figure 3
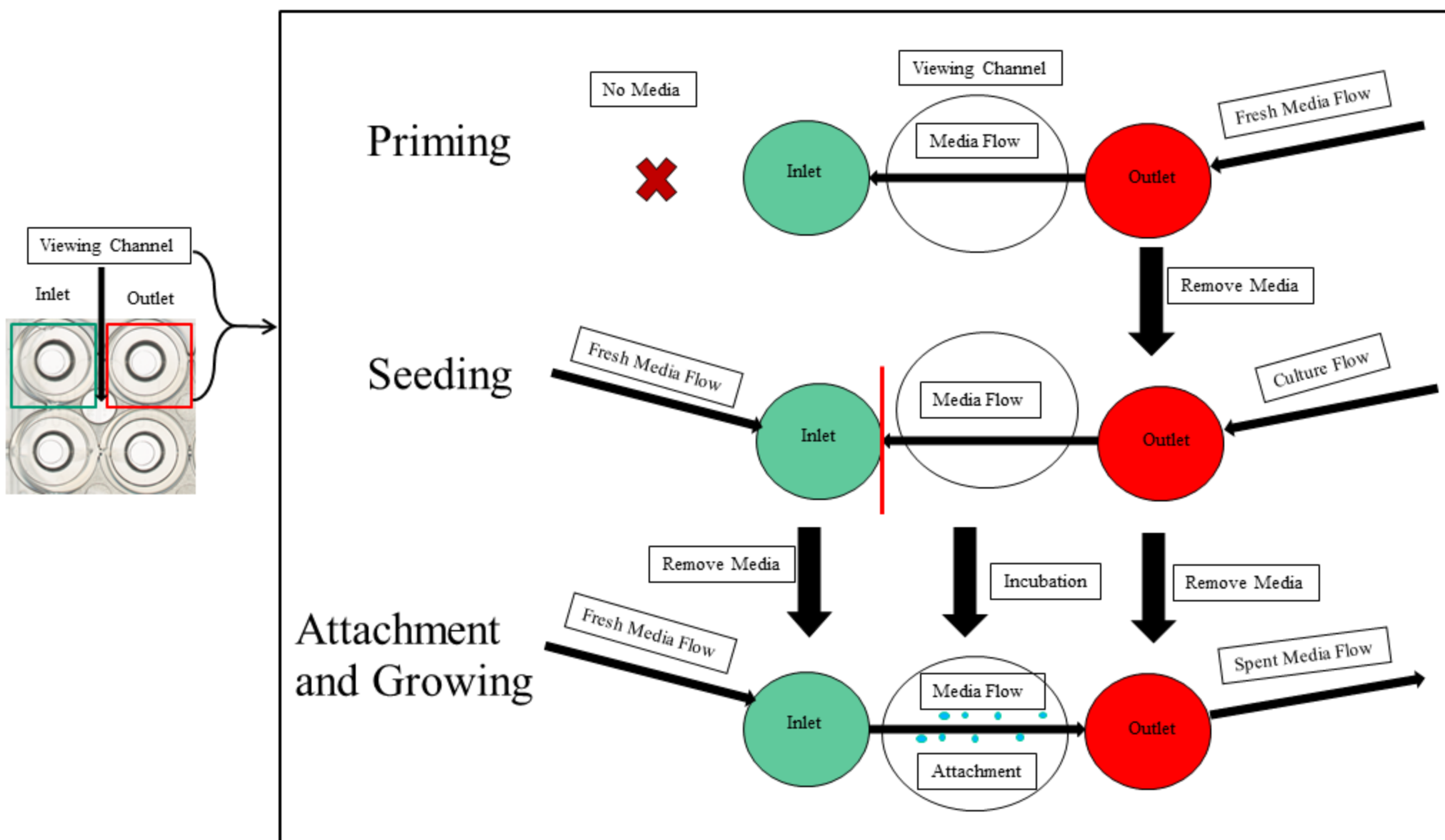
Figure 4
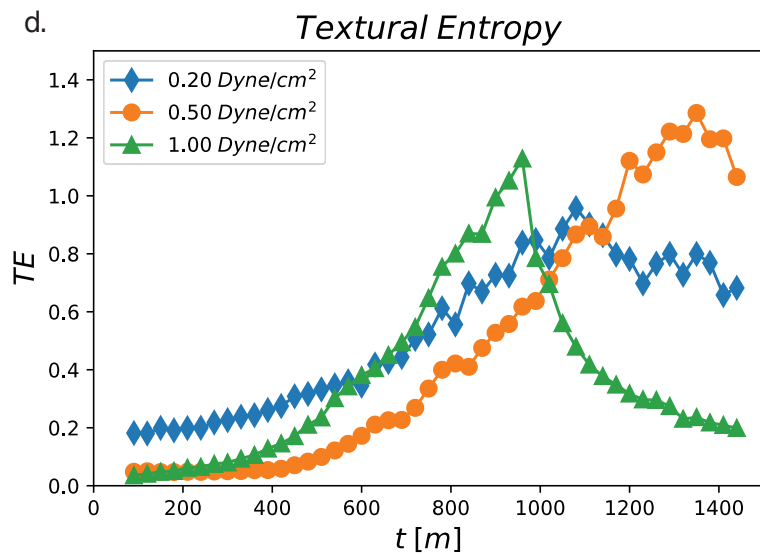