# High-throughput bacterial aggregation analysis in droplets

**Merili Saar-Abroi, Karoliine Lindpere, Daniel Kascor, Triini Olman, David Gonzalez, Fenella Lucia Sulp, Katri Kiir, Immanuel Sanka, Simona Bartkova, Ott Scheler**

Antimicrobial resistance (AMR), a rapidly emerging health risk, is knowingly accelerated by bacterial aggregation and biofilm formation. We present a robust, cost-effective method using microfluidic droplets and texture-based image analysis via open-source software CellProfiler™ to assess bacterial aggregation. The developed pipeline offers high accuracy, easy setup, and broad applicability for AMR studies and beyond.

**Introduction**

Antimicrobial resistance (AMR) is a global threat that can result in millions of deaths if preventive measures are not implemented. The global prevalence of deaths associated with bacterial AMR rose to 4.71 million in 2021, and a progressive upsurge is projected to reach up to 8.22 million deaths due to AMR in 2050 [1].

Bacterial aggregation and biofilm formation is seen as one of the key concerns in addressing AMR [2]. Aggregated lifestyle of bacteria ensures the survival of the innermost bacteria in this multicellular matrix even in hostile environments, while simultaneously providing a fruitful ground for horizontal gene transfer [3]. Once unfavourable conditions subside, bacteria could separate from the aggregate matrix and successfully colonise new surfaces [3]. This phenomenon is known to be fueled by constant antibiotic exposure and the presence of contaminants as aggregation platforms [4]-[7].

Altogether, these figures underscore a critical need for the exploration of novel techniques to monitor and study bacterial aggregation and AMR evolution. As the burden of AMR is considered the greatest in low-income and middle-income countries, cost-efficient and innovative techniques pleasing a variety of surveillance strategies of antimicrobial resistance are in high demand [8].

The most common methods for bacterial aggregation studies are sedimentation assays or flow cytometry [9]-[11]. However, these methods have the major limitation to be time-consuming or expensive [10]. Microfluidic droplets, acting as nanoscale bioreactors, exhibit great potential at increasing the speed and precision of microbiological experiments [12]. Coupling microfluidic droplet techniques with image analysis tools, this tandem can provide deep insight in the dynamics of bacterial aggregation and antibiotic resistance [13][14].

Due to high-throughput nature of droplet-based techniques, visual inspection of images is laborious and therefore, not a viable option for droplet image analysis. So far, automated image analysis often relies on sophisticated software or high proficiency in programming skills using Python, MATLAB, and other advanced tools [14]. Paradoxically, these proficiencies may fall outside the typical training of many biomedical professionals. Therefore, a clear need to bridge the gap between advanced bioinformatics and droplet image analysis for broader user base comes sharply into focus.

In this paper we propose a solution to this need by using water-in-oil droplets in combination with user-friendly image analysis tools to assess the aggregation patterns of bacteria in high-throughput. The applicability of the method is demonstrated by easy-to-use image analysis pipelines established using a free, open-source software CellProfiler™ to quantify bacterial aggregation in response to suboptimal concentrations of antibiotics, microplastic beads or metals in both mono- and polydisperse droplets. Our research group has previously shown high accuracy, precision and wide applicability of CellProfiler™ for various droplet experiments compared to similar software [14].

**Results and Discussion**

An image analysis pipeline utilizing textural characterisation within CellProfiler™ was specifically designed to identify and aid classifying droplets based on bacterial aggregation severity detected inside droplet (Figure 1a). The severity of aggregation is quantified by assessing the sum variance of droplets as defined by Haralick [15]. This involves

constructing a grey-level co-occurrence matrix of the droplets to assess the spatial relationships of neighbouring pixels, which as a result, determines the measurement for each droplet. The principles and protocol for implementing the pipeline is outlined in Supplementary file 1.

