Comparison of standard and droplet-based methods for assessing antibiotic susceptibility patterns in bacteria

Bachelor thesis

Student: Katri Kiir

Supervisor: Simona Bartkova, PhD

Department of Chemistry and Biotechnology

Co-supervisor: Ott Scheler, Professor

Department of Chemistry and Biotechnology

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Standard ja tilkpõhiste meetodite võrdlus bakterite antibiootikumitundlikkuse mustrite hindamiseks

Bakalaureusetöö

Üliõpilane: Katri Kiir

Juhendaja: Simona Bartkova, Järeldoktor-teadur

Keemia ja biotehnoloogia instituut

Kaasjuhendaja: Ott Scheler, Professor

Keemia ja biotehnoloogia instituut

Õppekava: LAAB17/17

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously presented for grading.

Author: Katri Kiir

(digitally signed)

The paper conforms to requirements in force.

Supervisor: Simona Bartkova

(digitally signed)

Permitted to the defence.

Chairman of the Defence Committee:

[Signature, date]

Abbreviations

Abs	-	Absorbance
CFU	-	Colony-forming unit
csv file	-	Comma-separated values file
CV	-	Coefficient of variation
DNA	-	Deoxyribonucleic acid
E.coli	-	*Escherichia coli*
LB	-	Luria-Bertani broth
MIC	-	Minimum inhibitory concentration
OD	-	Optical density
PDMS	-	Polydimethylsiloxane
scMIC	-	Single-cell minimum inhibitory concentration
TIFF	-	Tagged Images File Format
vs	-	versus

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Introduction

Droplets are like nanoscale test-tubes. They are generated by mixing two immiscible fluids, for example water and oil. They can be used to encapsulate substances or organisms and perform different experiments, such as the investigation of single-cell bacterial response to antibiotics.

Antibiotics are used to inhibit or block the growth of bacteria and can have different inhibitory mechanisms. Bacteria can have a heterogeneous response to antibiotics. Even genetically identical bacteria can individually respond differently. The majority can be susceptible cells, the minority can consist of antibiotic resistant subpopulations.

The standard method gives results only population-wide not analysing cells individually. That does not give an overview of possible resistant subpopulations. The single-cell method allows to see trends of the minority. It gives more detailed information because each individual cell is analysed separately.

The main aim of the thesis is to compare standard and droplet-based methods for assessing antibiotic susceptibility patterns in *Escherichia coli*.

The objectives of the thesis are (i) to find the minimal inhibitory concentration (MIC) of each tested antibiotic using the standard 96-well plate method, (ii) to use polydispersed droplets technology to find single-cell minimal inhibitory concentration (scMIC) for each tested antibiotic and (iii) comparing the MIC and scMIC of each tested antibiotic.

The literature chapter of the thesis gives an overview of (i) MIC, (ii) how MIC and scMIC methods are performed, and (iii) the antibiotics used in this thesis, including their specific inhibitory mechanisms.

The methods and materials chapter describes the steps included in the standard 96-well plate MIC and droplet-based scMIC methods respectively. This also includes imaging of droplets with Zeiss LSM 900 confocal microscope and analysis of droplets with CellProfiler™ and EasyFlow.

The last part of the thesis is a summary of the results and comparison of MIC and scMIC for each antibiotic and a discussion about the significance of these results.

1. Literature overview

1.1 Droplet microfluidics

Droplet microfluidics enables the capability to perform laboratory experiments on a small scale. Using this technology requires a smaller amount of reagents than standard laboratory methods, thus reducing costs and also protecting the environment from having an unnecessary amount of waste [1].
Droplet-based technology has helped making advancement in many research fields, for example single-cell analysis tools, small-scale cell cultures, in-droplet chemical synthesis, high-throughput drug screening and nanodevice fabrication [1].
In this thesis droplet-based technology is used as „micro-reactors“, where antibiotic and bacteria are encapsulated to see the response of bacteria to the antibiotic. In order to generate droplets, one has to have two immiscible phases: the continuous phase (medium in which droplets are formated) and the dispersed phase (droplets themselves). The generation method can be divided into active and passive. For the active method, there are usually electric, magnetic or centrifugal forces to direct cells/particles to the droplet-producing region and actively create droplets. The passive method is described under monodispersed droplets paragraph [2].

1.1.1 Monodispersed droplets

Monodispersed droplets are uniform in size and thus all have the same volume. The three most common microfluidic chip geometries used for generating monodisperse droplets are cross-flowing, flow focusing and co-flowing (**Figure 1**). For passive monodisperse generation some sort of external pressure source (for example syringe and pressure-driven pumps) is used and cells/particles arrive at the droplet formation region randomly [2]. The random arrival is based on Poisson distribution. It is a probability distribution, which expresses the count of independent events that are likely to occur over a fixed period of time and can be used to estimate the likelihood of these events [3].

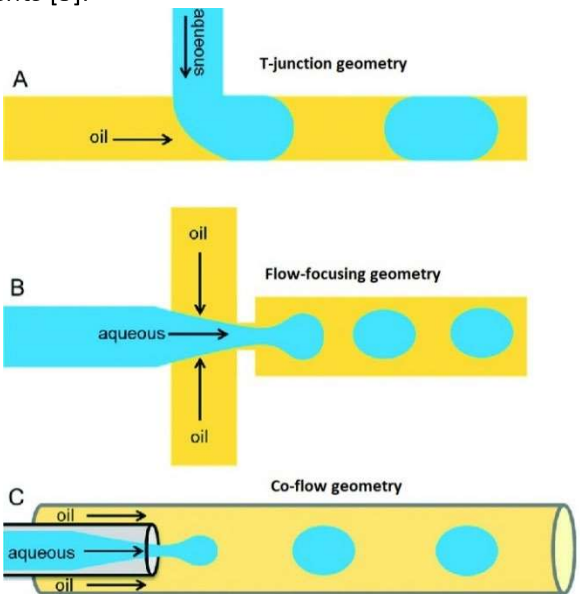

Figure 1. *Different droplet generation geometries: A) T-junction geometry; B) Flow-focusing geometry; C) Co-flow geometry. Two immiscible fluids are used: water (blue) and oil (yellow). Water is directed through oil flow and water-in-oil droplets are made. Adapted from* [4]

In the laboratory, microfluidic chips are used to mix two immiscible fluids through microchannels. The fluids for example can be broth with bacteria and oil/surfactant mixture. Surfactant is used, because it lowers surface tension and makes the droplets stable over a longer period of time[5]. To delivery all the fluids to microchannels, for example used pressure-driven pumps or syringe pumps connected to the chip with microtubes.
Chips are commonly made of PDMS from glass chip mold. Glass chips are the best ones that can be used in making monodispersed droplets. It have the temperature and pressure-resistance, chemical resistance (easy to clean), highly transparent, biocompatible and excellent quality control. However, they need to be thrown away after every experiment, which can become expensive. In the laboratory, a much cheaper way is to make a mold out of a glass chip and fill it with PDMS and make as many chips as needed for the experiments. PDMS chips are inexpensive, have high elasticity, easy to make prototypes, gas permeability and non-toxicity [6] [7].

1.1.2 Polydispersed droplets

Polydispersed droplets vary in volume. To form droplets two phases need to be mixed together as in monodispersed droplets but it can be done withput use of a microfluidic chip. To form droplets the same volume of oil/surfactant and sample are added to a tube and vortexed. The tube can also be just shaken with a hand, but vortexing is a more robust method, which is the reason for using it during experiments in this thesis. This method is much quicker than monodispersed one and can be done in any laboratory and does not require any special equipment like, for example pressure-driven pumps [8].

1.1.3 Comparison of making mono- and polydispersed droplets

Both presented methods have advantages and disadvantages (**Table 1**). Polydispersed droplets are a good option if there is a need to have many different samples to generate. For example if many different concentrations of each antibiotic were tested like in this thesis. For example 20 samples would have taken around 4 hours to make using the chip, while using the polydispersed method it took around two minutes. Making of polydispersed droplets does not require any special equipment or devices. It is a good choice for those, who have no knowledge of microfluidics [8].

Table 1. *Comparison of mono- and polydispersed droplets*

Monodispersed droplets	**Polydispersed droplets**
Volume control	Volume control not possible
Requires knowledge and much practice for beginners	An easy method to learn and perform for beginners
Slow generation, one sample takes minutes and prior equipment set-up	Fast generation, one sample takes seconds
Special equipment is required	No special equipment is needed
The software can detect droplets using a simple pipeline	Harder for software to detect droplets correctly. It usually requires a more complicated pipeline.

1.2 Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) is the lowest antibiotic concentration of an antimicrobial drug that will inhibit the visible growth of a bacteria after overnight incubation. MICs can be determined on plates of solid growth medium (agar) or broth dilution methods (in liquid growth media) after a culture is isolated [9].

A MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. Because a lower MIC value indicates that less of the drug is required to inhibit the growth of the organism, drugs with lower MIC scores are more effective. By identifying appropriate drugs and their effective concentrations, MIC scores aid in improving outcomes for patients and preventing the evolution of drug-resistant bacterial strains [9].

Single-cell minimal inhibitory concentration (scMIC) is also the lowest antibiotic concentration of an antimicrobial drug. However, single-cell inhibition of bacteria can not be seen without the use of a microscope.

It is important not to only analyse antibiotics and their resistance using the standard MIC experiments, but also the single-cell MIC tests, because commonly antibiotic resistance is based on and analysed using the standard method, which gives an overview of heteroresistance. It is a phenomenon in which a preexisting subpopulation of resistant cells can rapidly replicate in the presence of a given antibiotic, whereas the majority population of susceptible cells is killed [10].

Unfortunately, knowledge about heteroresistance is limited and even the definition is commonly used wrongly. It includes not only population-wide variation in antibiotic resistance but also observation and methods used to determine resistance, which vary depending on the laboratory.

Heteroresistance has subpopulations with different levels of resistance, but only one term is used to describe it. Antibiotic prescriptions that are not considering highly resistant subpopulations can possibly lead to the development of resistance. As result, it can lead to the elimination of sensitive cells and replace them with more resistant ones [11].

That is thought to be one of the reasons why the number of pathogenic bacteria that have resistance to one or multiple antibiotics has increased and are not responding to traditional treatments and/or to even last-resource antibiotics. Linking the mechanics of bacterial resistance with drug approaches help to get a better overview of antibiotic resistance. To avoid the development of resistance novel strategies for antibiotic treatment are needed [12].

One way to get a piece of information about susceptible cells individually is to perform scMIC. The single-cell method allows to get more detailed information about possible resistant subpopulations [10].

1.3 Antibiotics and their inhibitory mechanisms

It is important to have antibiotics with different inhibitory mechanisms because each type works only against specific bacteria or parasites. That is the reason why certain antibiotics are used to treat different types of infections [13].

