MACHINE LEARNING-ENHANCED DYNAMIC LASER SPECKLE IMAGING FOR RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING

A Thesis in Electrical Engineering

by

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ABSTRACT

Lacking rapid antimicrobial susceptibility testing (RAST) methods has become a big challenge in the 21st century. Recently, a series of RAST methods have been reported, including genotypic and phenotypic approaches. In this thesis, we first compared different phenotypic RAST methods based on bacterial morphology and motion, then present an innovative method combining dynamic laser speckle imaging (DLSI) and machine learning to achieve highly accurate prediction of minimum inhibitory concentration (MIC) of antibiotics – ampicillin and kanamycin as model antibiotics – in one hour. The time-varying motion of *Escherichia coli* (*E. coli*) treated with different antibiotic concentrations was captured using speckle imaging system and then utilized to train an artificial neural network model. Finally, the trained model is used to predict the bacterial susceptibility to antibiotics and the results are compared with gold standard broth microdilution result.
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Chapter 1

Introduction

Combating outbreaks and pandemics of infectious diseases has been a critical challenge in the 21st century. Infectious pathogens such as malaria, *mycobacterium tuberculosis*, Salmonella, AIDS, Ebola, and SARS have resulted more than 39 million fatality. Just recently, the 2019 novel coronavirus has emerged to be a pandemic. It has spread in more than 160 locations internationally, including in the United States, caused more than 550,000 people infected worldwide in just 3 months.

While virus threatens the survival of human, antimicrobial-resistant bacteria, either naturally occurring or intentionally developed as bioterror agents, are of growing concern to the public health. Antimicrobial resistance (AMR) stultifies all previous drug development efforts and causes persistent infections and threatens patients’ lives. Annually causing at least 2.8 million infections and 35,000 deaths in just the United States, the fatality rate is expected to reach 10 million death per year by 2050 if efforts are not made to slow down the spread of AMR.

1.1 Antimicrobial Resistance Issue

AMR is the ability of a microorganism to survive and reproduce in the presence of an antimicrobial agent by a variety of mechanisms. To combat AMR, two approaches can be envisioned: first is developing brand-new antimicrobial agents and the second method is to enforce antimicrobial stewardship.\(^1\) The first approach can only alleviate the crisis temporarily because the
microbe will eventually acquire resistance to the new drug due to natural evolution. In addition, the misuse and overuse of the antibiotics may exacerbate the spread of AMR. Thus, the second methodology has been preferred and implemented as a preventive strategy. To realize this, physicians need to only prescribe the necessary amount of antibiotics in antibiotic therapies, which require finding the most efficient antibiotics in a short time.

1.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) is a laboratory procedure to identify which type of antibiotics is specifically effective for a patient.\(^2\) AST is a critical procedure in infectious disease therapies, as it enables the healthcare providers to decide the treatment regimens, i.e. both the antibiotic type and dosage. Clinical laboratories currently use two gold-standard methods, including the disk diffusion and broth microdilution methods (Figure 1-1).\(^2\) Those methods need to incubate samples overnight and check the results in the second day, at the earliest, posing huge risk to the patients. Such delayed therapy can pose significant risk to patients in critical conditions, such as those in Intensive Care Units (ICUs) who are prone to sepsis. Indeed, every hour of delay in therapy of sepsis within the first 6 hours results in a 7.6% decrease in survival.\(^3\) Therefore, developing rapid antimicrobial susceptibility testing (RAST) technologies can be a game changer in clinical settings.

1.3 Rapid Antimicrobial Susceptibility Testing

As mentioned earlier, the development of RAST methods is a major challenge considering the worldwide escalation in antimicrobial resistance. Recently, a series of RAST technologies have
been developed which can be categorized into genotypic and phenotypic approaches, as summarized in this section.

Figure 1-1. Gold-standard methods for antimicrobial susceptibility testing. (a) Disk diffusion method, and (b) broth microdilution method. Despite being low-cost and highly sensitive, these methods are very slow.

