PROMPT AND IN SITU DIAGNOSIS OF LIVE/DEAD BACTERIA

An Undergraduate Research Scholars Thesis

by

MATTHEW WALCK

Submitted to the Undergraduate Research Scholars program
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Peter Rentzepis

May 2016

Major: Electrical Engineering

TABLE OF CONTENTS

ABSTRACT

Prompt and In Situ Diagnosis of Live/Dead Bacteria

Matthew Walck
Department of Electrical and Computer Engineering
Texas A&M University

Research Advisor: Dr. Peter Rentzepis
Department of Electrical and Computer Engineering

The goal of this project is to develop a method for rapid and in situ identification of live and dead bacteria using absorption spectroscopy, emission spectroscopy, and principal component analysis. To achieve this goal, we have designed a portable, handheld fluorescence spectrometer utilizing a UV-diode laser as an excitation source and a CCD for detection. This work presents evidence that principle component analysis can be used to successfully differentiate living from dead bacteria on site, quickly, with hand-held, portable equipment.

CHAPTER I

INTRODUCTION

Many infections are caused by living bacteria. As such, methods for quickly detecting and differentiating live bacteria from dead are desirable for many applications. Examples of such applications include verifying if water is potable, determining whether or not a wound is infected, and quickly confirming if a method of bacterial elimination was successful.

Bacteria are single cellular prokaryotic organisms. Bacteria can be pathogenic, like bacillus anthracis which causes the disease anthrax, or beneficial to human life, like cyanobacteria which produce oxygen through photosynthesis. Bacteria are protected by a cell envelope which comprises an outer polysaccharide structure called a cell wall and an inner lipid and protein filled cell membrane, both of which serve to protect the cell. They also contain a nucleoid which houses their DNA (Thanbichler, Wang, and Shapiro 2005). Bacteria are classified as either Gram-negative or Gram-positive. This classification refers to the bacteria's cell envelope and how it responds to crystal violet dye. Gram-negative cells, when exposed to crystal violet dye, do not retain the dye; whereas graham-positive cells do retain the dye. Gram-negative cells, due to their membrane, are more resistant to antibiotics("CDC - Gram-Negative Bacteria Infections in Healthcare Settings - HAI" 2016). Reproducing asexually, bacteria multiply quickly, with colonies doubling in size every 10-20 minutes depending on the species and the environment. This reproduction occurs through cell division: the bacterium grows until it is large enough, and then its body and DNA split to become two separate bacteria. UV light destroys bacteria by

damaging the DNA and preventing it from splitting, causing the bacterium to be unable to reproduce.

Infected wounds can have significant consequences if untreated or undiagnosed. An untreated infection can spread to the surrounding skin and bone, as well as enter the blood vessels and, from there, spread to the rest of the body. In extreme cases, infections can require amputation of a limb or lead to the death of the host. The treatment of wounds needs to begin quickly to eliminate the risks of infection, but this need conflicts with the current methods for determining the viability of bacteria in a wound, which require sophisticated equipment and long periods of time. This conflict is especially apparent in wounds that occur away from traditional medical settings, such as rural areas or battlefields, where the need for laboratory settings requires a sample from the wound to be transported to a location capable of analyzing it, further lengthening the time required for analysis. Any technology that seeks to aid in the determination of the infection potential of bacteria in wounds needs to be quick and portable.

The most common methods for determination of living and dead bacteria involve incubating the bacteria for 10 hours or more and visually measuring the colony forming units(JM et al. 1995). Because incubation requires a significant amount of time, such methods are not suitable for applications that necessitate immediate knowledge of the percentage of living bacteria. Other methods, involving the use of fluorescent dyes, such as CTC, during incubation (Créach et al. 2003) or the automation of the CFU counting process (Brugger et al. 2012), make the process more efficient in terms of labor, but the requirement of incubation facilities and long time periods make such processes non-feasible for many tasks.

To alleviate this problem, we have designed a portable, hand-held instrument which is capable of immediate determination of the concentration of live and dead bacteria.

CHAPTER II

METHODS

Materials and equipment

An Oriel mercury lamp was used for bacterial elimination via ultraviolet radiation. UV light was used for the inactivation of the bacteria, as opposed to alternative methods such as antibiotics, due to both its effectiveness and to bacteria's inability to develop a resistance to it. Many bacterial strains develop immunities to antibiotics, which makes the usage of antibiotics a less certain method of elimination.

The bacteria that were employed were Escherichia Coli provided by AgriLife scientists. The bacteria were stored at 4 degrees Celsius until they were used in experiments, when they were diluted in water.

