Rapid ultra-sensitive and high-throughput bioburden detection: Microfluidics and instrumentation

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Abstract

Contamination detection often requires lengthy culturing steps to detect low-level bioburden. To increase the rate of detection and decrease the limit of detection (LOD), a system featuring microfluidics and a multichannel fluorometer has been developed. The 8-channel fluorometer enables parallel testing of multiple samples with LOD as low as < 1 CFU/mL. This low-cost system utilizes the slope of fluorescence intensity that serves as the criterion for bioburden detection. The redox indicator dye resazurin is used to monitor the presence of viable cells, in this study and is reduced to resorufin with a high quantum yield at 585 nm. The sample under investigation is spiked with resazurin, loaded in a special-design microfluidic cassette and the rate of change is observed via the fluorometer. The method was validated using primary *E. coli* culture in comparison with a spectrophotometer which served as the gold standard. An optimized assay based on LB media was developed. The impact on the assay sensitivity based on incubation and filtration steps was also explored. The assay is shown to pick up inadvertent contamination from test tubes and pipette tips showing its applicability in real-world settings. The data analysis demonstrated a comparable performance of the multichannel fluorometer vis-a-vis the conventional plate reader. The multichannel system is shown to detect bioburden presence in as low as 20 seconds for bacterial concentrations ≥ 5 CFU/mL after 6 hours of incubation. Considering its portability, low cost, simplicity of operation and relevant assay sensitivity, the system is well-positioned to detect low-level bioburden in the laboratory, pharmaceutical and field settings.

Introduction

Thousands of lives are lost every year, and millions are infected due to food, water, or medicine contamination [1]. The World Health Organization reports that infectious diseases cause 26% of all deaths globally, and account for 45% of the global disease burden [2]. The lack of rapid low-cost tools for pathogen identification results in misdiagnoses that worsen the outcome and promote over-prescription of antibiotics as well. As the threat of antibiotic-resistant bacteria continues to rise [3], there is an increasing demand for diagnostic devices that can quickly identify bacteria and their antibiotic susceptibilities in a wide range of applications. [4,5].

In addition to that, bioburden contamination is a major concern in biopharmaceutical manufacturing [6]. One contamination incident can lead to a loss in production time, material, batch, as well as possible facility closure. In these cases, it is of vital importance to be able to monitor sterility at various stages of the manufacturing process in real-time. There are many potential analytical methods for bioburden detection– ATP bioluminescence, flow

cytometry, nucleic acid amplification, respiration, impedance methods, antibody detection, to name a few [7–13]. Unfortunately, different methods have variable sensitivity, detection rate and cost. The detection process is still relatively slow with most of the techniques (3 hours to 7 days) [14]. Further, the cost and sensitivity come as limitations when the detection rate is sufficiently fast [15–18].

The advantage over more sophisticated, molecular biological techniques such as quantitative polymerase chain reaction (qPCR) with fluorescence detection [19,20] or colorimetric measurements is that they provide rapid and easy handling detection of bacteria [21]. In this regard, cell viability assays are widely used in the development of new drugs for studying growth factors, cytokines, and cytotoxic agents that can detect viable cells [22,23]. Recently, resazurin has been used as an oxidation-reduction indicator for the measurement of cell viability in a given sample [24]. Resazurin is a weakly fluorescent dye, and it is noncytotoxic to bacteria, yeast and mammalian cells [25]. Resazurin-based formulations have found applications for determining susceptibilities of microorganisms to antimicrobials, especially for use in antibiotic susceptibility testing (AST). Thus, resazurin formulations generated signals are advantageous [26,27]. The resazurin-based assay gives a linear curve over a wide range of cell concentrations [28]. Viable cells continuously convert resazurin (532nm) to resorufin (590 nm), increasing the overall fluorescence and chromaticity of the cell culture media surrounding the cells.

In this study, we present a highly sensitive, accurate, fast, USB-powered portable fluorometer that can detect the presence of viable cells in a given sample based on the detection of high quantum yield resorufin. *E. coli* has been used in this study as a metabolically versatile bacterium, able to grow under aerobic and anaerobic conditions. The assay was optimized for different culture media, varying resazurin concentrations. The effect of sugar (glucose and sucrose) addition to the culture media, incubation period and filtration effect were explored. The investigations and optimization of the assay based on these factors led to <1 CFU/mL limit of detection with 6 hours of incubation and filtration. Our study presents that with the combination of incubation and filtration, it is possible to reach even lower LOD. Monitoring the viability of cells is particularly crucial in predictive toxicology studies. We have also shown the distinguishment of the live and dead cells showing that our assay is capable of detecting viable cells. The intraspecies variation within bacteria is extensive [29]. An understanding of bacterial growth kinetics leading to intraspecies variation in a culture medium is important from both research and commercial perspectives. The incubation and filtration of bacterial samples also need to be explored to understand the intraspecies variability and the different phases of growth in bacterial samples. The primary cell cultures find more relevance from basic research to drug discovery than secondary cell cultures. Hence, primary cell cultures have been used to validate the multichannel fluorometer in this study. Generally, we have analyzed the whole study with 3 minutes of fluorescence data, but extensive data analysis revealed it is possible to detect contamination within as low as 20 seconds and 90 seconds for bacterial concentrations of ≥ 5 CFU/mL and ≥ 1 CFU/mL respectively with a statistically significant difference.

Most of the existing portable fluorometers [30–34] have a very higher limit of detection (7 to 10^5 CFU/mL) requiring complicated procedures i.e., preparation of magnetic nanoparticles and detection time is usually higher (around 30 minutes). On the other hand, our multichannel fluorometer has a LOD of <1 CFU/mL with incubation and filtration and detection can be done within as low as 20 seconds. This multichannel device has the ability for parallel testing of samples, which presents a significant improvement over our earlier prototype [16,35]. It can feature inherent negative control and has the potential to detect anaerobes. The device has proven to be accurate and fast, compared with a standard spectrophotometer which makes it suitable for rapid detection of contamination applications with the optimized assay for in-field applications.

