Signal Optimization and Enhancement of Laser Induced Breakdown Spectroscopy for Discrimination of Bacterial Organisms

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Masters Defense

Outline

- Motivation
- LIBS Technique
  - Laser Induced Breakdown Spectroscopy
  - Previous Group Results / Limitations
  - Methodology
- Experimental Results
  - Background Reduction and Library Preprocessing
  - Signal Optimization and Outlier Rejection
  - Silver Microparticle LIBS Signal Enhancement
- Conclusions
- Future Work
- My Progress
- Acknowledgements

Motivation

- Current methods of bacterial identification in a clinical setting
  - Require transferring the sample to a lab
  - Require expertise in microbiology
  - Are expensive / labor-intensive
  - May only be useful for certain types of bacteria
  - Are **Slow** **(can take hours to days)**

- Immediate targeted treatment with LIBS could
  - Save lives
  - Lower health care costs
  - Reduce risk of bacterial infection to microbiologists, clinicians, and patients
  - **Aid in preventing the emergence of antibiotic strains of bacteria**

Laser-Induced Breakdown Spectroscopy (LIBS)

- Spectrochemical technique
- Provides a **near-instantaneous** measurement of the elemental composition of a target – solid, liquid or gas
- Little to no sample preparation
- Requires only **μg** to **ng** of sample
- Has potential to detect and identify bacteria in clinical specimens (i.e. blood, urine, csf samples, throat, cheek, nasal swabs, etc)
- Can be done with ns, ps or fs lasers
- Laser allows for **point sampling** & **elemental mapping**

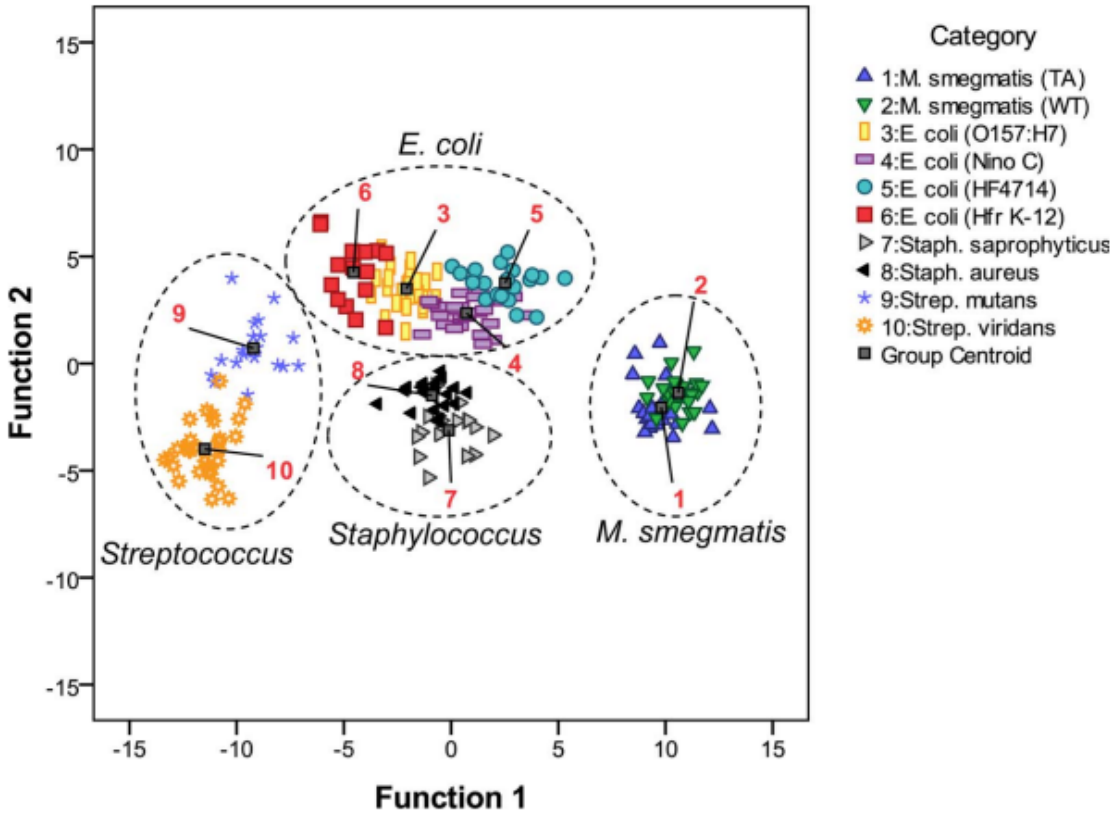
Goal

- Demonstrate the feasibility of LIBS as a **rapid point-of-care diagnostic tool** in a clinical environment
- Develop **quick bacterial collection** and **preparation methods** prior to testing on **inexpensive disposable substrates**
- Utilize equipment and techniques that are **familiar** to **clinicians** that would be **available** in a **medical setting**
- Rapid and accurate diagnosis could lead to advancements and improvements in many areas interested in bacterial detection diagnosis
  - **Medical**
  - Environmental
  - Food and water
  - Military

Previous Results for LIBS on Bacterial Samples

- Discriminate between different **strains** of a single species of bacteria
- LIBS spectrum of a certain bacterial species **does not change over time**
- LIBS spectra from different species and strains **naturally group together** according to **genus**

Bacterial classification based on elemental composition measured by LIBS



S. J. Rehse *et al.*, Appl. Opt., **49** (13), C27 (2010)

- Bacterial identification is **independent** of **growth conditions** of bacteria

