Ms. Ref. No. JoVE57823   
Title: *In situ* microscopy for real-time determination of single-cell morphology in bioprocesses  
JoVE

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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. |

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| **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](#_ENREF_2)).” |
| **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 here in more detail. Also: DoE: Included in the software package? If not: Any suggestion which program to use? | See section 2 “*Off line* Measurement” were this point has been specified.  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](#_ENREF_1)), 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 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) | 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 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). |