Signal Optimization and Chemometric Analysis of Laser-Induced Breakdown Spectroscopy Bacterial Spectra to Quantify Detection Limits and Improve Classification Accuracy

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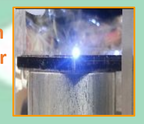
Introduction

We investigate the use of Laser-Induced Breakdown Spectroscopy (LIBS) for rapid bacterial detection, identification, and diagnosis. Clinical conditions are replicated by **swabbing trace bacteria off a target** and centrifuging the collected bacteria through a **nitrocellulose filter.** The filter is **analyzed by LIBS** in an argon environment.

Bacteria swabbed off target

Bacteria centrifuged onto filter

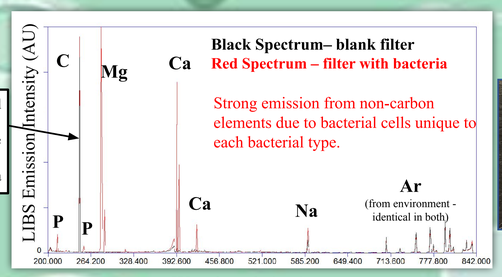
LIBS plasma on a bacteria filter



The result of this analysis is a **broadband optical emission spectrum** which allows us to determine the trace elements present in the bacterial cell, which are specific to a given bacterial species. A LIBS spectrum of *E. coli* and the filter medium is shown below. The intensity of the spectrum is proportional to the number of bacterial cells ablated.

Optical LIBS Spectrum from 200 - 840 nm
Revealing the Elements Present in a Bacterial Cell

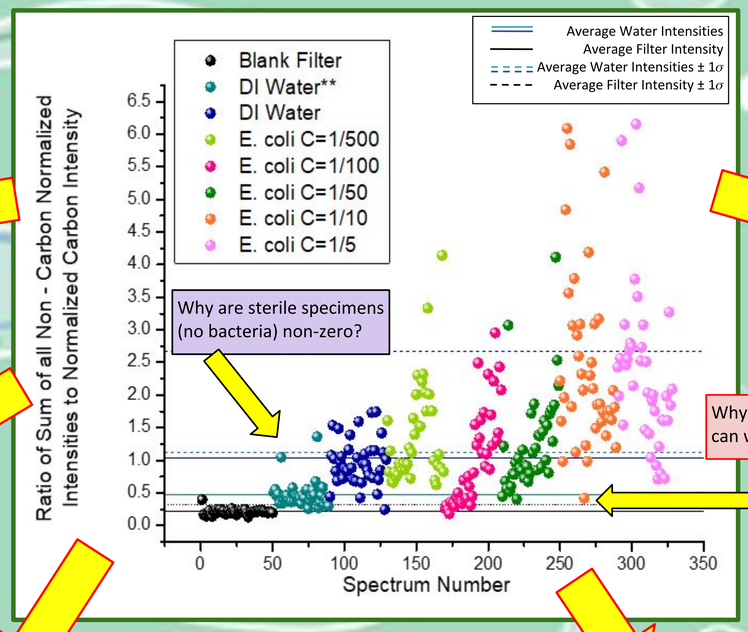
Strong background carbon emission from nitrocellulose filter even in absence of bacteria



Wavelength (nm)

Motivation

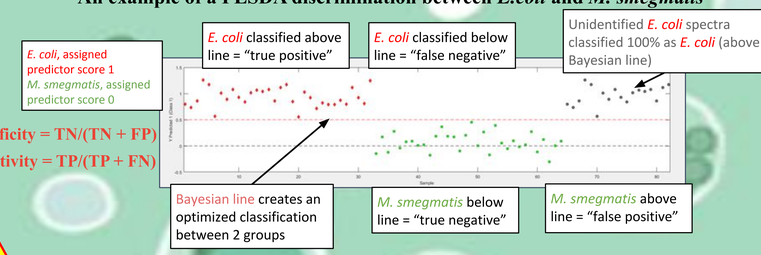
The intensities of emission lines in the LIBS spectrum provide a **unique elemental spectral fingerprint** for each type of bacteria which can be classified using **chemometric algorithms**. Our goal is to accurately identify/classify as small a number of bacterial cells as possible, while maximizing the rates of true positives and minimizing the rates of false positives. This research focuses on the **improvement of classification accuracy** by reducing background signal in the spectrum, investigating **data pre-processing**, and the **use of silver microparticles** to produce a more repeatable and robust spectrum.



