1	Smartphone-based lateral flow imaging system for detection
2	of food-borne bacteria
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4	Youngkee Jung ¹ , Yoojung Heo ² , Jaejoong Lee ³ , Amanda Deering ² , and Euiwon Bae ¹ *
5	¹ Applied Optics Laboratory, School of Mechanical Engineering, ² Department of Food
6	Science, ³ Department of Computer Science, Purdue University, West Lafayette, Indiana 47907,
7	USA
8	*Corresponding author: ebae@purdue.edu
9	Abstract: We report an application for the smartphone as an accurate and unbiased reading
10	platform of a lateral flow assays for food safety application. In particular, this report focuses
11	on detection of food-borne bacteria in samples extracted from various food matrices. The
12	lateral flow assay is a widely accepted methodology owing to its on-site results and low-cost
13	analysis, even though sensitivity is not quite equivalent to that of standard laboratory
14	equipment. An antibody-antigen relationship is transduced into a color change on a
15	nitrocellulose pad; interpretation of this color change can result in uncertainty, particularly
16	near the detection limit of the assay. Employing the high resolution integrated camera,
17	constant illumination from light source, and computing power of a smartphone, we provide
18	an objective and accurate method to determine the bacterial cell concentration in a food
19	matrix based on the regression model from the color intensity of test lines. A 3D-printed
20	sample holder was designed for representative commercial lateral flow assays and an in-

21	house application was developed in Android Studio to solve the inverse problem to provide
22	cell concentration information from the color intensity. Test results with <i>E.coli</i> O157:H7 as
23	a model organism suggests that smartphone-based reader can detect 10^4 - 10^5 CFU/ml from
24	ground beef and spinach food matrices.
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26 27	OCIS codes: (330.1730) Colorimetry; (150.1708) Color inspection; (280.1415) Biological sensing and sensors;(120.0120) Instrumentation, measurement, and metrology
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30 **1. Introduction**

31 The lateral flow assay (LFA) is a widely used analyte-detection platform such as pregnancy tests, chemical residue determination, and toxin detection (Anfossi et al., 2013). The strength of the LFA 32 33 is that detection can be performed on site; the cost of analysis is very low, since users need only 34 to read out the presence or absence of certain color lines. These color lines are typically designed to capture target analyte and using a specific antibody and antigen relationship. In addition, some 35 form of nanoparticles is used to generate a distinctive color, which is caused by the surface 36 37 plasmon effect (Hsieh et al., 2017, Ngom et al., 2010). LFA has also been used for detection of microbial contamination ranging from Bacillus (Posthuma-Trumpie et al., 2009, Sajid et al., 2015), 38 39 Legionella species (Posthuma-Trumpie, Korf and van Amerongen, 2009), Enterobacter (Sajid, 40 Kawde and Daud, 2015), and E. coli O157:H7 (Fisher et al., 2009, Gumustas et al., 2018) to Listeria monocytogenes (Gumustas, Caglayan, Eryilmaz, Suludere, Soykut, Uslu, Boyaci and 41 Tamer, 2018). One of the limitations of LFA detection is that reading the test line depends on the 42 user's eyesight and can become challenging, particularly when the analyte concentration is close 43

to the detection limit of the assay and interpreting colors in a different illumination conditions. In
addition, since color intensity cannot be accurately quantified, the reported outcome is simple
binary information, and analyte concentration cannot be retrieved in a reliable manner.

Nowadays, the smartphone has become a necessity of everyday life. Smartphone handsets have 47 become widely available, and their cost dramatically decreased owing to emergence of a multitude 48 49 of companies, while the performance specifications continue to improve. These favorable features make the smartphone more than a simple communication device. Characteristics such as large 50 random access memory (RAM), high-speed central processing unit (CPU), high-resolution 51 52 complementary metal oxide semiconductor (CMOS) sensor, and wi-fi network are similar to the modern-day computers needed for laboratory-based testing. Now these features enable the 53 smartphone to be the basis for a field-deployable instrument. One requirement for this application 54 is to design an optical attachment that can convert the color information into analyte concentration 55 so that default smartphone cameras can measure the concentration of the sample of interest. Many 56 authors have explored this idea and introduced diverse attachments that transduce the signal of 57 interest (Geng et al., 2017, Kılıç et al., 2018, Pohanka, 2017, Rasooly et al., 2016, Shin et al., 58 2018). Smartphones have been transformed into microscopes (Kim et al., 2013, Wareham et al., 59 60 2016), colorimetric devices (Jung et al., 2015, Kılıç, Alankus, Horzum, Mutlu, Bayram and Solmaz, 2018, Pohanka, 2017, Suria et al., 2015, Wang et al., 2017), luminometers (Arpa et al., 2012, Kim 61 et al., 2017, Switz et al., 2014), and spectrometers (Das et al., 2016, Jung et al., 2017, Wang et al., 62 63 2016, Zhang et al., 2016) Among those applications, we focus here on the design and application of the smartphone-based lateral flow assay (SLFA). The main design aim was to provide adequate 64 65 illumination to the sample strip, design a sample container to hold the LFA strips, and develop an 66 app that can quantify the color intensity in the region of interest.

