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An eHealth Android Application for Mobile Analysis of Microplate Assays

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Abstract: Drug efficacy assays with microplate readers are a key aspect of the modern drug discovery process. However, microtitre plate readers are expensive laboratory equipment and not easily transportable. We have created a prototype Android smartphone application that enables smartphones to measure the concentration values in microplate wells. We find that the smartphone camera measures concentrations of red and yellow solutions more accurately than green. Further, concentration readings are most accurate when microplates are backlit and sources of noise (such as glare and shadows) are removed. Therefore, we designed a simple dark box to control ambient light, which reduces the error in measurements to within 7% of a laboratory microplate reader. An affordable and mobile alternative to a microplate reader is expected to support drug research in developing countries by enabling rapid, cheap drug assays in small laboratories and in field work with a standard Android smartphone.

Keywords: Android, eHealth, mobile, infectious disease, Colourimetric assay, Smartphone, Plate Reader, microplate

1. Introduction

The persistent high global mortality associated with diseases such as tuberculosis and malaria in Sub-Saharan Africa and Asia, combined with the emergence of drug-resistant disease strains, indicates a pressing need for the development of new drug treatments or therapies involving more effective combinations of existing antimicrobial agents. However, although the World Health Organisation (WHO) has identified laboratory-based surveillance of antibiotic resistance as a "fundamental priority" in the development of strategies to contain antibiotic resistance and for assessment of the impact of interventions, laboratories remain a very under-resourced health system component in developing countries, especially in Africa [1].

Drug screening assays are used to identify antimicrobial compounds that effectively inhibit the growth of cellular pathogens (such as the tuberculosis bacterium). A common experimental assay assesses cellular response to a particular compound by placing varying concentrations across the wells in a microtiter assay plate or 'microplate' - a plastic tray containing a grid of small wells (Fig. 1, left). The wells function as an array of small test tubes: cells are stained with a dye and added to the wells. The efficacy of the drug combination in each well is measured as a function of the strength of the colour of the solution (absorbance): a strong colour implies that the drug (or drug combination) at that particular concentration was not effective, but a weaker colour indicates few remaining cells and therefore an effective drug or drug combination. The concentrations in each well are determined via light absorbance readings with a microtitre plate reader (Fig. 1, right). The combination of a microplate with a plate reader enables multiple, small-volume

experimental assays to be conducted simultaneously: it is a high-throughput method with minimal reagent usage that has become integral to drug screening.

Microplate readers are a vital component of a modern drug research laboratory, but are expensive for small research groups and impractical for fieldwork. A low-cost, portable alternative to the expensive microplate reader that enables small, underfunded laboratories or independent field researchers to perform microplate assays quickly and cost-effectively would enable more researchers and clinicians in developing countries to participate in the drug screening process, enabling more rapid identification of drug resistance and possibly accelerating identification of drug candidates for further research.

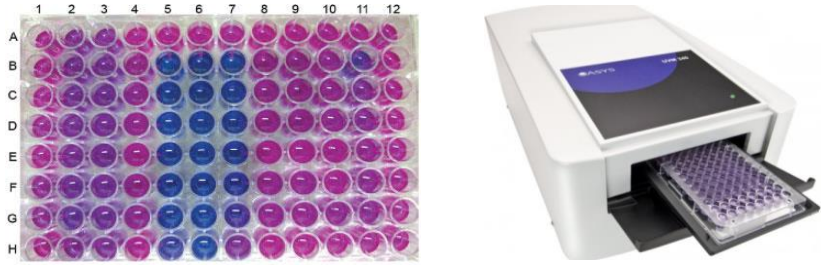

Figure 1. The concentrations in each well of a 96-well assay tray (left) are read very accurately through absorbances by a laboratory microplate reader (right).

A number of studies have demonstrated that a smart device with a built-in camera can perform high-quality colourimetric analysis. Yetisen et al. developed an application that performs accurate colourimetric analysis of paper-strip urine tests for pH, protein and glucose [2]. The analysis algorithm converts the average RGB value of 100 pixels to the C.I.E. chromaticity colour space. The closest point on a calibration curve is interpolated and the associated concentration reported. This application captures one point of data at a time, which is acceptable for a paper-strip test, but a laborious process for a 96-well microplate assay.