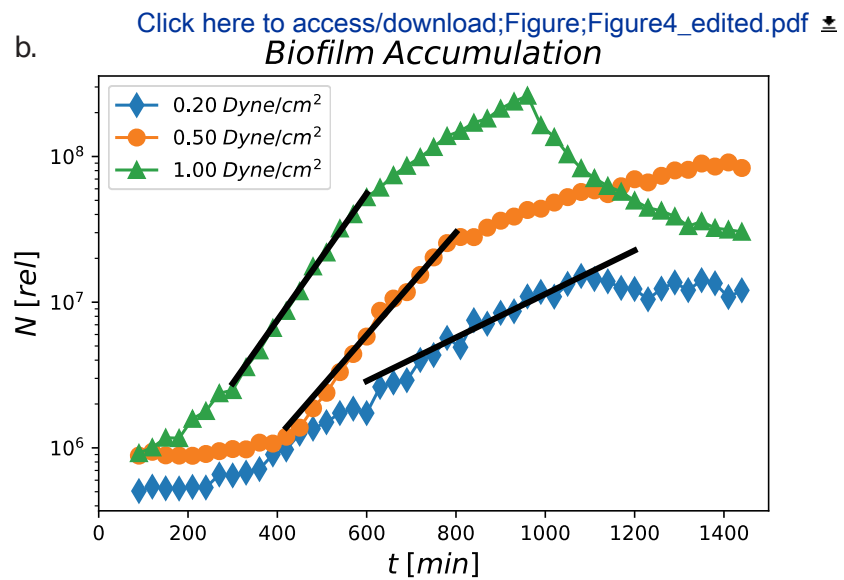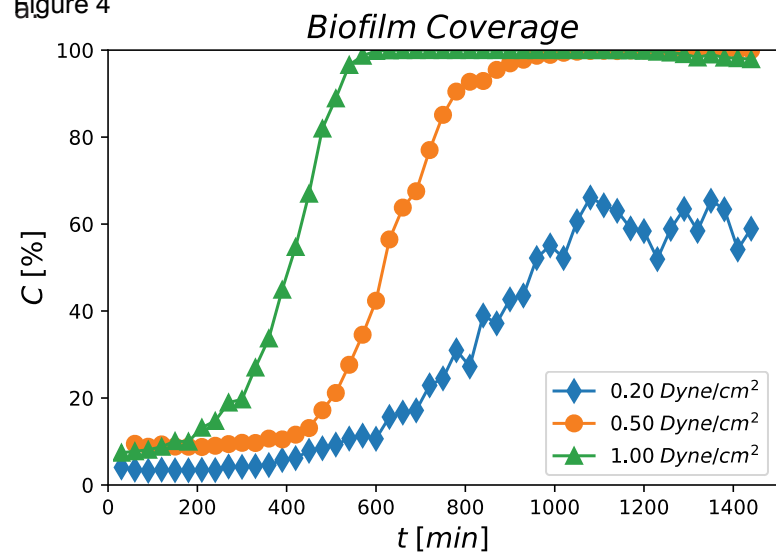
Figure 4

