Convolutional Neural Network Identification of Heteroresistance in E. Coli

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Abstract

Maintaining the efficacy of current antibiotic treatments is of utmost importance. Rapid detection mechanisms are needed to combat a global increase in antibiotic-resistant infections. We propose a machine learning classifier that utilizes microscopy data to classify resistance at the cell level. While other methods use change in cell area as a proxy for resistance, we build upon previous work showing that calcium fluorescence and cell membrane potential are earlier phenotypic markers of resistance.

Introduction

Antibiotic resistance is a persistent problem in the clinical treatment of bacterial infections. The overuse and misuse of antibiotic drugs have led to an increasing proportion of clinical cases that are resistant [6]. The deployment of antibiotic susceptibility tests (AST) that identify which drugs will and won't be effective on a specific infection is needed to combat this problem. In addition, the use of AST can prevent ineffective drug prescription, increasing the resistance of an infection []. In addition, AST identifies the best treatment on a patient-specific resolution, leading to better patient outcomes. However, current AST methods still have room for improvement. The two methods of AST most common in the literature are genomic, and imaging-based solutions [5]. Many genomic-based solutions require sequencing, which is both time and resource intensive. Imaging approaches seek to identify phenotypic indicators of resistance to classify bacteria. One drawback that both methods share is the necessity of a 24h preculture step to provide the method with enough bacteria to make a classification as to the resistance of the population [5]. Clinical infection is defined as any concentration greater than 100 CFU/mL urine [4]. This paper will not address the preculture bottleneck of AST but will highlight the importance of addressing this problem. All current AST requires this step, representing the most significant time overhead in determining antibiotic resistance. More research is required to find ways to shorten or eliminate this step. Current FDA-approved imaging-based methods, for example, VITEK2 (bioMérieux)and MicroScan WalkAway (Siemens Healthcare Diagnostics), classify samples as resistant or susceptible to a drug in at least (3.5–16h) [2]. More rapid methods like those developed by Accelerate Diagnostics can make predictions from a blood sample in an average of 7hr [7]. When considering specifically imaging-based approaches to AST, there is a distinction between methods that utilize changes in cell area as a proxy for antibiotic resistance versus those that leverage aspects of cell physiology to determine the minimum inhibitory concentration (MIC). Yu et al [12] utilized phenotypic changes in free-moving cells to

determine resistance. In addition, our previous work showing that calcium dynamics precedes cell growth as an early indicator of resistance [8] led us to distinguish between sensitive and resistant cells more rapidly than using cell area.

In this paper, we utilize our previous findings that calcium dynamics precede cell growth to create a machine learning model that can identify resistant/susceptible mixture populations. These heteroresistant populations are of interest because any cell area-based classification technique would be less accurate when determining a mixture of resistant and sensitive cells. Importantly, heteroresistance is suspected in cases where antibiotic treatment fails [3]. Pereira et al. showed that resistant subpopulations are enriched at sub-MIC concentrations. These enriched populations then quickly devolved to majority susceptible when antibiotic pressure was removed [10]. Current gold-standard techniques such as broth dilution methods often fail to identify resistant subpopulations [10]. Therefore, new tests are needed to meet the challenge of clinical identification of heteroresistance. We show that our model can make accurate predictions at the single cell level, and the model can determine the overall proportion of cells in a population resistant to a specific drug. Our methods include calcium fluorescence imaging, rapid image preprocessing, and machine learning to create an accurate classifier of heteroresistant populations of bacteria.

Materials and Methods

All cells were cultured overnight (>12h) in Luria-Bertani (LB) broth. The E. Coli containing carbenicillin-resistant plasmid were cultured in LB with 100ug/mL carbenicillin. Out cultures were performed for 2.5h in Plantarum Minimal Media (PMM) with no added drug. The PMM recipe was as follows; 7600 parts deionized water, 20 parts 1M Magnesium Sulfate (ID), 200 parts 20% glucose (ID). 200 parts 50x MFM Amino Acids (ID), 1 part 1M Calcium Chloride (ID), 2000 parts 5x M9 Salds (ID). At 2h, 1:200 of 50uM SYTOX™ Orange nucleic acid stain (Thermo Fisher) was added to out cultures to assist in cell segmentation.

