EasyFlow: User-friendly Workflow for Image-based Droplet Analysis with Multipurpose Modules

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Abstract

Droplet-based experimental platforms allow researchers to perform massive parallelization and high-throughput studies, such as single-cell experiments. Even though there are various options of image analysis software to evaluate the experiment, selecting the right tools require experience and is time consuming. Experts and sophisticated workflow are required to perform the analysis, especially to detect the droplets and analyze their content. There is need for user-friendly droplet analysis pipelines that can be adapted in laboratories with minimum learning curve. Here, we provide a user-friendly workflow for image-based droplet analysis. The workflow comprises of a) CellProfiler-based image-analysis pipeline and b) accompanied with web application that simplifies the analysis and visualization of the droplet-based experiment. We construct necessary modules in CellProfiler (CP) to detect droplets and export the results into our web application. Using the web application, we are able to process and provide basic profiles of the droplet experiment (droplet sizes, droplet signals, sizes-signals plot, and strip plot for each label/condition). We also add a specific module for growth heterogeneity studies in bacteria populations that includes single cell viability analysis and probability distribution of minimum inhibition concentration (MIC) values in population. Our pipeline is usable for both poly- and monodisperse droplet emulsions.

Background

[Water-in-oil droplet emulsions have become common tools for high-throughput chemical or biological analysis over the last decades]. In general, droplet emulsions enable massive parallelization with small amount of reagents[1] and miniaturization of laboratory[2]. Droplets with different sizes, usually called as polydisperse droplets, have been used for advanced parallel studies, e.g., for nucleic acid amplification[3,4]. This method allows an absolute quantification in digital droplet polymerase chain reaction (ddPCR) [5,6]. The technology not only eases the complexity of massive experiments but also improves the accuracy of the detection or quantification[7]. However, polydisperse droplet formation is usually avoided due to bias which arise and affect the quantification[8]. Moreover, it requires sophisticated software or experts to perform the droplets detection or the statistical correction for its analysis[3]. On the other hand, microfluidics technology is able to generate monodisperse droplets and

make analysis easier[9]. Since the early 2000s, droplet microfluidics has been developed in wide range of research[10]. Recent studies show that droplet applications allow wide range of microbial studies[11], including massive parallel experiments for microbial community studies[12], and investigating heteroresistance in antimicrobial resistance[13]. Unfortunately, droplet microfluidic requires delicate integrative equipment and setup to perform such experiments[14]. Nevertheless, droplet-based technology requires detection methods to analyze the results. For example, the droplet detection can be performed using image-based methods[15] and signal-based detection[16]. In this article, we focus on the image-based method.

[Imaging or image-based method is often used for the analysis of both mono- and polydisperse droplets]. Imaging is usually performed with microscopes using wide range of techniques[17], from fluorescent microscopy[16] up to high resolution electron microscopy[17]. Some people also use smartphone technology to capture or preserve their object of interest into image data[18] . In principle, the signals from the object of interest will be converted into pixels as the smallest units in image data[19]. From the pixels, image data can be processed to acquire the necessary information[20]. Image data can be used for various type of analysis, e.g. protein expression[21], bacterial counting[22], cell profiling[23], seeds analysis[24], single cell analysis[25], etc. In droplet-based experiment, imaging has been used both in mono- and polydisperse droplets. For instance, image analysis has been used to quantify droplet as a carrier[26], determine a concentration of metals in single droplet size[27], droplet identification in crystallization trials[28], nucleic acid amplification[4], and antibiotic susceptibility test in droplet assays[29]. However, these methods require either scripted programs, or specific pipeline that only work for their object of interest. Some software are only available commercially.