1.3.1 Genotypic Rapid Antimicrobial Susceptibility Testing

Genotypic ASTs rely on detection of specific nucleic acid sequences that can confer resistance of pathogens to antibiotics. Significant advancements have been made for developing different genotypic approaches in recent years, especially with the development of microfluidic chips which can reduce the starting volume and concentration needed to run the assays.4

In genotypic methods, amplification of target DNA strands using polymerase chain reaction (PCR)5, isothermal recombinase polymerase amplification (RPA)6, or loop-mediated isothermal amplification (LAMP)7, combined with fluorescence detection8 can provide the results within an hour in a single assay. Due to diverse genetic resistance determinants that do not have sufficient correlation with resistance phenotypes, culture-independent AST testing cannot be broadly applied to all pathogens without extensive prior knowledge of their biology and underlying
genetics. Furthermore, molecular methods still fail to detect resistance in cases where novel resistance mechanisms have not yet been characterized. Moreover, molecular-based methods require advanced analytical tools, high skill sets, and labeling agents which limit their application in resource-limited regions and field settings.

1.3.2 Phenotypic Rapid Antimicrobial Susceptibility Testing

Unlike genotypic methods, phenotypic ASTs measure change of physical characteristics of bacteria to antimicrobial agents. Change of bacterial viability can be effectively measured by monitoring its phenotypic features: population, shape, size, motion, and/or cell mass. Significant research effort has been made in recent years for developing phenotypic ASTs, as in many cases, they can provide information that is more relevant to clinical outcomes.

Currently, within the United States, the Food and Drug Administration (FDA) has cleared five automated phenotypic AST systems for clinical use, which include VITEK 2 (bioMérieux), Phoenix (Becton Dickinson Diagnostic Systems), Microscan WalkAway (Siemens Medical Solutions Diagnostics), Sensititer ARIS 2X (Trek Diagnostic Systems) and Accelerate Pheno (Accelerate Diagnostics, Inc., Tucson, AZ, USA). Among these phenotypic methods, Accelerate Pheno is the fastest (6-7 hours). The approach is based on measuring cellular morphokinetic changes. As shown in the Figure 1-2. This method combines fluorescence in situ hybridization (FISH) and automated microscopic imaging to obtain the AST results within 7 hrs. As another phenotypic method, Schoepp et al. showed that microbial growth can be measured based on quantification of nucleic acids and can determine MIC value for clinical urine samples in 30 min. This technique, coupled with digital LAMP, measures microbial growth based on quantification of nucleic acids using quantitative PCR (qPCR). One of the limitations of this ultra-fast technique is assuming that slowed or halted DNA replication after 15 min antibiotic exposure indicates
susceptibility of a bacterial population to a given antibiotic. However, the bacterial population response within the first 15 min is typically highly variable among assay replicates, which increases an error in prediction of susceptibility,\textsuperscript{11} especially when borderline MIC concentrations of antibiotics are tested.

![Figure 1-2. Accelerate Pheno™ ID/AST Technology process flow. Copyright 2020 Accelerate Diagnostics, Inc.](image)

There are other RAST methods which measure changes in bacterial viability in response to antibiotics by probing other phenotypic features, including shape,\textsuperscript{12} motion,\textsuperscript{13} size/mass,\textsuperscript{14,15} or respiration.\textsuperscript{16} Among them, AST methods which rely on monitoring bacterial motion are particularly attractive. Johnson \textit{et al.} used the phase noise of resonant crystal to prove that the motion of \textit{E. coli} was attenuated after treated with polymyxin B or ampicillin.\textsuperscript{17} This work makes the bacterial motion as a promising characterization for RAST. Yu \textit{et al.} reported a phenotypic AST method that utilized deep learning to analyze the freely moving \textit{E. coli} cells in urine and can determine the minimum inhibitory concentration (MIC) in 30 mins.\textsuperscript{12} However, these methods have poor sensitivity and/or require labeling\textsuperscript{18} or indicative markers such as redox-active chemicals, which can interfere with cellular physiology.\textsuperscript{16} In addition, these methods still need advanced imaging/analysis setups, such as high resolution optical microscopy, atomic force microscopy, or complex optical setups,\textsuperscript{4,12} which limits their broad application in clinical diagnostics. Hence, there is a need to develop RAST methods which are simple-to-use, label-free, low-cost, and still sensitive and accurate.
As summarized in Table 1-1, most phenotypic RAST methods require bulky, expensive instrument which hinders their application for resource-limited areas. Moreover, some methods utilize labeling reagents, which can interfere with antibiotics. Furthermore, the existing ultra-rapid methods, such as the work by Volbers et al., do not address possible adaptation of bacteria to antibiotics, which can lead to false interpretation of resistance. As discussed in Chapter 2 and 3, in this thesis we developed a low-cost, label-free method based on dynamic laser speckle imaging which can deliver a high AST accuracy comparable to the standard clinical methods by leveraging machine learning.

