Bio-LIBS and the Role of Trace Metals When Laser-Induced Breakdown Spectroscopy is Used to Study Biological or Biomedical Systems

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LIBS in geoscience: The applications of Laser-Induced Breakdown Spectroscopy in Earth and Planetary Sciences

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Quick Overview of LIBS at UWindsor

- Rapid bacterial identification and experiments on cellular composition (biomedical diagnostic)
- Measurement of zinc in fingernails to determine zinc nutriture (biomedical diagnostic)
- Salmon otolith composition (ecological application)
- Laser-induced fluorescence and branching ratio determinations in lanthanide and transition metal plasmas (atomic physics)
- Mining and mineral composition determination (joint project with Earth and Environmental Science)

Current Method of Bacterial identification

Our Method of Bacteria Classification

Bacteria is cultured using trypticase soy agar (TSA).

Colonies are removed and placed in 1.5 mL distilled water.

30 μL of vortexed sample are deposited on a standard 0.22 μm cellulose filter in contained wells.

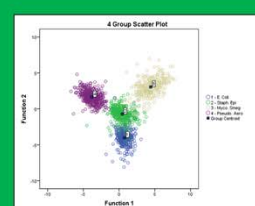
Colloidal solution is dried forming a bacteria lawn on the clinician-friendly filter.

Filter is placed in an argon environment and ablated using a pulsed 1064 nm Nd: YAG laser.

Average time to complete bacterial classification = 1 hour

Échelle diffraction grating spectrometer is used to obtain the atomic spectrum and composition of sample.

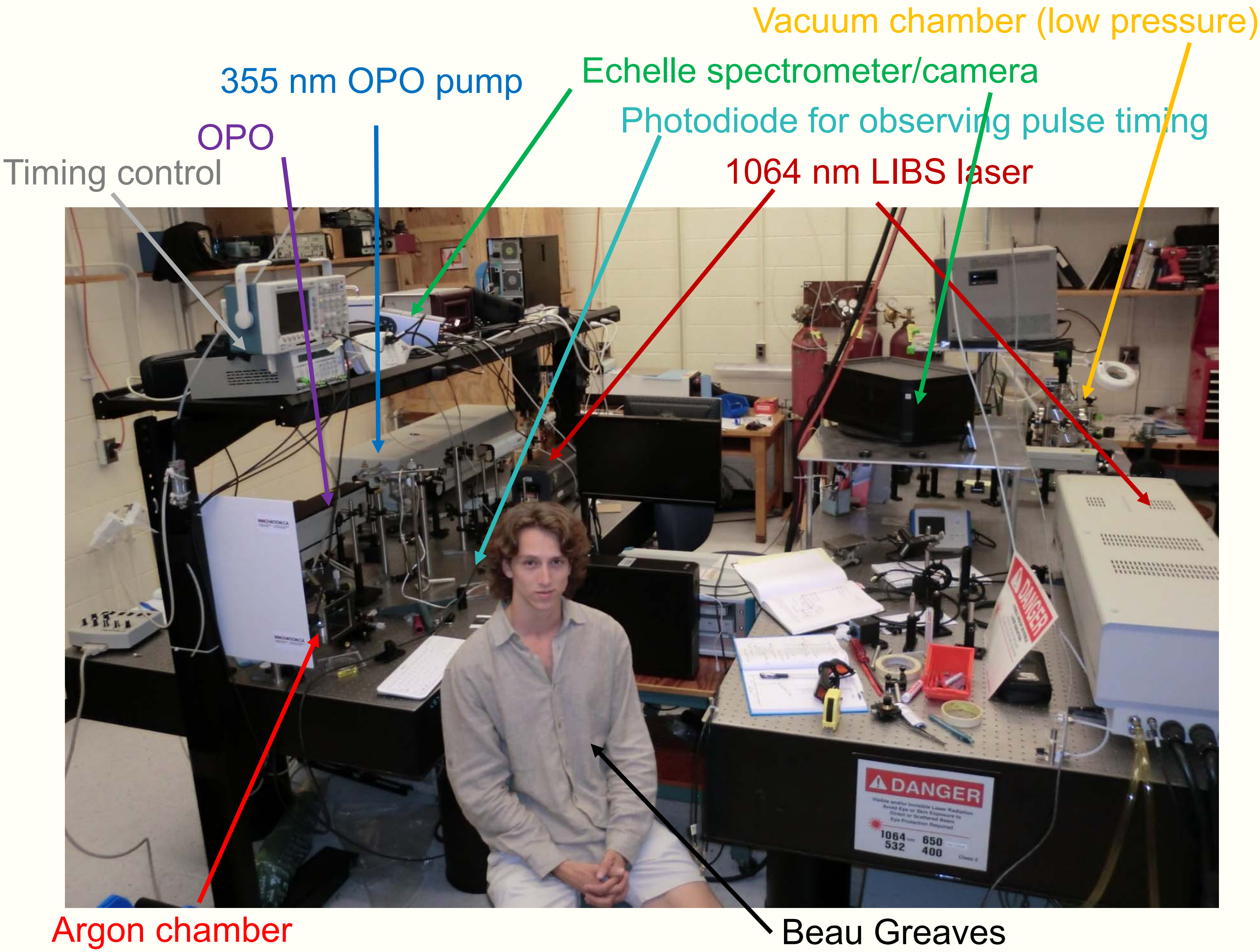
Atomic composition is used to discriminate bacteria against pre-existing library.



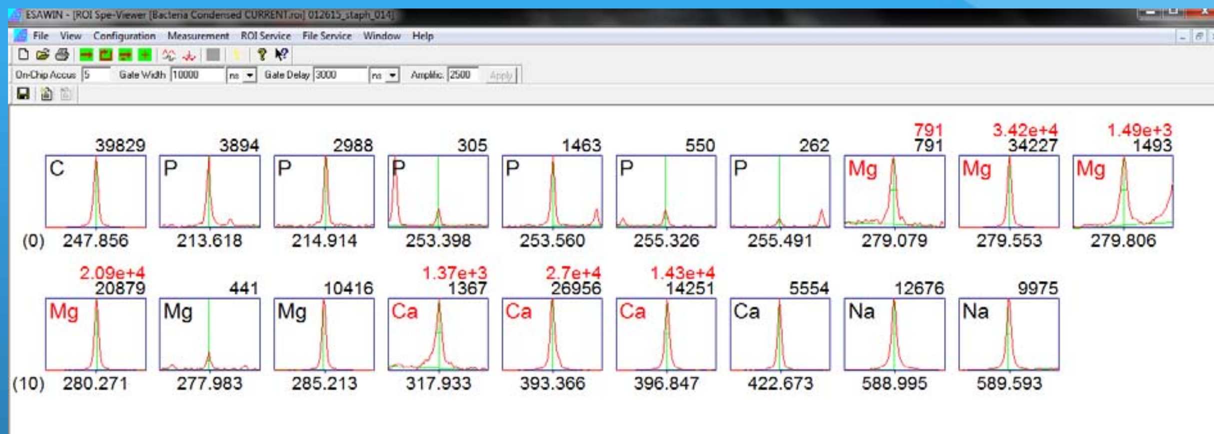
Element	% of fixed salt fraction
Sodium	2.6
Potassium	12.9
Calcium	9.1
Magnesium	5.9
Phosphorus	45.8
Sulfur	1.8
Iron	3.4

from "*The Bacteria: A Treatise on Structure and Function*"
I.C. Gunsalus and R.Y. Stanier, eds

LIBS at UWindsor



Variable Down Selection



- Classification model based on <u>inorganic</u> elements
- 164 independent variables
  - 19 line intensities (all divided by sum)
  - 145 ratios of intensities

A comparison of multivariate analysis techniques and variable selection strategies in a laser-induced breakdown spectroscopy bacterial classification, Russell A. Putnam, Qassem I. Mohaidat, Andrew Daabous, Steven J. Rehse, Spectrochimica Acta Part B **87** (2013) 161–167

Variable Down Selection

- No other metals. Beware?
  - Farooq (2014) sees S, Cl, Mn, Fe, Al, Cu, etc.
  - Sivakumar (2015) only sees Ca, Na, Mg, K, O, H, C, P
  - We see Ni, Fe, Ti only when contaminated!

Manzoor et al., in air,
Talanta 121 (2014)

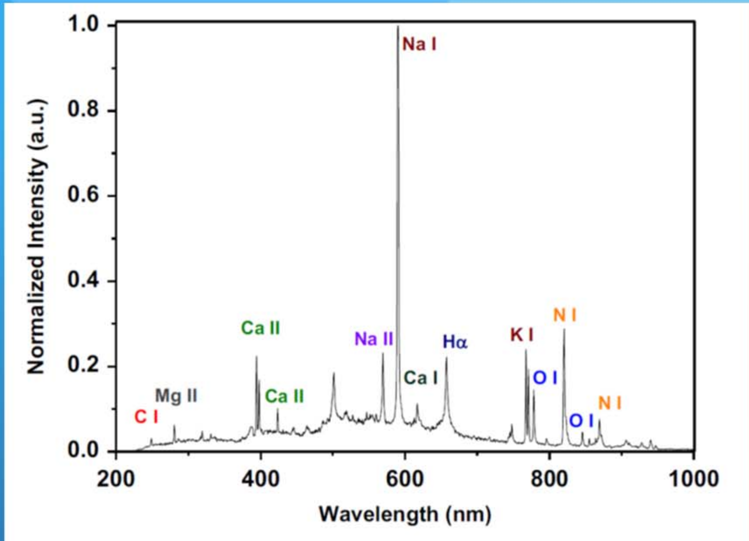


Fig. 1. Normalized LIBS spectrum of *Escherichia coli*.

Farooq et al.,
Plasma Science and Technology 16 (2014)

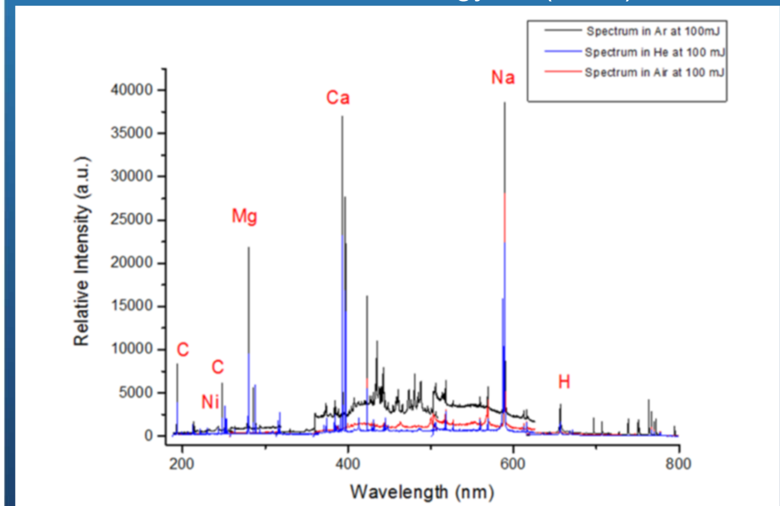
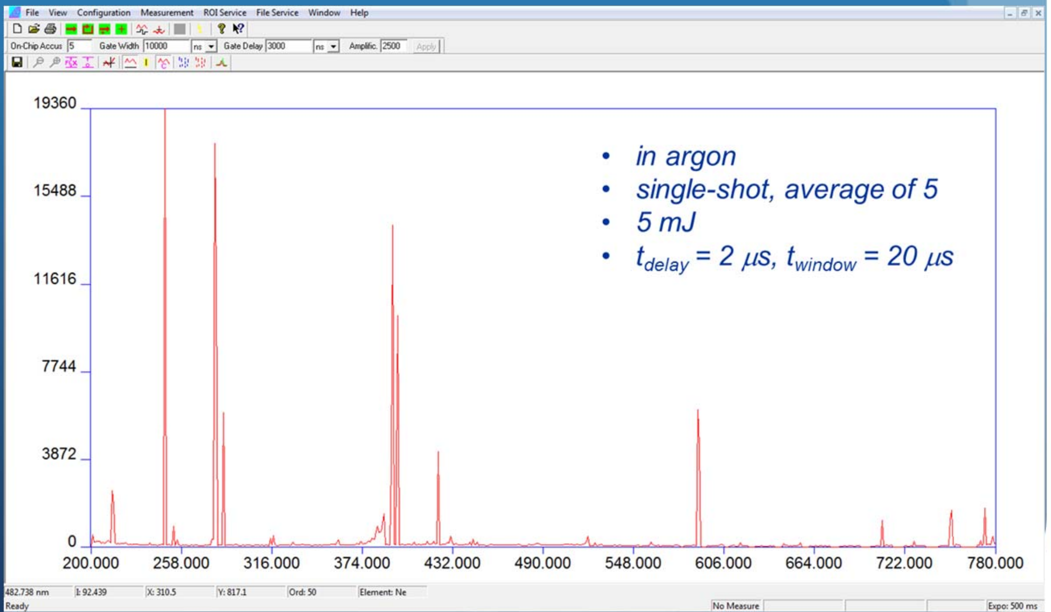
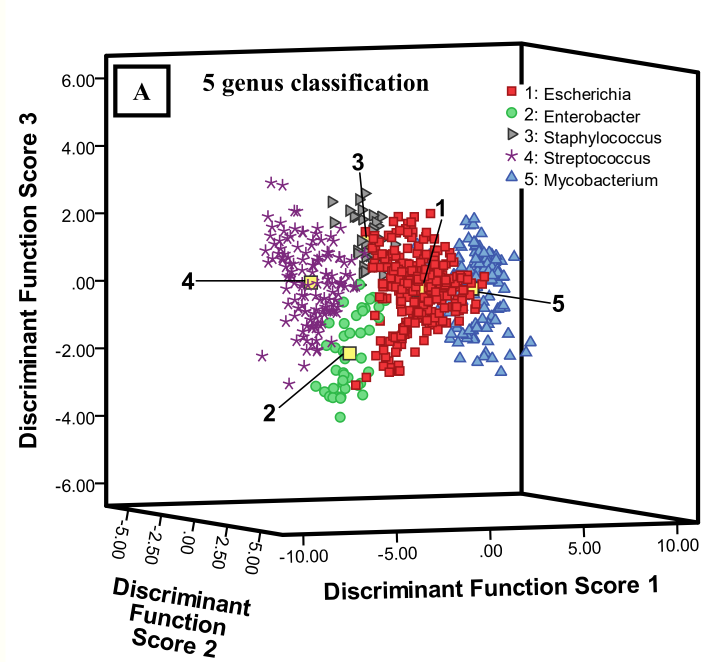


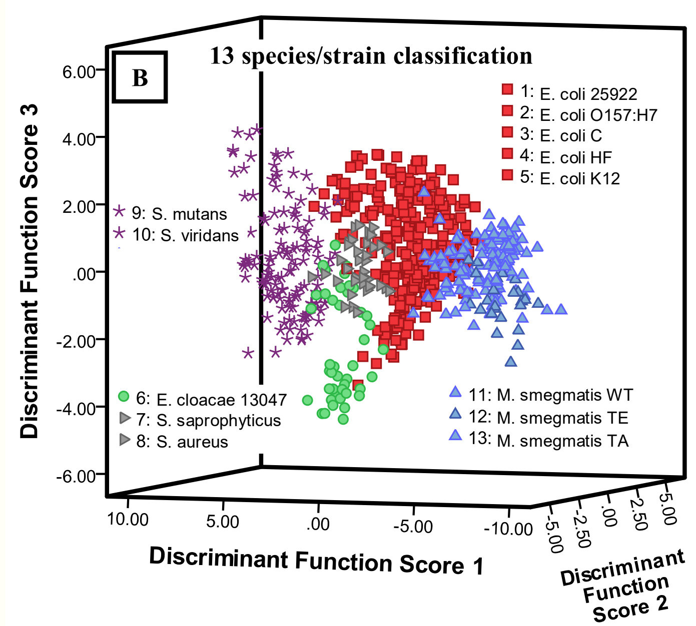
Fig.4 LIBS spectra of bacteria in air, Ar and He atmosphere



How unique is "unique"?

- ✓ We can identify a bacterial species, certainly its genus, with high sensitivity and specificity (confirmed by others).
- ✓ We can differentiate strains of *E. coli* (demonstrated by others in MRSA).
- ✓ Multiple multivariate techniques effective at discriminating spectra.





