Development of a Simple and Efficient Clinical Protocol for Identifying and Quantifying Bacterial Cells in Liquid Suspensions with Laser-Induced Breakdown Spectroscopy

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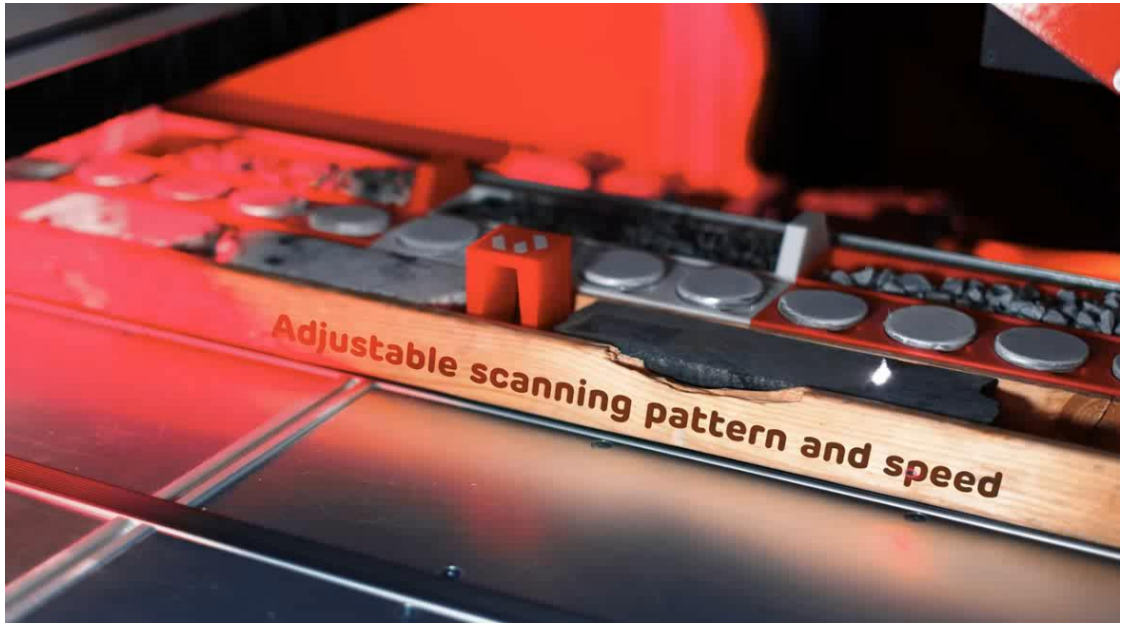
Presented in Symposium #001: Laser-Induced Breakdown Spectroscopy (LIBS) for the Benefit of the Global Community

Sunday, December 20, 2021

University of Windsor

Motivation

We all know the advantages that LIBS brings to an elemental analytical analysis…specifically speed and autonomous classification.



ECORE drill core scanner, by Elemission

SHREDDERSORT, by Lenz Instruments

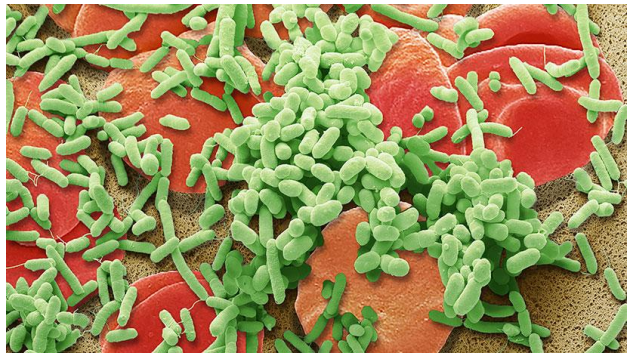
Motivation

A similar analysis of specimens for the detection and identification of pathogenic bacteria is badly needed.

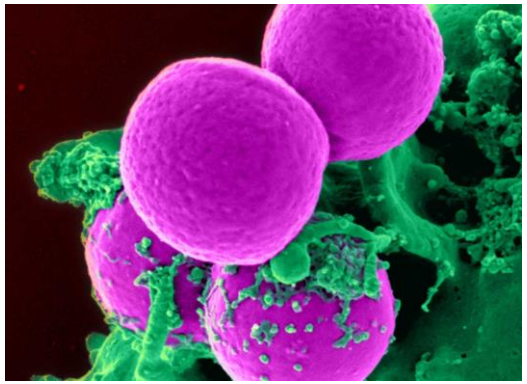
✓ Current “standard” methods of diagnosis takes ~1-3 days

Delaying the initiation of <u>appropriate</u> antibiotic therapy can lead to:

- Worsening patient outcomes, including death (sepsis, meningitis)
- Increased expense due to treatment time, hospitalization (hospital-acquired infections)
- Overuse/abuse of broad-spectrum antibiotics leading to antibiotic resistant strains (MRSA)



Composite coloured scanning electron micrograph (SEM) of sepsis. (Steve Gschmeissner/ Science Photo Library)



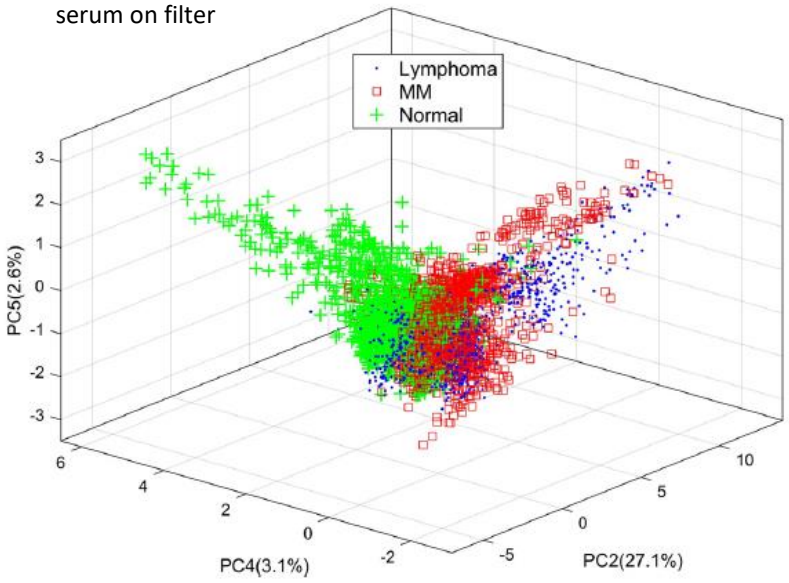
https://www.airius.bg/all-resources/how-can-medical-settings-reduce-the-risk-of-mrsa-transmission-with-airius-pureair/

Biomedical / Medical Applications

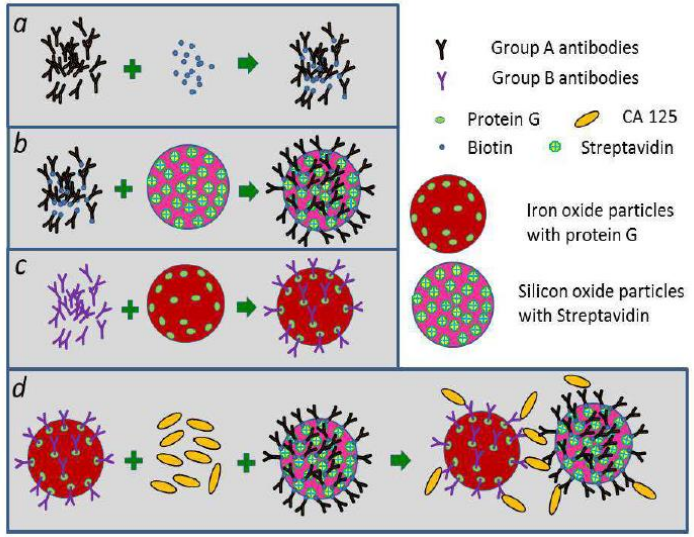
The idea to use LIBS on medical specimens is very reasonable:

Analysis of Biofluids

serum on filter



Chen et al., 2018



Markushin et al., 2012

CA125 ovarian cancer biomarker detected at the level of 10 U/mL (estimated LOD ~ 1 U/mL)

Biomarkers in fluid specimens

With a combination of appropriate substrate and algorithm, melanoma biomarkers in blood showed discrimination between healthy and diseased mice with accuracy up to 96%, but *direct analysis of LIBS spectra did not provide any conclusive results*. (*Gaudiuso et al. 2018)*

Multi-element micro- and nanoparticles labelling approach an attractive alternative (*Markushin et al. 2012*)

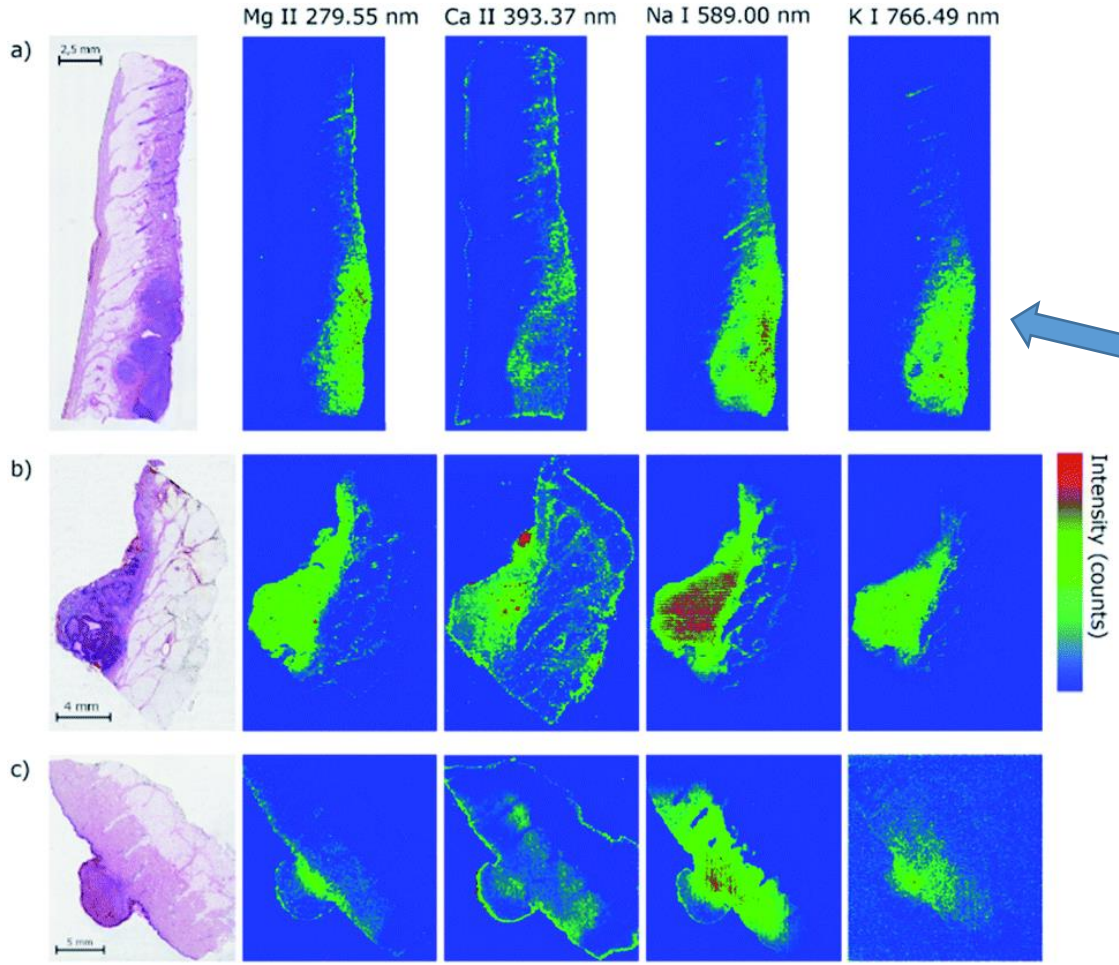
1 U or IU is defined as the enzyme quantity that liberates 1 microgram or micromole of product per ml of the reaction mixture per minute

Biomedical / Medical Applications

The idea to use LIBS on medical specimens is very reasonable:

Analysis of Tissues

Histological sections



<u>Tissues</u>

Higher concentrations of major and trace elements such as Mg, Fe, Ca, Na, and K in the neoplastic tissues.

Unfortunately a lot of work done *ex vivo* and in less-than-realistic experiments (caution)!

<u>Our next speaker</u>:

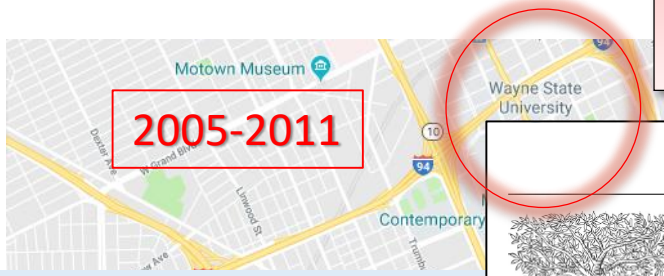
The imaging of biotic elements (Mg, Ca, Na, and K) provides the elemental distribution within the tissue.

The elemental images were correlated with the tumor progression and its margins, as well as with the difference between healthy and tumorous tissues

Imaging margins of skin tumors using laser-induced breakdown spectroscopy and machine learning, Kiss et al., J. Anal. At. Spectrom., 2021, 36, 909-916

Bacteria

Can all-optical technique of LIBS really identify bacteria in under a second?



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Invited Review

A review of the use of laser-induced breakdown spectroscopy for bacterial classification, quantification, and identification

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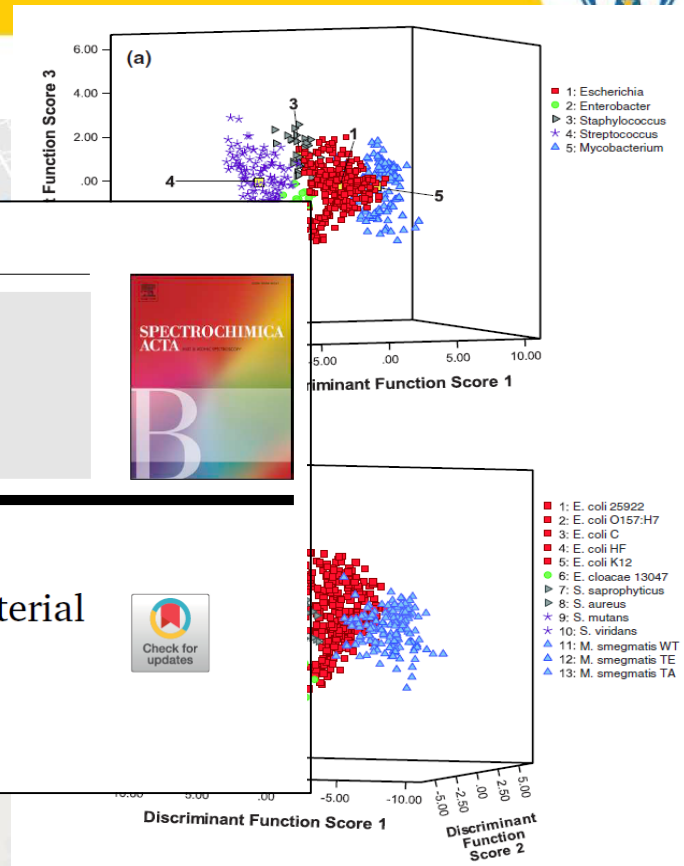
LIBS spectral finger

- growth-medium
- independent of s are)
- independent of v inactivated by UV light)
- obtainable even when other types of bacteria or contaminants are present (mixed samples)
- obtainable from urine specimens
- capable of strain discrimination
- obtainable from about 500 bacteria



PLSDA		
E. COLI	True	False
Positive	95.65%	9.17%
Negative	90.83%	4.35%
STAPHYLOCOCCUS	True	False
Positive	54.05%	0.51%
Negative	99.49%	45.95%
STREPTOCOCCUS	True	False
Positive	95.59%	1.02%
Negative	98.98%	4.41%
MYCOBACTERIUM	True	False
Positive	88.31%	1.06%
Negative	98.94%	11.69%