Here we propose to use the smartphone as a colorimetric and quantitative reader, thereby 67 improving classification of the minute changes resulting from different analyte concentration (You 68 et al., 2013). This will be particularly important for lower-concentration samples where, depending 69 on the color shades and illumination condition, the naked eye cannot confidently report the 70 presence of a test band. In addition, smartphones record the digital image of the experimental result; 71 72 time and date stamps are always recorded as well, a bonus for database purposes. Here we report the design, fabrication, and testing of smartphone-based LFA detection with special emphasis on 73 the detection of E. coli O157:H7. 74

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2. Materials and Methods

76 2.1 Bacterial sample preparation

E. coli O157:H7 was incubated in LB broth 18-19 h at 37 °C. The culture was diluted with 0.1 M 77 78 phosphate buffer (pH 7.0) and a concentration of 10⁴ CFU/ml was used to test the strips. To test the detection limit, Each culture was diluted with 0.1 M phosphate buffer (pH 7.0), and 79 concentrations of 10⁴, 10⁵, and 10⁶ CFU/ml were used to test the strips. To test specificity, E. coli 80 81 O157:H7, Salmonella typhimurium, E. coli K-12, and E. coli 99-1044 A1 were incubated in Luria-Bertani (LB) broth, and Listeria monocytogenes was incubated in Brain Heart Infusion (BHI) broth 82 for 18-19 h at 37 °C. For food-spike testing, E. coli O157:H7 and Salmonella typhimurium were 83 incubated in Luria-Bertani (LB) broth 18-19 h at 37 °C. Each culture was diluted with 0.1 M 84 85 phosphate buffer (pH 7.0); For the food test, 25 grams of spinach and 10% lean ground beef of sample was used and 10³, 10⁴,10⁵ CFU/ml of *E. coli* was added to each sample. After addition of 86 87 225 ml of 0.1 M phosphate buffer the homogenized liquid sample was used to test the strip. 1 mL 88 of non-inoculated samples and Salmonella-containing samples were used as negative controls. For

each strip, the procedure followed the instructions that came with the kit (Romber-Labs, 2019).
Test results were imaged with the cell-phone camera application.

91 2.2 Imaging attachment

92 The core of the smartphone-based imaging system relies on three major sub-components: sample 93 cartridges for each brand of LFA strip, interchangeable optical imaging box, and smartphone 94 cradle (Figure 1). Sample cartridges were designed to accommodate different strip dimensions to be imaged within the camera imaging area and repeatable lateral positions (Figure 1(A)). The 95 optical imaging box, which is an interchangeable module, was designed to be inserted into a 96 97 smartphone cradle (Figure 1(B), (C)). Smartphone cradles were designed separately for two different models tested (OnePlus One, model A0001, Shenzhen, China, and LG G4, model H811, 98 99 Seoul, Republic of Korea) since their overall dimension are different.



Figure. 1. Photo and schematic diagram of the overall smartphone based lateral flow imaging system. (A) Sample holder that was designed to match the dimension and location of the control and test band. (B) Optical imaging box that provide diffusive illumination and magnified image of the control and test band area. Dimension of the box is

2.5 x 3.8 x 2 cm. (C) Smartphone cradle that holds the phone in horizontal direction. Wire diagram in the middle
shows schematics of overall assembly.

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Detailed design of the proposed optical imaging box is shown in Figure 2(A). This box consists of a reflector to redirect the smartphone LED light onto the strip. To avoid specular illumination, an optical diffuser was inserted to spread out the incoming LED light. Sample LFA was imaged with a plano-convex lens that relays the image to the smartphone camera lens. Figure 2(B) shows a photograph of the imaging box where an individual Solidworks CAD model is shown in Figure S1.



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Figure. 2. Schematic diagram of the lateral flow imaging box. (A) Smartphone LED light is re-directed to a diffuser so that strong specular illumination becomes more diffusive illumination. (B) Break-down of the imaging box with lens, 3-D printed case, reflection mirror, and diffuser.