Materials and methods

Modification from single to the multichannel fluorometer

The multichannel fluorometer's circuitry is designed in the same way as the earlier in-house designed single channel fluorometer [16,35] (Figure 1a) with modifications to achieve a better signal-to-noise ratio and increase the sensitivity. The multichannel device can monitor several different samples in parallel or can be used to perform duplicates (or triplicates) for the confirmation of the tests as in Figure 1c. The device features a semiconductor light source for fluorescence excitation (green LED, center wavelength 525 nm, FWHM 20 nm, 12,000 millicandela brightness) and its emission is filtered with a bandpass interference filter with a center wavelength of 532 nm and bandwidth 40 nm. The LED is driven by a voltage-controlled current that can output up to 50 mA DC. The fluorescence is detected by a two stage transimpedance amplifier (OPA2301) and quantified by a 16-bit analog-to-digital converter (ADS8318). The photodiode is mounted at 90 degrees to the excitation beam, with the emission filter (600 ± 20 nm) mounted in front of it. Both interference filters were from Itor (Socorro, NM). To decrease the probability of the signal interferences, the photodetector, its amplifiers and the analog-to-digital converter are galvanically separated from the LED driver and the microcontroller which controls the detection process and an RF shield is installed on the board. The channels communicate with a single laptop computer via USB, and they are powered from the laptop. One hub board distributes power and brings back data between the laptop and the fluorometer.

The computer control program is written in LabVIEW. The initiation of measurement and recording of the fluorescence modulation can be controlled through the program. The computer software addresses the boards via the hub board and a set of multiplexers. The firmware also has some diagnostic functions, such as turning on the LED continuously to verify the photodiode's amplifier function, and the ability to set and control the LED brightness, which is useful when multiple boards are used in parallel. The control of the LED brightness allows for calibrating the boards to generate the same signal from the same target despite the large (up to 50%) variations in the LED output light flux. Furthermore, the firmware allows reading of the stored LED brightness to facilitate the calculation of the desired LED setting.

The cassette holder was cut out from several layers of black PMMA and glued together. The cassette holder is designed to support narrower cassettes that can be illuminated from the narrow edge and intentionally made taller to avoid the direct illumination of the emission filter at a shallow angle via reflections (Figure 1b).

Fabrication of microfluidic cassettes

The three layer microfluidic cassette was laser cut in the same way as our previous publications from PMMA using two 1.5 mm and one 0.2 mm thick layers [16,35]. High-throughput solvent-based thermal bonding was initiated in this study to reduce the bonding and curing time for each device. The inlet allows for the introduction of the sample from the same side of the cassette, which simplifies loading, especially for multiple samples. Also, needleless inserts can be introduced to safely add components to the device (as in Figure 1d), eliminating the sharps from the process completely and also making the cassette gas-tight suitable for both aerobic and anaerobic species. Its slim form factor is intended to decrease the total sample volume to ~ 350 µl, while maximizing the light-emitting surface.