DFA		
E. COLI	True	False
Positive	89.63%	15.95%
Negative	84.05%	10.37%
STAPHYLOCOCCUS	True	False
Positive	86.49%	5.85%
Negative	94.15%	13.51%
STREPTOCOCCUS	True	False
Positive	99.26%	13.32%
Negative	88.68%	0.74%
MYCOBACTERIUM	True	False
Positive	96.10%	4.08%
Negative	95.92%	3.90%

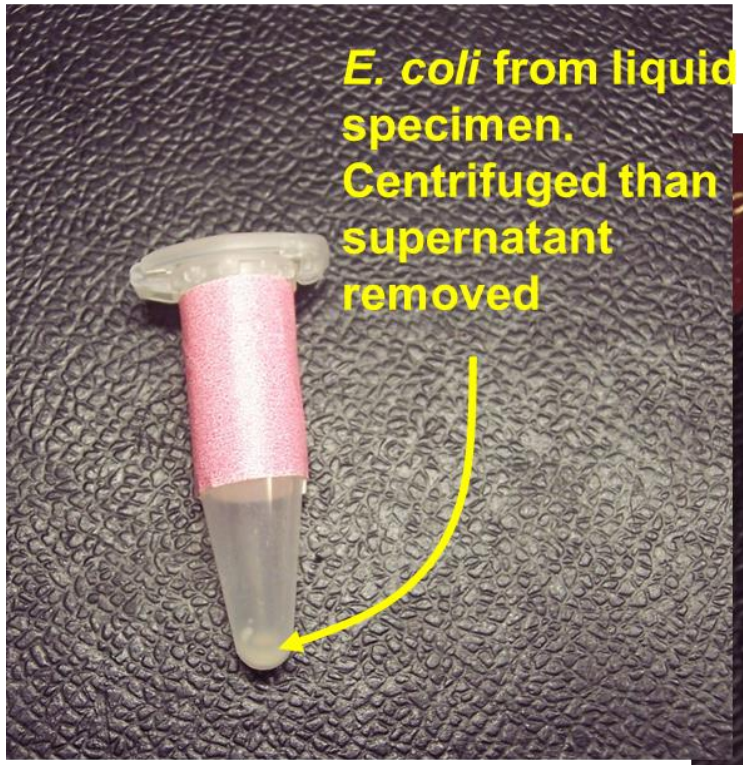
DFA: *Sensitivity*: 91.37 ± 16.39 % *Specificity*: 97.46 ± 9.35 %

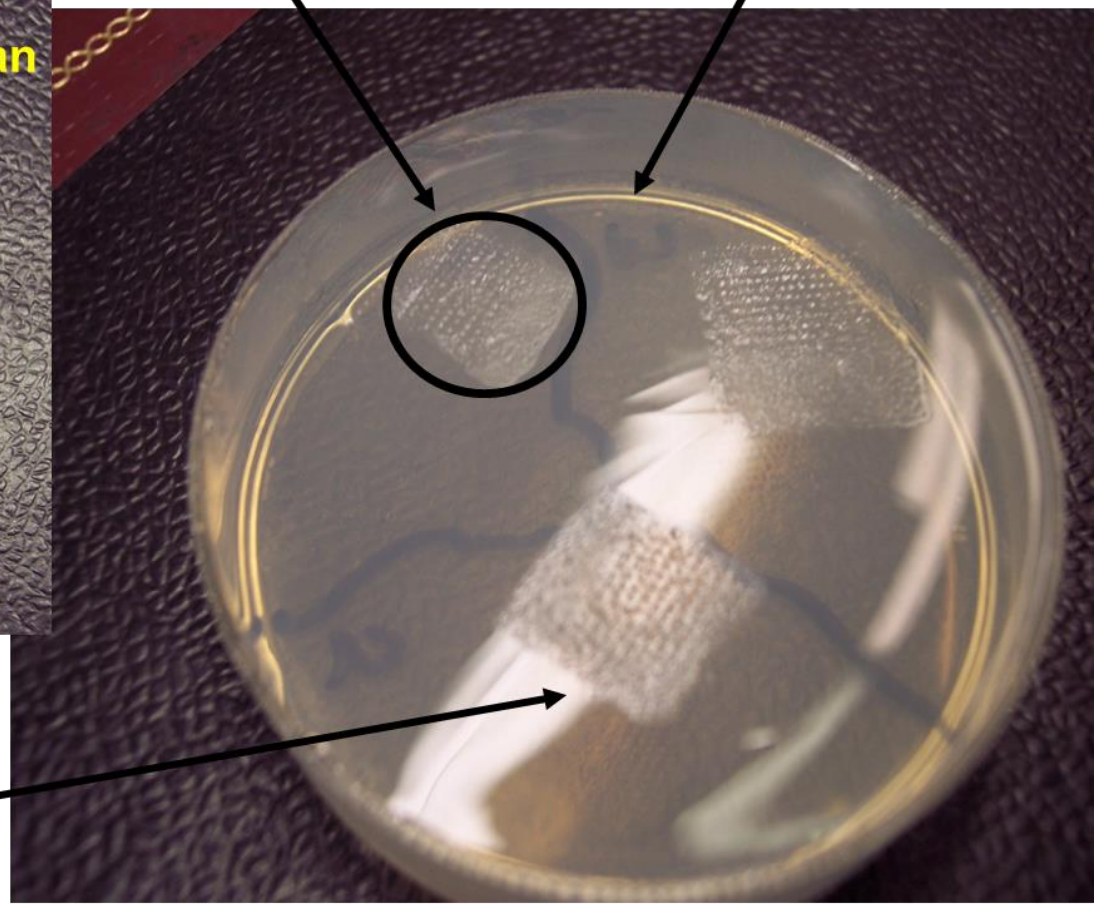
PLSDA: *Sensitivity*: 93.13 ± 10.25 % *Specificity*: 90.60 ± 21.33 %

Bacteria

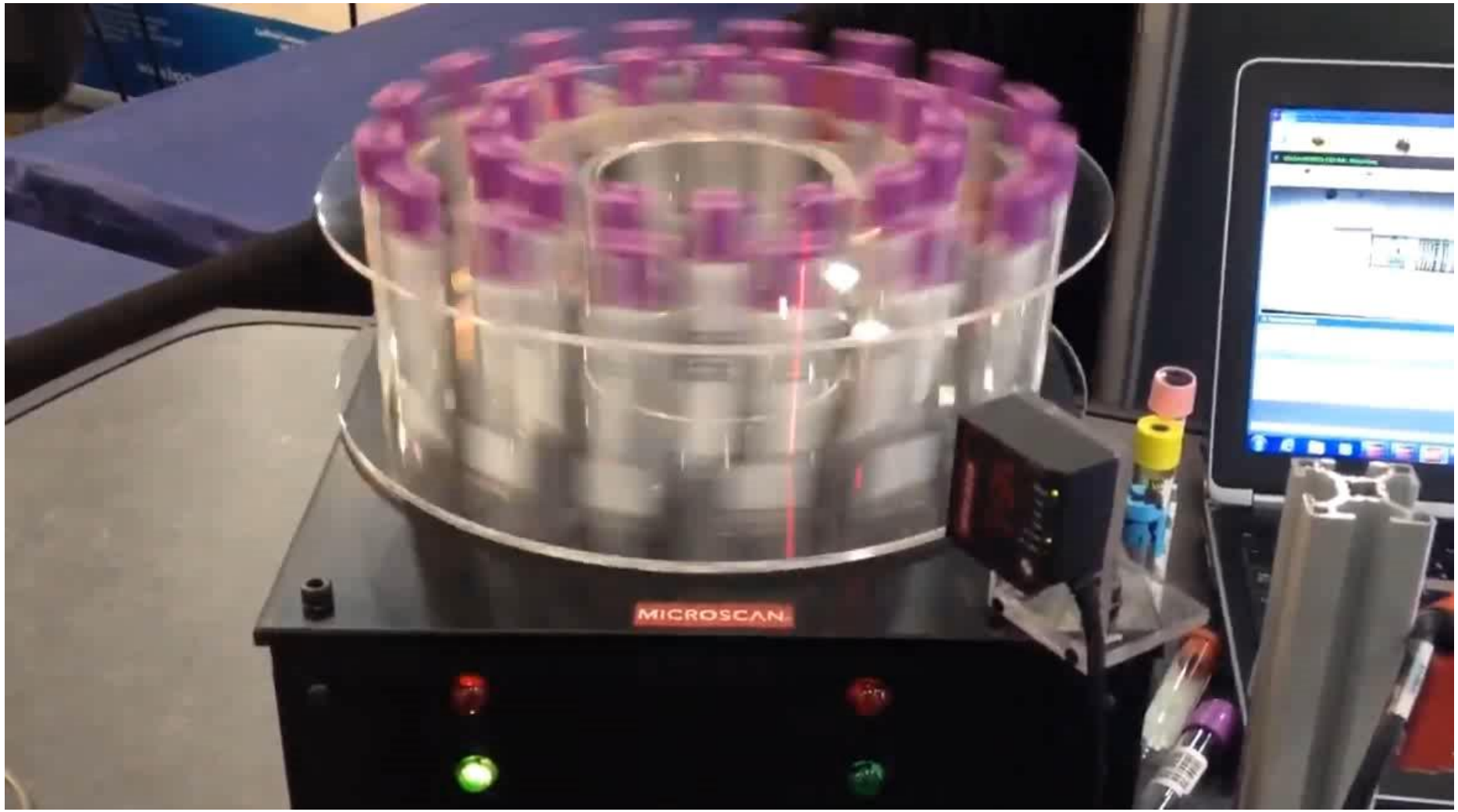

Motown Museum
Wayne State University
The Heidelberg Project
Museum of Contemporary Art Detroit
MIDTOWN
CASS CORRIDOR
EASTERN MARKET
Belle Isle
Belle Isle Park
Ford Field
MotorCity Casino
Greektown Casino
MGM Grand Detroit
Detroit
CORKTOWN
Detroit River
United States
Canada
Caesars Windsor
Ford Motor Company of Canada - Windsor...
RIVERS
MEXICANTOWN - SOUTHWEST DETROIT
Windsor
WALKERVILLE
Ambassador Bridge
University of Windsor
2011-2021
DELRAY
FCA Windsor
Zug Island
BP Canada - Windsor
Can we translate this to be a convenient and easy-to-use (for clinicians) test?
University of Windsor

Future


E. coli from liquid specimen. Centrifuged than supernatant removed


10 microliter of bacteria pellet
bacto-agar (99% water)
about 500-1500 bacteria per sampling location

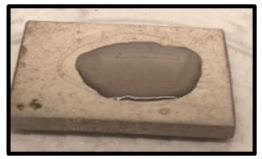
What We Need



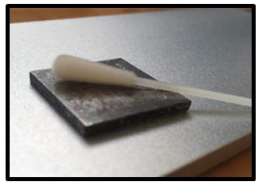
Our Approach

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.

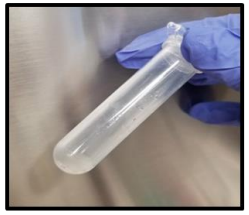


Place swab in centrifuge tube with 1 mL of water and vortex tube for 15 s.

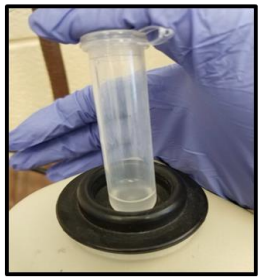
Place a 0.45 µm filter on the base piece. Connect the base. Place the cone in the insert.

Simulating infection in clinical specimen

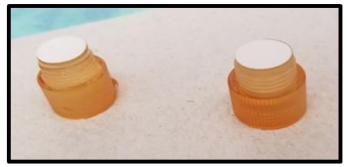
Pipette 100 µL of liquid contaminant containing bacteria suspension into a centrifuge tube.



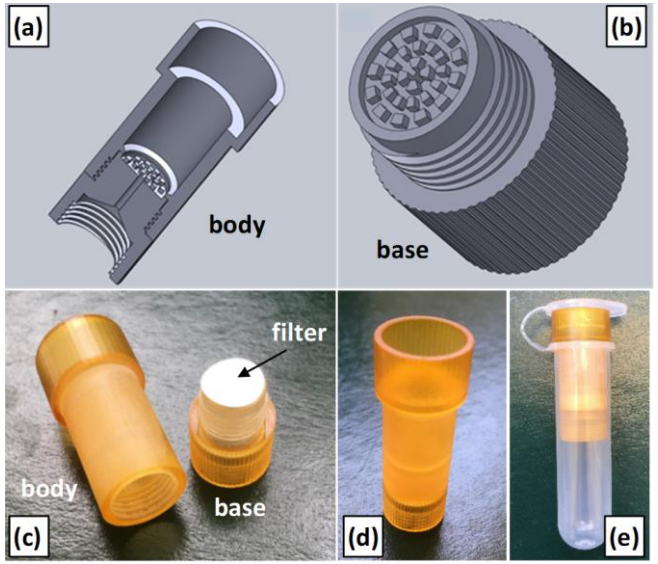
Add 1mL water to the tube and vortex for 15 s.



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



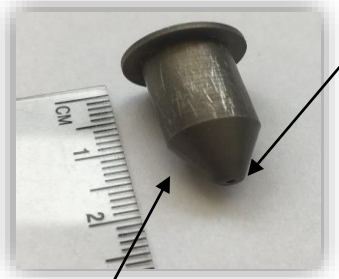
Connect both base pieces to the insert, with the 5 µm filter on top. Place the cone in the insert.

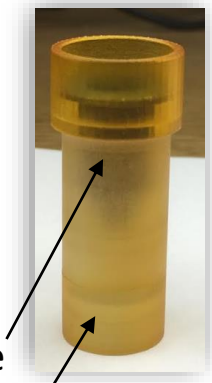


Bacterial LOD ~ **90 000 CFU** per laser ablation event

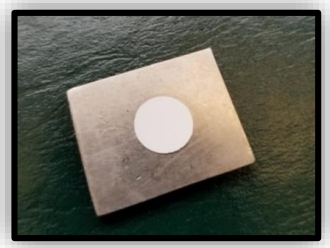
These LOD's are ***not clinically relevant***. Bacterial LOD with LIBS MUST be lowered.

