Journal of Visualized Experiments

In situ microscopy for real-time determination of single-cell morphology in bioprocesses.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57823R1
Full Title:	In situ microscopy for real-time determination of single-cell morphology in bioprocesses.
Keywords:	In situ microscopy, cell size, morphology, yeast, algae, population heterogeneity, budding, growth vitality, agglomeration, monitoring, image detection
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	TU Berlin, Ackerstrasse 76 ACK24, D-13355, Berlin, Germany

TITLE:

In Situ Microscopy for Real-time Determination of Single-cell Morphology in Bioprocesses

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KEYWORDS:

In situ microscopy, cell size, morphology, microbes, population heterogeneity, budding, growth vitality, agglomeration, monitoring, image detection

SUMMARY:

A photo-optical *in situ* microscopy device was developed to monitor the size of single cells directly in the cell suspension. The real-time measurement is conducted by coupling the photo-optical sterilizable probe to an automated image analysis. Morphological changes appear with dependence on the growth state and cultivation conditions.

ABSTRACT:

In situ monitoring in microbial bioprocesses is mostly restricted to chemical and physical properties of the medium (*e.g.*, pH value and the dissolved oxygen concentration). Nevertheless, the morphology of cells can be a suitable indicator for optimal conditions, since it changes with dependence on the growth state, product accumulation and cell stress. Furthermore, the single-cell size distribution provides not only information about the cultivation conditions, but also about the population heterogeneity. To gain such information, a photo-optical *in situ* microscopy device[1] was developed to enable the monitoring of the single-cell size distribution directly in the cell suspension in bioreactors. An automated image analysis is coupled to the microscopy based on a neural network model, which is trained with user-annotated images. Several parameters, which are gained from the captures of the microscope, are correlated to process relevant features of the cells, like their metabolic activity. Until now, the presented *in situ* microscopy probe series was applied to measure the pellet size in filamentous fungi suspensions. It was used to distinguish the single-cell size in microalgae cultivation and relate it to lipid accumulation. The shape of cellular particles was related to budding in yeast cultures. The microscopy analysis can

be generally split into three steps: (i) image acquisition, (ii) particle identification, and (iii) data analysis, respectively. All steps have to be adapted to the organism, and therefore specific annotated information is required in order to achieve reliable results. The ability to monitor changes in cell morphology directly *in line* or *on line* (in a by-pass) enables real-time values for monitoring and control, in process development as well as in production scale. If the *off line* data correlates with the real-time data, the current tedious *off line* measurements with unknown influences on the cell size become needless.

INTRODUCTION:

Morphological features of cells are often related to the physiological state, a connection between form and function exists for many applications. The morphology of a single cell is influenced by the state of growth, the cell's age, osmotic and other potential cell stresses or product accumulation. Morphological changes of cells are often a measure of the growth vitality of a culture. Intracellular product synthesis, lipid accumulation in algae and inclusion body formation in bacteria, among others, are related with the cell size as well. Cell agglomeration can be another factor that is worth investigating as summarized recently[2].

Population heterogeneities can be quantified based on morphological features of individual cells. Studies showed that heterogeneity within a culture might be significant, *e.g.*, under large-scale production conditions[3] the overall yield might be affected by a low performance of subpopulations[4].

Usually, the assessment of morphological features of cells is performed by manual sampling or with a by-pass flow chamber coupled to a photo-optical device. This leads to several restrictions: the limited amount of acquired data can hardly provide statistically reliable measurements; the time delay in between sampling and the accessibility of results may be too long in comparison to the dynamics of the process; and most important, the sampling procedure (location of the sampling port, pre-treatment of the sample before the measurement, unfavorable conditions in the sampling or bypass tube) can trigger a biased error as the sample procedure itself can already affect cell morphology. Finally, there exists always a high risk of contamination during sampling or in by-pass solutions, if they are not sterilizable in place.

The application of *in situ* microscopy (ISM) can circumvent several of these problems. If cells are detected automatically, a correct identification of their morphological features can be surveyed[5]. Until now, the main limitations of this method were (i) the evaluation time of images, which was too long for *in situ* applications, and (ii) the poor resolution of images, especially at high cell densities. Although first solutions of ISM included mechanical sampling, dilution of the probe, or were restricted to a by-pass system[6,7], further approaches allow capture of the cell suspension directly[8].

Recent advances in ISM allow for the *in line* or *on line* monitoring of cells on a single-cell basis, which provides the distribution of morphological parameters in real-time directly in cell suspensions at considerably high cell concentrations. Through *off line* analyses of the cells' key parameters, correlations with information provided by the coupled automated cell detection and

ISM can be identified. Then, new soft sensor designs are achieved, in which an unmeasurable parameter is estimated with the single-cell morphology.

In this report, the ISM is conducted by coupling a photo-optical probe to an automated image analysis. The ISM consists of a single-rod sensor probe that enables the capture of images within a known focus range in an adjustable measurement gap with a high-resolution CCD camera [MM-Ho = CCD GT2750 (2750x2200) and MM 2.1 = CMOS G507c (2464x2056)]. The flash light illumination is conducted by transmission. Therefore, the light originates from the opposite side of the camera[9]. Cells pass continuously through this gap with the liquid flow. Hence, a representative sample population is obtained. The probe can be mounted directly to the bioreactor so that it reaches into the cell suspension, or it can be used in a sterilizable by-pass. The sensor shell is connected to the system prior to sterilization, the optical parts are afterwards mounted into the shell.

Until now, relevant industrial microorganisms, *e.g.*, filamentous fungi (diameter of up to over 200 µm), the heterotrophic microalgae *Crypthecodinium cohnii* (average cell diameter of 20 µm), and the yeast *Saccharomyces cerevisiae* (average cell diameter of 5 µm), were investigated with this or similar devices, which is shortly described.

Filamentous fungi tend to form pellets under certain cultivation conditions. These are of a size of up to several hundred µm. The hyphae of the fungal cells develop different lengths in dependence to the hydrodynamic stress in the fluid phase. This has an influence on the metabolic and growth activity, substrate uptake and product release. ISM was applied to identify the pellet size distribution and the width of zones of lower biomass density at the edges of the pellets (own unpublished data).

The size of *C. cohnii* alters between 15 and 26 µm when cells accumulate the polyunsaturated fatty acid docosahexaenoic acid (DHA) under nitrogen limitation. This biotechnological DHA production process consists of two parts, the growth phase, in which cells divide and become smaller, and the production phase, in which cells accumulate the product and thus become larger. Therefore, the cell size was used to determine the process state, in which either growth or DHA production was favorable. Finally, a correlation between the cell size and the DHA content was found. In this case, ISM allows to monitor the intracellular DHA accumulation in real time without the requirement of sampling, cell disruption, and the common gas chromatography analysis[10].

Budding yeast is usually of a size between 3 and 8 µm. The proportion of cells that are in the maturation state at a time, as described with the budding index (BI), provides information about the growth vitality[11,12], and even a relation with recombinant protein secretion has been proven[13]. With the help of ISM, budding and non-budding yeast cells (cells with and without a bud) were distinguished[14]. Stress conditions can also lead to a broader variation of the cell size within a yeast population, as recently shown in scale-down cultivations, in which the conditions of large-scale nutrient-limited fed-batch cultivations were mimicked[3].

Therefore, ISM has the potential to monitor growth vitality and product formation on a single-cell level during all stages of a bioprocess for the identification of optimal cultivation conditions, or for the purpose of process control. The methods described here are focused on microbial applications with single cells, but are also applicable to larger particles like human and animal cells, cell agglomerates and pellets of filamentous organisms.

PROTOCOL:

Note: The following steps are necessary to adapt the parameters to the respective microorganism and culture conditions. The adjustment of probe settings lasts about 20 min for an experienced user. A detailed description of tools and steps is given in the corresponding manual. In general, the tools that are presented in the following protocol are needed: (i) *Probe Controller* for probe adjustments and image acquisition; (ii) *Fiji (ImageJ)* for annotations on acquired images; (iii) *SOPAT support* for artificial neural network (ANN) training and workflow creation; (iv) *Batcher* for data batch processing using already acquired images with a workflow; (v) *Result Analyzer* for result visualization and evaluation on batch processed images; and (vi) *Monitor* for automated real-time measurement and result visualization.

1. Setting Hardware Parameters

1.1. Prepare a culture with the highest cell concentration that might be achieved during the experiment or centrifuge and resuspend the pellet in order to achieve this concentration. In this case, choose 65 g L^{-1} of dry biomass concentration for *S. cerevisiae* cultivations.

1.2. Prepare different dilutions, which range from the highest to the lowest concentration so that the expected range is fully covered. A minimum of 4 different concentrations are recommended.

1.3. Identify the cell size range of the microorganism with conventional microscopy. Define the expected maximum diameter (d_{max}) of respective cells. This value is set to 8 µm in case of *S. cerevisiae*.

1.4. Choose two measurement gaps of 5x and 10x of the expected d_{max} of the cells.

1.5. Choose the maximum stroboscope intensity. Choose stroboscope intensities for both gaps with the highest cell concentration so that cells are still visible on the images with the lowest light intensity (darkest images).

1.6. Choose the minimum stroboscope intensity, and then choose stroboscope intensities for both gaps so that cells are still visible on the images with the highest light intensity (brightest images). Use the lowest cell concentration, which likely appears during the measurement period.

1.7. Choose one focus position, which yields the sharpest images for each measurement gap, for both stroboscope intensities and for the concentration range that need to be tested (see step

2 for details about focusing). Focus cells appropriately so that the image data can be annotated afterwards (see step 4).

1.8. Measure the previously prepared dilution series of the cell concentration (see step 2) with both gap widths and stroboscope intensities.