In this thesis antibiotics with the following inhibitory mechanisms were chosen (**Figure 2**):

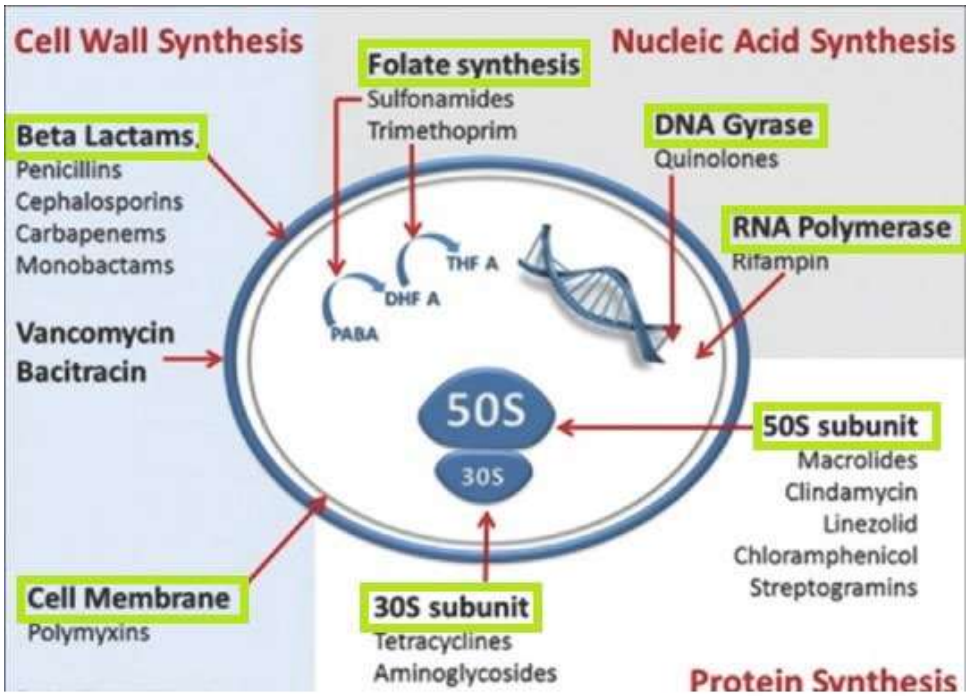

Figure 2. *Inhibitory mechanisms of action for different antibiotics. Three main inhibited bacteria cell syntheses: nucleic acid synthesis, protein synthesis and cell wall synthesis. Inhibition of nucleic acid synthesis consists of inhibiting folate synthesis (blocks dihydrofolate reductase and prevents the synthesis of DNA), DNA gyrase (prevents the DNA from supercoiling and replicating) or RNA polymerase (blocks RNA synthesis). Inhibition of protein synthesis consists of inhibiting the 50S subunit (inhibit peptidyl transferase and prevents peptide bond formation) or 30S subunit (suppresses translation and binding of tRNA). Inhibition of cell wall synthesis consists of inhibiting cell membrane (increases membrane permeability and leads to protein mistranslation) or beta-lactams (inhibits cell wall biosynthesis and blocks penicillin-binding proteins). Adapted from* [14].

In this thesis, the following antibiotics were chosen based on the above inhibitory mechanisms:

1) Trimethoprim antibiotic
   Trimethoprim is an anti-folate factor. It inhibits dihydrofolate reductase, the enzyme that catalyzes the formation of tetrahydrofolic acid from dihydrofolic acid. This prevents the synthesis of bacterial DNA and continued bacterial survival [15].
2) Fluoroquinolone antibiotic - Ciprofloxacin
   Ciprofloxacin inhibits bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. It prevents it from supercoiling the DNA and as a result, prevents DNA replication [16].
3) Rifampicin antibiotic
   Rifampin inhibits DNA-dependent RNA polymerase activity. It leads to the suppression of RNA synthesis and cell death [17].
4) Tetracycline antibiotic - Doxycycline
   Doxycycline inhibit protein synthesis. It suppresses translation by binding to the 16S rRNA of the 30S subunit, preventing the binding of tRNA, which is needed for the delivery of amino acids. As a result protein synthesis is blocked, which prevents the growth of bacteria [18].
5) Chloramphenicol antibiotic
   Chloramphenicol inhibits protein synthesis. It binds to the 50S subunit and blocks peptidyl transferase. The transfer of amino acids to growing peptide chains is blocked, which prevents peptide bond formation and protein synthesis [19].
6) Gentamicin antibiotic

Gentamicin binds to the cell membrane. It increases membrane permeability, which allows to enter the cytoplasm and get access to the 16S rRNA. This leads to the mistranslation of proteins. Inhibition is bothering translation, initiation, elongation, and ribosome recycling [20].

7) Beta-lactam antibiotic (third-generation cephalosporin) - Cefotaxime
   Cefotaxime is inhibiting cell wall biosynthesis through affinity for penicillin-binding proteins [21].

2. Aims of the study

The main aim of the thesis was to compare standard and droplet-based methods for assessing antibiotic susceptibility patterns in *E.coli*. During the process were analysed seven antibiotics with different inhibitory mechanisms.

Specific objectives of the study were:

1) To find the MIC of each antibiotic using the standard 96-well plate method
2) To find the scMIC of each antibiotic using the droplet-based technology method
3) Comparison of MIC and scMIC

This is part of the long-term research going on in the microfluidics group where different droplet-based technologies are used to investigate antibiotic susceptibility and the emergence of drug-resistance in bacteria.

3. Materials and methods

3.1 Standard 96-well plate method

The minimal inhibitory concentration of *E.coli* was tested for six antibiotics (trimethoprim, chloramphenicol, ciprofloxacin, rifampicin, doxycycline, gentamicin), chosen by their different inhibiting mechanism of action. For the antibiotic (cefotaxime) results were given by the supervisor, because it was already previously tested.

Materials:

- 1,5 mL (2 mL and 5 mL) test-tubes
- 50 mL Falcon tube
- Luria-Bertani (LB) broth (Biomaxima, Poland)
- Test-tube shakers Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
- Tested antibiotics (trimethoprim, chloramphenicol, ciprofloxacin, rifampicin, doxycycline, gentamicin- all stocks 10 mg/mL)
- Orbital shaker incubator ES-20 (Biosan, Latvia)
- Spectrophotometer (Shimadzu BioSpec-mini, DNA/RNA/Protein analyser)
- Cuvettes
- Well plate test analyser (GENios Pro, Tecan, Microplate reader)
- 96-well plates
- Pipettes

3.1.1 Preparations for the experiment

The approximate concentration range for each antibiotic was based upon the existing literature. The 96-well plate allows having 22 parallels of different concentrations of an antibiotic, as well as one positive control and one negative control.
Dilution steps are calculated based on antibiotic stock concentration and desired concentration range.

3.1.2 Performed experiment

Day 1:
One *E.coli* colony was picked from an agar plate and dissolved in 5 mL LB using 50 mL Falcone tube. Bacteria was incubated overnight at 37°C.
Day 2:

1. Overnight culture was diluted in 50 mL Falcon tube (1:100).
2. Bacteria grew to around 3-4 * 10^8 CFU/mL in density or higher.
   Around two hours after dilution, bacteria has reached the exponential phase, so when the suspension was added to the plate bacteria still had the ability to divide, which should be stopped with an antibiotic.
3. While bacteria grew, the 96-well plate was prepared and filled with all antibiotic concentrations, negative and positive control.
   24 rows: 22 rows for antibiotics+ one positive control + one negative control.
   One row has four parallel wells (analysing the average Optical Density).
   In each well: 100 µl LB with antibiotic and 100 µl of the bacterial suspension.
   Negative control: 200 µl of LB in each well.
   Positive control: 100 µl of LB (without antibiotics) and 100 µl of the bacterial suspension in each well (**Figure 3**).

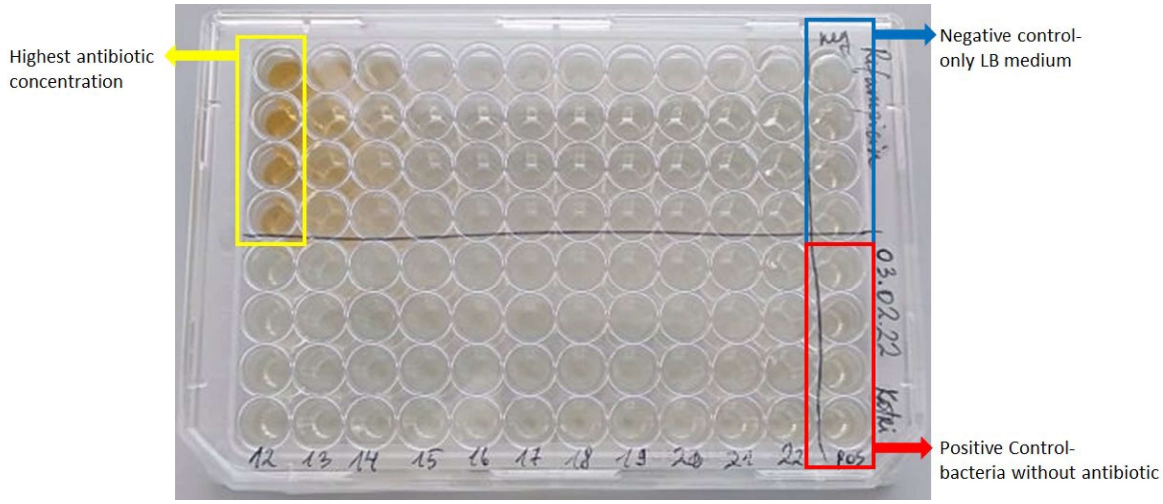

Figure 3. *96-well plate. It shows 22 rows of antibiotic concentrations (1-the highest concentration (yellow) and 22-the lowest concentration) and negative (blue)(only broth without bacteria) / positive (red)(growing bacteria without antibiotic) controls. Each row consists of four wells, which are four parallels of the same antibiotic concentration.*

4. The highest needed antibiotic concentration was made and then diluted in each following row. Needed to be calculated with consideration that concentration was halved after added bacterial suspension.
5. Overnight culture optical density was measured with a spectrophotometer.
   Autozero was performed with clear LB.
   Bacterial culture measurements gave results in Absorbance (Abs).
6. Using online calculator Abs was converted to CFU/mL = 6-8 * 10^8 CFU/mL.
   Needed to be diluted to 3-4 * 10^6 CFU/mL, because this concentration maximizes single-cell incubation according to Poisson (random) distribution.
7. The plate was secured with parafilm and incubated overnight at 37°C.

Day 3:

With Magellan 5 program and well plate test analyser (GENios Pro, Tecan, Microplate reader) results were obtained.

3.2 Droplet-based scMIC

96-well plate MIC results were further used to determine starting concentration of antibiotics in droplet-based experiments.

Materials:

- 1,5 mL (2 mL and 5 mL) test-tubes
- 5 mL test-tubes
- 50 mL Falcon tube
- Luria-Bertani (LB) broth (Biomaxima, Poland)
- Test-tube shaker Genie™ Vortex Mixer Model: Vortex-Genie® 2 (Scientific Industries, USA)
- Tested antibiotics (trimethoprim, chloramphenicol, ciprofloxacin, rifampicin, doxycycline, gentamicin- all stocks 10 mg/mL)
- Orbital shaker incubator ES-20 (Biosan, Latvia)
- Spectrophotometer (Shimadzu BioSpec-mini, DNA/RNA/Protein analyser)
- Cuvettes
- Pipettes
- Countess ™ cell counting chamber slides (Invitrogen by Thermo Fisher Scientific)

- Zeiss LSM 900 confocal microscope

3.2.1 Preparations

Based on the MIC curve determined in the 96-well plate experiment, nine concentrations were chosen for the droplet-based scMIC tests. The chosen range is calculated into nine different concentrations and one positive control (bacteria without antibiotic).