Table 1-1. Comparison of some of the reported recently RAST methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cell concentration</th>
<th>Sample</th>
<th>AST time</th>
<th>Validation method</th>
<th>Setup complexity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accelerate PhenoTest BC Kit</td>
<td>10^5 cell/mL</td>
<td>Clinical specimens</td>
<td>6h</td>
<td>Compared with disk diffusion result</td>
<td>High (require labeling)</td>
<td>20</td>
</tr>
<tr>
<td>OCelloScope</td>
<td>2×10^7 cell/mL</td>
<td>Veterinary samples</td>
<td>3h</td>
<td>N/A</td>
<td>Low</td>
<td>21</td>
</tr>
<tr>
<td>Drast</td>
<td>5 × 10^5 to 5 × 10^7 cell/mL</td>
<td>Bacteria add into fresh blood</td>
<td>6h</td>
<td>Compared with BMD</td>
<td>High (Microscopy)</td>
<td>22</td>
</tr>
<tr>
<td>SNDA–AST</td>
<td>5 × 10^5 cell/mL</td>
<td>Clinical isolates and urine samples</td>
<td>&lt; 5.5h</td>
<td>N/A</td>
<td>High (Microscopy)</td>
<td>23</td>
</tr>
<tr>
<td>Stress-based Microfluidic System</td>
<td>100-4000 cells in 12 nL</td>
<td>Four Gram-negative strains with MHB</td>
<td>&lt; 1h</td>
<td>MIC Determined using VITEK ®</td>
<td>High (Microscopy)</td>
<td>24</td>
</tr>
<tr>
<td>Laser Ablation Electrospray Ionization Mass Spectrometry</td>
<td>1.5–2.5 cells per 1000 µm2</td>
<td>Disk</td>
<td>21h</td>
<td>Compared with MIC database</td>
<td>High (Microscopy)</td>
<td>25</td>
</tr>
<tr>
<td>Nanomotion sensor</td>
<td>OD= 0.5</td>
<td>Clinical B. pertussis</td>
<td>3h</td>
<td>Compared with BMD</td>
<td>High (AFM setup)</td>
<td>26</td>
</tr>
<tr>
<td>Nanomotion-AST</td>
<td>100 cell/mL</td>
<td>Bacteria with human blood</td>
<td>&lt; 3h</td>
<td>N/A</td>
<td>High (AFM setup)</td>
<td>14</td>
</tr>
<tr>
<td>BacterioScan</td>
<td>$10^5$ cell/mL</td>
<td>Bacteria with MHB</td>
<td>6h</td>
<td>N/A</td>
<td>Low</td>
<td>27</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>-----</td>
<td>-----</td>
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<td>----</td>
</tr>
</tbody>
</table>

*MHB: Mueller-Hinton Broth; AFM: Atomic Force Microscopy*

1.4 Outline of Thesis

This thesis discusses design and development of a RAST technique based on dynamic laser speckle imaging (DLSI) and machine learning algorithms to achieve AST results in one hour. This work was supported through the Materials-Life Science Convergence Award supported by the Penn State’s Materials Research Institute, Huck Institute of Life Sciences, College of Engineering, and College of Medicine (Hershey). The outline of the thesis is as follows:

Chapter 2 discusses the DLSI setup, optimization, and utilization of this method as a novel RAST technology. Section 2.1 first introduces the principle of operation of DLSI and its application in life science and biomedical field. Then Section 2.2 discusses the optical setup and optimization. Section 2.3 summarizes the results of DLSI. Finally, Section 2.4 concludes the chapter. The contents of Chapter 2 is adapted/reproduced from Ref. 28.

Chapter 3 discusses data processing and development of a machine learning algorithm to process the time-resolved DLSI data, predict susceptibility vs. resistance of *E. coli* against two model antibiotics, and determine MIC based on cellular motion in 60 minutes. Section 3.1 provides a brief introduction on machine learning and its application in life science. Section 3.2 discusses the data processing procedure, the artificial neural network development, and the AST results obtained using machine learning analysis of the DLSI data. The chapter concludes in Section 3.3. The contents of Chapter 3 is adapted/reproduced from Ref. 28.