1.9. Evaluate the dilution series experiment.

1.9.1. Use the **Concentration Calibration (CoCa) Tool** (see **Figure 1**) to identify the optimal correlation between the extracted image features (brightness or sharpness) and the previously measured concentrations provided by the user, *e.g.*, dry biomass or cell counts. Follow the instructions in the software manual for further details.

[Place **Figure 1** here]

1.9.2. Identify the optimal correlation between the extracted information from the image features at various concentrations compared to any *off line* measurements. See the legend of **Figure 1**.

1.9.3. Choose the most reasonable measurement gap and stroboscope intensities under consideration of the concentration correlation curve with the features that result in the smallest weighted root mean square error (WRMSE).

Note: The measurement gap is fixed during the experiment, whereas the stroboscope intensity can be adapted according to the cell concentration.

2. *Off Line* Measurement

2.1. Adjust the desired measurement gap according to step 1 with the help of a thickness gauge.

2.2. Open the graphical user interface **Probe Controller** in the dashboard.

2.3. Connect the desired probe to the amplifier in the software subsection **Actions** and press **Connect**.

2.4. Open the tab **Probe control** and adjust the desired stroboscope intensity according to step 1.

2.5. Press the **Play** button to start streaming (**Live View**).

2.6. Clean the measurement gap by spraying ethanol into the gap and carefully wipe any dust or dirt with an optical paper. Check that the glass of the sensor is free of particles with the **Live View** in the **CamControl**.

Note: Particles and dust disturb measurements and the automatic cell identification.

2.7. Place a dry optical paper in the measurement gap and move the focus manually. Turn the binding screw until the single fibers of the paper are clearly seen.

2.8. Fill a tube with culture broth. Dip the microscope in the culture broth so that the gap is fully covered with cell suspension and focus on the cells by fine-tuning the focus binding screw. The focus must not be changed anymore during the experiment.

Note: 5-6 mL of culture broth are added to a 50 mL conical centrifugal tube to float the measuring gap sufficiently.

2.9. Define the number of frames per time point [-] in the user interface in the menu **Triggering** in the GUI **Frames per trigger**. Set the number of frames to 200 frames per trigger.

Note: The number of frames can be reduced to the lowest value, which is necessary for a statistical reliable result. This depends on the sample size required to obtain a representative morphological cell size distribution (see also step 5).

2.10. Define the frame rate [Hz] in the menu **Triggering** in the GUI **Frame rate**. Choose a frame rate that guarantees that moving particles from a previous frame will not appear in the following frame.

Note: This can be proven with a test trigger with 200 frames. Inspect the images for particles, which are captured repeatedly. If this is the case, decrease the frame rate. For *off line* measurements, 1 Hz is recommended.

2.11. Set the directory, in which the acquired images will be saved, in the menu **General**.

2.12. Perform an image acquisition by activating the **Start image trigger acquisition** button. Move the tube with culture suspension gently up and down to induce a flow through the measuring gap.

2.13. Repeat step 2.6 after each measurement.

2.14. Check the acquired images. Cells have to be sharp enough for annotation. Inspect the images for particles, which are captured repeatedly. If this is the case, decrease the frame rate.

2.15. Save the settings by selecting the following pathway: C:\Program Files\SOPAT GmbH\monitoringPrograms\camcontrol, and press **Save**.

3. *On Line* (By-Pass) or *In Line* Measurement

3.1. Perform the *off line* measurement procedure first (see step 2) in order to set the hardware and software settings as a function of the organism and process (concentration or media).

3.2. Upload the saved settings from the previous section by selecting the button **Load** and select the following pathway: C:\Program Files\SOPAT GmbH\monitoringPrograms\camcontrol.

3.3. Connect the probe to the flow cell or to the bioreactor.

Note: *In situ* measurements can be performed with a pinch flange.

3.4. Perform sterilization.

Note: Only the probe-wetted material of the instrument is sterilizable through steam sterilization. The probe wetted length can go from 6 to 222 mm (**Figure 2**).

[Place **Figure 2** here]

3.5. Define the image acquisition rate in the GUI **Triggering** in the field **Trigger interval [s]**.

Note: Depending on the process dynamics, the image acquisition rate can be adapted. For example, if a lag-phase of 3 hours is expected, the rate of acquisition can be lower, than if a metabolism shift or the accumulation of a product shall be monitored. Usually, this requires a much shorter acquisition time in the minute range. For example, an image acquisition sequence between 5 and 10 min during a batch yeast cultivation provides sufficient information to capture process dynamics.

3.6. Define the frame rate as explained in step 2.10.

Note: In *on line* and *in line* measurements, the frame rate can be increased, since the mechanical stirring can increase the flow rate through the gap.

3.7. Start image acquisition by activating the button **Start streaming of selected probe**.

3.8. Stop acquisition when the experiment is finished with the button **Stop streaming of selected probe**.

Note: For the 1st run, start the acquisition just before the inoculation of the culture and proceed to step 4. For the following runs, open the program **Monitoring** in the dashboard and select the created workflow (see step 4). Start the monitoring just before the inoculation of the culture by pressing the **Play** button.

4. Particle Identification

4.1. Annotate particles for the training of the artificial neural network (ANN) (training set).

4.1.1. Load the acquired images into the annotation tool "**Fiji, ImageJ**" by dragging and dropping the file into the Main "**ImageJ**" window (see GUI "**Fiji**" tool in **Figure 3**)

[Place **Figure 3** here]

4.1.2. Open the ROI Manager by selecting: **Analyze | Tools | ROI Manager**.

4.1.3. Choose a selection tool. **Wand (tracing) tool, freehand, oval, or elliptical** selections are recommended.

4.1.4. Draw a circle around the particle that shall be annotated with the selection tools mentioned before and then refine it with the brush tool.

4.1.5. Add the annotation to the **ROI Manager** by pressing **Add [t]**.

4.1.6. Mark all objects of interest (cells to be identified) on about 15 images.

Note: In order to cover all necessary information, *i.e.,* different shapes, sizes, concentration of cells, brightness, *etc.*, use five images from the start, five images from in between, and five images from the end of the experiment.

4.1.7. Decide, if cells need to be classified in different subclasses due to their shape (*e.g.*, different phases of a cell cycle), or if all cells are of the same class.

4.1.8. Change the name of each selected particles accordingly. Set one name or abbreviation for each class and a counter for each particle of the class (*e.g.*, cell_1, cell_2, *etc.*). Annotate at least 50 particles per class.

4.1.9. Do not annotate objects, which should not be detected, because they are not relevant for the process, such as gas bubbles or other particles like undissolved media components.

Note: Those events will not be included in the training procedure for the ANN and be regarded as background.

4.1.10. Do not annotate cells that are out of focus.

4.1.11. Annotate the images as consistent as possible. If there are doubts, the label **Ignore** can be applied. It is highly recommended not to abuse its use, since the ANN will only recognize structures that are labeled.

4.2. Save the annotated objects and the image to a ZIP format and send the file to the training network, either *via* upload to the platform or by sending the ZIP file *via* e-mail.

Note: Usually, it takes a number of iterative training rounds to identify adequate predictions of the classified objects on the images. Each training round leads to a workflow that is returned by the program.

4.3. Use the workflow (*.wf) with the trained object recognition algorithm to analyze test images with the data batch progressing program **Batcher** that can be started in the dashboard.

4.4. Check object detection on the test images through the quantification of false positive and negative events.

4.4.1. Quantify the detection of false positive events: particles erroneously detected as cells, cells that are not correctly classified, and cells of which the contour has not been well identified.

4.4.2. Quantify the false negative events (cells that are not recognized as such).

4.5. Visualize the results in the tool **Result analyzer** by starting the program in the dashboard.

4.5.1. Import the desired results files with **File | Import file** or **File | Import folders**.

4.5.2. Visualize the results by **Chart | Create chart** on the Charts GUI.

4.5.3. Select one of the following options: Distribution chart, Sensitivity plot, Characteristic over time, Characteristic over survey points, and Feature *vs.* feature.

Note: A manual regarding the utilization of the **Result Analyzer** comes with the system and is also available from the support.

4.5.4. If the results are acceptable, run the workflow in the **Batcher** on all acquired images of the experiment. At the same time then the monitoring program can be created by combining the saved settings from the **Probe Controller** (*.pcfg) with the workflow (*.wf), see also the manual.

Note: The workflow can also be used for monitoring future experiments for this culture media.

4.5.5. If the results are not acceptable, check the annotation on the training set and/or continue with another iterative training round (see step 4.2).

5. Sample Size Quantification

5.1. Set the standard deviation (σ), which is acceptable among the detected particles.

Note: The standard deviation changes in parallel to the cell size homogeneity. The maximum standard deviation indicates the sample with the highest degree of size heterogeneity.

5.2. Set the amplitude of the confidence interval or the desired accuracy in relation to the expected variance of measurements.

5.3. Set the admitted error (α) between 5% ($z_{1-\alpha/2}$ = 1.96) and 10% ($z_{1-\alpha/2}$ = 1.64).

5.4. Calculate the number of cells to be identified from each class from equation 1.

$$n = \left(\frac{\sigma \cdot z_{1-\alpha/2}}{d}\right)^2$$ [Equation 1]

Note: Based on the number of cells, the number of images that needs to be acquired can be defined for each data point.

5.5. Perform a sensitivity analysis on random time points of the experiment to check that the analysis of n particles leads to a variability of the mean Feret diameter and the Dv90 of less than 5%. It can be calculated automatically in the **Result Analyzer**.