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 %**

Results: We have already demonstrated…

- LIBS spectral fingerprint is a *sensitive* and *specific* (high rates of true positives, low rates of false positives) test to identify an unknown bacterial specimen or to differentiate between possible identifications

- This spectral fingerprint is *robust* and *reliable*, and exists through time (multiple tests spanning years on same strains of bacteria)

- In addition…

10 publications in Applied Physics Letters, Journal of Applied Physics, Applied Optics, Applied Spectroscopy, Spectrochimica Acta B, and others – confirmed by multiple other groups

Results: We have already demonstrated…

LIBS spectral fingerprint is:

- growth-medium independent
- independent of state of growth (how “old” the bacteria are)
- independent of whether the bacteria are live or dead (or 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

10 publications in Applied Physics Letters, Journal of Applied Physics, Applied Optics, Applied Spectroscopy, Spectrochimica Acta B, and others – confirmed by multiple other groups

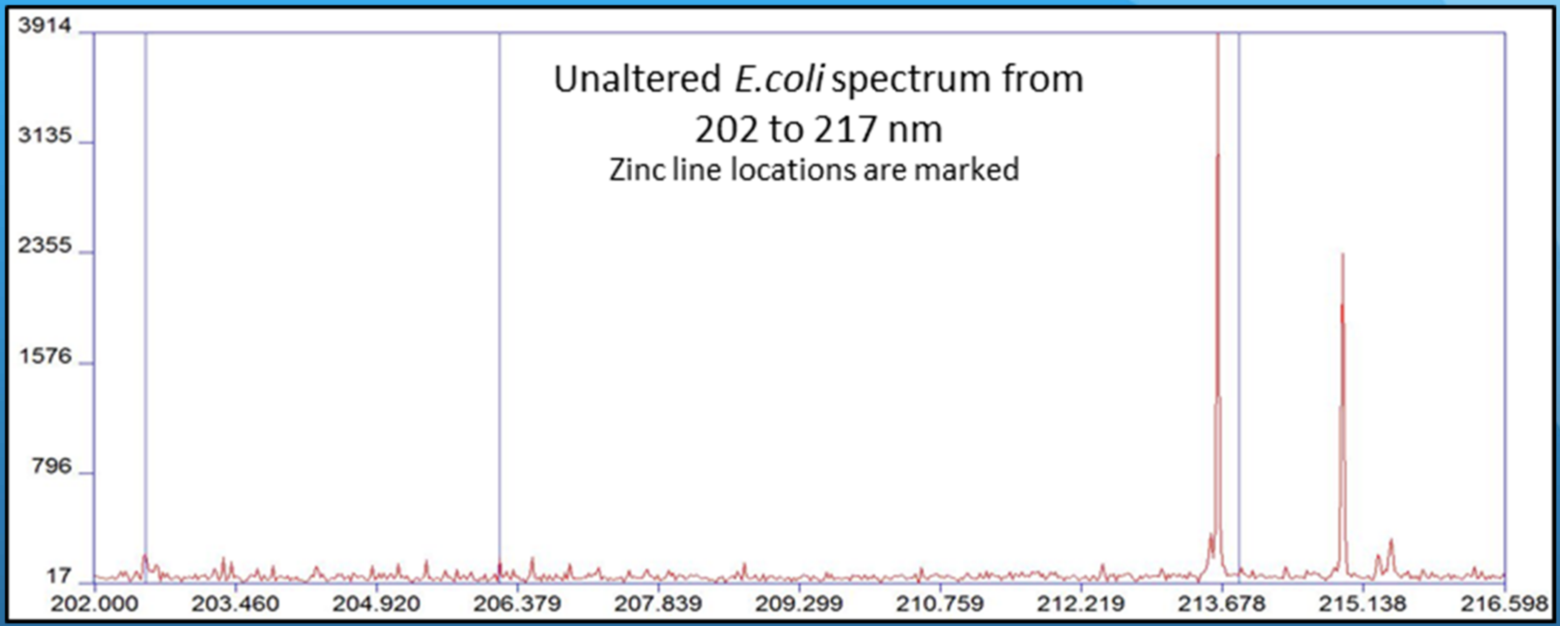
Challenges

1. Can we determine what the presence of other trace metals in the local environment will do to the LIBS spectrum?

2. Can we simply separate bacteria in a liquid suspension from larger cells for “clean” LIBS testing?

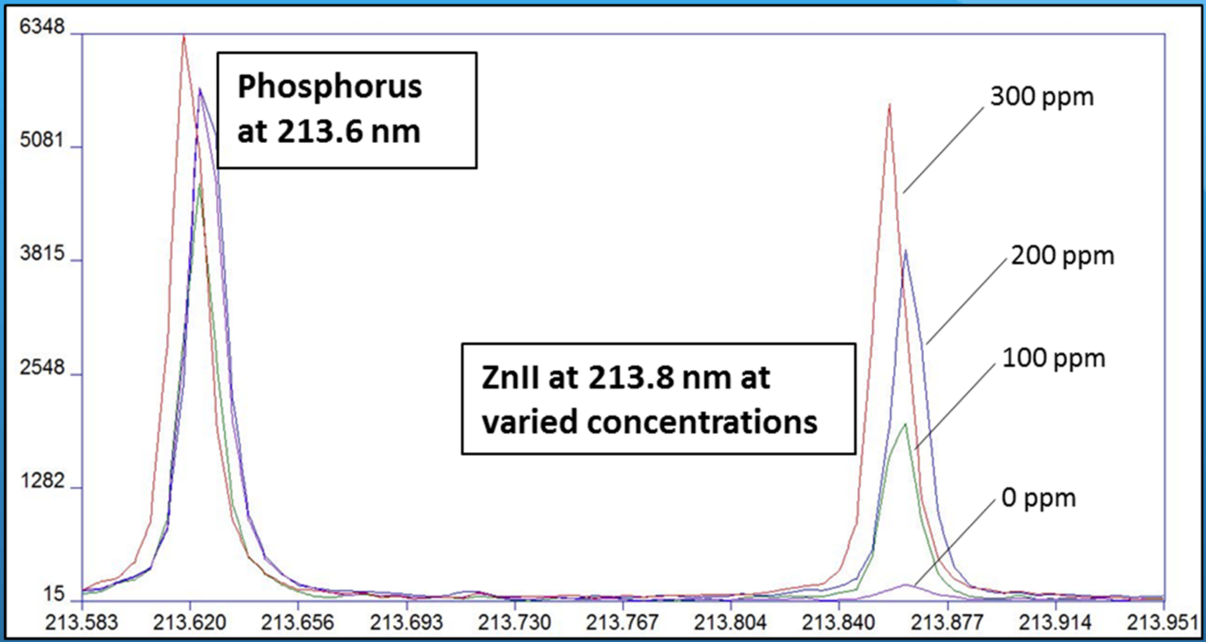
3. Can we lower our bacterial LOD to clinically realistic titers?

Altering Cell Metal Content: Zinc



Zinc lines are not distinguishable from noise at normal growth conditions using our testing protocol.

Altering Cell Metal Content: Zinc



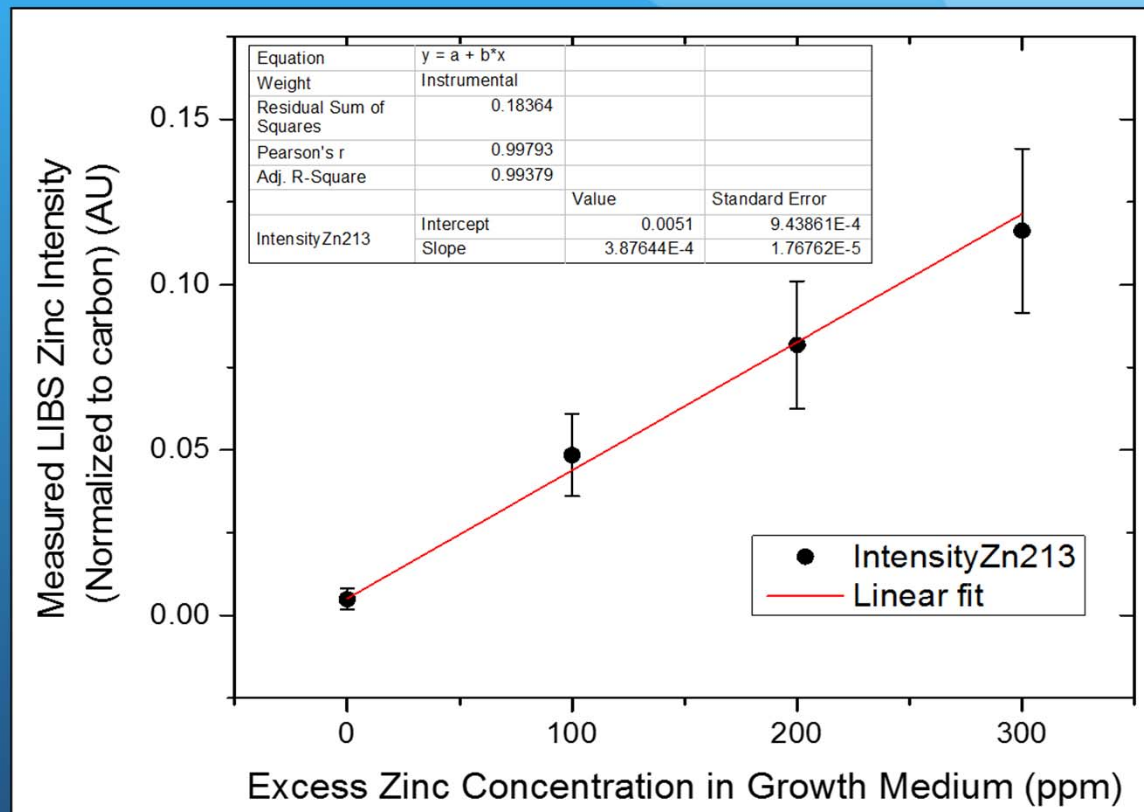
When zinc is added to the *E. coli* growth medium (TSA medium plates), cellular zinc is observed

Altering Cell Metal Content: Zinc

A linear fit of zinc line intensity to the excess zinc concentration gives an adjusted r^2 of 0.994.

The limit of detection (LOD) as calculated from this fit is 11 ppm.

The maximum concentration allowable for drinking water is 5 ppm.



Environmental Application

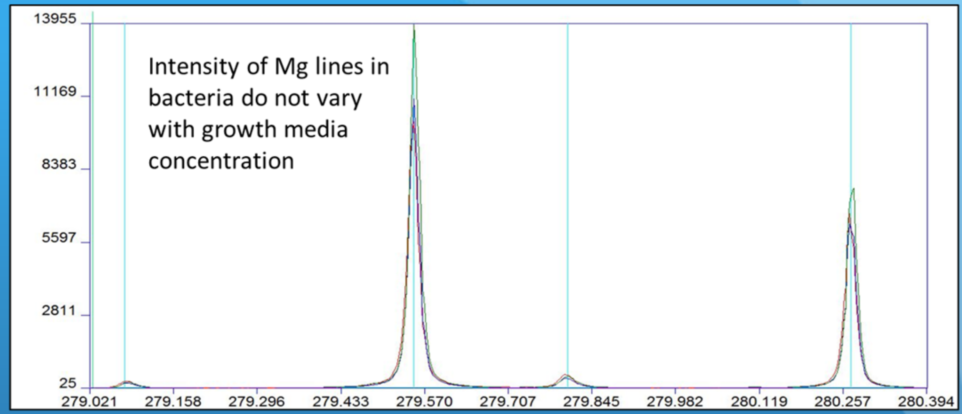
Since bacterial species take their nutrients from their environment, bacteria have been used as an indicator of environmental health, with trace metals in the cells being indicative of contamination of a water supply.

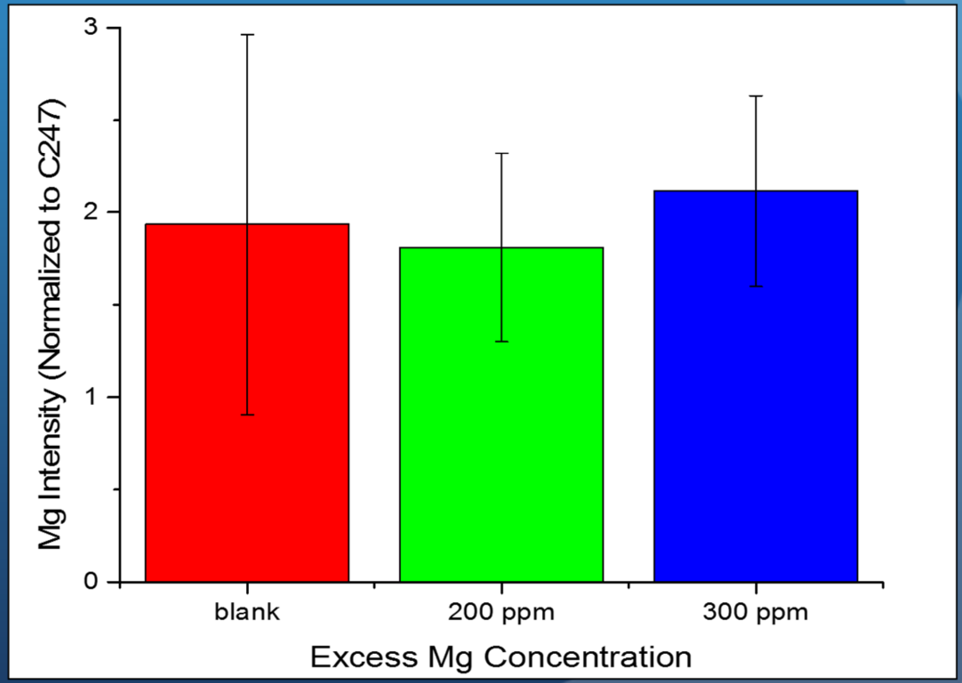
Altering Cell Metal Content: Magnesium

As excess Mg was added to the growth medium, the intensity of the Mg emission lines was largely unchanged.

The deviation in intensity reduced as the surplus increased.

A sample was prepared wherein Mg was precipitated out of the agar solution using HCl prior to autoclaving. This plate provided no bacterial growth.





What's Next

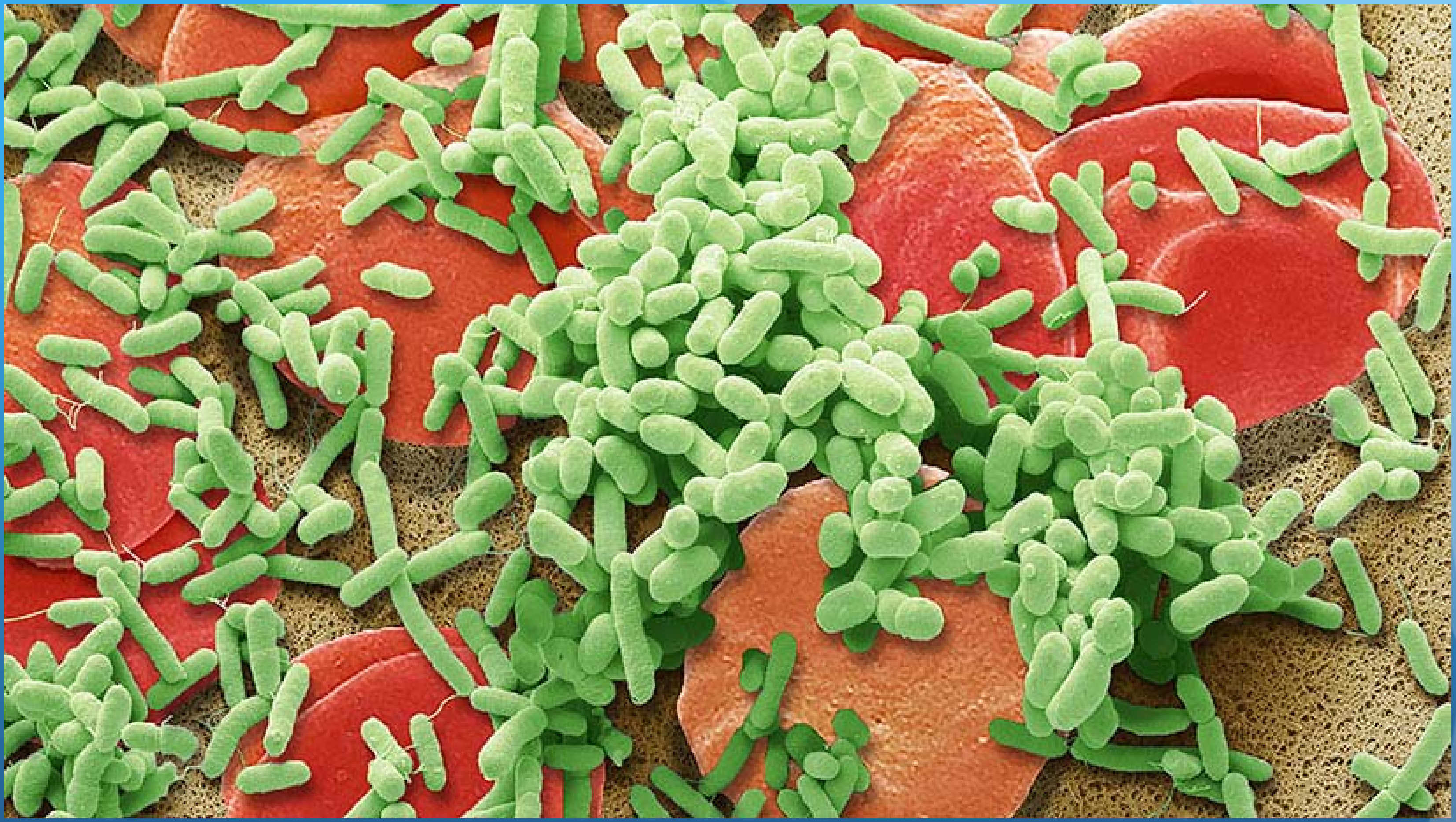
1. Attempt this with other organisms (although coliform bacteria is a standard) and heavy metals in water.