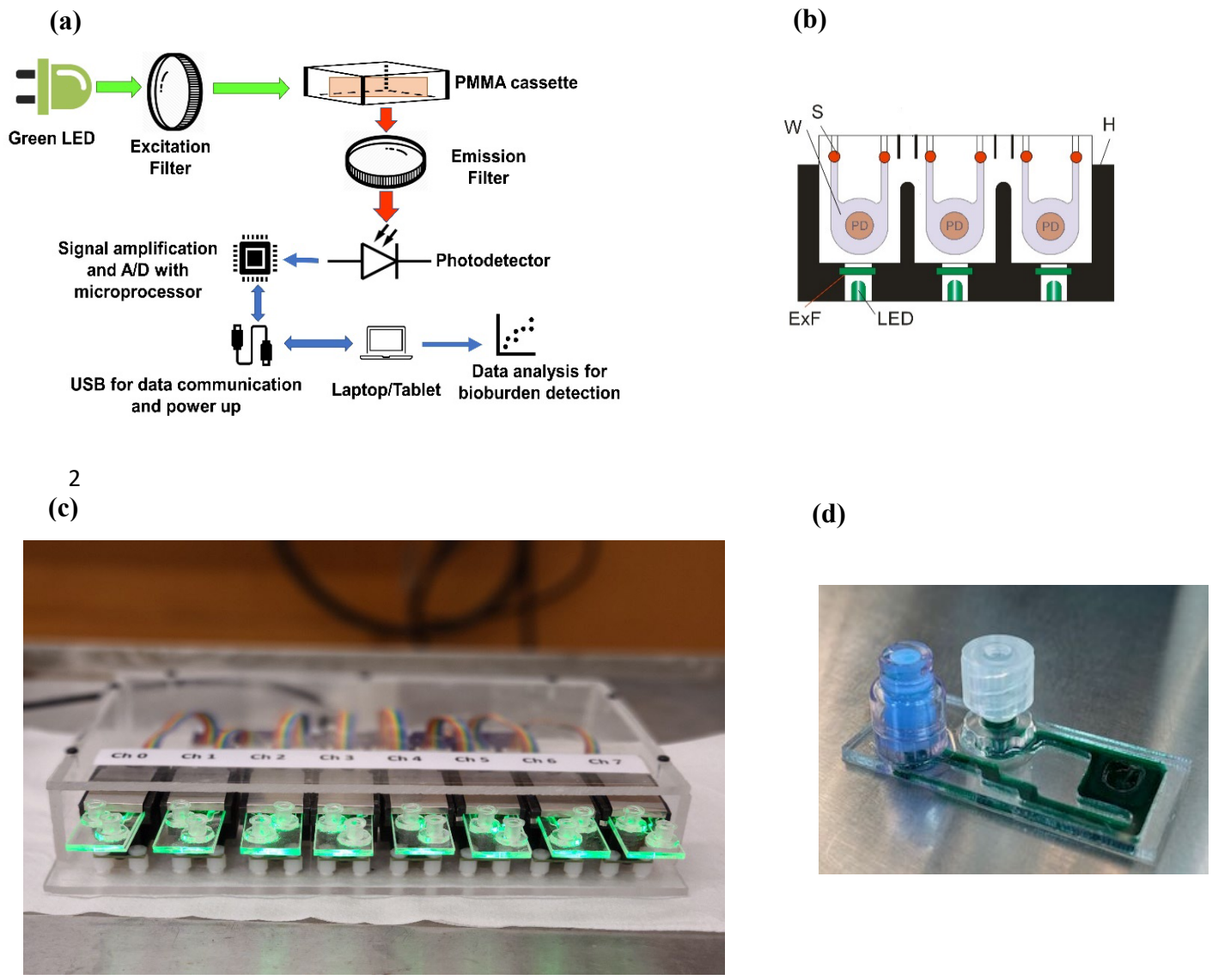


Figure 1. a) Schematic block diagram of single channel fluorometer. b) A graphical illustration of the multichannel fluorometer with cassette holders. The "W" is the microfluidic well where the sample is loaded, "S" represents inlet and outlet, "PD" is the photodetector lying underneath the well, "ExF" is the excitation filter mounted in front of LED and "H" is the cassette holder. c) Photograph of the multichannel fluorometer with microfluidic cassettes inserted. d) An image of the microfluidic cassettes with luer lock and needleless insert.

Preparation and optimization of the assay

In this study, we have carried out several experiments sequentially to optimize our assay in the spectrophotometer. After optimizing the assay, we have tested and validated the assay in the multichannel fluorometer and compared the results with the spectrophotometer. Figure 2a shows the experimental steps performed in this study.

Preparation of cell culture and resazurin

The preparation of primary and secondary culture cells is done in the same way as our previous publications [16,35]. Initially, 50 mL primary culture was prepared using 200 µL of *E. coli* NM303 cells, which was grown at 37 °C in a shaker at 150 rpm (Lab-line Instruments, Melrose Park, IL) overnight. The optical density of the primary culture was measured to be 2.5 at 600 nm (SpectraMax M5). The primary seed culture (1 %) was used to inoculate into 200 mL secondary culture and was grown at 37 °C in a shaker at 150 rpm to reach an optical density of 0.4 at 600 nm (~3×10^8 CFU/mL). This was centrifuged at 5000 RPM (Avanti J-25 I centrifuge, Beckman Coulter, Inc., Brea, CA) for 10 min. The cell pellet was washed with phosphate-buffered saline (PBS, pH 7.2) and the washed cells were re-suspended in PBS, pH 7.2. This sample is used for making serial dilutions (in 50 mL tubes) from 1:10 to $1:10^5$. The $1:10^5$ diluted sample is used to make further dilutions to make a final concentration of viable cells which is calibrated against a standard plate count. For this, the diluted *E. coli* samples from 1000 CFU/mL to 1 CFU/mL were prepared. 200 µL of each sample was spread on an LB agar plate, and the plates were placed in an incubator at 37 °C overnight. After about 18 hours, the number of colonies showing up on the plates was counted.

5µM resazurin is mainly used in our study for the reduction study. Additionally, we experimented with various resazurin concentrations for optimizing the assay. Generally, for making 31.85 mM concentration of resazurin, 100 mg (w/w) of resazurin was weighed and suspended in 10 mL of PBS, pH 7.2. This suspension was vortexed for 30 min (5 min on/off) at room temperature in the dark. Further, this suspension was sterile filtered through a 0.2µm filter, diluted to 3.185mM, and re-vortexed for 5 min (1 min on/off). This was filtered again and used for the present study and wrapped in aluminum foil in a tube. The sterile stock solution was stored in a refrigerator and was further diluted as per the experiment.

Assay preparation for the optimization of resazurin and culture media and effect of supplementary glucose

The optimized assay preparation involved varying resazurin concentrations, studying different culture media and monitoring the effect of supplementary glucose on the assay. These details have been incorporated in the materials and methods section of supplementary information.

Assay preparation to investigate the effect of incubation period on the assay sensitivity

Five samples of 100 CFU/mL were prepared by diluting the secondary culture to explore the effect of incubation time along with the consisting of negative control LB media. Other than the first sample, which was tested immediately, the other four samples were incubated in a 37°C incubator for 2, 4, 6, and 8 hours, respectively. 5µM resazurin was added after these time intervals, mixed well and were tested in 96-well plates in triplicates. The plates were then immediately read in the spectrophotometer.

Assay preparation for the investigation of the effect of incubation period combined with filtration on the assay sensitivity

A portion of both primary and secondary cultures was used to prepare samples by adjusting their OD_{600} to 0.4 with LB media. The primary and secondary samples were used to prepare 100, 50, 25, 10, and 5 CFU/mL cell samples named P-Group and S-Group, respectively. A particular volume (10 mL) of S-Group sample was filtered using a 70% ethanol sterile polyethersulfone (PES) filter, and the trapped cells were collected in 1 mL LB media by backflushing. The concentrated (10x) secondary culture group obtained by filtration is called the SF group. Four 96-well plates were prepared, each containing LB, P-Group, S-Group, and SF-Group samples in triplicates and were kept in the incubator for 5, 6, 7, and 8 hours and then tested sequentially in the spectrophotometer. After all the measurements were completed, the slopes of the fluorescence intensity profiles were calculated.

Assay preparation for the differentiation of viable (live) and non-viable (dead) cells

The detailed procedure of assay preparation for the differentiation of live and dead cells is stated in the materials and methods section of the supplementary information.

Assay preparation and procedure for the measurement in the multichannel fluorometer

Primary culture samples (LB, 5, 10, 25, 50 CFU/mL) were prepared and 1mL of each of the samples was placed in 24 well plates. The plates were incubated at 37°C for 5, 6, 7 and 8 hours, respectively for 25 CFU/mL samples with LB to validate the increasing assay sensitivity with modulation in incubation period. After each incubation period, 5 μM resazurin was added to the different wells and the mixture was injected into a cassette. The cassette was then inserted immediately into a channel of the 8-channel fluorometer and measured for 10 minutes. The same procedure was repeated after 6, 7, and 8 hours of incubation. A 24 well plate measurement was also completed in the spectrophotometer to determine the consistency and comparability of the measurements. The response for the different primary culture bacterial loads (LB, 5, 10, 25, 50 CFU/mL) was measured in the multichannel fluorometer in the same fashion at a fixed 6 hour incubation time, in triplicates.