REPRESENTATIVE RESULTS:

The cell size detection in yeast cultures with the ISM and automated image detection to distinguish between budding and non-budding cells was successfully conducted. Both the stroboscope intensity and the choice of the measurement gap have a range of tolerance, in which the particle identification is not affected. For example, *S. cerevisiae* cells were measured with various stroboscope intensities within a variation range of 11% at a dry biomass concentration of 4 g L^{-1}. The corresponding images provided sharp cell boundaries, therefore the particle identification was feasible with an acceptable variation of the cell size (1%) regardless of the stroboscope intensity. In case the stroboscope intensity is not properly adjusted, the images suffer from over-lighting and a proper cell identification will not be feasible (**Figure 4**).

[Place **Figure 4** here]

So far, the measuring gap cannot be re-adjusted during an *in situ* measurement. Therefore, the dilution series experiment is crucial in order to guarantee reliable data throughout a cultivation. The main concern is the occurrence of unidentifiable overlapping events due to the increment in cell concentration.

A sensitivity plot (sensitivity analysis of characteristic values, *e.g.*, mean cell diameter with respect to particle number n) of all detected cells from the loaded data file can be visualized (**Figure 5**). The user must decide which stability of a certain process parameter is needed. In this case the minimum number of cells needed for one valid data point. In consequence, more or less images can be analyzed for one data point.

[Place **Figure 5** here]

The annotation is the key point in order to achieve the desired accuracy of the particle identification. **Figure 6** shows an example of a "user annotation" (A), which is used as a training set for the neural network, as well as the particle identification on an image of the test set (unknown data for the neural network), which is used for its evaluation (B). Both images should have a similar rate of identified events.

[Place **Figure 6** here]

As an example of the effect of intracellular product accumulation on the cell size distribution, the accumulation of the polyunsaturated fatty acid docosahexaenoic acid (DHA) by nitrogen limitation was investigated in the heterotrophic microalgae *C. cohnii*. It was demonstrated that the accumulation of the product can be quantitatively detected by means of ISM[10]. The method is currently used to investigate the impact of shear forces in stirred bioreactors on the morphological heterogeneity of cells.

The maturation state of the budding yeast *S. cerevisiae* was quantified. In the case of budding, the proportion of cells that are in the maturation state at a time (described with the BI), provides information of the growth activity and population heterogeneity. The automatic cell recognition was able to identify and distinguish budding and non-budding (or daughter) cells successfully in cell suspension[15]. The cell size distribution of three samples is shown in **Figure 7**. A shift to smaller cells indicates a lower portion of budding cells within the population.

[Place **Figure 7** here]

FIGURE LEGENDS:

Figure 1. Concentration calibration tool. Left GUI: set image directories (minimum of 3) with known concentrations; central GUI: choose features to be calculated on the image directory; right GUI: choose the weighted root mean square error (WRMSE) to identify the minimum. WRMSE and the best correlation between any image feature and the cell concentration.

Figure 2. **Sketch of the ISM devices**. The probe MM-Ho (**A**) is installable directly in the bioreactor, whereas the probe MM 2.1 (**B**) can be used as a by-pass. The culture broth circulation is marked with arrows in each picture. The conversion factors are 0.166 µm pix^{-1} for MM-Ho and 0.087 µm pix^{-1} for MM 2.1.

Figure 3. **Fiji tool user interface**. A training set is created with the annotated images. A manual annotation consisting of two classes is depicted, the list of annotated particles is shown in the ROI Manager. Different names and colors can be set for different classes.

Figure 4. **Image acquisition features.** Examples of various stroboscope intensities (SI-%) and measurement gaps (MG-µm) for capturing *S. cerevisiae* cells (present values of SI and MG are indicated in brackets): **A** (25, 50); **B** (50, 50); **C** (25, 80); and **D** (50, 80).

Figure 5. **Mean cell diameter sensitivity plot.** Variability of the mean cell diameter in dependence of the number of detected particles. A constant value of the mean cell diameter is achieved with about 1,000 cells.

Figure 6. **Comparison of user annotation (training set) and automatic detection (test set).** Training set: annotated and original pictures are depicted in **A**, and **A'**, respectively. The information of this picture is used to train the ANN. Test set: the workflow created after training the ANN is applied to captures, which have not been used for the training: automatically identified cells (shown in **B**) from the original capture **B'**.

Figure 7. **Cumulative single-cell size distribution**. Cell size distributions measured during the time course of a cultivation at 3 h (straight line), 7 h (dotted line), and 13 h (dashed line).

DISCUSSION:

ISM as presented here with the same or very similar devices was used to measure morphologic dynamics of fungi, microalgae, and yeast cells, which enabled the determination of growth activity, and in case of algae, intracellular product accumulation. The sensor has no movable parts and is directly applicable in any standard stirred tank bioreactor, either through a standard port or in a sterilizable by-pass. Since yeast is much smaller than algae, the reduction in cell size required some recent hardware adaptions like a higher camera resolution and illumination by transmission in order to get a sufficient pixel resolution of the yeast (for technical details, see[15]). However, there exists still a limitation for measuring even smaller cells like bacteria. The current *in situ* photo-optical instrumentation indicates limitations regarding overlapping particle information in high concentration and interference effects with structures below the UV/VIS spectrum. Up to date, image analysis algorithms for bacterial suspensions have not been applied, apart from brightness correlations[16].

Other previously applied ISM tools were used to determine the cell concentration in order to show their reliability. This, however, became crucial if cells overlapped each other at elevated concentrations. Therefore, the focus of this study was not cell quantification, but morphological feature detection, while image features like brightness intensity can be correlated to the cell density as performed with other devices, too[17]. Otherwise all these devices would be limited to low cell concentrations; a maximal concentration of ca. 20 g L^{-1} of yeast cells was evaluated when using a cell recognition approach *vs.* ca. 80 g L^{-1} when using a cluster size algorithm.

In order to track the morphological features of a cell, its edges need to be detected accurately. In the case of algae, this is rather simple as the size changes, but the form remains constant throughout the transition of process stages. In contrast, yeast cells provide a bigger challenge due to their form, which cannot be approximated to a sphere or ellipse when cells are budding. Nevertheless, until now the cell size was calculated under the assumption that a cell is a perfect sphere in ISM measurements[18]. Although this approximation is close to the reality for some cases, more complicated forms such as budding cells or rod-shaped cells cannot be properly assessed. In this study, however, different forms were analyzed successfully due to the flexible boundary

detection enabled through machine learning algorithms. Furthermore, overlapping events are still under investigation[19] to achieve a further development stage.

Currently, the gold standard for vitality and viability assessment is to count colony forming units or to stain a sample with a viability dye[20], *e.g.*, methylene blue or methylene violet[21]; however, this procedure can influence results. Whenever such features are related to the cell morphology[22], the potential of rapidly assess them should be explored by the increasing potential of ISM. Furthermore, critical process parameters and/or quality attributes[23] can be related to shape, agglomeration and pellet formation, which all can be monitored by ISM.

Investigations with other key microorganisms frequently used in bioprocess are currently performed. The time for the adaptation of the object recognition algorithms and feature extraction for feature analysis depends mainly on the complexity of the images and the expected accuracy of the results. In the future, colored image capture will be considered in order to further broaden the range of information, which could be obtained on a single-cell level, *e.g.*, if pigments are accumulated or in genetically modified organisms, in which colored markers were integrated.

ACKNOWLEDGMENTS:

The authors are thankful for the support of the German Federal Ministry of Economics and Energy within the framework ZIM-Koop, project "Smart Process Inspection", grant no. ZF 4184201CR5.

DISCLOSURES:

The authors have nothing to declare.

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Figure

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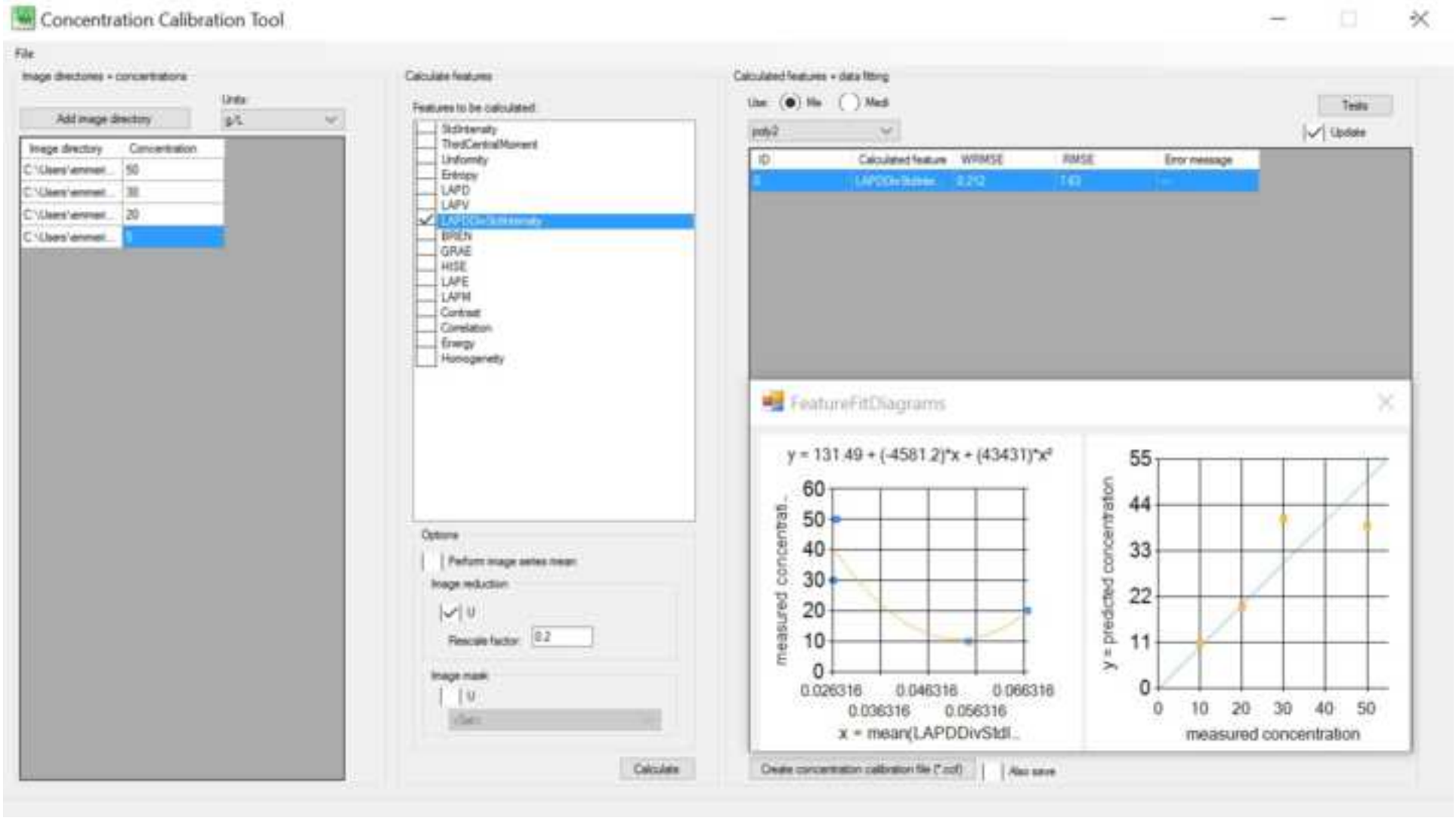