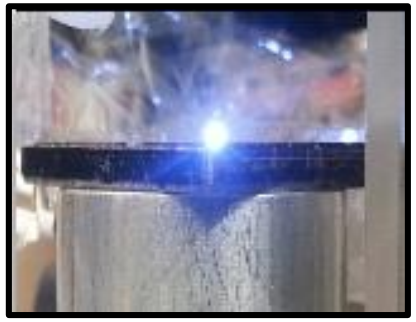
Our Approach

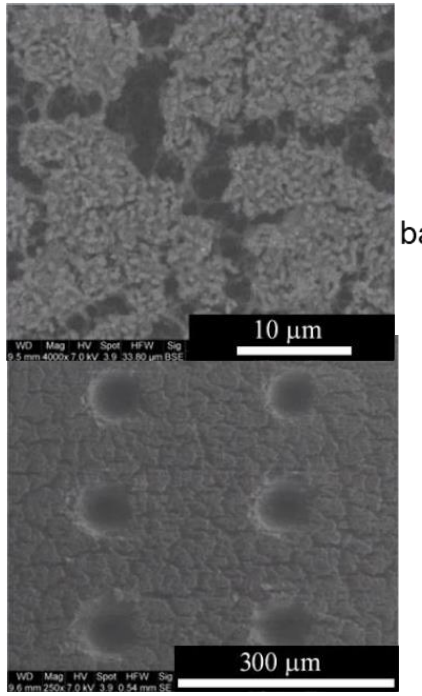




Bacteria is ***concentrated*** at center of filter paper





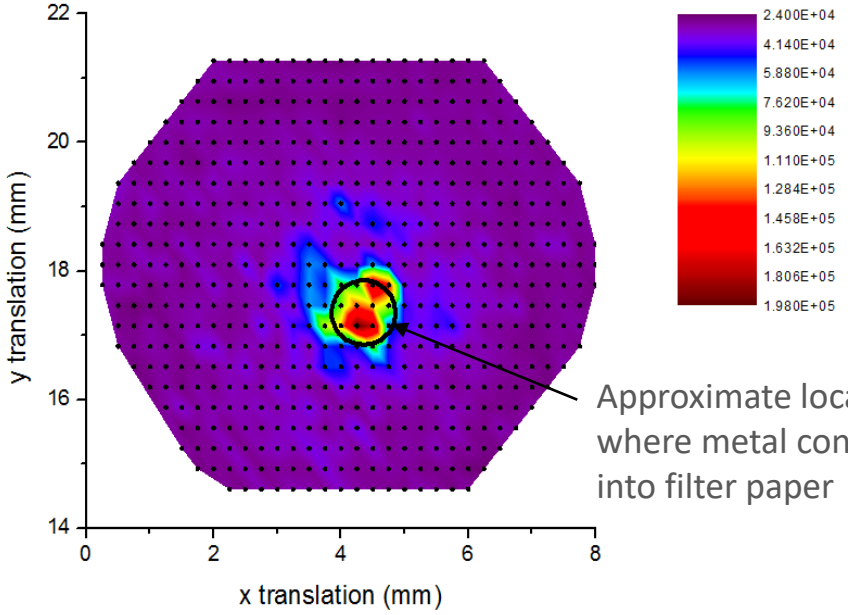


bacteria

Results

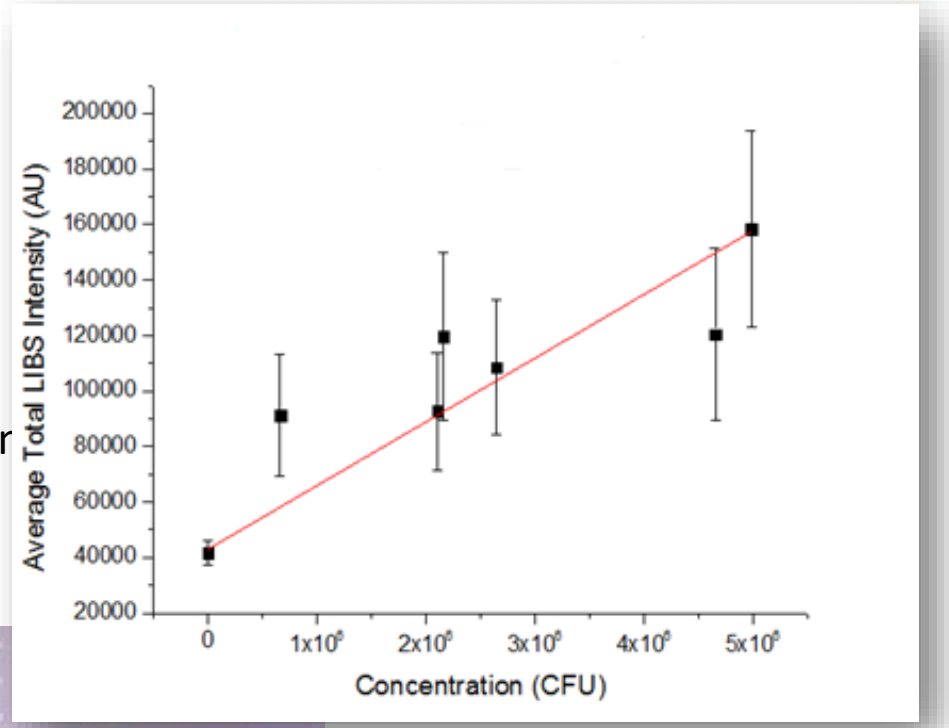
- *E. coli* deposited on filter paper with metal cone
- 569 LIBS spectra acquired across filter

Intensity map depicting bacterial deposition on filter paper for bacteria deposited with metal cone

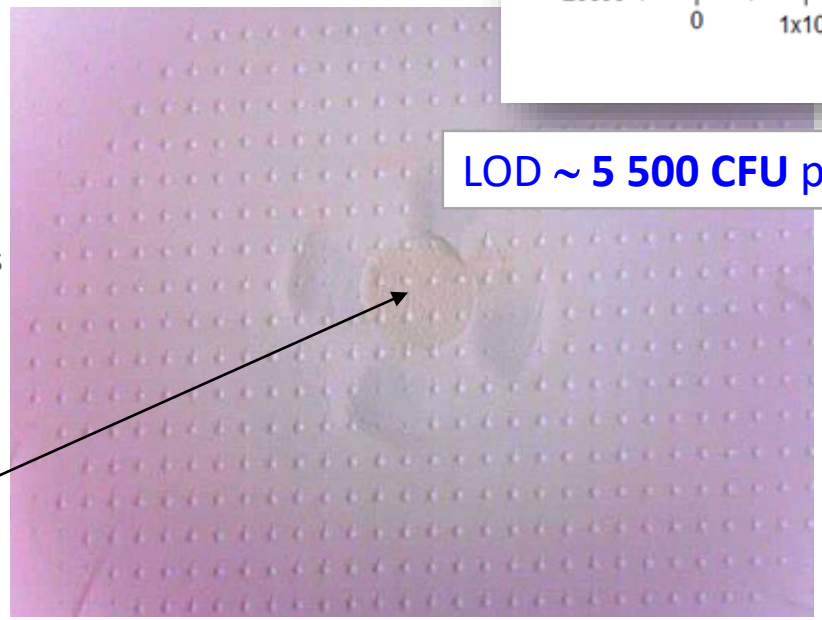


Approximate location of where metal cone presses into filter paper

Colour indicates bacterial LIBS ir
→ purple: no bacterial signal
→ red: strong bacterial signal

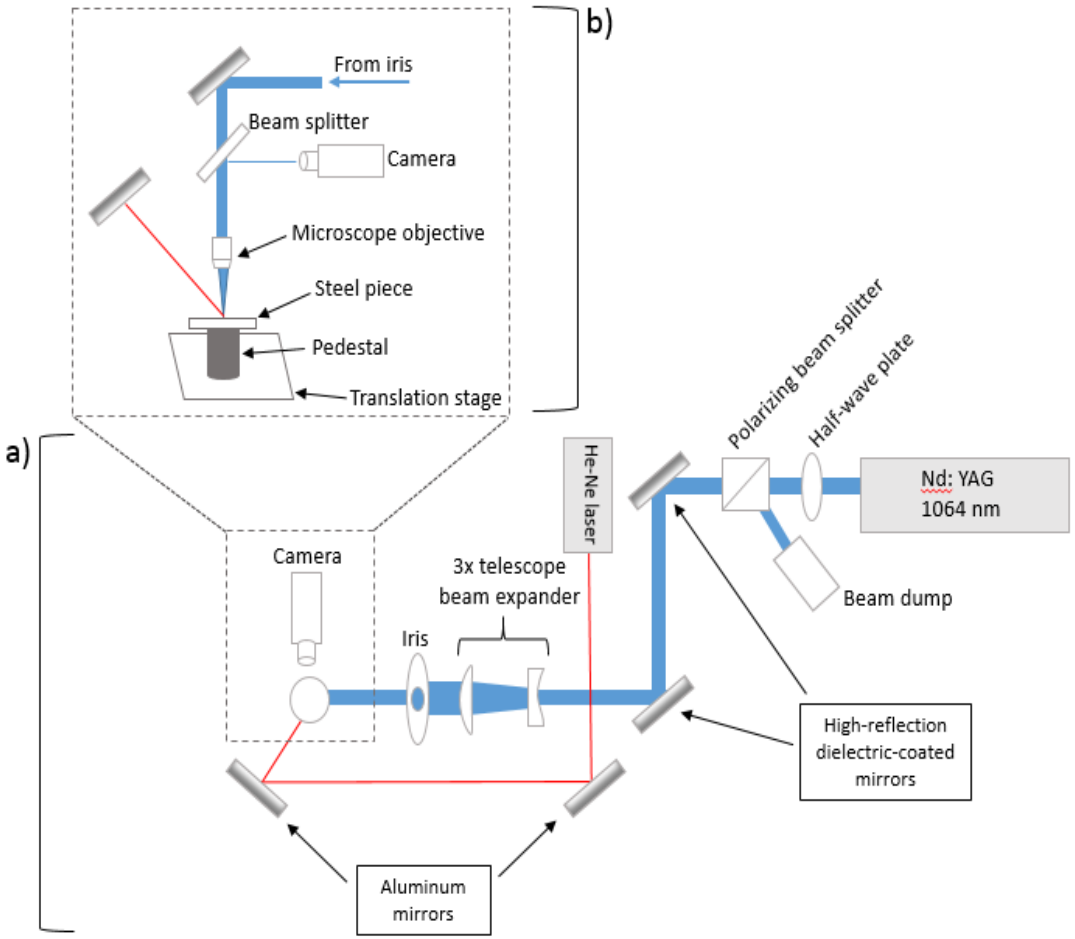


LOD ~ **5 500 CFU** per laser ablation event

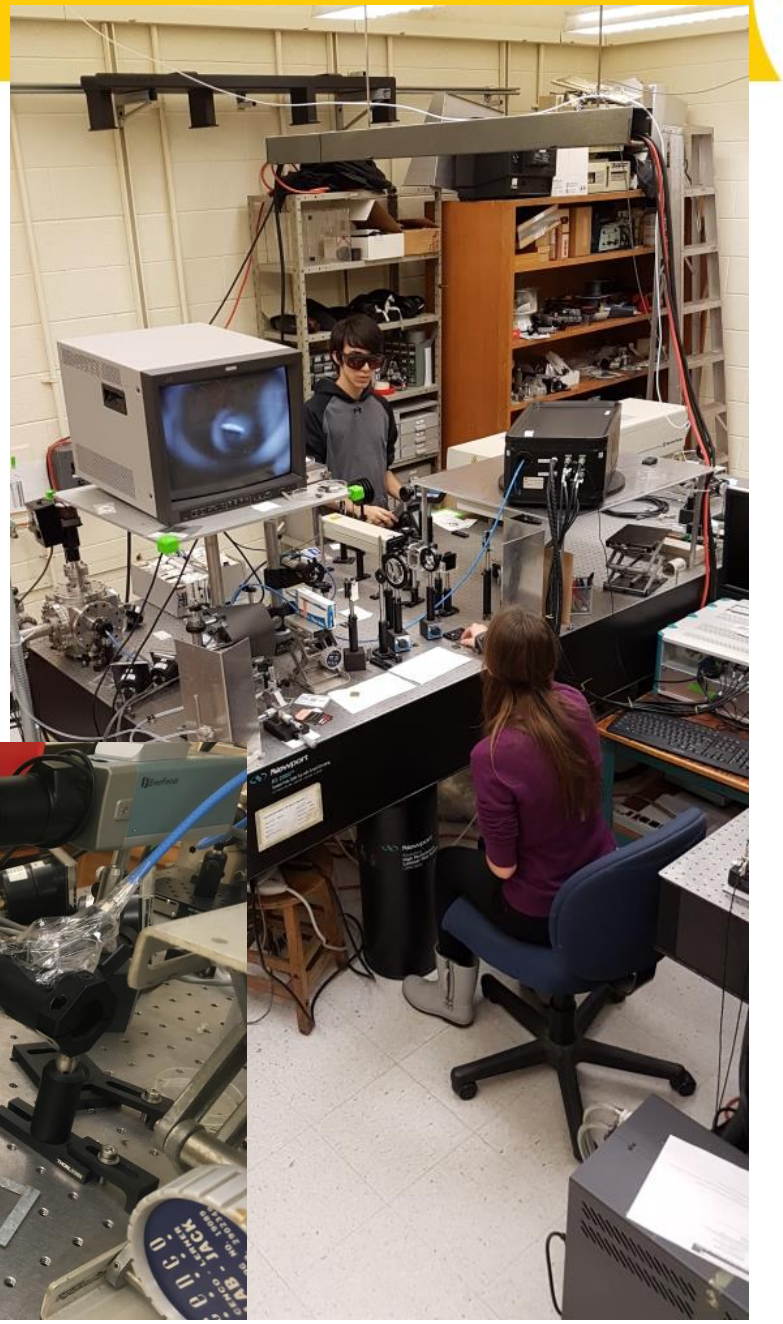


Discolouration due to presence of bacteria

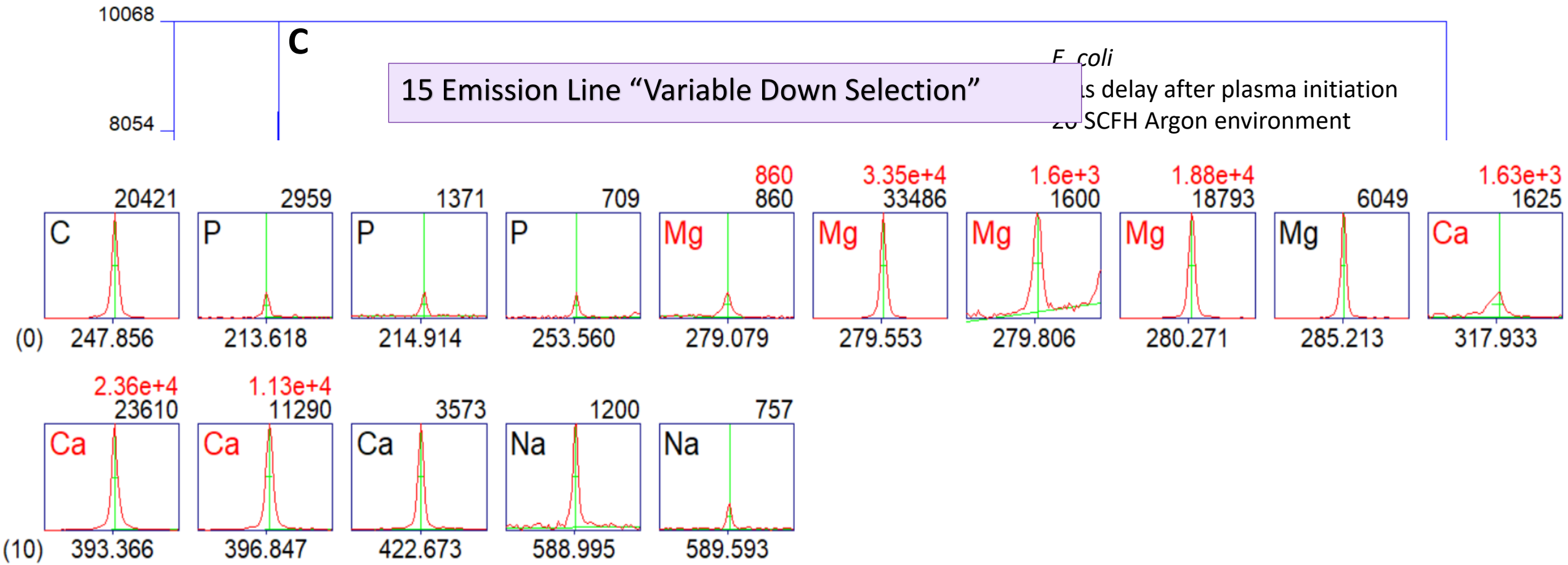
Apparatus



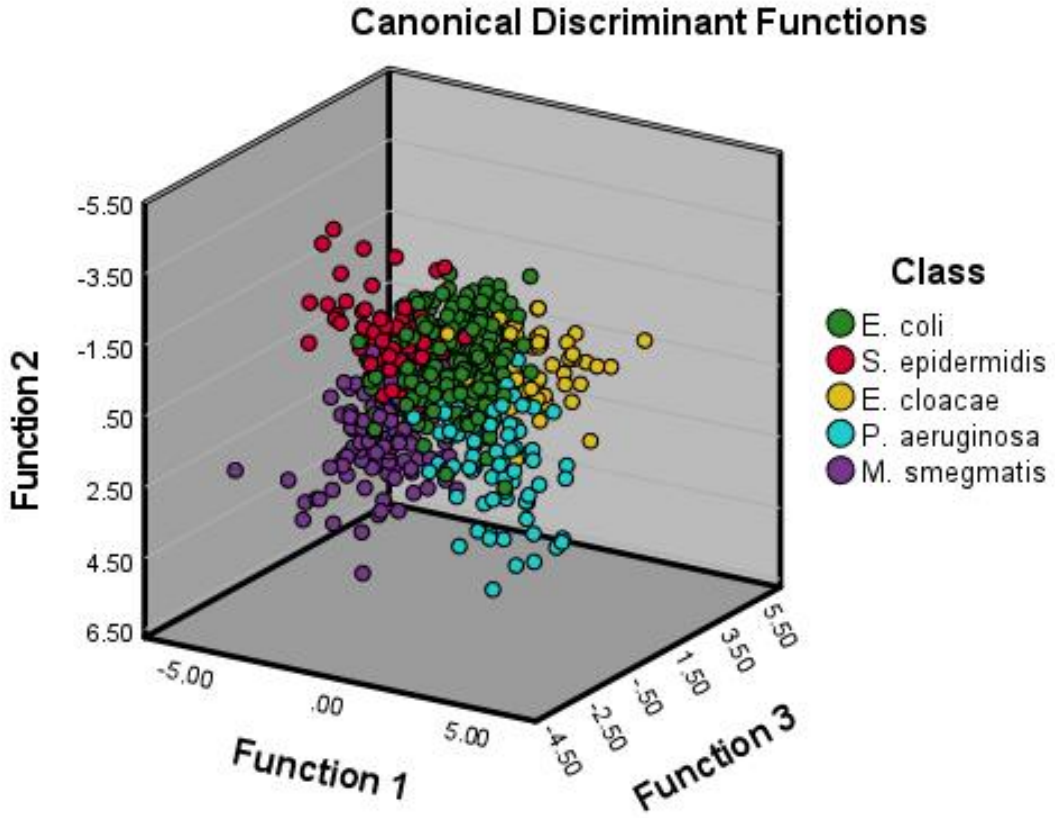
(a) Overhead schematic of the optical train used to direct laser pulses to the target. (b) Schematic side view of laser pulses emerging from the iris and directed to a target which is mounted on a steel piece