3.2.2 Performed experiment

Day 1:
One *E.coli* colony was picked from an agar plate and dissolved in 5 mL LB using 50 mL Falcone tube and incubated overnight at 37°C.

Day 2:
1. Overnight culture diluted in 50 mL tube in LB (1:100).
   Bacteria grew to 3-4 * 10^8 CFU/mL in density or higher.
2. While bacteria grew nine tubes with antibiotics were prepared.
   5 mL tubes were filled with 10 µl of antibiotics and 190 µl of Alexa dye + bacterial suspension.
   Ten tubes were labeled (nine antibiotics tubes and one positive control).
   Antibiotic was added before bacteria + dye (addition needs to be 20x higher than the final tube concentration).
3. In each tube was 10 µl of antibiotics (only LB in positive control).
   Antibiotic concentration was calculated with consideration that after adding 190 µl of dye/bacteria final concentration needs to match the calculated range.
4. With a spectrophotometer overnight culture optical density was measured.
   Autozero was performed with clear LB.
   Bacterial culture measurements gave results in Abs.
5. Using an online calculator Abs was converted to CFU/mL.
   Suspension grew around to 6-8 * 10^8 CFU/mL
   Needed to be diluted to 3-4 * 10^8 CFU/mL.
6. Diluting overnight culture 100x using Alexa/LB.
   Taking 23 µl of 3-4 * 10^8 CFU/mL + made 2250 µl of Alexa/LB.
   Get 2273 µl of 3-4 * 10^6 CFU/mL of bacteria + Alexa dye.
7. Pipetted 190 µl from 2273 µl Alexa/bacteria into all 10 tubes (added to 10 µl that were already in tubes).
8. 200 µl of oil/surfactant were added to each tube and vortexed for 5 seconds.
   The final concentration of dye in droplets was 1 µg/mL and bacteria had a concentration of 3-4 * 10^6 CFU/mL.
9. Incubated tubes overnight at 37°C.

Day 3:
Using Zeiss LSM 900 confocal microscope and Zen 3.3 (blue edition) program droplets imaging was performed.

3.3 Droplet imaging with a confocal microscope

After overnight incubation, droplets were imaged.

Materials:
- 5 mL test-tubes with antibiotics/dye/bacteria
- Pipette
- Countess ™ cell counting chamber slides (Invitrogen by Thermo Fisher Scientific)
- Zeiss LSM 900 confocal microscope

3.3.1 Preparations

Ten tubes were taken from the incubator. Pipet of 20 µl and Countess™ cell counting chamber slides (**Figure 4**) were taken to a confocal microscope.

Figure 4. *CountessTM cell counting chamber slide. Upper: Package of chamber slide. Lower: Slide with two chambers. The sample is pipetted into one of the chambers through a bigger (not the ones in the middle of the slide) hole and as a sample is spreading it forms a monolayer, for example of droplets.*

3.3.2 Performed experiment

1. Approximately 20 µl of the sample was used to fill one of two chambers of the Countess ™ cell counting chamber slide.
2. Equal amount of droplets and oil were pipetted from the tube (**Figure 5**).

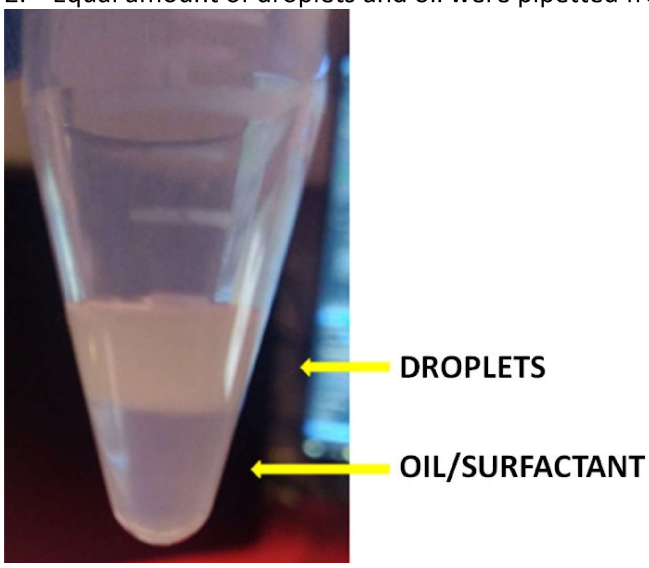

Figure 5. *5 mL test-tube with oil/surfactant mixture (lower) layer and droplets with growing E.coli (upper) layer after 24 hours incubation.*

3. Each sample (one chamber) provided 45-55 images.

3.3.3 File conversion

Imaging was done using Zeiss LSM 900 confocal microscope and a program called „Zen 3.3 (blue edition)“.

1. To take images of sample „Zen 3.3 (blue edition)“ program was opened on the computer.
2. Then to make images „Zen system“ was used or if it was needed to look at before made images „Zen image processing“.
3. Needed to open one of the older experiment file to be able to use the same parameters.
4. When the old file was opened under the tag „Acquisitions“ a „Reuse“ button was pressed (**Figure 6** – 1,2).
5. This allowed to use all the parameters from previous experiments for a further performed ones.
6. Then used the tag „Live“ (**Figure 6** - 3) manually with joy-stick chamber sizes were staked.
7. Focusing on the droplets was also manually performed using a microscope adjustment knob.
8. After the chamber was fixed and focused the „Start experiment“ button was pressed (**Figure 6** - 4).
9. Pressing tag „Processing“ images are exported as TIFF files (**Figure 6** - 1).

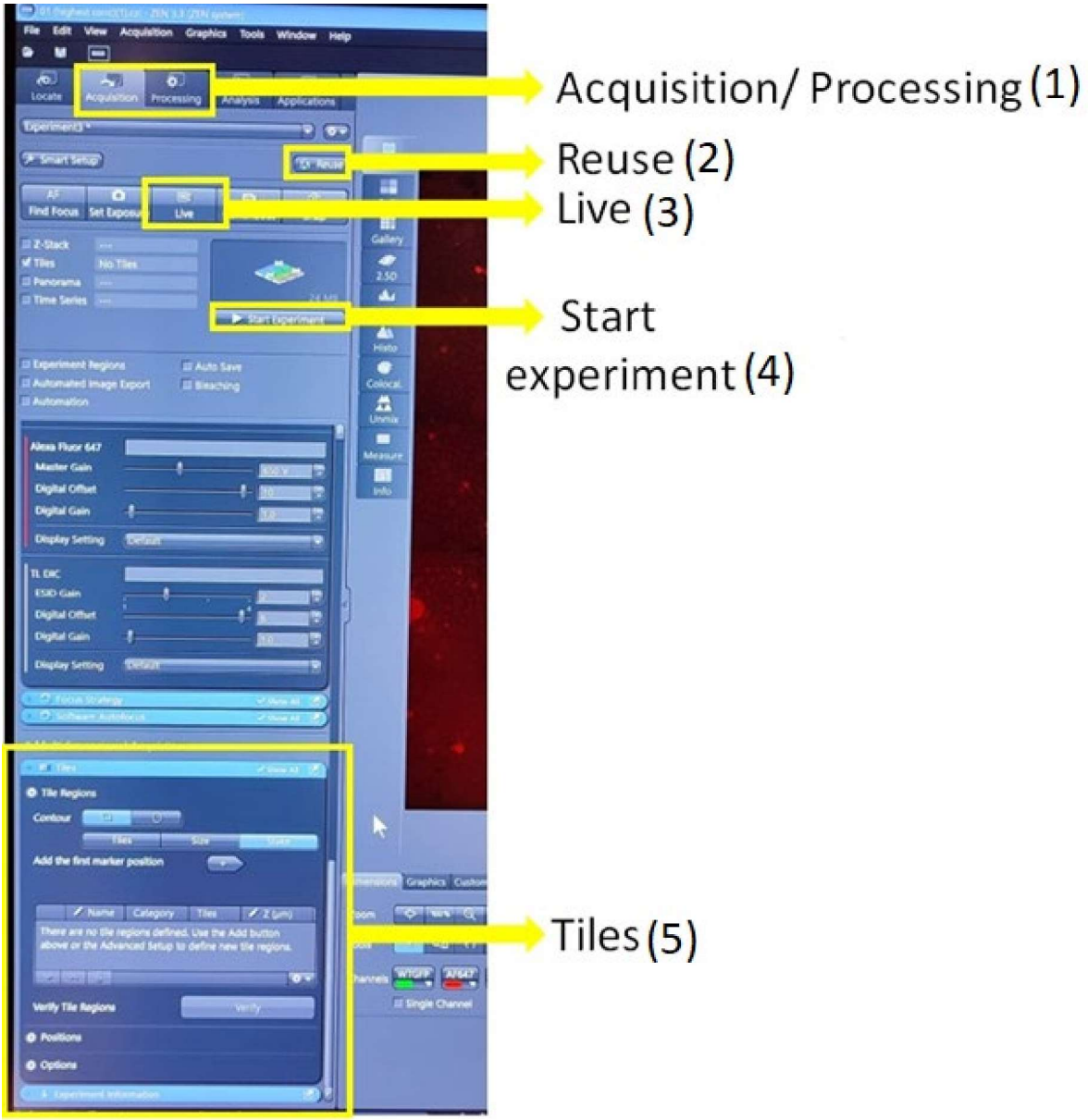

Figure 6. *Zen 3.3 (blue edition) layout and mainly used functions. (1) Acquisition/Processing – are used to open parameters and export made images; (2) Reuse – allows to copy and use the same parameters from older experiments; (3) Live – shows live picture from under the microscope; (4) Start experiment – starts imaging the sample; (5) Tiles – shows have many tiles will be made and helps to fix chamber size.*

3.4 Image analysis with CellProfiler™ and EasyFlow

After all images (**Figure 7**) were looked over and saved as TIFF they were analysed in the CellProfiler™ program.