Challenges

1. Can we determine what the presence of other trace metals in the local environment will do to the LIBS spectrum?

2. Can we simply separate bacteria in a liquid suspension from larger cells?

3. Can we lower our bacterial LOD to clinically realistic titers?


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

Adapted from "*Blood ties: The inspiration behind a potential sepsis breakthrough*," Vanessa Hrvatin, Maclean's, June 6, 2017

SciX poster: **Bacterial Mounting and Concentration Techniques to Translate Laser-Induced Breakdown Spectroscopy into a Clinical Setting**; Alexandra Paulick

Dual stage centrifugation insert prototype

5 μm filter

0.45 μm filter

An *E. coli* suspension with tungsten powder (12 μm APS) as the contaminant was deposited in the insert with the 5 μm filter paper on top and the 0.45 μm filter paper below it.

The tungsten powder was caught by the 5 μm filter while ~ 80% of the bacteria passed through it and settled onto the 0.45 μm filter.

SciX poster: **Bacterial Mounting and Concentration Techniques to Translate Laser-Induced Breakdown Spectroscopy into a Clinical Setting**; Alexandra Paulick

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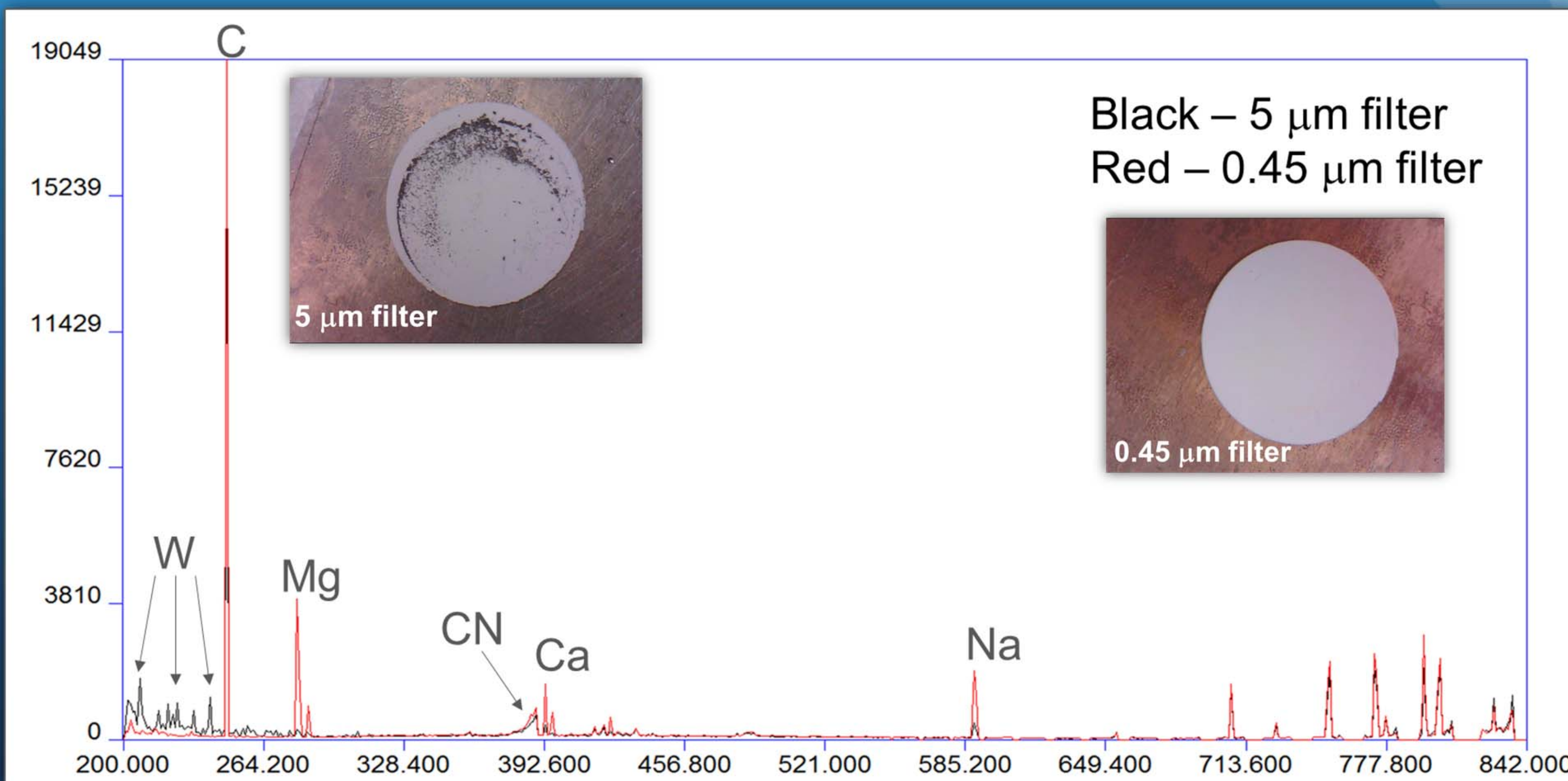


What's Next

1. Replace the inorganic metal powder with biologicals (probably yeast cells).

2. Eventually test blood/cerebral spinal fluid.

Challenges

1. Can we determine what the presence of other trace metals in the local environment will do to the LIBS spectrum?

2. Can we simply separate bacteria in a liquid suspension from larger cells?

3. Can we lower our bacterial LOD to clinically realistic titers?

Bacterial LOD

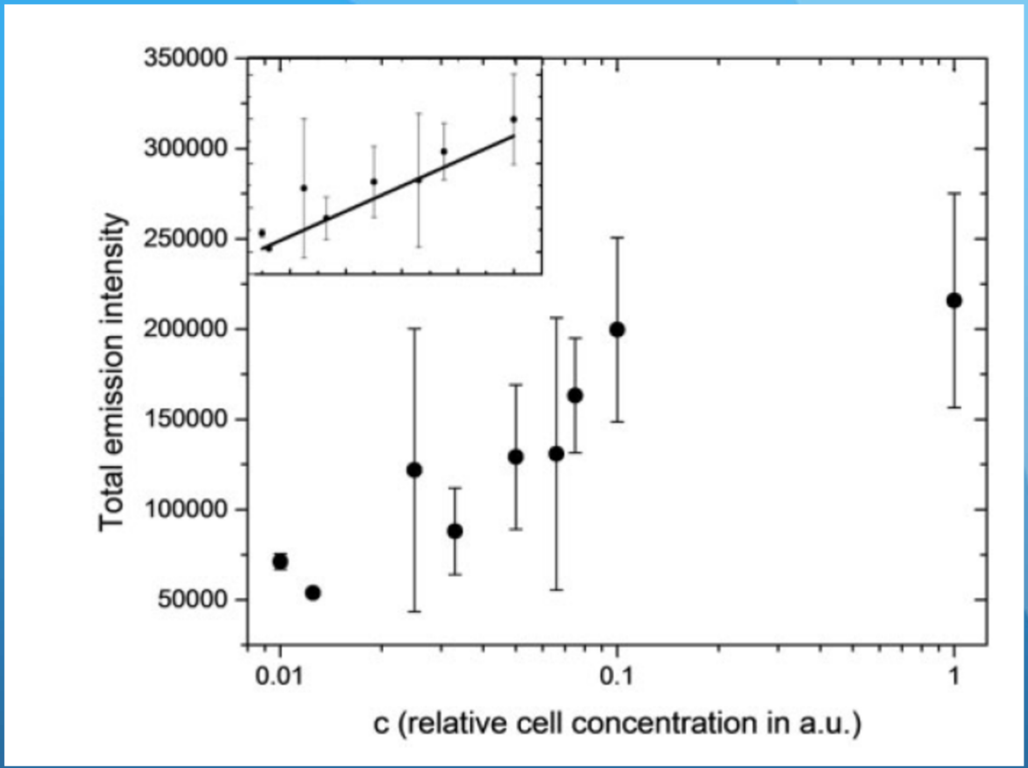


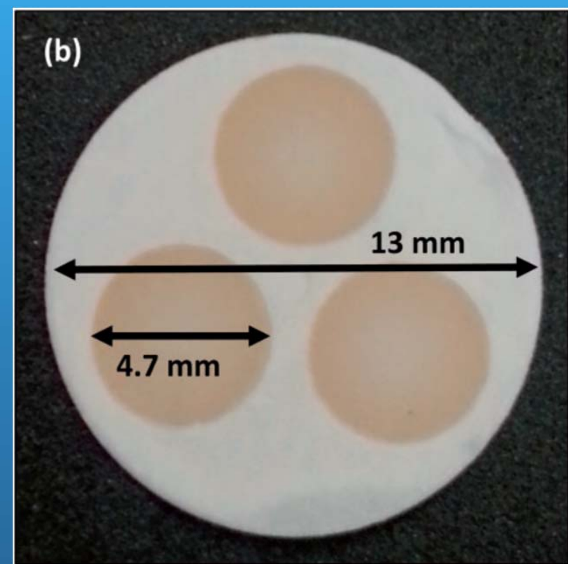
Figure 6. A log-lin calibration curve for bacterial samples. A concentration of $c = 1$ corresponded to 10^{11} cells/mL as determined by optical densitometry resulting in approximately 10^6 cells per ablation. This was the concentration achieved by transferring 24 h of growth for *E. coli* from a TSA plate to 1.5 mL distilled water. The inset shows a linear fit to the lowest eight concentrations on a lin-lin plot.

Bacterial Suspensions Deposited on Microbiological Filter Material for Rapid Laser-Induced Breakdown Spectroscopy Identification, Dylan J. Malenfant, Derek J. Gillies, and Steven J. Rehse, Applied Spectroscopy 2016, Vol. 70(3) 485–493

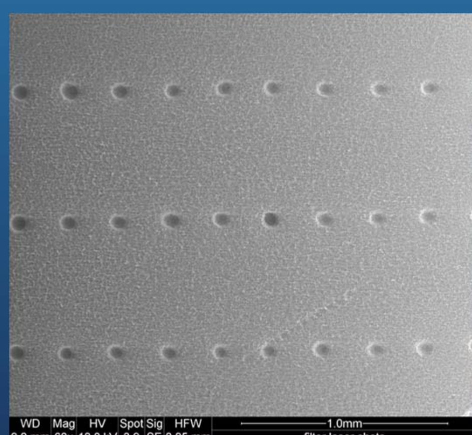
SciX poster: **Bacterial Mounting and Concentration Techniques to Translate Laser-Induced Breakdown Spectroscopy into a Clinical Setting**; Alexandra Paulick

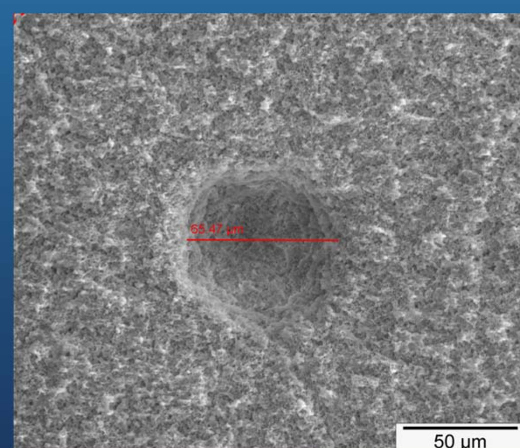
30 μL per spot

$>10^9$ cfu/ml



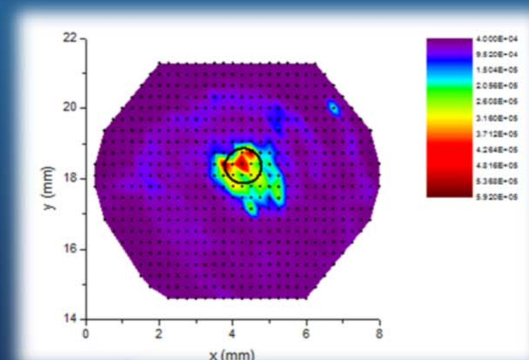
This is a LOT of bacteria!



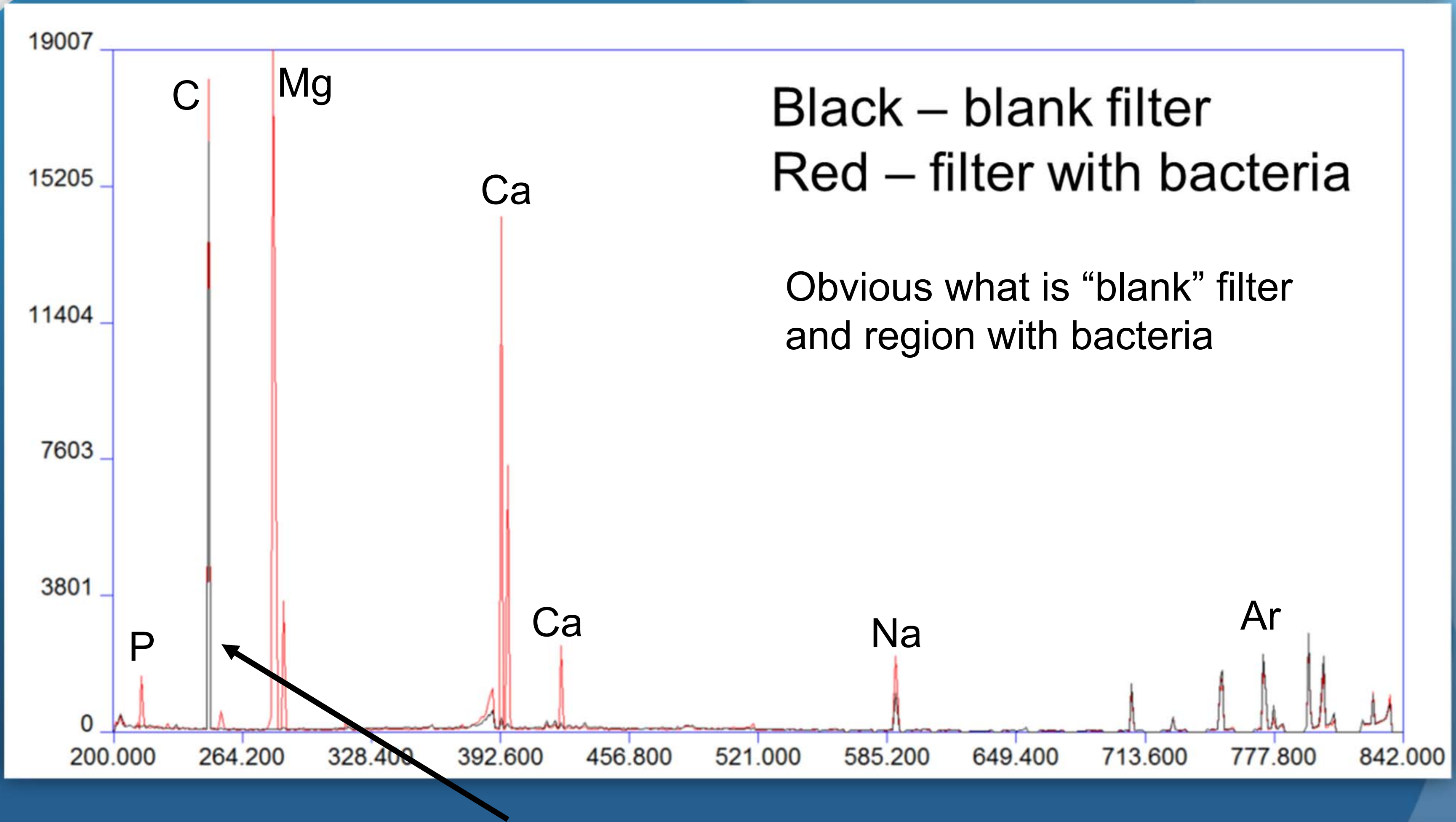


SciX poster: **Bacterial Mounting and Concentration Techniques to Translate Laser-Induced Breakdown Spectroscopy into a Clinical Setting**; Alexandra Paulick

To concentrate all the bacteria into one spot (one laser shot) a custom funnel was constructed for our centrifuge insert



Each point on the map corresponds to a single laser shot, and the color indicates the LIBS bacterial intensity, with purple indicating no LIBS bacterial signal, and red indicating the region with the strongest LIBS bacterial signal.



One of our biggest challenges: the C247 line dominates the spectrum at low / no concentration.

This large constant intensity limits the amplification we can use on the ICCD before damage.

Suggestions?