Figure 4

Click here to access/download
Video or Animated Figure
Figure4_edited.svg

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ammonium Chloride, ACS	VWR	BDH9208-500G	Part of the minimal media composition
BioFlux 1000 48 Well Low Shear Plate	Fluxion Biosciences	910-0047	
BioFlux 1000Z Microfluidic Imaging System	Fluxion Biosciences	BF 1000Z	
Calcium Chloride Dihydride, ACD Grade	VWR	97061-904	Part of the minimal media composition
Dextrose, Anhydrous, ACS	VWR	BDH9230-500G	Part of the minimal media composition
Magnesium Sulfate ACS Grade	VWR	EM-MX0070-1	Part of the minimal media composition
Potassium Phosphate Monobasic, ACS Grade	VWR	BDH9268-500G	Part of the minimal media composition
Pseudomonas Aeurginosa GFP	ATCC	15692GFP	Pseudomonas aeurinosa bacterial strain PA01 with GFP modification used for this study.
Sodium Chloride, ACS	VWR	BDH9286-500G	Part of the minimal media composition
Sodium Phosphate, Monobasic, Anhydrous, Reagent Grade	VWR	97061-942	Part of the minimal media composition

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Title of Article:

| Live Cell Analysis of Shear Stress on Pseudomonas aeruginosa using the Automated High-Throughput BioFlux 1000Z System |

Author(s):

| Arin L. Sutlief, Helena Valquier-Flynn, Christina Wilson, Marco Perez, Hunter, Kleinschmidt, Brett Schofield, Elizabeth Delmain, Andrea E. Holmes, Christopher D. Wentworth |

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Dear Dr. Steindel,

We would like to thank you and the reviewers for the insightful comments and suggestions. We have implemented revisions, including the remaking and reordering of the existing images, review of the protocol, and we tried to address every single comment the reviewers pointed out. Please see our revisions based on the reviewer's comments as detailed below, with the reviewers comments in black text and our corrections shown in red. The manuscript has also been edited throughout to correct grammatical or spelling errors. All changes are highlighted in red font color within the revised manuscript.

Sincerely,

Arin Sutlief

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The article was proofread to avoid any spelling or grammar issues.
2. There are missing articles throughout.
Missing articles were added throughout the manuscript where appropriate
3. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.
Files have been converted to .svg files.
4. Figures 3 and 5 can be made supplemental. Please note that there is a video here.
These were revised as described.
5. Figures 6-9 can be combined into one figure with 4 panels.
Images were reformatted as described.
6. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
The table of essential supplies, reagents, and equipment was revised as described.
7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: BioFlux Montage, etc.
The single mention of the company Fluxion Biosciences was removed. BioFlux is not the company and BioFlux Montage was used in previously published articles about the BioFlux including in JoVE.
8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
The protocol section was revised to contain only the imperative mood, except for a small number of "notes".

9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The protocol has been reviewed to reword or remove all non-action items. The discussion has been modified to include items of priority removed from the protocol.

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All figures were originally made by the authors.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe the use of the commercially available microfluidics platform from Fluxion, the Bioflux Z1000 system. They describe the use of this system for analysis of PAO1 biofilm formation. The described protocol is reasonable complete and should be useful for readers in the present state. The cited literature is not very complete in my eyes as there have been more studies describing the use of this system that are not mentioned. e.g., this system has been used with Candida albicans.

Line 59: I suggest changing "….the method…" to "…a method…"

Revised as described.

Line 61 and general: I have some doubts whether the Bioflux system is truly a high throughput system. I suggest the term medium throughput.

Taking into consideration this comment, all mentions of the throughput of the BioFlux system was described as a "higher throughput".

Line 69. There is no doubt that microbial biofilms are present in all natural streams, rivers and lakes. Estimates suggest that near 80% of all microbial life resides in biofilms. Biofilms are therefore not an exceptional community, just exceptionally understudied…

We are unsure of the meaning of this comment since there is no mention of an exceptional community in the article but this section was reviewed and reworded.

Line 76: I object to the abbreviation of Pseudomonas aeruginosa as PA. There is an agreement on the use of species names for a long time already. Please use P. aeruginosa as the standard abbreviation throughout (with exception of first mention).

Throughout the article, this was revised as described.

Line 83: the term prototype strain is strange. Please use reference strain.

Revised as described.

Line 86: …This type of fluorescence analysis is advantageous for the study of biofilms because GFP does not interfere with cell growth and function… This is debatable as all heterologous expressed proteins, even presence of "extra" DNA poses a burden on the cell. The protein has to be synthesized at the expense of energy. I agree that the effects are generally marginal and probably not relevant for the described experiments, yet the above statement is scientifically incorrect.

This was revised to "...GFP does not interfere significantly with cell growth and function…".

Line 102: Please replace…film… with …biofilm…

Revised as described.

Line 121: Biofilms in nature and medicine are notoriously heterogeneous. It is therefore relevant

to include this heterogeneity in model systems, even though it does make the job harder…

███████This phrase was reworded for clarity "Biofilms do not grow evenly across the surface in a drip flow reactor because the low shear of media causes trailing along the larger conglomerates of P. aeruginosa bacteria therefore the biofilm growth is not very smooth and the uneven samples cannot be easily analyzed."

Line 125: A typical biofilm experiment in the Bioflux requires at least 0.5-1 ml medium

This was revised to read as "...where only milliliters of media are required…".