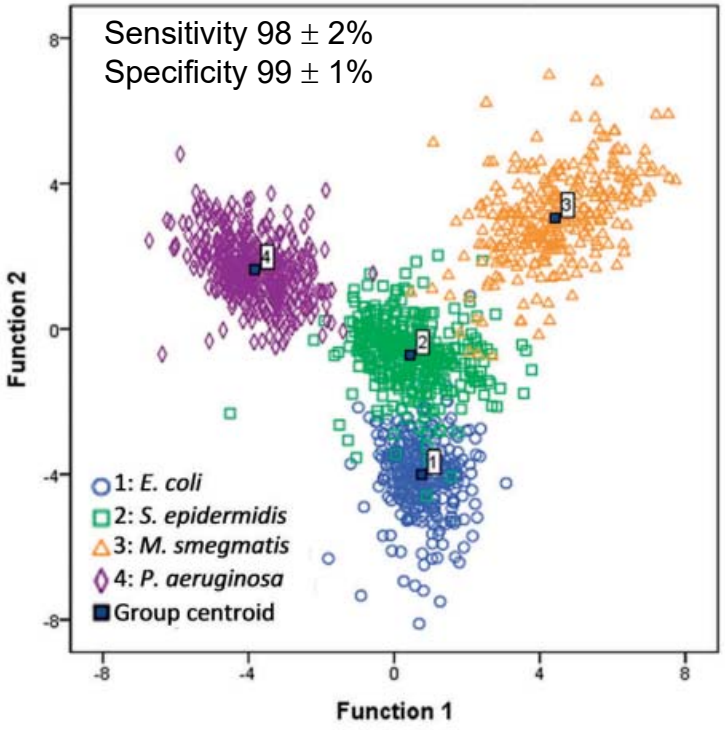
Previous Results for LIBS on Bacterial Samples

- Discriminate between **bacteria** and other **biotypes** (molds, pollens)
- Bacteria are discriminated based on **elemental composition**
- Bacterial spectra are classified using discriminant function analysis (**DFA**)
- Unknown spectra are classified against a precompiled library of known spectra

Bacterial library:

- 164 independent variables (intensities of elemental lines and ratios of these lines to each other)
- ~ 1500 spectra acquired over 3 months from 4 species of bacteria (*E. coli, S. epidermidis, M. smegmatis, P. aeruginosa)*

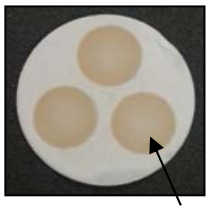
Bacterial classification based on elemental composition measured by LIBS



D. J. Malenfant *et al.*, Appl. Spectrosc., **70** (3), 485 (2016)

Previous Bacterial Deposition Procedures

1) Well-plate



Bacterial LOD **~ 50 000 CFU** per laser ablation event

2) Centrifuge tube insert

Bacterial LOD **~ 90 000 CFU** per laser ablation event

3) Metal Cone

Bacterial LOD **~ 5500 CFU** per laser ablation event

Number of bacterial cells present in clinical samples:

- < 100 CFU/mL in blood
- 0-200 CFU in typical nasal swab

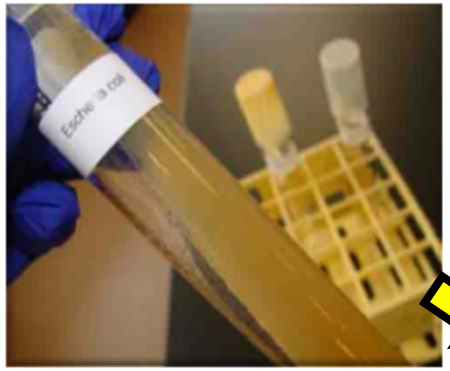
These LOD's are ***not clinically relevant***. Bacterial LOD with LIBS MUST be lowered.

Limitations of Previous Results

- Showed that LIBS is capable of bacterial identification in **idealized lab settings**
- Encountered sources of **contamination**
- Much of this work involved **proof-of-concept** experiments **actual clinical specimens**
- Things considered for **in previous work** include:
  - Separation of unwanted material mixed in with bacteria (i.e. tungsten powder)
  - Amount of bacteria present in a specimen (absorbance values)
  - Fabrication of metal cone to concentrate bacterial cells to small region
  - Investigation of bacteria collection and release with swabs

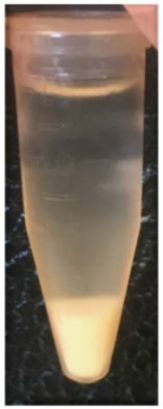
Methodology: Sample Growth

Initial bacteria sample



Bacteria is cultured on TSA plates

Bacterial cells are removed and suspended in 1.5 mL deionized water



Bacteria collection step stored in fridge

Methodology: Sample Preparation

Simulating bacteria on a surface

Deposit 100 µL of bacteria suspended in DI water onto metal plate and heat for 2 min and 20 s.

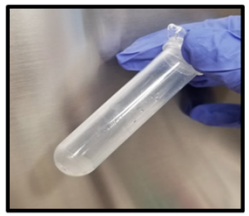
Deposit 10 µL water onto swab tip and swab metal plate.

Place swab in centrifuge tube with 1 mL of water and vortex tube for 15 s.

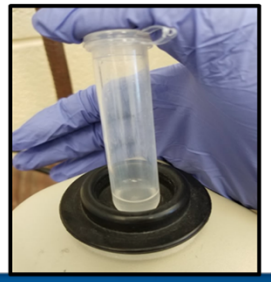
Place a 0.45 µm filter on the base piece. Connect the base. Place the cone in the insert.

Simulating infection in clinical specimen

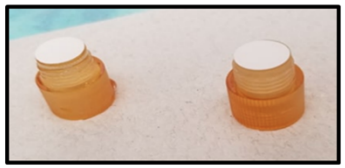
Pipette 100 µL of liquid contaminant containing bacteria suspension into a centrifuge tube.



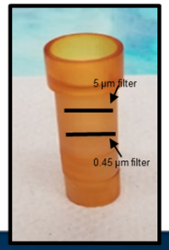
Add 1mL water to the tube and vortex for 15 s.



Mount a 5 µm filter and a 0.45 µm filter on two separate insert base pieces.

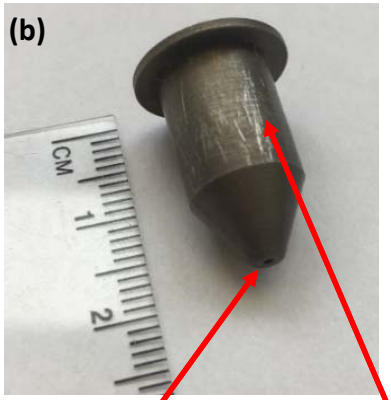


Connect both base pieces to the insert, with the 5 µm filter on top. Place the cone in the insert.