What's Next

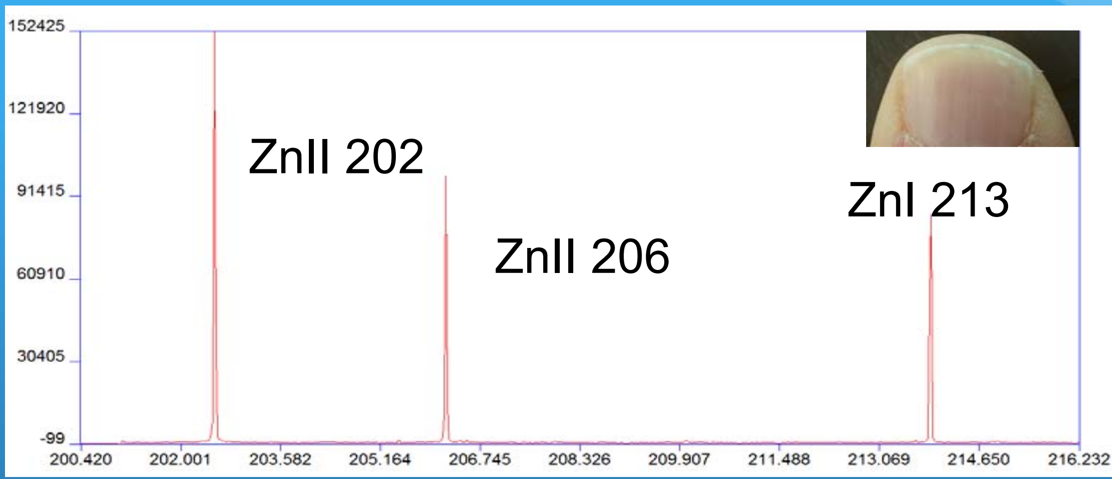
At low concentrations, bacteria do not deposit uniformly, they clump together.

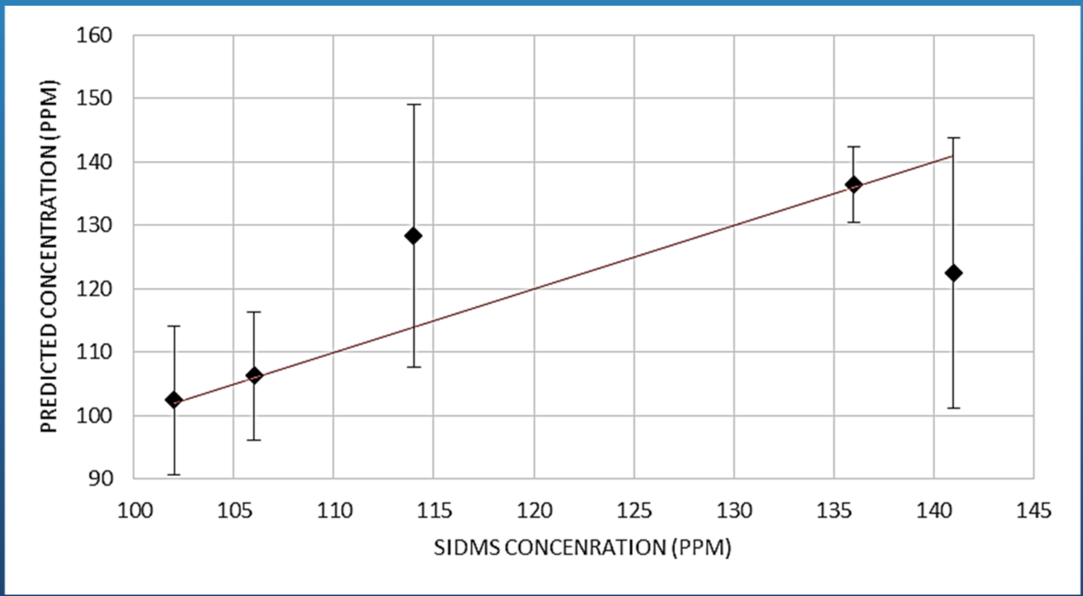
1. Try different types of bacteria (cocci versus rods).

2. Culture bacteria in liquid media. Keep lowering titer.

3. Further study use of Tween 20 to prevent sticky clumping of cells which should improve repeatability and increase transfer efficiency.

A real time assay for nutritional zinc deficiency





We have shown that LIBS fingernail zinc is related to dietary zinc.

LIBS spectra were acquired from clipped, buffed, cleaned nails.

PLS-DA was used to create a calibration curve versus the MS-measured zinc concentration.

Determination of the Zinc Concentration in Human Fingernails Using Laser-Induced Breakdown Spectroscopy, Vlora A. Riberdy, Christopher J. Frederickson, and Steven J. Rehse, Applied Spectroscopy 2017, Vol. 71(4) 567–582

Conclusions

- LIBS provides an accurate, fast, spatially resolved, remote spectrochemical analysis of almost any type of target (solid, liquid, gas, powder)
- It is particularly good for detecting/quantifying metals in biospecimens (due to their composition)
- Bacterial cells possess unique ratios of metals that allow their sensitive and specific identification
- Metals in human tissue can be quickly assayed

Funding and Acknowledgements

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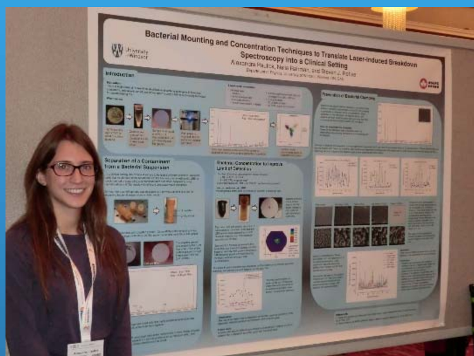
- A *Natural Sciences and Engineering Research Council of Canada* Discovery grant and a Research Tools and Instruments grant
- A *Canada Foundation for Innovation* Leaders Opportunity Fund grant
- An *Ontario Research Fund* Small Infrastructure Funds grant
- *University of Windsor* Outstanding Scholars program
- *University of Windsor* Faculty of Science

All Credit to the Students!

Allie Paulick



Anthony Piazza

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Vlora Riberdy

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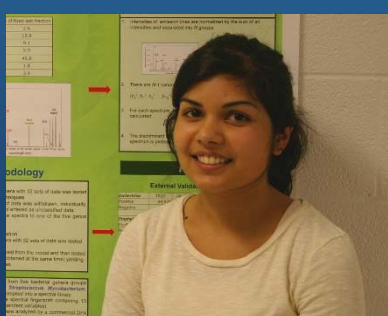

Russell Putnam

Paul Dubovan

Chris Heath

Beau Greaves

Erica Rustico

Courtney Jones

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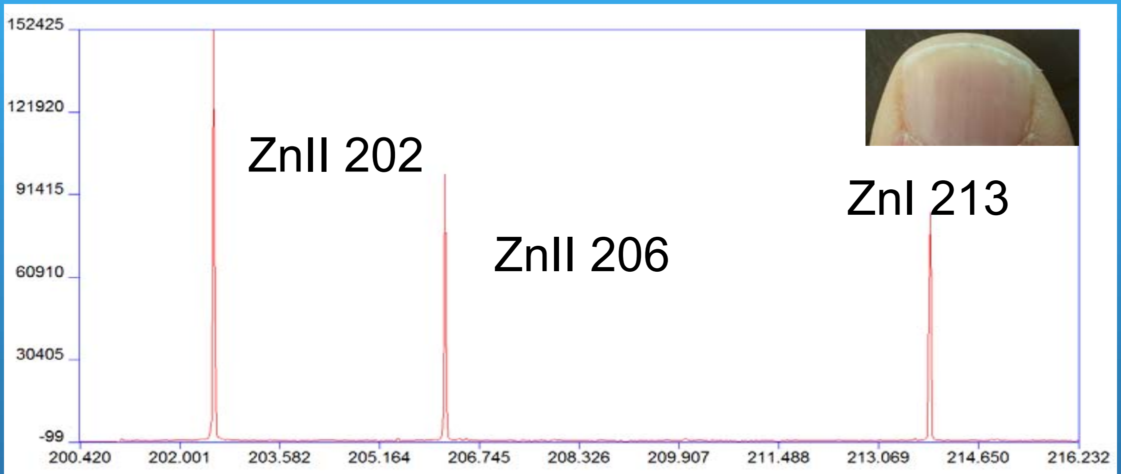

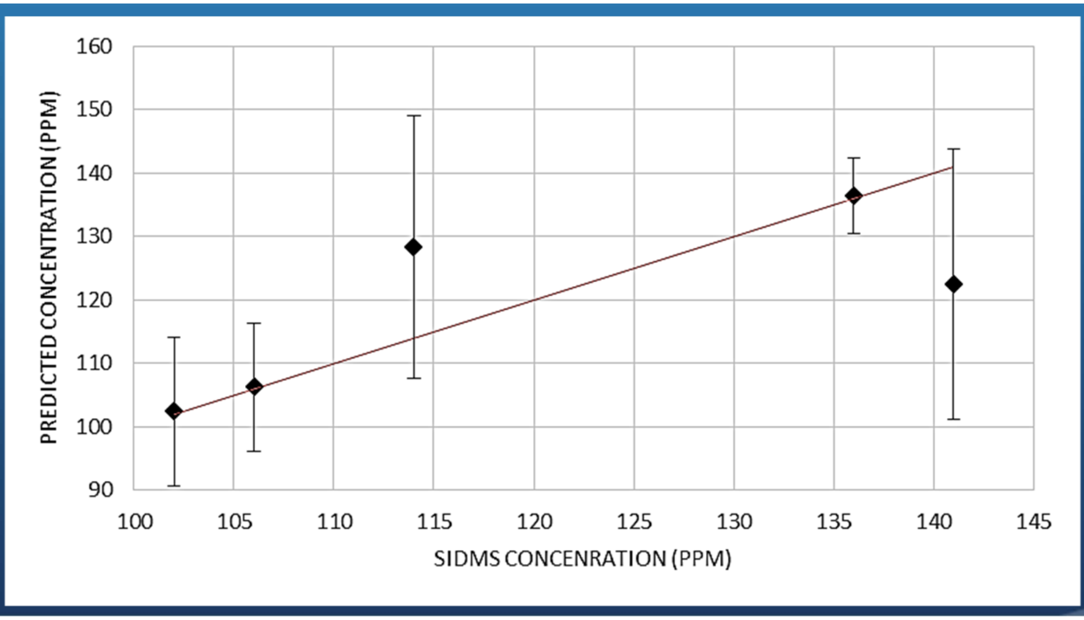

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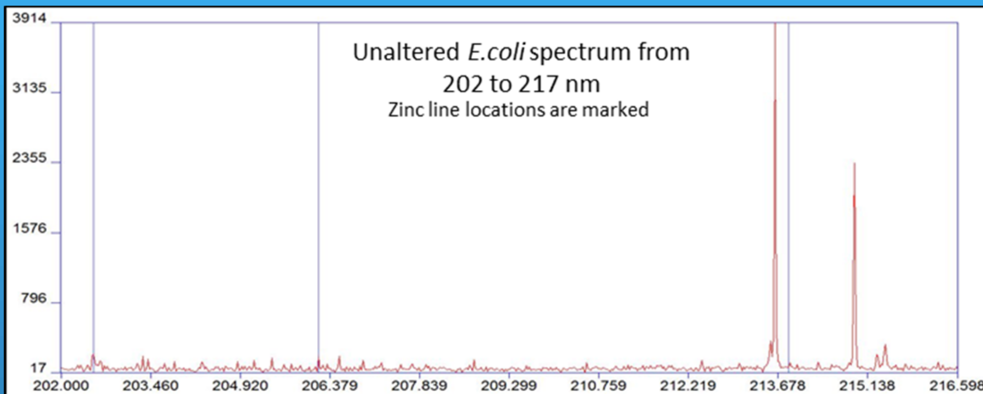
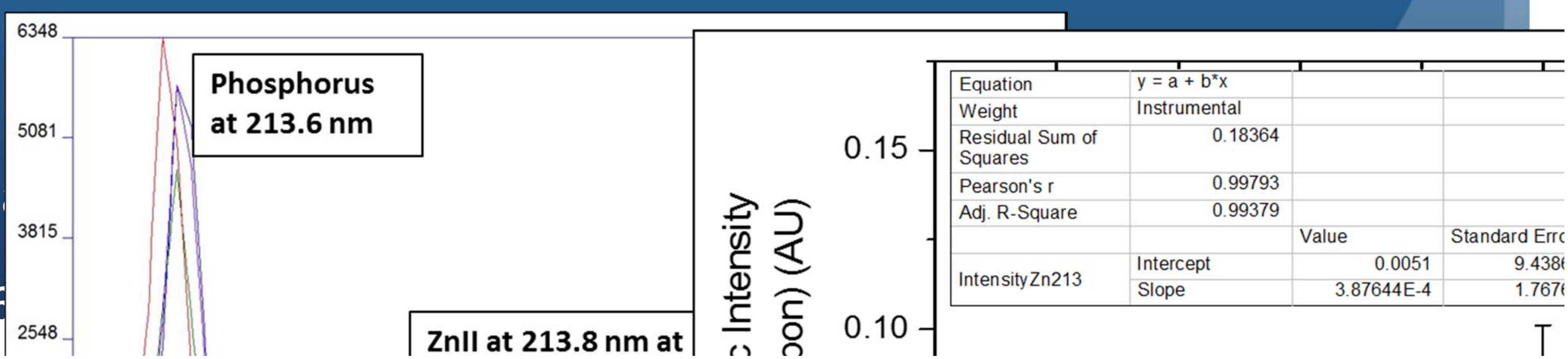


Zinc lines are not distinguishable from noise at normal growth conditions

- Cl
- 16



Outline

1. Introduction of the Method. Laser-induced breakdown spectroscopy (LIBS)

2. Advantages of LIBS over other analytic methods

3. Biomedical Applications of LIBS
   a. A new paradigm for rapid pathogen identification
   b. A real time assay for nutritional zinc deficiency

4. Concluding Thoughts

Outline

- Review of Current State of the Art
- Update on Our Group's Methodology
- New Results / New Questions?
- Concluding Thoughts

Progress on Using LIBS for Bacterial Identification

2007-2012 The bacterial LIBS spectrum for a given species is stable and does not change with time (experiments conducted on the same *E. coli* strain over the course of multiple years).

2007& 2012 Bacterial identification appears to be independent of the growth condition and culture medium in which the bacteria were grown.

2011 This result confirmed (*Marcos-Martinez et al. Universidad Complutense, Madrid*) on three similar growth media

2011 *Salmonella enterica* serovar Typhimuriumin identified at various concentrations in various liquids such as milk, chicken broth, and brain heart infusion. (*Barnett et al. Alabama State*)

Progress on Using LIBS for Bacterial Identification

2011 Bacterial LIBS spectra do not change with time as the bacteria age/sit on an abiotic surface

2013 This result confirmed (*Multari et al. ARA*) on cutting board, sink drainer

2012 Bacteria can be identified with high sensitivity and specificity when specimens are obtained from clinical samples (e.g. sterile urine containing organic and inorganic solutes) without the need to remove other compounds present in the sample.

2012 Live pathogenic *Bacillus anthracis* Sterne strain and *Francisella tularensis* can be differentiated regardless of mounting protocol (as lawn and/or colonies on agar, dilutions on agar, and dilutions on glass slides.) (*Multari et al. ARA*)

Progress on Using LIBS for Bacterial Identification

2011
2012

Bacteria in mixed samples are identifiable. The dominant or majority bacterial component of a two-component bacterial mixture is reliably identified provided it comprises 70% of the mixture or more. Trace mixture or contamination is insignificant.

2011

Bacterial LIBS spectra can be obtained from killed (via autoclaving) or inactivated (via UV light) specimens, and such treatment (which renders the specimen completely safe for handling) does not decrease identification specificity and does not decrease LIBS spectral intensity.

2013

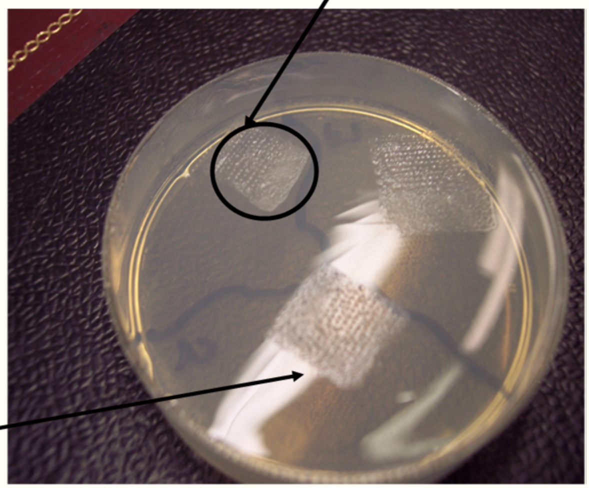
Heat killed bacteria are differentiable (*Multari et al., ARA*)

2015

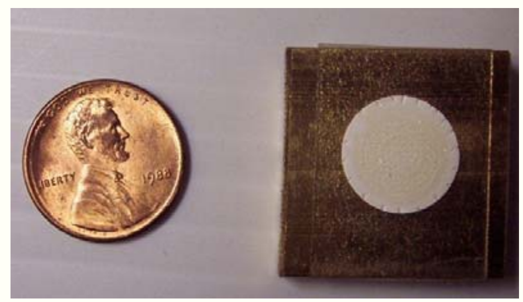
Inactivation by sonication / autoclaving is differentiable (*Sivakumar et al., Delaware State*) with fs-LIBS & ns-LIBS. Changes in spectral intensity were observed.