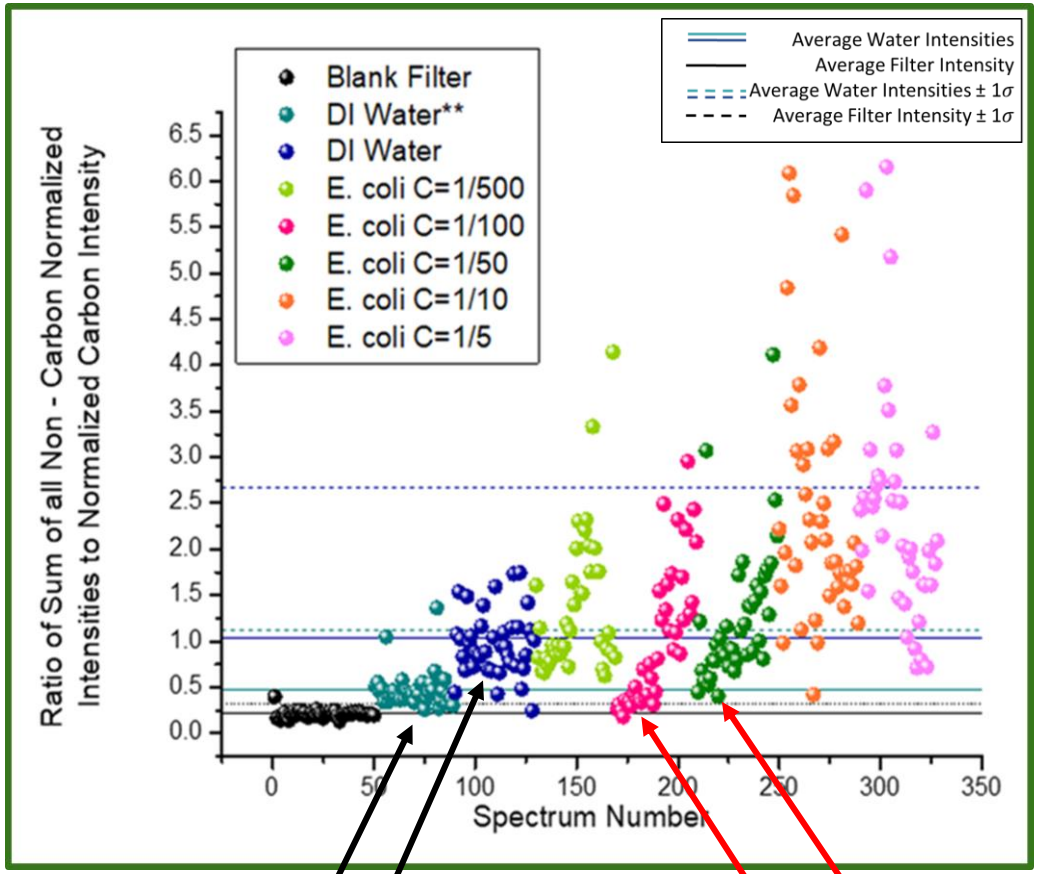


Typical LIBS Bacteria Spectrum



Spring 2021



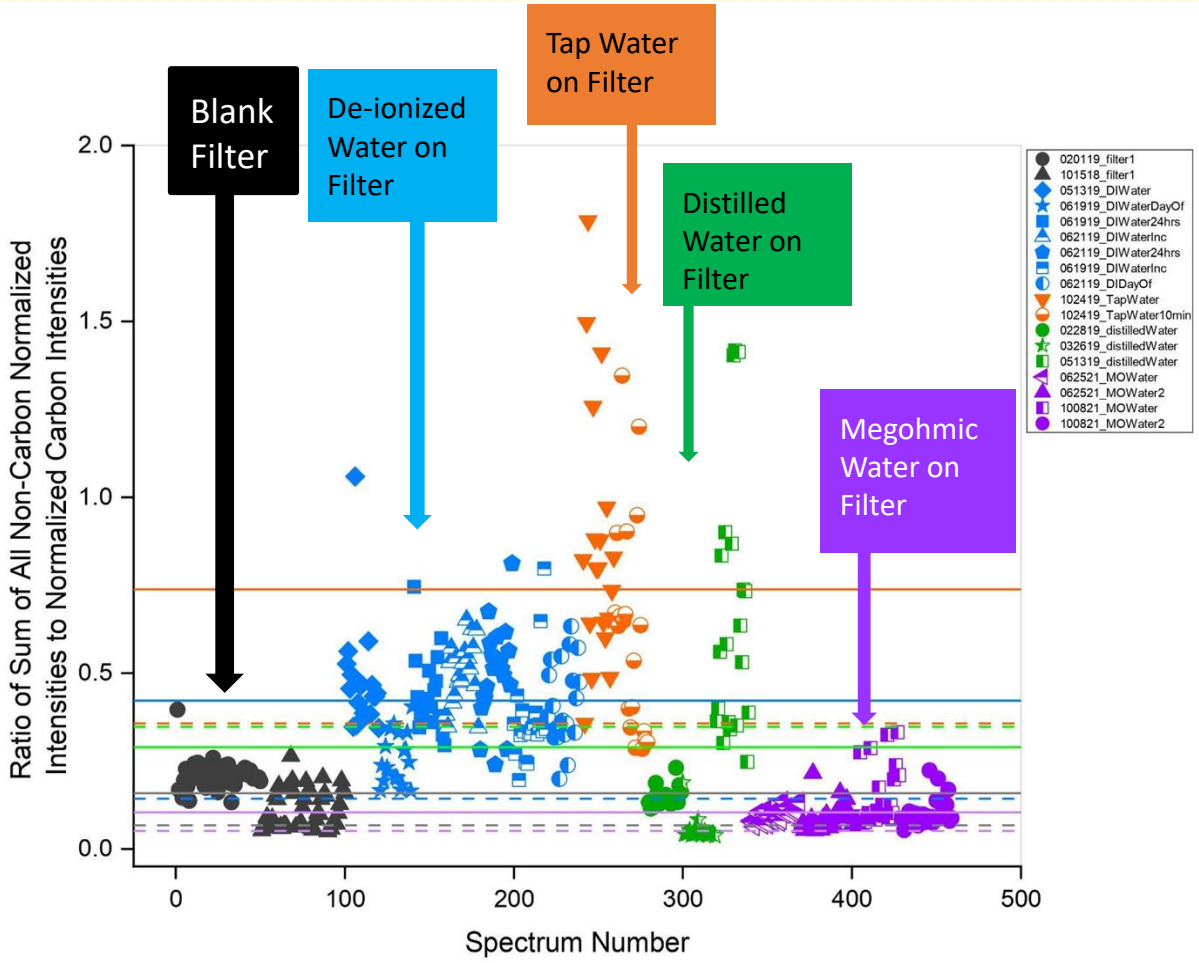


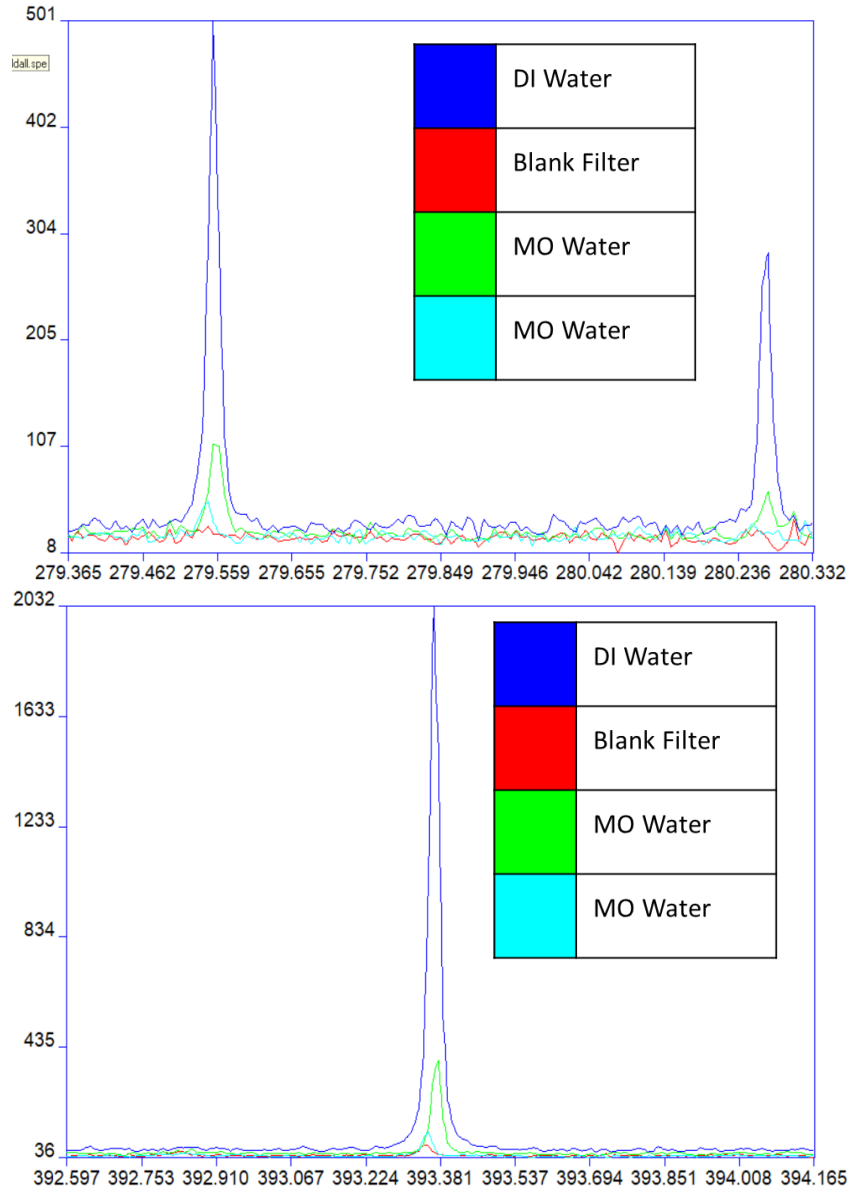
Bacteria	Sensitivity	Specificity	Classification error
E.coli	60 %	79 %	31 %
S. epidermidis	64 %	91 %	23 %
E. cloacae	50 %	91 %	29 %
P. aeruginosa	66 %	94 %	20 %
M. smegmatis	65 %	82 %	27 %

Why are sterile specimens (no bacteria) non-zero?

Why is this zero? What can we do about it?

To Reduce Background, Use Better Water

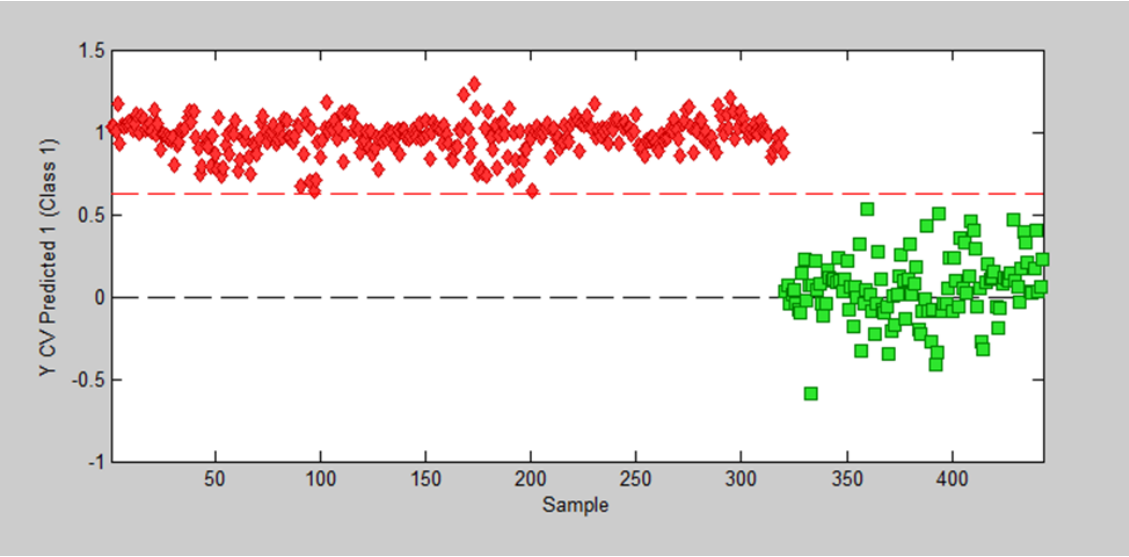




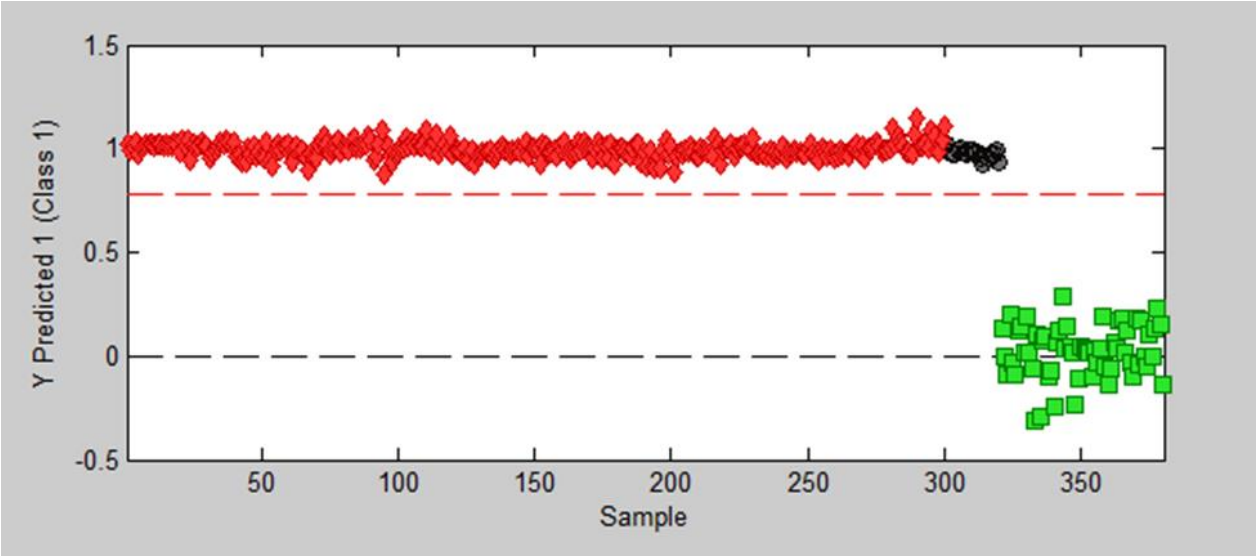
- Megohmic Water is consistent with filter
- Megohmic Water adds no extra background signal (as opposed to DI Water)