Concentrating and Shooting Bacteria on Filters

19 mm long Al cone



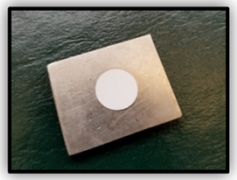
Centrifuge tube cap presses cone into filter

1 mm hole at apex

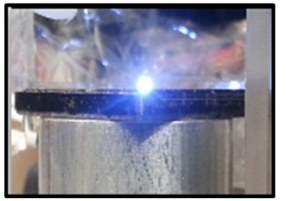
Holds 1 mL of fluid

Deposit the 1 mL of sample from vortexed tube into insert, place insert into clean centrifuge tube. Centrifuge insert at 5000 rpm, 2500 g's for 5 min.

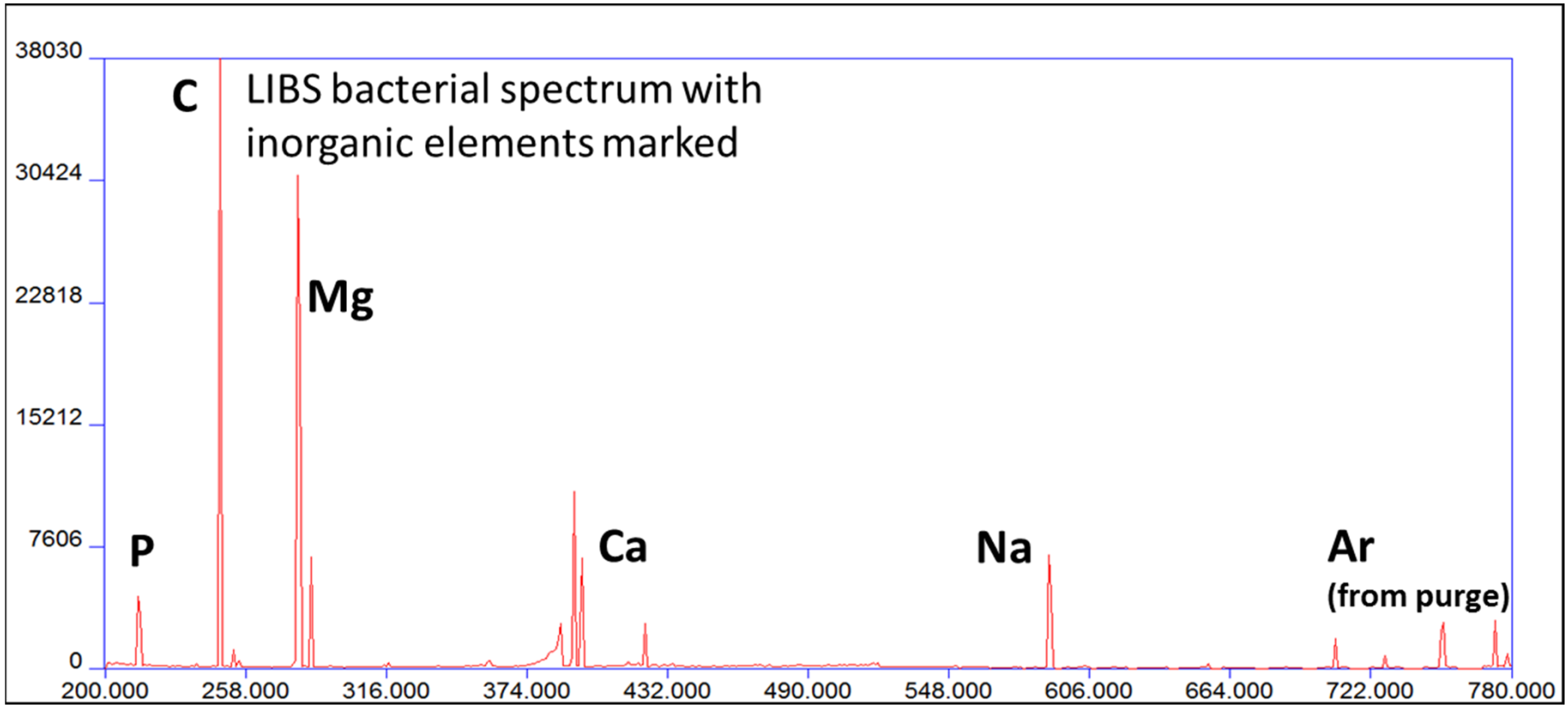
Remove insert(s) from centrifuge, then remove filter(s) from insert. Mount 0.45 µm filter on metal plate with double - sided tape.



Place filter in an argon environment and ablate using a pulsed 1064 nm Nd: YAG laser.