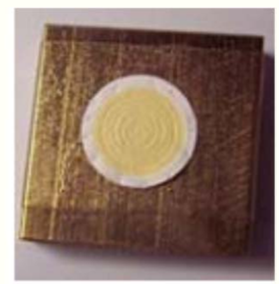
New Bacteria Testing Procedure

Previously: mounted on agar



Currently: mounting on nitrocellulose bacteriological filter



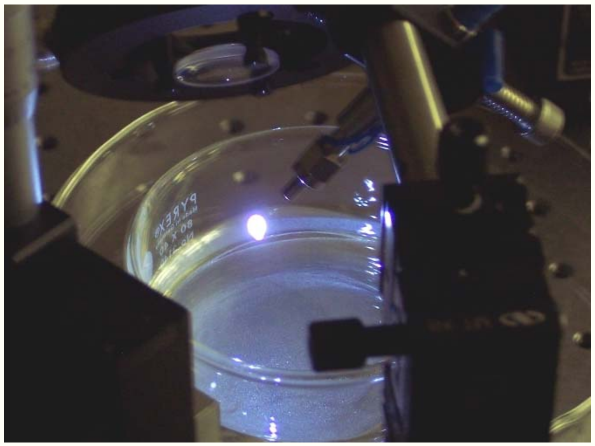


✓ Advantages: Flatter. Easier to do. More reproducible. Less time.

x Disadvantages: Carbon background.

New Bacteria Testing Procedure

Previously: light collected by fiber



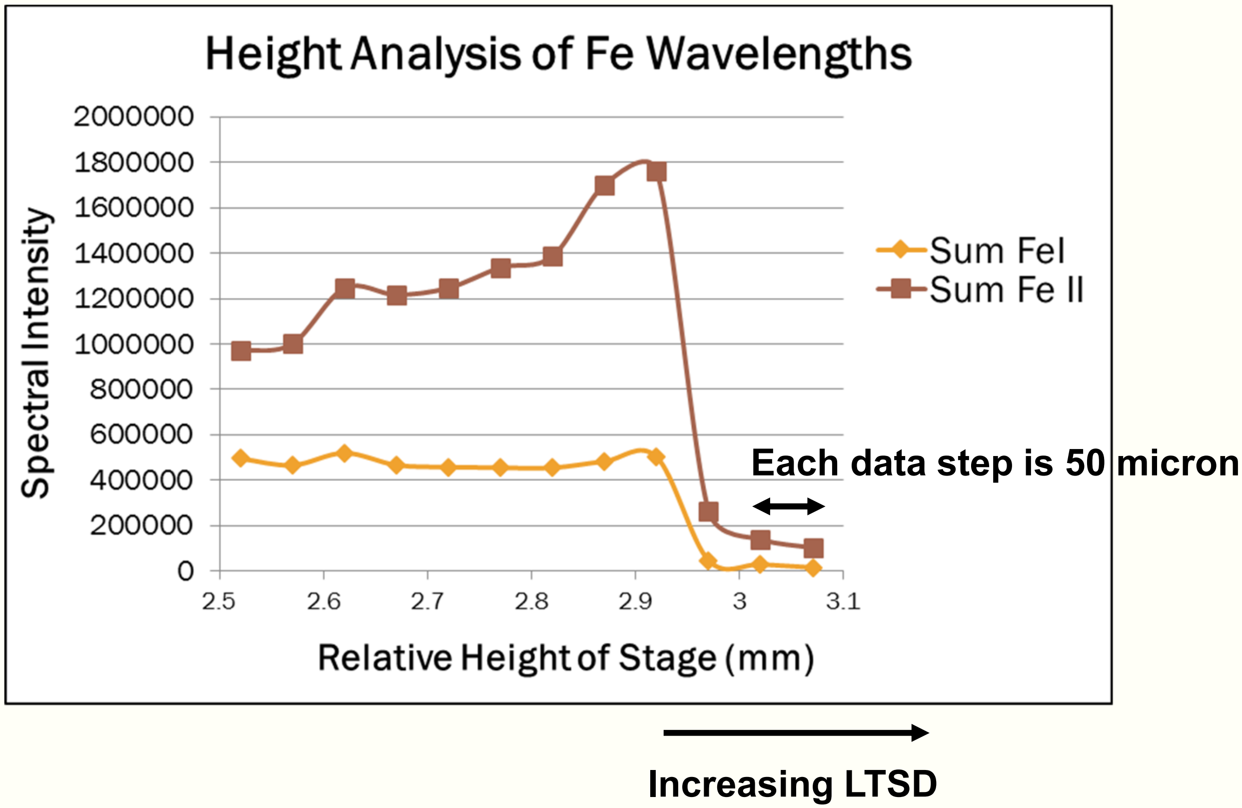
Currently: using matched parabolic reflectors into fiber

- ✓ Advantages: More light collected (solid angle)
- x Disadvantages: More sensitive to vertical position

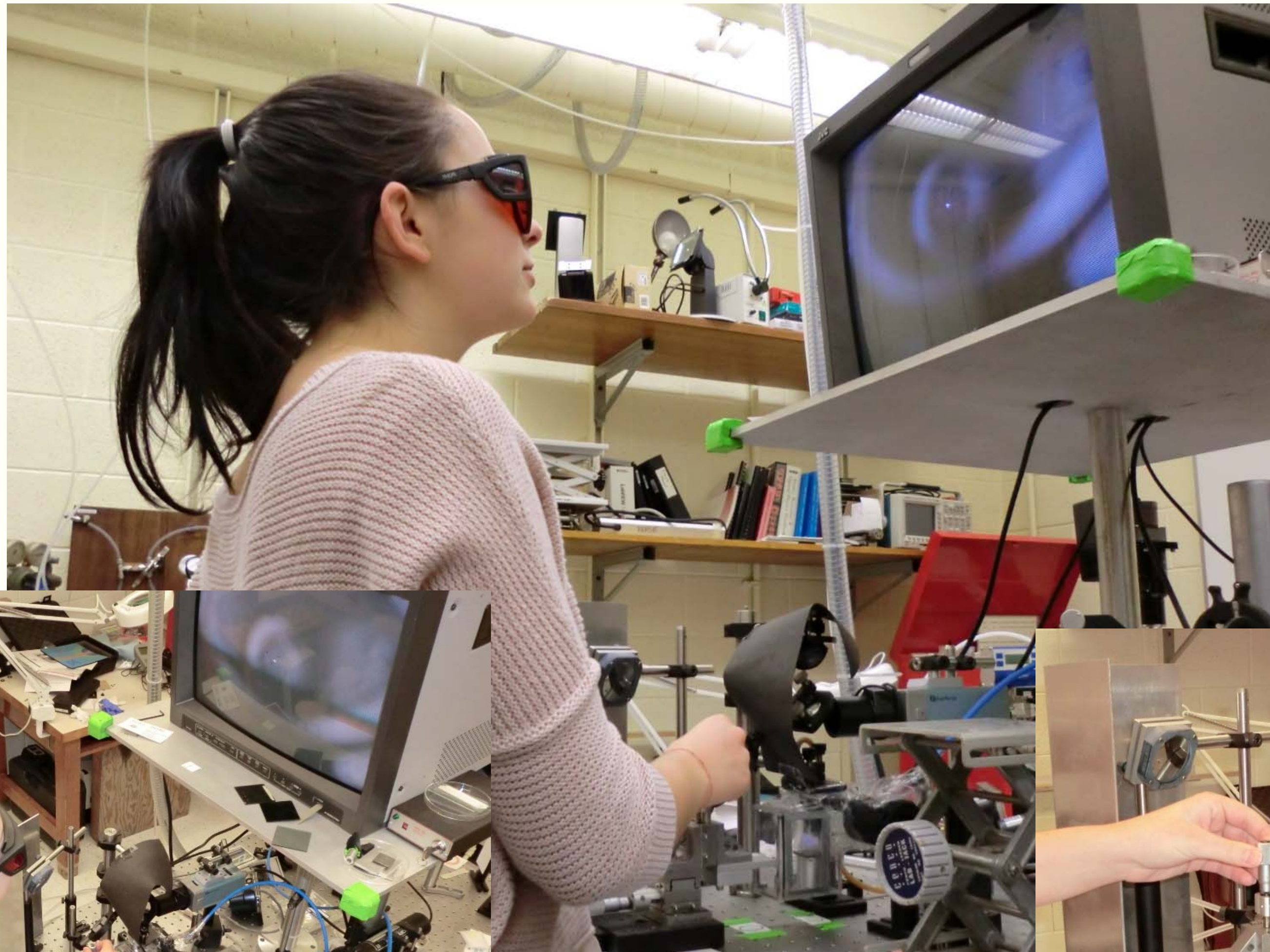
New Bacteria Testing Procedure

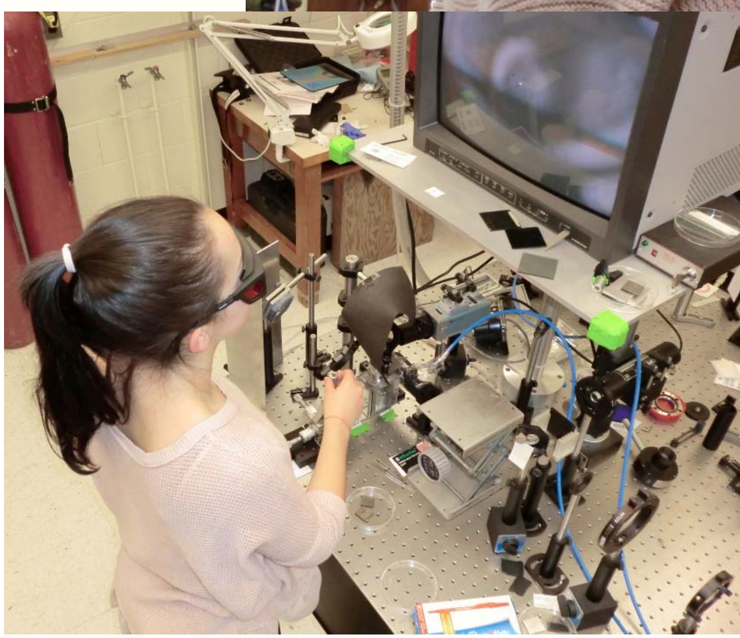
Previously: proper LTSD found by trial and error

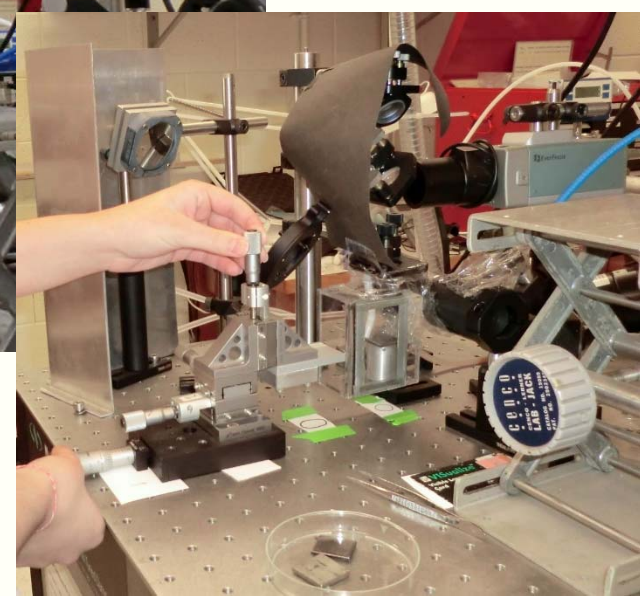
Currently: Appropriate LTSD/focus found with laser indicator.
Calibrated steel sample tested every day for intensity & spot size.
Unnormalized intensities very reproducible and controlled.



Student-Actuated Focus Finder



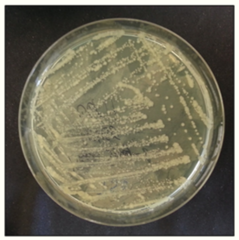




Current Method

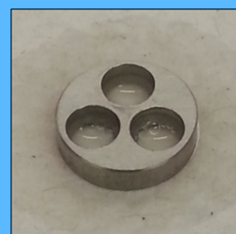
Our Method of Bacteria Classification

Bacteria is cultured using trypticase soy agar (TSA).



Colonies are removed and placed in 1.5 mL distilled water.

30 μL of vortexed sample are deposited on a standard 0.22 μm cellulose filter in contained wells.



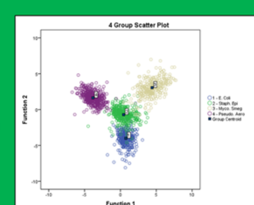
Colloidal solution is dried forming a bacteria lawn on the clinician-friendly filter.

Filter is placed in an argon environment and ablated using a pulsed 1064 nm Nd: YAG laser.

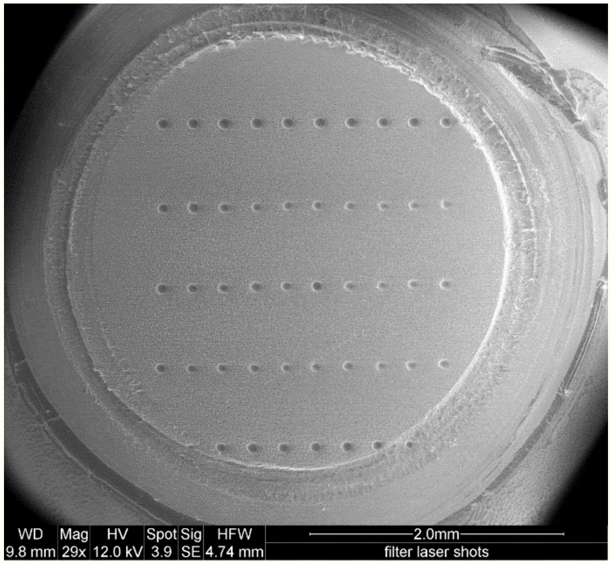
Average time to complete bacterial classification = 1 hour

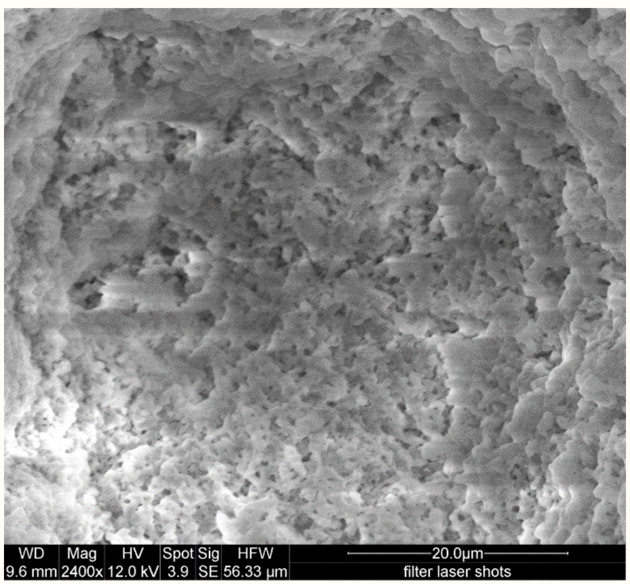
Échelle diffraction grating spectrometer is used to obtain the atomic spectrum and composition of sample.

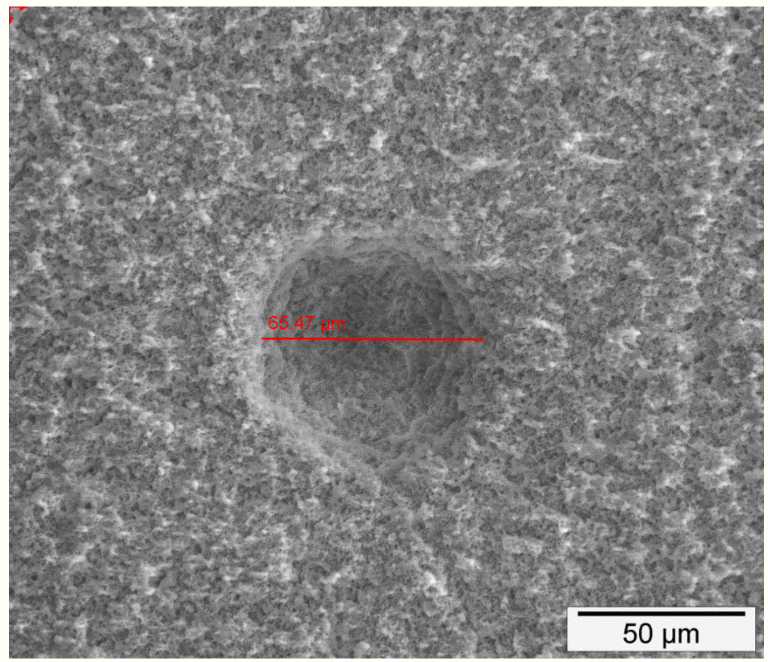
Atomic composition is used to discriminate bacteria against pre-existing library.