Chapter 4 provides a summary of the contributions of this thesis and an outlook for further development and utilization of the DLSI technique in biomedical applications.
1.5 List of Associated Publications

Journal article


Patent


Conference presentation

Chapter 2

Dynamic Laser Speckle Imaging for Rapid Antimicrobial Susceptibility Testing

In this Chapter*, we discuss the dynamic laser speckle imaging (DLSI) and utilize this method to design a novel RAST method. In Section 2.1, we first introduce the principle of operation of DLSI and some of its applications reported in the literature. Then we will discuss the DLSI setup and optimization to enable highly accurate and rapid AST using machine learning (discussed in Chapter 3). In Section 2.3, we will discuss the experimental results. Finally, we will conclude the chapter in Section 2.4.

2.1 Introduction

Compared to optical methods that visually assess changes in shape, length, and/or motion of bacterial cells, measuring light scattering off of bacterial populations is a simpler method that eliminates the need for advanced optical setup. One of such methods is laser scattering, which has been utilized in developing RAST. For example, BacterioScan system (BacterioScan Inc.) measures optical density of a liquid sample as well as the low-angle laser scattered intensity which enables measuring significantly lower OD levels compared to traditional ratiometric transmittance measurement. However, this method is based solely on OD, which does not provide for substantial improvement of the RAST turnaround time (~ 6 hrs).

In order to further reduce the detection time, we utilized dynamic laser speckle imaging to quantify bacterial micromotion and correlate the inactiveness in micromotion with the inhibitory

* The contents of this chapter are adapted/reproduced from Ref. 28.
effects of antibiotics. Light impinging on turbid media, such as biological tissues, experiences multiple scatterings. Interference of the scattered beams produces a speckle pattern. Scattered light from a static turbid medium generates constant laser speckle patterns because of its deterministic nature. On the contrary, if particles in the media instinctively move, the scatter pattern will vary with time. hence, a dynamic pattern is generated. Collecting time-varying speckle patterns contains information can be used to understand the effect of various environmental triggers on their motion.30

Dynamic laser speckle imaging (DLSI) has been widely used in monitoring mobility of particles in optically inhomogeneous media by analyzing time-varying laser speckle patterns. Murialdo et al. used DLSI to detect different degrees of motility and chemotaxis in bacteria swarming plates. Ramirez-Miquet et al. proposed a technique combining speckle imagining with digital image information technology (DIT) to track multiplying E. coli cells and S. aureus cells deposited on agar plates at high concentration of $1.5 \times 10^9$ cell/mL and $10^9$ cell/mL, respectively.31 They compared the mean activity of each pathogen after exposed to antibiotics for 15 mins, showed that speckle imagining method has the potential as a rapid pathogen assessment method.

Despite these efforts, to the best of our knowledge, there is no report of an application of time-resolved dynamic laser speckle imaging for antibacterial susceptibility testing in liquid samples. Moreover, previously reported assays are not sensitive enough for rapid analysis of cells at approximately $5 \times 10^5$ cell/mL, a concentration required by the gold standard protocols. Here, we show that analysis of the time-resolved dynamic laser speckle images using artificial neural network (ANN) was able to identify the MIC of ampicillin and gentamicin for E. coli K-12 in only 60 minutes with high accuracy comparable with gold standard methods using a voting strategy for the ANN predictions. The predictions were validated using gold standard broth microdilution. DLSI eliminates the need for advanced microscopy systems, hence significantly simplifying the AST.
2.2 Experimental Setup and Method

A schematic of the optical measurement setup consisting of a laser source, a lens, a cuvette holder, and a camera is shown in Figure 2-1 with the physical photo. A helium-neon laser (wavelength: 632.8 nm, power: 0.8 mW, HNLS008L, Thorlabs Inc., USA) was used as an illumination source. The laser beam was slightly expanded by using a concave lens (focal length=25.0 mm) before illuminating a cuvette (Fisherbrand, CAS#14-955-129) containing 3 mL of bacterial suspension. The resultant speckle pattern was captured by a CMOS camera (Zyla, ANDOR), that was controlled by a computer.

Distances between the components of the system are listed in the Table 2-1. We have optimized the setup in terms of the distance between different components and the scattered light angle, θ. We investigated three configurations: Setting #1-3. The important parameter in the setup is the angle between the axis of the camera and the laser beam (θ). The setup with Setting #3, which collects the speckle patterns at 11°-22° scattering angle, provides high sensitivity for laser speckle imaging (consistent with the Mie scattering model) in the current study, while also prevents the direct incidence of the laser beam on the camera.

