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.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

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 OD600 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 OD600 should be checked again immediately before seeding to insure that it has not changed significantly from the target OD600, 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.