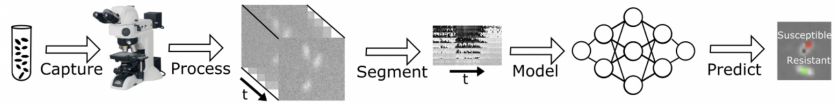
Imaging was performed by immobilizing cells under a 2% Agarose PMM pad on a 96-well glass plate (ID). Agarose SFR™(VWR Life Sciences) was dissolved in PMM (2g/100mL = 2%). All Agarose pads contained 10uM of Thioflavin T (ThT) fluorescent die (ID) and appropriate drug concentration. The Kanomyacin training data was at a concentration of 500ug/mL drug. The titration for both Kanomyacin and Carbenicillin was performed at concentrations of 25, 10, 5, 3, and 1ug/mL. Titration for Polymixin B was performed at concentrations of 10 and 1ug/mL. Controls of 0ug/mL were performed for all of these experiments. After these concentrations were added to liquid agarose and mixed, agar solutions were pipetted onto a 3D printed mold of the 96 well plate. These were covered with glass plates to make the pads uniform in shape. These pads were allowed to dry for at least 0.5h. Scientists flipped the molds, and 2uL of cells were added and allowed to spread over the pad for 10min. The scientist flipped the mold again, and pads were pressed onto the 96 well plates, fully contacting the glass bottom. Cells were immobilized between the glass bottom of the plate and the agarose pad. The scientist recorded the time between plating cells and the beginning of imaging to determine the interval of exposure to a drug before imaging.

Imaging was performed on Nikon Ti inverted microscope with capture performed by Slidebook software (3i Inc). Laser wavelengths of 488nm and 561nm were used to excite ThT and SYTOX™ dies, respectively. We used a 40x NA 0.95 air objective for all imaging.

Training data for model speed was performed using BW25113 (ID) and mntH KanR (ID) cell lines. We measured four conditions. We captured 1 image/min for intervals of 120, 240, and 480min depending on the experiment.

We saved images as sequential 16-bit TIF (.tif) files via the Slidebook software. Jitter correction via Fourier image registration was performed using the Python programming language (3.9) the Scikit-Image [11] and Imreg-DFT [] packages. Segmentation was performed using the determinant of hessian (DOH) blob segmentation in the Scikit-Image package. Centroids were determined for each segmented area, and the computer took a small circle to be the trace of each segmented cell. These traces were then transformed into .png images, where each column represents the trace of one pixel in the centroid at each timepoint.

Fig. 1 Experimental and Computational Procedure



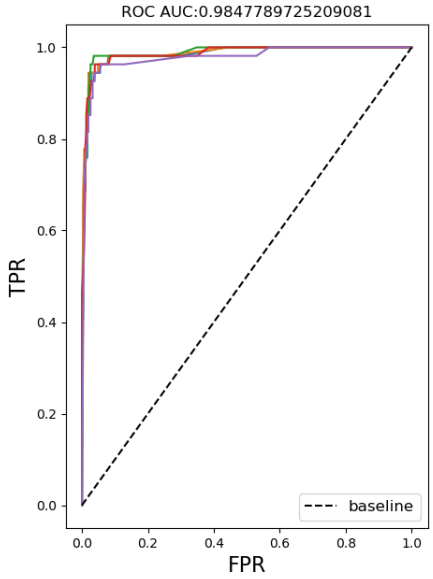
We labeled these images as sensitive or resistant for training data based on known genotype. We shuffled the data given to the model randomly so that a particular condition from a specific experiment would only appear in the training or validation/test set and not any two or three of these sets. This methodology was essential to ensure the model did not classify based on features specific to perturbations in each experiment.

A convolutional neural network (CNN) was built using the Tensorflow (2.5) package by Google Inc [1]. We compared results for accuracy and ROC AUC across architectures, and linear discriminant analysis (LDA) model was realized using the Scikit-Learn [9] package.

