July 7th, 2018

Dear Editors,

On behalf of the authors, we appreciate your recent invitation to resubmit our manuscript entitled “Real-time imaging and quantification of fungal biofilm development using a two phase recirculating flow system” (Manuscript #58457). We thank the reviewers for providing valuable input that we have used to improve the manuscript. Following are our specific responses (highlighted in bold) to the reviewers’ concerns. We hope that you now find our manuscript suitable for publication in the Journal of Visualized Experiments.

Sincerely,

Andrew McCall, Ph.D.

**Editorial comments:**  
Changes to be made by the Author(s):  
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.  
2. S Video 1: It is mentioned in line 316 but not provided. Please upload the video as “Animated/Video Figure” to your Editorial Manager account in the form of an .mov, .mp4, .m4v file.  
3. Please define all abbreviations before use.  
4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.  
5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.  
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.  
7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).  
8. Lines 71-119, 137-139, 155-157, 178-180, 280-312: Please revise the protocol so 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 should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.  
9. Line 225: Please specify the high speed used in this step.  
10. Please include single-line spaces between all paragraphs, headings, steps, etc.  
11. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.  
12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.  
13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.  
14. Line 351: Please remove commercial language: ibidi®.  
15. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

**We have reviewed our manuscript and made the changes suggested above.**

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
This manuscript will be tremendously useful to the fungal biofilm community. The apparatus developed by the authors is slightly complex (as they acknowledge) but also quite inexpensive. A major strength is the ability to quantify several key biofilm parameters. The presentation of green and orange sides was very helpful. I also very much appreciated the authors' even handed presentation of the apparatus, though I felt that they were perhaps a little more negative about their approach than necessary. (The fact that a Bioflux sends you to the poorhouse, and that it clogs really fast when hyphae are used, are major problems with this alternative.) I also very much appreciated the section on cleaning the apparatus. Overall, bravo!  
  
Major Concerns:  
None.  
  
Minor Concerns:  
There were some minor English usage errors.  
- "couple feet" or "couple ml" should be "couple of feet" or "couple of ml" I believe.  
- "Ensure all valves are open" should be "Ensure that all valves are open" I believe.

**We have made these changes.**  
  
**Reviewer #2:**  
Manuscript Summary:  
This manuscript describes a method for the real-time imaging of fungal attachment, growth, and detachment using reticulated flow of growth medium. The authors acknowledge that there are a number of systems available for studying the growth of fungi under flow conditions, but provide a valid justification for the visualisation of their protocol. Their arrangement has advantages in that cells do not have to be fluorescently labelled for visualisation, the reticulation of medium allows prolonged growth experiments, and the equipment should be relatively inexpensive to install. Thus there should be interest in the adoption of this technology. The authors provide detailed instructions and discuss the limitations of the system. I provide the following comments that could help improve the manuscript.  
  
Minor Concerns:  
1. Rather than simply stating that the fungal cells do not need to be fluorescently labelled, I think an unstated advantage of the system is that it allows the study of unmodified clinical isolates increasing the relevance of growth studies.

**We have added this to the introduction.**

2. I think the protocol section would benefit by beginning with a general description of the system. It is not clear at the outset that some of the equipment is within a temperature-controlled cabinet and some is outside the cabinet, but uses a hot plate.

**We have added a brief general description to the end of the introduction, just prior to the protocol.**

3. It is unclear how or why fungal cells should adhere in the chamber. Is it treated in some way to promote adherence (such as coating with poly-L-lysine)?

**The ibidi slides have a proprietary cell-adhesion coating (ibiTreat) that allows the cells to adhere. We have added this information to the manuscript.**

4. It is not clear what FB ('filter bottle') refers to. There is no 'filter bottle' on Figure 1, although the 'attachment flask' has a filter as does the 'filter flask'.

**We have revised figure 1, changing the filter flask to filter bottle.**

5. In protocol sections 1.6 - 1.8, feet and inches are used. As this is an international journal I think that SI units should be used. Likewise, in sections 16, 18, 23 and cleaning section 5, 'm' is used as an abbreviation for minute whereas 'm' is the SI unit for metres. Usually 'minute' is abbreviated to 'min'.

**We have made these changes.**

6. I don't think the protocol, in section 1.12, should introduce the possibility of alternative untried configurations. Either the authors should test the alternative set-up or they should limit the protocol to the set-up they used.

**In principle we agree, however I do not think everyone would be as comfortable drilling a hole in a rubber stopper, as it can be a little tricky to do (they may also not have access to a drill). This is why we mentioned the alternative configuration. We have made this more clear.**

7. It is unclear why large portions of the manuscript are highlighted in yellow.

**This is part of the JoVE submission process. The highlighted sections are for preparing the script for the video.**

8. In protocol section 6.1 the 0.2 µm filter is referred to as 'X' does this mean '10' because I think the 0.2 µm filter in Figure 1 is filter '11'.

**Our apologies, the X was meant as a place filler until Fig. 1 was finalized, this was supposed to be ‘11’.**

9. In section 14.7.2, I think it should be clarified that the 'dripping media' should be coming from the input tube.

**We have added this to the manuscript.**

10. In section 4, line 236, do the authors mean 200 ml undiluted bleach? This is quite a lot of bleach to be pumping around the system.

**Yes, undiluted bleach. We have tried using diluted bleach, but it does not clear the tubes of debris as well as undiluted bleach (based on the amount of debris in the ibidi slide at the start of the next run). We realize this is a lot of bleach, but bleach is cheap while small caliber tubing, PTFE membranes and micron pore size filters are not. Thus, we tend to err on the side of clearing the tubing as much as possible. We have clarified the use of undiluted bleach.**  
11. Cleaning section 6.1, what is meant by "into the water", what water?

**The water being used to rinse the remainder of the flow system, from step 6. This has been clarified in 6.1.**

12. Quantifying the videos section 3.7, should 'step 2.4' be 'step 3.4'?

**We have fixed this typo.**

13. In the analysis descriptions section 5, it would help if the analysis features, such as Complete Analysis were in italics to show that they are an analysis function.

**Great idea, we have made this change.**

14. Results section, lines 318-9, what does "normalized to the imaging area" mean?

**It simply means dividing by the imaging area. We have clarified this in the manuscript.**

15. I think the discussion section overemphasises potential difficulties with the system. In lines 390-2 I don't think it is necessary to say that it is difficult to use the system in a university - many facilities will allow long bookings of equipment. In lines 397-402 I don't think the authors should raise the possibility of forgetting steps. As scientists we should be trained to follow protocols precisely how ever many steps are involved. You can also forget steps in simple protocols with dire consequences and it is not necessary to warn people about forgetting them.

**We have removed these sentences.**

16. The clogging of filters by cells detached from the slide occurred to me early on in the reading of the manuscript. It is mentioned in the discussion, but it would be good to know how long growth can be followed before filters clog.

**The max time varies substantially, and this is one of the reasons we didn’t specify a value. Sadly, there are many variables that can influence when the filters clog.**

17. I think that the bubble trap in Figure 1 should have a line to vacuum marked on the plan.

**We have added a vacuum to the figure.**