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TITLE:

Measuring the Shape and Size of Activated Sludge Particles Immobilized in Agar with an Open Source Software Pipeline

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KEYWORDS:

Digital image analysis, particle size, particle shape, activated sludge, granular sludge, floc, morphology, microscopy, software, FIJI

SUMMARY:

The size and shape of particles in activated sludge are important parameters that are measured using varying methods. Inaccuracies arise from non-representative sampling, suboptimal images, and subjective analysis parameters. To minimize these errors and ease measurement, we present a protocol specifying every step, including an open source software pipeline.

ABSTRACT:

Experimental bioreactors, such as those treating wastewater, contain particles whose size and shape are important parameters. For example, the size and shape of activated sludge flocs can indicate the conditions at the microscale, and also directly affect how well the sludge settles in a clarifier.

Particle size and shape are both misleadingly 'simple' measurements. Many subtle issues, often unaddressed in informal protocols, can arise when sampling, imaging, and analyzing particles. Sampling methods may be biased or not provide enough statistical power. The samples themselves may be poorly preserved or undergo alteration during immobilization. Images may not be of sufficient quality; overlapping particles, depth of field, magnification level, and various noise can all produce poor results. Poorly specified analysis can introduce bias, such as that produced by manual image thresholding and segmentation.

Affordability and throughput are desirable alongside reproducibility. An affordable, high throughput method can enable more frequent particle measurement, producing many images containing thousands of particles. A method that uses inexpensive reagents, a common dissecting microscope, and freely-available open source analysis software allows repeatable, accessible, reproducible, and partially-automated experimental results. Further, the product of such a method can be well-formatted, well-defined, and easily understood by data analysis software, easing both within-lab analyses and data sharing between labs.

We present a protocol that details the steps needed to produce such a product, including: sampling, sample preparation and immobilization in agar, digital image acquisition, digital image analysis, and examples of experiment-specific figure generation from the analysis results. We have also included an open-source data analysis pipeline to support this protocol.

INTRODUCTION:

The purpose of this method is to provide a well-defined, repeatable, and partially-automated method for determining size and shape distributions of particles in bioreactors, particularly those containing activated sludge flocs and aerobic granules[1,2]. The rationale behind this method were to enhance the affordability, simplicity, throughput, and repeatability of our existing in-house protocols[3,4], ease particle measurement for others, and facilitate sharing and comparison of data.

There are two broad categories of particle measurement analysis – direct imaging and inferential methods using such qualities as light scattering[5]. Although inferential methods can be automated and have large throughput, the equipment is expensive. In addition, while inferential methods can accurately determine the equivalent size of a particle[6], they do not provide detailed shape information[7].

Because of the need for shape data, we have based our method on direct imaging. While some high-throughput imaging methods exist, they have traditionally required either expensive commercial hardware or custom built solutions[8,9]. Our method has been developed to employ common, affordable hardware and software that, although suffering from a reduction in throughput, produces far more particle images than the minimum needed for many analyses[10].

Existing protocols may not specify important sampling and image acquisition steps. Other protocols may specify manual steps that introduce subjective bias (such as ad hoc thresholding[11]). A well-defined method that specifies sampling, immobilization and image acquisition steps combined with freely available analysis software will enhance both within-lab image analysis and comparisons between labs. A major goal of this protocol is to provide a workflow and tools that should lead to reproducible results from different labs for the same sample.

Apart from normalizing the image analysis process, the data produced by this pipeline is recorded in a well-defined, well-formatted file[12] suitable for use by popular data analysis

packages[13,14], easing experiment specific analyses (such as custom figure generation) and facilitating data sharing between labs.

This protocol is especially suggested for researchers who require particle shape data, do not have access to inferential methods, do not wish to develop their own image analysis pipeline, and wish to share their data easily with others

PROTOCOL:

1. Collect samples for particle analysis

1.1) Determine the sample volume for specific reactors that will produce sufficient particles for statistical analysis[10] (>500) while avoiding particle overlap.

1.1.1) Assume that a range of 0.5 to 2 mL per sample of mixed liquor is sufficient for activated sludge samples with a mixed liquor suspended solids (MLSS) between 250 and 5000 mg/L.

1.1.2) Otherwise, prepare three test agar plates using 0.5, 2, and 5 mL of sample (steps 1.2 through 2.7).

1.1.3) Visually estimate which (if any) sample volumes best meet the criteria listed in step 1.1.

1.1.4) If particles still overlap for the 0.5 mL sample, repeat steps 1.1.2 and 1.1.3 with three 0.5 mL samples diluted with an added 0.5, 1, and 2 mL of phosphate buffered saline to determine the degree to which a 0.5 mL sample must be diluted prior to step 2.1.

NOTE: Steps 1.1 − 1.1.4 need only be performed once per experiment, or if the reactor contents change such that subsequent measurements no longer meet the criteria listed in step 1.1.

1.2) Acquire a representative sample from a well-mixed portion of the reactor by grabbing ~40 mL in a beaker or 50 mL centrifuge tube, gently mixing, and immediately pouring the determined sample volume of the well-mixed grab into a 15 mL centrifuge tube. Dilute the sample, necessary, as determined by step 1.1.4.

NOTE: The protocol can be paused here and the sample may be stored under refrigeration (4^{o}C) for up to 48 hours. Do not freeze the sample.

CAUTION: Common preservation media (e.g., formaldehyde/formamide) are not suitable. The large surface area of the plate, in combination with the open container, heat from the light source, and potentially poorly ventilated microscopy setup produce unnecessarily hazardous conditions for little gain in image quality.

2. Prepare agar plates of stained, immobilized particles

2.1) Add 5 µL of 1% (w/v) methylene blue to each sample, then cap and gently invert at 3 least times to mix. Allow samples to stain for at least 5 but no more than 30 minutes at room temperature.

2.2) Prepare approximately 10 mL per sample of 7.5% (w/v) agar in deionized water.

NOTE: Agar may be produced ahead of time and stored indefinitely if sterilized. Agarose may be substituted, but does not substantially improve images.

2.3) Melt agar using a microwave or water bath and allow to cool slightly before use. Ensure the agar is completely melted and pours easily. Solid globules of agar will stain differently, producing poor quality images.

2.4) Transfer sufficient melted 7.5% (w/v) agar to the centrifuge tube to bring the total tube volume to between 6.5 and 9 mL.

2.5) Recap centrifuge tubes and gently invert at least 3 times to mix.

2.6) While pointing the cap away from oneself or in a hood, open the cap. Pour the tube contents into a 100 mm plastic Petri dish while gently rocking the dish to achieve a full, smooth coating and a visually uniform distribution of particles.