This study utilized a Shimadzu UV160U UV-visible absorption spectrometer to measure the absorption spectra of bacteria. The absorption spectrum is a plot of the light absorbed by a sample, known as optical density, as a function of the wavelength of light, and can be used to identify bacteria and find the wavelengths of light absorbed by it.

A Shimadzu RF-5301PC fluorescence spectrometer was used to take the fluorescence and synchronous spectra of bacteria. The fluorescence spectrum of a sample is the plot of the intensity of light emitted by the sample, after it is excited by incident light of a particular wavelength, as a function of the wavelength of the emitted light. This spectrum can be used to

identify a bacteria and to find the emission wavelength maxima. The synchronous spectrum of a sample is a fluorescence spectrum where the excitation wavelength changes with the emission wavelength being measured so that the difference between the excitation wavelength and the emission remains constant. This difference is known as delta lambda. The synchronous spectrum can be used to identify specific chemical components within a bacterium.

Determining proper excitation wavelength for E. Coli

The absorption spectrum was taken of the E. Coli and used to find the maximum, peak absorption wavelengths of the bacteria. Then the fluorescence spectrum of the sample was taken using those peak wavelengths as the excitation wavelengths.

In order to further optimize the excitation wavelength, the peak from the fluorescence spectra was then used as the target wavelength for the measuring of the excitation spectrum of the E. Coli. The excitation spectrum is a plot of the light emitted at a particular wavelength as a function of the excitation wavelength. Through the use of this spectrum, the ideal excitation wavelength was able to be determined.

Plotting the excitation emission matrix for living and dead bacteria

The excitation-emission matrix for living E. Coli was plotted by taking 21 fluorescence spectra, with excitation wavelengths increasing from 220nm to 320nm in 5nm steps, and plotting them in three dimensions. The sample was then irradiated at a power level of 0.8 mW and time period of 10 minutes which is known to destroy the bacteria, and the same process was followed to record

the excitation-emission matrix for the dead E. Coli. The excitation emission matrix allows the data of both the living and the dead bacteria to be examined in three dimensions.

PCA of a single sample

A sample of live E. Coli, diluted with distilled water, was prepared. Then 10 separate synchronous spectra, with a delta lambda of 5 nm, and fluorescence spectra, with an excitation wavelength of 280nm, were recorded. Subsequently, the sample was irradiated at a power level of 0.8 mW and time period of 10 minutes which is known to eliminate the living bacteria. Ten more synchronous and fluorescence spectra were taken of the sample, which now contained only dead bacteria. This data was plotted and standardized. Principal component analysis, which has been used successfully in previous research to distinguish different types of bacteria (Héctor Enrique Giana et al. 2003), was then preformed on the both the raw and standardized data from the two samples.

PCA of multiple samples

Five samples of E. Coli were procured. A synchronous spectrum, with delta lambda 5nm, and an emission spectrum, with an excitation wavelength of 280nm, was taken of each. After this, the bacterial samples were irradiated at a power level of 0.8 mW and time period of 10 minutes which is known to eliminate the living bacteria, and the above process repeated. The spectroscopic data was then plotted and standardized, and principle component analysis was preformed upon both the raw and standardized data.

CHAPTER III

RESULTS

Determining proper excitation wavelength for E. Coli

Figure 1 shows the absorption spectrum of E. Coli. The peak absorption wavelength is 260 nm. This wavelength was used as the excitation wavelength for the fluorescence spectrum shown in figure 2A. The peak wavelength in figure 2A is at 338 nm, which was used as the target emission wavelength to take the excitation spectrum shown in figure 2C. The sharp vertical line occurring at between 330- 350 nm is the spectrometer detecting the excitation beam and should be ignored. The second largest peak, occurring at 280 nm, is the true excitation peak. Figure 2B shows the fluorescence spectrum for the sample excited at 280 nm. The intensity of the peak is significantly higher than the original spectrum with an excitation wavelength of 260 nm.