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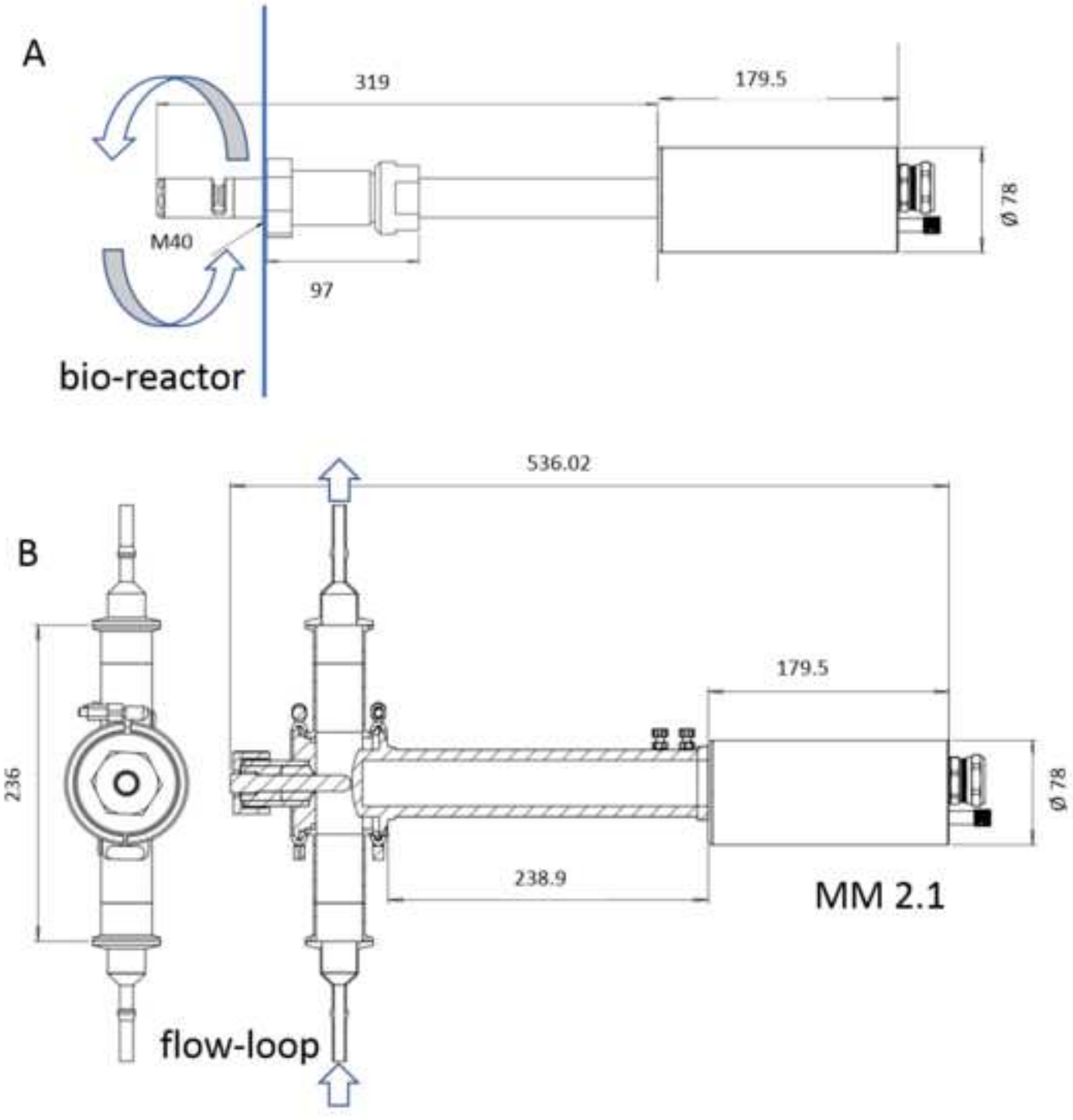

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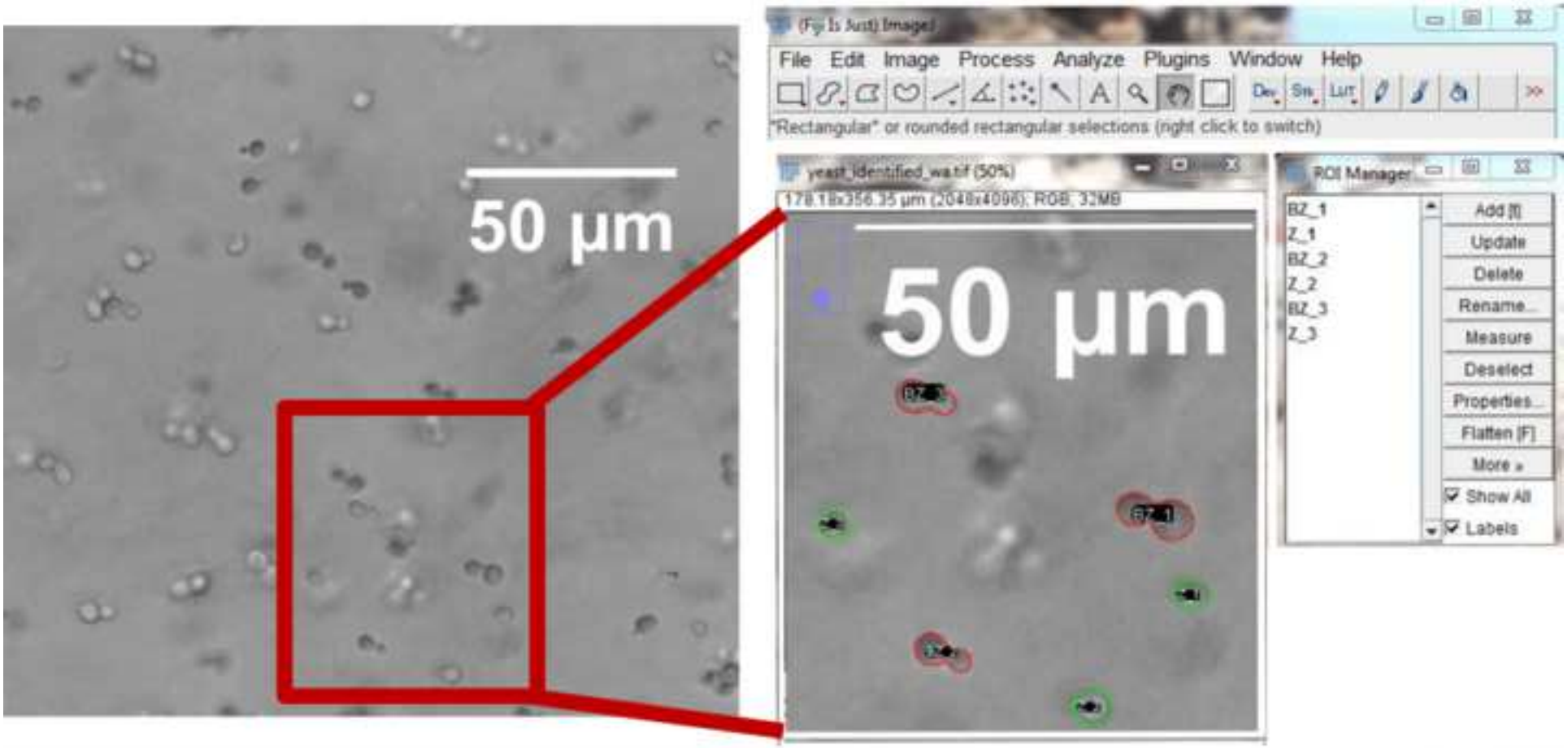