Vashist et al. developed a smartphone-based colourimetric reader for analysis of assays in 96- or 24-well microplates [3]. A secondary mobile tablet is required for illumination, as well as plastic housing for the smartphone to shield the setup from ambient light and to keep a constant angle and distance between the camera and samples. The analysis algorithm selects only one colour channel of the RGB spectrum, depending on the type of assay to be analysed. Once the centre of each well is located, a 40-pixel averaged colour value in the specified colour channel is used to determine the concentration by reference to a calibration curve. This system was evaluated with three different protein-based colourimetric assays and has good accuracy, at a 95% decrease in cost compared to modern plate readers.

Control of ambient light and effective illumination is key to accurate colour readings. The iTube system developed for food allergen assays, employs a specialised 3D printed attachment to the back of a phone to hold two vials [4]. The vials are enclosed to remove ambient light and illuminated with LEDs. A sample is loaded into a vial and photographed alongside the blank control vial. This level of environmental control enables the iTube system to detect peanut concentrations as low as 1 part per million.

Although providing proof-of-concept for colourimetric analysis with a mobile device, none of the prototype applications listed above is a viable prospect for a mobile microplate reader, where portability and rapid analysis of many samples across a variety of microplate sizes and brands is required.

2. Objectives

We aim to develop a prototype mobile application that enables a smartphone to act as an inexpensive, mobile microplate reader. We require a fast, responsive application that is not

computationally expensive. The application should be usable in a laboratory setting, automatically detecting all microplate wells in the camera view, extracting colour readings for each well and analysing these colourimetrically to produce concentration values for each well. The goal is to provide a robust software implementation that works efficiently across many microplate brands.

We aim for accuracy in calculated concentration to within 10% of a standard laboratory plate reader's measurements. To this end, we also aim to identify the image capture variables that have the greatest impact on accuracy in the plate detection and concentration readings.

Our goal is to create an affordable and mobile alternative to microplate readers for drug assays in small laboratories and for fieldwork to support drug research in developing countries.

3. Methodology

The plate reader application has two main functions: location of the wells in a microplate and transformation of the colour readings for each well into a concentration value.

3.1 – Microplate Well Detection

Well detection focuses on identifying the wells; the microplate outline is ignored, as microplate dimensions differ across brands. Different plates will have different sized gaps between wells and different well radii, and the angle of the camera can have a considerable affect on circle dimensions.

For well detection, the image frames are converted to greyscale and smoothed with a 3x3 Gaussian kernel. Wells are detected using the Open CV Circle Hough Transform (CHT) method, which first uses a Canny Edge Detector (CED) [5] to remove all non-structural information from the image of the microplate and then follows with a Circle Hough Transform [6] to identify potential circles on the edge map.

The OpenCV implementation of the CHT requires the following parameters: the Inverse Accumulator resolution, the minimum distance between circles (to avoid overlapping circles), the maximum circle radius, the minimum circle radius, the higher threshold of the edge detector and the accumulator threshold for the circle centres.

As microplates are transparent, well edges are difficult to detect and a sensitive edge detection algorithm was required. This sensitive algorithm is extremely susceptible to noise (such as glare and shadows) that causes detection of false circles. Therefore, it is important to minimize all sources of visible noise on the microplate. Further, in order to remove false circles detected by the sensitive algorithm, the average radii of the circles are determined and circles with radii outside two standard deviations of the mean are excluded. The maximum number of circles is limited to the plate size, as specified in the user input.

3.2 – Colour Detection

Plate readers calculate the concentration of compound (or compounds) in a solution through absorbance measurements: the concentration of the compound is proportional to the absorbance. The plate reader passes light of a specified wavelength and known intensity, I, through a well and a detector measures the intensity of light that has transmitted through to the other end of the well, I_0. The absorbance, A, is then calculated using

$$A = \log(I_0/I) \qquad \text{Equation 1}$$

A colour will appear darker for wells containing a greater volume of solution because of increased absorption along the longer path length. Modern plate readers scale the absorbance value to a standardized path length of 1cm. For a specific assay, the absorbance of a series of known concentrations of the compound is measured to plot a standard curve.