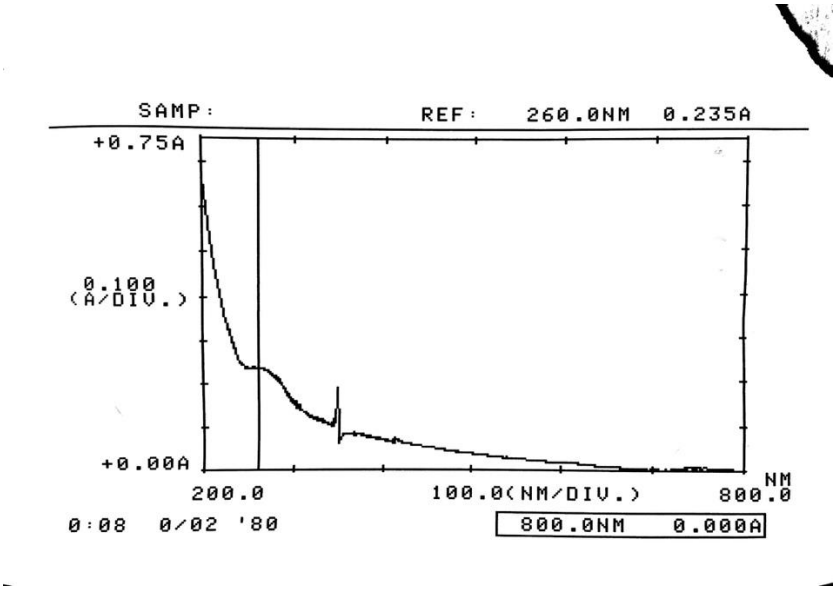

Fig. 1. E. Coli absorption spectrum. The above graph shows the absorption spectrum of a sample of E. Coli. The vertical line indicates the absorption peak at 260 nm.