Water Studies (Bacterial Control)

PLS-DA: *E. coli* ⅕ vs. MO-Water



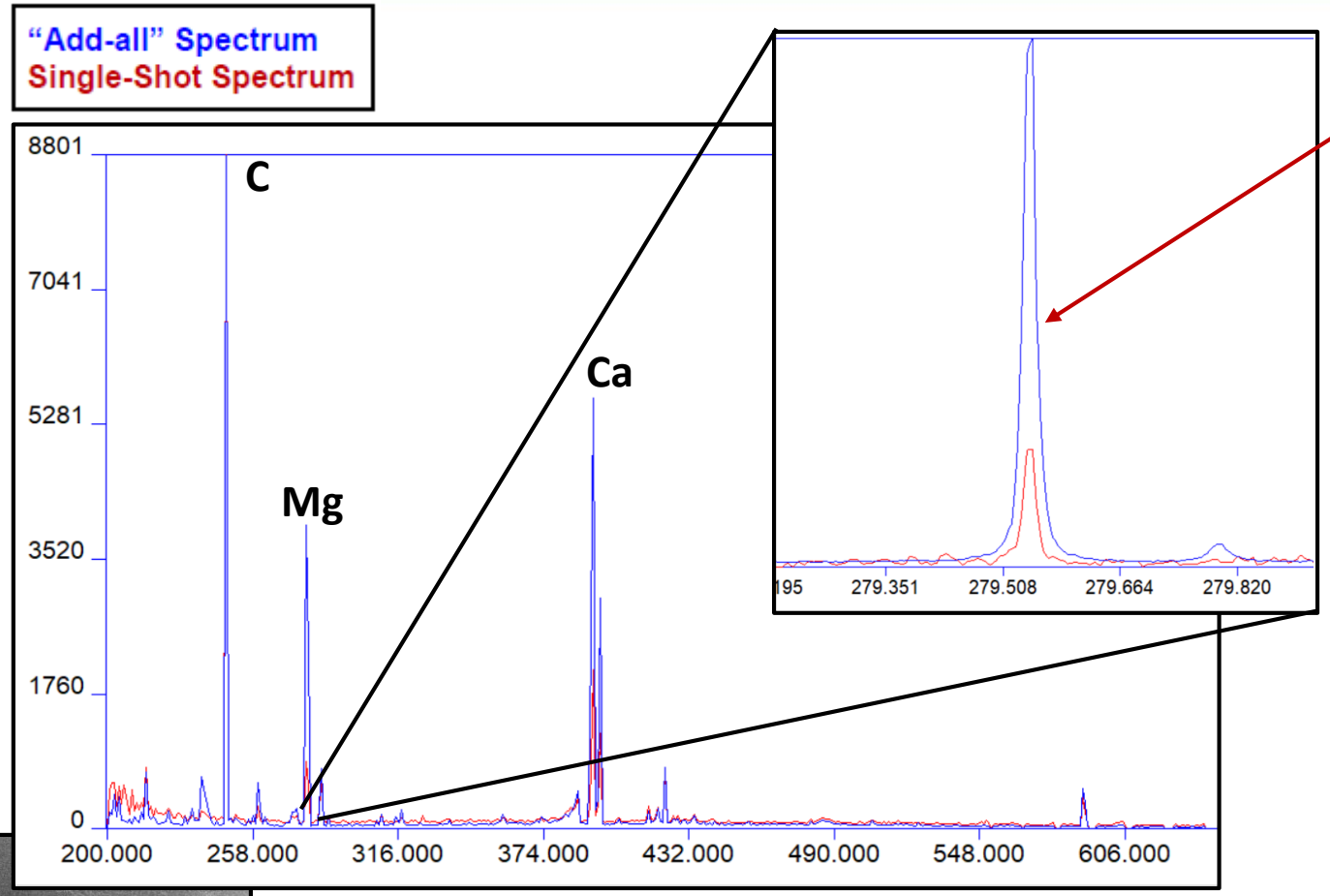
PLS-DA: All *E. coli* concentrations vs. MO-Water

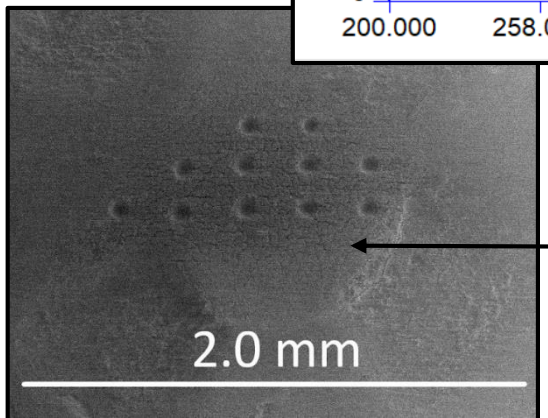


- Megohmic water allows for high discrimmination accuracy for all bacterial species we work with
- Megohmic water is indistinguishable from filter background

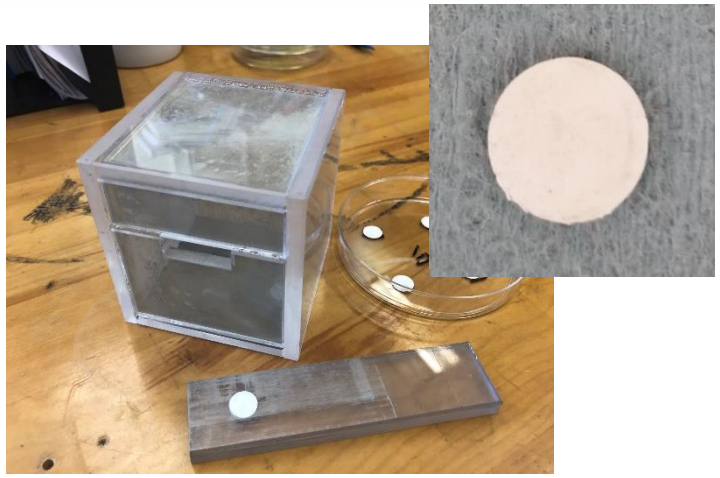
Averaging / Summation of Shots to Eliminate “Empty” Shots

- We need lots of data “shots” to build our chemometric model, but we only need ONE measurement per filter (patient sample)
- We can sum all the spectra using the “add all” feature of the spectrometer.
- Eliminates many “empty” spectra, but then data is very slow to accumulate and publish.





Using silver to amplify the LIBS spectrum: microparticles



Method for filter preparation

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

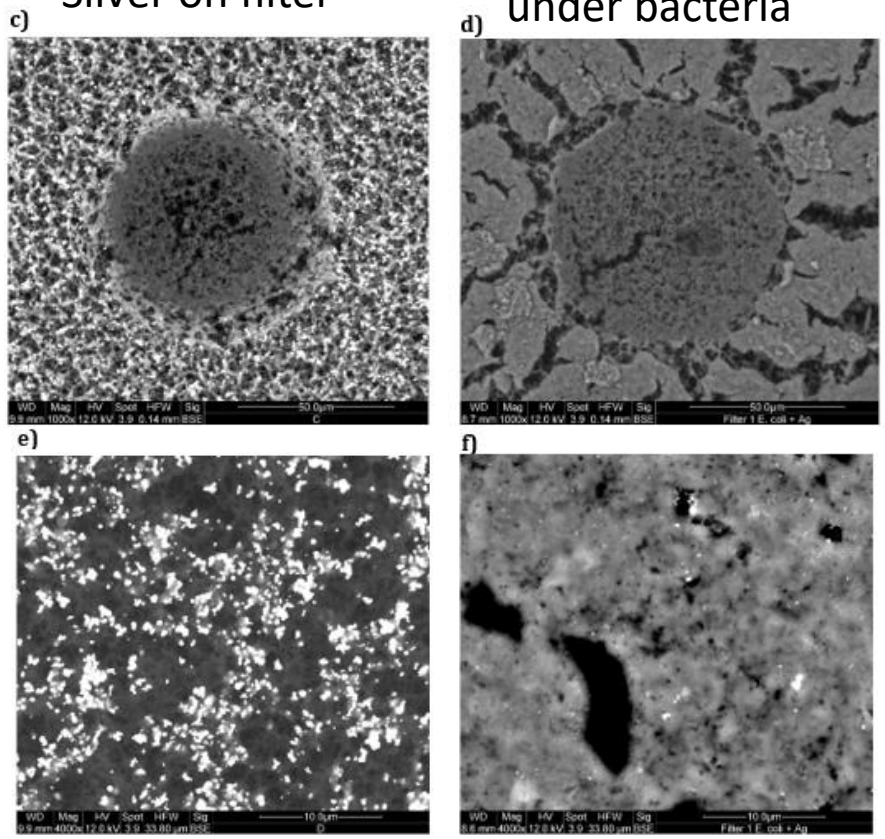
Trace uniform spread (with custom chamber) *approx. 1 – 50 ng deposited*

Surface coverage density rate 2.6 x 10^{-8} µg/µm^2·s

110 pg of Ag ablated per shot

Silver on filter

Silver on filter under bacteria



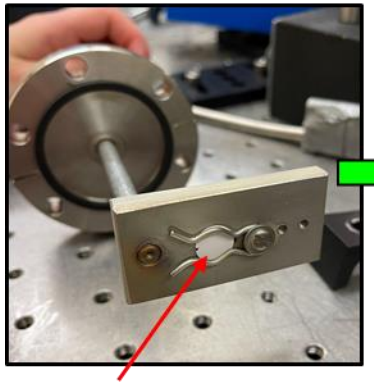
Average Elemental Enhancement of 4 Bacteria Species with the Addition of Silver Microparticles

	Elemental LIBS Spectral Emission Enhancement				
Bacteria Species	**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

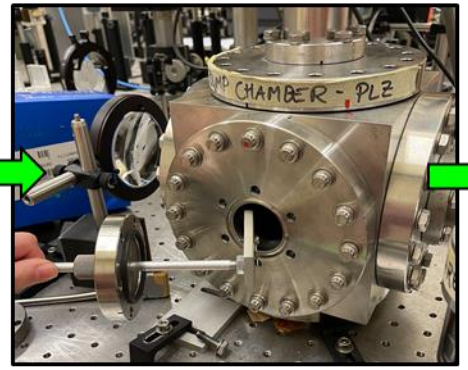
Using silver to amplify the LIBS spectrum: Ag sputtered filters

Method for filter preparation

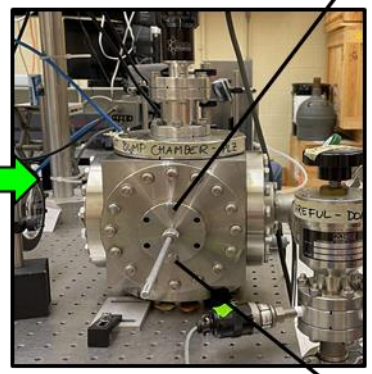
- 99.9% pure Ag foil on rotating target
- 1064 nm pulsed laser sputtering (*approx. 60 mJ / pulse)*
- Vacuum environment
- Blank filters places in various geometries around sputtering source
- Sputtering time variable (*20 s – 12 min)*



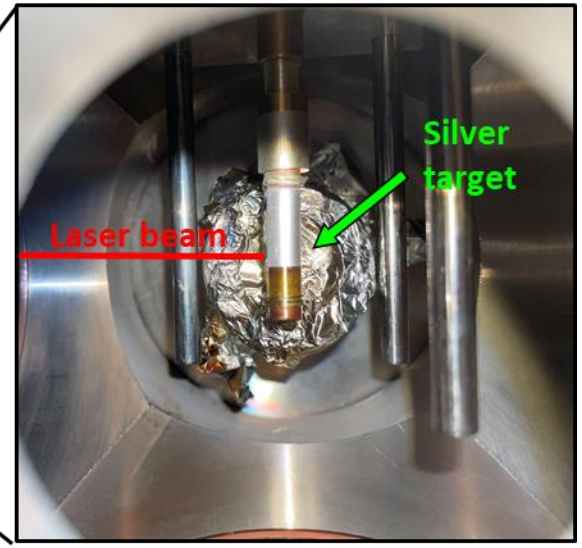
Filter on custom **aluminum filter holder**



Filter holder being inserted into **10 mTorr evacuated chamber**

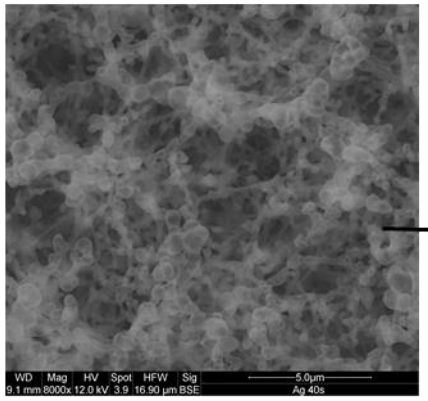


Closed vacuum chamber apparatus

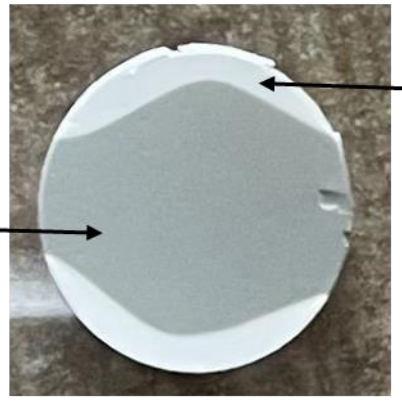


Inside the chamber

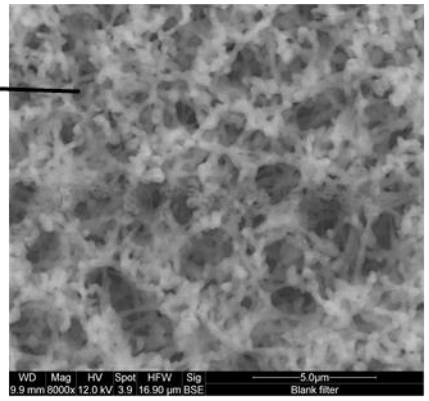
The deposition is so uniform and thin it appears the same as a blank filter