Line 130: Please replace fluorescence spectroscopy with microscopy. Also, do you measure GFP or visualize. I think this is an important drawback of microscopic methods, you can't really get objective quantification of GFP fluorescence. It should be made clear that the use of the bioflux method allows for semi-quantitative measurements at best.

This section was reworded to "It is coupled with fluorescence microscopy to visualize the fluorescence of the EGFP tag in PA01…"

Line 138: Important to mention is that temperature will be constant over the entire plate and not a variable within an experiment. Also, shear and thus flow is managed per column limiting the variation to 4 different values within a single experiment.

The section was reworded to the following to enhance its description: "This allows for 24 simultaneous experiments that can be performed with various experimental conditions, such as bacterial strains, antimicrobial treatments, and media varied from channel to channel and controlled shear flow for each column of six channels. The experimental temperature is also controlled with one temperature setting throughout the plate."

Line 147: Please cite literature on the use of the bioflux for studying Candida albicans and other species. I would not state…It is hoped that… However it is the aim of this protocol/report/manuscript…

This section was reworded and three references were added: This report will allow for the expansion of experiments involving other microorganism besides bacteria, such as fungi and algae that have medical and environmental applications.[41–43]

Line 155: I disagree with this statement. We always use partial plates without any problems. It is better to use partial plates in light of observed slight differences in flow within a column depending on the position of the well (near air connection on top or far away from it).

This statement has been removed in order to stay neutral to the company's claims.

Line 176: Does this strain require Antibiotic pressure to maintain eGFP? If so, please mention this.

No chemicals or antibiotic is required for the expression of eGFP and a reduction in eGFP signal has not been observed within the strain.

Line 186: The order of PC, Microscope and Bioflux is not really important, if the CCD is not on when the software is started, it will mention this.

In order to make this a more neutral statement the wording was adjusted to "In order to avoid an error in the connection of the instrument to the software, it is advised that the order of turning on the instrument is performed in the following order:…."

Section 4.2 and further: airbubbles will always form, Our experience is to always make sure to remove any bubbles prior to sealing the plate. Maybe this is important to mention, also how to do that…

To address this we added the following wording "Bubbles in the wells prior to sealing can lead to bubbles in the microfluidic channels and should be removed by either popped or sucked out with a pipette tip."

Also, we always prime from the input well. This is not really an issue with the 48-well plate, however when using the 2 inlet system (24-well plate) this is critical to prevent air to be caught in the system.

It was recommended by Fluxion by representatives and the manual to prime from the output well. In this case, the procedure described is a system for a 48-well plate system.

Specialty plates such as the 24-well plate may require modification to the set-up process. The following sentence at the end of introduction section was modified to make sure it was clear we were describing a method for a 48-well plate system: "The detailed approach describes how to culture PA01-EGFP, inoculate a 48-well plate and set up the microfluidic device and software, set up the fluorescent microscope, and demonstrate the software analysis to obtain the biofilm coverage, growth rate, and morphological properties such as surface roughness."

Section 4.5: it is critical to monitor this by eye due to slight differences throughout the length of the column. It is better to not give a time for the flow as this might vary depending on temp. medium and organism used. Also, maybe it is good to mention that all channels need to be checked individually to ensure all of them are inoculated. The microscope only visualizes 1 or 2 channels.

Given that this is a paper describing a protocol for a specific experimental set-up with a single experimental temperature and organism, a time frame would be appropriate for the protocol to act as a frame of reference. The phrase (below) was reworded to reflect that the live camera feed should be used but a note (below) was included to caution that the time frame would be optimal for our conditions only and across the plate there may be variation which would require additional flow time for certain columns of channels.

"On the Montage Control Module, focus on a single channel using the live camera feed after placing the plate stage onto the microscope stage. Visually monitoring by the live feed, resume flow at 1.00-2.00 dyne/cm2 for approximately two to four s to allow cells to enter the experimental channel but not in the serpentine channels."

"Note: The amount of time needed for seeding will vary with media and organism so it should be monitored closely by the live feed until optimized and used as a general time frame. Seeding throughout the plate may vary which would require more time of applied flow to certain columns of channels for complete seeding."

Section 6.1: We were advised never to use more than 1 mL of liquid per well…

Another group that regularly use the BioFlux advised us that 1.3 mL maximum could be put into the well as long as certain cautions were made such as the closer the media is to the cover the more slight movements could cause bacteria to come into contact with the cover. This would contaminant the cover which could be significant in the case of organisms not susceptible to ethanol killing. The amount of volume can be adjusted based on desired experimental time/flow rate but would be advised to keep it a consistent variable.