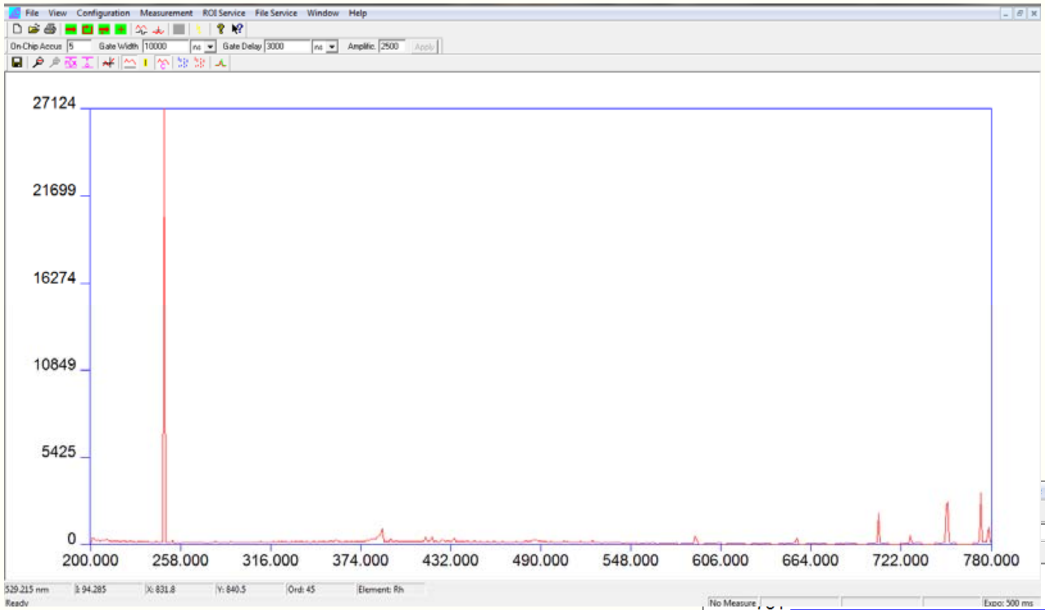
Filter


WD Mag HV Spot Sig HFW
9.8 mm 29x 12.0 kV 3.9 SE 4.74 mm
2.0mm
filter laser shots


WD Mag HV Spot Sig HFW
9.6 mm 2400x 12.0 kV 3.9 SE 56.33 µm
20.0µm
filter laser shots


65.47 µm
50 µm

Filter


File View Configuration Measurement ROI Service File Service Window Help
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21699
16274
10849
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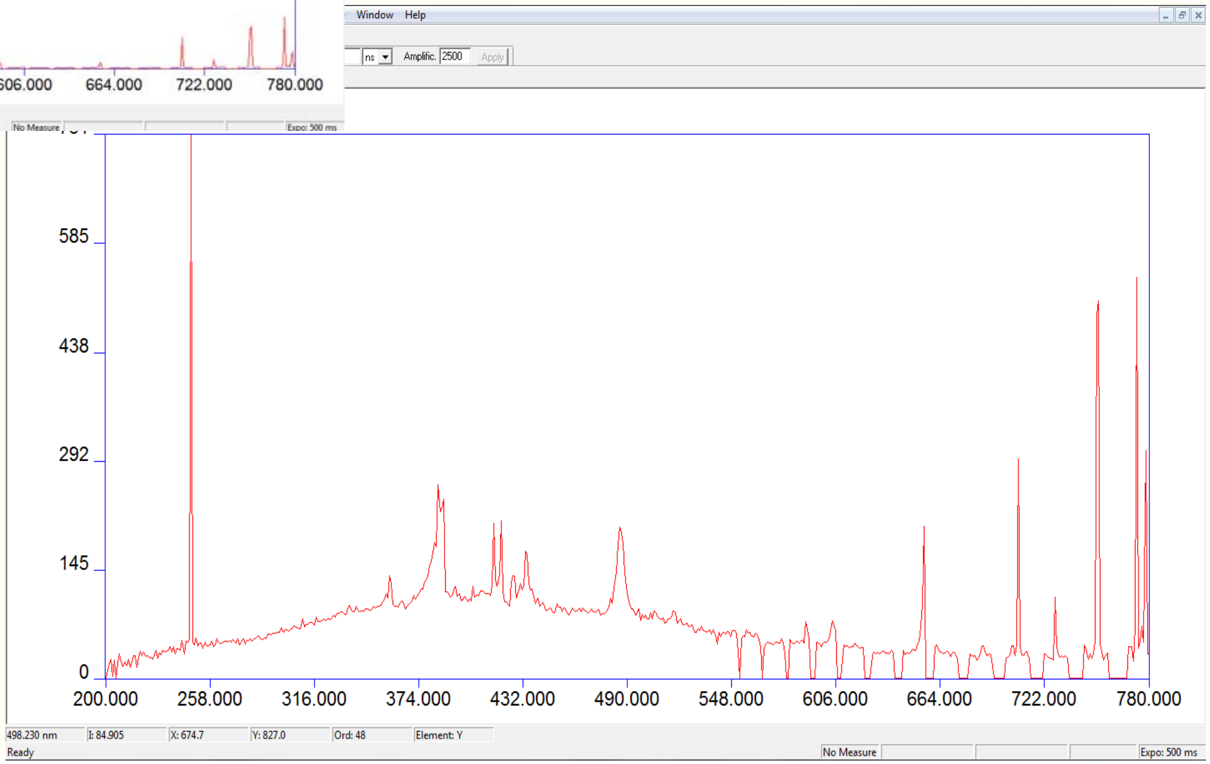
200.000 258.000 316.000 374.000 432.000 490.000 548.000 606.000 664.000 722.000 780.000


Window Help
Amplific. 2500 Apply
585
438
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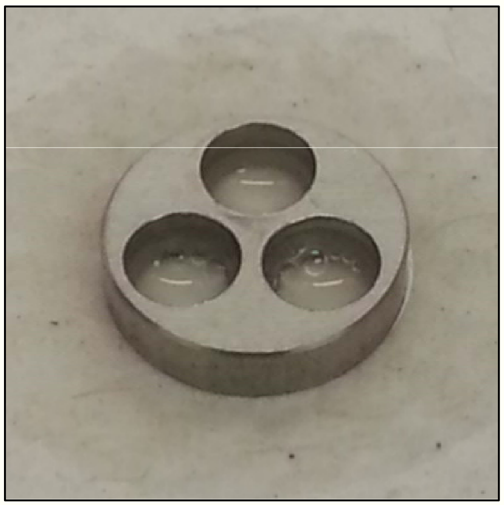
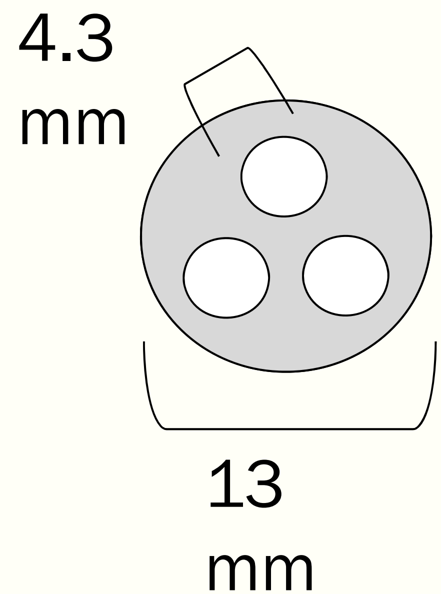
0
200.000 258.000 316.000 374.000 432.000 490.000 548.000 606.000 664.000 722.000 780.000
Ready
No Measure
Expo: 500 ms

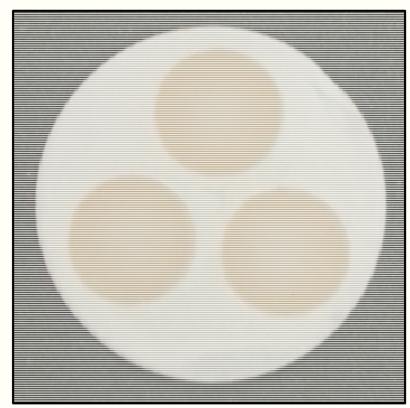
Can bacteria be deposited in a controlled manner?

A steel disk was designed in order to create a reproducible area for bacteria to be placed on.



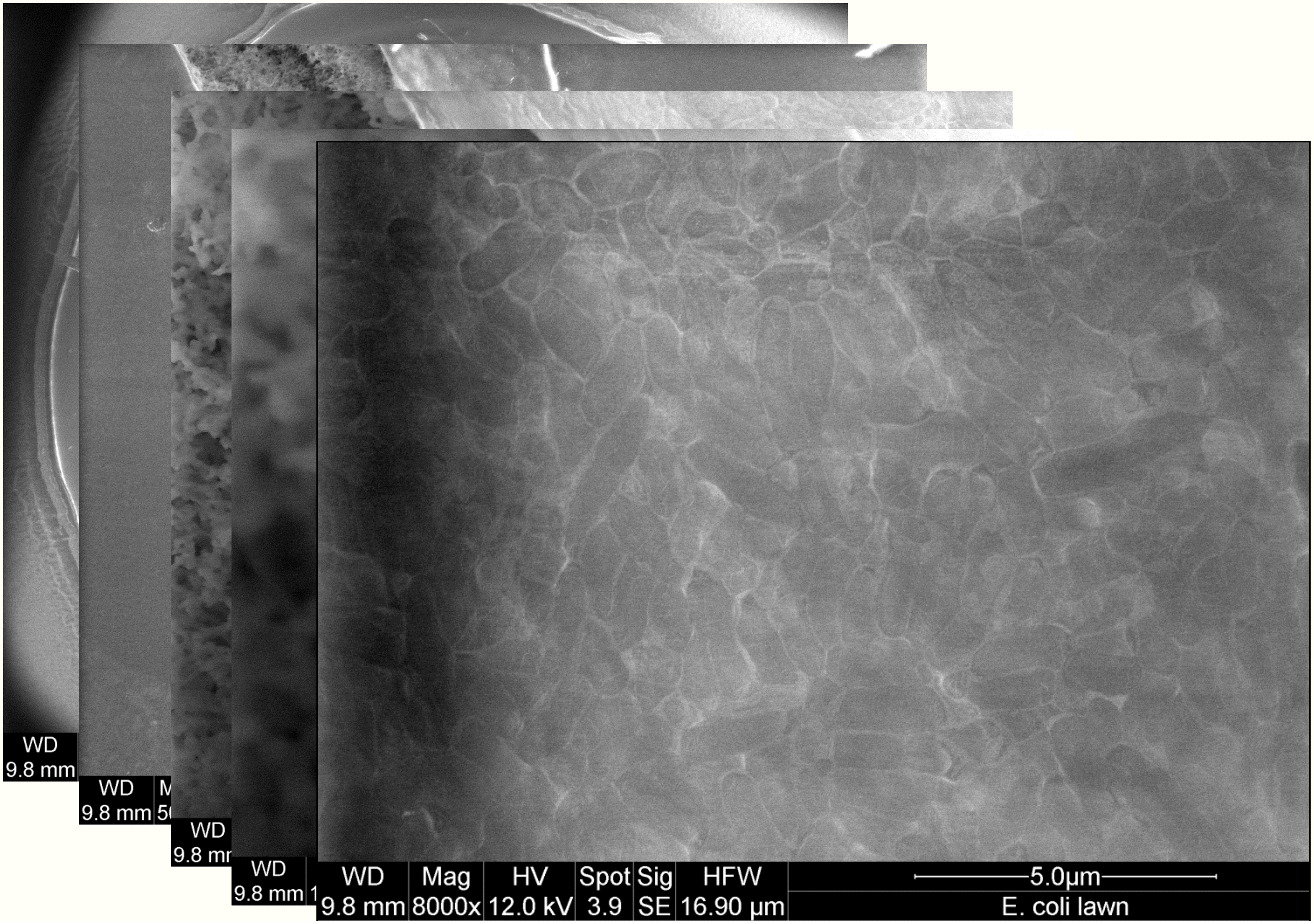


Scanning electron microscopy images verified complete coverage of the deposited bacterial lawn.

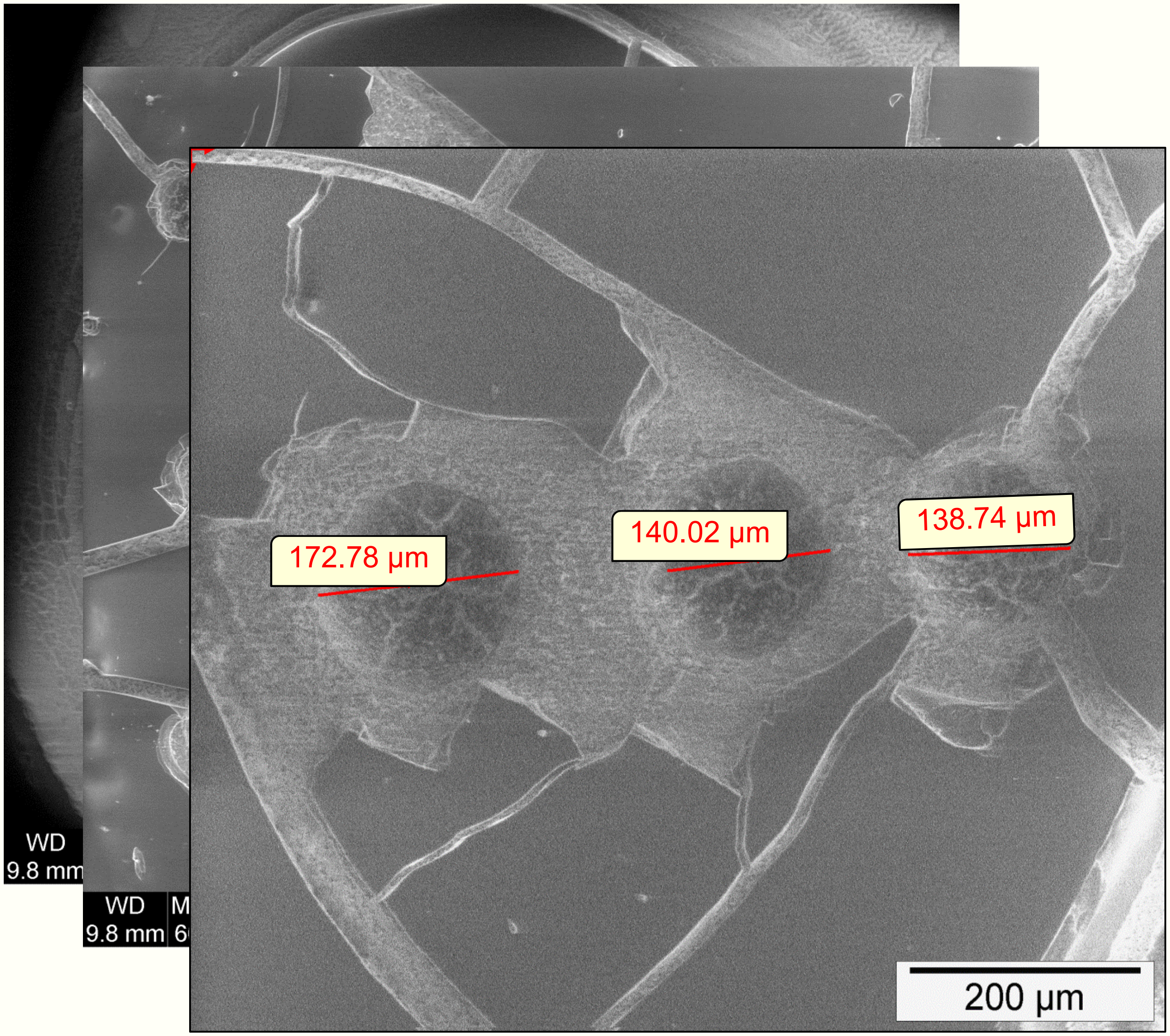


Can bacteria be deposited in a controlled manner?

Can bacteria be deposited in a controlled manner?