[There is a need for accessible and user-friendly image analysis and data visualization tools that can be used for both mono- and polydisperse droplets]. Some imaging tools come as a package with the microscope equipment and its software, e.g., Zen Imaging[30] or NIS-Elements[31]. Unfortunately, these kinds of bundle are not accessible in every laboratory. In our previous study, we have reviewed some popular freely available open-source software which can be used for the image data analysis[32], e.g., CellProfiler™ (CP) [33], ImageJ[34], Ilastik[35], and QuPath[36]. We also explored each of the software's pipeline to detect droplets and found that each of the software has different name or option but host the same principle[32]. Despite of the mentioned tools, there are some other software which can be used to process image data and perform the analysis afterward, e.g., Python[29,37], C++[38], MATLAB[39], or R[40,41]. Yet, these software require programming skills while not every lab has the experts for implementing the available library or scripted code. In our recent studies, we used CP and CellProfiler Analyst (CPA) to detect and analyze monodisperse droplets [29,30]. Nonetheless, there are some limitations to process the data, e.g., to implement a specific-case formula or calculation. This requires other software for data management or visualization, such as Excel[42], Libre office[43], Tableu[44], and Looker[45]. Notwithstanding, it takes a lot of computing power to process high-throughput data and they are mostly not freely available. Moreover, each of the software require some experience to be used optimally.

[Herein, we provide user-friendly droplet analysis workflow that is universally usable for detecting droplets and visualizing the results]. The workflow consists of two parts which can work independently (Fig. 1). Each part is modular and can be substituted with other software to generate suitable output. In

brief, the first part is for image-based droplet analysis or detection. We use CP to detect droplets and generate the data. For the second part, we have built a web application consists of necessary Python libraries for processing the data and visualize the results in figures. The web app also hosts modules which can be used for multipurpose analysis. This workflow will lower the boundary for individuals with no programming skill to analyze and visualize their data.