CAUTION: The heat from the agar may produce a slight overpressure in the tube. This often produces an audible hiss and has the potential to expel small droplets of hot agar.

2.7) Allow the plates to cool at room temperature for at least 5 minutes, until the agar solidifies.

NOTE: The protocol may be paused here. Store plated inverted and sealed (e.g., in a sealing plastic bag or with paraffin film) for up to 48 hours under refrigeration (4 °C).

3. Acquire particle images using a stereomicroscope and digital camera

3.1) Place the uncovered plate face up on the microscope stage of a stereomicroscope capable of 10x to 20x magnification. Illuminate the sample from below with even, diffuse light using equipment such as an LED illuminator stand or light plate.

3.2) Open the image capture software, ensure the microscope light path is set to **Photo**, and click on the appropriate camera from the camera list.

3.3) Adjust the microscope so that multiple particles appear in the software in the focal plane with large, well-defined edges. Use a magnification of 10-20x to measure particles while maintaining a relatively deep focal plane.

3.3.1) Temporarily remove the agar plate and place the micrometer on the stage. Adjust the fine focus until the graduations on the micrometer appear sharply focused in the image capture software.

3.3.2) If not previously calibrated, record the pixel to micro ratio for the current magnification.

3.3.2.1) Set the zoom to 100% by clicking **Zoom > Actual Size** and select **Options > Calibrate** then align the red calibration bar in the main viewport along the long axis of the micrometer, with the vertical bars centered on the 0 and 200 µm graduations. In the **Calibrate** dialog box, enter the current magnification level and actual length of 200 µm.

3.3.3) If already calibrated select **Magnification** from the menu bar, and the select the current magnification level and confirm the calibration.

3.3.3.1) Select **Measurements > Line > Arbitrary Line**. Click on the intersection of the 0 graduation and long axis of the micrometer. Click again on the intersection at 200 and the long the axis. A correct calibration should display approximately 200 µm. Delete the line by clicking on it, pressing delete, and pressing **Yes** on the confirmation box.

NOTE: The instructions given for selecting the camera and calibration are specific to the software used for this hardware. Similar functions should be available in other imaging software. The goal is to determine the pixel to micron ratio of the image for accurate particle size measurement.

3.4) Replace the agar plate and adjust the fine focus to achieve maximum detail in the imaging software.

3.5) Adjust the imaging software so that maximum image quality is achieved.

3.5.1) Increase the bit depth to the maximum value allowed, by selecting the radio button in the **Bit Depth** panel of the **Camera** sidebar. Set the software to acquire grayscale images by selecting the appropriate radio button in the **Color/Gray** panel of the **Camera** sidebar.

3.5.2) Collapse any open sidebar panels between exposure and histogram. Reduce the gain to 1.0 and increase the exposure until a clear image appears in the viewport and until the histogram appears as a distribution that is not clipped by either end of the histogram box.

3.5.3) Adjust the histogram to avoid over and underexposure. In the histogram panel of the camera side bar, slide the left boundary of the histogram to just outside the lowest values and the right boundary to just outside the highest values.

3.6) Save the image as an **uncompressed TIFF**, including magnification information in the image metadata, using the **File > Save as** dialog box, selecting the TIFF format, and ensuring that the **Save with calibration information** box is checked.

NOTE: Saving image metadata, including spatial calibration, may vary between acquisition programs. FIJI[15], the underlying software used by the pipeline, understands most common variants. The important information to record is the pixel height, width, and associated unit(s).

3.7) Using either a mobile stage or manually moving the plate itself, select another area, which does not overlap previous images, following a path which alternates between left-to-right and right-to-left as one moves down the plate; also known as a 'lawnmower search pattern'. Repeat step 3.6 until sufficient images are produced to capture at least 500 visually estimated particles, more are better.

NOTE: Alternatives patterns (e.g., circular, random) are acceptable but should be reported. Acquiring multiple overlapping images for combination into a mosaic via digital stitching produces a resulting file size which greatly hinders downstream processing and artifacts from stitching may be introduced and is not currently recommended.

3.8) Retain plates until after image analysis for potential follow-up imaging. After final imaging, discard as appropriate for biological waste.

4. Measure and analyze particle silhouettes

4.1) Install the required image analysis software packages

4.1.1) Install FIJI (an enhanced version of the National Institutes of Health's ImageJ v1.52e following the instructions at: https://imagej.net/Fiji/Downloads

4.1.2) Install git, if not already present, by following the instructions at: https://git-scm.com/downloads

4.1.3) Acquire the particle analysis code from by cloning the git repository[16].

4.1.3.1) At the command line, retrieve the latest version of the code by typing:

git clone https://github.com/joeweaver/SParMorIA-Sludge-Particle-Morphological-Image-Analysis.git

NOTE: Using git is preferred, as it will automatically retrieve the latest version of the code. If git is not available, it is also possible to download the code as a zip file on the release page at: https://github.com/joeweaver/SParMorIA-Sludge-Particle-Morphological-Image-Analysis/releases

4.1.3.2) Install the analysis code following the instructions in the README.md text file found in the top-level directory of the cloned repository.

4.1.4) Edit a text file listing the directories to be processed, along with optional parameters. Refer to the examples/analysis subdirectory for a list of parameters and examples.

4.2) Run the analysis on the command line by typing:

<FIJI-PATH>\ImageJ-win64.exe --console -macro SParMorIA-SludgeParticle_Morphological_Image_Analysis <paramsfile>

where <FIJI-path> is the directory in which ImageJ-win64.exe is located and <paramsfile> the location of the text file describing the analysis setup

NOTE: The name of the executable may differ, depending on which operating system FIJI is installed on. Depending on the number and size of images, the analysis may take a few minutes to hours and will run automatically.

4.3) Perform a quality control check

4.3.1) Examine the quality control files located in the overlay subdirectory of the specified output directory. Note images with spurious, missed, and poorly captured particles, all apparent as shaded outlines which do not match the background. Refer to **Figure 3** for examples of such. The particle data are now ready for experiment-specific analysis figure generation.

4.4) Reject either whole plates or individual particles by specifying in the analysis code the noted files and/or particle IDs to be ignored. Refer to examples/censoring in the repository for relevant R and Python code.

4.5) Generate experiment specific figures using the image analysis results for each image are stored in a TIDY[12] comma separated text file in the results subdirectory of the specified output directory. Refer to the examples/figures/R and examples/figures/Python subdirectories for examples of how to read the results files.

REPRESENTATIVE RESULTS:

Files Generated

The process illustrated in **Figure 1** will produce two files per image analyzed. The first file is a comma separated value (CSV) text file where each row corresponds to an individual particle and the columns describe various particle metrics such as area, circularity, and solidity and defined in the ImageJ manual[17]. Example CSV files are included as supplemental information and in the examples/data directory.