Chemometric Analysis

Classification algorithms are used to externally classify spectra based on their similarity to a reference library. From a LIBS spectrum, 164 unique and independent variables are created from 18 emission lines. These variables are then reduced to a smaller number of discriminant scores by DFA or a single 'yes' or 'no' prediction classification by PLSDA. Both techniques result in a **sensitivity** and a **specificity** for each classification group which define an overall accuracy. The **accuracy of an external validation can be improved** by training a library through objective data rejection techniques to delete spectra with an unacceptably low intensity and "pathological" spectra which do not classify correctly.

An example of a PLSDA discrimination between *E.coli* and *M. smegmatis*



Current Total Library

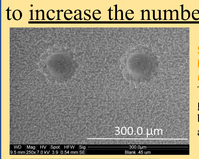
To classify bacteria using chemometrics, an extensive collection of "known" spectra with hundreds of data points is required. This is called a "library."

	Current Total Library	Total
Bacteria	Escherichia coli (180)	653
	Mycobacterium smegmatis (150)	
	Enterobacter cloacae (113)	
	Pseudomonas aeruginosa (100)	
	Staphylococcus aureus (110)	
Blank	DI Water (260)	510
	Nitrocellulose Filter (250)	

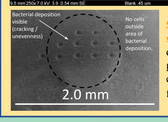
Bacteria currently fingerprinted in library (# of spectra)

To compile a large library, a study was conducted on the **laser shot spacing** to maximize the number of spectra taken from one filter (nominally 20). In this study, pairs of single laser-shot spectra were acquired side-by-side and the ratio of total emission intensity of the second spectrum to the first was calculated.

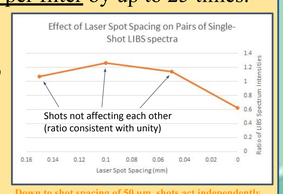
Independent shots not affecting each other had a **ratio of 1** (within uncertainty). Shots on top of each other possessed a **reduced ratio** due to the removal of bacteria from the first shot. Shot spacings down to 50 µm did not significantly effect each other. To preserve a safe margin, the shot spacing was reduced from 250 µm to 150 µm, which increased the sampling density. Further investigation with this technique will allow us to increase the number of spectra per filter by up to 25 times.



SEM image of single laser shots in blank nitrocellulose filter. The shot spacing is 250 µm - no overlap, but bacteria in-between not analyzed.



SEM image of single laser shots in *Staphylococcus* cells deposited in circular area 900 µm in diameter by centrifuge cone. One shot of "blank filter" also obtained.



Down to shot spacing of 50 µm, shots act independently. Shot spacing of zero does not yield a ratio of 0 due to presence of unablated material, emission from ablated filter, and redeposition of ablated cellular debris.

Library Preprocessing

The library was trained to improve the values of sensitivity by removing all spectra that did not classify correctly in a PLSDA discrimination between bacteria and water. A discrimination was done between *E. coli* and DI water with an unaltered library (**Library 1**). Spectra were removed from either *E. coli* or DI water one at a time and externally classified against the library of water and *E.coli.* Any spectra that were classified incorrectly were removed from the library in one of two ways:

1. Spectra were removed sequentially from the library, and a discrimination was performed after each removal to see if any improvement in sensitivity was observed. (**Library 2**).
2. All spectra that did not classify correctly were removed at the same time, and a discrimination was performed after this to see if any improvements in sensitivity was observed (**Library 3**).

There was no significant improvement upon removing spectra of DI water that classified incorrectly. There was some improvement for individual data sets of *E. coli,* however those data sets that improved markedly had the majority of spectra removed.

External Validation of DI Water	Average Sensitivity
Library 1	78.40%
Library 2	78.40%
Library 3	75.90%
External Validation of E. coli	**Average Sensitivity**
Library 1	72.50%
Library 2	88.80%
Library 3	88.80%

No improvement was observed for the classification of DI water. Some improvement was observed for *E. coli*, however further investigation needs to be done with all the sets of *E. coli* in the library.