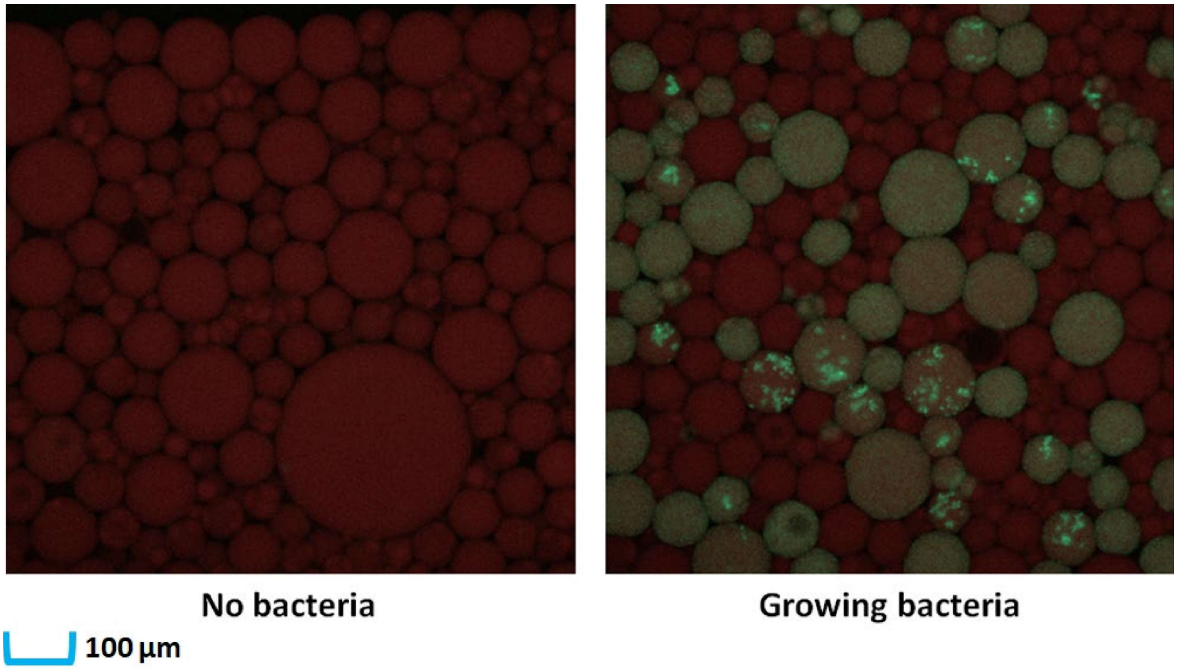

Figure 7. *Example of droplet images. Left: Sample without any bacteria. Right: Sample with E.coli bacteria. Red droplets are empty (only Alexa background dye inside droplets). Green droplets have viable bacteria after growth for approximately 24 hours in them.*

1. Imported previously developed pipeline for polydispersed droplet analysis.
2. Imported TIFF converted images from the experiment.
3. Pressed the „Analyse“ button.
4. Got a .csv file which needed to be changed to excel worksheet file type.
5. Data got sorted into the tables.
6. Calculated average droplets diameter.
7. Made graphs diameter vs intensity.
8. Made graphs fraction positive droplets standardized.

3.4.1 CellProfiler™ pipeline functions

In CellProfiler™ there was a developed pipeline which helped to convert tiles from a confocal microscope and to perform measurements from images that can be exported to Excel for further analysis and making graphs. The pipeline was previously designed and tested by the TalTech Microfluidics group. The main functions of the given pipeline were:

1) Converted all red (dye) and green (growing bacteria) colour (**Figure 10**) to gray, so CellProfiler™ was able to analyse it.
2) Experimentallytly was figured out the approximate diameter range for droplets, so droplets that are outside the range were discarded as well as droplets that touched the edge of the image.
3) Droplets were filtered by these criteria in order to eliminate any falsely identified droplets:
    1. Eccentricity – circularity of droplets (for example if detected droplets had weird shapes they were filtered out). Filter used value range from 0 to 0,5.
    2. Solidity - if chosen droplets were keeping their round shape, for example if a rubber band was wrapped around a round droplet, the solidity factor would be high since it would fit around tightly. However, if droplets were wrongly identified and had alternative shapes, the solidity factor would be low since there would be space

between the rubber band and the oddly shaped droplet/object. Filter used value range from 0,93 to 1,0.

4) Droplets that pass all the filters were exported to a spreadsheet in Excel, where they were further analysed.

3.4.2 EasyFlow

EasyFlow is an user-friendly web application for image-based droplet analysis with multipurpose modules, which was made and developed in TalTech [22], [23]. This platform allows visualization and calculations of poly- and monodispersed droplets. It allows to easily find the threshold between positive versus negative droplets and also calculate the total amount of bacteria and how the amount divides between two classifications. It also calculates parameters like volume CV and the fraction of positive droplets. The platform uses the CellProfiler™ data-set and provides basic profiles and graphs of the droplet experiment [24].

EasyFlow allows to make graphs ([22]**)**:

1) Droplet Signal Plot (Intensity vs Number of droplets)
   Using this plot, you can see the average pixels distribution within your data. Usually, this plot is used to define the threshold for classification.
2) Droplet Sizes Plot (Volume vs Number of droplets)
   Plot generates size distribution among your sample.
   Also gives the mean of total volume and coefficient of variation from the total volume.
3) Sizes-Signals Plot (Volume vs Intensity)
   Used to help find a good threshold for classification.
   Also gives the total amount of droplets and how many of them are positive/negative.
4) Label-based Plot (Concentration vs Intensity)
   Groups pixel intensities from the available data.

Example:
Trimethoprim is used as an example to illustrate graphs generated by EasyFlow (**Figure 8**).
The rest of the graphs can be found in the „Extras“ chapter.

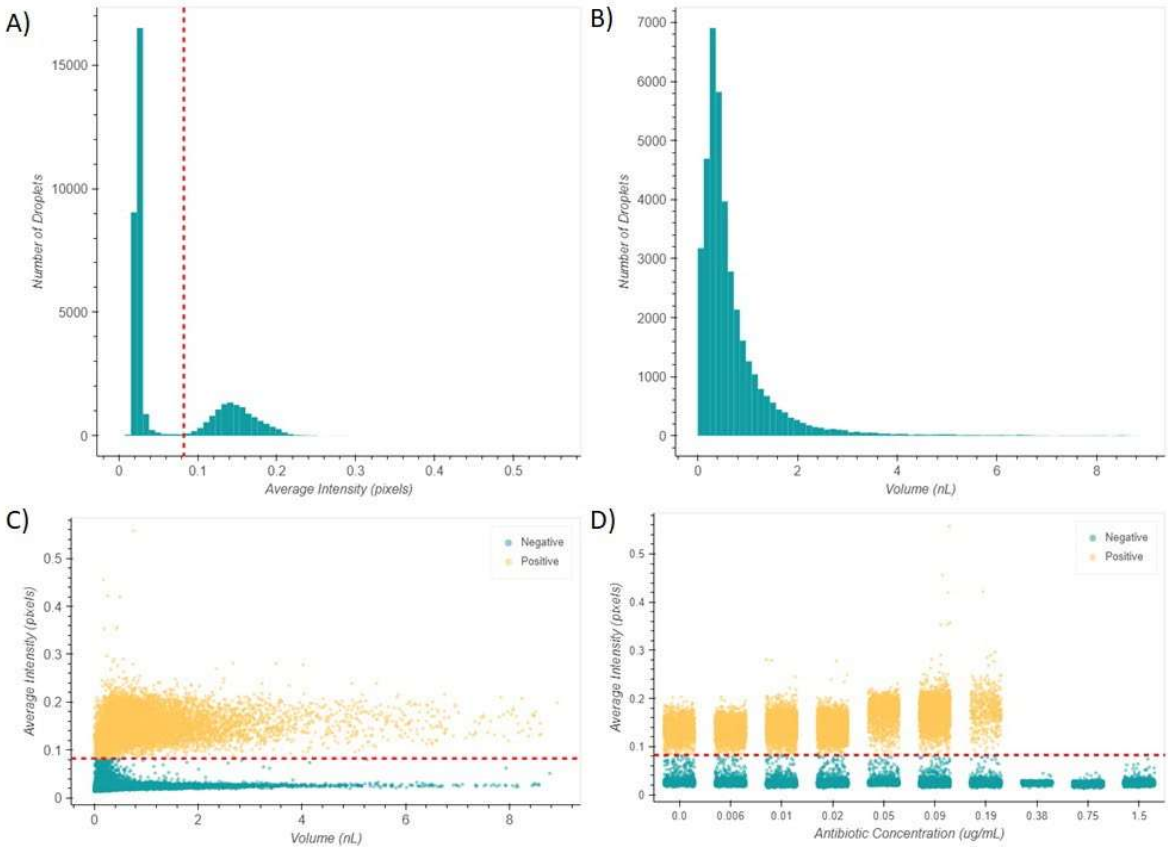

Figure 8. *EasyFlow Trimethoprim. Graphs: A) Average Intensity (pixels)(x-axis) vs Number of Droplets (y-axis) - shows pixels distribution within the data; B) Volume (mL)(x-axis) vs Number of Droplets (y-axis) – shows size distribution within the data; C) Volume (mL)(x-axis) vs Average Intensity (pixels)(y-axis) – shows correlation between each droplet size and signal intensity; D) Antibiotic Concentration (ug/mL)(x-axis) vs Average Intensity (pixels)(y-axis) - shows droplet distribution at different antibiotic concentrations.*

Graph (**Figure 8** – A, C, D) threshold is shown as a red line within the figures. Threshold determines the classification between two types of droplets (negative and positive droplets). It is found manually by analysing the **Figure 8** - A. Threshold is usually around 0,07 pixels. In this case threshold is 0,08 pixels (**Figure 9**). To determine the threshold on the graph it is zoomed in and found the lowest bin between two peaks, where the first and highest peak (to the left from the red line – under 0,08 pixels) is negative droplets and the second lower peak is positive droplets (to the right of the red line - above 0,08 pixels).

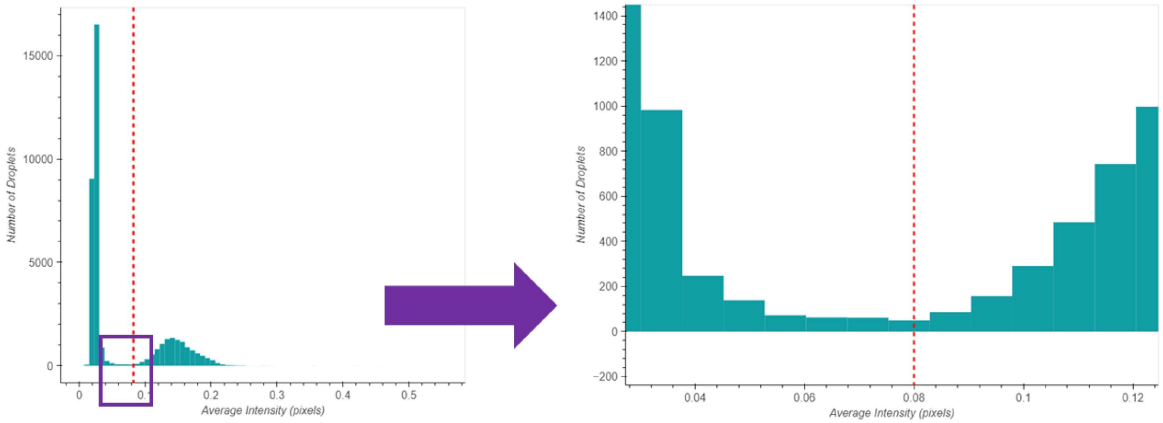

Figure 9. *Left: EasyFlow Trimethoprim threshold (red line) shown on graph Average Intensity (pixels)(x-axis) vs Number of Droplets(y-axis) and square of which graph part is zoom in on the right. Right: Zoomed in graph part that shows the lowest bin between two peaks, which is determined as the threshold value.*

Figure 8 - B shows the size distribution among the sample. The graph shows that most of the made droplets were under 1 nL volume. The application also calculated the mean of total volume, which is 0.7 nL, and CV from total volume, which is 114.52%. CV is so high, because it is calculated in relation to the mean, but polydispersed droplets were used, which vary a lot in size. It means that the level of dispersion around the mean is really high.