[Place **Figure 1** here]

The second file is intended for use in quality control (QC) and is a GIF image file which overlays the original image with semi-opaque regions representing identified particles, as in **Figure 2**.

The quality of particle identification and segmentation can then be quickly manually evaluated. Although no particle thresholding method is perfect[18], **Figure 2** is presented as an example of an acceptable result. Poor quality images can either be retaken, or if sufficient data is available, simply removed from further processing.

[Place **Figure 2** here]

When evaluating QC images, there are three common errors found:

1. failure to accurately conform to the particle boundaries
2. failure to identify particles
3. artifact inclusion due either to: non-particle components (e.g., bubbles), or errors in thresholding

Examples of these errors are illustrated in **Figure 3**. Poor particle boundary identification and segmentation between particles often is a result of over-dying, as seen in **Figure 3a**. Poor illumination can lead to both failure to identify particles (**Figure 3b**, blue solid circle) and artefact false particles (**Figure 3b**, red dashed circle). Non-particle matter, such as bubbles, protozoa, fungi, and metazoans, such as the tardigrade in **Figure 3c** can also be spuriously identified as particles.

[Place **Figure 3** here]

It is easiest to reject the entire image. However, it is possible to use the particle identifier in the QC image (**Figure 2**, inset) to reject individual particles. This approach is particularly useful when there are a handful of issues in an otherwise useful image (such as the inclusion of non-particles) **Figure 3c**. Examples of doing so in a reproducible and reportable manner are included in the examples/censoring directory of the github repository.

When a small minimum diameter is specified (<10 pixels), image noise may be spuriously identified as a particle. In those cases, the image may be still be accepted when further downstream analysis is removes their presence. As a guideline, shape data should be treated with skepticism when particles are composed of less than ~200 pixels[19].

Figure Generation

The CSV files resulting from the image analysis are Tidy[12] and may be easily combined and analyzed in the researcher's preferred software package (such as pandas[20] with seaborn[21] in Python or dplyr[22] with ggplot2[23] in R). However, the exact figure type required will necessarily vary with research questions and result. An example of a possible figure is included below (**Figure 4**) and the corresponding code to generate it from the CSV files is available on github[16].

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1. Graphical workflow describing the four major steps of the protocol.

Figure 2. Example of a Quality Control (QC) gif generated by the image analysis pipeline. Magnification of main image 15x. Excerpt is digitally zoomed to show numbers identifying individual particles in the image.

Figure 3. Common errors detected during QC analysis. (**a**) Poor particle boundary detection. (**b**) spurious particles (red dashed ellipse) and unsegmented particles (blue solid ellipse). (**c**) Foreign non-particle object. Magnification 15x.

Figure 4. Example of experiment-specific figure generated from CSV data produced by the image pipeline. In this example, the particle distributions between two experimental reactors over time are displayed and combined with qualitative metadata noted by the researcher. See examples/figures/R for the generating code and data.

Figure 5. Reference images showing particle concentrations which are too concentrated, acceptable, and overly-diluted. Magnification 15x.

Figure 6. Increased stain concentration improves particle contrast, but also distorts the observed boundary. Magnification 15x.

Figure 7. Reference images illustrating overgrowth signaling that a plate has been stored beyond its useful lifetime. Magnification 15x.

Figure 8. Using excessive amounts of agar will produce a sample thicker than the focal plane, resulting in blurry particles. Magnification 15x.

Figure 9. Overly-vigorous swirling during plate preparation will appear as non-uniform particle distributions (**a**), biasing sections of the plate towards larger (**b**) and smaller (**c**) particle distributions. Plate is 100 mm diameter, micrographs magnified 15x.

Figure 10. Reference images showing poor and acceptable image exposures.

DISCUSSION:

Although the image analysis system is fairly robust and QC steps are taken to ensure poor images are removed, proper attention to specific issues in sampling, plate preparation, and image acquisition can improve both the accuracy of the data and the proportion of images passing QC.

Sampling concentration

Assuming a representative sample has been taken, the most important step is to ensure that sufficient particles are present for representative[9] and efficient analysis while not so concentrated that particles overlap.

This has corresponded to approximately 0.5 to 2 mL of mixed liquor over a wide range of total suspended solids, but experiment-specific determination may be necessary. Examples of overly concentrated, overly -diluted, and appropriate particle concentrations are shown in **Figure 5** as a reference. Staining is also affected by particle concentration. Over-dilution can result in overly stained, blurry particles while under-dilution may not produce particles with sufficient contrast for optimum thresholding.

[Place **Figure 5** here]

Dye concentration

The amount of stain added to the sample is crucial and the correct amount may vary between sludges. Approximately 5 µL of 1% (w/v) methylene blue per 0.5 to 2 mL of sample provides sufficient contrast for thresholding without causing 'bleeding' and obscuring the particle's shape.

There is no single ideal concentration; a balance between contrast and clarity must be chosen. **Figure 6** illustrates this tradeoff in three samples stained with 5, 25 and 50 µL of 1% methylene blue per 2 mL of sludge. When weighing this tradeoff, the occasional poorly contrasting particle (**Figure 6a**) is preferred over poorly resolvable blobs (**Figure 6c**).

[Place **Figure 6** here]

Plate storage

After immobilization, plates can be stored under refrigeration (4 °C) for at least 3 days. This is a conservative period during which it is unlikely that contaminating growth and dye diffusion will occur. Plates not showing any of the issues described below may still be imaged after 3 days. When stored for too long, existing particles may continue to grow and will appear in the focal plane of other particles while retaining the hue of the stain, as can be seen in **Figure 7a**. Surface contaminants, such as fungal spores, may also grow after long periods of storage. These generally will not take up the color of the stain and will appear in a different focal plane, as can be seen in **Figure 7b**. In some cases, it is unclear whether overgrowth or diffusion of the stain has occurred, such as in the bottom of **Figure 7b** and center of **Figure 7c**. Regardless of the cause, spots such as those indicate the plate has aged beyond its useful lifetime

[Place **Figure 7** here]

Plate preparation

There are two issues associated with physically preparing the agar plates – overly thick agar and excessive swirling. In the first case (**Figure 8**), the particles become suspended at various depths, making it difficult to acquire images with the majority of particles in focus.

[Place **Figure 8** here]

In the second case, swirling produces a non-uniform distribution of particles (**Figure 9a**), biasing results from different sections of the plate (**Figure 9b,c**. Generally, no more than 7 mL of agar is required to cover a 100 mm Petri dish and only gentle hand motions are needed to evenly cover the dish.