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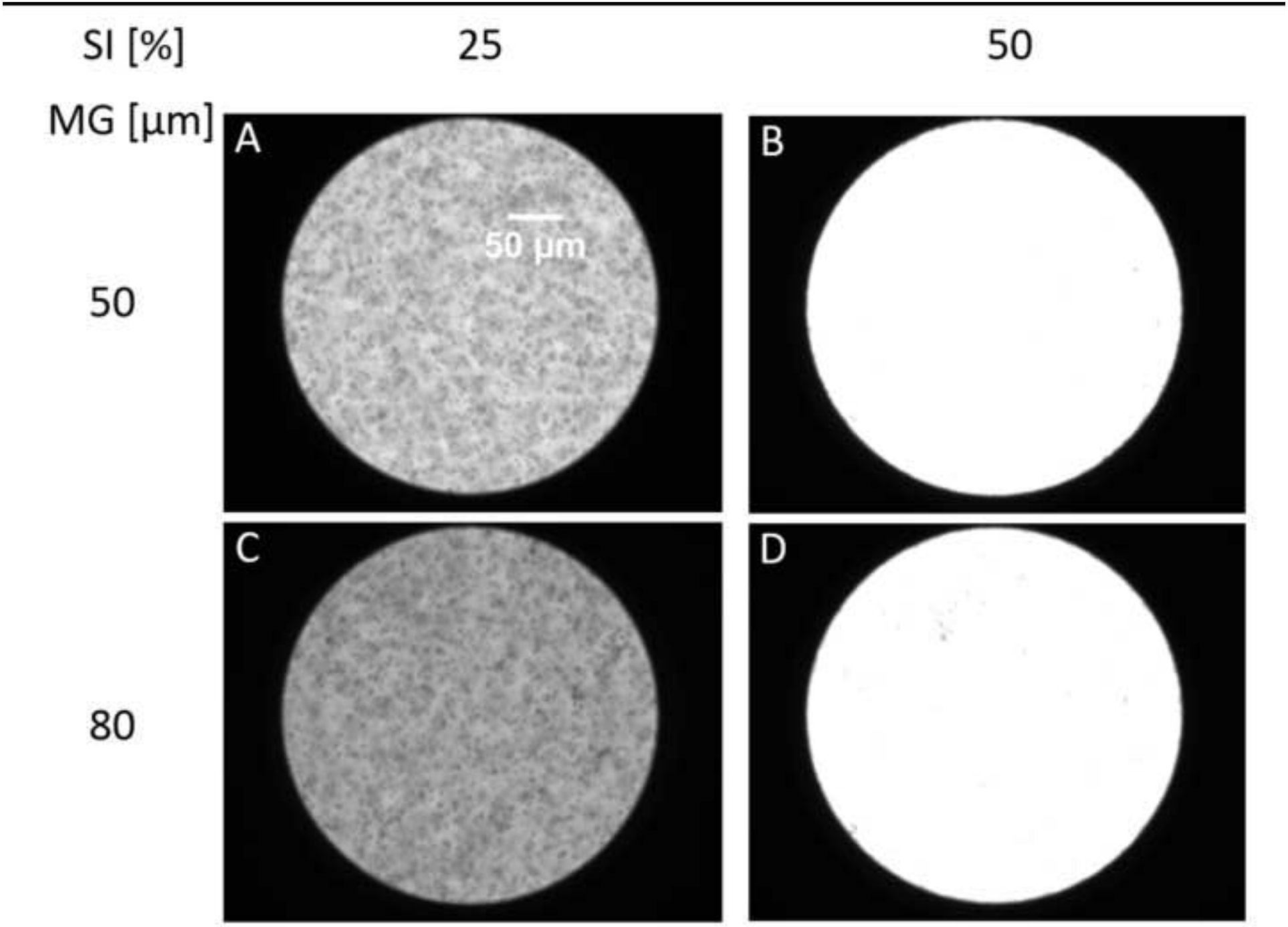

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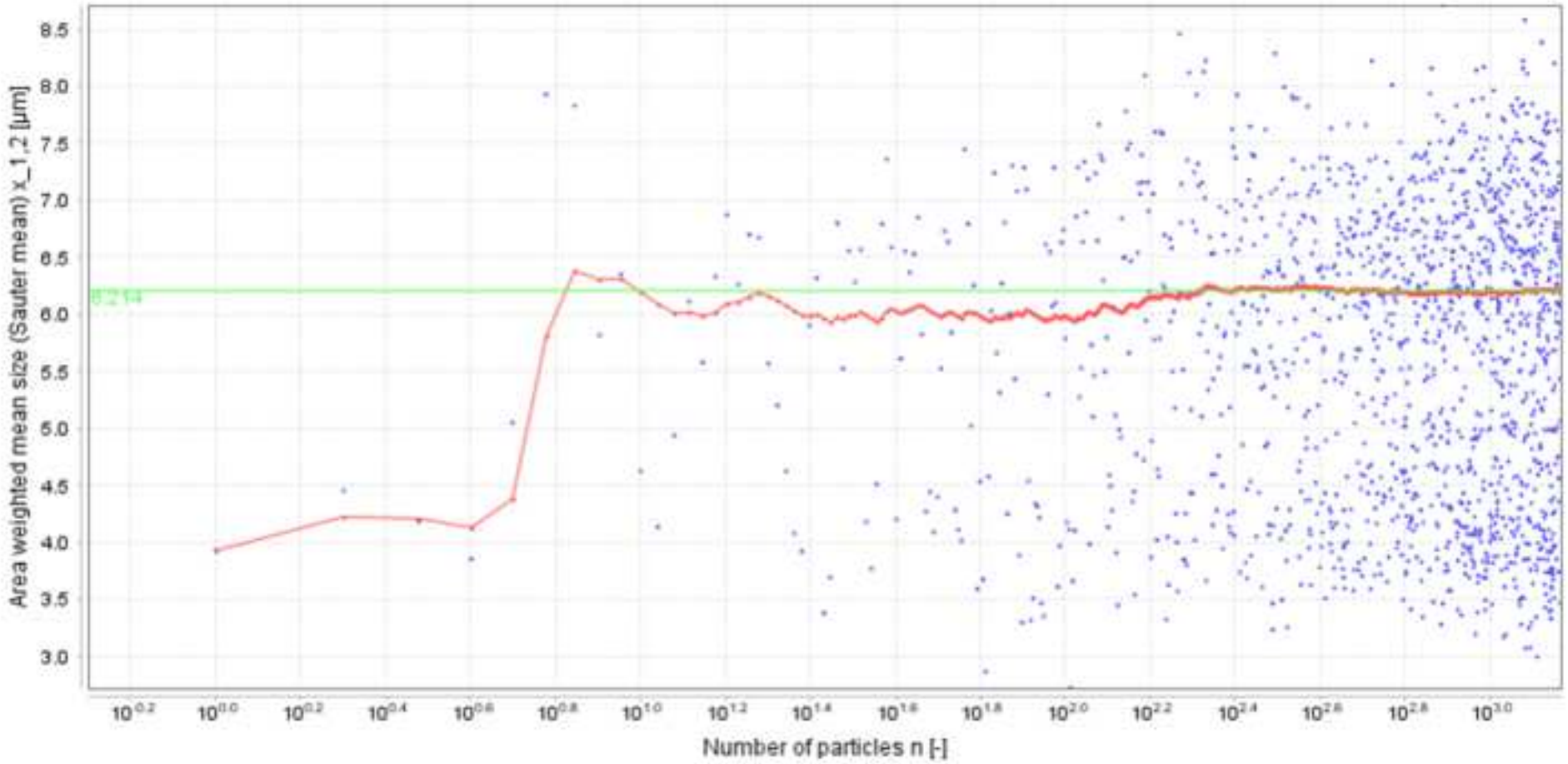

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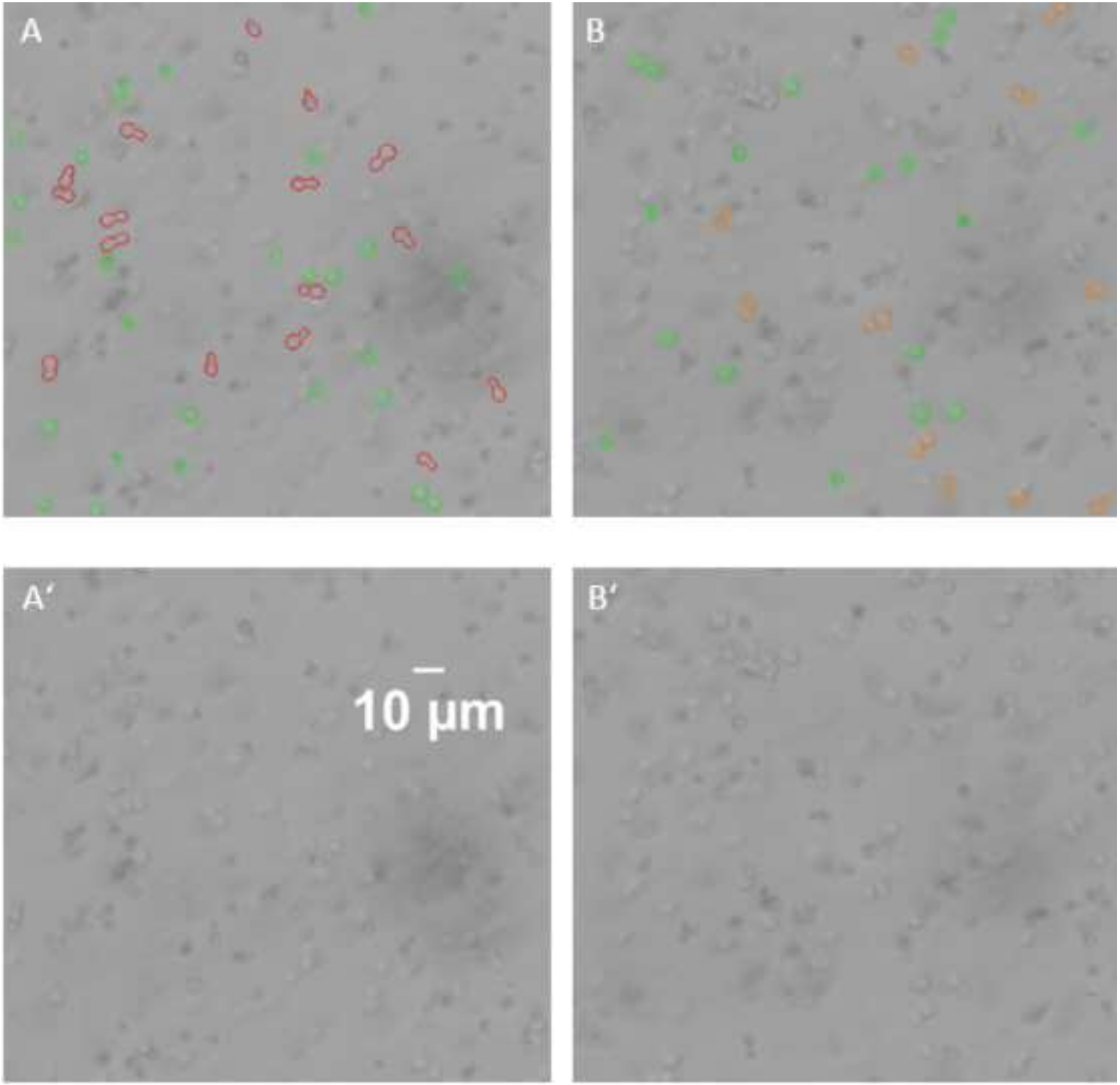

