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

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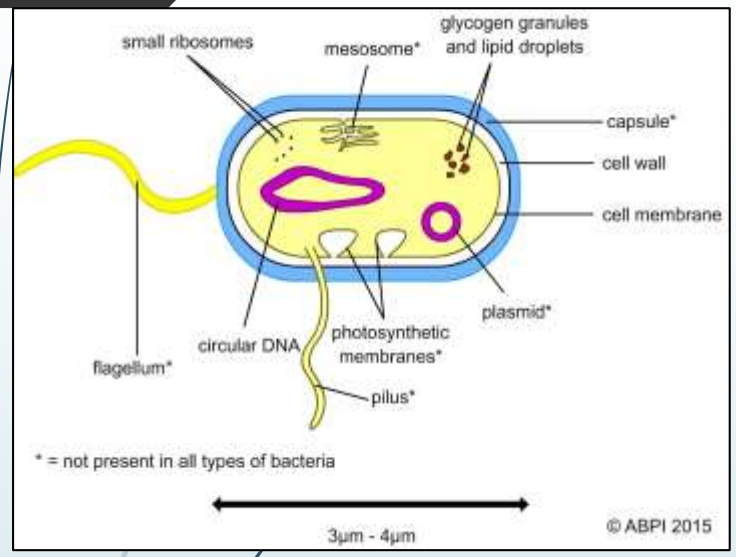
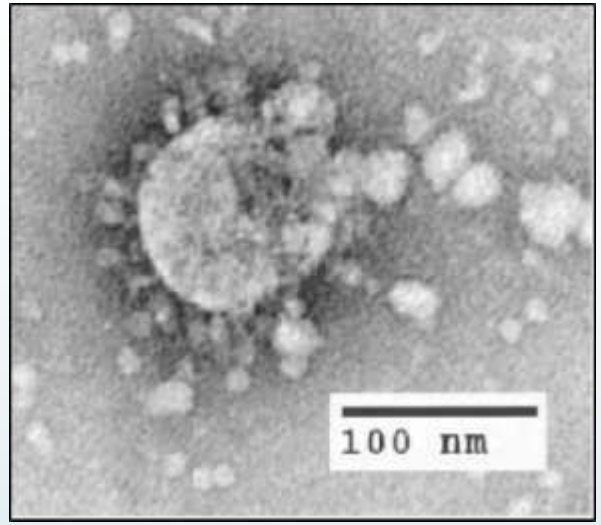
LIBS on Viruses? Size matters!



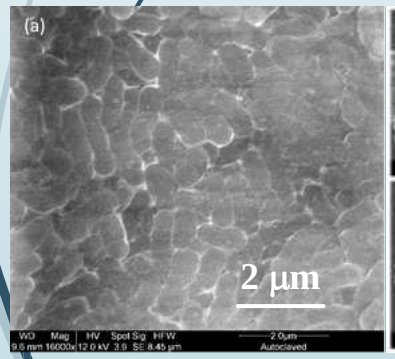
https://www.abpischools.org.uk/topic/pathogens/2/1

- Bacteria are ~1-3 µm
- Corona viruses are ~100-300 nm
- Volume is roughly 1,000 – 10,000 lower!
- Also, viruses are not rich / don't contain trace metals, as bacteria do.



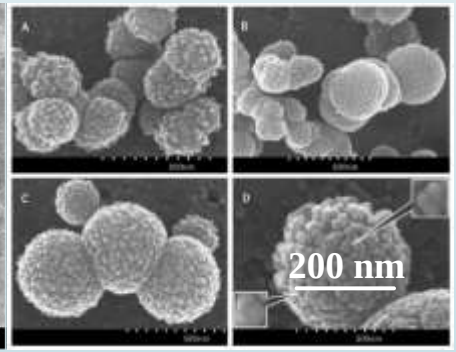
C.S. Goldsmith, CDC,

https://www.cdc.gov/sars/lab/images.html



SEM of *E. coli* specimen from our lab

vs.



SEM of SARS corona-virus, Antiviral Therapy 9:287-289, 2004

Two known papers on the use of LIBS to identify viruses:
(full details in S.J. Rehse, Spectrochimica Acta Part B 154 (2019) 50–69)

detect the presences of an MS-2 bacteriophage (smallpox surrogate)
J.L. Gottfried, Anal. Bioanal. Chem. 400 (2011) 3289–3301,

differentiation with LIBS of four strains of live *hantavirus*
R.A. Multari et al., Appl. Opt. 51 (2012) B57–B64,

Motivation

- Current methods of bacterial identification in a clinical setting
  - require transferring the sample to a lab
  - require expertise in microbiology
  - expensive/labor-intensive
  - may only be useful for certain types of bacteria
  - **slow**

  For example: standard culturing techniques for bacterial identification take **1-3 days**

- Patients are treated with broad-spectrum drugs that have given rise to the crisis of antibiotic resistant bacteria

- Rapid and accurate diagnosis of bacterial infection are required so that **more targeted treatment can begin as soon as possible**

Goal

- Develop LIBS as a **rapid point-of-care diagnostic tool** in a clinical setting

- This includes developing **quick bacterial preparation methods** prior to testing that utilizes equipment and methods that are **common or easy to implement in a clinical setting**

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

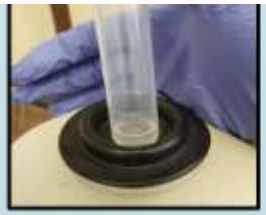
Pipette 100 µL of liquid contaminant containing bacteria suspension into a centrifuge tube.

