Automatic Image Analysis for Rapid Drug Susceptibility Testing

Andrey Grushnikov,*, # Kazuma Kikuchi, Yoshimi Matsumoto, Takeo Kanade, Yasushi Yagi*

Abstract In recent years, a rapid increase in bacterial strains resistant to modern antibiotics has been observed. This alarming rise in drug-resistant organisms has emphasized the importance of identifying new effective antimicrobial agents. Since traditional approaches for drug susceptibility testing are time-consuming and labor-intensive, more efficient methods are urgently needed. Here, we report an automatic image analysis system for drug susceptibility testing that provides results within 3 hours using a drug susceptibility testing microfluidic (DSTM) device. The device consists of five sets of four microfluidic channels prepared by soft lithography. The channels are in close proximity to allow simultaneous observations. The antimicrobial agent and bacterial suspension to be tested are added to the channel and incubated for 3 hours. Previously, microscopic images of the DSTM device were analyzed manually by an expert to evaluate the susceptibility of a strain. In this work, we present an automatic computer vision algorithm for processing images and performing analysis. The algorithm enhances the quality of the input image, detects cells in each channel, extracts a variety of cell-related characteristics, and estimates drug susceptibility using a pre-trained support vector machine. We address the issue of overlapping cells by incorporating a graph-based cell separation algorithm. The minimum concentration of a drug for which the proposed method predicted susceptibility represents the minimum inhibitory concentration (MIC). The novel method was implemented as a standalone application and tested on a dataset containing images of 101 clinically isolated strains of Pseudomonas aeruginosa incubated in the presence of five different drugs. The estimated MICs correlated well with the results obtained using the conventional broth microdilution method.

Keywords: susceptibility testing, microscopy image analysis, image processing, support vector machine.


1. Introduction

The recent increase in bacterial strains resistant to multiple antimicrobial agents is alarming. Resistant microorganisms such as Pseudomonas aeruginosa have already caused nosocomial outbreaks in Japan and other countries [1, 2]. Despite considerable research efforts to determine the genetic basis for drug resistance in bacteria, no significant progress has been achieved. This is mainly due to the weak correlation between the expression levels of resistance genes and the actual resistance levels in bacterial strains, making it difficult to identify intrinsic genes responsible for drug resistance [3]. This emphasizes the necessity to develop alternative methods for drug susceptibility testing that are rapid and accurate.

Traditional approaches such as optical density and colorimetric methods require long incubation periods (often overnight) for the control culture to become visible. To overcome this issue, a novel drug susceptibility testing microfluidic (DSTM) device has been developed [4, 5]. The DSTM device consists of five sets of four microfluidic channels (100 μm wide and 50 μm deep) printed in polymer on a glass cover by a conventional soft lithography process. The channels in each set share an inlet hole and are gathered in such a way as to allow concurrent observations (Fig. 1). This design facilitates simultaneous testing of five antimicrobial agents at three different concentrations plus the control.

In our previous report [4], the structure of the DSTM device was introduced for the first time. We presented that the device can be used for successful evaluation of the effects of inhibitors on multidrug efflux pumps. Using microfluidic devices with different shapes, we attempted to develop a procedure for determining the susceptibility of bacterial isolates by observing visible changes in cell growth during incubation. This procedure was initially carried out manually. This approach provided useful qualitative information concerning alteration in cell number or morphology. However, it was labor-intensive, prone to errors due to subjective biases, and lacked reproducibility. To overcome these drawbacks, we focused on developing dedicated image analysis software to provide tools for quantitative analyses. We accomplished this goal, and a brief overview of an image analysis system that we designed and results of testing using P. aeruginosa were presented in a report [5]. The automatic system was primarily used for evaluating cell features, while the decision of strain susceptibility was done by a human expert.

In the present study, we extended the software by adding an algorithm for automatic analysis of cell characteristics and estimation of susceptibility using a support vector machine (SVM). Here we give a detailed explanation of the final design of the algorithm and present results of rigorous testing.