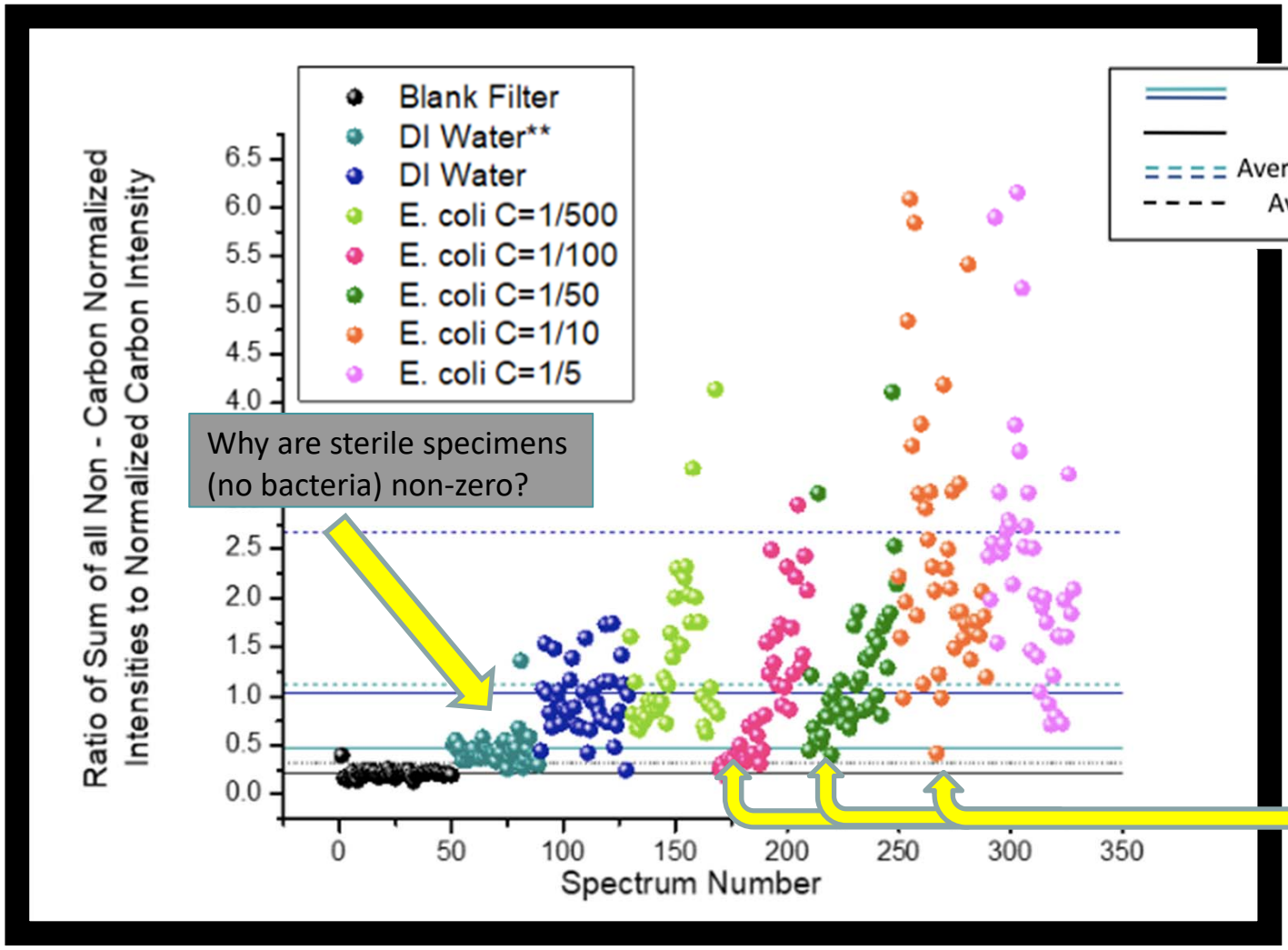
LIBS Emission Spectra



After laser ablation, light from the plasma is dispersed, revealing the sample's elemental composition. Measured intensities of emission lines in the LIBS spectrum provide a unique elemental "spectral fingerprint" for each type of bacteria.

Background Reduction and Library Preprocessing

LIBS bacterial curve-of-growth



All data points are the intensities of single shot LIBS spectra

My Plan

- 1st Approach
  - **Data Analysis** and **Signal Optimization**
  - Can we do something to improve our collected data
  - Explored **preprocessing** methods and **outlier rejection**
  - *Conducted during Covid shutdown*

- 2nd Approach
  - **Alternate** the current technique
  - Investigated cleaning techniques for **Background Reduction**
  - **Increased** Shot Density on filters
  - **Silver Microparticle** LIBS Signal **Enhancement**
  - *Conducted before and after returning to lab*

Current Total Bacterial LIBS Spectral Library

	LIBS Spectral Data Library	
Bacteria Species	Number of Acquired Bacteria Spectra	**Total**
Escherichia coli	797	**1665**
Mycobacterium smegmatis	430	
Staphylococcus aureus	148	
Pseudomonas aeruginosa	160	
Enterobacter cloacae	130	
Blank	Number of Acquired Bacteria Spectra	
Deionized water	260	**510**
Nitrocellulose Filter	250	

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

1st Approach

Signal Optimization and Outlier Rejection

- **Goal**: to accurately identify and classify as small a number of bacterial cells as possible in order to improve the LOD and to also maximize the rates of true positives while minimizing the rates of false positives during classification

- Removal of misclassified spectra to improve overall library and accuracy

- Two tests were also investigated to identify outliers and remove weak spectra

- PLSDA discrimination performed on *E. coli* and water: **"Library 1"**
- All misidentified spectra were removed in one of 2 ways

- Removed sequentially in a leave-one-out analysis: **"Library 2"**
  - Does removing a subset of data points improve quality of library ?
- All spectra of a filter that did not classify correctly were removed simultaneously: **"Library 3"**
  - Do we have to remove them all in order to improve the quality of the library ?

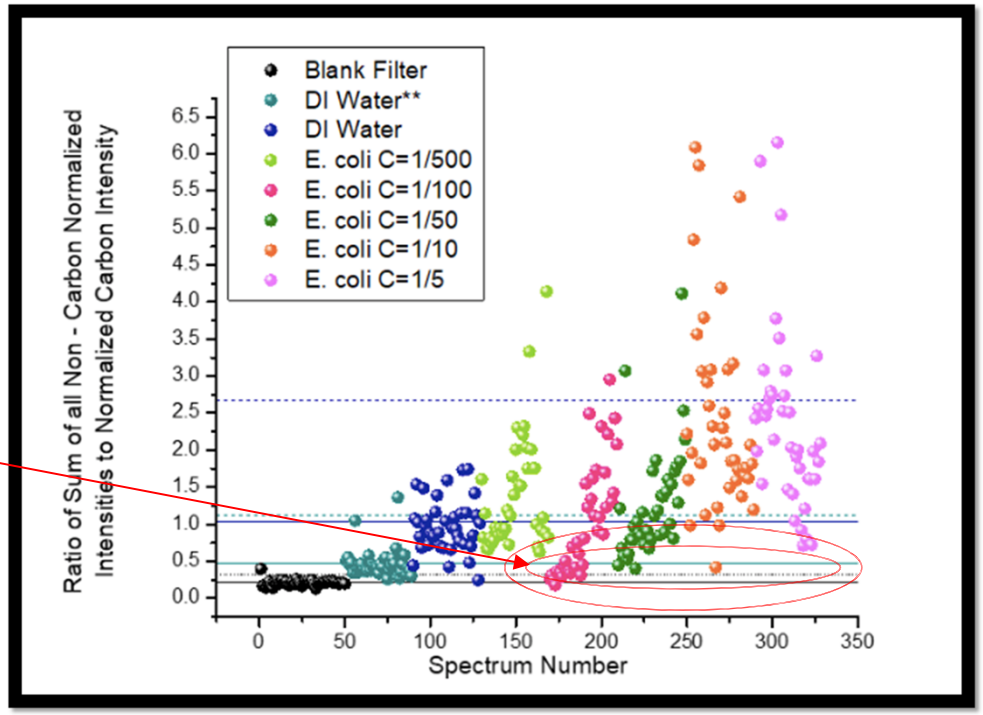
External Validation of DI Water	Average Sensitivity
Library 1	78.40%
Library 2	78.40%
Library 3	75.90%
External Validation of E. coli	**Average Sensitivity**
Library 1	72.50%
Library 2	88.80%
Library 3	88.80%

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.