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

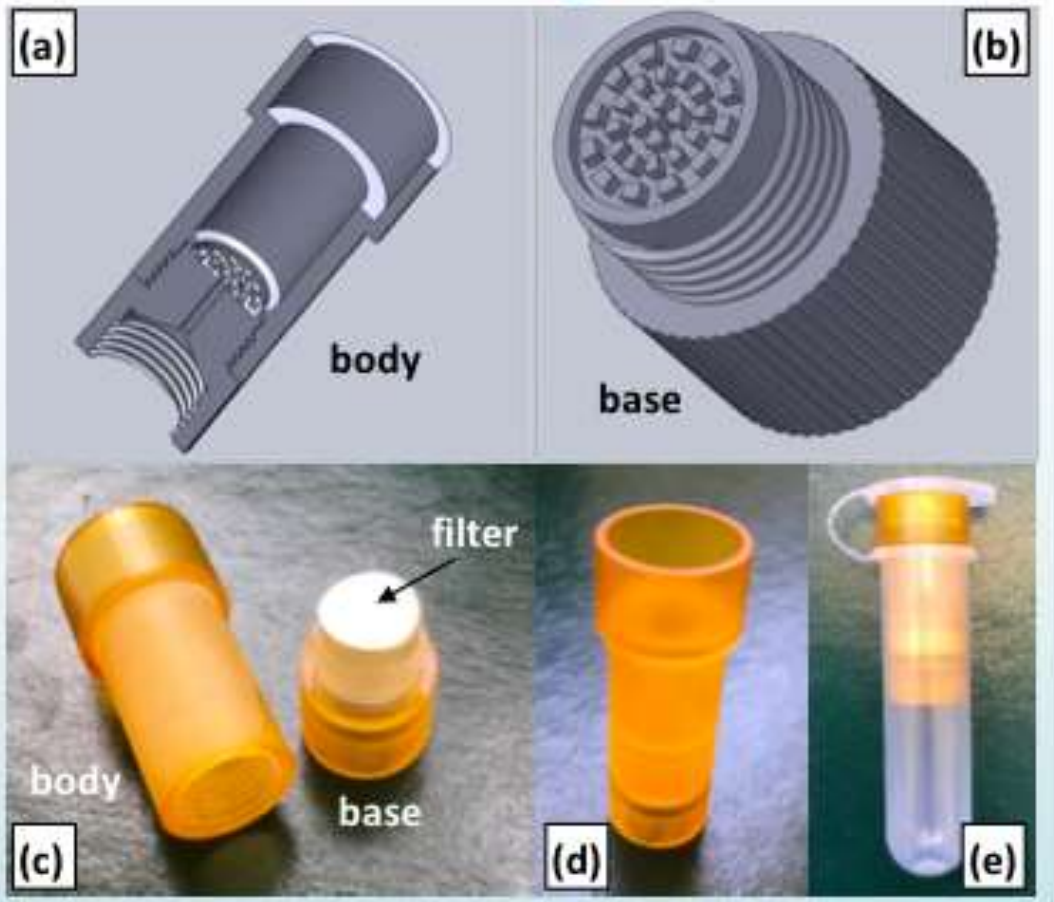
Mount a 5 µm filter and a 0.45 µm filter on two separate insert base pieces.