The input image for the proposed algorithm is a photograph of one set of the DSTM device microfluidic channels, captured with a camera attached to a phase contrast microscope. The method automatically locates each channel, detects cells, extracts a
variety of cell characteristics, and estimates the susceptibility of the strain to each concentration of the test drug with a pre-trained SVM. At the final stage, the device determines the MIC.

The most challenging and important step of the algorithm is cell detection. Following exposure to certain drugs, some bacteria appear elongated, causing partial overlap or occluded. We addressed this issue by including a procedure for separating such cells.

The algorithm we developed for MIC estimation is straightforward and applies thresholding to extract cells from the background. Simplicity of the algorithm achieves high computational efficiency without decreasing accuracy of estimation.

2. Drug susceptibility testing using the DSTM device

To determine the MIC of a drug using the DSTM device, firstly, the drug is added at three different concentrations into three test channels of a channel set. The remaining channel in the set is the control sample for observation. The drug is then dried and a bacterial solution is added to all the channels using a micropipette. Empirical observations show that despite the variable growth rate of bacterial cells after 3 h of incubation, differences in cell morphology become visible and can be inspected by phase contrast microscopy [5]. These differences in shape, number, and other characteristics of the bacterial cells (Fig. 2) in the test channel compared with the control signify that the input strain is susceptible to the test drug at a particular concentration. The minimum concentration of the drug to which the strain is susceptible constitutes its MIC.

3. Algorithm

The algorithm developed for quantitative analyses and susceptibility assessment of a bacterial strain from a microscopic image of the DSTM device channels is divided into five stages: noise reduction, localization of the channel region, cell detection, feature extraction, and susceptibility estimation using the SVM (Fig. 3).

3.1 Noise reduction

During the first stage of the algorithm, the input image is corrected for brightness variation caused by uneven illumination, a com-
mon problem with phase contrast microscopy. Since the light intensity can be assumed to be quadratic [6], it is calibrated by fitting a two-dimensional quadratic polynomial and then subtracting the calculated surface from the original image.

3.2 Channel localization
The second stage of the algorithm is the localization of the regions in the input image occupied by the microfluidic channels. The DSTM is placed under a microscope manually without any precise control of alignment, orientation or position of the channels. Thus, it is necessary to identify their positions individually for each input image.

To achieve this, we detect channel border candidates by performing Hough transform for the output of the Canny edge detector applied to the original image. Among all the lines detected, only those satisfying additional constraints, i.e. equality of channel widths and parallelism, are chosen.

3.3 Cell detection
For each channel located during the second stage, cell detection is performed. Since bacteria display considerably lower intensity than the background, we extract pixels corresponding to cells by applying adaptive thresholding technique proposed by Otsu [7]. Cell candidates are obtained by utilizing connected component labeling. Then, the procedure is performed to separate overlapping cells.

For the binary image acquired in the first stage, thinning is applied. Then, for each connected component, points of intersection are located. We model the thinned connected component as a graph where end points and points of intersection become vertices. Next, a set of all possible routes connecting each of the two vertices is built. For each route, the smoothness constraint is checked, and the route that does not satisfy the constraint is removed from the set. The smoothness constraint is defined as the maximum allowed angle between nodes. A set of routes with the maximum number of nodes to cover the entire graph is chosen. The identified routes represent individual cells. Finally, for all pixels removed in the thinning stage, a corresponding cell label is assigned.

3.4 Feature extraction
During the previous step, the algorithm labels all the cell pixels allowing the extraction of various morphological cell features to analyze the effect of a particular concentration of drug. The features chosen represent two typically observed changes in response to drug exposure: cell elongation and decreased cell number. The calculated characteristics include total number of cells, length statistics (average, deviation, min, max), area statistics (average, deviation, min, max, ratio of area occupied by cells to total channel area), and an eight-bin histogram representing the occurrence of cells of particular lengths. Since the extracted features need to represent changes in cell growth in the test sample in relation to the control sample, all calculated statistics are divided by the corresponding statistics of the control sample.