Details on the blinding protocol implemented

The blinded tests were performed by two individuals using both 96 well plates of the spectrophotometer and the multichannel fluorometer. Primary cell culture was used to prepare 50, 25, and 5,1,0.2 CFU/mL and corresponding concentrated (10x) cell samples by filtration with negative control to test in the 96 well plates parallelly (one blinded, one unblinded). In addition to that, the above-prepared cell samples were tested in the multichannel fluorometer in a blinded manner. After the tests have been performed, the results from the 96 well plates and the fluorometer were verified to see the consistency and comparison. All the experiments have been performed at least twice with only primary culture samples for imitating real-world phenomena and to have statistically significant values.

Analytical methods

The analytical methods used to calculate sensitivity, noise, LOD and minimum time for detection are described in the materials and methods section of the supplementary information.

Results and Discussion

Reproducibility and robustness of the device

The repeatability and robustness of the device were tested with 5 μM resazurin dye in triplicates. More details and results are presented in the results and discussion section in the supporting information. The results indicate the fluorometer is a robust device having good repeatability and significantly low relative standard deviation and fluorescence decay rate.

Effect of incubation period on the sensitivity of the assay

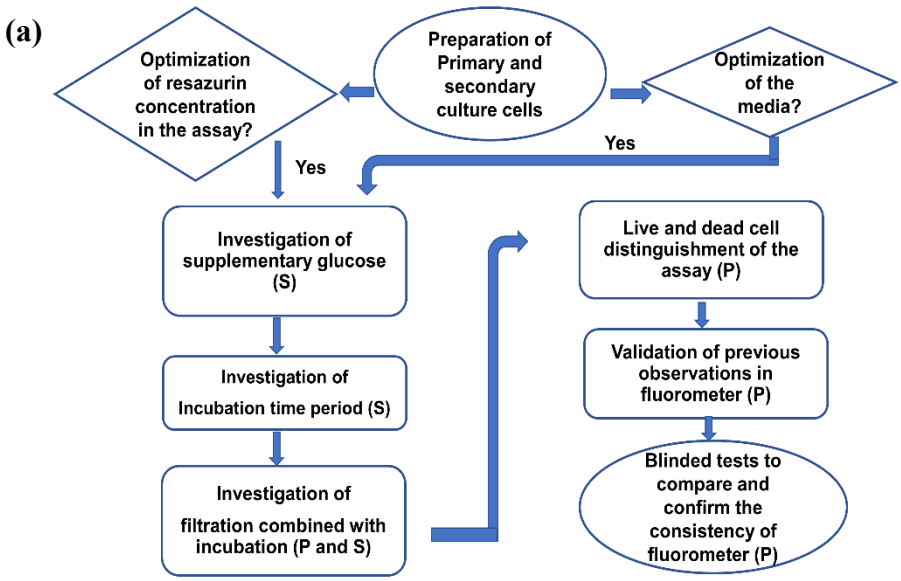
The secondary culture was diluted to prepare 100 CFU/mL samples and the samples were incubated for 0 to 8 hours with an interval of two hours with LB media. 5μM resazurin was added and fluorescence intensity was measured as before. The fluorescence slope and LOD are plotted in Figure 2b. It is evident from the figure that incubation can significantly increase assay sensitivity. By incubating the samples for 4, 6 and 8 hours, the slopes of their fluorescence intensity profiles increased to 0.59, 1.24, and 22.12, and the LOD was reduced to 149, 22, and 4 CFU/mL, respectively. As the incubation period increases, the growth rate of bacteria increases as a result of the metabolic rate increment. We hypothesize that for a lower incubation period, in this case for 0 to 4 hours, the bacterial growth is in the lag phase where the cells are increased in size, but no cell division occurs resulting in a very low fluorescence signal. But after that incubation period, the bacterial growth enters into the exponential

(log) phase where the increasing incubation period results in the increasing number of viable cells [36–38]. This in turn increases the fluorescence signal and effectively the LOD is significantly improved. This difference for a single bacterial strain with different incubation periods leads to a better understanding of intraspecies variation. Thus, our assay can play a vital role to determine the different bacterial growth stages leading to intraspecies variation initiated by altering the incubation periods. The LOD for no incubation time was also calculated with different cell concentrations and the data is included in the supplementary Table S2.

Effect of incubation combined with filtration on the assay sensitivity

A portion of primary and secondary culture cell samples were prepared, and the secondary culture cell samples were manually filtered and retrieved using the backflushing technique. All these samples named P, S and SF samples were incubated for 5 to 8 hours (in 1 hour intervals) chosen to reach lower LOD and higher sensitivity. The sensitivity and LOD for different incubation hours are presented in Figure 2c. The sensitivity for the S-Group samples is higher than that for the P-Group samples resulting in lower LOD. This can be accounted for based on the primary culture having entered the stationary phase, as there are fewer active cells in the primary culture although their OD values were adjusted to be the same as the S-group samples. However, the S-group samples were in the log phase of growth for [36–38] this period. The assay sensitivity for the SF-Group samples is higher than that for the unfiltered samples as can be seen from the LOD values. After filtration, the number of cells recovered from the filter paper is usually more than the unfiltered samples. This in turn leads to higher assay sensitivity and achieving significantly lower LOD values for the concentrated samples after fixed hours of incubation. This is despite the fact that it is not possible to recover all the cells because of the backflushing technique.