To evaluate the performance of DLSI, we studied the effect of a β-lactam ampicillin and an aminoglycoside gentamicin on Escherichia coli strain K-12 as a model microorganism. We first used a common laboratory method to grow the E. coli, and then capture the time-resolved speckle patterns by DLSI. Finally analyze the data and compare the results with standard AST methods. Specifically, the culture grown was adjusted to $5 \times 10^5$ CFU/mL was further incubated at 37°C, 210 rpm for 1 h to reach the logarithmic growth phase before an antibiotic was added to the culture in concentration equal to 0×MIC (control), 0.5×MIC, 1×MIC, and 2×MIC. Three mL of each culture was collected at each time point, $t_i$ ($i = 0, 1, ..., 4$) at 0, 30 min, 60 min, 90 min, and 120
min, respectively, and transferred into the cuvette used for DLSI in the imaging system described above. At each \( t_i \), a dynamic speckle patterns series (16 bits, \( 1000 \times 2000 \) pixels, 50 frames per second) were collected. The exposure time for each frame was 1 msec (millisecond) and the full capture period was 10 sec at each time point, \( t_i \). The OD\(_{600}\) of bacteria suspension is obtained using a BioPhotometer D30 (Eppendorf, Haupauge, NY). All experiments were conducted in independent triplicates.

Figure 2-1. Schematic and optical image (top-left corner) of the setup for dynamic laser speckle imaging. \( a \): distance between the lasers and lens. \( b \): distance between the lens and cuvette. \( c \): distance between the cuvette and camera. \( \theta \): the angle between the camera optical axis and the laser beam.

Table 2-1. The optical setup parameters studied for optimization of the developed AST method.

<table>
<thead>
<tr>
<th>Setting</th>
<th>( a )</th>
<th>( b )</th>
<th>( c )</th>
<th>( \theta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>9 cm</td>
<td>6 cm</td>
<td>8 cm</td>
<td>20°</td>
</tr>
<tr>
<td>#2</td>
<td>9 cm</td>
<td>6 cm</td>
<td>4 cm</td>
<td>10°</td>
</tr>
<tr>
<td>#3</td>
<td>9 cm</td>
<td>5 cm</td>
<td>6 cm</td>
<td>15°</td>
</tr>
</tbody>
</table>
2.3 Results and Discussion

The raw speckle images of Setting #1-3 are shown in Figure 2-2, 2-3 and 2-4, respectively. In Setting #1, the scattered light is too weak that it can only distinguish the control group and experimental group but fail to give the susceptible test results.

![Fig 2-2. Raw speckle images obtained using Setting #1.](image)

To improve the resolution of our system, we moved the camera slightly closer to the cuvette and decreased the angle $\theta$. This time, the speckle images are clearer compared with the previous setting because the light intensity increased. But it brought new problem that the intensity is out of the detection range of the camera in some cases, e.g., in Figure 2-3, when after 3 hours, the image of the control group showed a uniform red color, meaning almost all the pixel got a very high intensity. It can damage the camera sensor and the data are not usable.
Figure 2-3. Raw speckle images obtained using Setting #2.

Figure 2-4. Raw speckle images obtained using Setting #3. Which gains the best results on machine learning analysis (discussed in Chapter 3).
To balance the intensity range, in Setting#3 we changed the parameter c and θ to average value of Setting #1 and #2, which collects the speckle patterns at 11°-22° scattering angle, provides high sensitivity for laser speckle imaging (consistent with the Mie scattering model) in the current study, while also prevents the direct incidence of the laser beam on the camera. The results presented in the next sections are based on this configuration.

Using Setting #3, we compared the DLSI data with the optical density (O.D.) measurement (which is a popular method to determine turbidity) and traditional colony counting method, as shown in Figure 2-5(a-c) with ampicillin and Figure 2-5(d-e) with gentamicin. The details of Microbiology Steps are provided in Appendix B. Intensity data measured using speckle imaging can differentiate between different antibiotic concentrations in 2 hours which is better than conventional O.D. measurements. The dashed lines in Figure 2-5(b) and (e) indicate the detection limit (0.05) of the O.D. measurement system.