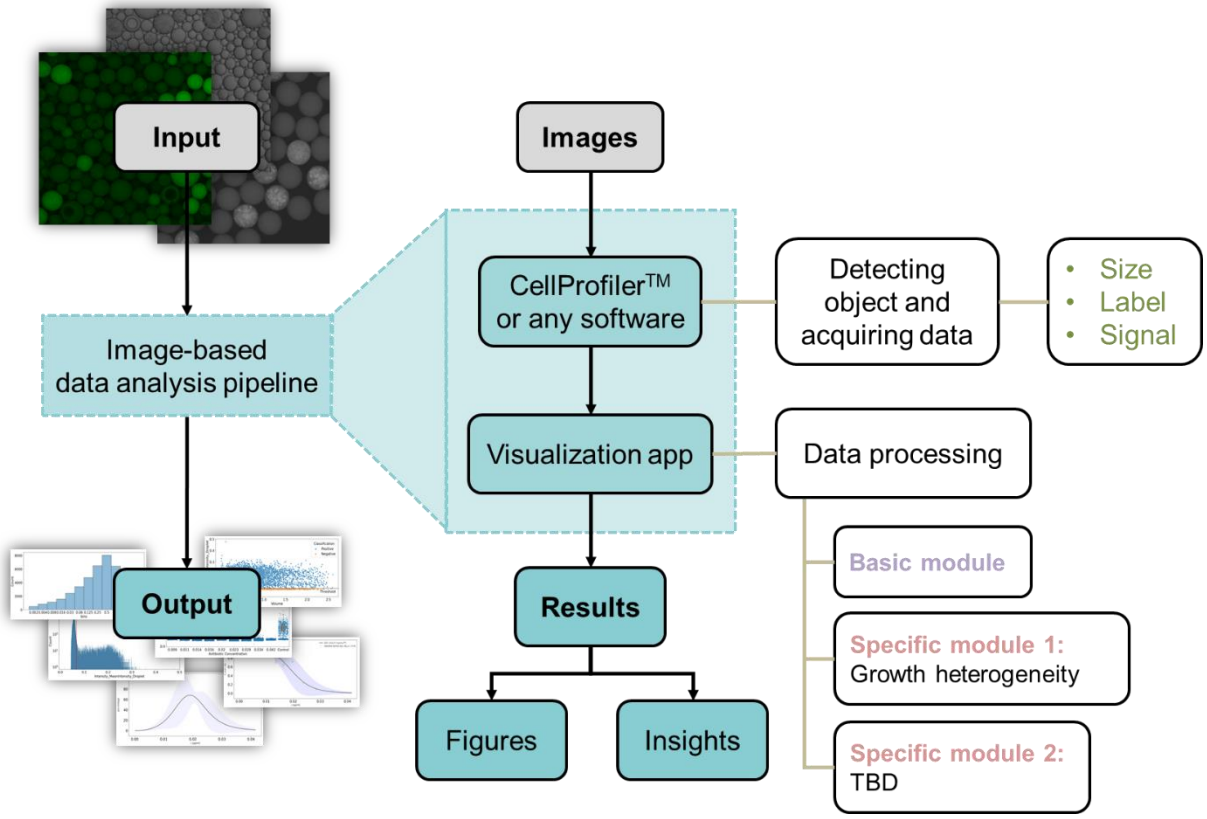


Figure 1. Pipeline structure of our image-based droplet detection and web application for the analysis. Data is obtained from images which processed in CP or any other software. There are three data which now our web app can process, Size, Label, and Average Pixels' Intensity. Since our web app is independent, the source data is not limited from image-based data but is required for having three group of data mentioned previously. In the web application, there are basic module which can be used to visualize general information from the uploaded data. For the specific module, we tested our previous experiment data to analyze bacterial growth in polydisperse droplets and its heteroresistance profile.

Result and Discussions

[Adaptive Otsu thresholding and eccentricity filter improve the polydisperse droplets detection pipeline]

We generate polydisperse droplets by vortexing oil, surfactant, and bacteria in Luria-Bertani broth together in a tube. The encapsulation method is adopted from Byrnes et al.[4] For obtaining the data, we image the droplets under confocal microscope and detect the droplets in CellProfiler™ (CP) using the pipeline which is illustrated in Fig. 2. We perform optimization due to complications, such as different sizes and overlayed droplets in polydisperse droplets image data. These situations result in additional variables which need to be added in the pipeline, such as the typical droplets' shape. In this experiment,

we use CP to detect droplets with available modules and generate the data which consists of labels, sizes (volume is recommended), and average pixel intensities. This can be generated with any available software as long as it generates mentioned data with comma separated value (.csv) or Microsoft Excel (.xlsx) file type. We have demonstrated the first part in our previous article [46] and add additional necessary modules to analyze polydisperse droplets in this article.

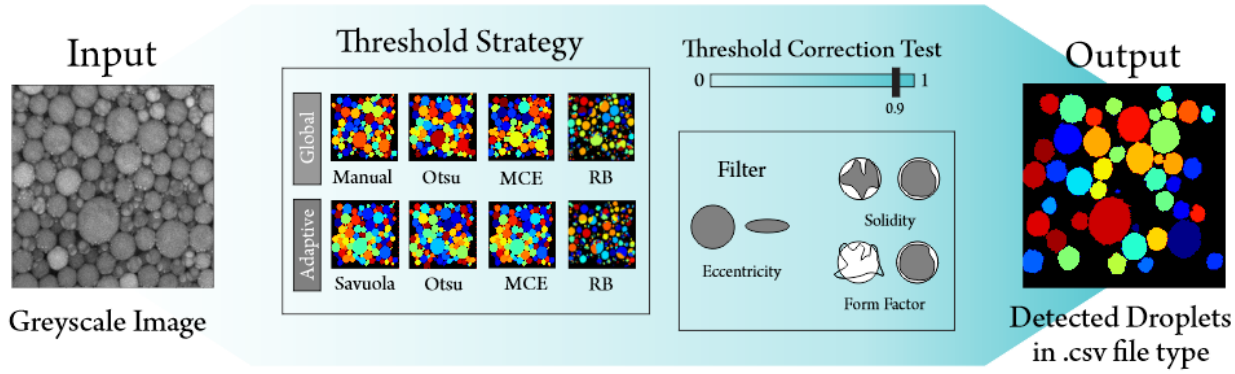


Figure 2. CellProfiler™ provides modules for constructing image-based droplet detection pipeline. We use fluorescent image from microscopy as an input. We test the available thresholding strategy, e.g., Global/Adaptive with different algorithm, e.g., Manual, Otsu, Savuola, Robust Background (RB), Minimum Cross Entropy (MCE). We also find the optimal threshold correction at 0.9 within the 0-1 scale. For improving the droplet detection, we filter the object using eccentricity, solidity, and form factor.