[Place **Figure 9** here]

Microscopic imaging

There are two major image acquisition issues affecting quality. The first issue is ensuring that the majority of particles are in the focal plane. Even at low magnification, the size of many activated sludge particles is such that without minor adjustments to coarse focus, many particles will be slightly out of focus, introducing inaccurate particle measurement. No image will contain 100% perfectly focused particles; **Figure 8** and **Figure 5b** are respective examples of poor and acceptable focus.

Exposure levels constitute the second major issue. Poorly exposed images result in lost data and poor segmentation[11]. Further, the high contrast of the dye can produce a narrow histogram, reducing the effective dynamic range of the data. The upper and lower bounds of the histogram may be adjusted prior to capturing an image to both prevent poor exposure and increase the dynamic range. Examples of over, under, and acceptable exposures are included below in **Figure 10**.

[Place **Figure 10** here]

This method's advantages are that it provides specific criteria encompassing the entire process. Further, we have provided a software pipeline easing within-lab analysis and promoting comparable between-lab data. 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. Future directions of this method could incorporate advanced image analysis techniques (specifically noise reduction[24,25], high dynamic range imaging, focus stacking[26,27], and machine-learning assisted thresholding, segmentation, and classification[28]. The major image acquisition improvement would incorporate software to control mechanical stages[8] and produce 'whole plate' mosaic archives.

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DISCLOSURES:

The authors have nothing to disclose.

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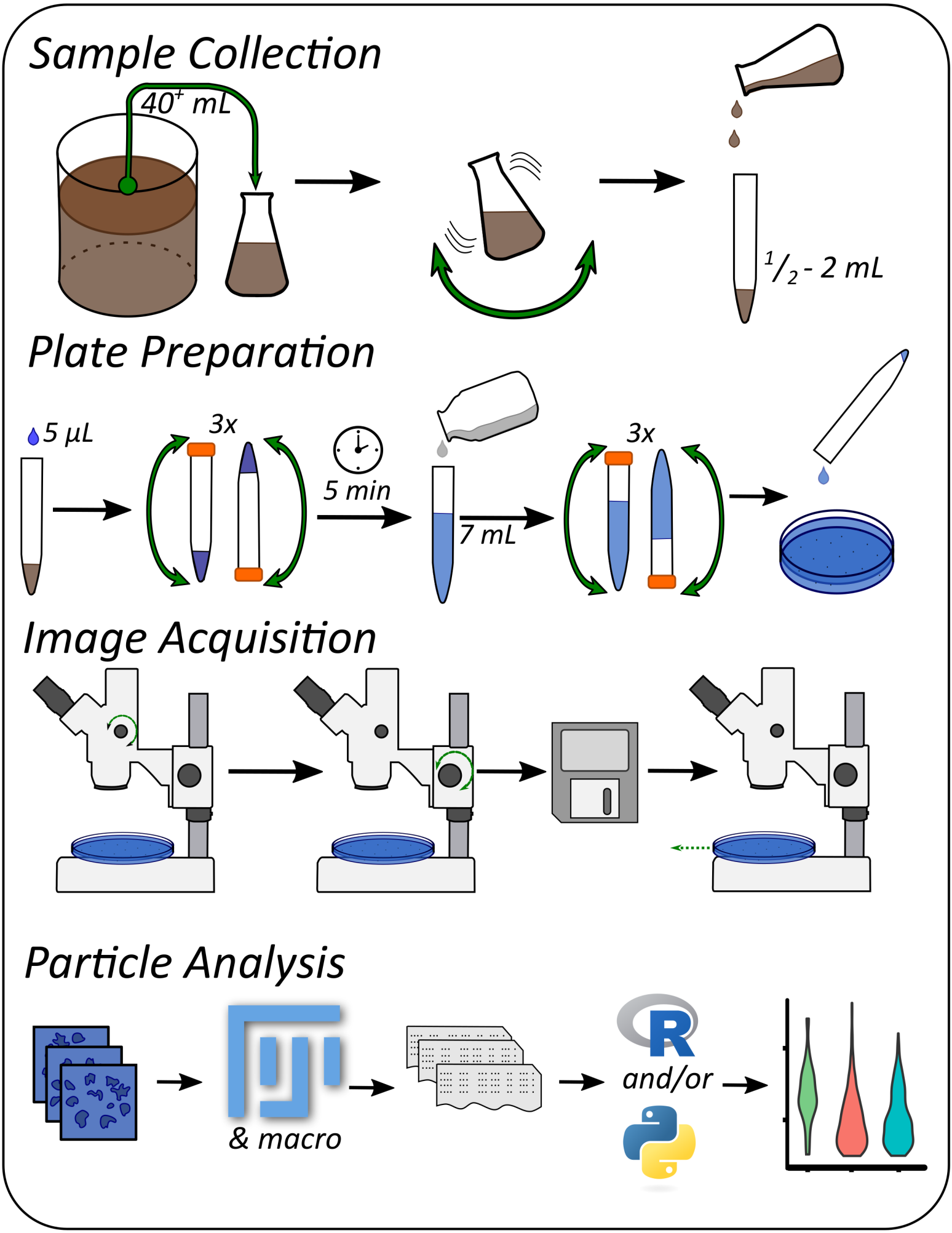