Outlier Rejection: Water Threshold Analysis

- Spectra were excluded if their intensity was consistent with the **average water signal intensity +/- 1σ** displayed below in **red** of tables.



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

C = 1/5 dilutions only

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

All bacterial concentrations

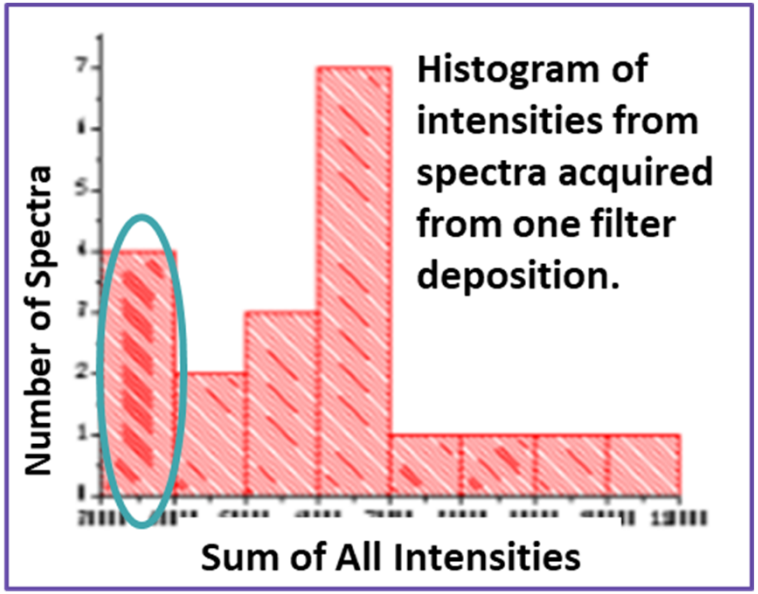
Outlier Rejection: Histogram Analysis

- All the spectra in the bin containing the weakest intensities were taken to represent **'empty shots'** and were removed from the library
- The binning was chosen automatically
- Results shown in gold of the table

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

*Still analyzing this method when applied to all concentrations of data

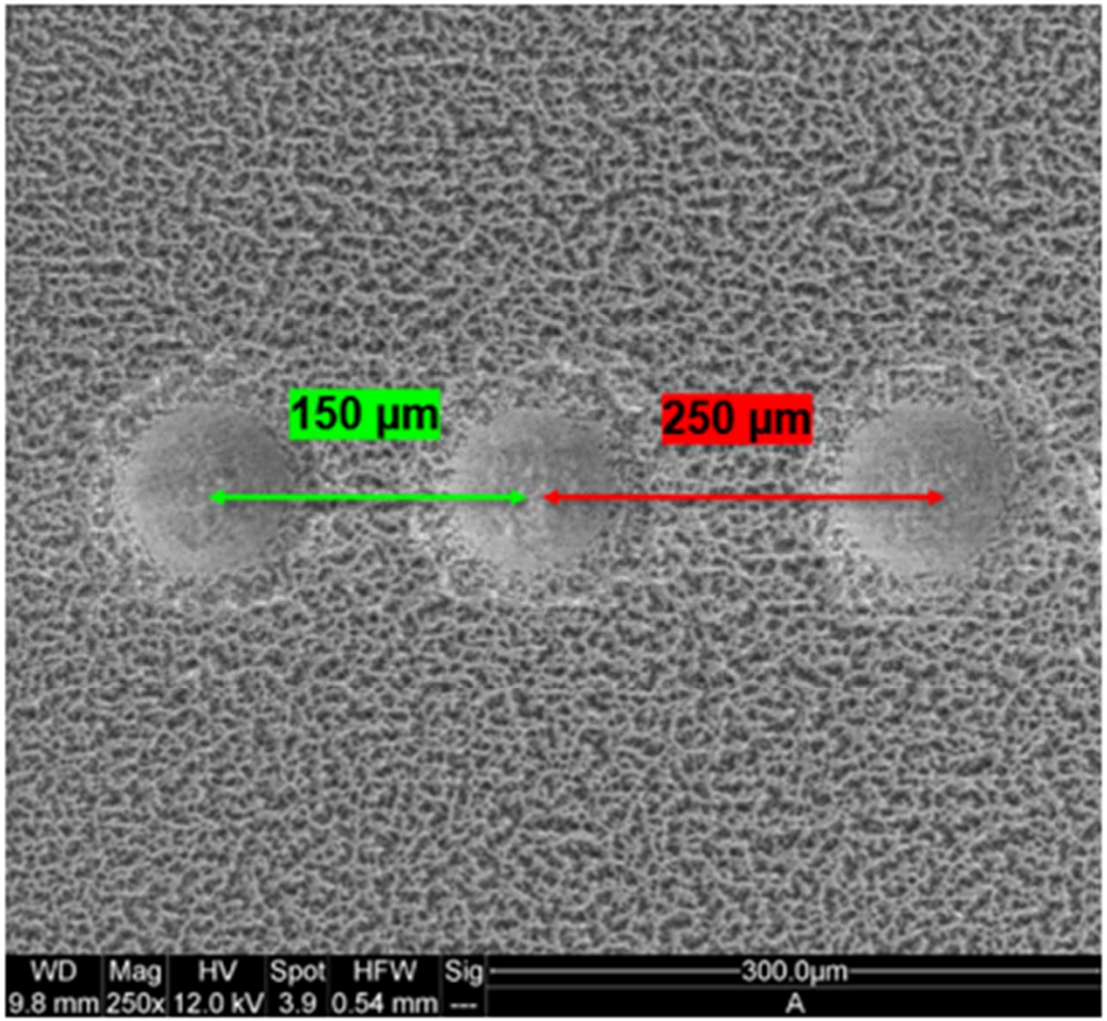
Histogram of intensities from spectra acquired from one filter deposition.