**Figure 2-5.** Comparison of the optical density measurement (a), (d), the average intensity of the speckle images (b), (e), and the colony counting results (c), (f), with ampicillin and gentamicin, respectively.
2.4 Conclusion

The DLSI data was collected using a simple-to-use, low-cost optical setup, with no labeling or advanced imaging/optical setup required. However, simple intensity measurement is “blind” to the overnight (18 h) colony counting results, and hence prone to false positive; for example, determining ampicillin MIC to be 2 μg/mL (Figure 2-5(b)), while the correct MIC based on the overnight data is 4 μg/mL. The intensity measurement also neglects the time-resolved motility information of cells collected at each time point (i.e. 500 frames per second captured for 10 sec). Moreover, absolute intensity is sensitive to setup specification (such as laser power, exposure time, camera gain, etc.), and hence present challenge for calibration and expansion to a general case. In order to overcome this problem, in Chapter 3, we utilized machine learning and trained artificial neural network (ANN) using the overnight gold-standard results to analyze the time-resolved DLSI patterns and accurately determine MIC in 60 minutes.
Chapter 3

Machine Learning-Based Analysis of Dynamic Laser Speckle Imaging Data for Rapid AST

In order to overcome the shortcomings of AST based on analysis of absolute DLSI intensity (Chapter 2), in this Chapter*, we will use machine learning to process the time-resolved DLSI data and predict susceptibility and determine MIC based motion of the cells in 60 minutes. Machine learning offers systems the ability to automatically learn and improve their performance based on experience without explicit instruction. After a brief introduction about machine learning in Section 3.1, in Section 3.2 we will demonstrate the data processing procedure, the artificial neural network that was developed, and the AST results obtained using machine learning-based analysis of the DLSI data. Finally, we will conclude the chapter in Section 3.3.

3.1 Introduction

Machine learning (ML) spans a broad set of algorithms and statistical models that computer systems use to perform a specific task without using explicit instructions, relying on patterns and inference instead.32 As a subset of artificial intelligence, machine learning algorithms build a mathematical model based on sample data – known as "training data" – in order to make predictions or decisions without being explicitly programmed to perform the task. Machine learning algorithms are used in a wide variety of applications, such as email filtering and computer vision, where it is difficult or infeasible to develop a conventional algorithm for effectively performing the task. In life science, machine learning has been used extensively in genomics, proteomics, and other omics

* The contents of this chapter are adapted/reproduced from Ref. 28.
fields. Haas et al. conclude a review with successful setup of the omics experiments and the multitude of interactions between the multi-omic system. For example, using the genomes sequence to predict RNA and protein sequences, investigating the interactions between the metabolome and the proteome to identify the cellular mechanisms. More specifically, a recent study used the DNA sequences obtained from stool microbiota to predict postprandial glucose responses and give personalized dietary interventions based on the prediction.

3.2 Data Processing and Machine Learning Results

The raw images (1000 × 2000) were first resized (100 × 200) using the nearest neighbor method to reduce the computational cost. Fourier Transform (FT) was performed along the time axis of the measured data cube. Only the spectral intensity was used in our analysis since a spectral intensity distribution with significant high frequency content generally corresponds to more active motion, whilst the spectral phase is not directly linked to motion activeness and presents a challenge in quantitative interpretation. The DC term was first normalized to 1 for each individual pixel spectrum and was subsequently removed. Moreover, the negative frequencies provide identical information as their positive counterparts. Essentially, each measurement captures 20,000 spectra and each of the spectra contains 249 frequency features. This large amount of data provides a basis for machine learning based analytics.

We trained the ANN models with same data in Section 2.2. The raw images (1000 × 2000) were first resized (100 × 200) using the nearest neighbor method to reduce the computational cost. Fourier Transform (FT) was performed along the time axis of the measured data cube. Only the spectral intensity was used in our analysis since a spectral intensity distribution with significant high frequency content generally corresponds to more active motion, whilst the spectral phase is not directly linked to motion activeness and presents a challenge in quantitative interpretation. The
DC term was first normalized to 1 for each individual pixel spectrum and was subsequently removed. We conducted experiments in three independent replicates with groups exposed to four concentrations for each antibiotic. The four concentrations yielded a total of 80,000 spectra for each time point. As shown schematically in Figure 3-1, we split the samples randomly to training, validation, and test groups with a ratio of 70%, 15%, and 15%, respectively. The validation was carried out to avoid model overfitting, and the test group was used to verify the predictive power of the trained neural networks. Two independent experiments where each experiment was completed with four concentrations of the antibiotic resulted in a combined data size of 160,000 samples at each time point, were used in building the learning models. We then used a third, independent experiment that consisted of 80,000 spectra at each time point for a final comprehensive test to evaluate the robustness and accuracy of trained models.