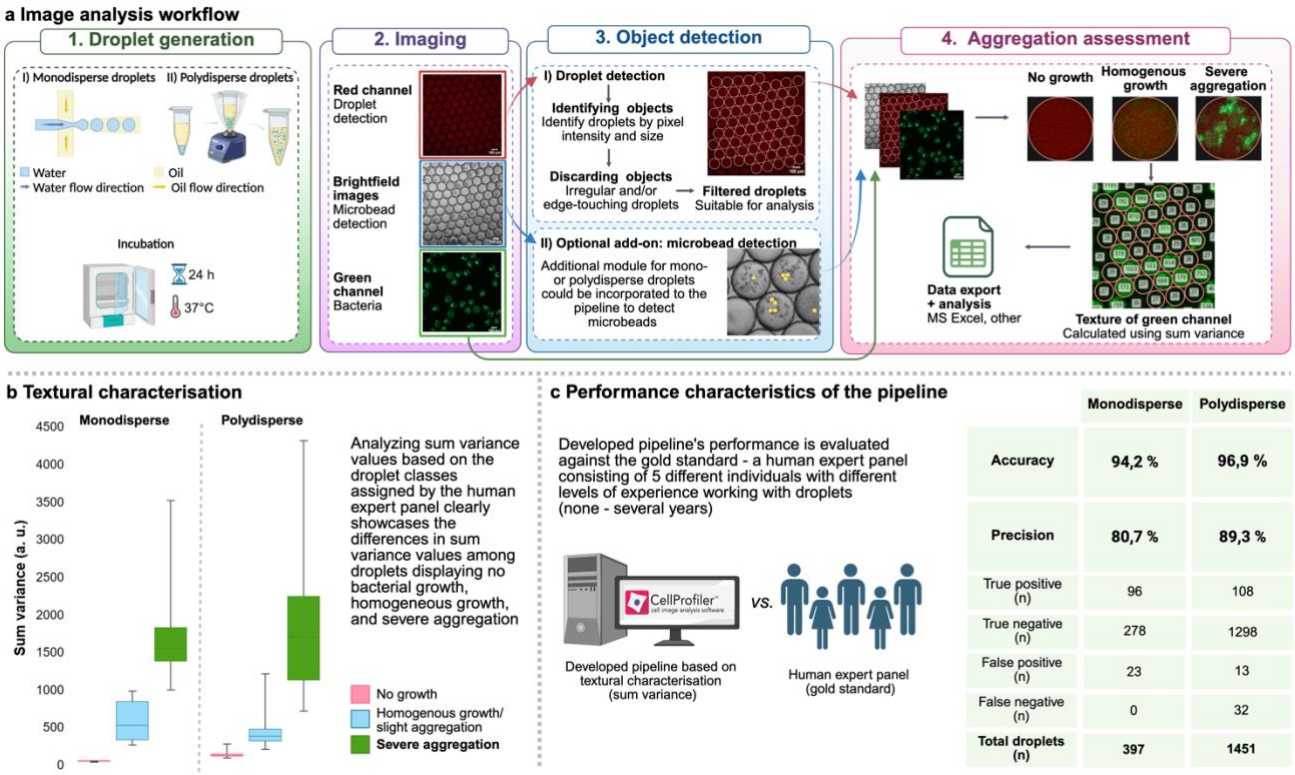


**Fig. 1 | Droplet generation and image analysis workflow utilising textural properties in CellProfiler™ showcases a fit tool for bacterial aggregation evaluation in droplets. a** Protocol overview of the bacterial aggregation assessment using CellProfiler™. Droplets of mono- or polydisperse nature can be used to analyse bacterial aggregation. Generation of droplets is followed by further incubation to encourage bacterial growth and study the phenomenon under investigation. Droplets are visualised via microscopy, where three types of images are acquired – brightfield, red, and green channel. Droplets are detected using the red channel of the images, microbeads via brightfield images and textural characterisation is performed in the green channel. All data gathered during the analysis is exported as a .csv file and could be analysed using any spreadsheet software. **b** Higher values of textural characteristic sum variance measurements reveal severely aggregated droplets. Although Haralick initially proposed several other textural characterisation formulas, sum variance proved to be the most suitable for aggregation analysis as shown in Supplementary file 3. Shown data is applicable to experiments performed with sub-optimal concentrations of cefotaxime in mono- and polydisperse droplets. Textural characterisation data for other experiments is found in Supplementary file 3. **c** Developed pipeline can assess aggregation severity with great accuracy and precision in both mono- and polydisperse droplet data. Shown data is applicable to experiments performed with sub-optimal concentrations of cefotaxime and kanamycin in monodisperse- and cefotaxime, chloramphenicol, ciprofloxacin, doxycycline and trimethoprim in polydisperse droplets. Figure created with biorender.com.