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

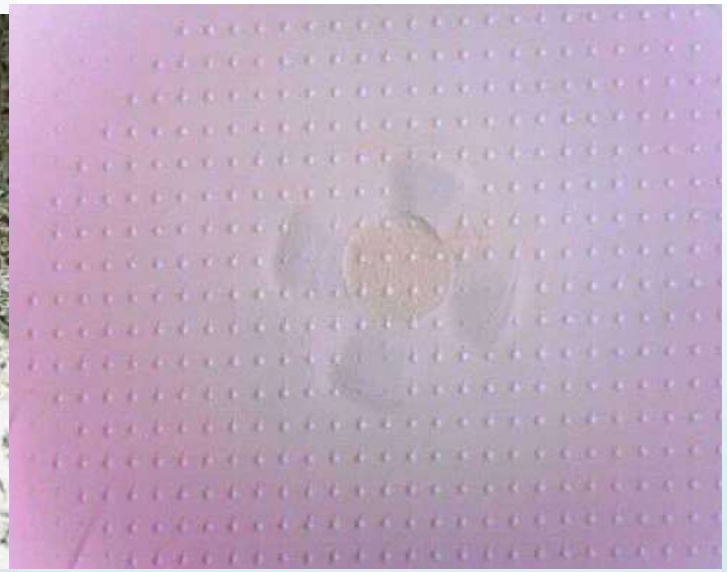
Sample Preparation

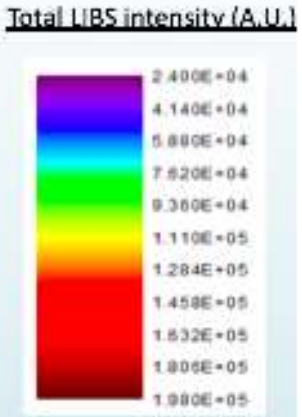


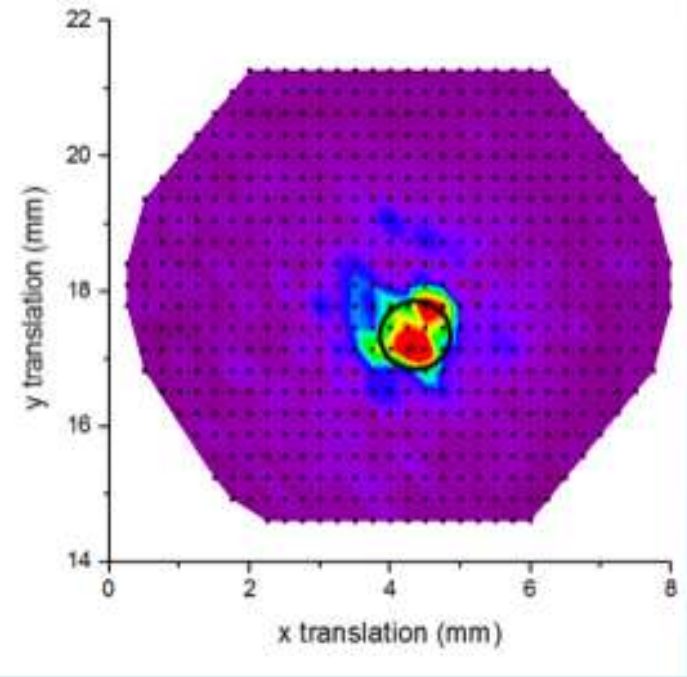
The Centrifuge Insert



Concentrating and Shooting Bacteria on Filters





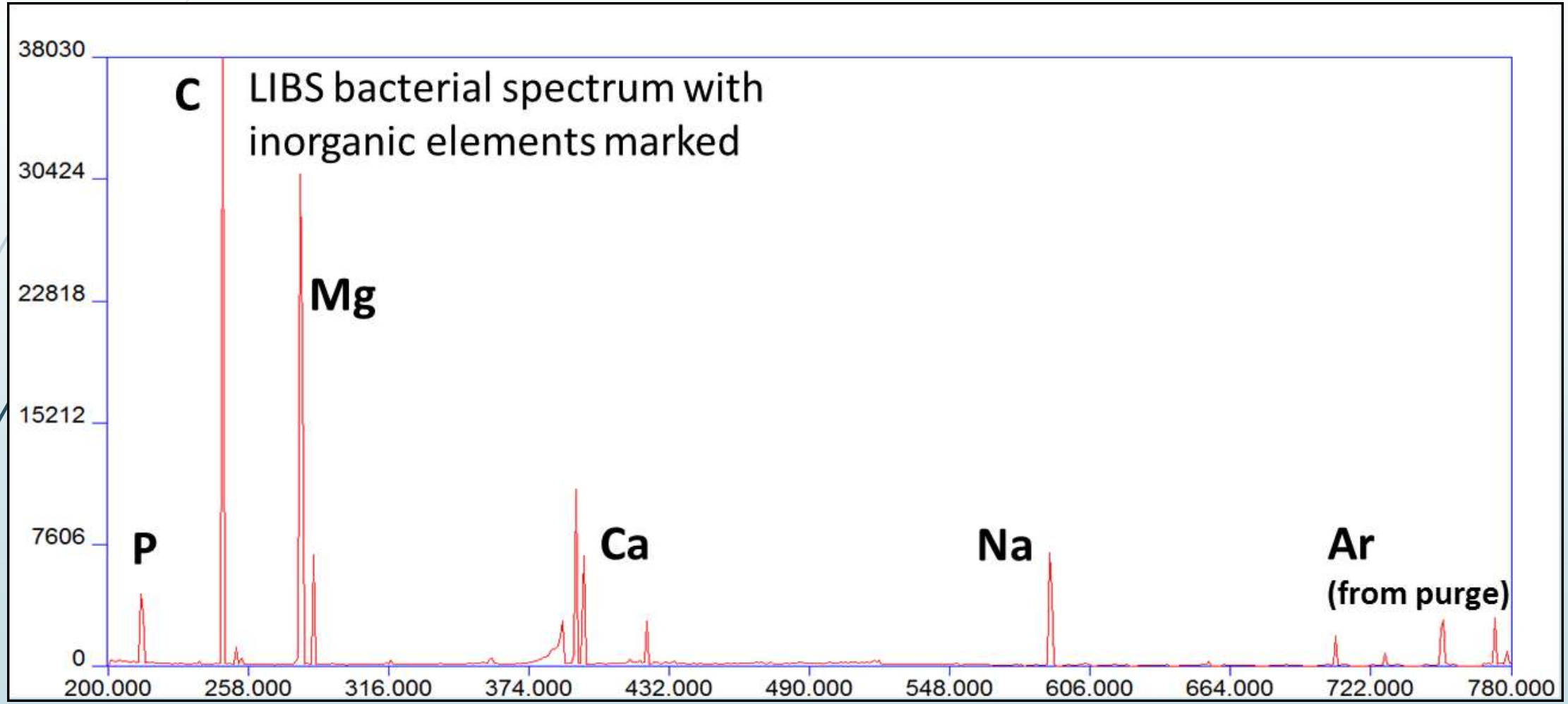


Deposit the 1 mL of sample from vortexed tube into insert, place insert into clean centrifuge tube. Centrifuge insert at 5000 rpm, 2500 g's for 5 min.

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.