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.

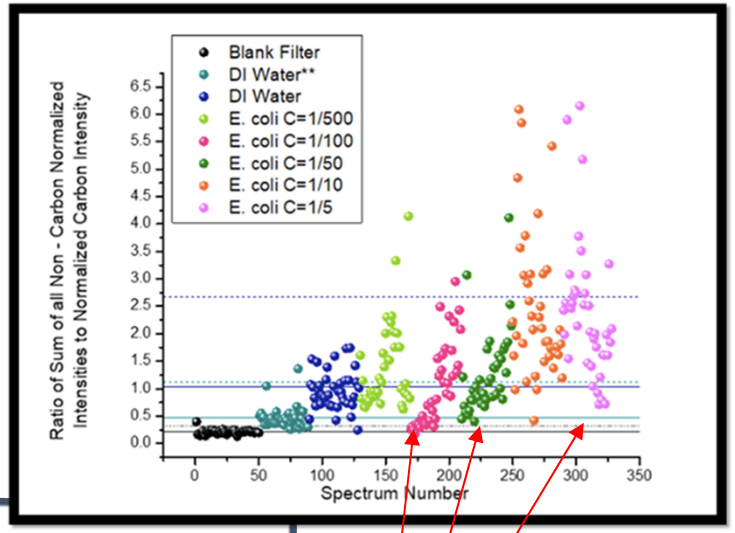
2nd Approach

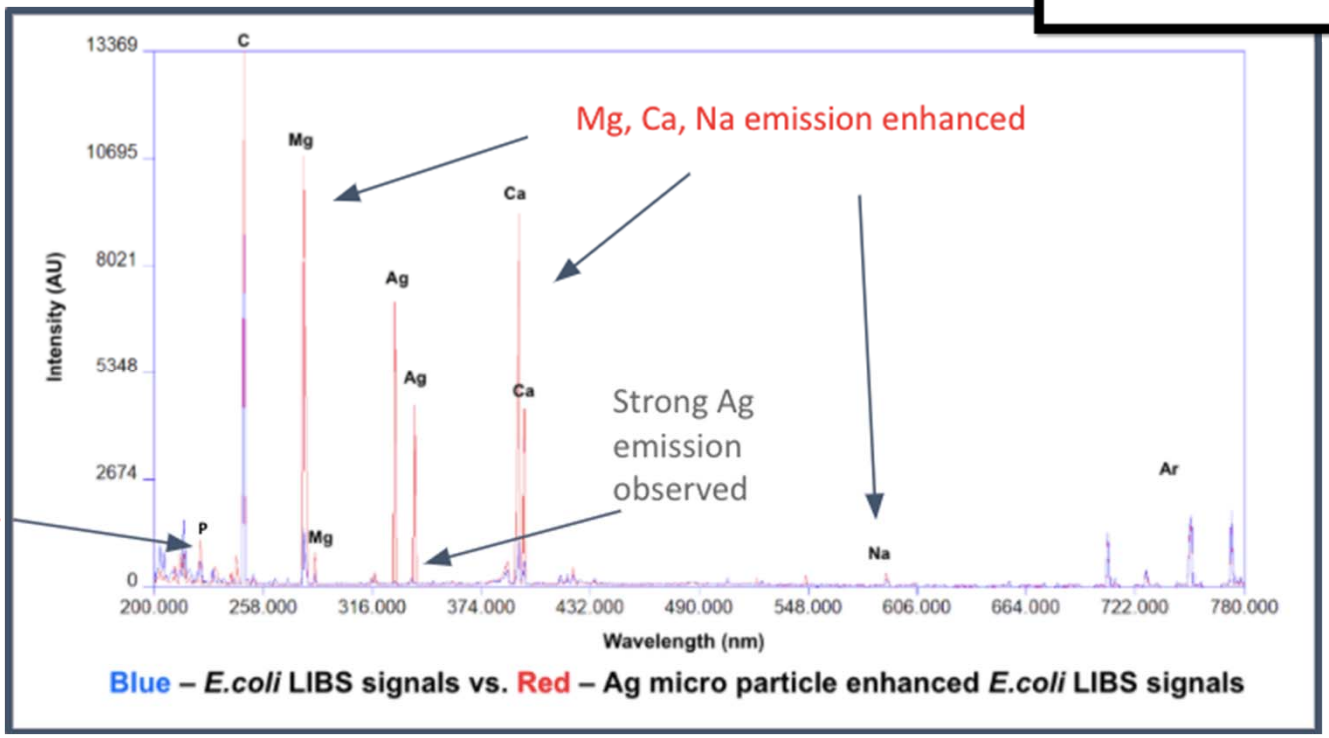
Improving the Technique



Silver Microparticles

- It is known that **Ag** and **Au** nanoparticles effectively enhance LIBS emission.
- **Ag microparticles appear to enhance bacteria spectra as well.**