The overnight turbidity of the bacteria culture was utilized to label the dataset. If the culture stays transparent as the initial broth, the bacterial group was labeled as “Inhibited”. On the other hand, if the culture changes turbid, indicating that the antibiotic did not inhibit the growth of the bacteria, the group was labeled as “Non-Inhibited”. In our experiments, 0.25× and 0.5×MIC were labeled as “Non-Inhibited”, while 1× and 2×MIC were labeled as “Inhibited”. For each group at a specific time point, $t_i$, since all the 20,000 pixel-level spectra have the same label, the result of the test should be interpreted as a voting process where the percentage of each prediction category indicates the likelihood for this group to be classified as the said category. For example, 30% predicted as “Inhibited” means that 30% out of the 20,000 spectra are predicted as “Inhibited”, while the remaining 70% is predicted to be “Non-Inhibited”. Therefore, this group is classified as “Non-Inhibited”. It should be noted that the ML method does not rely on the absolute speckle intensity which could be difficult to calibrate, as the DC frequency component is normalized to 1.

All the image pre-processing and machine learning computations were performed with a desktop computer (Intel Core i9-9900k, 32GB RAM).
The machine learning results for prediction of ampicillin and gentamicin susceptibility and MIC are shown Figure 3-2: The accuracy of both ampicillin (a) and gentamicin (c) are relatively low at first 30 mins, but it increase over 80% after expose to the antibiotics for an hour. Consider this accuracy was obtained from 20,000 pixel-level spectrum. We used a voting strategy to make final decision: use 50% as the voting threshold to predict antimicrobial susceptibility. We expect a classification “Inhibited” for $1 \times \text{MIC}$ and $2 \times \text{MIC}$, while at the same time, we expect to a classification “Non-Inhibited” for $0.25 \times \text{MIC}$ and $0.5 \times \text{MIC}$. For example, at 30 min, the pixel-level predicted percentage of classification into “Inhibited” or “Non-Inhibited” for $2 \times \text{MIC}$ of ampicillin is approximately 50%, making it difficult to decide which category the sample is more likely to fall into. While at 60 min, the correct category (“Inhibited”) is predicted in more than 80% of the votes. Interestingly, by comparing Figure 3-2 (a) and (c), it is observed that the method can predict MIC for gentamicin in 30 min (compared to 60 min for ampicillin). As a result, in order to
confidently identify MIC in current experiments, a minimum of 60 min is required by DyRAST for accurate analysis. It is worth noting that by counting viable cells using the broth microdilution technique (a sensitive yet slow and laborious method), differentiation among 0.5×MIC, 1×MIC and 2×MIC is possible only after 5 hours for ampicillin and after 2 hours for gentamicin (see Figure 2-5 (c) and (f)).

![Figure 3-2.](image)

Figure 3-2. The prediction accuracy of machine learning algorithm improves with time for ampicillin (a), (b) and gentamicin (c), (d).

### 3.3 Conclusion

In summary, after using machine learning method and adapting a voting strategy for analysis of the pixel-level prediction values obtained using artificial neural network models, the method can predict MIC with a high accuracy comparable with the gold standard method in all
tested conditions, but just in 60 minutes (as opposed to the gold standard which requires at least 16 hours). By leveraging machine learning, the method overcomes the shortcoming of simple intensity data collected using DLSI, yet keeps the simple-to-use and cost-effective setting. Compared with the gold-standard broth microdilution method or other FDA-approved methods (e.g. Accelerate Pheno™ system), this method achieves the AST results in just one hour (at least a 6x improvement in detection time). In addition, by training a machine learning algorithm with the overnight turbidity data, the present method eliminates the possible adaptation of bacteria to antibiotics.
Chapter 4

Thesis Summary and Outlook

This work presents a rapid, phenotype-based antibacterial susceptibility testing method capable of identifying MIC in 60 minutes. The method leverages machine learning analysis of time-resolved dynamic laser speckle imaging patterns to predict antimicrobial susceptibility and MIC in a rapid and reliable manner. The DLSI data was collected using a simple-to-use, low-cost optical setup, with no labeling or advanced optical setup required. To demonstrate the capabilities of the method, we studied the effect of two antibiotics, ampicillin and gentamicin, which have different mechanisms of action. DLSI captures change of bacterial motion/division in response to antibiotic treatment. The method was validated against the gold standard AST methods using *E. coli* K-12 as a model microorganism. By adapting a voting strategy for analysis of the pixel-level prediction values obtained using artificial neural network models, DyRAST can determine MIC with high accuracy comparable to gold standard methods, yet at least 16 times faster, in all tested conditions.