Additionally, the assay sensitivity for the concentrated secondary samples did not increase significantly with the increase in incubation time as the cells had already entered the stationary phase with 5 hours of incubation. We have also shown the concentrated primary culture via filtration samples have higher sensitivity and lower LOD than the primary sample which is included in the supplementary Table S3. Hence, the assay with the combination of incubation and filtration has the capability of reaching significantly low LOD and needs to be further investigated.



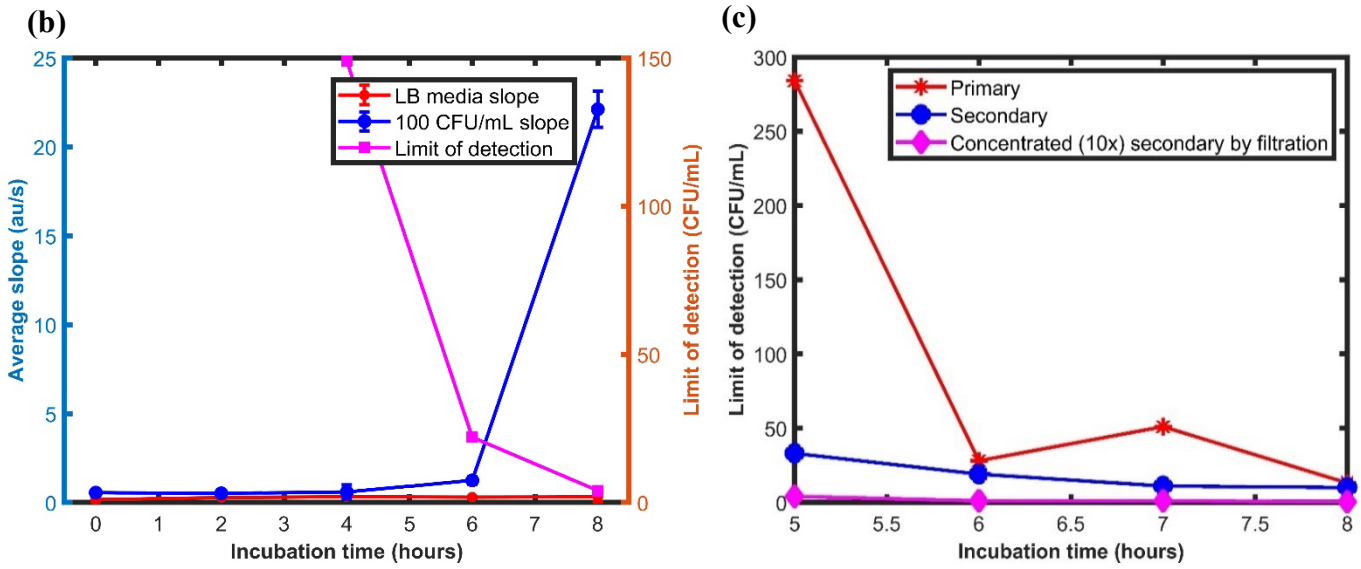


Figure 2. a) Flow chart of the experiments performed from optimization of the assay to testing and validating it in the multichannel fluorometer. The "S" and "P" in the parentheses represent experiments performed with secondary and primary culture cell samples respectively. b) LB media and 100 CFU/mL slope for different incubation periods with LOD. c) Limit of detection of primary, secondary and concentrated secondary culture cell samples for varying incubation hours.

Assay based differentiation of the viable and non-viable cells

To determine the capability of the assay to differentiate between live (viable) and dead cells, three sets of samples were prepared along with the negative control LB media based on concentration via filtration samples and primary culture samples. These samples were boiled before and after incubation for 5 minutes and the fluorescence intensity was recorded in the spectrophotometer along with their non-boiled samples. The results and analysis summary are presented in supplementary Table S4.

A statistical analysis of the samples was conducted using the samples boiled before incubation as reference. It can be seen that all 3 LB samples (boiled before incubation, unboiled and boiled after incubation) have no significant difference with 4 to 6 hours of incubation. With 4 hours of incubation, all same-group samples have no significant difference, showing that 4 hours of incubation is not long enough to distinguish between the boiled and unboiled samples. With 5 and 6 hours of incubation, the slopes for all unboiled samples are significantly higher than the boiled samples and all the p-values for t-tests are ≤ 0.5. This shows that with ≥ 5 hours of incubation time, the assay can differentiate between live and dead cells. With pre-filtration, the concentrated assay sensitivity is higher for ≥ 5 hours of incubation time.

Comparative study on assay performance in multichannel fluorometer and conventional plate reader

After the optimization of the assay and the exploration of the effect of incubation and filtration, the optimized parameters have been employed to put into test using the multichannel fluorometer. A primary culture sample of 25 CFU/mL has been incubated for 5 to 8 hours along with the negative control LB media and after that resazurin was added and the sample was injected into the microfluidic cassettes for measurement in triplicates. A parallel 24 well plate was tested in the spectrophotometer to determine the consistency of the multichannel fluorometer and compare their performance. The sensitivity and calculated LOD for the incubation hours are plotted and presented sidewise in Figure 3a. The LOD and the sensitivity of the two different detectors are quite comparable.

The lower LOD in the multichannel fluorometer despite lower sensitivity is accounted for by the lower noise in the fluorometer. The lowering in LOD with increasing incubation time and improved sensitivity is consistent with our previous observations. Hence, the intraspecies variability with variable incubation periods is also observed using the in-house designed multichannel fluorometer.

Different primary culture samples LB, 5, 10, 25, 50 CFU/mL have been incubated for a fixed 6 hour incubation period. The 6 hour incubation time has been chosen to reach lower LOD as confirmed by previous experiments and is sufficient to differentiate live and dead cells. The fluorescence response of the multichannel fluorometer is shown in Figure 3b**.** Though all the samples have been tested in triplicates only one sample detail is presented here. The figure shows that with increasing bacterial load there is a higher slope observed than LB media which exhibits very little or no slope. For 25 and 50 CFU/mL, a decreasing slope is observed after some time that is due to the reduction of resorufin to colorless dihydroresorufin. The decaying occurs faster and at a lower time for higher concentrations. Thus, our multichannel fluorometer can distinguish between bacterial and nonbacterial samples with similar or lower LOD than the spectrophotometer and can be implemented to explore intraspecies variability in field studies.