Results

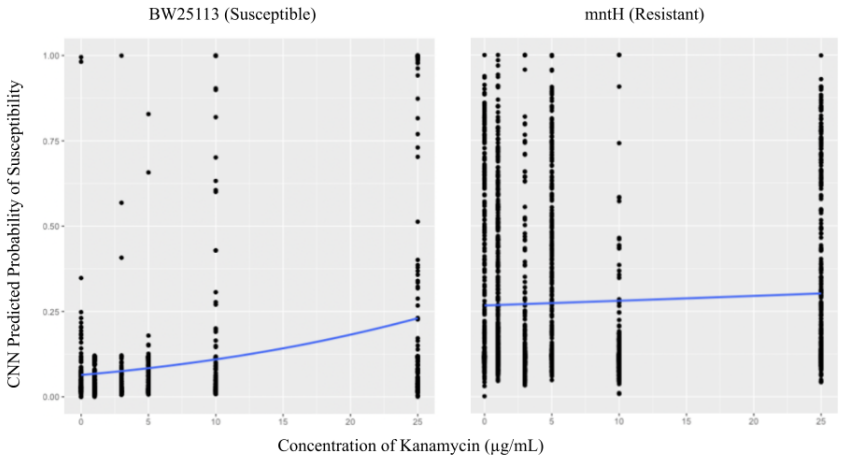
The model attained high accuracy (>0.95) on 2h video footage of training data for Kanomyacin. Notably, the model could distinguish only between treated susceptible cells and the three other conditions tested (untreated susceptible, untreated resistant, and treated resistant). The model did not differentiate between untreated susceptible and treated resistant cell lines. This result implies the features learned by the model were unique to the treated susceptible line and not more generally unique to the susceptible line. The training data represented an ideal case, where all cells in each well were known to have a genotype that allowed either resistance or susceptibility.

Fig. 2 Receiver Operating Characteristic for CNN Model



Next, this model was applied to predict the susceptibility of sensitive and resistant cells when exposed to different levels of Kanomyacin. The concentrations of drug tested were 25ug/mL, 10ug/mL, 5ug/mL, 3ug/mL, 1ug/mL and 0ug/mL. Scientists added the drug to the agarose pad, and cells were exposed for 0.5h to the drug before imaging began due to experimental considerations.

Fig. 3 Application of CNN Model to Novel Data



The model performed modestly on previously unseen data at different concentrations of Kanamycin. However, the model could differentiate between susceptible cells at lower and higher concentrations of Kanamycin. The fact that it failed to classify resistant cell lines correctly is likely due to the nature of the training data. Qualitatively, cells that are treated with $500\mu g/mL$ of Kanamycin are visually different than those treated at $25\mu g/mL$.

Overall, these results demonstrate that it is possible to identify resistance in mixed populations of cells rapidly. This technology has myriad applications in research and medical settings. These include but are not limited to drug prescriptions, environmental sampling, education, and remote antibiotic prescription.

Discussion

In our previous work [8] we showed that cell calcium fluorescence preceded cell area in predicting antibiotic resistance. By applying a neural network to this metric, we sought to create the most predictive model in the shortest time. As noted in [] the time in which antibiotic tests are performed is of utmost importance in clinician decision-making around antibiotic prescription.

Features are measurable properties of a data point. For example, a feature of a cat picture would be the prominent triangular ears. A convolutional neural net can detect these by something approximating an edge detection filter.

Neural networks work by self-learning essential features of a dataset to gain a high predictive ability. This differs from other modeling techniques that require features to be pre-generated before the modeling process begins. Therefore, machine learning is a double-edged sword, as the model can learn features intrinsic to how researchers acquired the data instead of the desired characteristics. On the other hand, it will also potentially find features that researchers could not have imagined. Another drawback of neural networks is their poor interpretability. It is difficult for humans to fully understand the numeric optimization that caused a model to generate the features it did or determine their importance. For this reason, researchers should not apply neural networks in areas where the predictive characteristics of a process are poorly understood. Instead, neural networks can be best used where the processes are understood but difficult to quantify into features. Image recognition is a prime example of this, with neural networks performing better than humans at some image classification problems [].

Our previous work showed that calcium fluorescence preceded and predicted better than predicted better than cell area. The features that were identified as necessary by the support vector machine, moving standard deviation with a 25min window and 49min window, were ripe to be

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