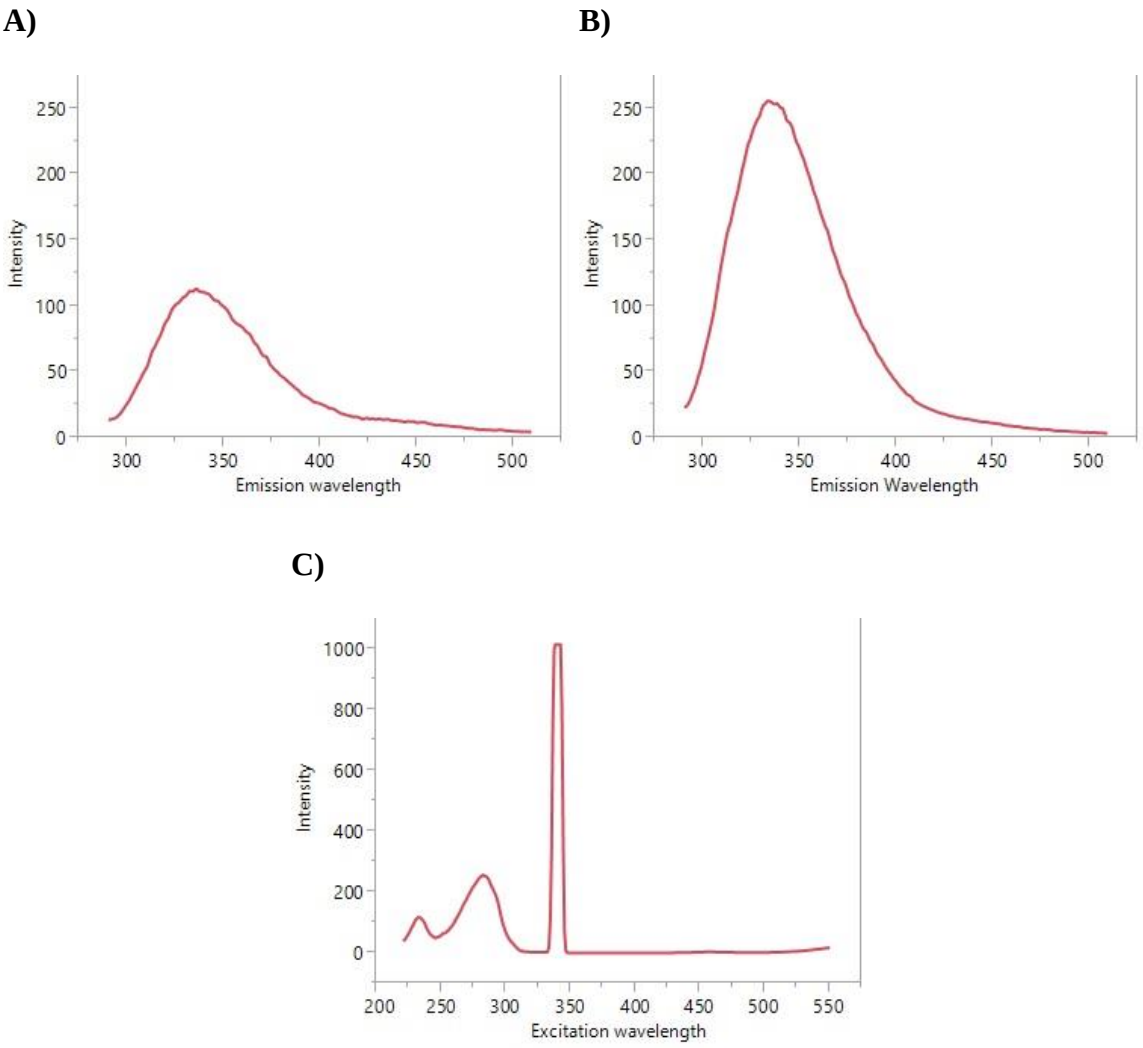

Fig. 2. Fluorescence and excitation spectra of E. Coli. The top left graph (A) shows the fluorescence spectrum of a sample of E. Coli that is being excited at 260 nm. The top right graph (B) shows the fluorescence spectrum of a sample of E. Coli that is being excited at 280 nm. The bottom graph (C) shows the excitation spectrum of E. Coli with the target emission wavelength being 338 nm.

Plotting the excitation emission matrix for living and dead bacteria

Figure 3 and 4 show the three dimensional plots of the excitation emission matrices of the living and dead bacteria taken at excitation wavelengths between 220 nm and 320 nm. This three dimensional plot allows the fluorescence spectra to be displayed as a function of excitation wavelength.

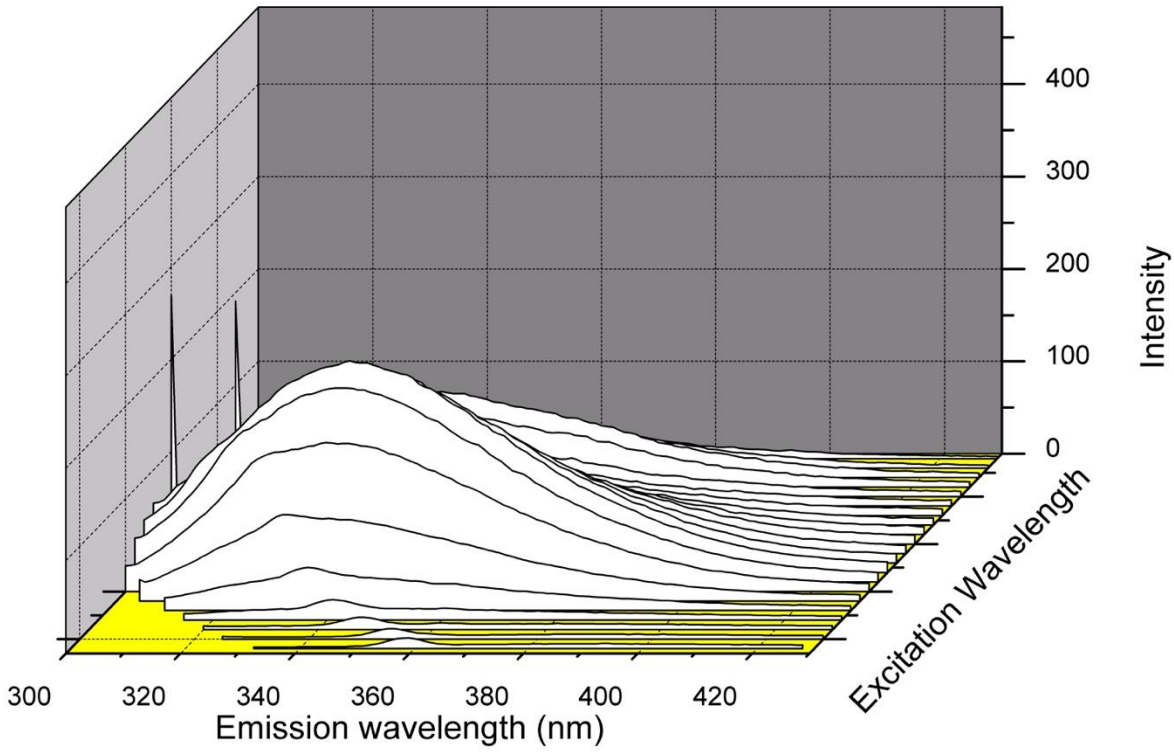

Fig. 3. Living Bacteria EEM. Three dimensional plot of living bacteria's fluorescence intensity vs. excitation wavelengths between 220 nm and 320 nm.