Pipeline for image analysis is composed of modular components, in a puzzle-like fashion[47,48]. In CP, we construct the modular components to detect droplets. We also test different thresholding strategies, including between Global (Manual, Otsu, Minimum Cross-Entropy, and Robust Background) and Adaptive (Savuola, Otsu, Minimum Cross-Entropy, and Robust background). The difference between two thresholding strategies are that Global will consider all pixels when implementing the settings (including threshold, size range, etc.), while in Adaptive, the setting corresponds to the spatial variations in illuminations within a specific size of window[49]. We also test different threshold correction in each of the thresholding strategies and found that 0.9 works best in our detection (Suppl. Table 1). Since the detection including the irregular typical shape of droplets, we implement filter module to find the similar shape of droplets with measurable parameter. This includes eccentricity (conic section), solidity (overall concavity), and form factor (ratio between the object's area and circumscribed circle)[50,51]. We put the specific range between 0-0.5 or 0.5-1 for each of the filter to help us finding the best setting for droplets detection (Supp. Table 1). Based on the optimization, the best setting for our droplet detection is using Adaptive Otsu with 0.9 threshold correction with 0-0.5 eccentricity filter. Using the setting, we detect the droplets and obtain the result in .csv format.

[We have built the web application with multipurpose modules for visualization of basic droplet data and specific experimental case]. In the web application, there are 1) basic and 2) specific modules (Fig. 3). At the moment, the data should contain three variables for maximizing the feature in our web application. This web application consists of necessary Python libraries that help users to visualize their data. In the web application, three variables (labels, sizes and signals) can be processed with a basic

visualization profile (e.g., plot of size distribution, fluorescent signals, comparison between sizes and the signals, and condition/label-based variable) and specific experiment profile in growth heterogeneity (e.g. Gompertz fitting, single cell viability, and minimum inhibition concentration probability density). Furthermore, each of the profile or visualization is also independent for each other and ease user to select the necessary profile.

This application will lower the boundary for individuals with no programming skill to analyze and visualize their data. The web application will automatically treat the data as the best fit, e.g., size calculation will be performed automatically with maximum and minimum Ferret Diameter. The basic module can visualize the droplet sizes, droplet signals, sizes-signals plot, and plot data based on conditions or labels. In specific module, we currently have heteroresistance module which can generate the Gompertz fitting, single cell viability and minimum inhibition concentration (MIC) probability density. These modules can be run once the .csv or .xlsx data is uploaded into the application. To demonstrate the functionality, we use the platform to visualize our recent experiment in bacteria heteroresistance. We conduct an experiment with 10 different label or condition (nine different antibiotic concentrations with one control). We test some thresholds ranging from 0.067-0.076 to generate average classification value between two types of droplets in the population (Supp. Table 2). We use this value to generate the results in growth heterogeneity module. We also need to include the antibiotic concentration's range in the web application to run the growth heterogeneity module.

Heteroresistance is a type of antibiotic resistance in isogenic bacteria within sub-population level[52,53]. Wrong treatment toward this phenomenon results a treatment failure and death[54]. In 2019, Centers for Disease Control and Prevention (CDC) reported that more than 2,8 million individuals have been infected by antibiotic resistant bacteria and more than 35.000 deaths are caused by it each year[55]. The heteroresistance can be observed through disc diffusion, E-test methods and population analysis profile[56]. However, these methods are tedious and not feasible for clinical use because of complexity and long duration[54]. Droplet-based experiments can accommodate such analysis with massive parallelization and high-throughput results[57]. Using polydisperse droplets, we perform an experiment with nine different antibiotic concentrations and produce 1451 images for the analysis. We perform the droplet detection using the modules in the CP and upload the result (in .csv) in the web application.