The future works based on this technique can be optimization for analysis of other pathogenic bacterial and fungal species and their response to antimicrobial treatment. The envisioned automated platform including identify, separate the isolate and then perform AST will be compatible with existing clinical laboratory measurements, e.g. 96 microwell plates and petri dish. The test can be multiplexed to analyze multiple antibiotics at once, giving patients the ideal antibiotic in a faster time. In addition, considering no complicated optical components are required for DLSI, a portable version of this method can be potentially adapted by using consumer-level components, such as smartphone camera and laser diodes. It was reported the next generation iPhone will integrate a laser with the imaging system. That means the data collection processing
and results prediction can be done by a smartphone, permitting applicability at point of care and remote location of this system.
REFERENCES


9. Ellington, M. J. et al. The role of whole genome sequencing in antimicrobial susceptibility...


Appendix A

List of Abbreviations

Antimicrobial Resistance ................................................................. AMR
Antimicrobial Susceptibility Testing .................................................. AST
Dynamic Laser Speckle Imaging ...................................................... DLSI
Fluorescence in Situ Hybridization .................................................. FISH
Intensive Care Unit ................................................................. ICU
Machine Learning .............................................................................. ML
Optical Density ................................................................................ O.D.
Polymerase Chain Reaction .......................................................... PCR
Rapid Antimicrobial Susceptibility Testing ........................................ RAST
Recombinase Polymerase Amplification ............................................ RPA
Appendix B

Microbiology Protocols

**Bacterial culture:**

*Escherichia coli* (*E. coli*) strain K-12 was used as a model bacterial strain in all experiments. The culture was stored as a frozen stock at -80 °C and resuscitated every 14 days to maintain a fresh inoculum. The culture was resuscitated by streaking onto Muller Hinton Agar (MHA) and was then incubated at 37 °C 20+/−2 h. A single colony from the MHA agar was re-streaked on another MHA plate and incubated at 37 °C 20+/−2h. A single colony from the MHA sub-streak plate was inoculated into 10 mL Muller Hinton Broth (MHB) and incubated at 37 °C, shaking at 210 rpm, for 20+/−2 h. The overnight culture was diluted in MHB based on the OD$_{600}$ to obtain $5 \times 10^5$ CFU/mL using a BioPhotometer D30 (Eppendorf, Hauppauge, NY). An OD$_{600}$ of 1 was assumed to be equal to $8 \times 10^8$ CFU/mL.

**Preparation of the antibiotics:**

Ampicillin (Sigma Aldrich, CAS# 7177-48-2) and gentamicin (Sigma Aldrich, CAS# 1405-41-0) stock solutions were prepared by dissolving antibiotic powder in sterilized MilliQ ultrapure water to achieve 5 mg/mL and 10 mg/mL stock solutions, respectively. All the stock solutions were frozen in 0.1 mL aliquots and stored at −20 °C.

**Antimicrobial susceptibility testing using broth microdilution:**

The $5 \times 10^5$ CFU/mL culture was used for broth microdilution and speckle imaging. For broth microdilution, the standard methods recommended by the Clinical and Laboratory Standards Institute (CLSI) guideline M100-S22 were followed to determine the MIC of ampicillin (AMP) and gentamicin (GEN) for *E. coli* strain K-12. Briefly, 50 μL of MHB were aliquoted in wells of
96-well microtiter plates (Greiner bio-one). Antibiotics were added to the wells of the first row and sequentially diluted two-fold down the row. 50 μL of culture prepared as described above were added to each well and incubated for 16-20 hours. Negative and positive controls were included in each test plate and each test was carried out in a triplicate in at least two independent experiments. MIC was determined by visually inspecting wells for turbidity resulting from culture growth.

**Verifying antimicrobial susceptibility using time-kill kinetics:**

To determine the time-kill kinetics of *E. coli* K-12, $5 \times 10^5$ CFU/mL culture was first grown for 1 hour at 37°C in a shaking condition (210 rpm) and was then exposed to antibiotic concentrations of 0×MIC (control), 1×MIC, and 2×MIC determined via broth macrodilution. Viable cells were quantified at times 0, 1, 2, 3, 5, 8, 16, and 24 hours by spiral plating (easySpiral, Interscience) with 10-fold dilution onto MHB agar. Inoculated plates were incubated at 37°C for 16-20 hours and counted to determine the CFU/mL at each time point. All experiments were completed using three biological replicates and three technical replicates per plate.