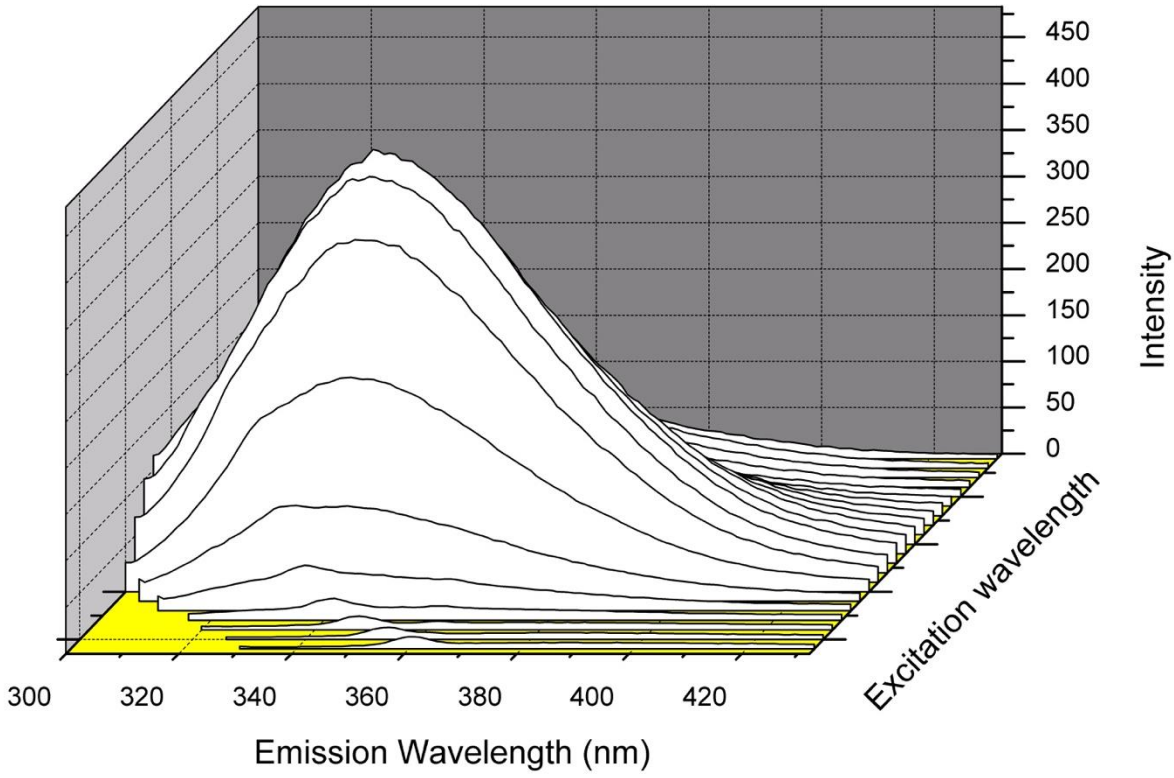

Fig. 4. Dead Bacteria EEM. Three dimensional plot of dead bacteria's fluorescence intensity vs. excitation wavelengths between 220 nm and 320 nm.

PCA of a single sample

Figure 5 shows the two plots generated using PCA on the emission spectra from a sample of live bacteria and a sample of dead bacteria. Both plots show clear separation between the living sample and the dead sample.