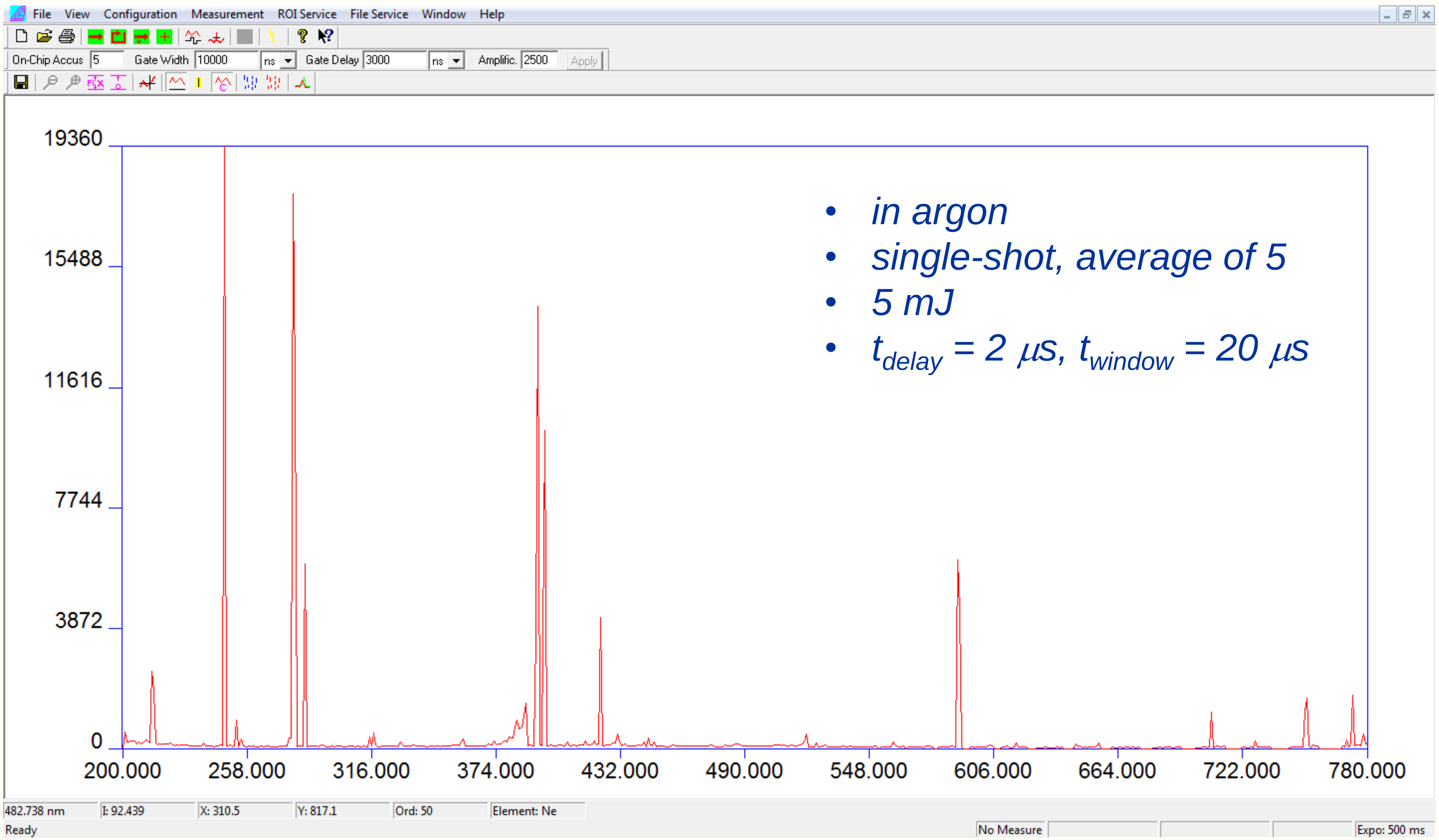


Can laser ablate in a controlled manner?



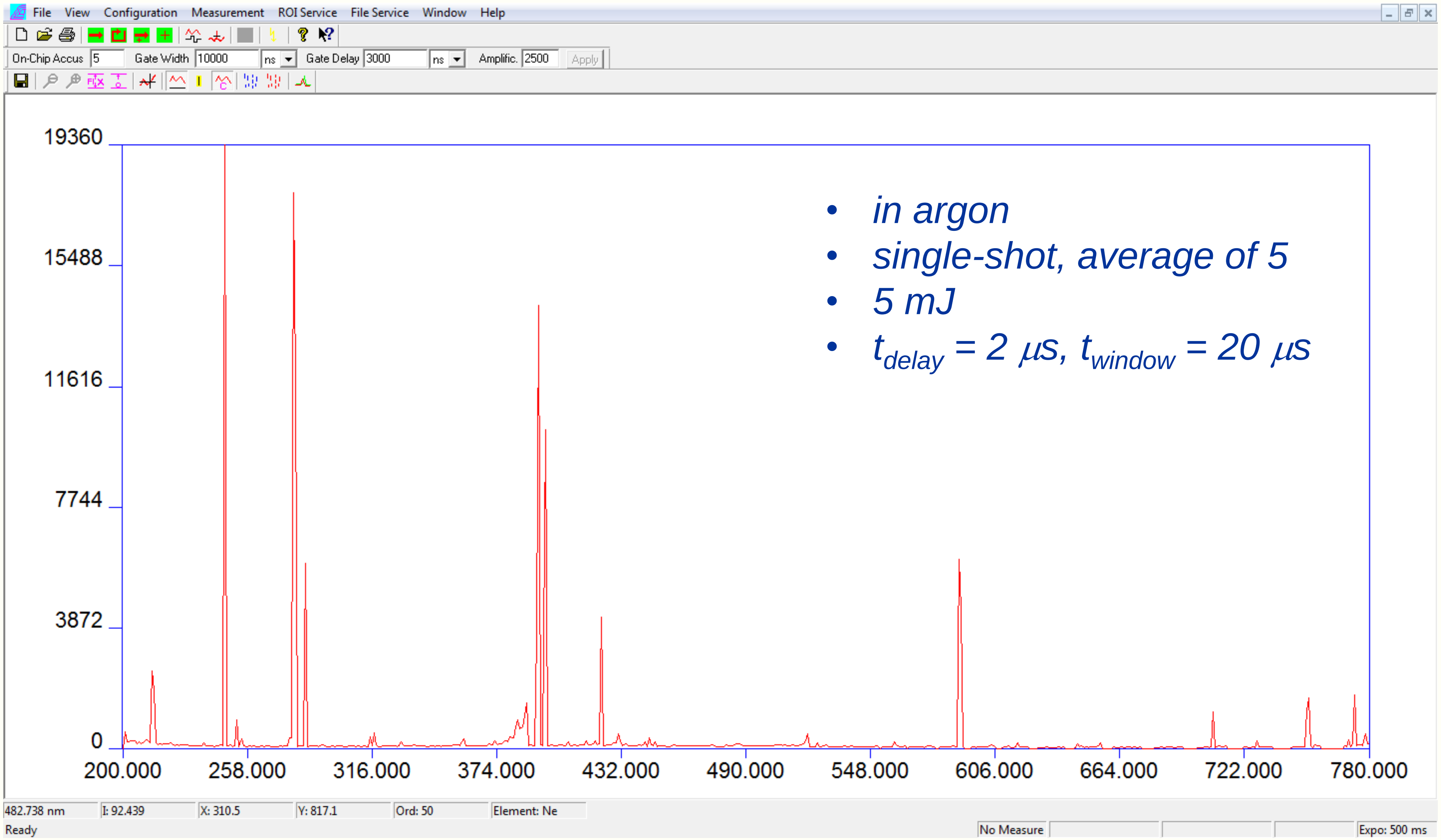
With crater diameters of about 150 μm, the quantity of cells vaporized for each spectrum was estimated at 10^6 (verified using optical densitometry.

Typical Bacterial Spectrum

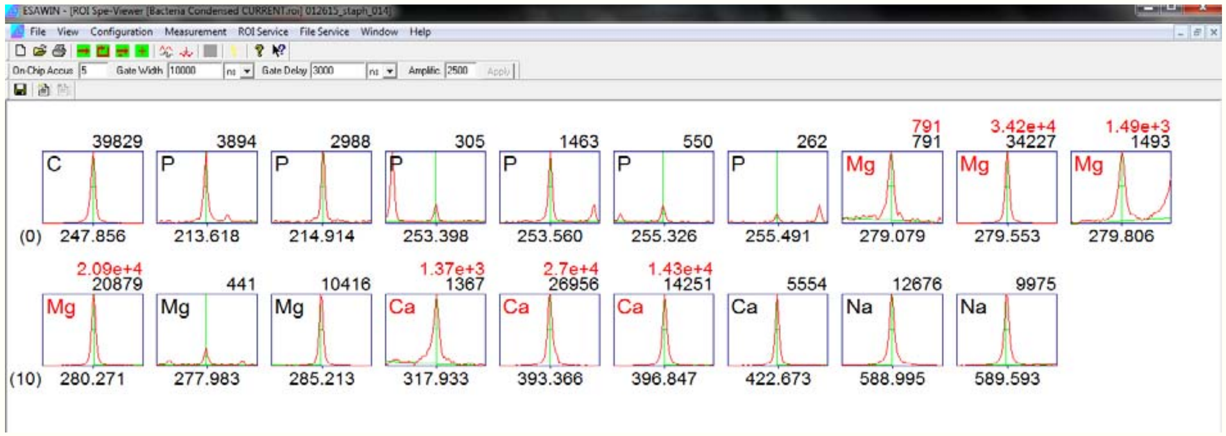


- *in argon*
- *single-shot, average of 5*
- *5 mJ*
- $t_{delay} = 2\ \mu s$, $t_{window} = 20\ \mu s$

Typical Bacterial Spectrum

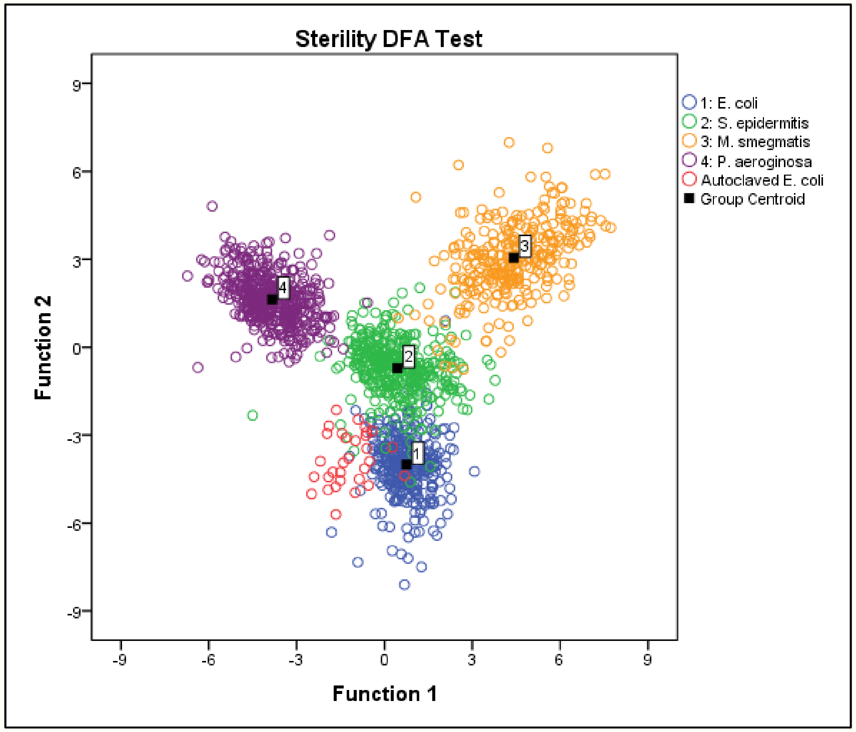


Variable Down-Selection



- New classification model
- 164 independent variable
  - 19 line intensities (all divided by sum)
  - 145 ratios of intensities
- No other metals. Beware?
  - Farooq (2014) sees S, Cl, Mn, Fe, Al, Cu, etc.
  - Sivakumar (2015) only sees Ca, Na, Mg, K, O, H, C, P
  - We see Ni, Fe, Ti only when contaminated!

Performance With New Library



DFA Classification Grouped by Species

Escherichia	TRUE	FALSE	***Staphylococcus***	TRUE	FALSE
Positive	98.28%	0.77%	Positive	97.75%	1.44%
Negative	99.23%	1.72%	Negative	98.56%	2.25%
Mycobacterium	TRUE	FALSE	***Pseudomonas***	TRUE	FALSE
Positive	95.36%	0.33%	Positive	99.57%	0.22%
Negative	99.67%	4.64%	Negative	99.78%	0.43%

Sensitivity: 98 ± 2% Specificity: 99 ± 1%

PLS-DA Classification Grouped by Species

Escherichia	TRUE	FALSE	***Staphylococcus***	TRUE	FALSE
Positive	96.55%	1.12%	Positive	96.75%	1.53%
Negative	98.88%	3.45%	Negative	98.47%	3.25%
Mycobacterium	TRUE	FALSE	***Pseudomonas***	TRUE	FALSE
Positive	97.02%	0.41%	Positive	98.92%	0.33%
Negative	99.59%	2.98%	Negative	99.67%	1.08%

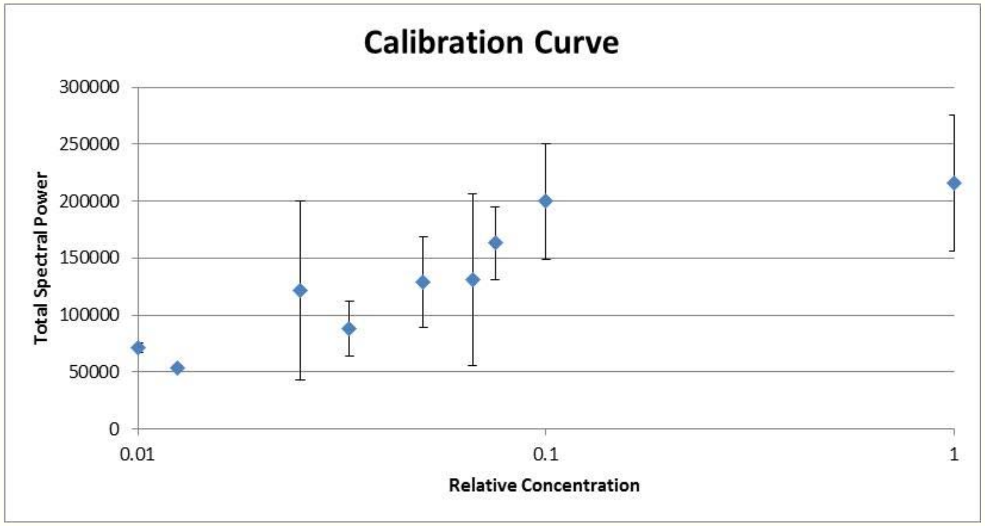
Sensitivity: 97 ± 3% Specificity: 99 ± 2%

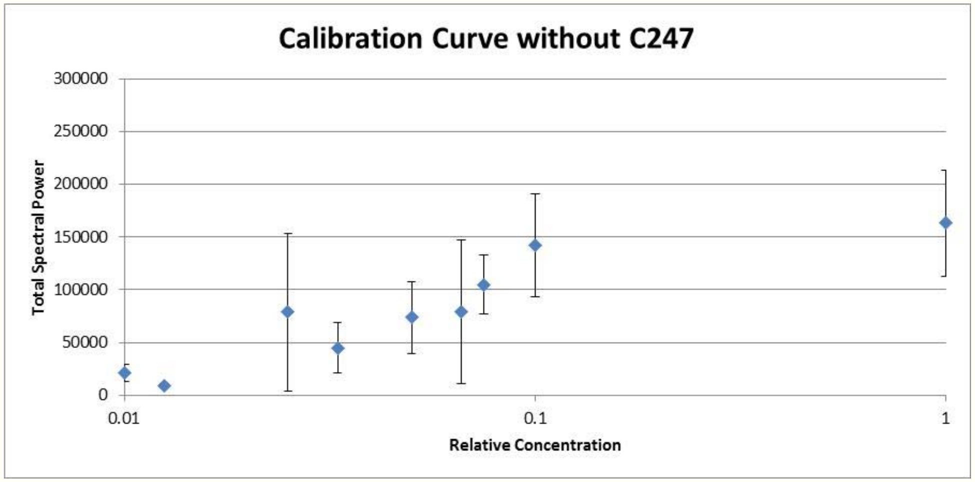
All external validation results

	DFA (by filter)	**DFA (by species)** above	**PLSDA (by species)** above
Sensitivity	0.93±0.07	0.98±0.02	0.97±0.03
Specificity	0.98±0.03	0.99±0.01	0.99±0.02

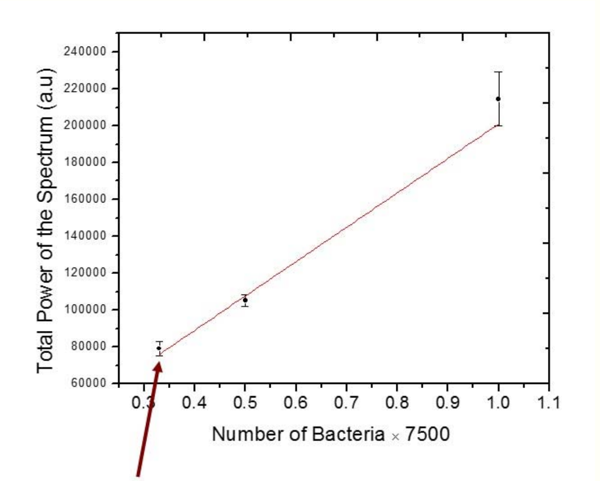
"by filter" means approximately 30 groups in DFA, no relationships between groups assumed

