High-throughput bacterial aggregation analysis in droplets

ABSTRACT

The communal lifestyle of bacteria manifested as bacterial aggregation and potential biofilm formation is posing as a significant contributor to antimicrobial resistance. A key approach to adress the emerging pandemic is to implement novel techniques that enhance the precision and speed of analysis in various microbiological experiments.

Droplet-based platforms coupled with image analysis techniques exhibit great potential in advancing the precision of investigating bacterial behaviour and increasing the speed of microbiological experiments. As most of the image analysis techniques require advanced programming skills or implement high-priced fees, open-source software interweaving user-friendly interface is in great demand.

In this paper, we introduce two image analysis pipelines constructed in CellProfiler™ to quantify bacterial aggregation responding to a presence of microplastics as well as suboptimal concentrations of antibiotics in droplets.

INTRODUCTION

Extensive exposure to antibiotics in tandem with rapidly shifting environmental reservoir compositions have significantly increased the incidence of antimicrobial resistance (AMR). Bacterial aggregation and subsequent biofilm formation, fueled by constant antibiotic exposure and the presence of microplastics as aggregation platforms, are one of the key concerns in addressing antimicrobial resistance [1][2]

The year 2019 marked a record high, with approximately 865 000 infections and almost 39 000 deaths in the EU/EEA countries attributed to AMR bacterial strains [3]. Globally, this number escalates to 4.95 million deaths linked to bacterial AMR outcomes [3]. In the face of the global concern of AMR, increase in healthcare costs and reduced productivity present a major economic burden estimated to cost approximately 1.1 billion euros every year between 2015 and 2050 [4].

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 [5].

Microfluidic droplets, acting as nanoscale bioreactors, exhibit great potential at increasing the speed and precision of microbiological experiments. Coupled with image analysis, the method can provide deep insight in the dynamics of bacterial aggregation and antibiotic resistance. [6]

In this paper a novel approach to analyse images of droplets and their contents via texture characteristics is offered. The applicability of the method is demonstrated by two 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 or microplastic beads. Previous works by our research group have shown the highest accuracy, precision and wide applicability of CellProfiler™ for various droplet experiments [6]-[8].

RESULTS AND DISCUSSION

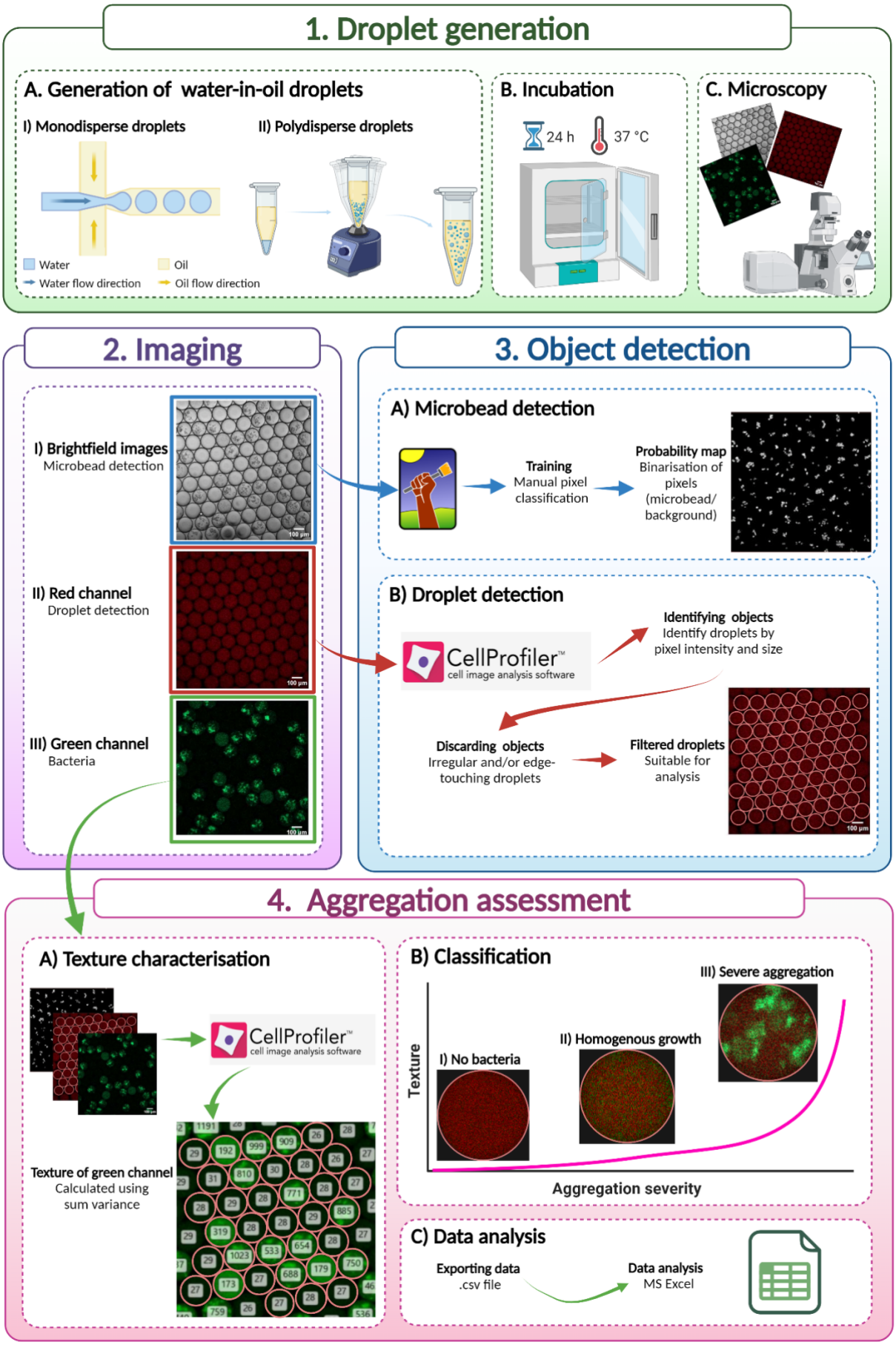
Texture analysis showcases a useful tool to assess bacterial aggregation in droplets