Interpolation from this standard curve is then used to determine the concentration of an unknown solution from its measured absorbance. It is important to note that, while plate readers have a very accurate independent error range of 1% for absorbance measurements, the human error in sample preparation for standard curves is still relatively large, even with the use of mechanical pipettes [7, 8].

Conversely, colour detection in a smart phone is dependent on the accuracy with which the phone camera measures the intensity of light in the visible range. The camera measures the intensity of light transmitted from each well at three individual wavelengths – wavelengths corresponding to red, green and blue – which vary slightly between mobile devices. Smartphone cameras capture and store images in the red-green-blue (RGB) colour scheme. A factor to consider is that digital cameras have two green photosensors for each red and blue sensor to correspond to the increased sensitivity of the human eye to green light: a smartphone camera may therefore be more accurate for colours with a large green component.

3.3 – Microplate Housing

To improve the accuracy of both well detection and colour measurements in the microplate readings, we designed and tested an inexpensive DIY box to house the smartphone and remove ambient light. The microplate environment greatly affects the accuracy of the readings. Glare and shadows can hide circle edges and affect the colour readings. Therefore, the microplate should be well and evenly backlit and the camera distance and angle should remain constant for best results. Our housing comprises a simple cardboard shoebox (Fig. 2) with a cut-out at the bottom to allow illumination of the plate from below. This hole is 3mm smaller in width and height than the dimensions of an assay plate. The hole is covered by 2 layers of A4 printer paper cut to fit and glued down onto the inside of the box. This allows a plate to have a firm, flat foundation on which to rest, while allowing for backlighting that will be diffused by the paper. On the top of the box, a 3.0cm x 2.5cm hole allows a space for a camera but is small enough so that the body of the mobile phone blocks out ambient light. The box is propped up by two smaller vertical boxes, 10cm in height, to allow a light source to be placed underneath the setup and light up the underside evenly.

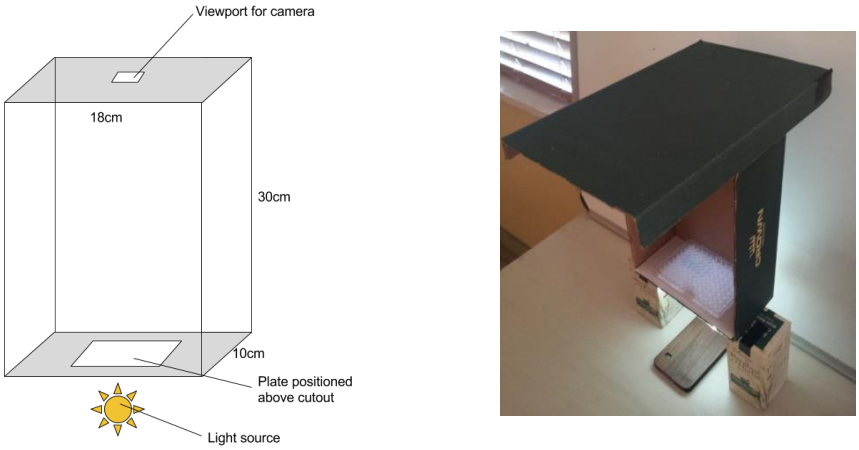

Figure 2. Left: diagram of the box designed to control the microplate lighting and camera. Right: experimental box setup with bottom illumination from a smartphone flash.

Our DIY housing is simple to make, low-cost, portable and optional: a user may choose not to use the housing for initial assays, when accuracy is not of key importance.

4. Technology or Business Case Description

Our microplate reader application comprises two main modules: a *Plate Detection Module* to detect the wells in the plate and a *Colour Analysis Module* to convert colour readings to concentration values. The prototype application was primarily developed for a Samsung Galaxy S4 or S5, running Android Jellybean 4.3 or subsequent Android operating system releases. The software is written in Java with computer vision tools from the OpenCV library.

The application assumes a microplate containing a single assay comprising varying quantities of either one or two compounds. Two compounds can be tested per plate, but the user can choose to only test one. The microplate must be transparent and have all wells completely filled. In addition, it is assumed that the first row in the plate is used for calibration and is a row of wells of known concentrations. These wells provide a necessary reference for the *Colour Analysis Module*. For this prototype, the application user interface focuses on functionality; with the expectation that usability will be addressed in later iterations.