Filter with 40s Ag

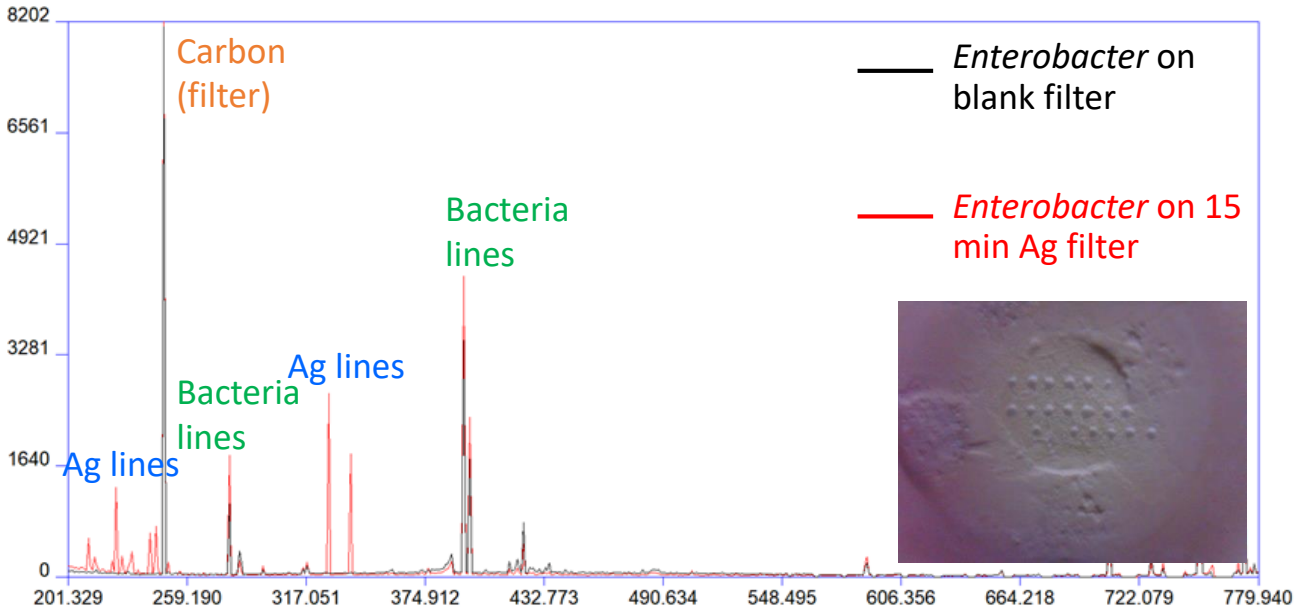


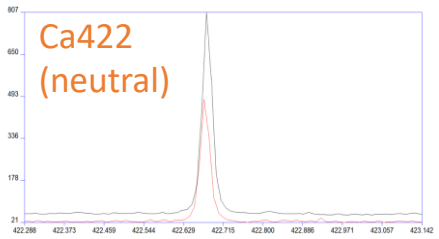
40 s deposition time Ag filter

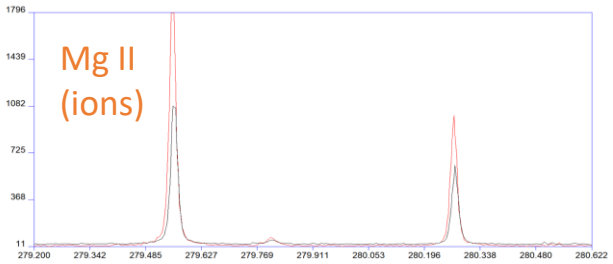


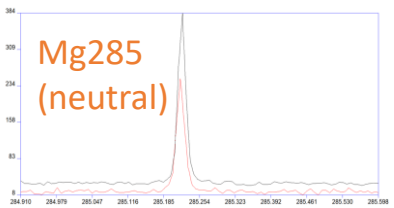
Blank Filter

Using silver to amplify the LIBS spectrum: Ag sputtered filters



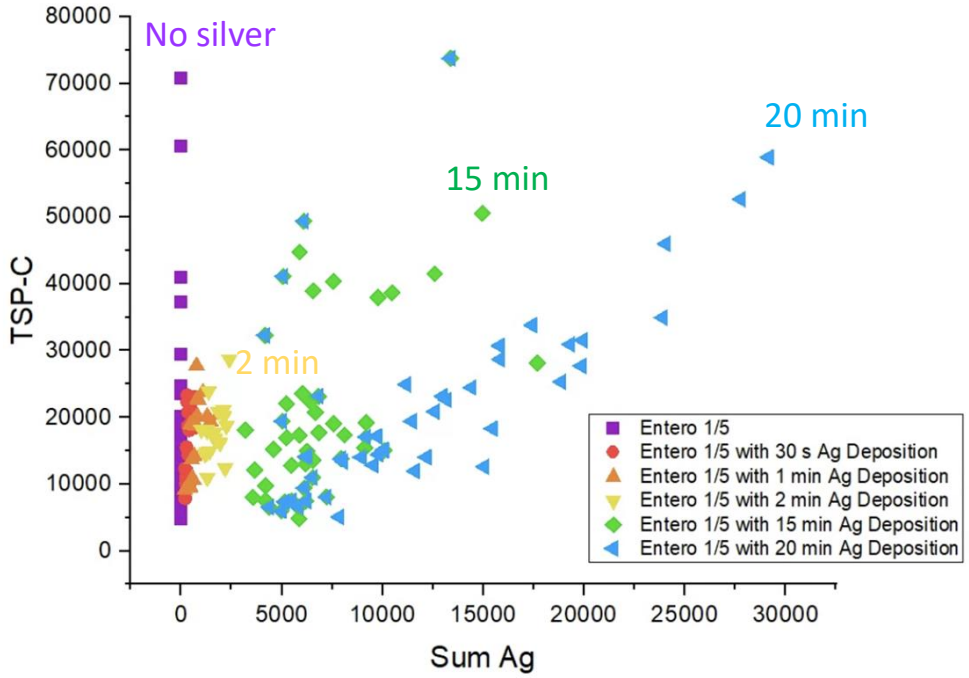




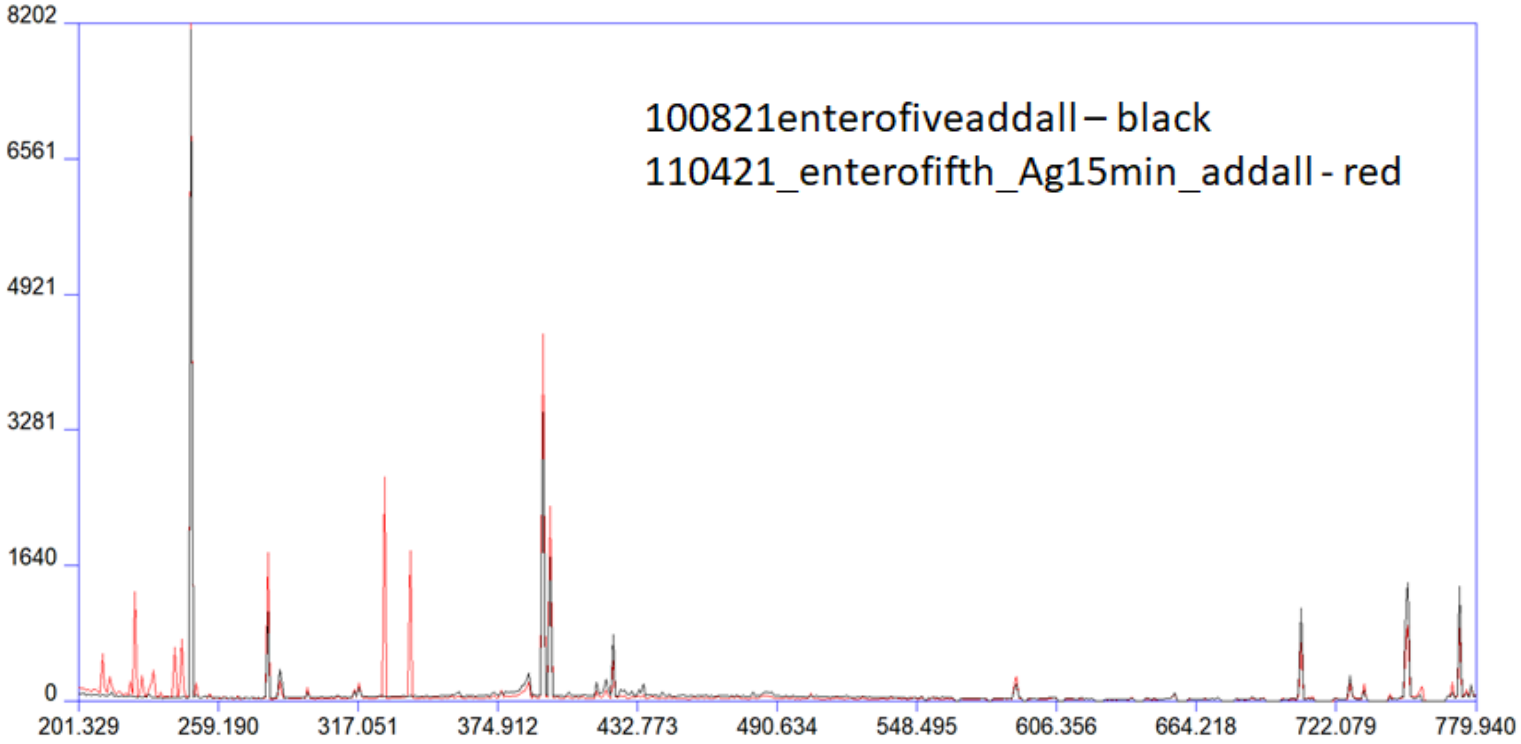


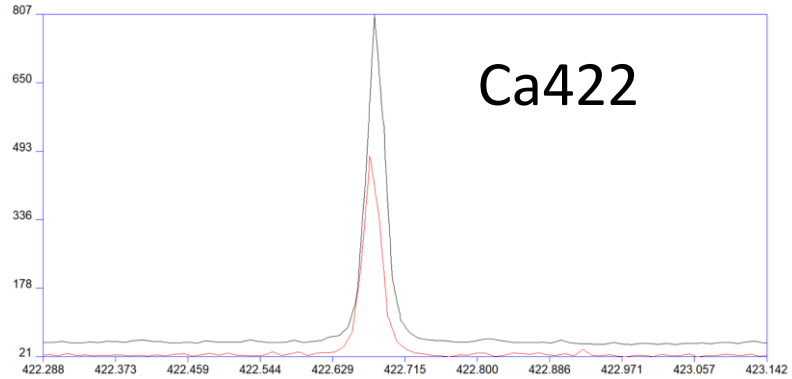
- Plasma temperature is increased.
- Enhancement was not observed until 15 min deposition time.
- 20 min gave the same amount of enhancement as 15 min.

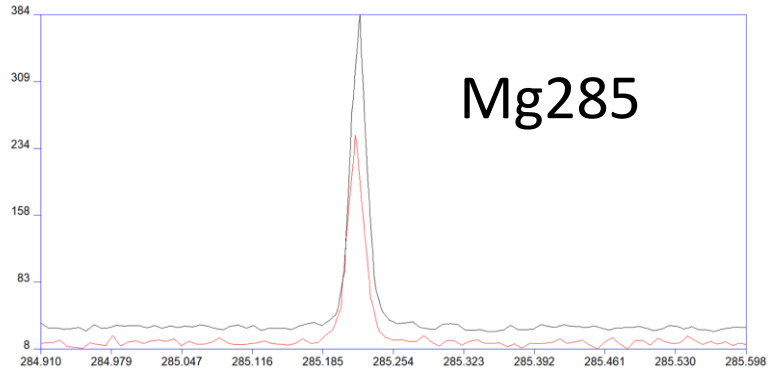
- Bacterial lines strongly correlate with Ag lines
- But they are not enhanced relative to use of “blank filter” (purple)



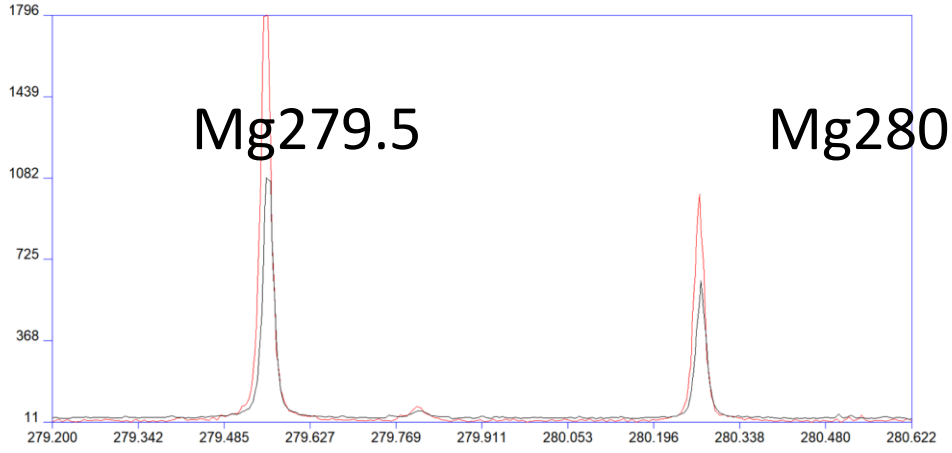
The x-axis is the sum of all Ag lines and the y is sum of all lines other than silver excluding carbon.





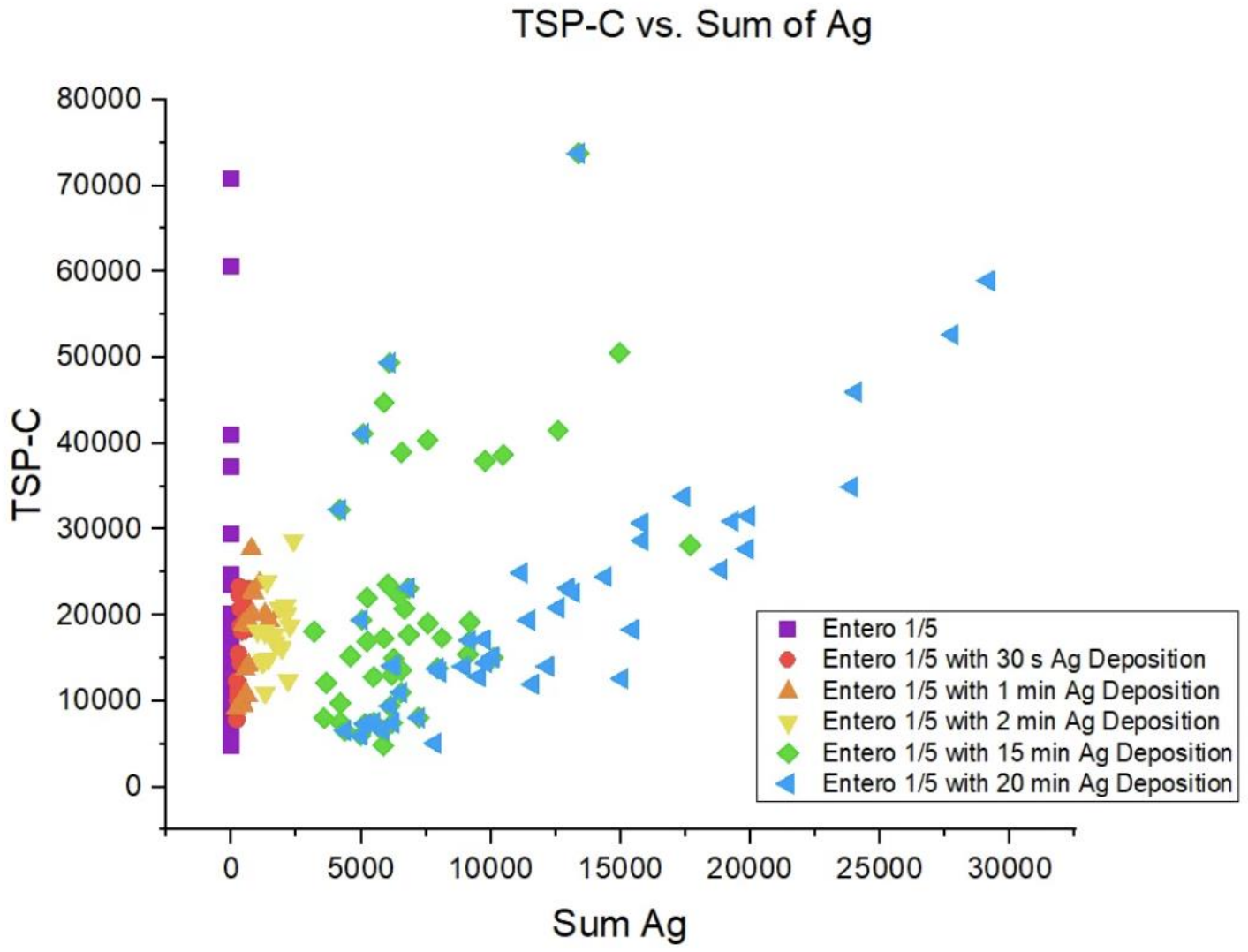


Intensity is enhanced for all lines except C247, which is the same, and Mg 285 and Ca422 which are lower because they are neutrals. This proves that our plasma temperature is increased.
Enhancement was not observed until 15 min deposition time. 20 min gave the same amount of



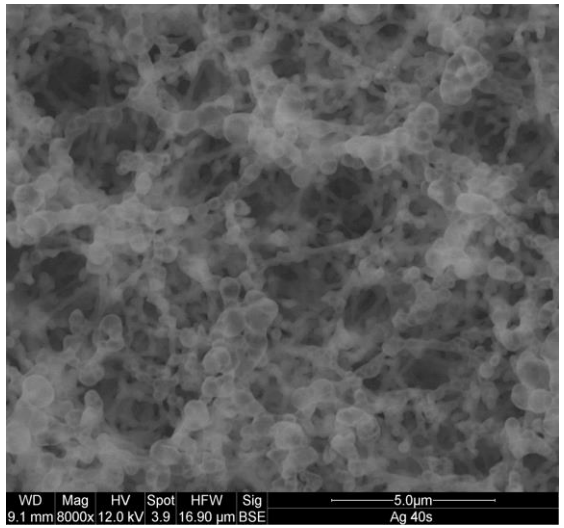
Silver Enhancement vs. TSP-C

The x-axis is the sum of all ag lines and the y is sum of all lines other than silver except for carbon.