Empirical experimentation in our laboratory has shown universality and effectiveness of the pipeline. In our experience, texture characterisation a) does not rely on defining thresholds to determine the boundaries of objects under interest (aggregates) inside droplets and b) the droplets contents are characterised by textural properties independent of the experiment's nature or conditions. The method proves to be user-friendly and computationally efficient, due to eliminating the need for additional modules within the pipeline to perform multiple measurements.

Furthermore, CellProfiler™ is equipped to detect and exclude extraneous objects during the analysis of the objects under interest. The main analysis of target objects remains unaffected while simultaneously providing valuable information on the presence of additional objects to the user. This translates to the capability of assessing the presence of additional objects (e.g. microplastic) inside the droplets that can affect the aggregation patterns of bacteria. An easy setup of the pipeline for microplastic beads alongside bacteria in droplets is assisted with a Supplementary file 2.

Calculating the texture of droplets via sum variance correlates well with aggregation severity in droplets as demonstrated in Figure 1b. Figure 1c demonstrates the accuracy of our pipeline to assess the severity of aggregation up to 94.2 % (ranging from 93.5 % to 94.8 %) and precision of 80.7 % (ranging from 78.2 % to 82.8 %) in monodisperse droplets. Similar performance could be achieved in polydisperse droplets with an accuracy of 96.9 % (range = 94.0 – 98.6 %) and precision of 89.3 % (range = 65 – 100 %). However, users must be aware that the polydisperse data may be more unpredictable - additional assessments may be valuable to determine an appropriate texture scale for analysing larger droplets in the polydisperse dataset.

Our pipelines can quantify bacterial aggregation in different experimental scenarios as demonstrated in Figure 2a. The software was tested with droplets infused with *Escherichia coli,* antibiotics in varying concentrations and additionally, metals or

microplastic beads. Further data analysis of these testing results revealed diverse aggregation patterns.