3.5 Susceptibility estimation with SVM
Cell features extracted in the previous step are used to determine whether the strain is susceptible to a particular concentration of the drug using a pre-trained SVM. SVM is a widely used technique commonly applied to solve binary classification problem. To separate data into two classes, SVM builds a separation hyperplane between classes so that the distance from it to the nearest data points is maximized [8]. We use a soft-margin SVM that allows classification of non-linearly separable classes.

4. Implementation
We developed software implementation of the algorithm described above in Java. It provides a graphical interface to facilitate analysis of the input images (Fig. 4). The software is capable of processing a single image or a batch of files one-by-one in a fully

![Graphical interface of the developed software. Calculated data can be viewed using the interface and saved in a format supported by Excel.](image-url)
automated manner. To increase the execution speed of the algorithm, it is parallelized to allow simultaneous detection of all channels presented in the image. The average time for processing a single input microscopic image does not exceed one minute (Core i7–6700K, 4 GHz, 4 Cores, 16 GB RAM). The code can be accessed via GitHub link (https://github.com/Akazora/ACA-RaST/tree/master/ACA-RaST).

5. Performance evaluation

5.1 Materials and reagents

The DSTM used in our study consisted of four microfluidic channels. The channels were printed by a conventional soft lithography method [9] in polydimethylsiloxane (PDMS; Silpot184, Dow Corning Toray Co., Ltd., Tokyo, Japan) on a glass cover (Matsunami Glass Ind., Ltd., Osaka, Japan) [4]. *Pseudomonas aeruginosa* was chosen for the tests as the species is inherently resistant to many antibiotics and disinfectants [10].

Five antimicrobial agents were selected: amikacin (AMK; Nichi-Iko Pharmaceutical Co., Ltd. Toyama, Japan), ciprofloxacin (CIP; Meiji Seika Kaisha, Ltd., Tokyo, Japan), meropenem (MPM; Meiji Seika Kaisha, Ltd.), ceftazidime (CAZ; Sawai Pharmaceutical Co., Ltd., Osaka, Japan) and piperacillin (PPC; Toyama Chemical Co., Ltd., Tokyo, Japan). These drugs were applied to 101 clinically isolated strains at three concentrations: AMK 4, 8 and 16 mg/L; CIP 1, 2 and 4 mg/L; MPM 1, 2 and 4 mg/L; CAZ 4, 8 and 16 mg/L; and PPC 4, 8 and 16 mg/L.

5.2 Dataset

To construct the dataset, microscopic images were obtained for each of 101 strains after 2 and 3 h of incubation in the presence of one of the five drugs. Images were captured with MIR-MDCE-5C USB digital camera attached to a phase contrast microscope MT4210L with a 10× objective lens. The photographs obtained were JPEG images with resolution of 1280×1024 pixels. The reference MIC for each strain was obtained using the conventional broth microdilution method.

5.3 Accuracy of cell detection

To measure the reliability of the cell detection method, we compared the number of cells counted manually with those detected by the algorithm. For this purpose, we used 25 images, providing 100 sample channels, and counted the number of visible bacteria. A scatter plot representing the relationship between the number of cells identified automatically with those detected manually is presented in Fig. 5. The average accuracy of cell detection was 93%. Misdetection of cells mainly occurred in highly overlapped regions where the algorithm labeled fragments of elongated cells as individual cells (Fig. 6).

5.4 Susceptibility estimation

To predict susceptibility of a bacterial strain to a particular concentration of a drug, we trained separate SVMs for each antibiotic. To determine if a drug had an effect, various extracted cell features were used. Testing was performed with cross validation, choosing 80% of the dataset for training and 20% for testing. Average accuracy of susceptibility estimation exceeded 90% (Table 1).