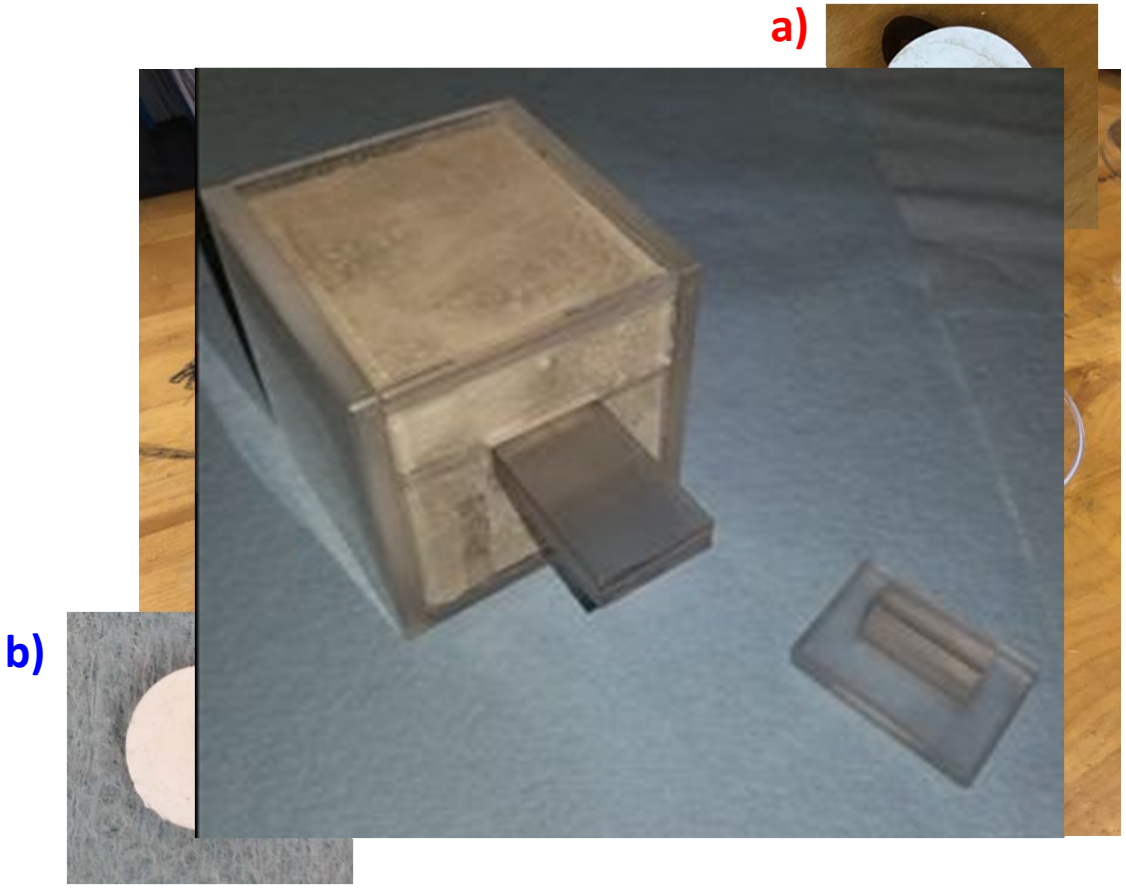


P signal becomes evident

If we can't eliminate them with outlier rejection, maybe we can stop these seemingly blank bacteria data from ever occurring using enhancement.

Silver Microparticle Deposition

- 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, waiting and settling time were adjusted to obtain a uniform coverage.



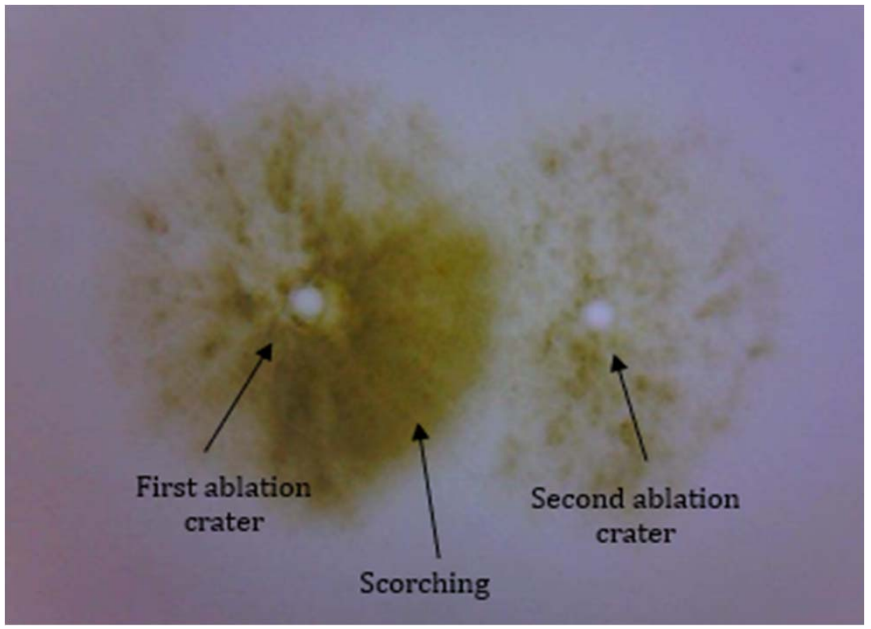
<u>Two methods for filter preparation</u>

0.5 - 1 micron spherical silver (99.9%) powder:

a) Spread on filter (without chamber) **vs**

b) Trace uniform spread (with chamber) *approx. 1 – 50 ng deposited*

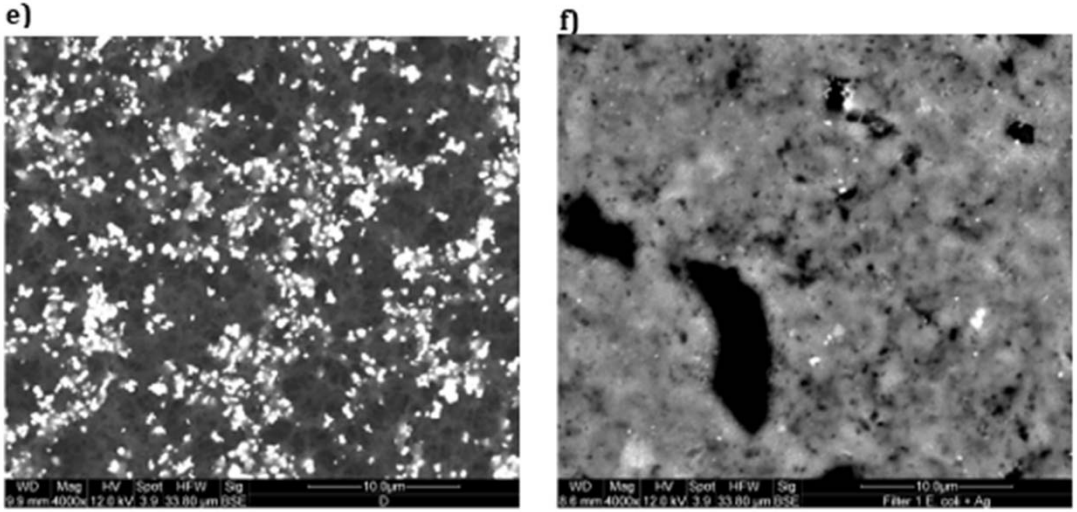
Silver Microparticle Mass



- Filters prepared in this way did not produce scorch marks during laser ablation