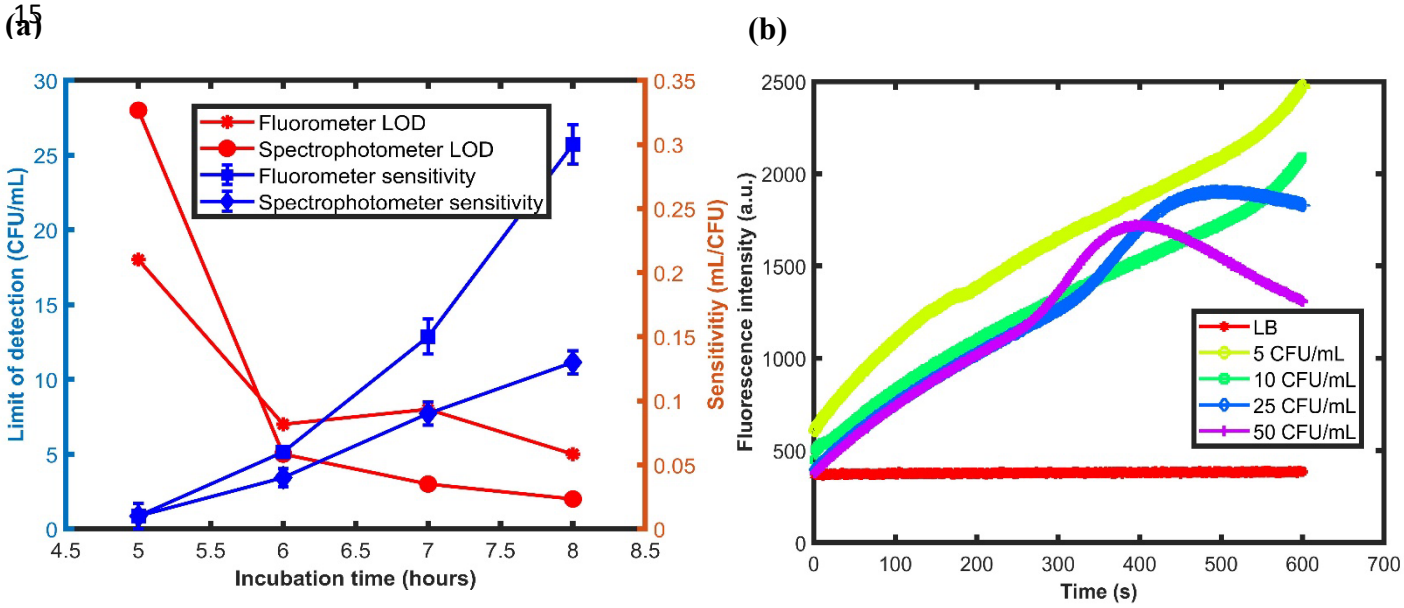


Figure 3. a) LOD and sensitivity in the spectrophotometer and multichannel fluorometer. b) Multichannel fluorometer response for different bacterial cell concentrations.

Results and analysis of the blinded tests performed

The blinded tests were performed using different primary culture bacterial concentrations with their negative control by two individuals to avoid experimental bias. The blinded tests were performed in two different groups: lower (LB, 0.2,1 CFU/mL) and higher cell concentration (LB, 5, 25, 50 CFU/mL) groups in duplicates. One set of complete data analysis for LOD, F-test and t-tests for the higher and lower cell concentrations in the fluorometer is included in Tables S5, S6. The corresponding spectrophotometer response is enlisted in supplementary Tables S7, S8. The calculated LOD for the two parallel tests in the spectrophotometer and corresponding blinded test in the fluorometer is shown for higher (Figure 4a, 4b) and lower (Figure 4c, 4d) cell concentrations. From the bar plots, we see that the LOD values for the concentrated samples after filtration are lower than the unfiltered samples in both the detectors which is coherent with our previous findings except for the spectrophotometer response in Figure 4a. Though the sensitivity is higher for the concentrated samples in this case, as evident from the

supplementary Table S7, the higher LOD can be attributed to a higher noise value. The calculated LOD for the concentrated samples is < 1 CFU/mL and for non-filtered primary samples is < 2 CFU/mL in both the detectors. Hence, the response of the multichannel fluorometer is quite comparable and consistent with the conventional plate readers. Additionally, the p values of the t-tests between blinded and unblinded samples in the spectrophotometer of all the experiments are all > 0.5, presenting no significant difference. One set of data for the comparison of blinded and unblinded samples is provided in the supplementary Table S9. Our multichannel fluorometer with the microfluidic cassettes can hence confidently and consistently reach LOD of < 2 CFU/mL for the primary culture samples with 6 hours of incubation. The highlight is that with filtration, it is possible to reach < 1 CFU/mL. With more incubation period combined with filtration, it is possible to reach even lower LOD with this in-house designed fluorometer.