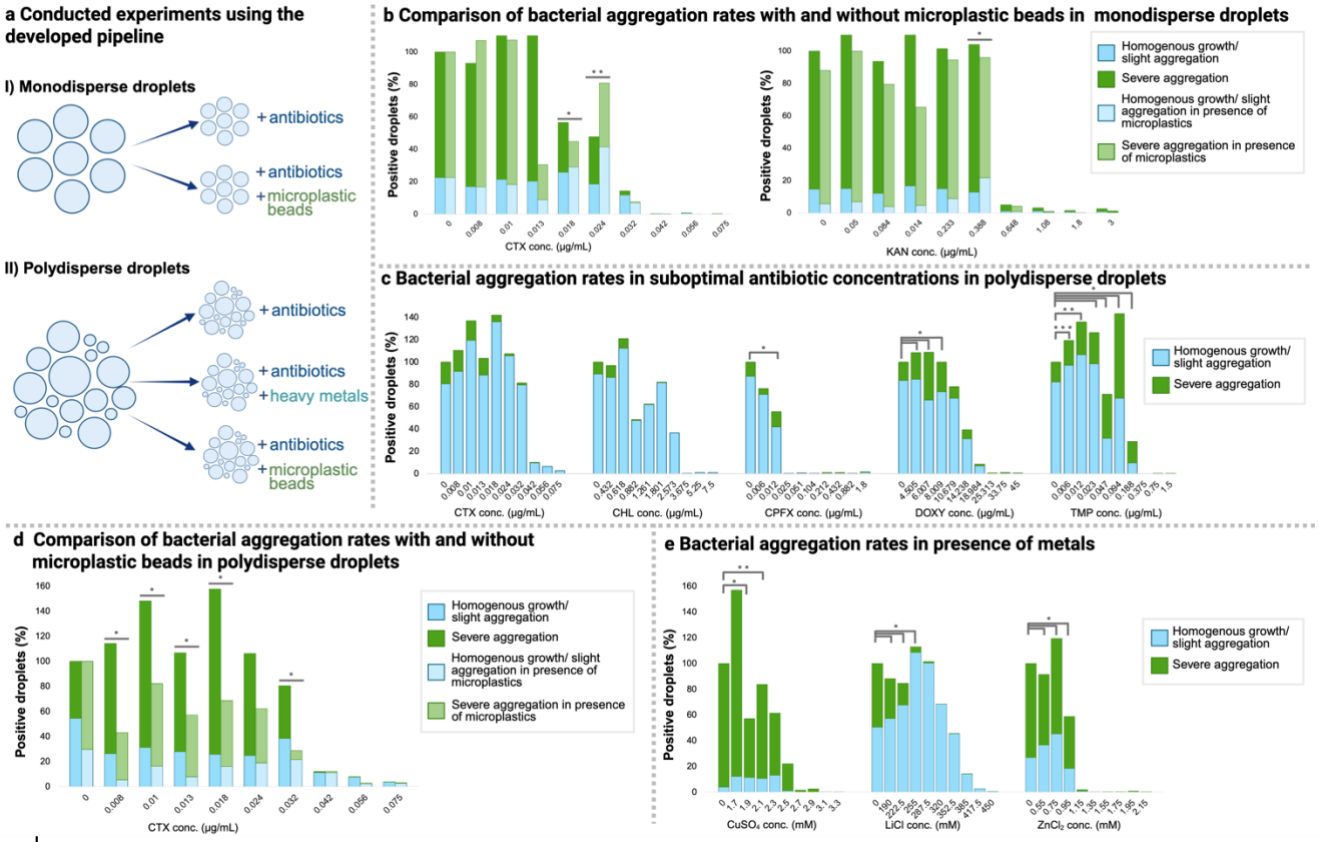


**Fig. 2 | Texture-based aggregation assessment is applicable in various experimental setups. a** The aggregation pipeline described in the current paper was applied to various datasets using *E. coli*. **b** Monodisperse droplets harbouring bacteria were infused with suboptimal concentrations of antibiotics and in an additional experiment, with microplastic beads. Microplastic beads are seen to significantly decrease aggregation where cefotaxime (CTX) concentration is 0.018 µl/mL ($p<0.001$) and 0.024 µl/mL ($p=0.026$). In experiments conducted with kanamycin (KAN) with a concentration of 0.388 µl/mL, aggregation is decreased in the presence of microplastics ($p<0.001$). **c** In polydisperse droplets, a significant increase in aggregation compared to droplets without any antibiotics is seen in some concentrations of ciprofloxacin (CPFX at a concentration of 0.012 µl/mL, $p<0.001$), doxycycline (DOXY at a concentration of 4.505 µl/mL, 6.007 µl/mL, 8.009 µl/mL and 10.679 µl/mL, $p<0.001$) and trimethoprim (TMP at a concentration of 0.006 µl/mL, $p=0.016$; 0.012 µl/mL, $p=0.008$ and 0.023 µl/mL, 0.047 µl/mL, 0.094 µl/mL, 0.188 µl/mL, $p<0.001$). **d** In polydisperse droplets using parallel experiments of cefotaxime-infused droplets with and without microplastic beads, CTX at concentrations of 0.008 µl/mL, 0.01 µl/mL, 0.013 µl/mL, 0.018 µl/mL and 0.032 µl/mL decrease aggregation significantly ($p<0.001$). Similarly to experiments conducted with monodisperse droplets shown on Figure 2b, microplastic beads appear to have an antagonistic effect on the bacterial natural defence mechanisms against antibiotics, resulting in increased sensitivity to antibiotics. **e** Droplets containing *E. coli* and metal solutions demonstrated a significant decrease in aggregation. In experiments conducted with $CuSO_4$, a reduced presence of aggregates was shown in concentrations of 1.9 mM and 2.3 mM with p values of 0.002 and <0.001, respectively. A similar effect was observed with experiments using LiCl and $ZnCl_2$, where aggregation and overall viability is effectively reduced in response to increasing levels of LiCl and $ZnCl_2$ (• - $p<0.001$). Figure created with biorender.com.