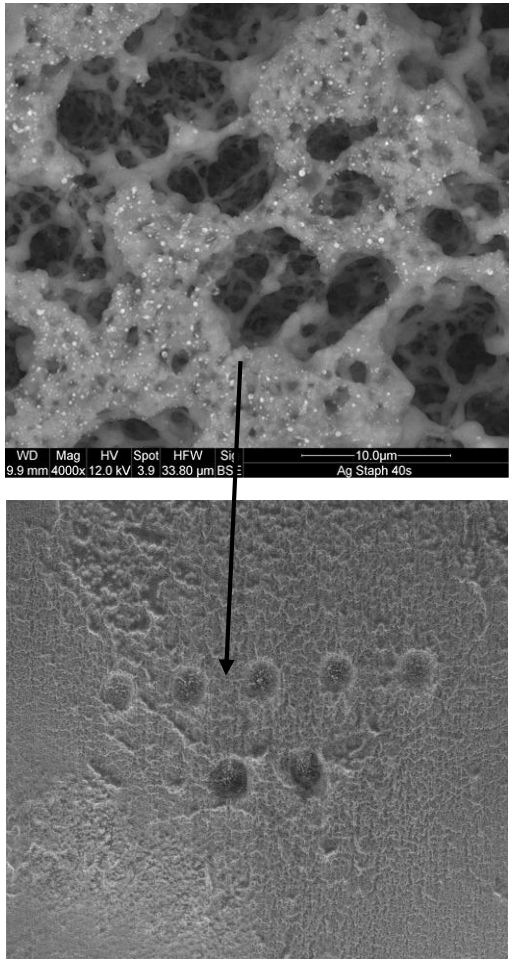
Using silver to amplify the LIBS spectrum: nanoparticles

Silver is present, but not visible



Filter with 40s Ag

White specks = Silver nanoparticles



Filter with 40s Ag, then *Staphylococcus* deposited on top

Sputter deposition in vacuum produces no nanoparticles.

LIBS on the sputter targets DOES produce nanoparticles.

Subsequent shots on areas coated with nanoparticles shows enhancement (NPELIBS).

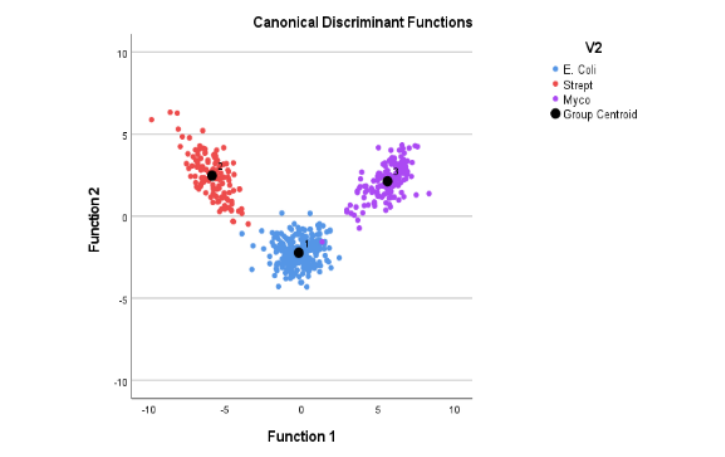
Using silver to “boost” signals

- We might get rid of carbon

We Are Also…

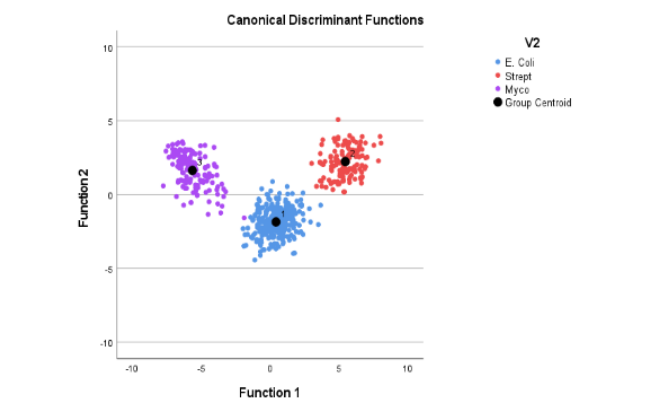
- Investigating the removal of the C247 line from all analysis (most of all the carbon comes from the filter)
  - Preliminary results show this will not significantly reduce sensitivity or specificity

Includes carbon line and three bacteria classes



All results obtained from analysis of previously-obtained data (not from filters)

Carbon line excluded and three bacteria classes



Sensitivity and Specificity results:

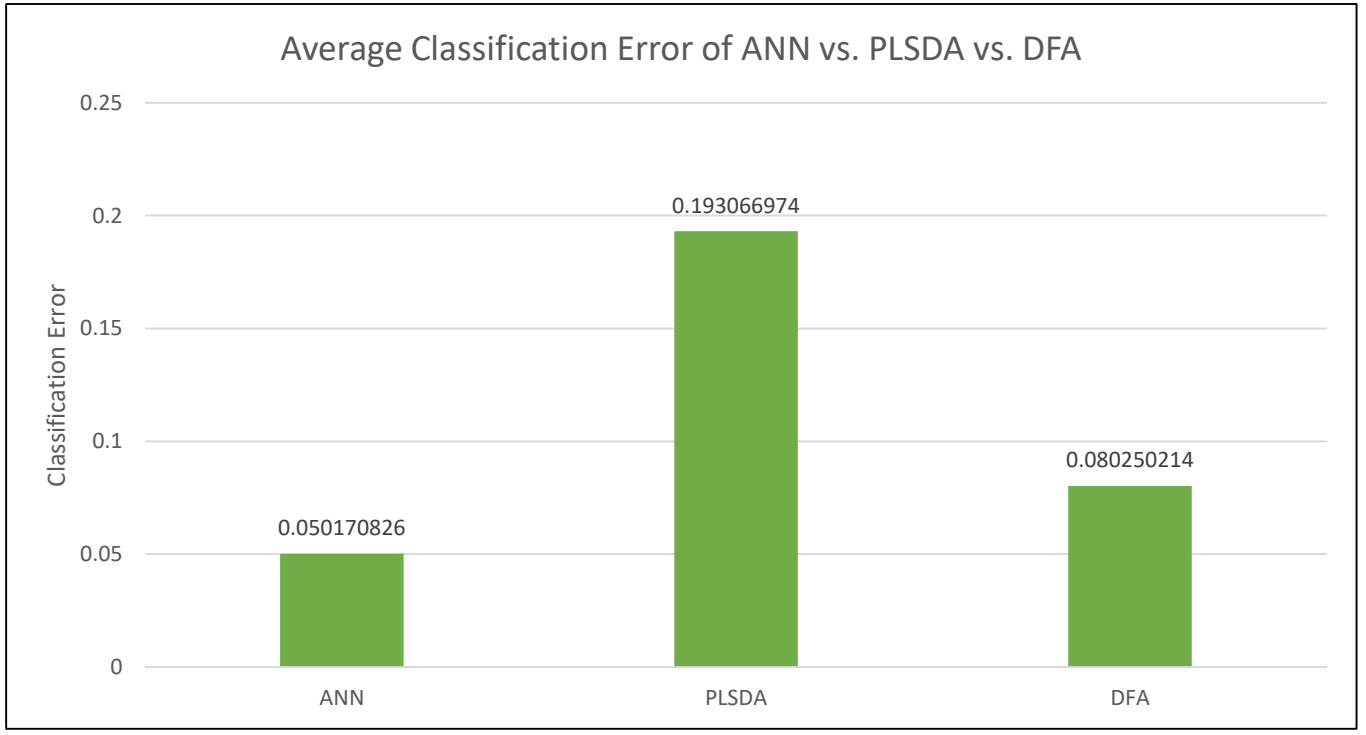
E. Coli			Myco	
Sensitivity with Carbon:	**0.983277**		**Sensitivity with Carbon:**	**0.9025974**
Specificity with Carbon:	0.9448275		Specificity with Carbon:	0.9908045
Strept				
Sensitivity with Carbon:	**0.992647**			
Specificity with Carbon:	0.997792			

Sensitivity and Specificity results:

E. Coli			Myco	
Sensitivity:	**0.969899**		**Sensitivity:**	**0.8961038**
Specificity:	0.931034		Specificity:	1
Strept				
Sensitivity:	**0.970588**			
Specificity:	0.980132			

We Are Also…

- Investigating the use of ANN* to replace PLS-DA or DFA
  - Most groups have shown improved performance when using SVM, ANN, or KNN as opposed to DFA, LDA, PLS-DA
  - We need <u>much</u> more training data to properly develop this model (number of epochs, number of nodes in hidden layer, use of validation, batch size, etc.)

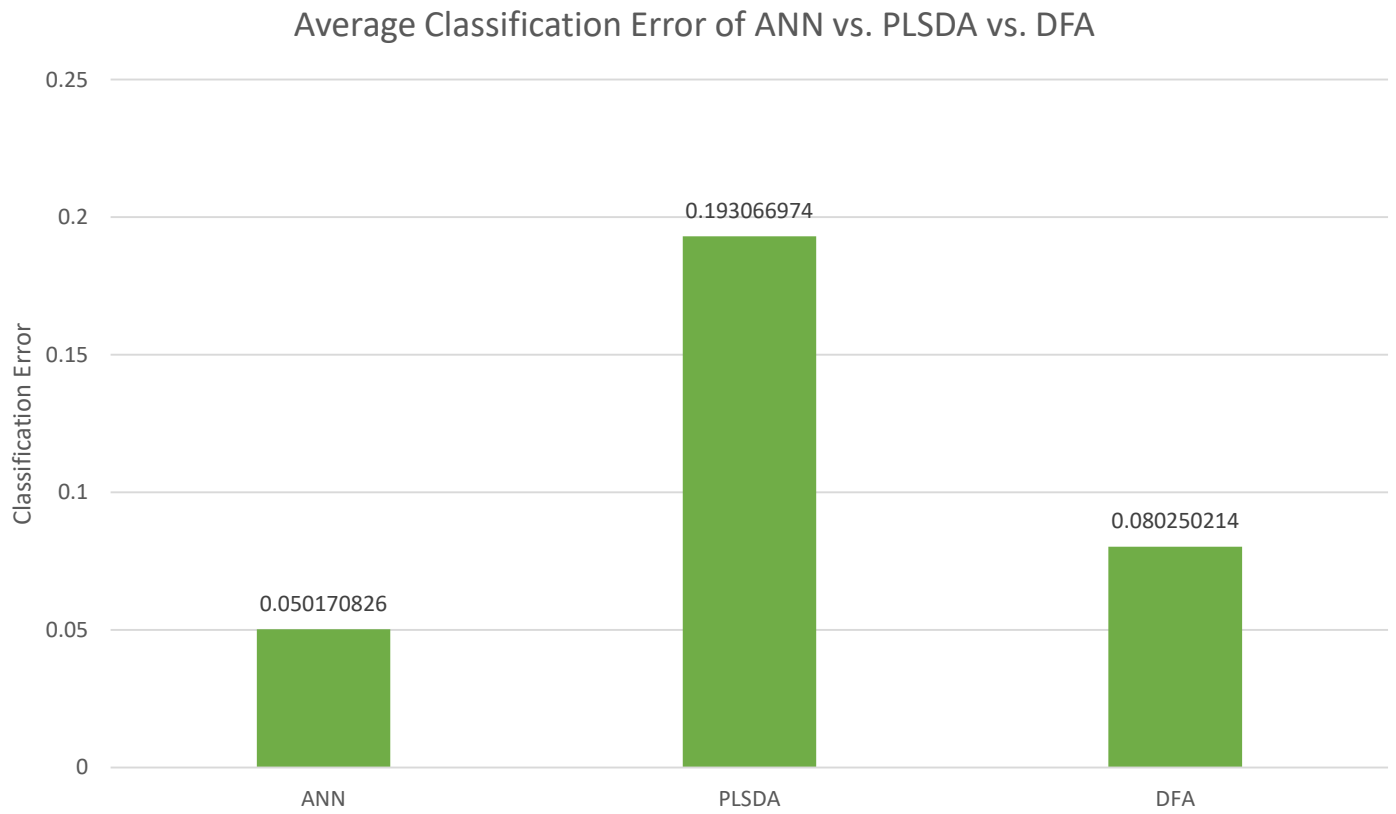


Using our previously obtained data (not on filter)
5 Class test

- Escherichia
- Enterobacter
- Staphylococcus
- Streptococcus
- Mycobacterium

*Built an artificial neural network (ANN) in python using Keras which acts as an interface of Tensorflow (open-source platform for machine learning)

- Using silver

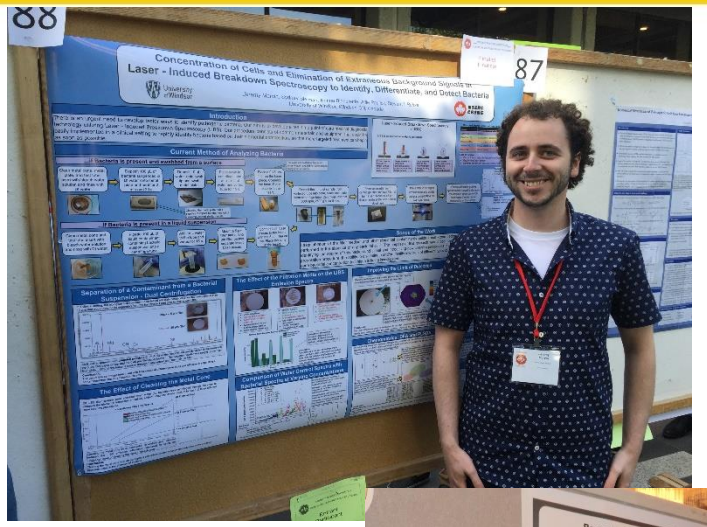


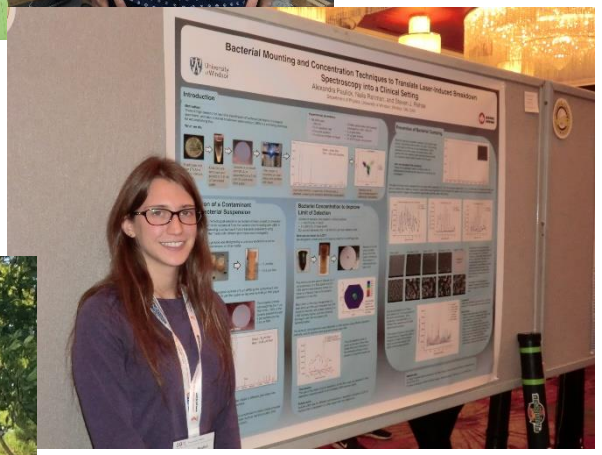
Conclusion

- We have a very flexible, rapid way to deposit bacteria on a testable disposable filter
- Use of ultra-pure water imporoves specificity
- The modification of filters with metallic silver shows promise
- The removal of carbon from the elemental analysis may improve performance
- The use of ANN may improve performance
- We are now beginning to test blood and urine clinical specimens obtained from our clinical colloarborators at Windsor Regaion hospital.