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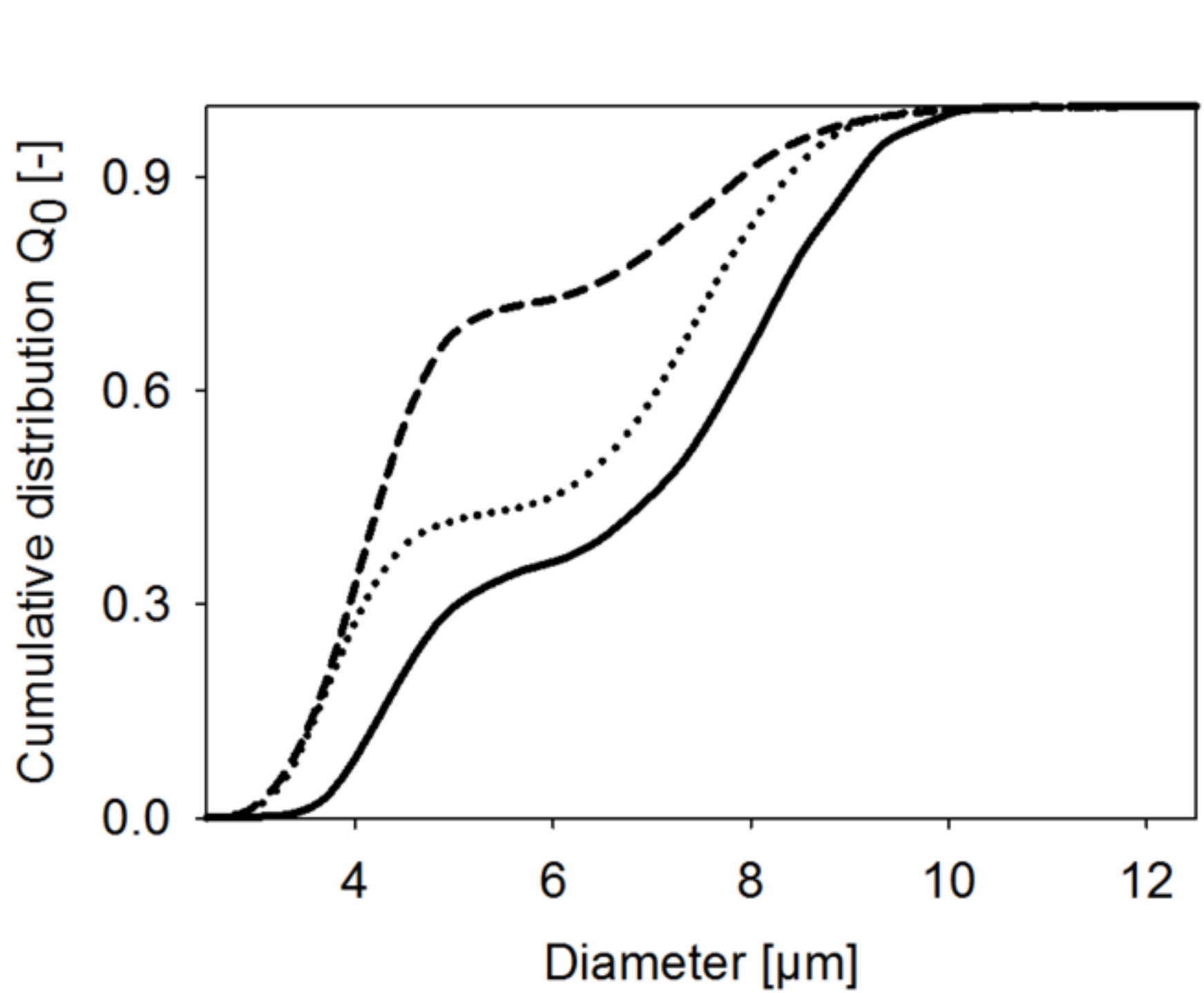

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sensor MM 2.1 - MFC	SOPAT GmbH, Germany	n.a.	Inline Monocular Microscopic probe Version 2.1 with a M
Sofware version v1R.003.0092	SOPAT GmbH, Germany	n.a.	
Thickness gauge	n.n.		It can be any supplier, DIN 2275:2014-03
Ethanol 70%	n.n.		It can be any supplier
SOPAT manual Version 2.0.5	SOPAT GmbH, Germany		
Optical lense paper	VWR	470150-460	
Fiji, ImageJ	open source		
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Author(s): Anna-Maria Marbà-Ardébol, Jörn Emmerich, Michael Muthig, Peter Neubauer, Stefan Junne

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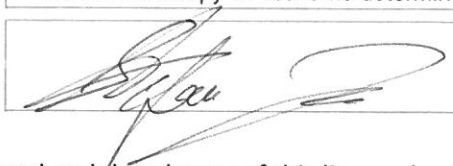
Signature:	
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Editorial Office
Journal of Visualized Experiments

Berlin, 05.09.2018

Manuscript re-submission JoVE 57823

Dear Editor,

on behalf of all authors, I'm re-submitting our manuscript, entitled:

In-situ microscopy for real-time determination of single-cell morphology in bioprocesses.

We have tried to accomplish a re-submission that addresses all remarks of reviewer 1 and most concerns of reviewer 2, although the vast amount of comments and criticism let us to discuss whether it makes sense to continue this process. This is the reason for the late re-submission. I think we achieved a good compromise between our freedom to compose the protocol and the opinion of reviewer 2. Each concern is commented in the attached table. For readability, we did not mark changes in the manuscript.

On behalf of all authors, I declare that there exists no conflict of interest and that the content of the manuscript has not been submitted or published elsewhere before, neither by the authors nor by others.

Please do not hesitate to contact us in case of open questions.

Sincerely yours on behalf of all authors,

Dr. Stefan Junne

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Editorial' comments	**Author's 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 manuscript was thoroughly checked for spelling mistakes and partly checked for grammar by a native speaker.
2. Figure 4/5/6: Please increase the size of the scale bar for visibility.	This was changed.
3. Figure 5: Is the unit for MG correct? This figure is not very clear.	Yes, it is correct.
4. Figure 7: Why is panel C to the right of Panel A when in prior figures, it is Panel B? Please be consistent.	This was changed.
5. 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 Fiji tool from ImageJ software and 50 mL conical centrifuge tubes have been added to the list.
6. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.	The signed ALA was uploaded.
7. 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.	This was checked throughout the manuscript. Several notes have been added when necessary.

8. 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 manuscript was checked and discussions sections in the method part were removed.
9. Please add more details to your protocol steps. 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.	We think we provided exactly the information that is needed to conduct the method w/o mentioning all potential additional steps, where we refer to the handbook. Otherwise the idea of a protocol, which is applicable for lab work, is not achieved.
10. Section 1 of the protocol: Please provide specific values to be used here as we need them in order to be filmed. We cannot film a generalized protocol; we need specific values of a specific experiment.	We are explaining a general protocol, since the technology can be used for various organisms, as well as for different processes. We therefore added "in case of *Saccharomyces cerevisiae…"* phrases to add specific information to the protocol, which is related to the content of the film.
11. What is the sample?	As pointed out above, the sample is culture broth containing any yeast or larger cells. This was specified in the manuscript.
12. Please provide all experimental parameters.	This is part of the method itself and cannot be pre-defined. The first two sections of the protocol are covering this issue.
13. Please provide all volumes and concentrations used throughout.	For *off line* test 5-6 mL are necessary. Afterwards, the measurement can be performed directly in the bioreactor or through a by-pass. This was added in line 196: "Note: 5-6 mL are added to a 50 mL conical centrifugal tube to float the measuring gap sufficiently".
14. 1.4: How are these measurements made?	Each step was specified in section 2: "*Offline* measurement".
15. 2.2: What is this cleaning procedure?	The cleaning procedure is to ensure that the measurement gap is free of visible particles. The following information has been added.