Consistent with earlier findings [4][5], antibiotic exposure modulates the aggregation of bacteria depending on the antibiotic type. Experiments conducted with polydisperse droplets demonstrate a significant increase in aggregation levels, with the most pronounced levels observed in the presence of doxycycline, trimethoprim and ciprofloxacin (Figure 2c). The cause of the phenomenon is likely the release of small amounts of extracellular DNA into the bacterial environment, which is well known for its aggregative characteristics [5].

Strikingly, our data is indicating that the presence of microplastics in the bacterial environment could have a negative effect on the bacterial aggregation. A significant reduction in aggregation rates and increased sensitivity to cefotaxime was shown in both mono- and polydisperse droplet experiments (Figure 2b and 2d). The effect may be linked to oxidative stress-inducing properties of microplastics, which could enhance antibiotic effect on bacterial cells [16]. Our preliminary findings highlight the need for more systematic studies on bacterial aggregation dynamics in the presence of microplastics.

Our experiments revealed a significant influence of metals on bacterial aggregation patterns. As shown on Figure 2e, introduction of metals to bacterial environment rapidly decreases the aggregation levels as well as the viability of bacteria. Although $CuSO_4$, LiCl and $ZnCl_2$ salts are known for their innovative biotechnology applications prospects in microbiology, not all is known in terms of physiological response of bacteria to these substances. For these experiments, droplet technologies may prove valuable to further determine the cellular and molecular responses at single-cell resolution.

## Conclusion

Texture based image analysis pipeline was established in a user-friendly and open-source software CellProfiler™.The method is considered a fit tool to unveil bacterial aggregation mechanisms in response to different environmental factors, such as suboptimal antibiotic concentrations and microplastic bead presence. The solution addresses the unmet need for efficient analysis of aggregated bacteria within microfluidic droplets in bulkpaving the way to improve or develop novel antimicrobial strategies.

Providing high-throughput methods for droplet image analysis bridge the gap in bioinformatics expertise among laboratory professionals, enabling the advancement of novel antimicrobial strategies and evaluating their efficacy. User-friendly approach of the method promotes the automation of image analysis in droplet microfluidics, making it nearly an instantaneous process while ensuring reproducible and quantitative results. While the current study design used bacterial aggregates as an object of interest inside droplets, texture characterisation could be applicable in various other experiments, such as cellular (platelets, tumor cell clusters) or protein aggregation experiments. Moreover, the calculation of droplet content texture values in general are easily applicable in other software (e.g. ImageJ/Fiji) or programming languages (e.g MATLAB®, Python). This ensures that the method is accessible and easy to employ regardless of the preferred tool used for image analysis.

A major limitation is seen in the classification capabilities within Cellprofiler™ - the software provides the most basic functionality of a strict threshold to classify data into bins. For more advanced classification demands, users must incorporate a sister-software CellProfiler Analyst incorporating machine learning methods, or alternatively, employ other specialised software or programming languages with the necessary advanced capabilities.

It is recommended that further research be undertaken regarding experimentation with different tools, particularly script-based tools. This may aid in establishing a greater degree of understanding of droplet analysis in microbiology and other fields, perhaps leading to the development of advanced tools dedicated to droplet image analysis. Additionally, it would be beneficial to develop an image analysis pipeline to determine the size and shape of aggregates, which would provide further valuable insight on bacterial aggregation patterns and -behaviour influenced by various contaminants in their environment.