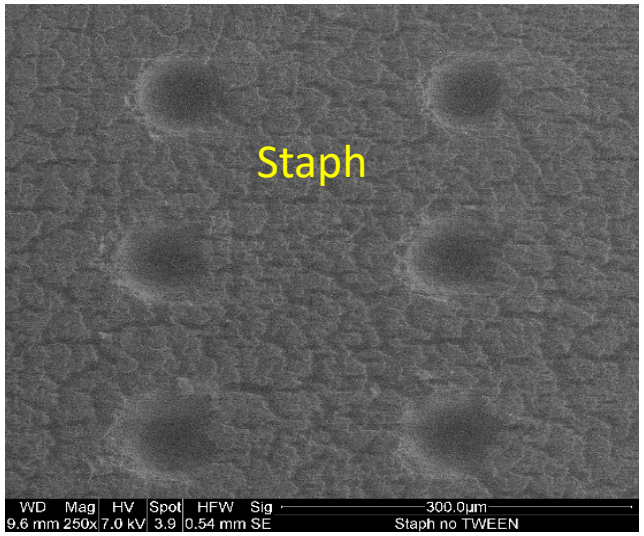
Thanks To

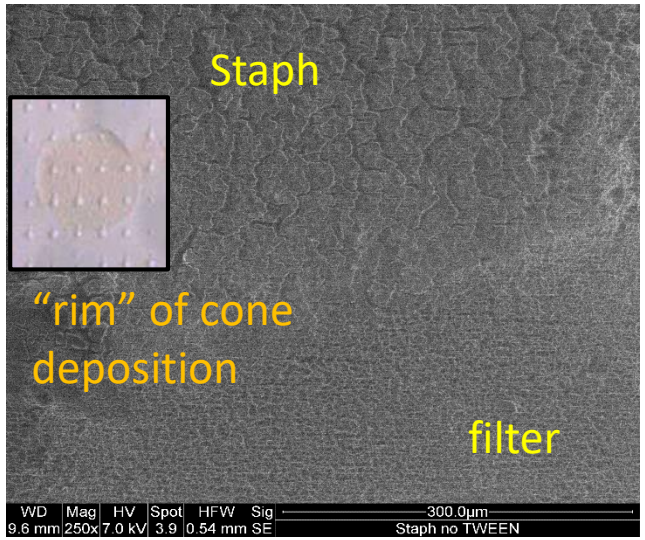
Thanks To

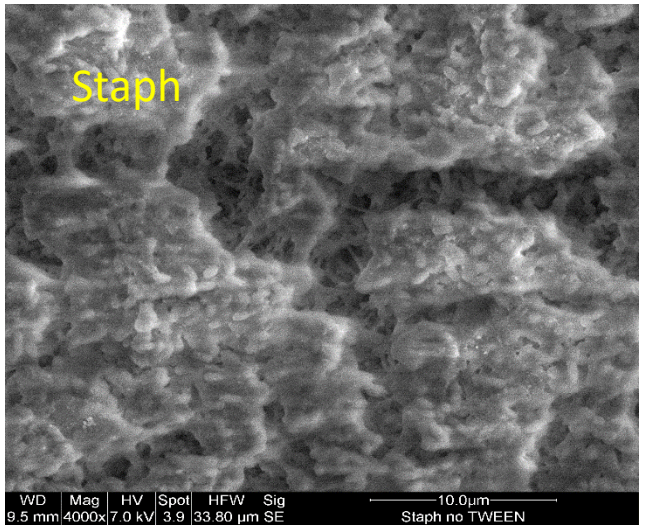

88
87
Concentration of Cells and Elimination of Extraneous Background Signals in Laser - Induced Breakdown Spectroscopy to Identify, Differentiate, and Detect Bacteria

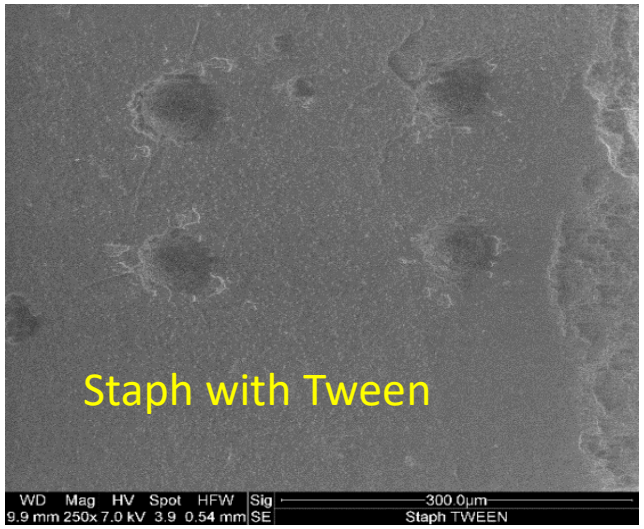

Bacterial Mounting and Concentration Techniques to Translate Laser-Induced Breakdown Spectroscopy into a Clinical Setting

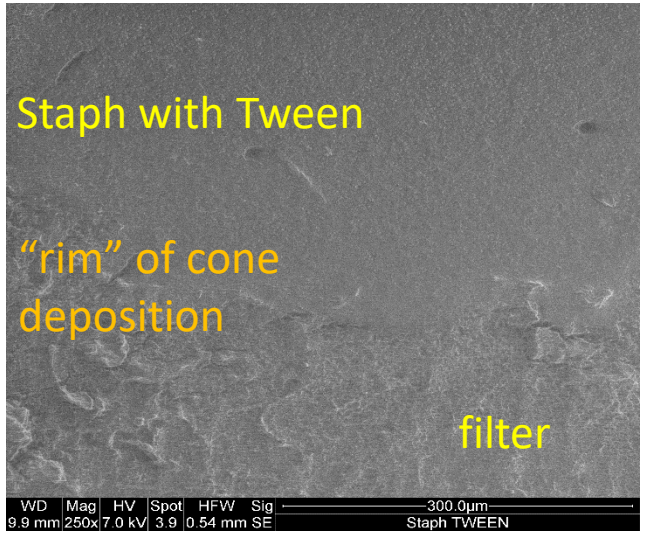
The Scatter in the Data

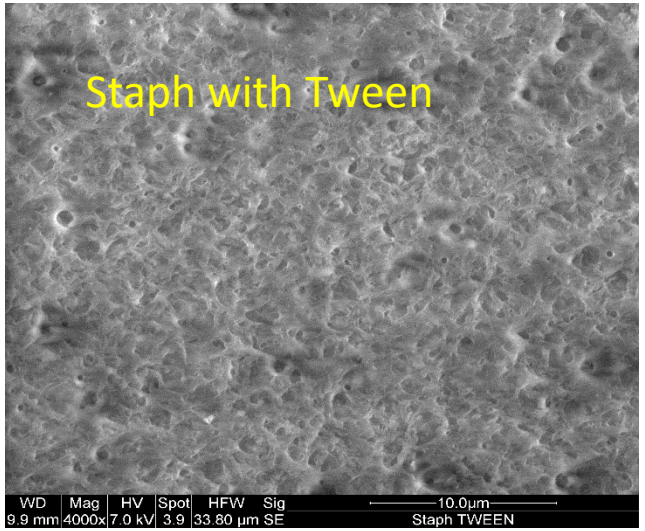






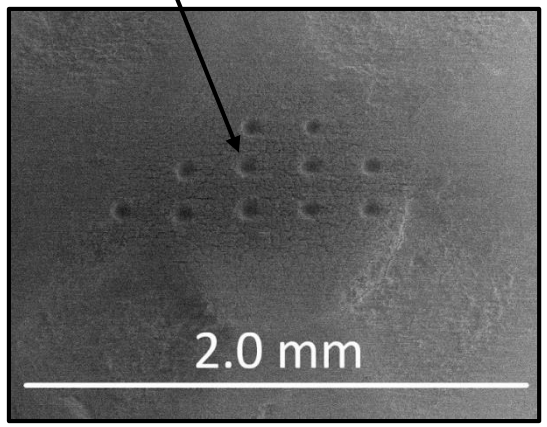


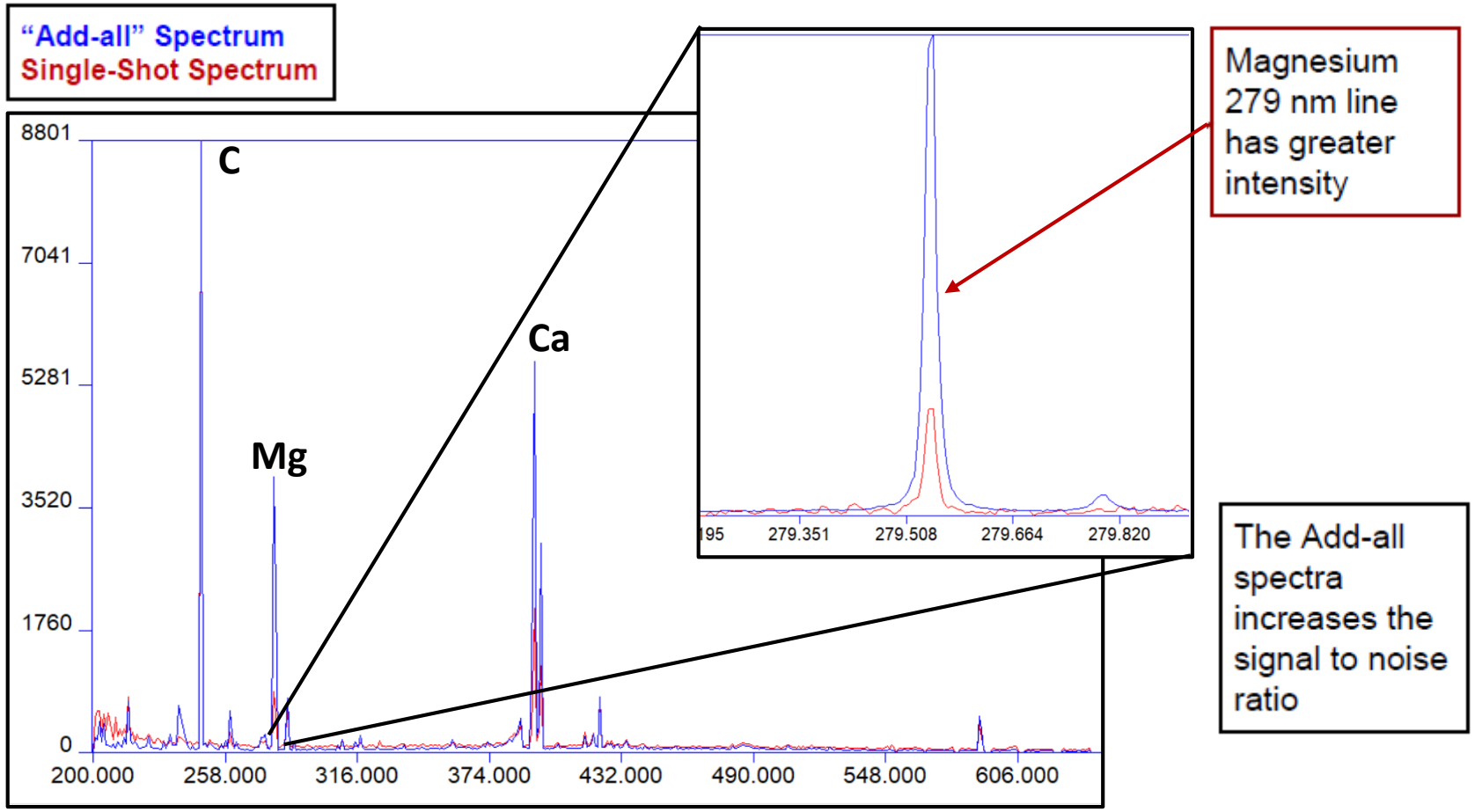




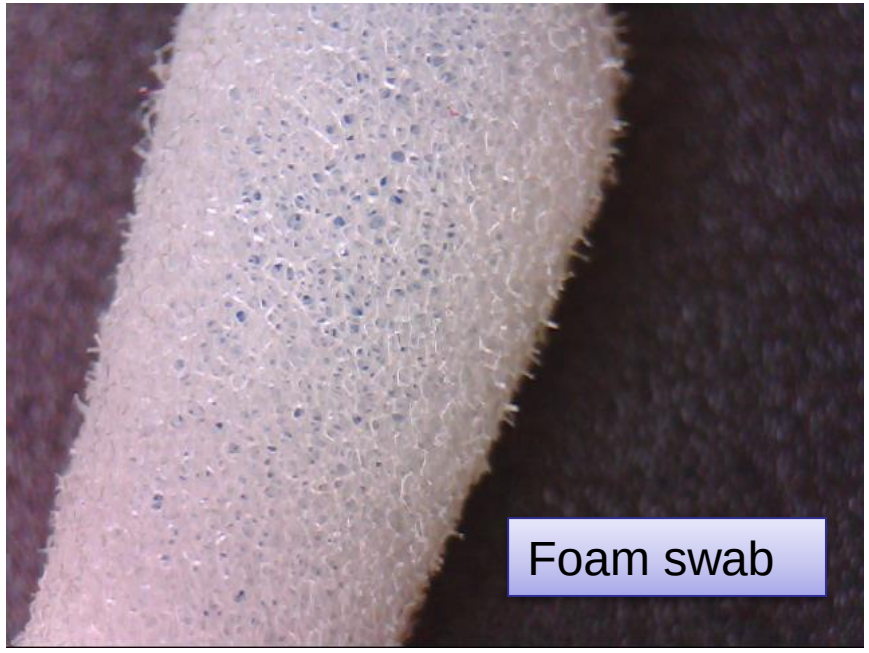
Effect of Adding Spectra

Circular area of filter containing 10 single-shot spectra.



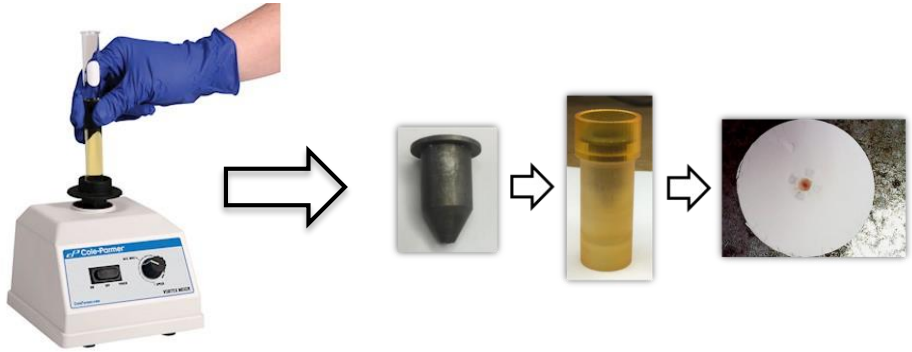


Method 4



Cannot shoot right on the swab

- Far too irregular (almost no plasma)
- Cells not concentrated



Method 4

Cannot shoot right on the swab

- Far too irregular (almost no plasma)
- Cells not concentrated