Spectra


LIBS bacterial spectrum with inorganic elements marked
C
Mg
P
Ca
Na
Ar
(from purge)
38030
30424
22818
15212
7606
0
200.000
258.000
316.000
374.000
432.000
490.000
548.000
606.000
664.000
722.000
780.000

Focus of our Work

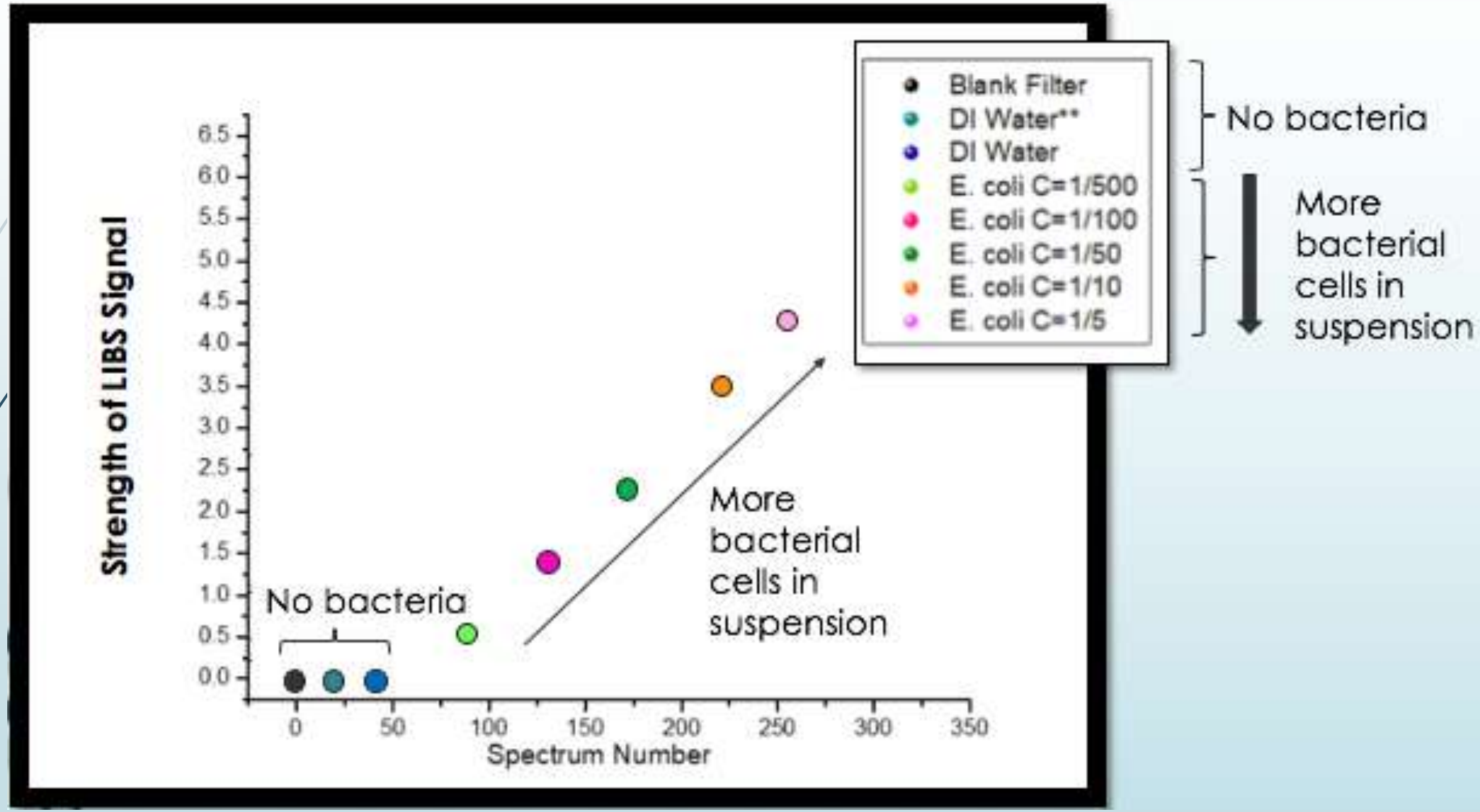
- The intensities of emission lines in the LIBS spectrum provide a **unique elemental spectral fingerprint** for each type of bacteria which can be classified using **chemometric algorithms**.

- Our goals:
  - accurately identify/classify as small a number of bacterial cells as possible (lower LOD)
  - maximize the rates of true positives while minimizing the rates of false positives.

- How can we **improve classification accuracy**?
  - reducing background signal in the spectrum
  - investigating **data pre-processing**, and the
  - **use of silver microparticles** to produce a more repeatable and robust spectrum.