## Methods

### Microbiology

*E. coli* strain JEK 1036, is a wild-type *E. coli* W3310 strain labelled with green fluorescent protein [17]. To prepare *E. coli* for the study, one colony of *E. coli* grown on Luria-Bertani (L-B) agar (BioMaxima, Poland) was dissolved in 5 mL of L-B broth (BioMaxima, Poland) and incubated overnight at 37° C. The culture was diluted with the mixture of fluorophore Alexa Fluor™ 647 (Invitrogen, Thermo Fisher Scientific Inc., USA) dye and L-B broth (BioMaxima, Poland) to reach the target bacterial density value of yielding roughly 50:50 ratio of positive and negative droplets.

### Droplet generation

To generate monodisperse droplets sized 120 nL, a PDMS microfluidic chip was prepared and connected with appropriate sizes of PTFE tubing as described by Bartkova et al. [6]. To generate polydisperse droplets sized between 20 − 200 nL, test tubes containing oil, Alexa Fluor™ 647 and bacterial suspension were vortexed using Vortex-Genie® 2 (Scientific Industries, USA) for 5 seconds. Novec HFE 7500 fluorocarbon oil with 2% concentration of perfluoropolyether (PFPE)−poly(ethylene glycol) (PEG)−PFPE triblock surfactant was used for both mono- and polydisperse droplets.

### Imaging setup

For imaging, Countess™ (Invitrogen, Thermo Fisher Scientific Inc., USA) cell counting chamber slides were prepared with droplets. Approximately 18–20 µL of droplets with oil were pipetted into one chamber of the slide to fill the chamber. Images of droplets were captured using the LSM 900 Laser Scanning Microscope (Zeiss, Germany) equipped with ZEN 3.3 (blue edition) software with following settings: Plan-Apochromat 10x/0.45 objective, diode lasers 488 and 640 nm, DIC light, pinhole size 460 µm. In experiments using monodisperse droplets and polydisperse droplet experiments with solely antibiotics, 8-bit images were acquired. Polydisperse droplet experiments with microplastics and -metals were imaged in 16-bit format. All images were acquired at a resolution of 2048×2048 pixels and exported as TIFF files.

### Software and data analysis

All acquired images were analysed using CellProfiler™ software version 4.2.6 developed in Python environment (Broad Institute, Inc., USA) [18]. All experiments were carried out on MacBook Air (M1, 2020) with a macOS Monterey system version 12.0.1 (Apple Inc., USA). Data analysis was performed using Excel (Microsoft Inc., USA). For statistical testing, $\chi^2$-test was employed with a significance level set at a p-value of $<0.05$.

### Figure assembly

Composite figures were compiled using Biorender (Biorender.com).

### Data availability

All data generated or analysed during this project are included in this published article and its supplementary information files.

### Acknowledgements

This work was supported by Estonian research council grants PRG620 and MOBJD556, and by Tallinn University of Technology grant GFLKSB22.

**Author contributions**

The author's responsibilities were as follows: MSA, KL, DK, IS. were responsible of analytical pipeline development, performance analysis and validation; MSA, DG, TO were involved in data analysis, MSA, SB and OS participated in manuscript drafting, review and editing; FLS, KK, TO, and SB performed droplet generation and imaging, OS and SB were the principal investigators and participated in the design of the study, coordination and funding acquisition. All authors contributed to the interpretation of data and in writing and approval of the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

[text]

**References**

[1] Antimicrobial Resistance Collaborators, " Global burden of bacterial antimicrobial resistance 1990 – 2021 : a systematic analysis with forecasts to 2050," 2021, doi: 10.1016/S0140-6736(24)01867-1.

[2] Stevenson, E. M., Buckling, A., Cole, M., Lindeque, P. K. and Murray, A. K., "Selection for antimicrobial resistance in the plastisphere," *Science of the Total Environment*, vol. 908, no. September 2023, p. 168234, 2024, doi: 10.1016/j.scitotenv.2023.168234.