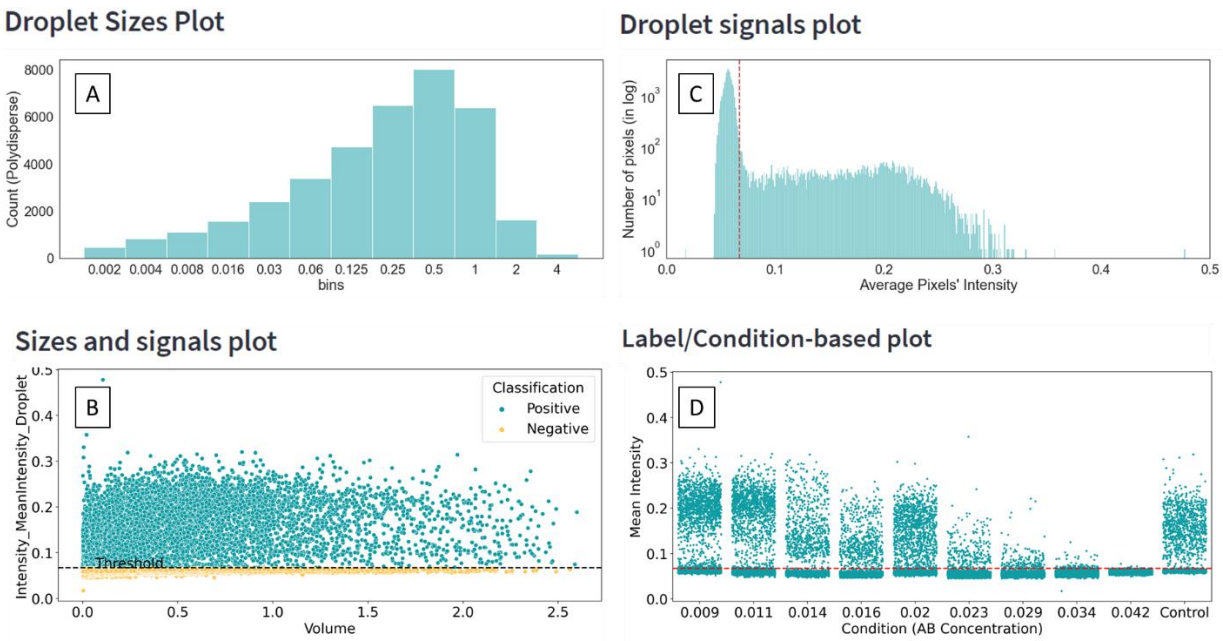


Figure 3. Basic module in the web application depicts (A) droplet sizes distribution, (B) whole droplet signals, (C) fluorescent distribution in different sizes, and (D) individual experiment profile. Each of the plot can be generated independently.

[Growth heterogeneity module analyzes microbial growth at single cell level in response to antimicrobial exposure conditions]. The growth heterogeneity module provides single cell viability graph (Fig. 4A) which utilize Gompertz fitting. Usually, this fitting is used to explain the nature of the phenomenon, specifically the correlation between time series or particular variable in sigmoid function[58,59]. We also provide the minimum inhibition concentration (MIC) probability density plot (Fig. 4B) to show the probability distribution of bacteria proliferation at a given antibiotic concentration.

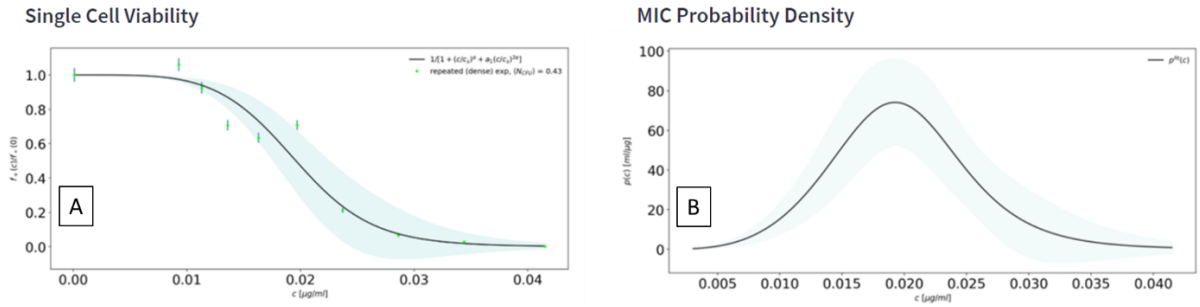


Figure 4. Heteroresistance module gives the option to produce fitting using (A) Gompertz model where it shows the decremental in sigmoid curve towards high antibiotic concentration. We also provide the partial derivative of the fitting and add error propagation calculation to find the best fit for single cell viability. For finding the MIC probability density, we use the negative numerical derivative from the fitting parameter from (B) with negative derivative of the fitting to define the error. The calculation is adopted from Scheler et al.[57]