4.1 – Data Input

Before taking a microplate reading, the user must specify necessary data, viz. the initial concentration of the first well in the calibration row; the name of the compound(s); the initial concentration of the compound(s); the dilution step-size; the plate size/number of wells and the initial/starting well (i.e the well with highest concentration). A help function provides information on how to use the application.

4.2 – Plate Detection

The *Plate Detection* module then locates the wells on the microplate and displays a live preview of the wells identified as green circles overlaid on the image of the plate. The user may then add circles/wells, by touching empty places in the image, or delete circles by touching existing ones. During plate detection, the user may zoom to fill the preview window with the plate and tap-to-focus to improve the performance of the CHT when detecting circles. The user may start or stop well detection on demand. Well detection lowers the frame rate, which can make it difficult to align the camera with the plate. Stopping detection allows the user to align the camera so the microplate fills the preview window, and then activate detection.

At any time, the user can change the parameters of the detection algorithm to better suit the environment in which they are capturing the plate. The settings activity allows for adjustment of the default Canny Edge and Hough Circle parameters. A user can vary the parameters and immediately see the effect of their changes on well detection.

Once the user approves the capture, the image is saved to the device in the lossless PNG format in order to preserve all colour information. The circles identified are sorted into their respective rows according to the layout of the microplate and the well locations and radii are passed to the *Colour Analysis* module for processing.

4.3 – Colour Analysis

The *Colour Analysis* module captures the colour information of each well and converts it to a concentration value by reference to a standard curve constructed from the first row of wells on the plate. The first row of the plate is assumed to have the wells to be used for the standard curve, while the remaining wells are treated as experimental wells.

Each well in the plate is processed in sequence. The well's colour is measured using a representative sample of pixels that are not image noise or artefacts (such as glare). A pre-processing step determines the pixels to be analysed, calculating the coordinates of a square

placed into the centre of the well circle. This simplifies calculations and removes the pixels on the edge of the well, which are the most likely to contain skewed colour values. Pixels with brightness greater than 0.9 (glare) or less than 0.15 (shadows) are excluded.

The standard curve is then calculated from the wells in the first row of the microplate. The *Colour Analysis* module converts colour information into a concentration value by reference to a standard curve constructed from the first row of wells on the plate. We use the Hue-Saturation-Value (HSV) colour space for detection, where the *hue* parameter stores the specific colour in an angular value stored between [0;360] degrees, *saturation* stores the colour intensity and *value* stores the brightness of the colour.

Average hue and saturation values are calculated for each of the wells and two standard curves are then constructed by linear interpolation between each pair of points.

Then the remaining wells on the plate, which are assumed to be test wells, are analysed in sequence. The component of their HSV colour that is relevant to the selected standard curve is used to calculate each well's concentration by linear interpolation from the standard curve.

5. Results

The *Well Detection* module successfully detects the wells of a range of microplate sizes, ranging from 4 to 96-wells. Best results are obtained on a clean, white background (such as a blank sheet of paper) in order to reduce noise. The DIY box housing with back lighting was found to be necessary for accuracy (Fig. 3). With the use of the box, the algorithm was able to detect all wells on an entirely blank plate - the most difficult scenario.

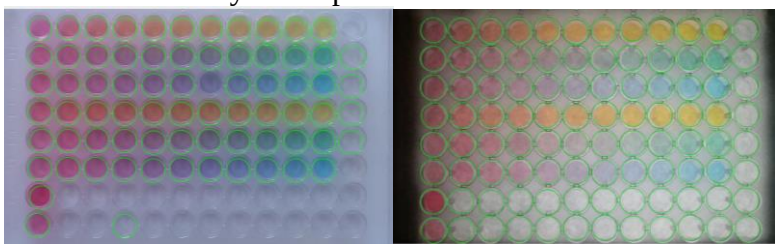

Figure 3. A test plate with constant ambient lighting (left), and the same plate with backlighting (right) – the green circles are superimposed where a circle is detected

We evaluated the *Colour Analysis* module by measuring the accuracy to which it can measure concentrations of solutions of different colours, as compared to the values obtained with a plate reader (Fig.4). Solutions of red, yellow and blue Moirs food colourants (Table 1) were used to test the applications accuracy with different colours.