Figure 2

Click here to access/download;Figure;figure_2_qc_example.eps

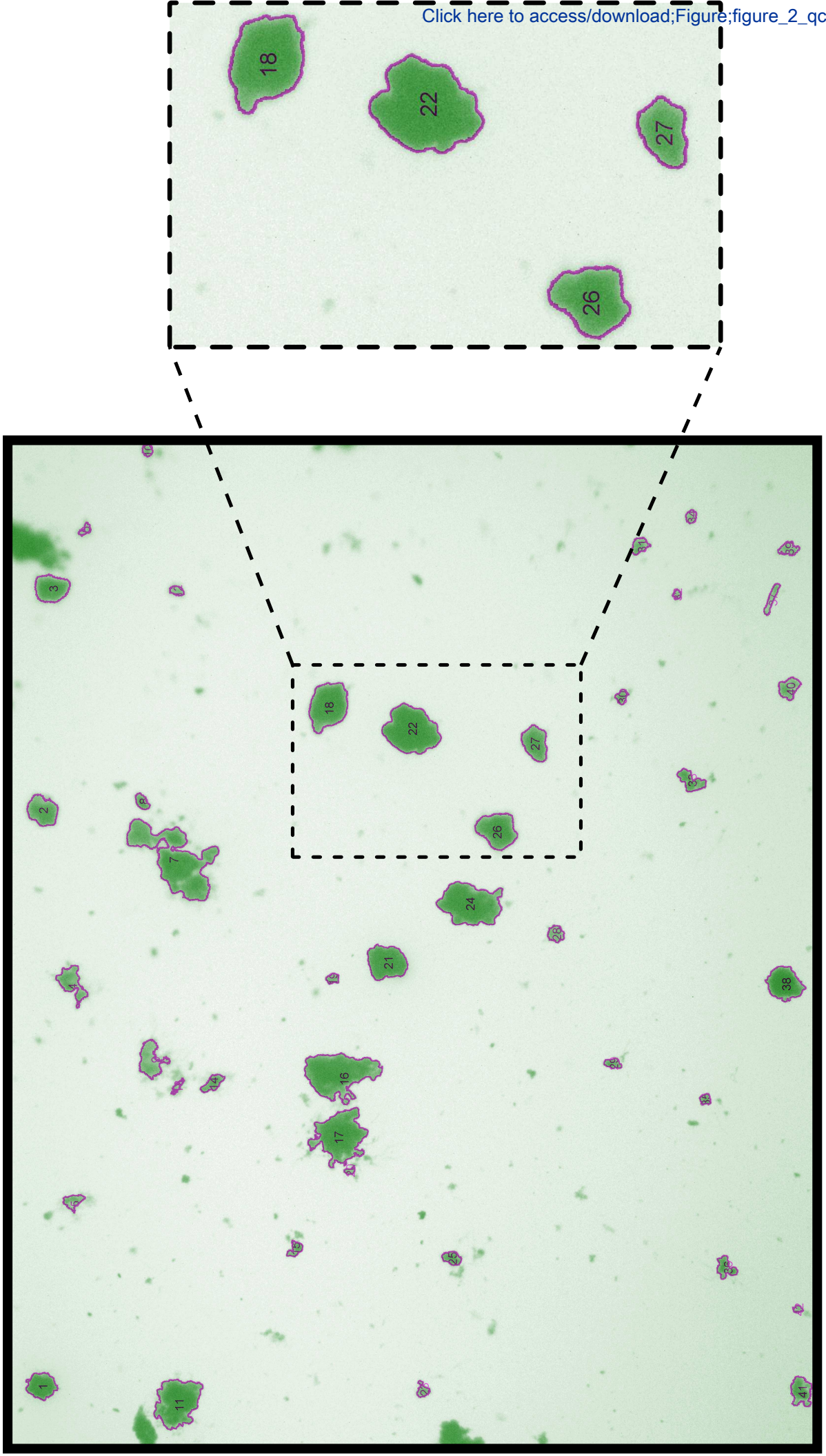
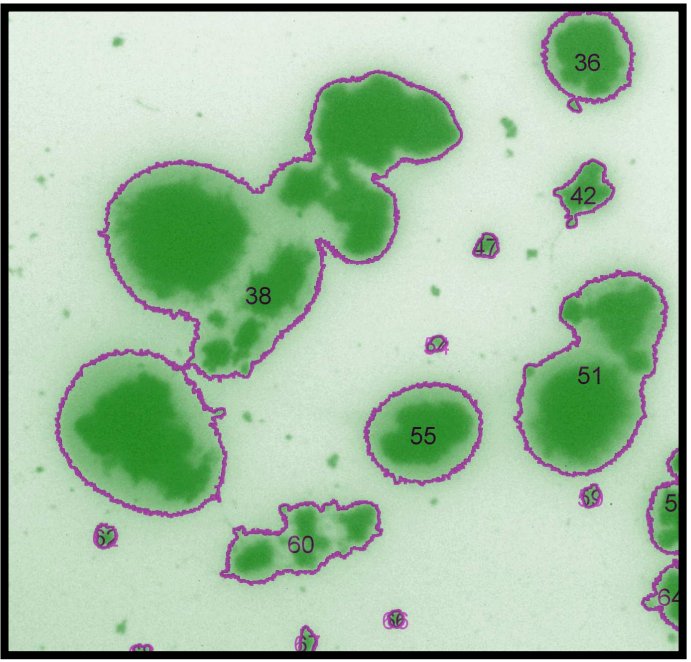
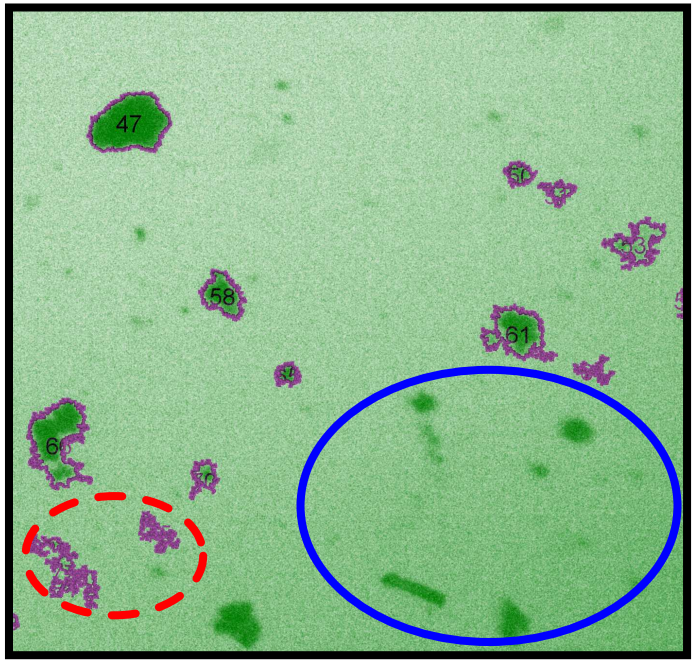


Figure 3

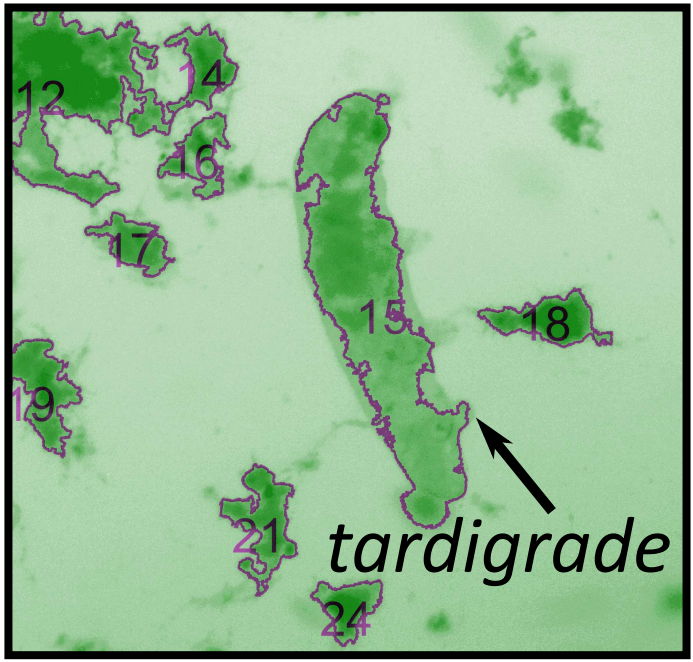
Click here to access/download;Figure;figure_3_common_errors.eps