	Line 187: “Clean the measurement gap by spraying ethanol into the gap and carefully wipe any dust or dirt with an optical paper. Check that the glass of the sensor is free of particles with the “Live View” in the CamControl.” Line 190: “Note: Particles and dust disturb measurements and the automatic cell identification.”
16. 2.4: How many cells are used to fill the tube?	The cells are suspended in the culture broth. A maximum concentration of 65 g L of *Saccharomyces cerevisiae* was measured. Threshold (maximum) values for individual applications have to be identified by adjusting the stroboscope intensity. The volume was specified above (point 13).
17. Please note that for computational steps, we can only film if there is a graphical user interface with explicit user input commands: File | Save | etc.	There is a graphical user interface, which description has been added to the protocol at several points. Images of the graphical user interface were added to the original manuscript.
18. Much of the protocol in step 3 onwards is very abstract. We need more specifics in order to film.	The particle identification section (now section 4) has been modified, there are no further specifications to be added.
19. 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.	This was done.
20. 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.	This was done.

Reviewers' comments: 1	Author's comments
21. There are just some small critics/changes that had to be done from my point of view. General the importance of the adaption (via calibration) to the microorganism of interest should be discussed and mentioned earlier in the manuscript. General the introduction is written well and considered all important facts.	The adaptation to the organism is performed by adjusting the measuring gap, the focus and the stroboscope intensity, which are described in the very first parts of the protocol. We add a sentence to make this clearer at the very beginning of the protocol section: Line 130: "The following steps are necessary to adapt the parameters to the respective microorganism and culture conditions."
22. In chapter 1 of the protocol may a note of the time expanse for the calibration could be helpful for applicants.	The calibration file is provided by SOPAT GmbH depending on the probe used, but the settings of the hardware and software parameters need to be adapted to the organism, as well as to the concentration range. A sentence was added: Line 131: "The adjustment of probe settings lasts about 20 min for an experienced user." The adaptation of the automated image recognition was added to the discussion part: Line 406: "The time for the adaptation of the object recognition algorithms and feature extraction for feature analysis depends mainly on the complexity of the images and the expected accuracy of the results."
23. Point 2.10 (line 177): A comment in chapter 1 at which point the calibration file had to be saved would be helpful.	The calibration file is provided by SOPAT GmbH depending on the probe
24. Point 3.1 (line 185-186): Fig.8 should also include a marked region of an object of interest.	This has been added (now figure 3).
25. Fig 4 and 7 should be shown in higher resolution	Figure 4 has been removed and figure 7 (now figure 5) has a higher resolution.
26. In Fig 9 mark the curves (which one are A, B or C)	This was added to the legend of Figure 9 (now figure 7). "A (straight line), B (dotted line), C (dashed line)".
27. In the discussion part also the state of process development concerning smaller microorganism (bacteria etc.) can be discussed shortly.	Line 377: "However, there exists still a limitation for measuring even smaller cells like bacteria. The current *in situ* photo-optical instrumentation indicates limitations regarding overlapping particle information in high concentration and interference effects with structures below the UV/VIS spectrum. Up to date, image analysis algorithms for bacterial

	suspensions have not been applied, apart from brightness correlations (Marquard, Schneider-Barthold et al. 2017)."
Reviewers' comments: 2	**Author's comments**
28. Language and spelling of the article need improvements. Maybe have it checked by a native speaker (I am not one but even I can see it is necessary).	Although it becomes not obvious in the specific comments of this reviewer and this statement is not in line with the editorial comments and the comments of reviewer 1, we have checked several critical passages together with a native speaker.
29. Some of the steps are not described with enough care. Figure legends are too short and not descriptive. Some figures do not help / do not contain meaningful information. Relevant pieces of literature are not cited. Altogether, the manuscript at present is not prepared with an acceptable degree of diligence.	It does not become clear, which steps are not described with enough care and which legends are not descriptive. This Reviewer comment is not specific enough to correct the manuscript in a meaningful way. This manuscript is about the monitoring of yeast or similar cells and not about in situ microscopy in general. It makes no sense to write a protocol and cite a lot of work about the cultivation of cell lines. Such cells are 10 times larger. A sketch of the sensors has been added (Figure 2), whereas Figure 3 (Screenshot of SOPAT Results Analyzer) has been removed. More literature has been added: Belini, V. L., Wiedemann, P. & Suhr, H. In situ microscopy: A perspective for industrial bioethanol production monitoring. *Journal of microbiological methods.* **93** (3), 224-232, (2013). Havlik, I. *et al.* Monitoring of microalgal cultivations with on-line, flow-through microscopy. *Algal Research.* **2** (3), 253-257, (2013). Lemoine, A., et al. Tools for the determination of population heterogeneity caused by inhomogeneous cultivation conditions. *Journal of biotechnology.* **251** 84-93, (2017). Marquard, D. et al. Online monitoring of cell concentration in high cell density Escherichia coli cultivations using in situ Microscopy. *Journal of biotechnology.* **259** 83-85, (2017).

	Suhr, H. & Herkommer, A. M. In situ microscopy using adjustment-free optics. *Journal of biomedical optics.* **20** (11), 116007-116007, (2015).
30. Abstract, last paragraph: "on line": online monitoring is mostly regarded as via bypass, FIA etc. The idea of your method is primarily in situ (or in-line) monitoring, right?	Indeed both is addressed. This was changed in line 48: “The feasibility to monitor changes in cell morphology directly *in line or on line* (by-pass) enables…”
31. P2, 61: cell agglomeration is very often interesting and by far not only "e.g. at (wording?) stem cell proliferation".	The sentence points out that cell agglomeration may be a factor to consider when measuring cell morphology. It is not meant as a discussion about the importance of cell agglomeration. This would not be appropriate (see comment 8). Since we see that this comment rises questions among some audience, we removed the statement completely.
32. 74: There is substantially more literature on ISM than the cited ref. 4. Although the focus here is on the method, a bit more background is necessary. In the case of yeast, maybe Belini et al. 2013 and literature found therein (Belini VL,Wiedemann P, Suhr H (2013): In situ microscopy: A perspective for industrial bioethanol production monitoring. J Microbiol Methods 93, 224-232).	We added more literature (see comment 29).
33. 77: again: on line - any particular reason? ISM includes in situ…	This was changed in line 80: “Recent advances in ISM allow the *in line* or *on line* monitoring of cells on a single-cell basis…”
34. P3, 97: please explain in more detail. Particle size? Or pellet size? Of course at the edges of pellets there is less biomass than in the center?	We talk about particle size (of single cells) throughout the manuscript if not particularly stated in case of fungi, as done in the manuscript.
35. P4, 128: Centrifuge? You mean centrifuge and resuspend? (This is not about being "picky" - it is a question of how precise you want to be or the journal wants you to be)	Changed in line 142: **“**Prepare a culture with the highest cell concentration that might be achieved during the experiment or centrifuge and resuspend the pellet in order to reach this concentration.”
36. 131: Measure…: How? You refer to the gap later on (even citing a DIN) but changing the gap and measuring with the instrument is just taking for granted here? Refer to the manual or describe the procedure	See section 2 “*Off line* Measurement” were this point has been specified.

here in more detail. Also: DoE: Included in the software package? If not: Any suggestion which program to use?	DoE is not mentioned anymore as this is not necessary and not in line with the editorial comments.
37. 142: What is the readout of conc. calib.: Concentration? Brightness? Assumingly, the "image feature" is cell number, but later on you state (p9, 305) intensity or brightness (by the way: what is the difference between the two?)?	As it is described in the discussion part, only for concentration analyses, additional image features (i.e. brightness, sharpness) have been extracted and correlated to cell dry biomass concentrations. “Intensity” was removed when it was mentioned in the context of cell intensity. This particular line was rephrased in line 165: “Use the “Concentration Calibration (CoCa) Tool” (see *Figure 1*) to identify the optimal correlation between the extracted image features (brightness or sharpness) and the previously measured concentrations provided by the user as e.g. dry biomass or cell counts. Follow the instructions in the manual of the CoCa tool of the SOPAT Software for further details.”
38. 145: What is the merit of Fig 1? In particular, with no explaining legend.	The legend has been modified: Figure 1. **Concentration Calibration Tool**. Left GUI: Set image directories (minimum of 3) with known concentrations; central GUI: choose features to be calculated on the image directory; right GUI: choose the weighted root mean square error (WRMSE) to identify the min. WRMSE and the best correlation between any image feature and the cell concentration.
39. 146: Again: What is "measurement information"? Cell concentration? Brightness?	There is no general image feature, which provides an optimal correlation. Therefore, the method was described as mentioned at the answer of comment 38. The wording was modified in line 170: “Identify the optimal correlation between the extracted information from the image features at various concentrations compared to any *off line* measurements. See the legend of *Figure 1*.”
40. 155: Most likely not "cleaning in place" - compare with the definition of CIP	CIP was removed, the wording was changed in Line 187: “Clean the measurement gap by spraying ethanol into the gap and carefully wipe any dust or dirt with an optical paper. Check that the glass of the sensor is free of particles with the “Live View” in the “CamControl”.”