Figure 8 - C shows the threshold for droplet classification. Under the red line are negative droplets and upper from the red line are positive droplets. EasyFlow also generates a table, where it shows that there are 27066 negative droplets and 11697 positive droplets. Also application made the table with the counted amount of the total droplets within the experiment, which is 38763, and the fraction positive of the experiment, which is 0.3.

Figure 8 - D shows droplet distribution at different antibiotic concentrations (µg/mL). The higher the antibiotic concentration gets the fewer positive droplets and at higher concentration there are no positive droplets, which means that around 0,4 µg/mL *E.coli* is inhibited using single-cell droplet technology.

4. Results and discussion

4.1 Results of performed standard method 96-well plate MICs

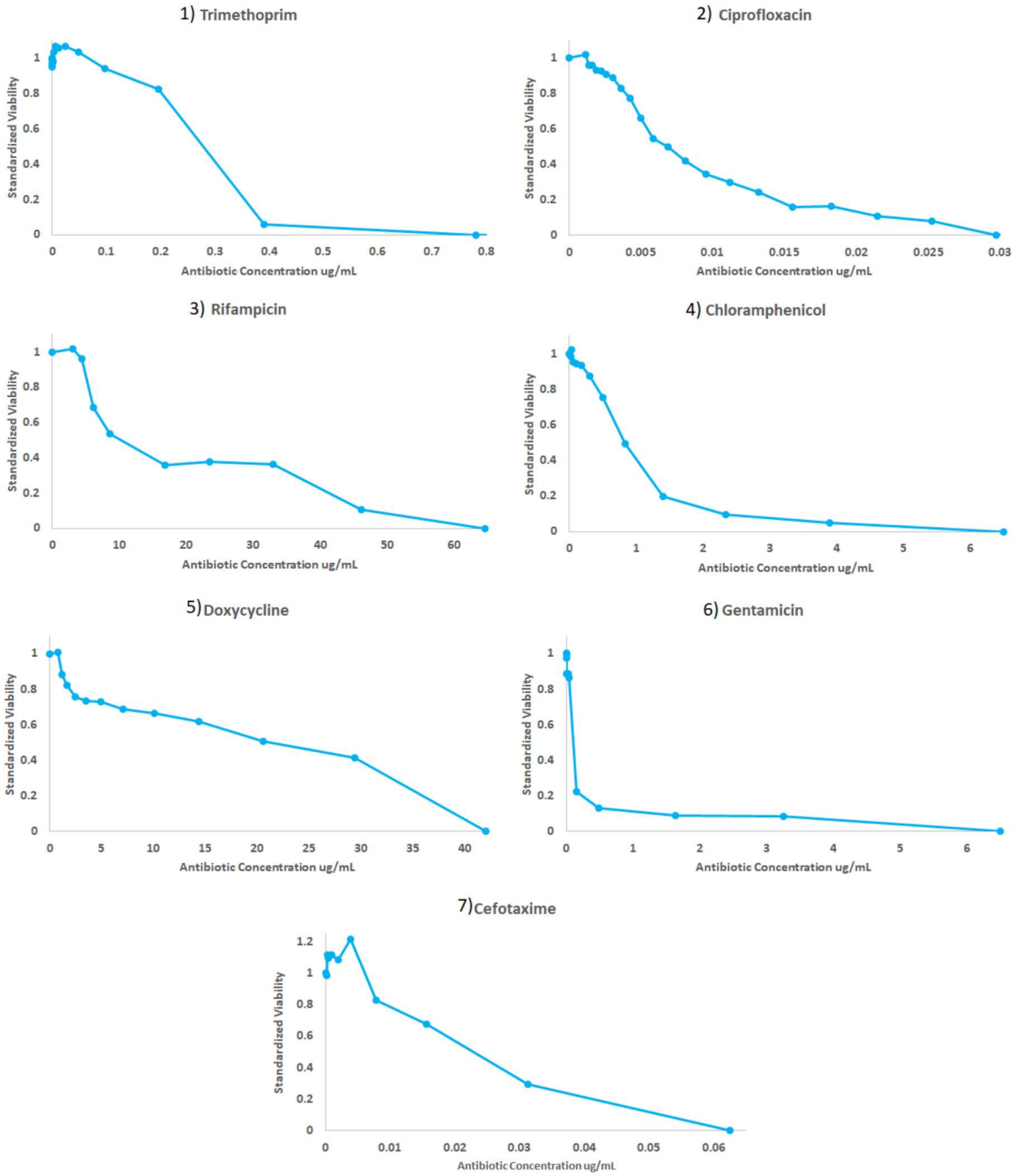

Figure 10. *MIC graphs (Antibiotic concentration (ug/mL)(x-axis) vs Standardized Viability(y-axis)): 1) Trimethoprim; 2) Ciprofloxacin; 3) Rifampicin; 4) Chloramphenicol; 5) Doxycycline; 6) Gentamicin; 7) Cefotaxime*

Minimal inhibitory concentration is determined by the viability of bacteria. When bacteria growth is totally inhibited, the viability trendline hits the x-axis (y-axis equals zero). This point shows the antibiotic concentration, where *E.coli* is inhibited, which is called the MIC value.
Some antibiotic 96-well plate experiments had to be repeated as the range differed greatly from what was expected by reading previous studies. Previous studies mostly used already clinically

resistant strains of *E.coli*, but in this thesis laboratory reference strain (friendly *E.coli*) was used. That is why the range in this thesis results can be different compared to the other literature.

Standard method MICs for the respective antibiotics are (**Figure 10**):

1) Trimethoprim – 0,78 μg/mL
2) Ciprofloxacin – 0,03 μg/mL
3) Rifampicin – 64,6 μg/mL
4) Chloramphenicol – 6,5 μg/mL
5) Doxycyline - 42 μg/mL
6) Gentamicin – 6,5 μg/mL
7) Cefotaxime 0,063 μg/mL

4.2 Results of performed droplet-based method single-cell MICs

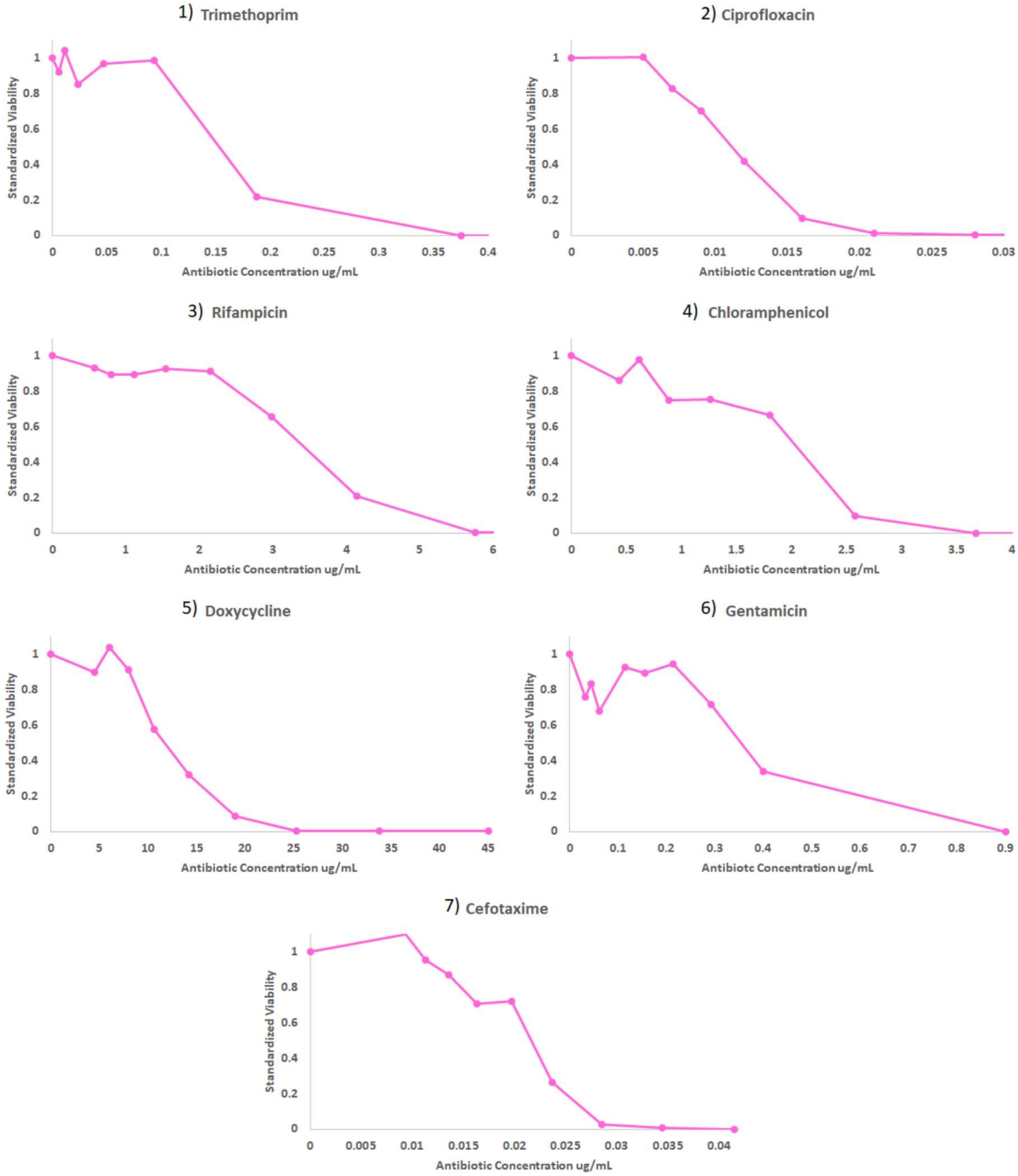

Figure 11. *scMIC graphs (Antibiotic concentration (ug/mL)(x-axis) vs Standardized Viability(y-axis)): 1) Trimethoprim; 2) Ciprofloxacin; 3) Rifampicin; 4) Chloramphenicol; 5) Doxycycline; 6) Gentamicin; 7) Cefotaxime*

Single-cell MIC on the graph was found the same way as standard MIC. Where the trendline hits the x-axis (y-axis equals zero) *E.coli* is totally inhibited and this is the scMIC value.
Some antibiotic droplet-based experiments had to be repeated, because the bacteria were a lot more or less resistant when isolated in droplets. The range in this thesis results can not be compared to the other literature, because experiments using droplet-based test are not commonly performed and that is why there are no sources that can be referred to.