(a)



(b)



(c)

Figure 4

Click here to access/download;Figure;figure_4_two_reactor_time__series.eps

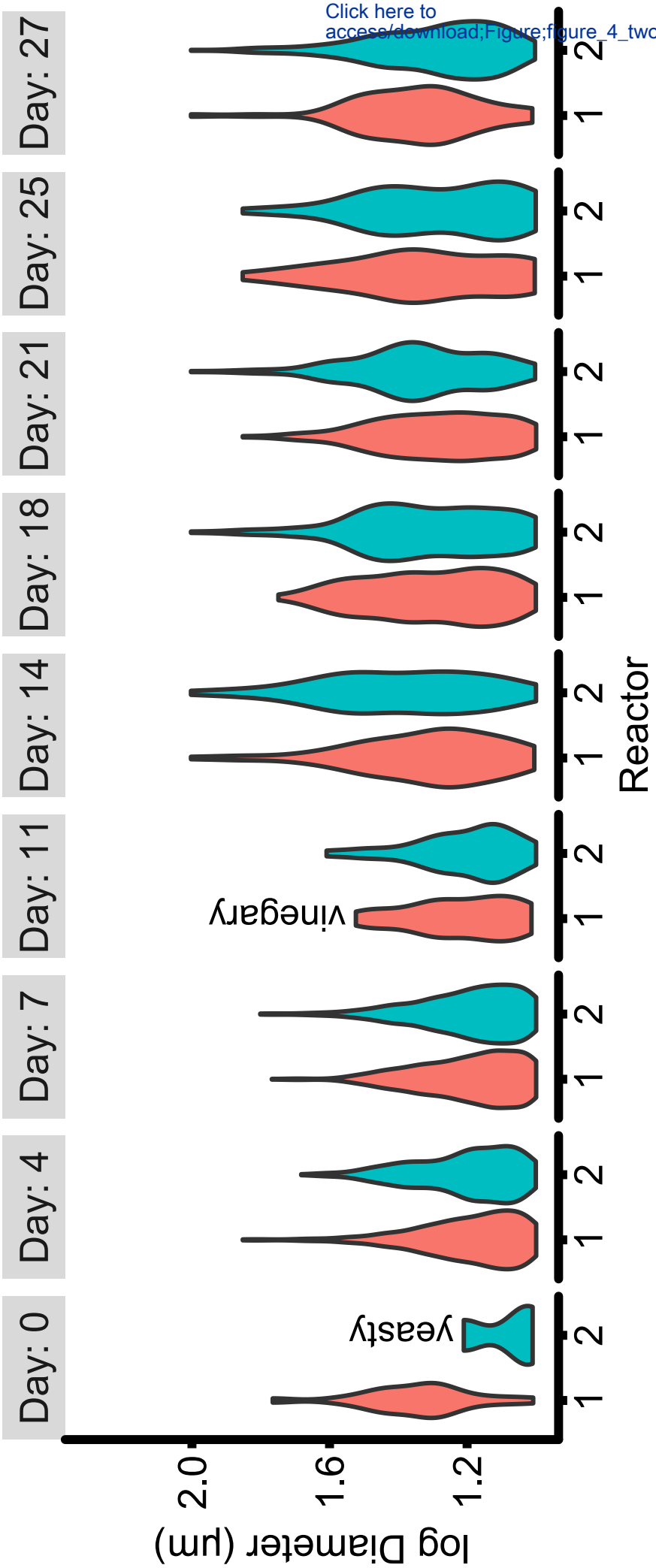


Figure 5

Click here to access/download;Figure;new_figure_5_goldilocks particle concentration.tiff

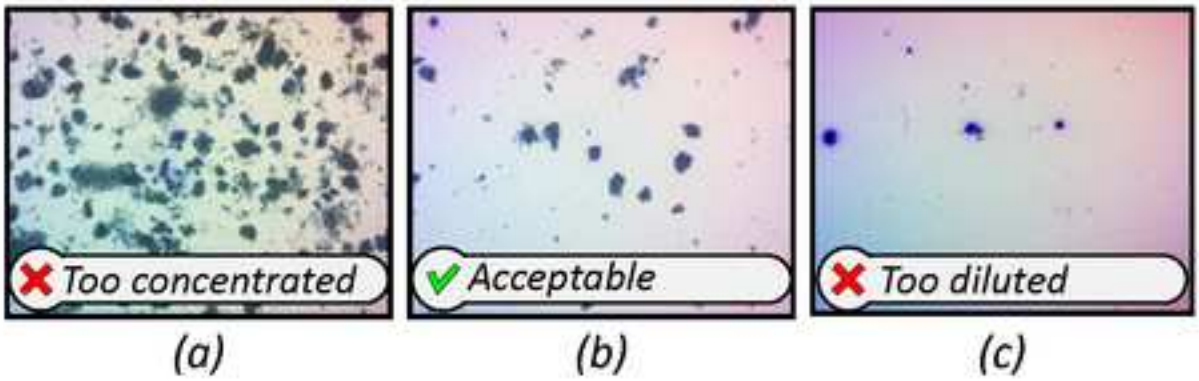
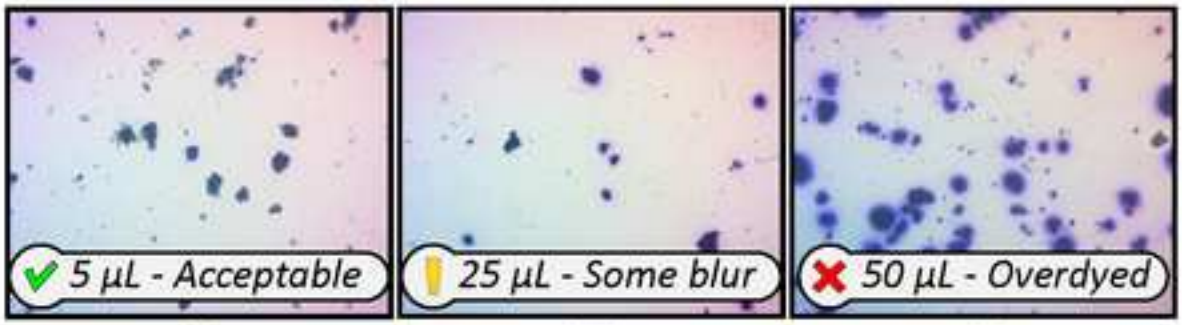