117 2.3 Experimental procedure

Two different brands of LFA strips were used for this model study: *E. coli* O157:H7 rapid detection
kit (Model ETLF-020, Bioassay, Ijamsville, MD, USA), Rapidcheck *E. coli* O157:H7 test kit
7000157 (Romer Labs, Newark, DE, USA). When bacterial samples were ready, each LFA strip
was prepared according to manufacturer's directions. Common preparation elements included

adding promoter from the kit and incubating the strip for approximately 15 min at 42 °C. After a 122 15-min cool down of strips, they were added to the appropriate individual cartridge and inserted 123 into the smartphone for reading (Figure 3). The overall procedure was divided into three steps. 124 First was conducting a standard dilution series test to acquire calibration data for color intensity 125 versus bacterial concentration. Second was conducting a specificity test in which four different 126 127 non-E. coli O157:H7 samples were challenged against the LFA strip. Third was conducting a foodspike test in which two representative food matrices (ground beef and spinach) associated with E. 128 coli O157:H7 outbreaks were selected. 129



Figure 3. Sample preparation steps. (A) Approximately, transfer 200 uL of enriched bacterial sample to a sample cup and prepare the LFA strip. (B) Add 1 drop of promoter reagent (C) Place the LFA into the sample cup and incubate at $42 \pm 1^{\circ}$ C for 15 minutes. (D) Remove the LFA from the incubator and wait for 15 min. (E) Perform visual reading of control and test line.

135 *2.4 Data analysis*

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Native images from the phone were transferred to computer to be analyzed by Matlab scripts. First
of all, original color RGB images were transformed to grayscale images and cropped to display
only the LFA assay portion for each standard-concentration, specificity, and food-spike sample.

With the longer dimension defined as the x axis and the shorter dimension as the y axis, 2-D image 139 intensity was spatially averaged in the y direction so that a 1-D linear intensity cross section was 140 achieved. Utilizing Matlab's curve-fitting toolbox, an 8th-order polynomial function was used to 141 model the background image from illumination and subtracted from the 1-D linear cross-sectional 142 plot. The result shows two peaks (control and test line); both peak area and intensity were used to 143 144 estimate the presence of the test line and concentration of the analyte. Since each brand of LFA strips had different locations and widths of the control and test lines, this process was separately 145 optimized for each brand. 146

147 2.5 App development

To implement the whole functionality independent of a separate computer, an Android app was 148 developed to implement similar processes and calculations on the smartphone. First off, the current 149 150 app asks the user to select the brand of the LFA (one of the three tested) because the LFA strip area and respective control and test-line dimensions were coded into the app development. Once a 151 brand is selected, the app automatically crops the white strip area and performs a spatial averaging 152 153 calculation as explained in section 2.4. Second, a fitted curve is created using a polynomial curve 154 fitter (Apache, 2019) with a degree-of -6 equation. Unlike Matlab's curve-fitting function, this library required careful coding to make a better fitting around the peak (control and test) areas. In 155 particular, signs of slope were checked on the each side of the peak locations. Data points were 156 157 estimated using a polynomial curve fitter, if there were no dramatic changes in its slope. However, if there were such changes, those points were estimated using linear regression. For graphing the 158 plot, a 3rd-party library called GraphView was used (Android, 2019). When the first time the app 159 is ran, the user is asked to provide calibration images for which bacterial samples of known 160 161 CFU/ml are deposited on the test strip. After processing by the same procedure described above,

a linear calibration curve from control to 10⁶ CFU/ml is constructed. When an unknown sample is inserted in the phone, the app compares the intensity value to the calibration curve to provide an estimated CFU/ml. For the on-phone database, Sqlite for Android was used (Android, 2019), in which the app records the test-strip image, date, time, and test location. This information can be tagged to provide geographical location of the test result using default map software such as Google Map.

3. Results





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Figure. 4. Core of the image processing and calibration step. (A) displays the concentration dependent LFA image taken with the proposed imaging attachment. Control line (left) is shown for every sample while test line (right) are less visible for lower concentration. (B) express the fitting function to remove the background reflectance from nitrocellulose paper. Top image shows the raw image which is 1-D average of the Y-direction of the LFA image. After

fitting the curvature with 8th order polynomial, the fitting function minimize the residue and provides flat response ofcontrol and test time.