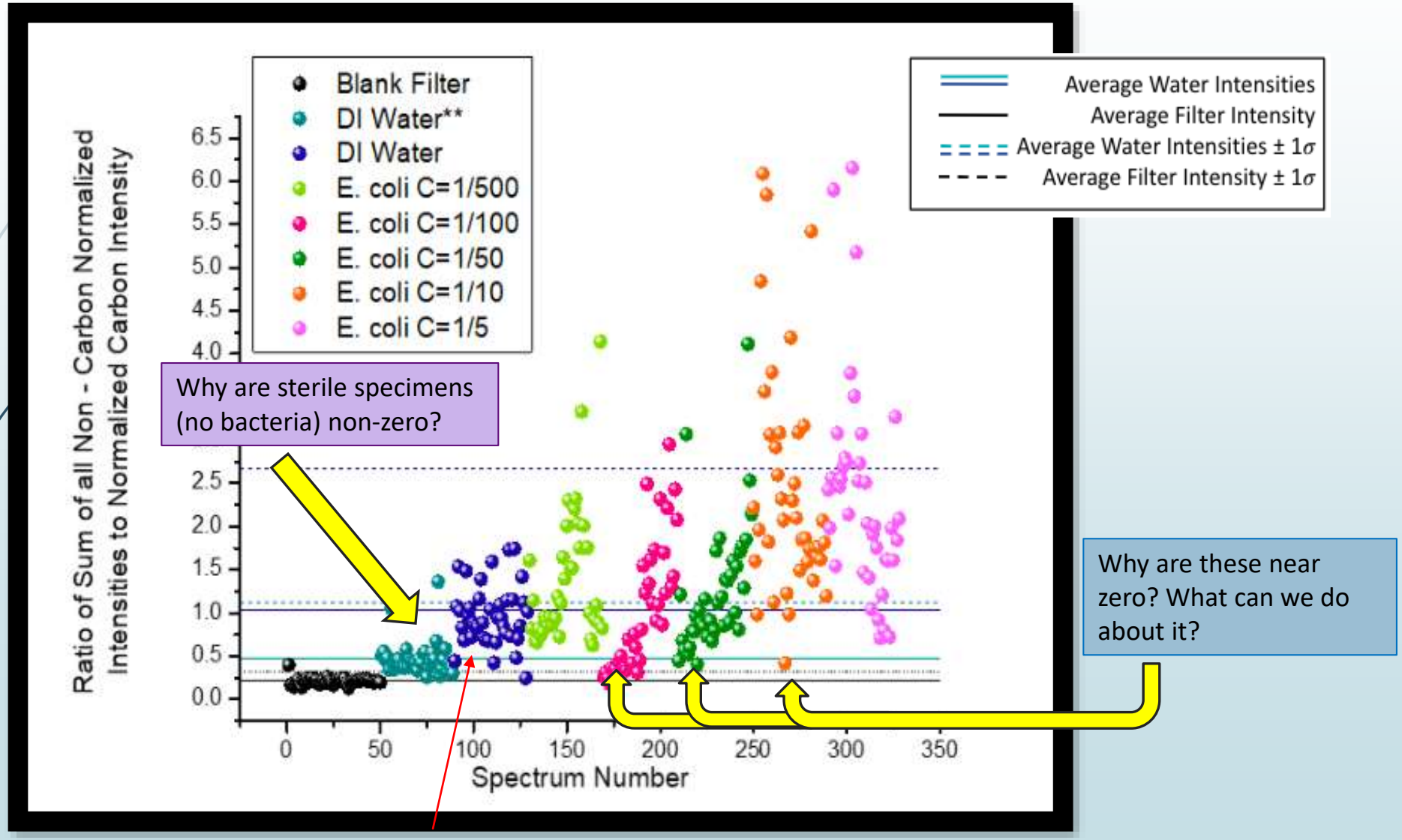
Our *Expected* Curve-of-Growth

A LIBS bacterial curve of growth **SHOULD** look something like this



Our "Confusing" Curve-of-Growth

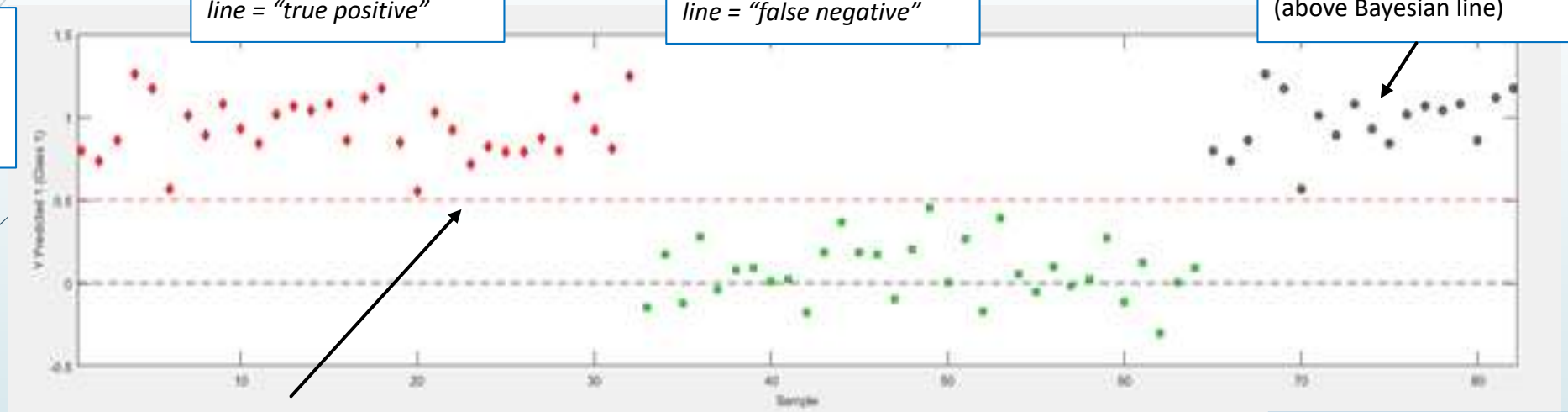
Our LIBS bacterial curve of growth (constructed with serial dilutions of *E. coli* and sterile water) looks like this…



"**Rigorous cleaning**" has eliminated these particular strong blue water data.