Figure 6

Click here to access/download;Figure;new_figure_6_goldilocks stain.tiff



(a) (b) (c)

Figure 7

Click here to access/download;Figure;new_figure_7_overstored.tiff

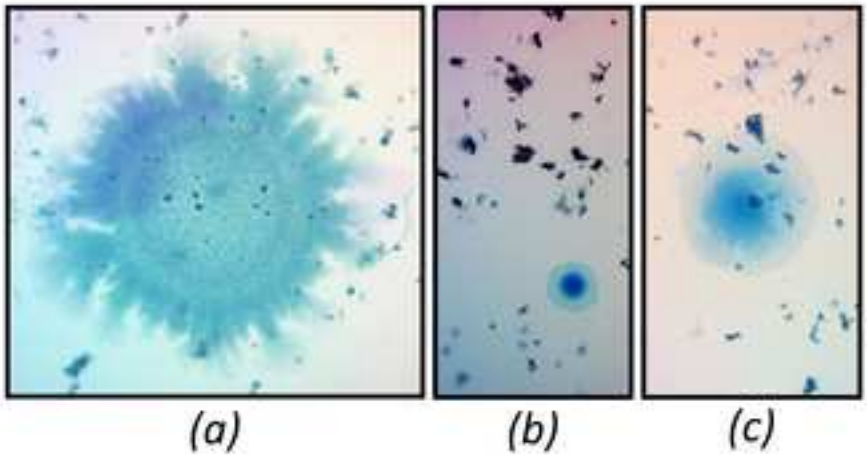


Figure 8

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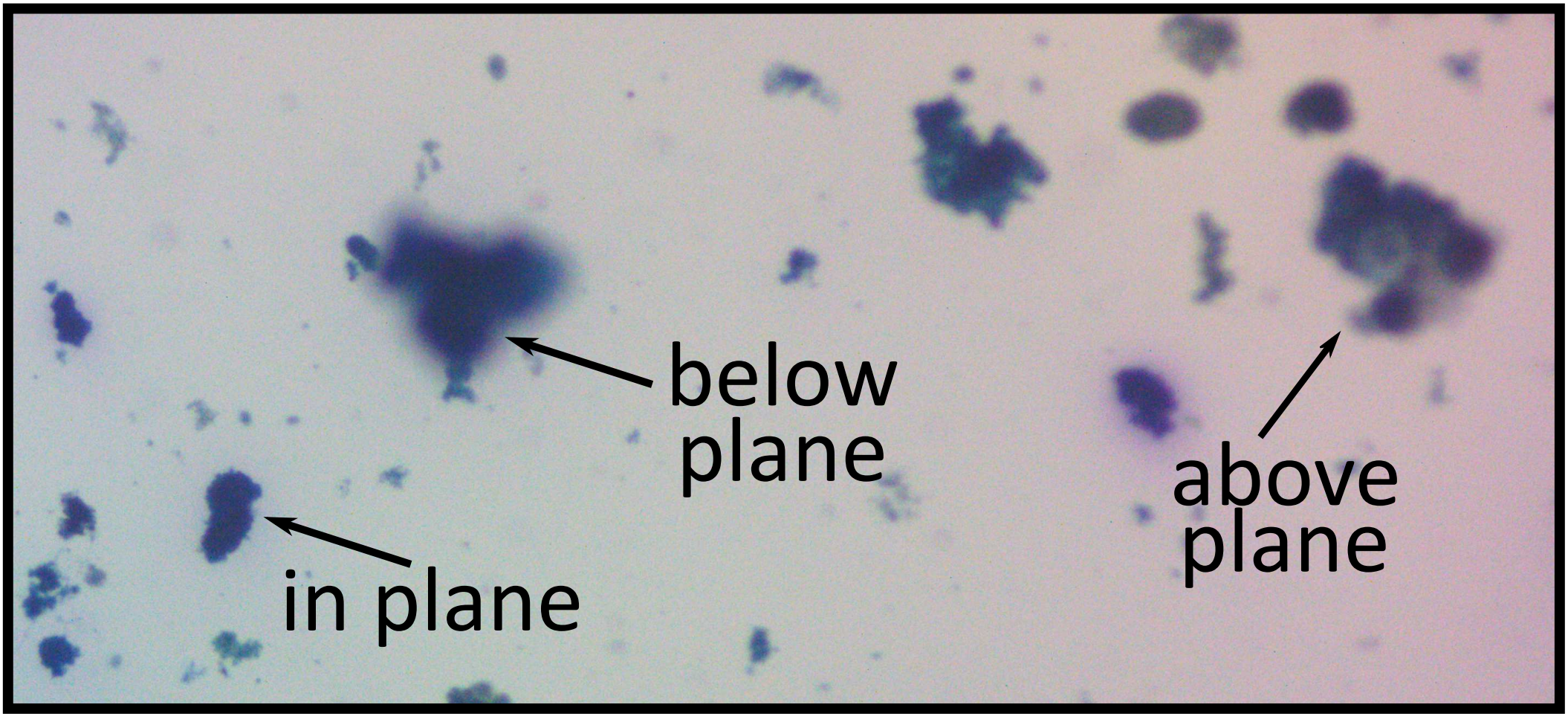