[3] Sauer, K. *et al.*, "The biofilm life cycle: expanding the conceptual model of biofilm formation," *Nature Reviews Microbiology*. 2022. doi: 10.1038/s41579-022-00767-0.

[4] Tavaddod, S., Dawson, A., and Allen, R. J., "Bacterial aggregation triggered by low-level antibiotic-mediated lysis," *Biofilms and Microbiomes*, vol. 10, no. 90, p. 2023.11.11.566689, 2024, doi: 10.1038/s41522-024-00553-1.

[5] Yu, W., Hallinen, K. M. and Wood, K. B. , "Interplay between antibiotic efficacy and drug-induced lysis underlies enhanced biofilm formation at subinhibitory drug concentrations," *Antimicrobial Agents and Chemotherapy*, vol. 62, no. 1, 2018, doi: 10.1128/AAC.01603-17.

[6] Pal, A., Bhattacharjee, S., Saha, J., Sarkar, M., and Mandal, P., "Bacterial survival strategies and responses under heavy metal stress: a comprehensive overview," *Critical Reviews in Microbiology*. 2022. doi: 10.1080/1040841X.2021.1970512.

[7] Pham, D. N., Clark, L., and Li, M., "Microplastics as hubs enriching antibiotic-resistant bacteria and pathogens in municipal activated sludge," *Journal of Hazardous Materials Letters*, 2021, doi: 10.1016/j.hazl.2021.100014.

[8] Lewnard, J. A. *et al.*, "Burden of bacterial antimicrobial resistance in low-income and middle-income countries avertible by existing interventions: an evidence review and modelling analysis," *The Lancet*, vol. 403, no. 10442, pp. 2439–2454, 2024, doi: 10.1016/S0140-6736(24)00862-6.

[9] Kragh, K. N., Tolker-Nielsen, T., and Lichtenberg, M. , "The non-attached biofilm aggregate," *Communications Biology*, vol. 6, no. 1, pp. 1–13, 2023, doi: 10.1038/s42003-023-05281-4.

[10] Crivello, G. *et al.*, "In Vitro Models of Bacterial Biofilms: Innovative Tools to Improve Understanding and Treatment of Infections," *Nanomaterials*. 2023. doi: 10.3390/nano13050904.

[11] Konieczny, M. *et al.*, "Imaging flow cytometry to study biofilm-associated microbial aggregates," *Molecules*, 2021, doi: 10.3390/molecules26237096.

[12] Ruszczak, A. *et al.,* "Droplet-based methods for tackling antimicrobial resistance," *Current Opinion in Biotechnology*. 2022. doi: 10.1016/j.copbio.2022.102755.

[13] Bartkova, S. *et al.,* "Droplet image analysis with user-friendly freeware CellProfiler," *Analytical Methods*, 2020, doi: 10.1039/d0ay00031k.

[14] Sanka, I. *et al.*, "Investigation of Different Free Image Analysis Software for High-Throughput Droplet Detection," *ACS Omega*, vol. 6, no. 35, pp. 22625–22634, 2021, doi: 10.1021/acsomega.1c02664.

[15] Haralick, R. M., Dinstein, I. and Shanmugam, K. "Textural Features for Image Classification," *IEEE Transactions on Systems, Man and Cybernetics*, vol. SMC-3, no. 6, pp. 610–621, 1973, doi: 10.1109/TSMC.1973.4309314.

[16] Wang, R. *et al.*, "Bacterial Interactions with Nanoplastics and the Environmental Effects They Cause," *Fermentation*. 2023. doi: 10.3390/fermentation9110939.

[17] Keymer, J. E. *et al.*, "Computation of mutual fitness by competing bacteria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 51, pp. 20269–20273, 2008, doi: 10.1073/pnas.0810792105.

[18] Lamprecht, M. R., Sabatini, D. M. and Carpenter, A. E., "CellProfiler™: Free, versatile software for automated biological image analysis," *BioTechniques*, vol. 42, no. 1, pp. 71–75, 2007, doi: 10.2144/000112257.