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The processing steps described in section 2.4 are shown in Figure 4. To provide a proof-of-concept 178 179 for a concentration dependent LFA, captured images from the dilution series of E. coli O157:H7 sample were analyzed. Figure 4(A) displays bacterial concentration of 10⁴ CFU/ml to 10⁶ CFU/ml 180 from the Bioassay LFA with the definition of Cartesian coordinate. Visual observation clearly 181 detects the presence of a test band on the 10⁵ and 10⁶ CFU/ml samples, while the 10⁴ CFU/ml 182 sample is not so certain. However, Figure 4(B) demonstrates that after curve fitting with an 8th-183 order polynomial and background subtraction, peak intensity from both control and test line was 184 185 visible at approximately 1200 and 2300 pixels, respectively.



Figure 5. Result of the dilution series for Bioassay and Rapidcheck. (A) Comparison of the 1-D cross-sectional intensity for control (left) and test (right) lines. (B) shows the integrated intensity versus log CFU number with it's

fitting function ($R^2=0.992$) for Bioassay (C) Shows the similar results for Rapidcheck with (D) fitting function ($R^2=0.997$).

Figure 5 shows the dilution series test for the two different brands of LFA for 10⁴, 10⁵, and 10⁶ 191 CFU/ml. Direction of flow is from right to left. The Bioassay LFA displayed sharp and narrow 192 193 peaks with asymmetric peak shape, whereas the other two brands show more symmetric peaks for 194 both control and test lines. Individual brands showed different peak intensities depending on the 195 concentration changes. Based on these results, intensity of the test bands from Bioassay with 196 similar sample concentrations resulted in better contrast than that of the Rapidcheck. To provide 197 quantitative assessment of the performance, average integrated intensity and standard deviation 198 were calculated for the test line as shown in Table S1 which shows detection limit of 10^4 CFU/ml for Bioassay and 10⁵ CFU/ml for Rapidcheck. Original images are tabulated on Figure S2. 199

200 3.2 Specificity test

To test the possibility of false positives from the assays, a specificity test was conducted. Four non-*E. coli* O157:H7 samples (*E. coli* 99-1044 A1, *E. coli* K12, *L. monocytogenes*, and *Salmonella typimurium*) were challenged; most of the strips provided good specificity. However, for the Rapidcheck LFA, *E. coli* 99-1044 A1, *E. coli* K12, and *Salmonella typhimurium* samples were detected with a positive test line that was not visually observable owing to weak intensity (Figure S3) but was picked up by the image analysis. *L. monocytogenes* did not provide any response on the same strip.



Figure 6. Result from specificity test using four different non-*E.coli* O157:H7 samples for (A) Bioassay and (B) Rapidcheck. 1-D cross-sectional intensity profile reveals that most of the strips provided good specificity while Rapidcheck *E.coli* 99-1044 A1, *E.coli* K12, and *Salmonella typhimurium* generated weak false positive response from the test line. This line was not easily observable by eye but smartphone imaging system can detect them.

213 3.3 Food-spike test

Ground beef and spinach artificially inoculated with *E. coli* O157:H7 were tested using LFA. Figure 7(A-B) displays the results from ground beef samples for Bioassay and Rapidcheck assays ,respectively. All samples displayed positive lines for 10^4 and 10^5 CFU/ml. Similar results were found for spinach (Figure 7(C-D)). In this case, all brands provided positive lines for 10^5 CFU/ml samples, while only the Bioassay LFA resulted in a positive line for 10^4 CFU/ml. Original image was tabulated in Figure S4.



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Figure 7. Food spike test results for ground beef samples with varying concentration of inoculum. (A) Bioassay (B)
 Rapidcheck. Similarly, spinaches were inoculated with *E.coli* O157:H7 and challenged with (C) Bioassay and (D)
 Rapidcheck.

224 3.4 App development



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227 Figure 8. Screenshot of app developed for lateral flow analysis with smartphone. (A) shows the first screen where 228 user can selected calibration, test, and access the database menu. (B) shows the screen for calibration process. Different 229 vendors can be added for separate calibration (C) During the calibration, each concentration of cell numbers in 230 CFU/ml is plotted for control and test line. Their average pixel values are integrated and saved. (D) shows the result 231 of test function when new image is loaded. Based on the linear calibration plot, app reports the estimated concentration in CFU/ml. (E) Screenshot of the history function where thumbnail image, brand, date, time, and geographic location 232 233 is saved. (F) once one of the data entry is clicked, map software automatically displays the location where the data 234 has been acquired.