Chemometric Analysis

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

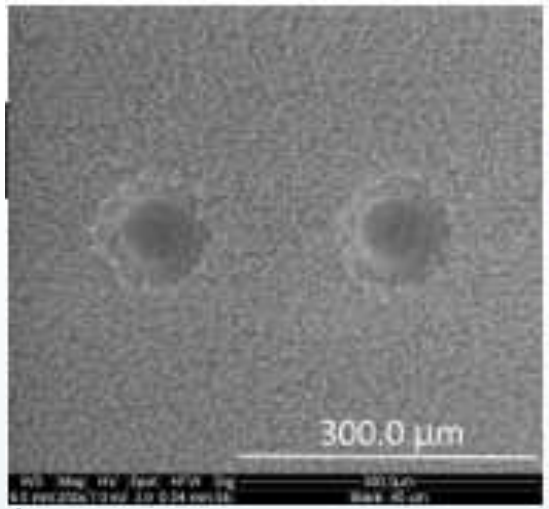


- 164 unique and independent variables are created from 18 emission lines.
- Classified by DFA or by PLSDA.
- Both techniques result in a **sensitivity** and a **specificity**
- Improve **accuracy** by training a library through objective data rejection techniques

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) Mycobacterium smegmatis (150) Enterobacter cloacae (113) Pseudomonas aeruginosa (100) Staphylococcus aureus (110)	653
Blank	DI Water (260) Nitrocellulose Filter (250)	510



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

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

Bacterial deposition visible (cracking / unevenness)

No cells outside area of bacterial deposition

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.

Library Preprocessing

- 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 ?

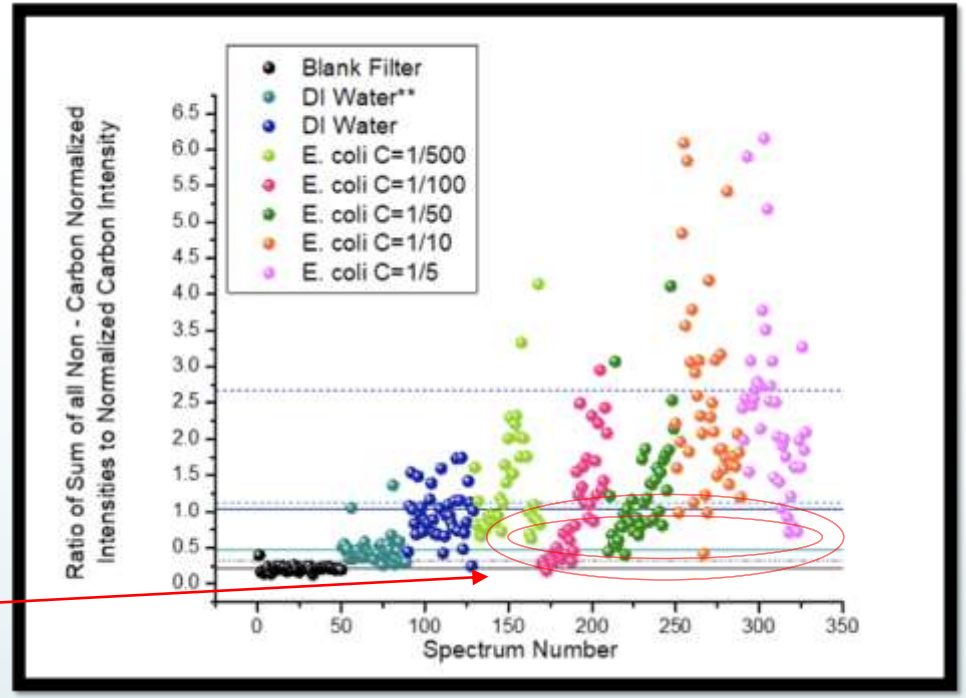
External Validation of DI Water	Average Sensitivity
Library 1	78.40%
Library 2	78.40%
Library 3	75.90%
External Validation of E. coli	**Average Sensitivity**
Library 1	72.50%
Library 2	88.80%
Library 3	88.80%

- 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

Two tests were investigated to identify outliers:

- **Method 1**: *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

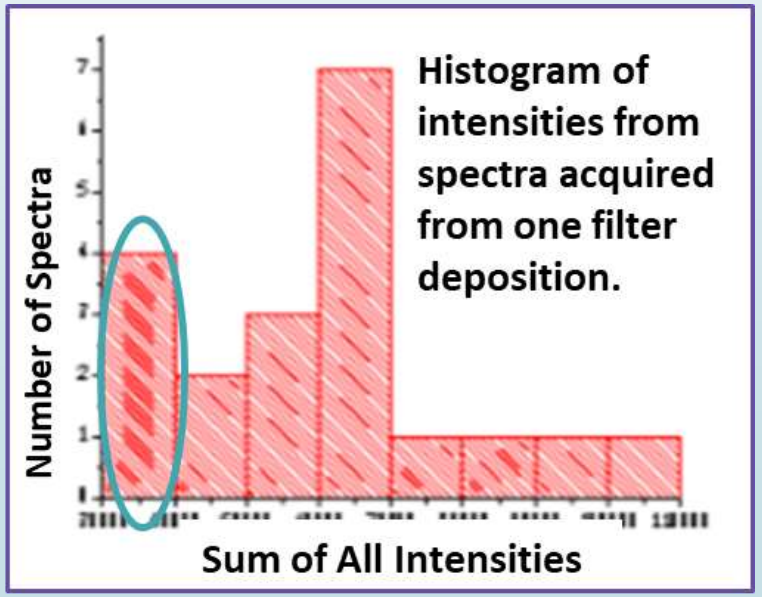
Outlier Rejection

- **Method 2**: *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

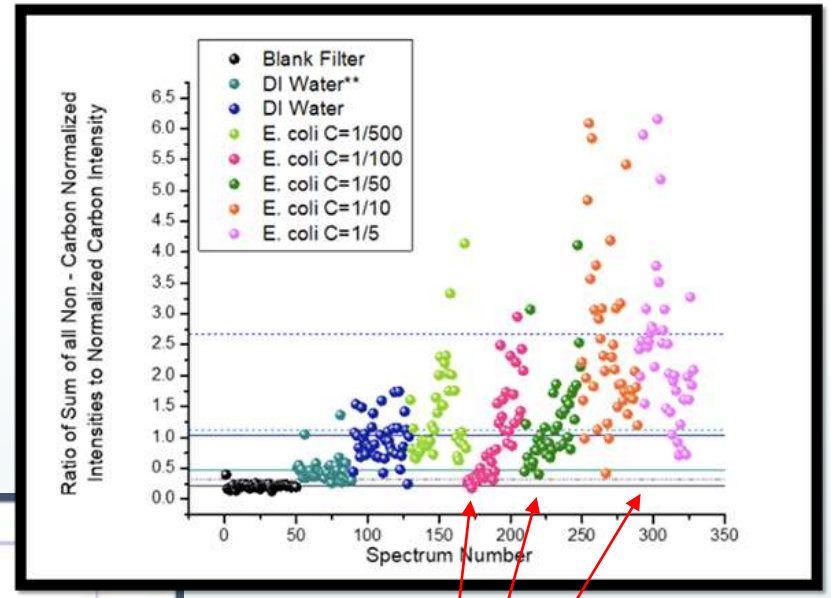
Histogram of intensities from spectra acquired from one filter deposition.