Reviewer #2:

Manuscript Summary:
This manuscript provides a clear procedure for the effective use of the BioFlux device to study P. aeruginosa PAO1 biofilms in more detail and in new ways that hasn't to my knowledge been described previously. This procedure will be extremely helpful to other researchers interested in using the BioFlux microfluidic device to study biofilms produced by P. aeruginosa as well as other bacterial species. The use of the enhanced GFP is very important as this eliminates the need for another fluorescent stain, such as propidium iodide (which is not that effective in determining dead cells in P. aeruginosa) to distinguish between live and dead cells in the biofilm. Also, the methodology for determining textural entropy and roughness coefficient are fascinating as is the concept that the amount of shear flow applied affects the formation of the biofilms. This will help other researchers maximize or optimize the conditions they may wish to use depending on what they are testing. Finally, the procedure described is detailed and clear and the figures help enhance the procedures and the results that are obtained.

Major Concerns:
Lines 180-182 - I found the OD used to be quite high, but maybe this is an optimal OD600

based on the number of hours the biofilms are allowed to develop. We had problems in the past and had to use PAO1 at a lower OD600 (around 0.3) or we would get over-development by 8 hours. Also I am wondering about holding the cells at 37 degrees Celcius, I am assuming this would keep them warm as I know we have to warm our media and such before adding to the device, but do you think the OD600 will increase if the cells are left at 37 too long before adding to the plate?

The following was added in the discussion under "protocol considerations" to clarify the choice of OD600 used for seeding:

"The specific value of OD_{600} used for seeding must be determined using trial runs of a growth experiment to see what works best for the particular set of conditions used."

The following sentence was added to the protocol to mitigate the concern about holding the cells at 37 [C] while waiting for the seeding procedure:

"The OD_{600} should be checked again immediately before seeding to insure that it has not changed significantly from the target OD_{600}, 0.8, in this case."

Figure 2 - the images for the biofilms could be a bit more resolved. I understand it is hard to see individual cells normally, but a bit more definition of the cells would be best. Also it would be nice to see some information on how many cells are living versus dead on the fluorescent image, if this can be distinguished. Finally, what objective was used on the microscope, are these images taken from a 10X lens?

The image has been remade with biofilm images that are a little clearer.

The GFP marker is known to dissipate upon the death of GFP modified P. aeruginosa and therefore, acts as a method to disguise live versus dead cells.

Step 5.3.1 states that the objective used for the experiment is a 20x lens. Wording was added to Figure 2 caption further clarification. "Shown are bright field (top) and fluorescent (bottom) microscopy images of a single channel with a PA01-EGFP biofilm using a 20x objective."

Minor Concerns:

Lines 184-306 - why are these sections (3-6) highlighted in yellow?

Sections of the manuscript are highlighted as instructed by JoVE requirements to indicate the sections the authors find important to address in the complimentary video.

Lines 647-648 - should this read biofilm characteristics?

The indicated lines does not include any biofilm characteristics so we are not sure what is being addressed with this comment. We reviewed the section to catch any possible errors.

Reviewer #3:

Manuscript Summary:
The manuscript entitled "Live Cell Analysis of Shear Stress on Pseudomonas aeruginosa using an Automated High-Throughput Microfluidic System" by Arin Sutlief and colleagues provides a comprehensive guidance to study microbial biofilms by time-lapse microscopy using the BioFlux 1000Z microfluidic system. The manuscript is clearly written and provides all necessary technical details required for successful and reproducible application of the described methodology. The subject described in the manuscript fits well within the scope of the journal and the methodology will undoubtedly be interesting to a broad audience. I have only a few minor comments:

-Lines 65-66 - This sentence requires rephrasing as it is a bit unclear in the present form. Specifically:
"…be beneficial to the bacteria and their environment…." - what kind of environments the authors are referring to?
"….they can also be highly harmful with undesirable consequences" - harmful to what?

This section has been reworded and references were added for clarity. "These biofilm communities can be beneficial to the environment, such as improving water quality in water supply lines and in bioremediation of recalcitrant compounds.[2, 3] However, biofilms can also be highly harmful to human health with undesirable consequences. For example, medical devices, such as hip and knee implants, are one type of surface where biofilm accumulation has been a challenge and causes severe medical complications.[4, 5]

-Lines 77-78 - "…community-acquired and hospital-acquired…" - can be changed to … community- and hospital-acquired…

Revised as described.

-Line 84 - "EGFP is a mutant of GFP…" - can be changed to -… EGFP represents a mutant form of GFP…

Revised as described.

-Line 93 - Can be rephrased to -…. Biofilms can grow under various environmental conditions including those with different flow rates.