Droplet-based method scMICs for the respective antibiotics are (**Figure 11**):

1) Trimethoprim – 0,375 µg/mL
2) Ciprofloxacin – 0,028 µg/mL
3) Rifampicin – 5,76 µg/mL
4) Chloramphenicol – 3,675 µg/mL
5) Doxycyline – 25,31 µg/mL
6) Gentamicin – 0,9 µg/mL
7) Cefotaxime - 0,042 µg/mL

4.3 Comparison of standard MIC vs single-cell MIC

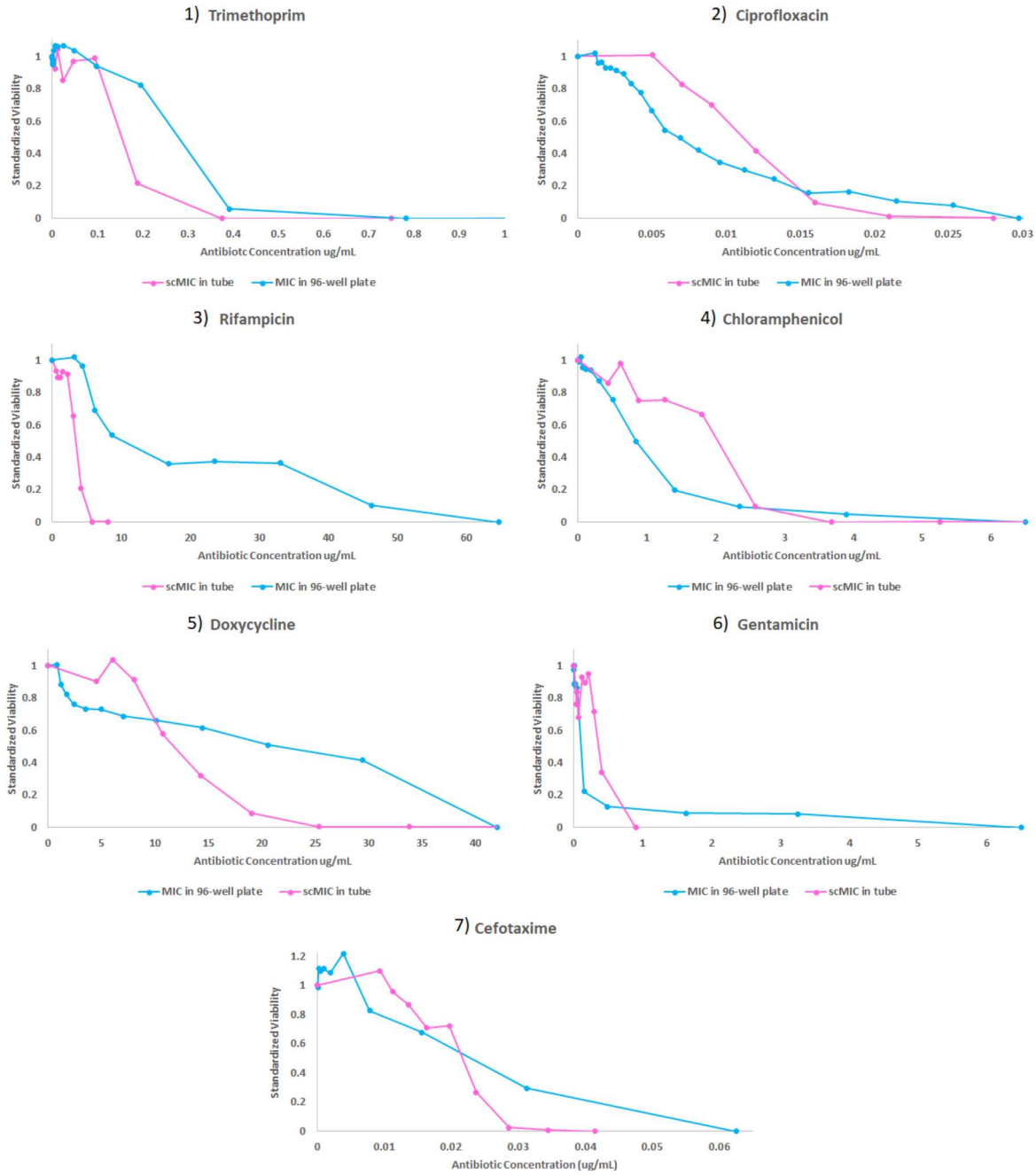

Figure 12. *MIC vs scMIC graphs (Antibiotic concentration (ug/mL)(x-axis) vs Standardized Viability(y-axis)): 1) Trimethoprim; 2) Ciprofloxacin; 3) Rifampicin; 4) Chloramphenicol; 5) Doxycycline; 6) Gentamicin; 7) Cefotaxime*

4.3.1 Results

Based on the results can be stated that for all seven antibiotics tested in this thesis scMIC was lower than standard MIC (**Figure 12**).

The difference between the standard MIC and scMIC varied among the antibiotics (**Table 2**). Ciprofloxacin had the smallest difference of 0,002 µg/mL (**Figure 12** Graph 2). Rifampicin had the largest difference of 58,84 µg/mL (**Figure 12** Graph 3). Individual value differences between MIC and scMIC for each antibiotic come from their mechanism of action. Each antibiotic were chosen by their inhibitory mechanism (**Figure 2**), which proved that different mechanism of action provide different results even while using the same methods and isogenic bacteria.

Table 2. *Standard MIC and droplet-based scMIC results for each antibiotic.*

Antibiotics	Standard MIC	Droplet-based scMIC	Difference between MIC and scMIC values
Trimethoprim	0,78 µg/ml	0,375 µg/ml	0,405 µg/ml
Ciprofloxacin	0,03 µg/ml	0,028 µg/ml	0,002 µg/ml
Rifampicin	64,6 µg/ml	5,76 µg/ml	58,84 µg/ml
Doxycycline	42 µg/ml	25,31 µg/ml	16,69 µg/ml
Chloramphenicol	6,5 µg/ml	3,675 µg/ml	2,825 µg/ml
Gentamicin	6,5 µg/ml	0,9 µg/ml	5,6 µg/ml
Cefotaxime	0,063 µg/ml	0,042 µg/ml	0,021 µg/ml

Heteroresistance that can be seen in the scMIC curves (**Figure 11**) most likely could not have been due to mutations on the genetic level, because the bacteria was incubated with antibiotics for only 24 hours. The more likely reason could be bacteria phenotype changes such as difference in protein expressions or possible being at different stages in their cell cycle stages. During the experiment the resistant cells were able to survive and grow in the presence of even high antibiotic concentrations, as demonstrated by their detectable fluorescence signal after 24 hours. That is one of the reasons why performing the scMIC test should be important because it allows to see this minority subpopulation, which should be further analysed [10], [25].

ScMIC values are much more accurate and can give more information about subpopulations, which is important for performing further experiments, especially related to the clinical field. It can also help to investigate antibiotic mechanism of action, to get an idea how to inhibit and stop bacteria growth in a more efficient way [10], [11].

4.4 Conclusion

The first and second objectives of the study were to find MIC using the standard 96-well plate method and scMIC of each antibiotic using the droplet-based method. All of the results were obtained and can be stated that tested antibiotics scMIC is lower than standard MIC. The difference of scMIC and MIC values among the antibiotics is most likely related to the antibiotic´s different inhibitory mechanisms.

The range from standard MIC sometimes varied greatly from the range found in literature. That is why when the experiments needed to be repeated, the 96-well plate tests took multiply tries to get an accurate graph for each antibiotic. While in the droplet-based experiments, obtained values from a 96-well plate experiment were used with the same *E.coli* strain. That is why scMIC tests only needed one redo for each antibiotic to get accurate results.

Values obtained from the experiment can differ from literature ones, because no proved information can be found about scMIC, and values related to standard MIC are usually collected by analysing *E.coli* with developed clinical resistance, while we used *E.coli* laboratory reference strain.

The last objective was the comparison of MIC and scMIC. For all seven antibiotics tested in this thesis scMIC required a lower antibiotic concentration than standard MIC. Standard method MIC is based on population susceptible cells, while droplet-based scMIC is based on resistant subpopulation. Both methods still work to determine antibiotic MICs for *E.coli*, but it would be the best to use both of them together to get as much detailed information as possible. If the droplet-based method will be more widespread then it will also help to gain more knowledge on bacterial heteroresistance. This knowledge could then be used to help prevent new resistant bacteria.

Abstract

The main aim of the thesis was to compare standard and droplet-based methods for assessing antibiotic susceptibility patterns in *E.coli*. Seven antibiotics were tested and compared: trimethoprim, ciprofloxacin, rifampicin, doxycycline, chloramphenicol, gentamicin and cefotaxime. Both the first (standard MICs) and the second (droplet-based scMIC) objectives were successfully performed even if both methods required repeating the experiment for each analysed antibiotic.

The comparison was made and showed that for all seven tested antibiotics scMIC was lower than MIC. The differences between the two values for each antibiotic varied and can be assumed that based on results most likely one of the reasons for it was different inhibitory mechanisms for each tested antibiotic.

It is important to analyse bacteria individually using, for example droplet-based method, because each cell can respond differently to antibiotics. By performing standard MIC tests results are given only population-wide. That gives an overview of the majority that are susceptible cells, but the minority remains and leads to an abrupt growth of resistant bacteria and the formation of a new resistant generation with new even stronger genes.

With use of the standard bulk method, one cannot be sure that all present bacteria are exposed to the antibiotic or if there is enough amount for each bacteria cell. This may give false-positive results, when bacteria actually do not have resistance for an antibiotic.

The single-cell method allows to see trends of minority and individually show the growth of each bacteria. It can give information about resistant subpopulations, which will help to thoroughly analyse antibiotic mechanism of action and how to more effectively inhibit and block bacterial growth.

The results from this thesis can be used for further analyses related to tested seven antibiotics. The methods described in this thesis could also be used to gain knowledge about bacterial heteroresistance and prevent new resistant bacteria. It is important especially in the clinical field where it is a serious problem.

Kokkuvõte

Tilgad on kui nanomõõtmelised katseklaasid. Need saadakse kahe segunematu vedeliku, näiteks vee ja õli, segamisel. Neid saab kasutada ainete või organismide kapseldamiseks ja erinevate katsete tegemiseks, näiteks üherakuliste bakterite vastuse uurimiseks antibiootikumidele.

Antibiootikume kasutatakse bakterite kasvu pärssimiseks või blokeerimiseks ja neil võivad olla erinevad inhibeerivad mehhanismid. Bakteritel võib antibiootikumidele olla heterogeenne reaktsioon. Isegi geneetiliselt identsed bakterid võivad individuaalselt reageerida erinevalt. Suurem osa võib olla vastuvõtlikud rakud, vähemus koosneda aga antibiootikumi resistentsetest alampopulatsioonidest.

Standard meetod annab tulemusi ainult kogu populatsiooni ulatuses, mitte analüüsides rakke eraldi. See ei anna ülevaadet võimalikest resistentsetest alampopulatsioonidest. Üherakuline meetod võimaldab näha vähemuse trende. See annab üksikasjalikumat teavet, kuna iga üksikut rakku analüüsitakse eraldi.

Lõputöö põhieesmärk on võrrelda standard ja tilkpõhiseid meetodeid *Escherichia coli* antibiootikumide tundlikkuse hindamiseks.

Antud töö alameesmärkideks olid (i) leida minimaalne inhibeeriv kontsentratsiooni (MIC) iga katsetatud antibiootikumi jaoks kasutates standardset 96-kaevu plaadi meetodit, (ii) leida üksikraku minimaalne inhibeeriv kontsentratsioon (MIC) iga katsetatud antibiootikumi jaoks kasutades tilkpõhist meetodit ning (iii) võrrelda MIC ja üksikraku MIC iga katsetatud antibiootikumi jaoks.

Töö raames katsetati ja võrreldi seitset antibiootikumi: trimetoprim, tsiprofloksatsiin, rifampitsiin, doksütsükliin, klooramfenikool, gentamütsiin ja tsefotaksiim. Kõigi seitsme antibiootikumidega tehti läbi standardsed 96-kaevu plaadi MIC katsed ja tilkpõhised üksikraku scMIC katsed.