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A screenshot of the developed app is shown in Figure 8. At first, users can select calibration, test, and history as a sub-menu. The calibration process is required only once to acquire standard concentration images to provide estimated concentrations from unknown samples (Figure 8(A)). Then users are asked to select the brand of LFA as their respective control and test line positions and strip dimensions differ (Figure 8(B)). Figure 8(C) displays the internal curve-fitting process
and plotting routing, while Figure 8(D) displays the result from an unknown sample interrogation.
Based on the previously conducted calibration routine, the app estimates the bacterial
concentration by CFU/ml and reports the result. Once the test is conducted, all of data are stored
in a local database (Figure 8(E)) in which time, date, image, and location information are recorded.
If the user clicks one of the listed items, any linked map software displays the geographical location
of the test site (Figure 8(F)).

247 **4. Discussion**

248 One of the important design parameters for smartphone-based imaging system is the ability to provide consistent and repeatable imaging of samples. Diffusive imaging provided better 249 illumination and less specular reflection of white LFA strips and a plano-convex lens enough 250 251 magnification to provide a sufficient number of pixels to cover the control and test areas. The design of the sample cartridge has an extended dimension at the end so that when users push the 252 cartridge into the imaging box, the sample is always positioned at the center of the camera image. 253 254 In addition, different brands of phone typically require separate cradles; designing the imaging box as an interchangeable module provides consistency. 255

A critical component of peak analysis was to ensure differentiation of the background (blank) strip and positive test line. Even with the diffuse imaging technique, some degree of uneven illumination across the horizontal direction still exists, and it is critical to not modify the peak intensity or location and to subtract only the illumination effect. This aim was achieved by a baseline subtraction method, which is a widely accepted processing technique in other spectrumbased methods such as Raman spectroscopy. In Raman spectroscopic data, fluorescence background is present along with the native Raman spectrum; this background signal is typically

removed so that only Raman peaks are left for analysis. Similarly, LFA images with illumination generates a consistent background signal from the white nitrocellulose pads and this signal was treated like the fluorescence background of a Raman spectrum.

In terms of assay sensitivity, tested brands showed different CFU/ml detection limits. While tested 266 brands claim to have a detection limit of 10⁴ CFU/ml after enrichment, our current test protocol 267 268 did not include sample enrichment for any of the three tess conducted (standard concentration test, specificity test, and food-spike test). Even without an enrichment step for E. coli O157:H7, two 269 brands have shown clear visual test bands for 10⁵ and 10⁶ CFU/ml. It is interesting to note that a 270 271 smartphone-based LFA system can provide more consistent readings for samples close to the detection limit of the assays. In addition, each brand of assay must have proprietary design 272 parameters in types of antibody, width of the test lines, type and size of gold nanoparticle used, 273 and construction of the flow strip. This was confirmed by variations in the width of control and 274 test peaks, slope of peaks, and location of peaks. For example, Bioassay tests have shown 275 asymmetric peaks when flow is moving from right to left. The first area that contacts an incoming 276 flow has a steep slope for intensity that gradually decreases. Based on the series of tests from 277 Figure 5, sensitivity of each assay without enrichment Bioassay was more sensitive than 278 Rapidcheck. Lastly, the intensity of the test band was roughly proportional to the CFU/ml; this 279 was used as the basis for the calibration curve for the Android app development. For specificity, 280 281 most of the samples tested showed no reaction from non-O157:H7 samples, but several test strips 282 from Rapidcheck resulted in false-positive results. As noted in Figure S3, visual inspection does not perceive this false positive result, whereas smartphone-based LFA sensitivity detects minute 283 color changes in the test band. 284

For food-spike testing, the matrix effect due to particulates from the food itself can adversely interfere with the detection limit of the LFA. In particular, testing with spinach samples visually colored the lateral flow strip green and overall negatively affected the detection limit, as shown in Figure 7 (C-D). All brands generated less intense peaks from the test bands with this sample matrix compared to the ground beef matrix. This result suggests a need for additional sample preparation to filter out large particulate from the matrix to maintain detection limits similar to those of standard pure-sample testing.

5. Conclusions

293 As an application of internet-of-things in research field, smartphone-based LFA reader provides various advantages compared to visual interpretation. The proposed method can provide 294 unambiguous determination of the positive results especially for low concentration samples, 295 296 interprets the color intensity to provide quantitative cell concentration information, and records digital image and geographic information of the LFA for future analysis and record-keeping. 297 Future plans include applying this concept to different types of bacterial species such as 298 299 Salmonella or Listeria, interrogating the same images with different color spaces such as YUV and L*a*b* (Kim et al., 2017), and increasing the dynamic range by utilizing the 10-bit digital-300 negative (DNG) format which has recently become available on most smartphones. 301

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Conflict of interest

All authors declare no conflict of interest.