Next, we focused on attempting to determine which features are the most useful for susceptibility determination. We trained SVM on the following feature sets: (F1) length statistics, (F2) area statistics, (F3) count and average length, (F4) count and average area, (F5) average length and area, and (F6) histogram (Table 1). Among various feature sets, those containing only cell count and average length showed the best accuracy, followed by the histogram. Features including length (F3, F4 and F5) were more reliable characteristics for estimation for CAZ and PPC, whereas features including cell count were better for AMK and CIP. For

### Table 1 Accuracy of susceptibility estimation for different feature sets.

<table>
<thead>
<tr>
<th>Feature</th>
<th>AMK</th>
<th>CIP</th>
<th>MPM</th>
<th>CAZ</th>
<th>PPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>0.91</td>
<td>0.96</td>
<td>0.94</td>
<td>0.95</td>
<td>0.9</td>
</tr>
<tr>
<td>F1</td>
<td>0.81</td>
<td>0.85</td>
<td>0.9</td>
<td>0.88</td>
<td>0.89</td>
</tr>
<tr>
<td>F2</td>
<td>0.83</td>
<td>0.88</td>
<td>0.89</td>
<td>0.86</td>
<td>0.87</td>
</tr>
<tr>
<td>F3</td>
<td>0.9</td>
<td>0.96</td>
<td>0.94</td>
<td>0.94</td>
<td>0.9</td>
</tr>
<tr>
<td>F4</td>
<td>0.85</td>
<td>0.88</td>
<td>0.87</td>
<td>0.85</td>
<td>0.88</td>
</tr>
<tr>
<td>F5</td>
<td>0.78</td>
<td>0.83</td>
<td>0.87</td>
<td>0.87</td>
<td>0.89</td>
</tr>
<tr>
<td>F6</td>
<td>0.89</td>
<td>0.95</td>
<td>0.94</td>
<td>0.92</td>
<td>0.9</td>
</tr>
</tbody>
</table>
was higher than 96% (Table 3).

Microdilution method, the matching rate of the proposed method was two-fold difference in MIC, as determined by the traditional broth concentration of susceptible channels. Including samples with average length. Then, the MIC was assigned as the minimum concentration in the corresponding image was calculated with the pre-trained SVM based on a feature vector containing only cell count and average length. For this feature set, we trained SVMs on 2 h and 3 h cultures. We used feature sets consisting of only two elements: cell count and average length. For this feature set, we trained SVMs on 2 h and 3 h cultures and compared the accuracy (Table 2).

The accuracy of estimating the susceptibility of a drug was dramatically reduced after 2 h incubation than after 3 h. This was simply because the degree of cellular changes was lower after 2 h incubation than after 3 h.

### 5.5 Susceptibility estimation: 2 h vs. 3 h incubation

In the previous section we showed that SVM is able to determine susceptibility with high accuracy for *P. aeruginosa* strains after 3 hours of incubation. Since reducing time of result acquisition is very important, we compared the efficiency of estimation for 2 h and 3 h cultures. We used feature sets consisting of only two elements: cell count and average length. For this feature set, we trained SVMs on 2 h and 3 h cultures and compared the accuracy (Table 2).

The accuracy of estimating the susceptibility of a drug was dramatically reduced after 2 h incubation than after 3 h. This was simply because the degree of cellular changes was lower after 2 h incubation than after 3 h.

### 5.6 MIC estimation accuracy

Our final aim was to predict how well our algorithm is able to determine MIC of a drug. To obtain the minimum concentration for each individual bacterial strain, the susceptibility of each channel in the corresponding image was calculated with the pre-trained SVM based on a feature vector containing only cell count and average length. Then, the MIC was assigned as the minimum concentration of susceptible channels. Including samples with two-fold difference in MIC, as determined by the traditional broth microdilution method, the matching rate of the proposed method was higher than 96% (Table 3).