Table 1. Moirs Food Colourants Used in This Study

Dye	Colour	Common Name	Absorption Maxima (nm)		Absorption Coefficient ($L.g^{-1}.cm^{-1}$)	Molar Mass ($g.mol^{-1}$)
			This Study	Literature		
E122	Red	Carmoisine	515	515	151	502.44
E104	Yellow	Quinoline Yellow	410	412	91.6	477.38
E133	Blue	Brilliant Blue FCF	625	630	169	792.85

A colour dilution assay (Fig. 5, left) tests the effect of a change in saturation with a relatively constant hue and allows for a comparison of the accuracy of measurements for red, yellow, green and blue colours. A 2D colour shift assay (Fig. 5, right) tested the module's response to a change in hue at constant saturation. This assay mimics common laboratory assays, which typically involve a colour change. The well colour fades from red to yellow, green and blue respectively across the microplate rows.

Figure 5. The Colour Dilution Assay (left) and Colour Shift Assay (right).

We found a marked increase in measurement accuracy with use of the DIY box, confirming that removal of ambient light is crucial. With use of the box, the average error in colour readings is reduced from 30% to around 7%. We found that the accuracy of the colour detection is affected by the colour of the solution. Red is the most accurate, with an average error of 5.6% as compared to the plate reader. Surprisingly, green solutions have the greatest associated average error of 8.7 %.

Table 2. Accuracy in the Colour Shift Assay

Approx red %	% Error in ratio determination		
	Red->Yellow	Red->Green	Red->Blue
90.00	0.66	4.46	5.29
80.00	2.07	6.99	5.81
75.00	2.51	0.94	8.42
65.00	1.60	7.24	7.30
60.00	1.64	4.12	5.86
50.00	3.97	3.42	2.96
40.00	6.98	7.42	1.16
30.00	7.07	25.08	0.67
20.00	5.62	56.18	3.39
10.00	3.63	83.04	7.58
Average	3.57	19.89	4.84
CCC Statistic	0.989	0.252	0.962

Our results reveal that mobile phones are only accurate in the upper half of a conventional plate reader's measurement range (Table 2). However, they are able to produce accurate measurements of concentrations corresponding to much higher absorbances than those measurable on a plate reader. A plate reader is able to accurately measure very small absorbances of light, but are limited by the Beer-Lambert Law in their range of measurement – they cannot be used for absorbances greater than one. Digital cameras do not measure absorbance values and are not bound by this law. Therefore, a mobile phone plate reader and the standard plate reader are not mutually exclusive – each can complement the other's strengths and weaknesses. The mobile phone application would allow a user to obtain rough results for high concentration experiments without requiring dilution. Importantly, the module is able to follow a colour trend (i.e. the halving of the colour concentration from well to well) and thus supports qualitative work where a precise concentration is not required, which is often the case in fieldwork.

We found very little significant hardware dependency for colour detection as similar results are produced on separate devices and at both ends of the resolution quality spectrum. This bodes well for future development of the application as it indicates that the algorithm can be run effectively on poorer quality phones.

6. Conclusions and Recommendations

Our Android prototype application indicates that a viable, transportable alternative to expensive microplate readers is possible on standard smart devices. The application is able to provide fast analysis of solutions across a range of microplate sizes with a reasonable degree of accuracy. With the use of our simple DIY box construction to remove ambient

light, our application has a concentration measurement accuracy of 7%. This is not yet high enough accuracy for modern laboratory use, but is potentially useful as a temporary replacement for a plate reader and sufficient for many fieldwork contexts. In addition, our mobile application is accurate for higher concentration solutions for which a plate reader cannot be used. The application can thus serve as a companion instrument for fast, efficient measurement of concentrated solutions.

Following on from this successful prototype, future work will develop a fully-functional plate-reader application. Usability and interaction will be a focus of the next iteration, as will be improvement of the detection algorithm. This application can be used in drug discovery, testing natural products, or even education and thus expedite research into neglected infectious diseases.

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