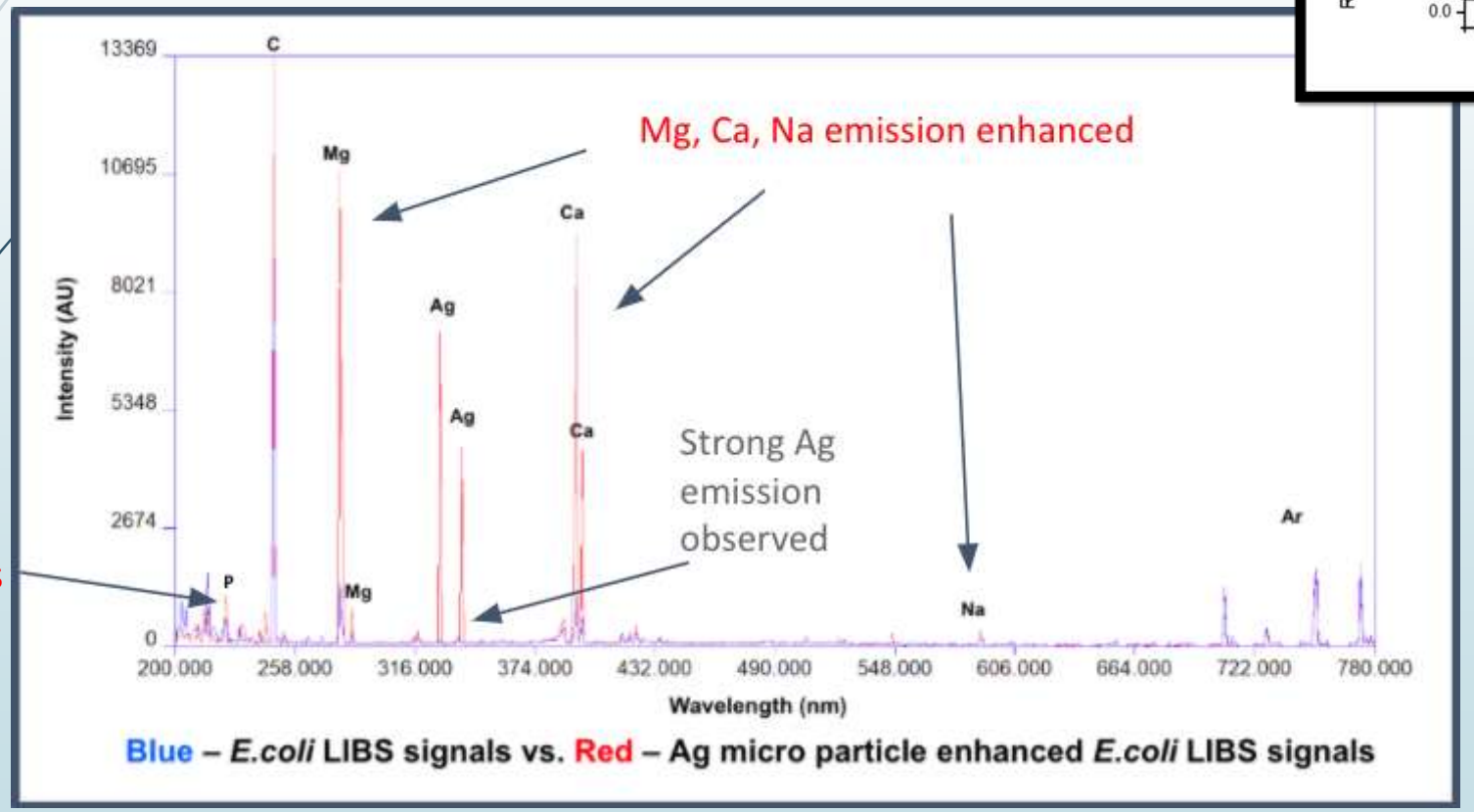


The column circled represents the 'empty shots' which clearly do not follow a normal distribution for bacterial spectra. In this case, 4 of 23 spectra were rejected.

Silver Microparticles

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





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

- *Note: this is not the same enhancement that was mentioned in the nanoparticle talk yesterday

Preliminary Data – Currently Under Investigation

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

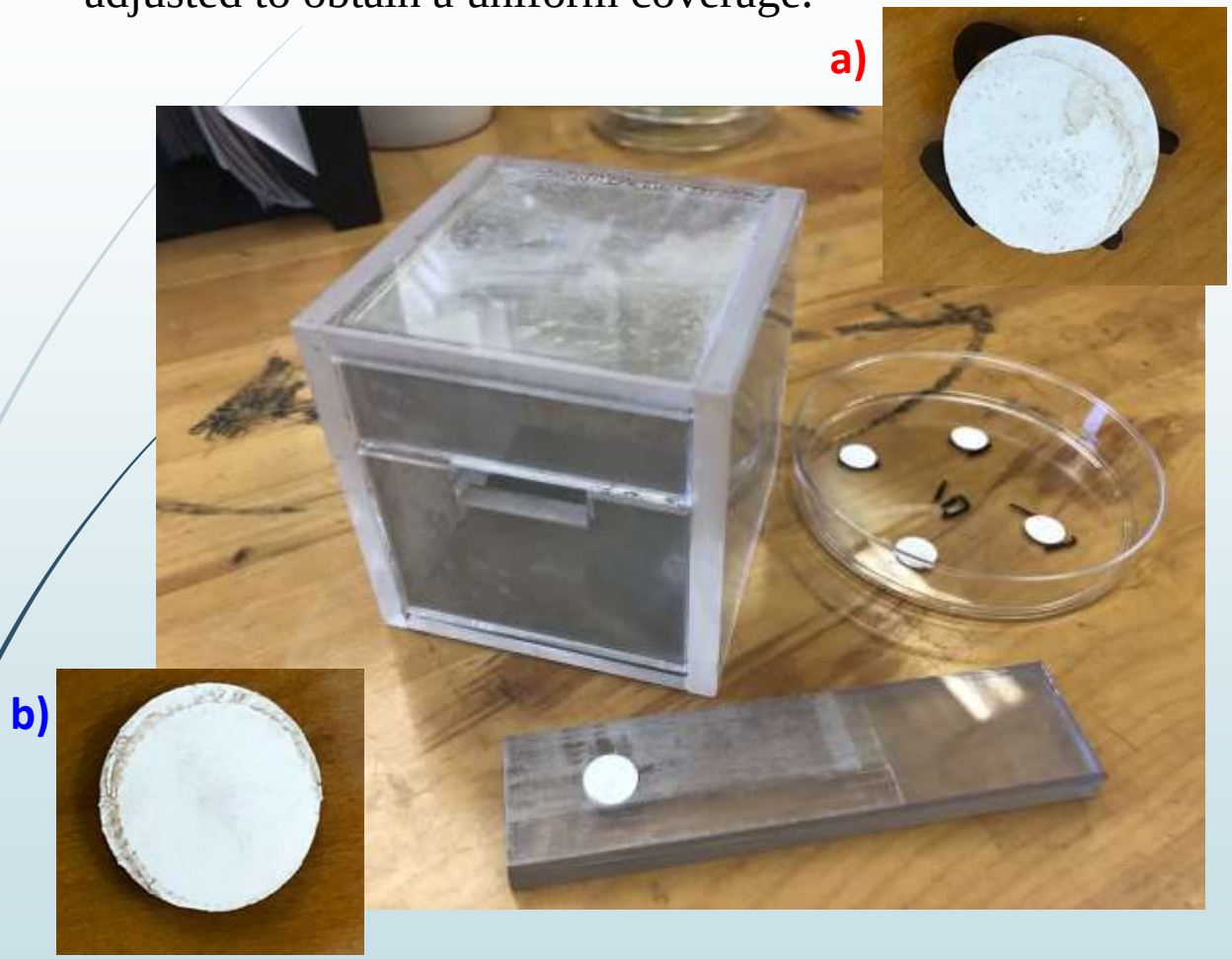
Not all elements are enhanced in the same way

https://www.reade.com/products/silver-powder-silver-metal

- Spectra appear to be stronger now

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

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.



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)

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. Test gold microparticles and nanoparticles.

The people who did the work…

NSERC Discovery Grant

Natural Sciences and Engineering Research Council of Canada

Conseil de recherches en sciences naturelles et en génie du Canada

CFI-LOF grant

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