Figure 9

Click here to access/download;Figure;figure_9_overswirled.pdf

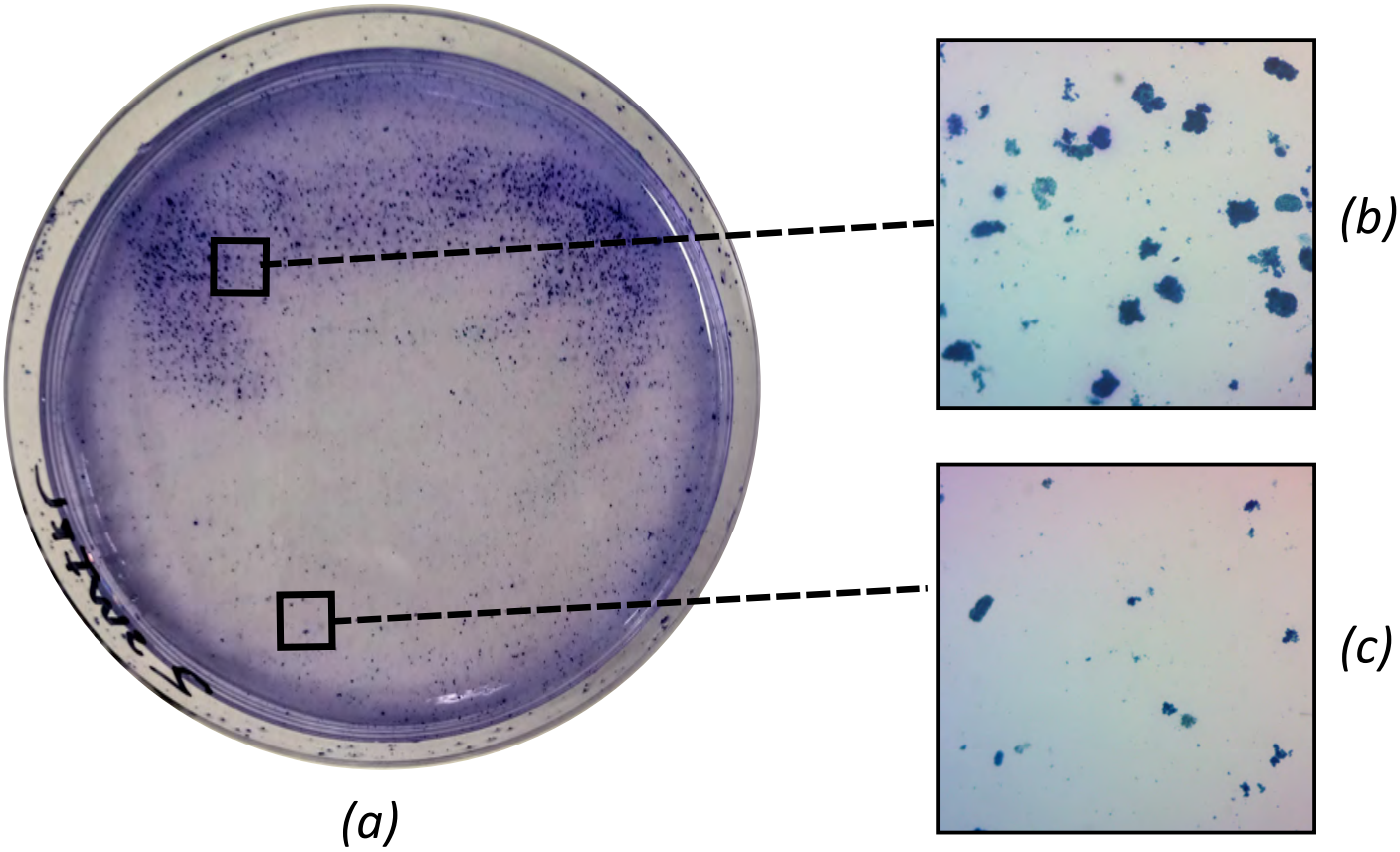
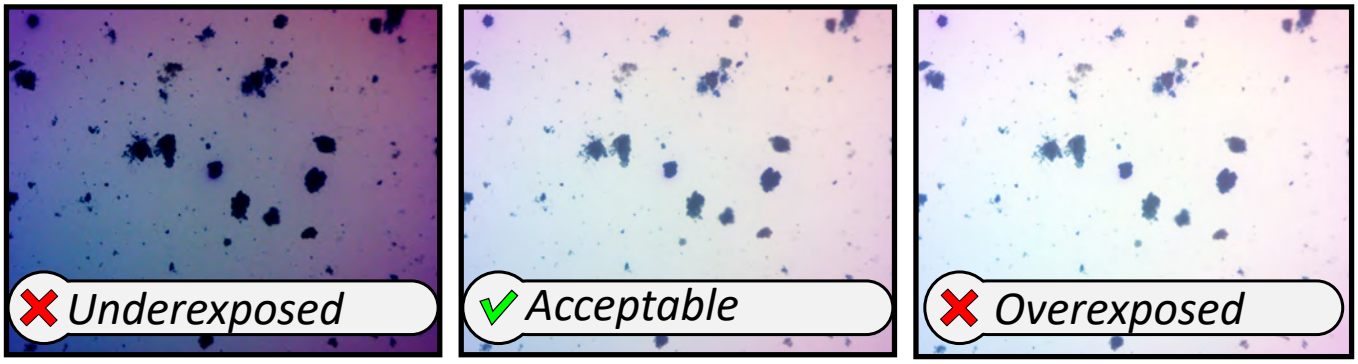


Figure 10

Click here to access/download;Figure;figure_10_goldilocks expsoure.pdf



(a) (b) (c)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
10% Bleach solution	Chlorox	31009
15 mL centrifuge tube with cap	Corning	430790
50 mL Erlenmeyer flask	Corning	4980-50
500 mL Kimax Bottle	Kimble-Chase	14395-50
Agar	BD	214010
Data analysis software	N/A	N/A
Deionized water	N/A	N/A
Desktop computer	N/A	N/A
External hard drive	Seagate	STEB5000100
FIJI	NIH	version 1.51d
GIT	Open Source	version 2.19.1 or later
Image capture software	ToupView	version 3.7.5177
Mechanical (X/Y) Stage	OMAX	A512
Methylene blue	Fisher	M291-100
Microscope camera	OMAX	A35140U
Optical Stage Micrometer	OMAX	A36CALM1
Petri dish, 100 mm	Fisher	FB0875712
PPE	N/A	N/A
Sparmoria macro	NCSU	version 0.2.1
Stereo/dissecting microscope	Nikon	SMZ-2T

Comments/Description

For workspace disinfection.
Per sample.
Other vessels are suitable so long as they can contain > 40 mL of sample and allow mixing
Or otherwise sufficient for agar handling
Solid, to prepare 7.5% gel. 7 mL per sample.
R or Python are suggested
Sufficient to prepare stain and agar. If unavailable, tap should be fine.
Image analysis is not CPU intensive, any 'ordinary' desktop computer circa 2017 should be sufficient.
Not fully required, but extremely useful given the number an size of images. 2 or more TB of storage suggested.
Version is ImageJ core. Plugins are updated as of writing. Available at: https://imagej.net/Fiji/Downloads
Available at: https://git-scm.com/
Any compatible with camera, may come with camera. Should allow saving TIFF images with spatial calibration data.
Not fully required, but greatly aids image acquisition.
Solid, to prepare 1% w/v solution. 5 uL solution per sample.
Any digitial camera compatible with microscope. Resolution providing at least 5 um per pixel at 10x magnification and a dynamic ran
Or otherwise sufficient for spatial calibration.
1 per sample.
Standard lab coat, gloves, and eyewear.
Available at github repository : https://github.com/joeweaver/SParMorIA-Sludge-Particle-Morphological-Image-Analysis
Should provide 10 to 20x magnficiation and allow digital photos either with a buit-in camera or profide a mounting point for a CCD.

ge of at least 8 bits per pixel per color channel is suggested.

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Author(s):	Joseph E Weaver, Jon C Williams, Joel J Ducoste, Francis L de los Reyes III

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Signature:	
Date:	2018-08-17

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Rebuttal Letter
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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.