Outlier Rejection

Water Threshold Analysis

All spectra acquired on one filter deposition were analyzed prior to the addition of these spectra to the library to eliminate outliers (intensity too large or too small). Two tests were investigated. All concentrations of *E. coli* were tested against all concentrations of *M. smegmatis*. Spectra were excluded if their intensity was consistent with the **average water signal intensity +/- 1σ**. Due to the large number of bacterial spectra possessing intensities consistent with water*, this resulted in the rejection of a large portion of the data from the library, reducing discrimination accuracy.

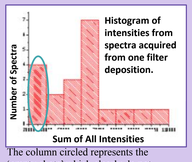
Outlier Rejection Method	Sensitivity (CV)	Specificity (CV)
Unprocessed	97.5%	100.0%
Water ± 1σ	94.4%	100.0%
Histogram	100.0%	96.9%

Sensitivity and specificity for a PLSDA discrimination between *E. coli* and *M. smegmatis* at a variety of concentrations with low intensity outliers removed. Left is the concentrated dilutions, right is all dilutions.

Outlier Rejection Method	Sensitivity (CV)	Specificity (CV)
Unprocessed	85.5%	87.2%
Water ± 1σ	67.8%	79.0%

Histogram Analysis

In an attempt to retain more spectra but remove the lowest intensity spectra acquired from every filter, histograms were constructed for each filter set of data based on the sum of all observed emission intensities. The binning was chosen automatically. All the spectra in the bin containing the weakest intensities were taken to represent **'empty shots'** and were removed from the library.

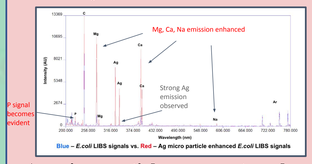


The column circled represents the 'empty shots' which clearly do not follow a normal distribution for bacterial spectra. In this case, 4 of 23 spectra were rejected.

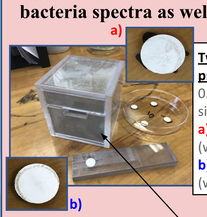
*the reason for low intensity spectra is under investigation, but is due to laser-matter interaction not non-uniform bacteria surface coverage.

Silver Microparticles

It is known that **Ag** and **Au** nanoparticles effectively enhance LIBS emission.



Ag microparticles appear to enhance bacteria spectra as well. ✓



Two methods for filter preparation.
0.5 - 1 micron spherical silver (99.9%) powder:
a) Spread on filter (without chamber) **vs**
b) Trace uniform spread (with chamber)

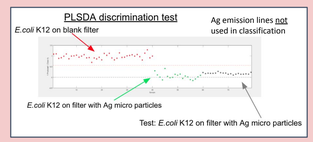
A **custom sealed chamber** was built to agitate the silver micro-powder. Filters inserted into the chamber collect trace powder as it settles. The amount of silver*, shaking, and settling time were adjusted to obtain a uniform coverage. ✓
*(Too much powder caused damage to the filter media)

Average Elemental Enhancement of 3 Bacteria Species with the Addition of Silver Microparticles

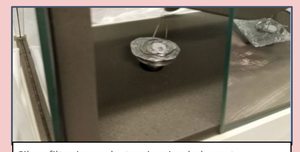
	C	P	Mg	Ca	Na
Enhancement of *E. coli*	1.3	4.6	3.9	5.3	3.9
Enhancement of *M. smegmatis*	1.1	1.1	0.8	1.9	2.1
Enhancement of *P. aeruginosa*	1.3	1.1	6.9	27.3	1.0

Not all elements are enhanced in the same way

Question: Could this eliminate empty spectra from occurring and improve our overall limit of detection?



The addition of metallic microparticles did not decrease the ability to accurately classify and discriminate bacterial samples. ✓



Silver filter in an electronic microbalance to determine mass of silver microparticles deposited.

Next Steps: Quantify enhancement in terms of surface coverage of silver microparticles using mass, density, and diameter of filter media. SEM images to confirm coverage.

Acknowledgements

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References

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