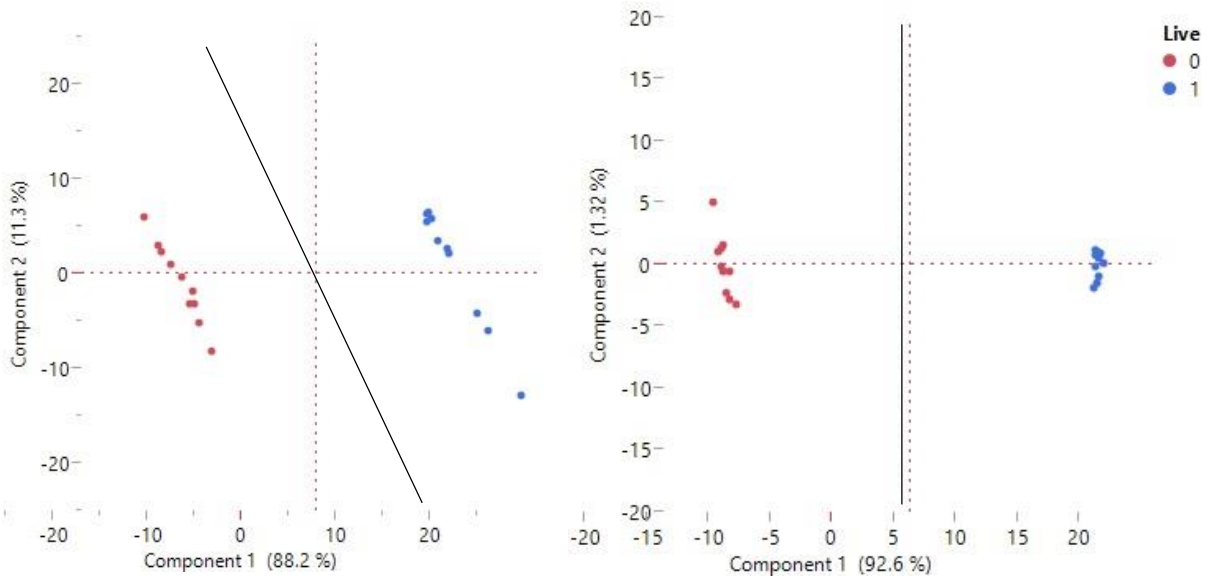

Fig. 5. Emission PCA of a single sample. The graph on the left (A) is the plot of principle component one and two of the fluorescence spectra from a sample of living bacteria and a sample of dead bacteria. This graph used the raw fluorescent data with no modifications as the dataset for PCA. The graph B is the plot of principle component one and two of the standardized fluorescence spectra from a sample of living bacteria and a sample of dead bacteria. In both graphs, red points represent the sample of dead bacteria and blue points represent the sample of living bacteria.

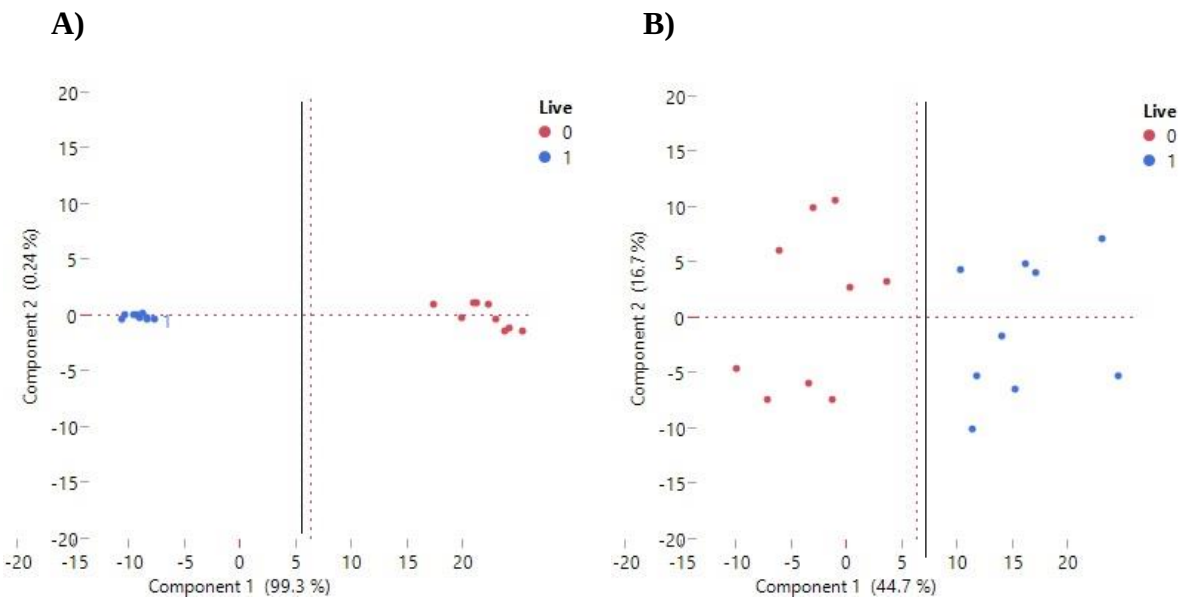

Fig. 6. Synchronous PCA of a single sample. The graph on the left (A) is the plot of principle component one and two of the synchronous spectra of the sample of living E. Coli and the sample of dead E. Coli. The graph B is the PCA of the synchronous data after it had been standardized. In both graphs, red points represent the sample of dead bacteria and blue points represent the sample of living bacteria.

PCA of multiple samples

Figure 3 shows the results of PCA on the fluorescence spectra from the samples of living and dead bacteria. In figure 3A, the data set used for PCA was the raw fluorescence data. There is separation between the samples of living and dead bacteria as indicated by the black line, although there is a sample of living bacteria misclassified as dead bacteria. In figure 3B, the dataset used for PCA was standardized. The separation of living from dead is extremely pronounced with all samples properly classified.