Mõlemad meetodid osutusid edukaks ning võrreldes tulemusi oli avastatud, et kõigi seitsme katsetatud antibiootikumide üksikraku MIC oli madalam kui standard MIC. Erinevused kahe väärtuse vahel iga antibiootikumi puhul varieerusid ja võib eeldada, et tulemustele toetudes oli suure tõenäosusega varieeruvuse põhjuseks erinevad inhibeerimismehhanismid iga katsetatud antibiootikumi puhul.

Tulevikus on väga oluline analüüsida individuaalselt baktereid, kasutades, näiteks tilkpõhist meetodit. See võib anda teavet resistensetest bakteri alampopulatsioonidest, mis aitab põhjalikult analüüsida antibiootikumide toimemehhanismi ja kuidas tõhusamalt pärssida ja blokeerida bakterite kasvu. Standardset MIC katset läbi viies saab ülevaate enamikust vastuvõtlikest rakkudest, kuid vähemus jääb alles ja toob kaasa resistentsete bakterite järsu kasvu ja uue resistentse põlvkonna moodustamiseni koos veelgi tugevamate geenidega. Antud meetodeid saab kasutada ka teadmiste saamiseks bakterite heteroresistentsuse kohta, et ennetada uute resistentsete bakterite teket. See on oluline eriti kliinilises valdkonnas, kus see on osutunud tõsiseks probleemiks.

Acknowledgments

Foremost, I would like to express my sincere gratitude to my supervisor Simona Bartkova and co-supervisor Ott Scheler for their support of my study and thesis. Their patience, motivation and helpfulness at any time and topic supported me in the practical and the theoretical part of the thesis.

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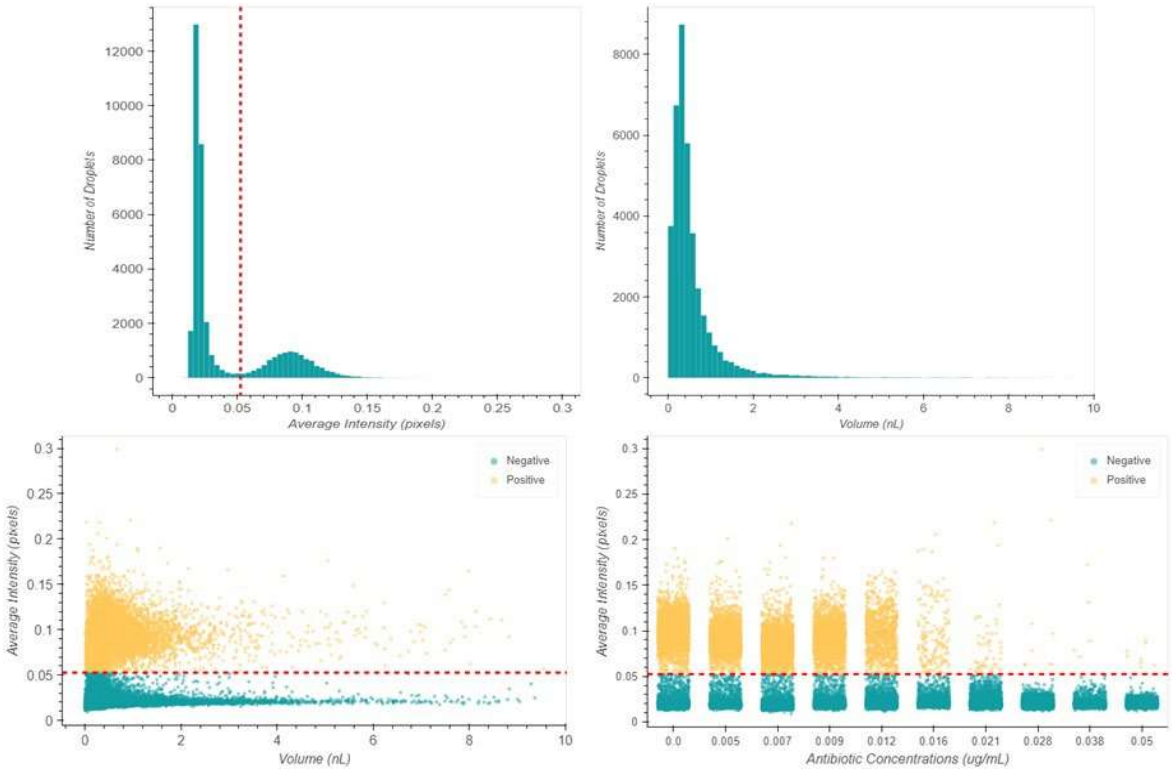
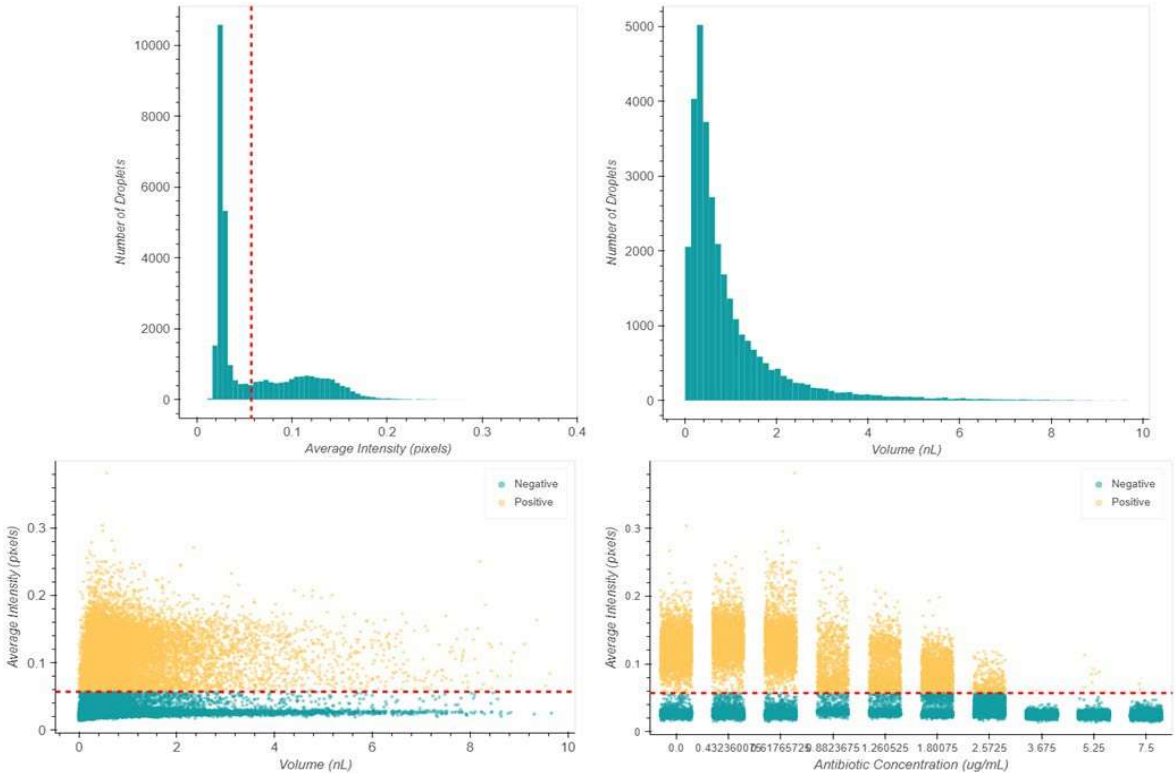
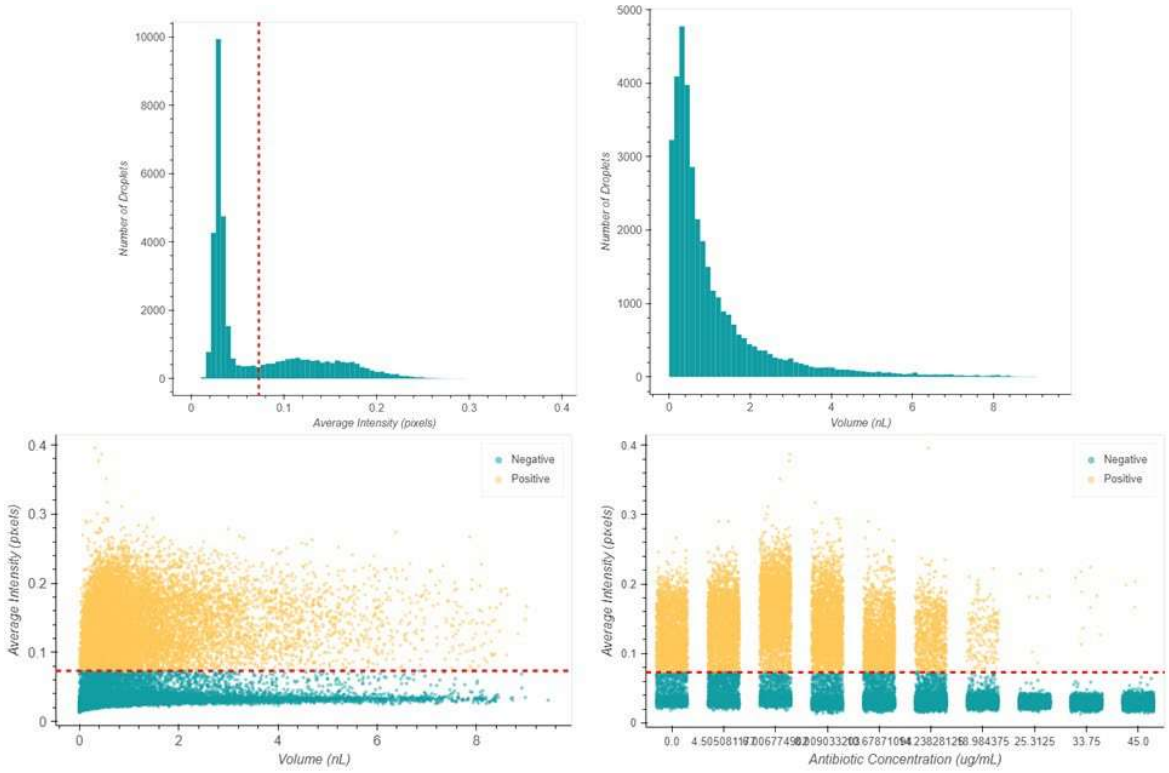
Extras