41. P5, 160: Not clear: Fill a tube and dip against microscope?	Changed in line 193: “Fill a tube with culture broth. Dip the microscope in the culture broth so that the gap is fully covered with cell suspension, and focus on the cells by fine-tuning the focus binding screw.”
42. 167: probe-wetted? Why does the probe wet anything? And: Where is the camera? Can it not be sterilised?	Wetted material is the term and it does mean any material in contact with the process media. Therefore, any part of the probe that is in contact with the media can be sterilizable. In order to clarify this point, a picture of the probe has been attached (Figure 2), as well as the following note: Line 226: “Only the probe-wetted material of the instrument is sterilizable through steam sterilization. The probe wetted length can go from 6 to 222 mm (See Figure 2).”
43. 168: Define… : Where? You show more or less meaningless pictures of software interfaces - but you do not tell the reader where to define parameters.	We have added steps regarding the SOPAT user interface all along the protocol. We do not agree that the pictures are meaningless, but very helpful for a quick guidance through the software applications.
44. 173: s.a.	We have added steps regarding the SOPAT user interface all along the protocol.
45. 177: s.a.	We have added steps regarding the SOPAT user interface all along the protocol.
46. 178: s.a.	We have added steps regarding the SOPAT user interface all along the protocol.
47. 185: Fiji is a version of Image J, right? If that is the one, it should be made clear that this software is not written by or part of the Sopat software. (and should also be included in the materials table)	Exactly, Fiji from ImageJ. Several points have been rephrased: Line 249: “Load the acquired images into the annotation tool Fiji, ImageJ (open source software product) by dragging and dropping the file into the Main ImageJ window (see GUI “Fiji” tool in *Figure 3*).”
48. 188: What is Fig 2 useful for? With hardly any legend?	Generally this tool is used for labeling certain object classes and producing a file format for further training of object recognition algorithms. We have added some steps for an intensified description of this application (object annotation and classification) in section *4. Particle identification*.

49. P6, 195: Annotate where / with what? Fiji? Description of the procedure? How detailed and structured is this whole protocol meant to be - at this stage, it is not possible to carry out an experiment on the basis of the current manuscript.	This general statement is not justified sufficiently. See also comment 47.
50. 199: annotate: s.a.	See comment 48.
51. 202: All of the sudden, here are details down to the level that a particular label can be set to "ignore". That is in contrast to the rest of the protocol, s.a.	See comment 48.
52. 216: Sense of Fig 3, with hardly any legend on top of that?	Figure 3 has been removed
53. 223/224: saved where? In general: When do you use which software to do what and how?	The directory for saving the images or settings can be selected by the user. In case a certain directory for a certain file is needed, it is described in the protocol. The following overview was added before starting the protocol in Line 133: In general, the tools that are presented in the following protocol are needed for: a) Probe Controller: probe adjustments and image acquisition. b) Fiji (ImageJ): annotations on acquired images Annotation. c) SOPAT support: ANN training and workflow creation. d) Batcher: data batch processing using already acquired images with a workflow. e) Result Analyzer: result visualization and evaluation on batch processed images. f) Monitor: for automated real-time measurement and result visualization.
54. P7, 241: "maximal" standard deviation? Is there more than one? In one class there should be one mean of diameter with one SD.	Each measurement (time) point has a standard deviation depending on the cell morphological heterogeneity. For a better understanding, a note has been added:

	Line 315: “Note: The standard deviation changes in parallel to the cell size homogeneity. The maximum standard deviation indicates the sample with the highest degree of size heterogeneity“
55. 241: please include (again) … where n is …, sigma is …, etc.	n is calculated from equation 1. The parameters needed for the calculation of n are explained in 4.1, 4.2, and 4.3, respectively.
56. 242: Where? In which software?	This section has been deleted, the information was added to the previous sections.
57. 247 - 250: acquisition rate or acquisition time? Is that how often a time point (5.1.) happens?	See point 3.5.
58. 257: A certain concentration…? Which?	Figure 4 has been removed.
59. P8, 271: sensitivity analysis: how? The essential amount of particles: essential for what? How much is essential?	This is part of the “Result Analyzer” manual. This information has been added at Line 343: “A sensitivity plot (Sensitivity analysis of characteristic values, e.g. mean cell diameter with respect to particle number n) of all detected cells from the loaded data file can be visualized (*Figure 5*). The user must decide which stability of a certain process parameter is needed. In this case the minimum number of cells needed for one valid data point. In consequence, more or less images can be analyzed for one data point.”
60. 274: Fig 7: No meaningful legend. Fig 7 A and B: How meaningful is the mean? There seem to be two groups of cells, one with smaller, one with larger mean diameter. Also: Cannot read axis description (too small font)	This figure (now figure 5) has been changed for showing better the axis description and make it understandable.
61. 278: Fig 8: not clear. There should be one original picture and then one manually and one automatically annotated one.	The comments about this figure were rephrased, as well as the legend: Line 350: “*Figure 6* shows an example of a “user annotation” (A), which is used as a training set for training the neural network, as well as an the particle identification on an image of the test set (unknown data for the neural network), which is used for its evaluation (B). Both images should have a similar rate of identified events.”

	Figure 6. **Comparison of user annotation (training set) and automatic detection (test set).** Training set: annotated and original pictures are depicted in A, and A', respectively. The information of this picture is used to train the ANN. Test set: the workflow created after training the ANN is applied to captures, which have not been used for the training: automatically identified cells (shown in B) from the original capture B'.
62. 284: ..morphological heterogeneity … of what?	Changed in Line 358: "The method is currently used to investigate the impact of shear forces in stirred bioreactors on the morphological heterogeneity of cells."
63. 298: Technical details are lacking. What is "higher resolution"? How is that achieved? What is the resolution of the instrument anyway? P9, 299: What is pixel resolution?	Added in Line 88: "The ISM consists of a single-rod sensor probe that enables the capture of images within a known focus range in an adjustable measurement gap with a high-resolution CCD camera, which is depending on the sensor model (e.g. MM1 = CCD GT2750 (2750x2200) and MM 2.1 = CMOS G507c (2464x2056))." More technical details are depicted in another publication (Marba-Ardebol, Emmerich et al. 2018), whose citation has been added in line 359. A new figure has been included to show a sketch of the probes (Figure 2). Legend of Figure 2: Figure 2. **Sketch of the ISM devices**. The probe MM-Ho (A) is installable directly in the bioreactor, whereas the probe MM 2.1 (B) can be used as a by-pass. The culture broth circulation is marked with arrows in each picture. The conversion factor of each probe is 0.166 µm pix^{-1} (MM-Ho), and 0.087 µm pix^{-1} (MM 2.1), respectively.
64. 304 etc: Examples? Figures? This would be meaningful for the reader (much more than some of the Figs. above).	We show a figure about the annotation. We do not add more to not blow up the protocol.
65: 316: There is more literature on this and it is not true that only perfect circles are used until now: See e.g. Belini et al. 2013 above, Sierra et al. 2017, Wiedemann et al. 2011 (Wiedemann P, Guez JS, Wiegemann HB, Egner F, Quintana JC, Asanza-Maldonado D, Filipaki M, Wilkesman J, Schwiebert C, Cassar JP, Dhulster P, Suhr H (2011): In situ microscopic cytometry enables noninvasive viability assessment of animal cells by	Only studies of small microbial cells are addressed in this protocol, not cell line cultivation. We also did not aim to provide a review about cytometry studies as this represents clearly another method with drawbacks for the application in microbial cultivation with complex media, if fluorescence markers cannot be applied. There might have been a

measuring entropy states. Biotechnol Bioeng 108(12), 2884-2993; Sierra F DA, Melzak KA, Janetzko K, Klüter H, Suhr H, Bieback K, Wiedemann P (2017): Flow morphometry to assess the red blood cell storage lesion. Cytometry A 91(9), 874-882)	misunderstanding of the reviewer of the purpose of this manuscript, therefore "cell proliferation" was removed. The paper clearly describes the application in microbial cultures, only one sentence will refer to the much larger animal/human cells in line 124: "The methods described here are focused on microbial applications with single cells, but are also applicable to larger particles like human and animal cells, cell agglomerates and pellets of filamentous organisms." The publication of Bellini from 2013 is now cited.
66. 321: What is the difference between viability and vitality? Define!	This is commonly known and content of a basic biotechnology class. If we start to explain definitions of such terminologies, this all becomes an endless manuscript.
67. 324: That is already published, e.g. in the case of animal cells, see Wiedemann et al. 2011 and 2011 (see above and Wiedemann P, Worf M, Wiegemann HB, Egner F, Schwiebert C, Wilkesman J, Guez JS, Quintana JC, Assanza D, Suhr H (2011): On-line and real time cell counting and viability determination for animal cell process monitoring by in situ microscopy. BMC Proceedings 5(Suppl 8), P77	It is clear to us that numerous literature and in line/on line cell counting methods were commercialized and are used for monitoring animal/human cell proliferation. The slow processes make it also doubtful, if in situ microscopy is necessary. As mentioned above, we don't want to include such a discussion, as it is not the focus of the application described here.
68. 327: Microorganisms: In the usual definition of the term, microorganism does not apply to microalgae, or does it? In general terms: ISM is applicable to more than microorganisms. Why not phrase it more general?	We changed the wording to point out that there is no restriction to microbial applications. Microalgae themselves cover a heterogeneous group of different species, thus the group is polyphyletic. In case of heterotrophic algae, a dominant group are dinoflagellates. These are microorganisms. In order to avoid misunderstanding, microalgae are not denoted as a separate group beyond microorganisms anymore.
70. P12, 401: Legend not understandable. What is the difference between A and B?	For a better understanding, the legend of figure 6 was changed. (See comment 61)
71. Fig 5: Would one not expect a higher concentration in C (gap is larger) than in A?	Correct, a larger gap leads to more particles in the measuring gap compared to a smaller gap and therefore darker images using the same light intensities The focus area was smaller (now figure 4).

72. Fig 9: Legend? What is A, B, C?	Added in the legend of Figure 7: Figure 7. **Cumulative single-cell size distribution**. Cell size distributions measured during the time course of a cultivation at 3 h (straight line), 7 h (dotted line), and 13 h (dashed line).