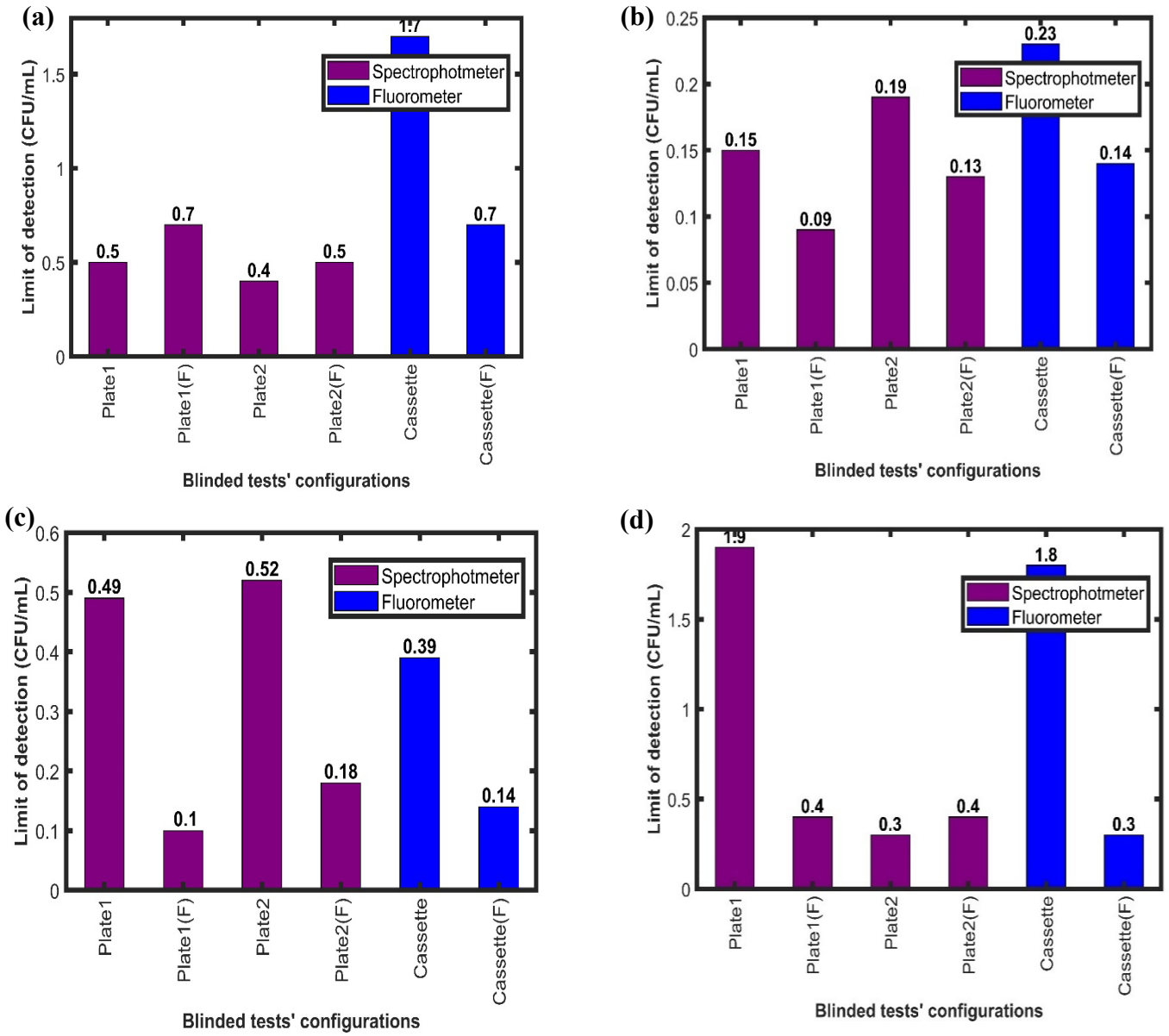


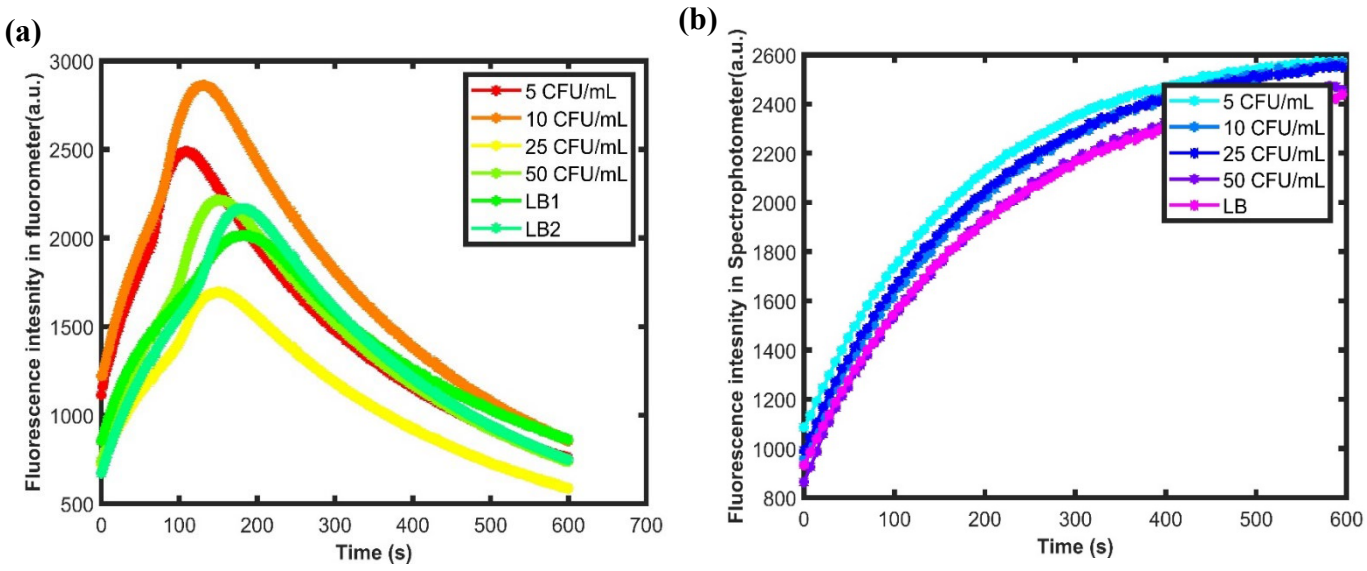
Figure 4. a) LOD for the first blinded test performed using higher cell concentrations. The "F" refers to the concentrated (10x) samples by filtration. Plate 1 and Plate 2 refer to the spectrophotometer readings in parallel. Cassette refers to the corresponding blinded fluorometer response for the same experiment. b) LOD for the second blinded test performed using higher cell concentrations. c) LOD for the first blinded test performed using lower cell concentrations. d) LOD for the second blinded test performed using lower cell concentrations.