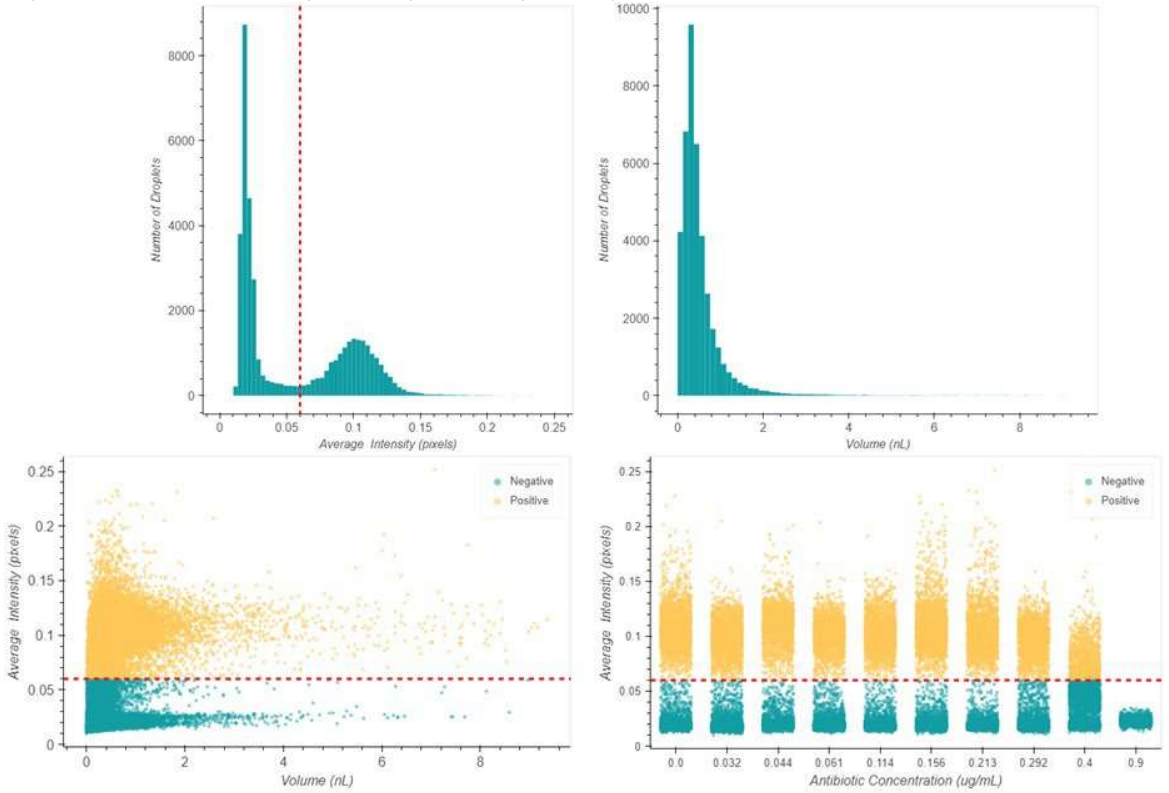
***Extra 1**. EasyFlow Ciprofloxacin. The mean of total volume is 0.57. The CV from total volume is 121.45%. Negative droplets: 27420. Positive droplets: 10424. The total droplets within the experiment is 37844. The fraction positive of the experiment is 0.28.*



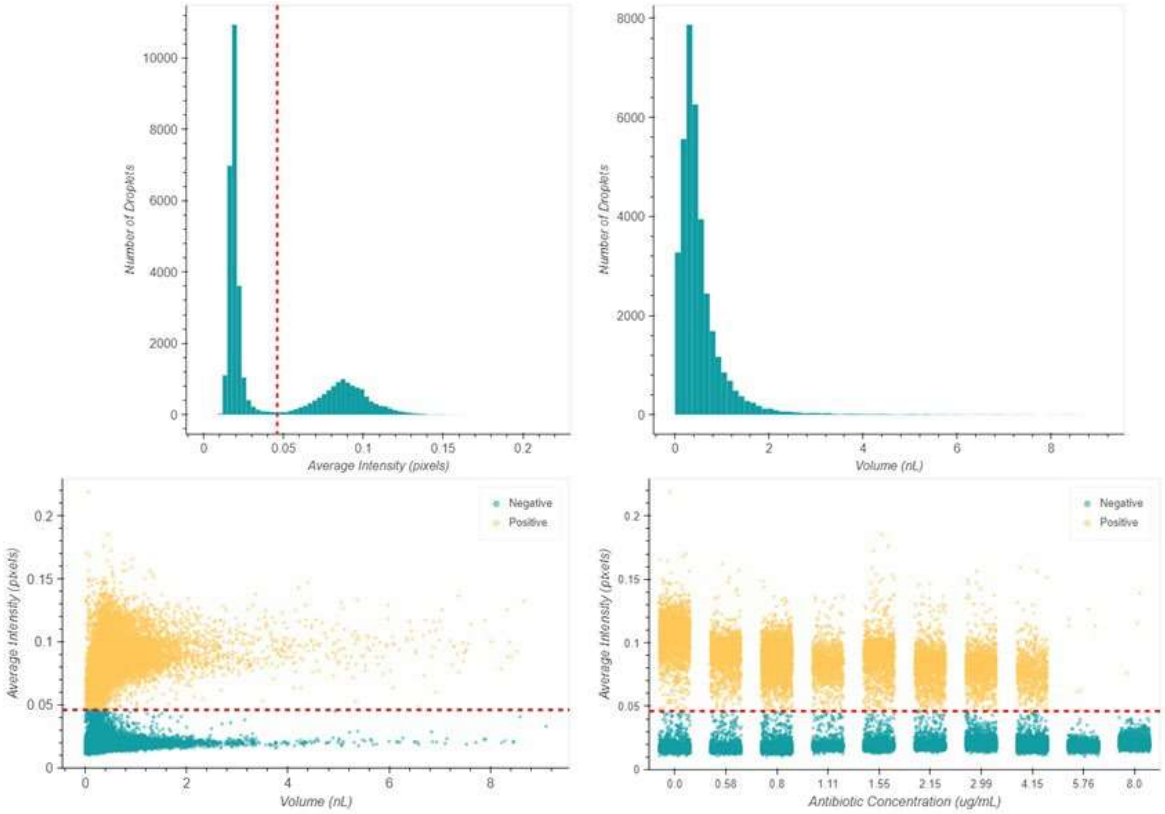
***Extra 2.** EasyFlow Chloramphenicol. The mean of total volume is 0.96. The CV from total volume is 116.34%. Negative droplets: 20120. Positive droplets: 11338. The total droplets within the experiment is 31458. The fraction positive of the experiment is 0.36.*



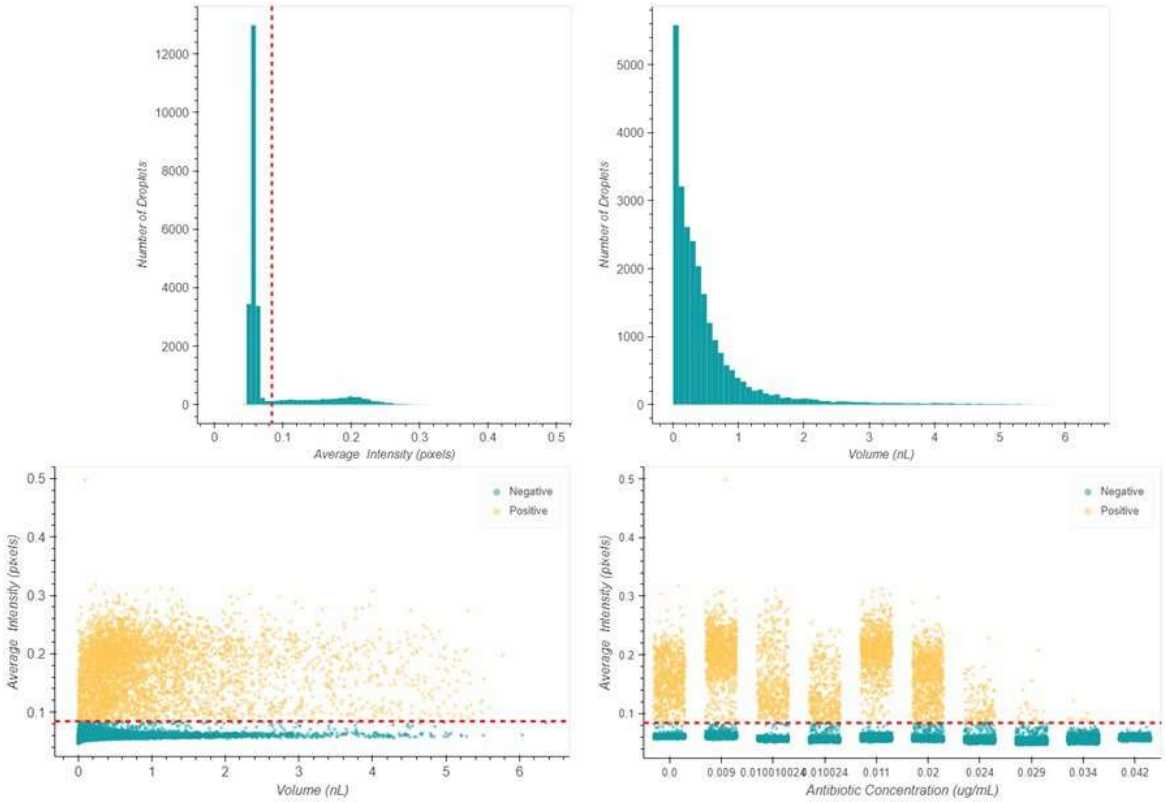
Extra 3. *EasyFlow Doxycycline. The mean of total volume is 1.07. The CV from total volume is 119.87%. Negative droplets: 23595. Positive droplets: 12218. The total droplets within the experiment is 35813. The fraction positive of the experiment is 0.34.*



Extra 4. *EasyFlow Gentamicin. The mean of total volume is 0.52. The CV from total volume is 115.49%. Negative droplets: 23506. Positive droplets: 17227. The total droplets within the experiment is 40733. The fraction positive of the experiment is 0.42.*



Extra 5. *EasyFlow Rifampicin. The mean of total volume is 0.54. The CV from total volume is 112.28%. Negative droplets: 24743. Positive droplets: 11556. The total droplets within the experiment is 36299. The fraction positive of the experiment is 0.32.*



Extra 6. *EasyFlow Cefotaxime. The mean of total volume is 0.5. The CV from total volume is 137.89%. Negative droplets: 20230. Positive droplets: 4545. The total droplets within the experiment is 24775. The fraction positive of the experiment is 0.18.*

Lisa
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Lihtlitsents lõputöö reprodutseerimiseks ja lõputöö üldsusele kättesaadavaks tegemiseks[1]

Mina Katri Kiir

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„Comparison of standard and droplet-based methods for assessing antibiotic susceptibility patterns in bacteria“,

mille juhendajad on Simona Bartkova ja Ott Scheler,

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3. Kinnitan, et lihtlitsentsi andmisega ei rikuta teiste isikute intellektuaalomandi ega isikuandmete kaitse seadusest ning muudest õigusaktidest tulenevaid õigusi.

(digitally signed)

[1] *Lihtlitsents ei kehti juurdepääsupiirangu kehtivuse ajal vastavalt üliõpilase taotlusele lõputööle juurdepääsupiirangu kehtestamiseks, mis on allkirjastatud teaduskonna dekaani poolt, välja arvatud ülikooli õigus lõputööd reprodutseerida üksnes säilitamise eesmärgil. Kui lõputöö on loonud kaks või enam isikut oma ühise loomingulise tegevusega ning lõputöö kaas- või ühisautor(id) ei ole andnud lõputööd kaitsvale üliõpilasele kindlaksmääratud tähtajaks nõusolekut lõputöö reprodutseerimiseks ja avalikustamiseks vastavalt lihtlitsentsi punktidele 1.1. ja 1.2, siis lihtlitsents nimetatud tähtaja jooksul ei kehti.*