A) **B)**

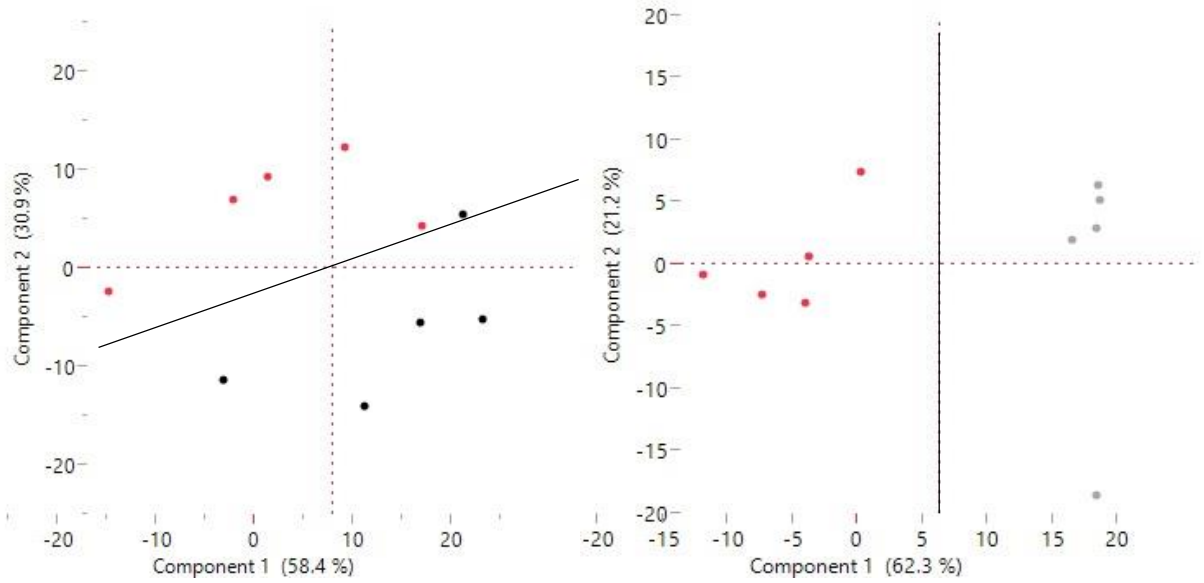

Fig. 7. Fluorescence PCA. The graph on the left (A) is the plot of principle component one and two of the unmodified fluorescence spectra of the samples. The red points are samples of dead E. Coli, and the black are samples of living E. Coli. Graph B is the plot of principle component one and two of the standardized fluorescence spectra of the samples. The red points are samples of dead E. Coli, and the grey are samples of living E. Coli.

The principle component analysis of the synchronous spectra of the samples shows similar results. In figure 8A, which is PCA preformed on raw synchronous data, there is separation between the living and dead, although one living bacteria is misclassified as dead. In figure 8B, after the data has been standardized, the separation becomes more clear, with a separation line able to be drawn to completely separate samples of living bacteria from samples of dead bacteria.