In comparison with the human-based judgement criteria [5], the SVM shows better accuracy in estimating MIC of AMK. However, a 1% decrease in accuracy was observed for samples exposed to CIP.

### 6. Discussion

Before implementing the algorithm described in this paper, no software was readily available that is able to perform drug susceptibility testing and estimate MICs directly from microscopic images of the microfluidic channels of a DSTM device. However, various tools and packages for automated cell image analysis have existed.

ImageJ [11] is one of the most popular open source biological image analysis packages. By virtue of its flexibility, it has been successfully employed in a range of different tasks. Macros can be written to automate individual steps of image processing for drug susceptibility testing, but it is difficult to combine these into one unified framework. A more dedicated tool, CellProfiler [12], was designed to count and classify cells in yeast colonies and measure tissue morphology. Another package, called CellC [13], is able to quantify fluorescent-labeled bacterial cells. Finally, CellScreen [14] measures cell growth of a culture in a small-volume cultivation vessel (a 96-well plate). We tried to apply these existing tools to our task, but they require significant modifications. Different packages need to be used in different stages of analysis, resulting in complex workflow and increased processing time. Several attempts to develop a method for judging susceptibility from microscopic images have been reported recently. For example, a method designed by Choi et al. [15, 16] used a customized 96-well plate and required the acquisition of individual microscopic images at certain time intervals. Price et al. [17] also automatically analyzed time-lapse images of cell growth of a single strain over a period of 2–4 h. Tailoring existing methods to solve our task was too burdensome. Thus, the primary goal was to develop our own method.

In comparison to existing methods, the proposed framework for drug susceptibility testing with a DSTM device has several important advantages. First, our method provides results within 3 h, which is shorter than any previously reported system. Second, to acquire the MIC, our algorithm processes only a single small-volume cultivation vessel (a 96-well plate). We tried to apply these existing tools to our task, but they require significant modifications. Different packages need to be used in different stages of analysis, resulting in complex workflow and increased processing time. Several attempts to develop a method for judging susceptibility from microscopic images have been reported recently. For example, a method designed by Choi et al. [15, 16] used a customized 96-well plate and required the acquisition of individual microscopic images at certain time intervals. Price et al. [17] also automatically analyzed time-lapse images of cell growth of a single strain over a period of 2–4 h. Tailoring existing methods to solve our task was too burdensome. Thus, the primary goal was to develop our own method.

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### Table 2 Accuracy of susceptibility estimation using a feature vector reflecting only cell count and average length, on samples incubated for 2 h or 3 h.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Drug</th>
<th>AMK</th>
<th>CIP</th>
<th>MPM</th>
<th>CAZ</th>
<th>PPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td></td>
<td>0.74</td>
<td>0.85</td>
<td>0.82</td>
<td>0.8</td>
<td>0.79</td>
</tr>
<tr>
<td>3 h</td>
<td></td>
<td>0.9</td>
<td>0.96</td>
<td>0.94</td>
<td>0.94</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### Table 3 Accuracy of MIC estimation for different drugs based on a feature vector containing only cell count and average length.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMK</td>
<td>0.97</td>
</tr>
<tr>
<td>CIP</td>
<td>0.99</td>
</tr>
<tr>
<td>MPM</td>
<td>0.97</td>
</tr>
<tr>
<td>CAZ</td>
<td>0.97</td>
</tr>
<tr>
<td>PPC</td>
<td>0.96</td>
</tr>
</tbody>
</table>
The current version of the system is used frequently in the laboratory environment for testing different strains of P. aeruginosa. We plan to extend the system by training SVMs for different bacterial species. We also intend to supply the DSTM device together with the developed system as a single package to be used in other research facilities and hospitals.

**Conflicts of interests**

We have no conflicts of interest relationship with any companies or commercial organizations based on the definition of Japanese Society of Medical and Biological Engineering.

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