With our web application, we can generate all the necessary analysis results after uploading the output from image analysis software. When building this module, we also found that the numerical derivative of the single cell viability[57] not always can be useful to determine MIC probability density. It happens when the measured cell viability has a non-monotonic dependence on antibiotic concentration. This correlates with the threshold that is used to generate the graph. Since we use ten thresholds to estimate the error, the variance can be observed from the averaging. We do not discuss this error here in depth as we focus on the presentation of the web application. Alternatively, we provide the Gompertz fitting function to show how it fits without considering the non-monotonical results in the growth heterogeneity module. We also have tested the software using our previous published data with monodisperse droplets in Bartkova et al.[46] (Supp. Fig. 4). For now, the web application can host three variables that contain the necessary data from CP or other software. We envisage to provide a more general visualization setup with other specific modules in near future.

Conclusion

We have implemented a user-friendly pipeline for image-based droplet analysis with two independent parts: image analysis software and our web application for visualizing the results. We simplify the data visualization by removing the demand of programming skills to run it. This will accommodate non-experts or researchers with no programming skill to perform droplet-based research and use image data for the analysis. For the image analysis part, we discuss the detailed settings to optimize the droplet detection using CellProfiler. The visualization web application is available online, and it is not limited to the user's computer power. Our app can be upgraded with new specific modules depending on the experimental data of the user. The pipeline can potentially be used not only for droplet-based analysis, but for any object analysis research in biotechnology and bioengineering or similar fields where data visualization application is needed.

Materials and Methods

Web application program construction

Basis script of our web application is written in Python[60] under libraries dependency, including Matplotlib[61] and Seaborn[62] for plotting and visualization, Numpy[63] for working with arrays, Pandas[64] to play with dataframe, Math[65] and Statistics[66] for embedding the formula, Re[67] for regular expression, and SessionState[68] to link each of the session within the program. The application is streamed using Streamlit library[69] and the Python script is adjusted accordingly. For hosting the application online, we use Heroku server (https://easy-flow.herokuapp.com/) and will be hosted in local server in Tallinn University of Technology. The full script will be provided and will be available in our GitHub on request.

Polydisperse droplet generation and imaging

Droplets generation is performed using the vortexing method from Byrnes et al.[3] Surfactant (perfluoropolyether (PFPE)–poly(ethylene glycol) (PEG)–PFPE triblock surfactant), Novec HFE 7500 fluorocarbon oil, bacteria (*Escherichia coli* JEK 1036 with a chromosome- incorporated gene encoding the green fluorescence protein (GFP) and medium (Luria broth mixed with Dextran, Alexa FluorTM 647) are vortexed at the 1,5 mL tube for 5s. Once droplets are formed, we put the droplets on the Countess™ slide (Invitrogen) for imaging. We use LSM 510 Laser Scanning Microscope (Zeiss, Germany) running on

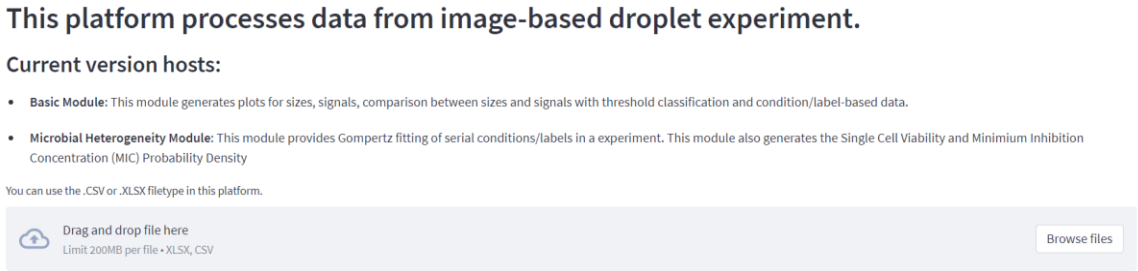
Zen 2009 software with the following settings: Plan-Apochromat 10X/0.45 objective, Argon/2 and HeNe633 lasers, Transmission light (Bright Field), pinhole size 452. We obtain 1451 images and convert them into grayscale TIF format before processing the data.