- Silver powder is uniformly distributed among the central region of the filters
- The ability to detect silver particles accurately below the nanogram range is not physically achievable with our current equipment
- Surface coverage density rate of 2.6×10^{-8} μg/μm^2

Silver Microparticle Surface Coverage



- *a) LIBS ablation on blank*
- *b) LIBS ablation on silver MP*
- *c) Zoomed in b)*
- *d) LIBS ablation of bacteria deposited on silver MPs*
- *e) Zoomed in c)*
- *f) Zoomed in d)*
- Ablation craters approx. 75 *μm*
- Silver MPs approx. 0.5 – 1 *μm*
- Majority of MPs covered by bacteria layer

- Assuming a uniform silver surface coverage density yields a silver ablation mass of 1.1 x 10^{-4} μg = 0.11 ng = 110 pg per laser shot
- **That is a very small amount of material**!

Silver Microparticle Enhancement

Average Elemental Enhancement of 4 Bacteria Species with the Addition of Silver Microparticles

Bacteria Species	Elemental LIBS Spectral Emission Enhancement				
	C	P	Mg	Ca	Na
Escherichia coli	1.3	4.6	3.9	5.4	3.9
Mycobacterium smegmatis	1.2	1.7	2.7	8.4	6.7
Pseudomonas aeruginosa	1.3	1.1	6.9	27.3	1.0
Enterobacter cloacae	1.2	4.4	6.9	2.2	1.3

- Spectra appear to be stronger now
- Not all elements are enhanced in the same way
- Could this eliminate empty spectra from occurring or improve LOD?
- **New field of MPLIBS**

Conclusions

- Background reduction and library preprocessing:
  - Identified sources of non-zero background signal
  - Introduced new cleaning procedures
  - Reduced unwanted contamination during sample preparation

- Signal optimization and outlier rejection:
  - Had limited effectiveness
  - Investigated removing weakest intensity spectra (histogram TSP)
  - Improved sensitivity and specificity trade-off between significant loss of data

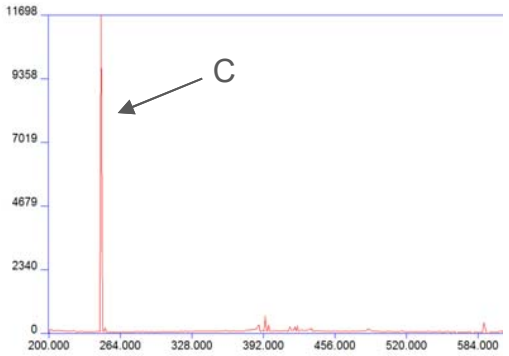
- Silver microparticle enhancement was effective:
  - Boosted all key elemental LIBS signals in bacteria spectra
  - Introduced reproducible Ag deposition technique, uniformity
  - Quantified mass and surface area coverage of microparticles, SEM images
- Next Steps:
  - Quantify enhancement for other bacteria species with Ag MPs to build library
  - Perform DFA and PLS-DA on these samples
  - Determine improved limit of detection (LOD)

Future Work

- Quantify LOD and LOI of current technique
  - Add bacteria spectra to build robust spectral library
  - Determine identification accuracy using chemometric techniques (DFA and PLS-DA)
  - How well can bacteria be classified when they are:
    - prepared using the methods developed in this work
    - obtained from different types of biological specimens (i.e. blood, CSF, urine, etc.)
- Optimize LIBS apparatus
  - Physical modifications
  - Automatic transition stage and data acquisition
  - Observe acoustic signatures of LIBS plasmas to indicate shot variation
- Clinical Method
  - Single (entire) filter ablation to analyze patient samples
  - Clinically 1,2 maybe 3 shots to confirm diagnosis
  - Portable or benchtop LIBS device for clinical setting

Future Work

- Modify Spectrometer
  - Remove physical limitation of amplification
  - Eliminate signal detection of C
    - Notch filter to attenuate emission from C (expensive
    - Multiple spectrometers with smaller wavelength cove (expensive)



- Classification utilizing Artificial Neural Networks
  - Train ANN by processing bacterial spectra
  - Excellent predictive abilities and performance

- MPLIBS and NELIBS
  - Shift towards technique using MPs and NPs for enhanced signals for very low CFU

Future Work

- Improved Bacterial Handling
  - Explore techniques to prevent stringiness and clumping of bacteria cells
  - Mechanically
    - Ultrasonic bath combined with glass beads
    - Pasteur pipette repeatedly
  - Chemically:
    - Tween 80 at concentration of 0.05 – 0.1%

- Dual Step Chemometric Analysis
  - Conduct classification and discrimination between all sets using chemometric techniques (PCA, DFA and PLS-DA)
  - Similar sets are retested again to provide sequential analysis

My Progress and Activities

- Aim of my work was to address some of the issues related to the LIBS testing of **actual clinical specimens**

1) Constructed an extensive **spectral library database** for the discrimination of multiple genera of bacteria organisms

2) Developed a new filter preparation and bacterial mounting technique to **enhance** weak LIBS emission signals

3) Investigated whether LIBS could be used as a **diagnostic tool** for samples collected with **swabs**

4) Utilized **easy, inexpensive,** and **fast** sample preparation methods that could be easily introduced into a clinical setting

Suggests that LIBS is a feasible diagnostic tool

My Contributions to the Field

- My research was presented at:
  - **UWill Discover Conference** (University of Windsor)
  - **CAP 2019** (Simon Fraser University)
    - Placed 1st place for the student poster presentation in Physics and Medicine category
    - Placed 3rd place for overall student poster presentation in all categories
    - Awarded by the Biophysical Society of Canada
  - **CAP 2020** (McMaster University Virtually)
  - **IOMLIBS Conference** (Europe Virtually)

- Publications that will be written:
  - Manuscript of Discrimination of swabbed bacterial specimens deposited through cone using current technique
  - Manuscript of Silver Microparticle Enhanced LIBS (MPLIBS)

Acknowledgments

Thank you to:

- Dr. Rehse
- Allie Paulick, Emma Blanchette and Sydney Sleiman
- Our research group

NSERC CRSNG