Water-in-oil droplets encapsulating *E. coli* JEK 1036 with chromosomal incorporated GFP were imaged and analysed via an open-source script-free image analysis software CellProfiler™ [9]. The pipeline developed accommodates all key modules with appropriate specifications for successful droplet content analysis and categorisation based on the severity of bacterial aggregation. The principles and protocol for implementing the pipeline are outlined in supplementary file 1.

Combining image texture analysis with detection of microplastic beads via machine learning enables advanced analysis of bacterial aggregation in plastic-saturated environments

The pipeline functionalities were further extended to enable the analysis of microplastic beads alongside bacteria in droplets. For this, the previously developed pipeline in CellProfiler was combined with Ilastik [10], another image classification and segmentation software focused on employing machine learning methods for image analysis. An easy setup of the pipeline for microplastic beads alongside bacteria in droplets is assisted with a supplementary file 2. A general workflow of the pipelines in demonstrated in Figure 1.

Figure 1. Protocol overview of the bacterial aggregation assessment pipeline. 1. a) Droplets of mono- or polydisperse nature can be used to analyse bacterial aggregation. 1. b) Generation of droplets is followed by further incubation to encourage bacterial growth and study of the phenomenon under investigation. 1. c) Droplets are studied via microscopy. 2. Three types of images are acquired - brightfield, red and green channel. 3. a) Microbead detection introduces another software - Ilastik - which uses machine learning principles to provide a probability map of the microbeads depicted on a brightfield image. 3. b) Droplets are detected using the red channel of the images. 4. a) The analysis then evaluates the texture of droplets based on green channel. 4. b) Higher values of sum variance reveal severly aggregated droplets. 4. c) All data gathered during the analysis is exported as a .csv file and could be analysed using any spreadsheet software. Created with BioRender.


1. Droplet generation
A. Generation of water-in-oil droplets
I) Monodisperse droplets
II) Polydisperse droplets
Water
Oil
Water flow direction
Oil flow direction
B. Incubation
24 h
37 °C
C. Microscopy
2. Imaging
I) Brightfield images
Microbead detection
II) Red channel
Droplet detection
III) Green channel
Bacteria
3. Object detection
A) Microbead detection
Training
Manual pixel classification
Probability map
Binarisation of pixels (microbead/ background)
B) Droplet detection
CellProfiler™
cell image analysis software
Identifying objects
Identify droplets by pixel intensity and size
Discarding objects
Irregular and/or edge-touching droplets
Filtered droplets
Suitable for analysis
4. Aggregation assessment
A) Texture characterisation
CellProfiler™
cell image analysis software
Texture of green channel
Calculated using sum variance
B) Classification
I) No bacteria
II) Homogenous growth
III) Severe aggregation
Texture
Aggregation severity
C) Data analysis
Exporting data
.csv file
Data analysis
MS Excel

CONCLUSION

Texture based image analysis pipelines were established in a user-friendly and open-source software CellProfiler™ to analyse aggregated bacteria compartmentalized in microfluidic droplets

The pipelines are considered fit tools to unveil bacterial aggregation mechanisms in response to different environmental factors, such as suboptimal antibiotic concentrations and microplastic bead presence, paving 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 design of the pipelines promote the automation of image analysis in droplet microfluidics, making it nearly an instantaneous process while ensuring reproducible and quantitative results.

More information on bacterial aggregation and further experiments with a similar setup would help to establish a greater degree of accuracy of the pipelines. Although there is room for improvement in the accuracy of the developed pipelines, the approach was confirmed as a quick and effective approach to assess bacterial aggregation in droplets

METHODS

Subparagraph 1: Microbiology

E. coli strain JEK 1036, is a wild-type *E. coli* W3310 strain labelled with green fluorescent protein as described in Keymer *et al.* 2008. To prepare *E. coli* for the study, one colony of *E. coli* grown on Mueller-Hinton agar was dissolved in 5 mL of Luria-Bertani (L-B) broth (BioMaxima, Poland) and incubated overnight at 37° C. The culture was diluted to reach target value of 3–4 · 10^8 CFU/mL and was then further diluted 100-fold with the mixture of fluorophore Alexa Fluor™ 647 (Invitrogen, Thermo Fisher Scientific Inc., USA) dye and L-B broth.

Subparagraph 2: Droplet generation

To generate <u>monodisperse droplets</u> sized 120 µL, a PDMS microfluidic chip was prepared and connected with appropriate sizes of PTFE tubing as described by Bartkova et al. [6]. To generate <u>polydisperse droplets</u> sized between 20 – 200 µL, test tubes containing oil, Alexa dye 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.

Subparagraph 2: 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 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. Imaging of the green and red channel were carried out using excitation wavelengths of 395 and 653 nm with emission wavelengths of 502 and 668 nm, respectively. 8-bit images were acquired at a resolution of 2048×2048 pixels and exported as TIFF files.

Subparagraph 3: Software

All acquired images were analysed using CellProfiler™ software version 4.2.6 developed in Python environment (Broad Institute, Inc., USA). 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).

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