Bioburden detection in real-world samples

During the blinded tests performed, we have come across several sources of contaminations, caused due to accidental bioburden exposure. The contaminations were caused by contaminated pipette tips, test tubes, ethanol reduction which is much more likely to be encountered in real-life scenarios. This serves as a proof-of-concept, presenting the capability of our technique in picking up bacterial contamination from swab samples, surface samples and different instruments used in this assay. Hence, all the tools used in the experiments need to be autoclaved prior to use, as per established protocol. Two scenarios are presented here as case studies.

Contaminated pipette tips in one blinded experiment were the source of bioburden. Different bacterial concentrations (LB, 5,10,25,50 CFU/mL) were incubated for 6 hours after using the contaminated pipette tips and tested in both multichannel fluorometer and spectrophotometer. Also, another parallel test was done using the same samples where pipette tips were the only difference. The fluorescence intensity plots from contaminated samples from both fluorometer and spectrophotometer are presented in Figure 5a, 5b. The fluorescence intensity plots from the non-contaminated pipette tips are presented in Figure 5c in the spectrophotometer. As from figure 5a, 5b, there is no way to distinguish from different bacterial samples and the negative control for the contaminated test coherent or both the devices. Whereas the non-contaminated samples clearly show the difference between the LB media and the bacteria samples studied.

Another contamination was caused by the presence of bioburden in the test tube. The experiment was conducted using LB, 0.2,1,5 CFU/mL samples. The samples were kept in the incubator in five different tubes and taken out after 6 hours of incubation for the test. The results from the multichannel fluorometer and spectrophotometer are shown in supplementary Figure S3. For lower bacterial contamination it is hard to see the slope difference between LB media and other bacterial concentrations (despite statistical difference). The 0.2 CFU/mL samples have an unusually higher slope than other bacterial samples. It is the same for both the detectors. As for the experiment, all the equipment used is the same except for the test tube used in this particular study, the source of contamination in this case which is picked up by the assay.



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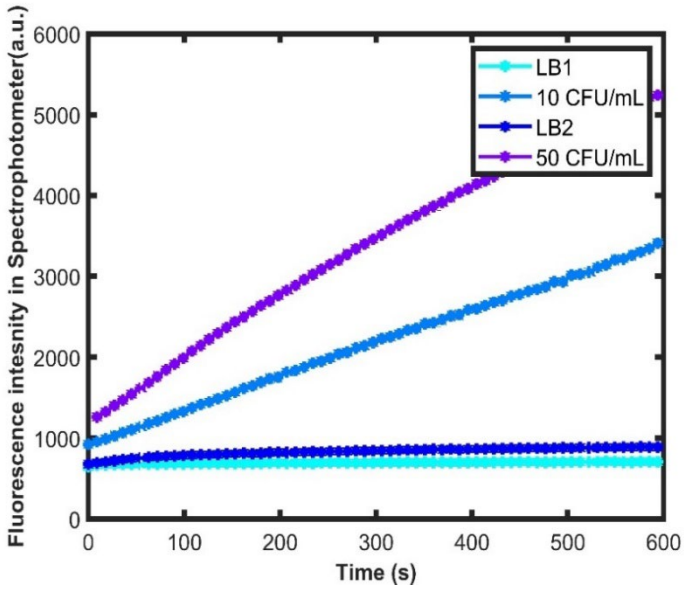

Figure 5. a) Multichannel fluorometer response for different bacterial cell concentrations with contamination from pipette tips. b) Spectrophotometer response for different bacterial cell concentrations with contamination from pipette tips. c) Spectrophotometer response of different bacterial cell concentrations with non-contaminated pipette tips.

Response time for threshold bacterial detection

The analysis between the LB media and different cell culture samples was done using bacterial samples mentioned in the analytical method section. The F-test and t-tests were performed for three maximum slopes obtained from the LB and three minimum slopes from different cell concentration samples for 10-300 seconds. The minimum time that gives the statistical difference was considered the time for detection. Tables S10, S11 in the supporting information shows the minimum time, F-test and t-test values for different cell concentration limits. From this data, the multichannel fluorometer can determine bacterial contamination as low as 5 CFU/mL with a minimum of 20 seconds. Additionally, concentrated bacterial samples of ≥ 1 CFU/mL can be detected within 90 seconds. This shows the potential of the fluorometer as a rapid device for determining bioburden presence.

Conclusion

In response to the increasing number of contaminations caused by the presence of bacteria in the food, water and pharmaceutical industry, it is of immense importance to detect these in a rapid manner accurately with a field-deployable device. In this study, we have developed a very sensitive and accurate assay to determine the bacterial contamination <1 CFU/mL primary cell culture samples with 6 hours of incubation time and followed by filtration. In addition to that, we have shown how the incubation and filtration steps on the assay can further increase the sensitivity with the additional advantage of comprehending different bacterial growth stages. The small, compact, mechanically robust multichannel fluorometer with the ability of parallel sampling can accurately detect the abovementioned range of bacterial contamination within as low as 20 seconds. The multichannel fluorometer can be further modified to determine the negative control of the assay using the filtration approach described in this study, with the potential of intraspecies variability detection, differentiating different bacterial strains along with the antibiotic susceptible strains. Our future study will focus on testing different gram-positive, gram-negative bacteria as well as yeast, molds and surface contaminations in the fluorometer. The low detection

limit, the simplicity and rapidness of the test, as well as the portability of the device with microfluidic cassettes make it a promising tool for the rapid detection of bioburden.

Supporting Information

Additional experimental details and results for the optimization of the assay, robustness of the device, details of analytical methods, results and comparisons for blinded and unblinded testing, and the minimum detection time.

Acknowledgment

This work was supported by the U.S. Food and Drug Administration under grant no. BAA 75F40119C10132.

Author contributions statement

The manuscript was written through contributions of all authors. All authors approved the final version of the manuscript.

Competing Interests

G.R. and Y.K. are listed inventors on US Patent 10,948,414 B2 "Methods and Apparatus for Rapid Detection of Bacterial Contamination"**.**

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