A) **B)**

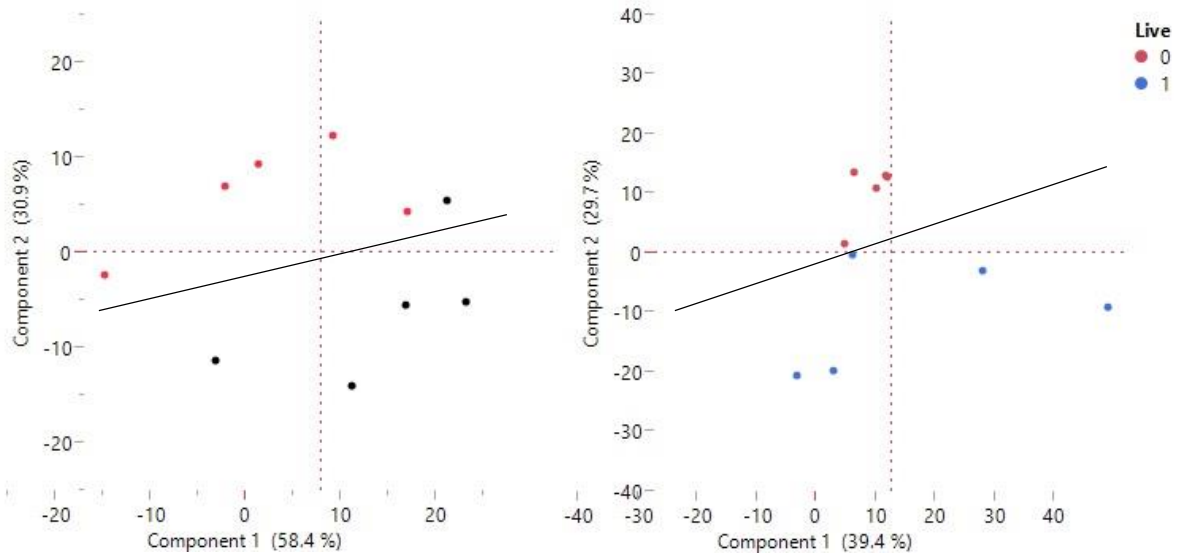

Fig. 8. Synchronous PCA. The graph on the left (A) is the plot of principle component one and two of the unmodified synchronous spectra of the samples. The red points are samples of dead bacteria and the black points are samples of living bacteria. The graph B is the plot of principle component one and two of the standardized fluorescence spectra of the samples. The red points are samples of dead bacteria and the blue points are samples of living bacteria.

CHAPTER IV

CONCLUSION

Discussion of results

Standardizing the data before principle component analysis improved the separation between living and dead bacteria in all cases except one (synchronous spectra from the single sample of dead bacteria and single sample of living bacteria) and in no cases prevented separation of the two. Through standardization of the data, the effects of concentration, and its subsequent effect on the intensity of the emitted light, are mitigated and the data is able to be compared despite the incompatibility of the original data.

The principle component analysis performed on the single sample of living and dead bacteria shows that the chemical change occurring in the bacteria when UV light damages their DNA can be detected with both fluorescence and synchronous spectroscopy. This chemical change is difficult to observe using only fluorescence and synchronous spectroscopy; however, PCA draws out the information contained within the spectra that is difficult to manually identify with just normal fluorescence spectra.

Both the emission and the synchronous spectra were used to differentiate living bacteria from dead bacteria. While, during the tests on multiple samples of living and dead bacteria, both emission and synchronous PCA had one misclassification, both were able to differentiate living bacteria from dead bacteria with no false acceptance or rejection after the data had been standardized.

Closing

PCA presents a novel method of in situ differentiation of living and dead bacteria. With minimal equipment, fluorescence spectroscopy can be preformed and the results analyzed quickly and on site. The results presented in this work show the potential for the construction of a hand-held, portable instrument that could revolutionize trauma care through the ability determine immediately whether or not a wound is infected with dangerous, living pathogens, without the need for traditional medical. Such a technology could drastically reduce harmful infections, unnecessary amputations, and preventable deaths that occur in the field, far from clinics and medical laboratories.

REFERENCES

Brugger, Silvio D., Christian Baumberger, Marcel Jost, Werner Jenni, Urs Brugger, and Kathrin Mühlemann. 2012. "Automated Counting of Bacterial Colony Forming Units on Agar Plates." *PloS One* 7 (3): e33695. doi:10.1371/journal.pone.0033695.

"CDC - Gram-Negative Bacteria Infections in Healthcare Settings - HAI." 2016. Accessed April 9. http://www.cdc.gov/hai/organisms/gram-negative-bacteria.html.

Créach, Véronique, Anne Claire Baudoux, Georges Bertru, and Bertrand Le Rouzic. 2003. "Direct Estimate of Active Bacteria: CTC Use and Limitations." *Journal of Microbiological Methods* 52 (1): 19–28.

Héctor Enrique Giana, Landulfo Silveira Jr., Renato Amaro Zângaro, and Marcos Tadeu T. Pacheco. 2003. "Rapid Identification of Bacterial Species by Fluorescence Spectroscopy and Classification Through Principal Components Analysis." *Journal of Fluorescence* 13 (6): 489.

JM, Gasol, del Giorgio PA, Massana R, and Duarte CM. 1995. "Active versus Inactive Bacteria: Size-Dependence in a Coastal Marine Plankton Community." *Marine Ecology Progress Series* 128 (November): 91–97. doi:10.3354/meps128091.

Thanbichler, Martin, Sherry C. Wang, and Lucy Shapiro. 2005. "The Bacterial Nucleoid: A Highly Organized and Dynamic Structure." *Journal of Cellular Biochemistry* 96 (3): 506–21. doi:10.1002/jcb.20519.