CellProfiler pipeline construction

We process the grayscale images in CellProfiler™ (version 4.2.1) using our constructed pipeline. The pipeline consists of IdentifyPrimaryObjects, MeasureObjectSizeShape, FilterObjects, MeasureObjectIntensity, another MeasureObjectIntensity, and ExportToSpreadsheet. For IdentifyPrimaryObjects, we set the range of diameter is between 20- and 350-pixel units. We test the Global and Adaptive thresholding strategies with different thresholding algorithm (Manual, Otsu, Savuola, Minimum Cross-Entropy, and Robust Background). For best detection pipeline, we use Adaptive thresholding strategy with three classes Otsu algorithm and 350 size of adaptive window with 0.9 threshold correction factor. The rest of the setting follows the default. The first ExportToSpreadsheet module provide measurement for the detected droplets. The FilterObjects module allow us to select droplets which have specific shape as spherical or spheroid. We use the range of 0-0.5 eccentricity for filtering the droplets. We put MeasureObjectIntensity and second MeasureObjectIntensity modules to obtain the mean pixel's intensity and droplets' size for the analysis. For making the output can be processed in our web application, we export the data as .csv using ExportToSpreadsheet. For a brief explanation of the module construction, we have described it as well in our previous work[46].

Web application visualization workflow

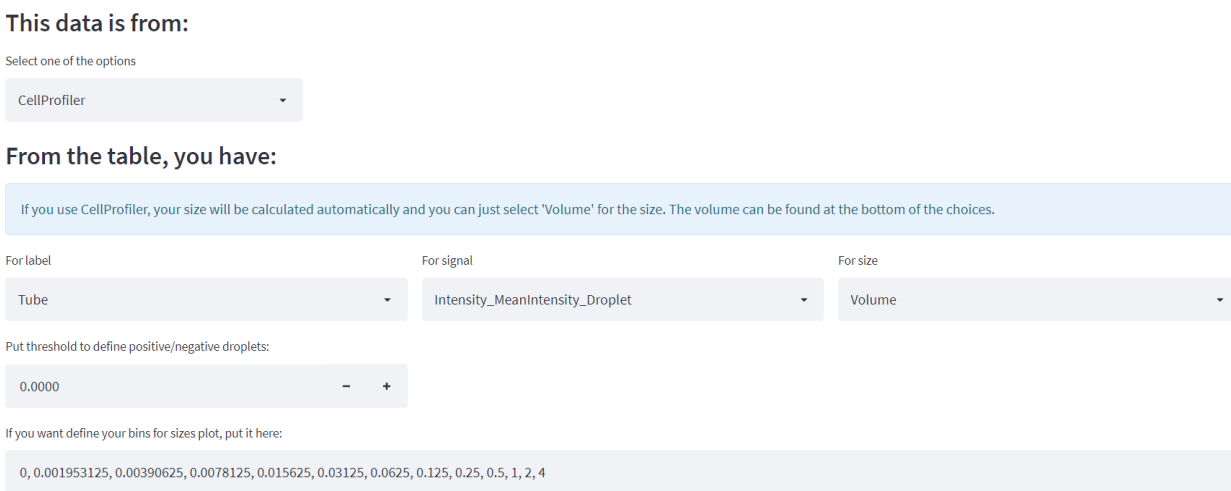
1. Our web app works after uploading the .csv or .xlxs in the app by dragging the file or browsing the file on the available box.



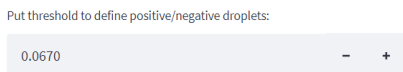
2. Once the file is uploaded, there will be a table for showing the uploaded files.

	Tube	AreaShape_MaxFeretDiameter	AreaShape_MinFeretDiameter	Intensity_MeanIntensity_Droplet
0	Tube01	123.4382	110.9539	0.0673
1	Tube01	100.6578	85.4427	0.0629
2	Tube01	98.5089	86.1700	0.0697
3	Tube01	122.5765	109.5837	0.0615
4	Tube01	165.1303	142.4778	0.2224

3. Depending on the uploaded data, here, we provide an example using CellProfiler as our image-based detection first part of the workflow. Therefore, the interface will show the tabs below.

