**JoVE Editor**

Dear Editor,

We have revised the manuscript in response to the Editorial Comments and Reviewers’ Comments, and provide a point-by-point response to the comments below.

**Editorial comments:**

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We have done so.

2. *Abstract: Please refrain from using indentations.*

Fixed.

3*. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*

Fixed

4. *Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*

We have reworded steps to be definite, imperative actions.

5. *Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:*

We have expanded the protocol to address the above concern, both for the examples listed below and for other cases.

*1.1.2: Please specify how to determine appropriate sample volumes using a dilution series. How are the particles counted, manually?*

*1.2: Please specify where the sample is collected.*

*2.1.1: Please mention how preliminary tests are done or add a relevant reference.*

*3.5: Please specify how the pixel:micron ratio for the microscope is determined in this protocol.*

6. *Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:*

We have added the appropriate software steps, including how to clone the repository, and updated the table of materials.

*3.5.1-3.7.1: Please list the software used in this protocol in the Table of Materials and explicitly explain ('click', 'select', etc.) how to perform these steps.*

*4.1.1.2: Please describe how to clone the git repository. Details are needed for filming this step.*

4.4 *and sub-steps: Please describe in imperative tense how to perform a quality control check. As currently written, the sub-steps do not contain specific actions that are required for filming.*

We have added instructions on how to do this.

7. After you have made all the recommended changes to your protocol (listed above), 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.

8. *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.*

Fixed.

9. *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.*

Fixed.

*10. Discussion: Please discuss any limitations of the technique.*

Added to lines 454-455: “The major limitation of this method is that the requirement to keep all particles focused prevents high magnifications, limiting its utility for particles with small minor dimensions – notably filamentous structures.”

11. References: Please do not abbreviate journal titles.

Fixed. In one case, ‘*AAPS Pharm SciTec’* appears to be the full journal title as far as we can determine.

**Reviewers' comments:**

**Reviewer #1:**

*Major Concerns:*

*This system looks like a shape and size analysis based on optical images. However, it can not judge the characteristics of particles, such as biological or nonbiological. So I don't think to define the system as the activated sludge measurement is correct. This is really like a particle analysis protocol.*

The underlying methodology is, of course, particle analysis. The protocol is specific to activated sludge because of the agar immobilization step, staining process, and the identified sample volumes and magnifications appropriate for these particles.

*Moreover, the agar functions as the fixing element for particles. Is it possible to replace it with the microscope cover glass? That will make the process more convenient.*

There is insufficient space under a cover glass to contain sufficient particles. Further, the cover glass alters their shape - especially so in the case of granular activated sludge.

**Reviewer #2:**

Major Concerns:

*1) The authors have discussed and shown particle size calculations but particle shape has not been explained explicitly anywhere, except that when other organisms like protozoa are present then these can be identified.*

The files generated contain shape information for each particle, using standard metrics such as solidity and circularity. We have added a reference on line 301 which describes how ImageJ/FIJI defines those shape factors.

*2) for Figure 2, the numbers represent which parameter, area, circularity?*

The numbers are the particle ID. We have reworded the caption to make this clearer.

3) *for image processing often the first step is noise removal, which has not been mentioned.*

The size of the particles and the contrast provided by the stain is such that noise removal is not necessary when using the suggested magnification. We did try out various noise removal methods (median filtering, erode-dilate, rolling ball, gaussian subtraction, image averaging, dark-field and white-field correction), and while they resulted in 'prettier' pictures, they did not improve particle detection.

4*) the imaging protocol is also not defined, like do you have to cover the full slide plate, or only a part. as the concentration of the sample may not be even so it may effect the result.*

The coverage pattern can be arbitrary, but we have added unambiguous directions for a default lawnmower pattern in step 3.7. We specifically point out the necessity of even particle coverage in the plate preparation section and have reworded step 2.6 of the protocol to emphasize this.

5*) if the whole plate is not covered, then how to decide which part of plate is to be covered*

With even particle distribution, it does not matter. We have, however, altered the protocol to suggest a default coverage pattern in step 3.7

*6) if the whole plate is covered then what will be the protocol of covering the plate , left to right, top to bottom, or in circular motion*

See above.

*7) authors have also mentioned image stitching, but has not been implemented, automatic image stitching may not give correct results and blur some part of image, any thoughts on it and same method may not apply on all images.*

We agree and share the reviewer's concerns about image stitching and did not implement it for those reasons. We have reworded the note following step 3.7 for clarity and to more explicitly caution against stitching.

8*) also to cover whole slide need to focus separately as the sample size is big so there could be problem of sample drying?*

Because we are imaging an agar plate, not a slide, there is no drying issue.

9) *No mention of vignetting problem it always appears in microscopic imaging?*

Vignetting has proven to not be a problem, for the same reasons we give regarding noise removal. We also performed image analysis on images with vignetting removed (via large sigma smoothing background removal and rolling ball removal), and saw no improvement in particle analysis.

*10) Z- stacking is mentioned for future work, but can automatic z- stacking can work for all samples, or it may need customization.*

That is to be determined in future work.