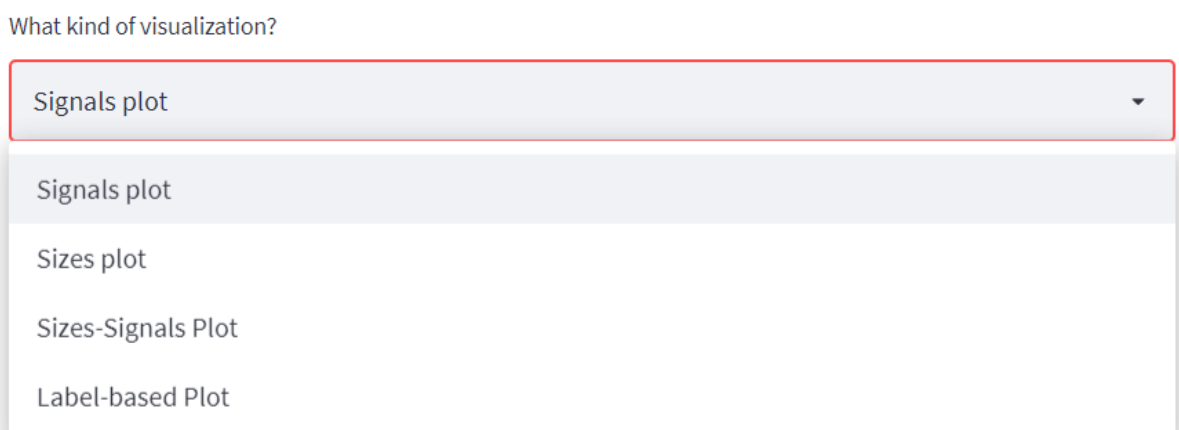


4. For droplet's classification, we use threshold to define both positive/negative droplets. We put 0.067 for the thresholding.



5. Once the tabs are filled, there will be Data Visualization tab with the available modules and visualization options.

6. For basic module, there are four options that are available.



7. For Growth heterogeneity, we put two options, Gompertz fitting and Single Cell Viability + MIC Probability Density. The MIC Probability Density plot requires scViability to run, therefore, we put both scViability and MIC Probability Density in one option to avoid the confusion.

What kind of visualization?

Gompertz fitting

Gompertz fitting

Single cell viability and MIC probability density

8. To make the Growth heterogeneity to run, antibiotic concentrations are needed. We provide example in the important note tab.

Requirement for Microbial Heterogeneity Module

Since the range of antibiotic concentration is needed, gives the antibiotic concentration range with comma here:

IMPORTANT NOTE: Write the antibiotic concentration separated with comma and put 0.0001 as a control. Put the list corresponds to the 'Labels' which has listed under your data above. Example: 0.0093,0.0113,0.0136,0.0163,0.0197,0.0237,0.0286,0.0344,0.0415, 0.0001

If there is no range of antibiotics in the required column, the results will not be generated.

9. We also provide the raw data which used to perform the Growth heterogeneity module.

See the raw data?

	label	Average Volume (nL)	Negative	Positive	Total	Fraction Positive	Occupancy	Viability
0	Tube01	0.4144	2160	1383	3543	0.3903	0.3232	1.1093
1	Tube02	0.3826	2533	1267	3800	0.3334	0.2835	0.9475
2	Tube03	0.3040	2034	673	2707	0.2486	0.2201	0.7065
3	Tube04	0.3254	1895	552	2447	0.2256	0.2019	0.6411
4	Tube05	0.3272	3123	1062	4185	0.2538	0.2241	0.7211
5	Tube06	0.2776	2891	253	3144	0.0805	0.0773	0.2287
6	Tube07	0.1802	5898	173	6071	0.0285	0.0281	0.0810
7	Tube08	0.2481	4598	99	4697	0.0211	0.0209	0.0599
8	Tube09	0.3096	4172	18	4190	0.0043	0.0043	0.0122
9	Control	0.5243	1630	885	2515	0.3519	0.2966	1.0000

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