New Concentration Study





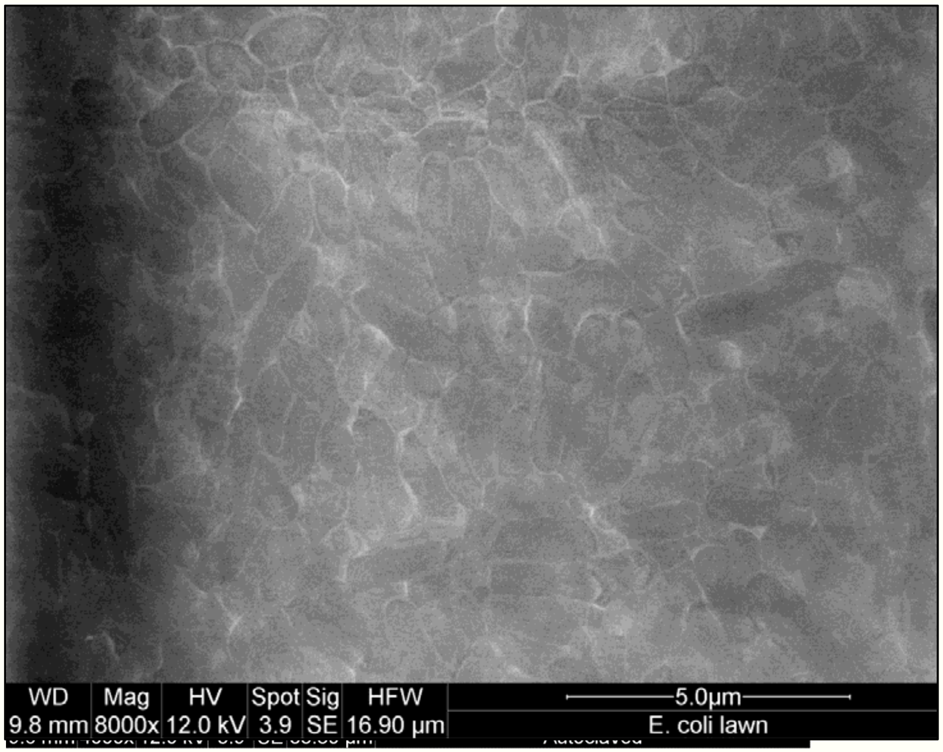
Previous result

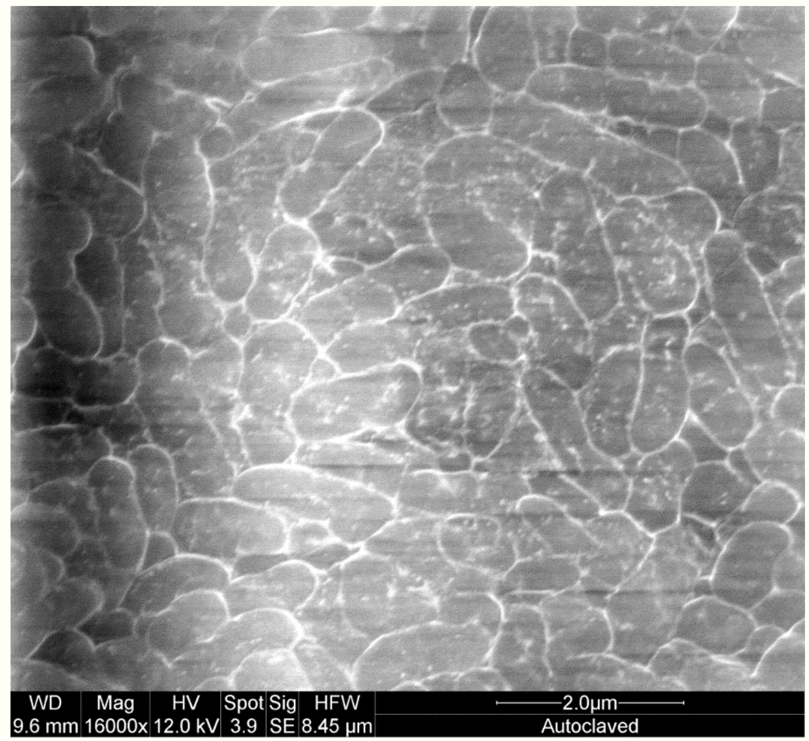


- Performed with serial dilutions.
- "Concentration 1" → harvest entire plate of colonies off TSA, suspend in 1.5 mL distilled H_20
- Measure with optical densitometry
- OD=0.1 measured for C=0.001 (from literature OD 0.1=10^8 cells/mL).
- C=1 → 10^{11} cells/mL
- Implies for C=1, 10^6/shot

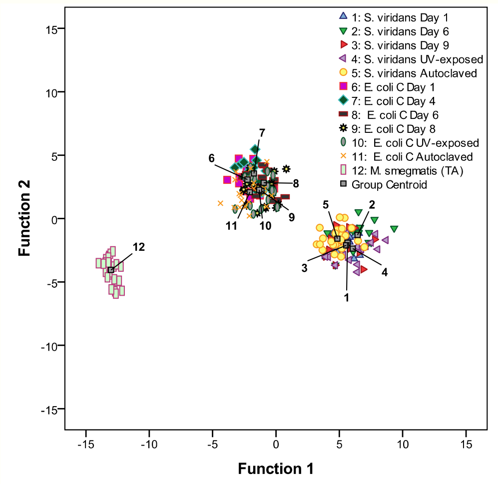
Viability study - Autoclaved

- Live cells were placed in 1 mL of distilled water (microcentrifuge tube).
- This was covered and placed in the autoclave on the liquid cycle.
- Deposited on the filter in the same procedure after vortexing (obtaining solution close to the bottom of the tube).
- Viability was confirmed by restreaking and growth for 24 hours.

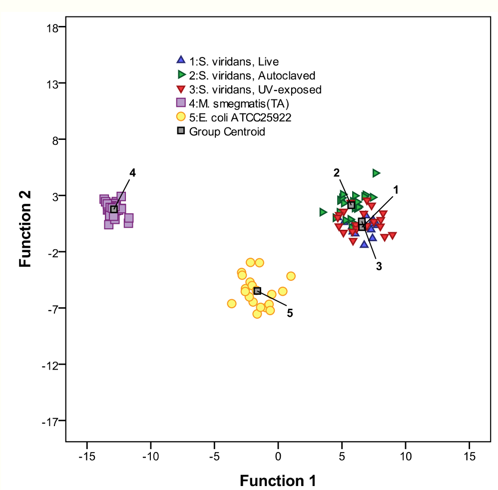




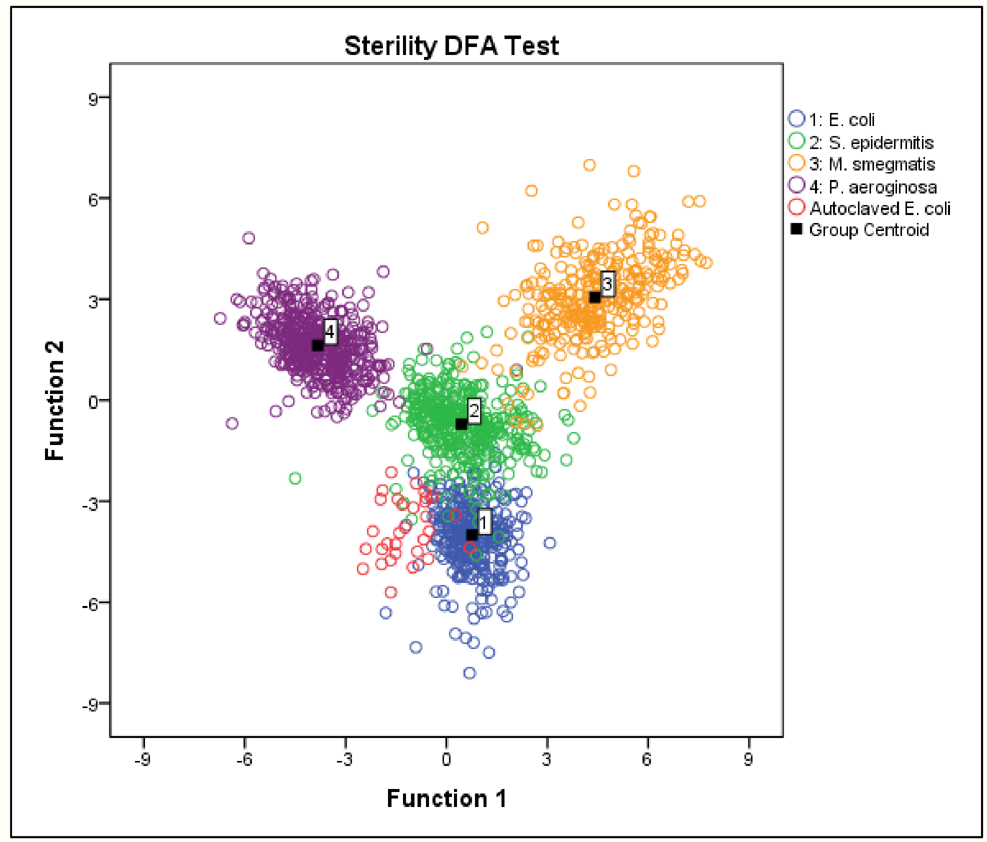
Viability study - Autoclaved



Previous result

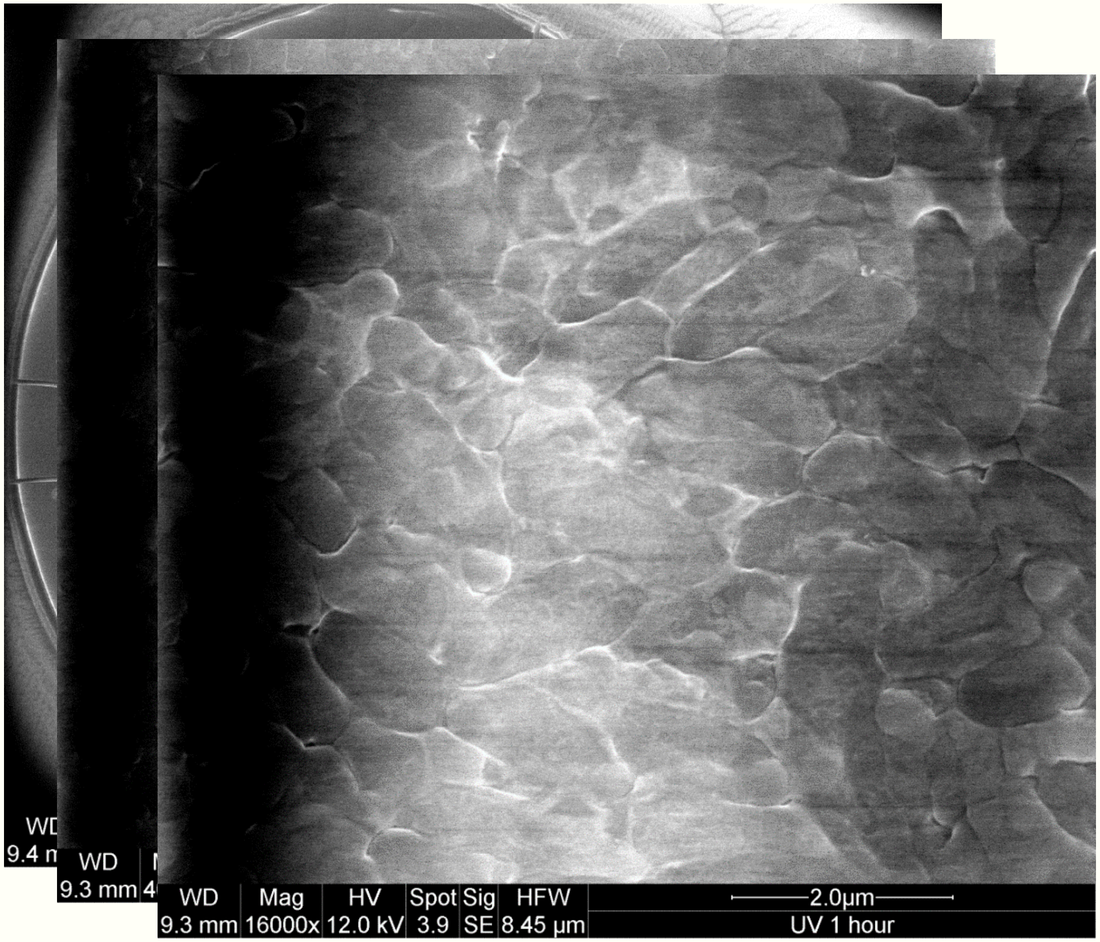


Autoclaved *E. coli* classify >98% as live *E. coli*, yet spectrum seems to be slightly distinct

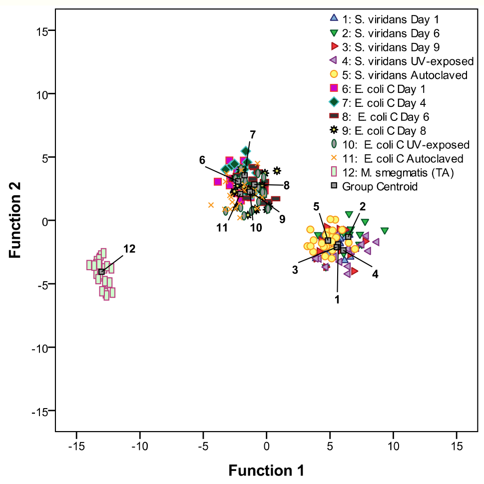


Viability study - UV

- Live cells were deposited on the filter, the whole filter was then placed under UV light (4 W, 254 nm at about 15 cm from the source) for 1 hr, 30 min, 15 min.
- Saw no difference in signal and they classified as live.
- 30 min was chosen for future experiments.
- UV non-viability confirmed via pressing into TSA growth plate and cultured for 24 hours.

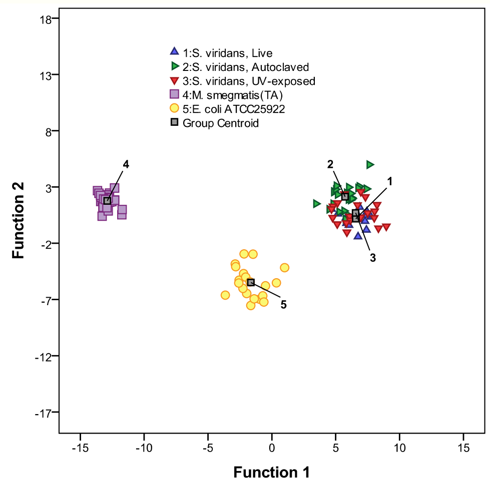


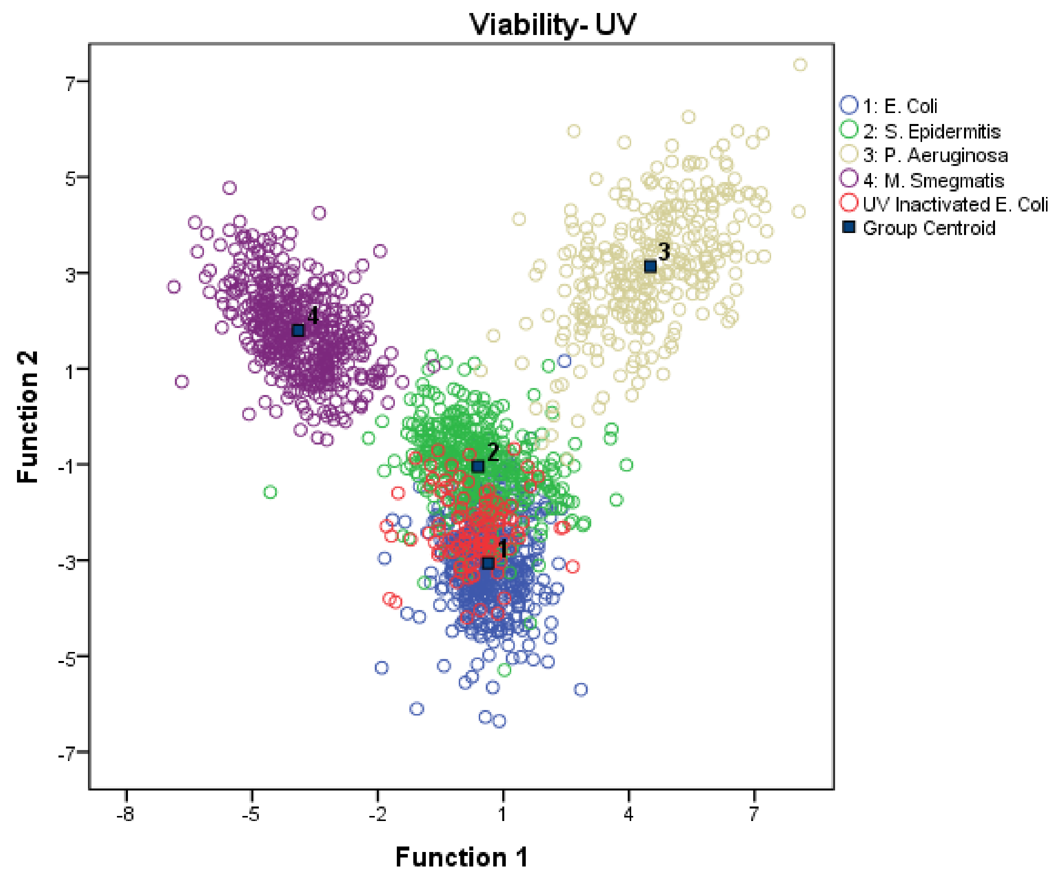
Viability study - UV



Of 118 UV'd *E. coli* spectra, 100% classified as live *E. coli*

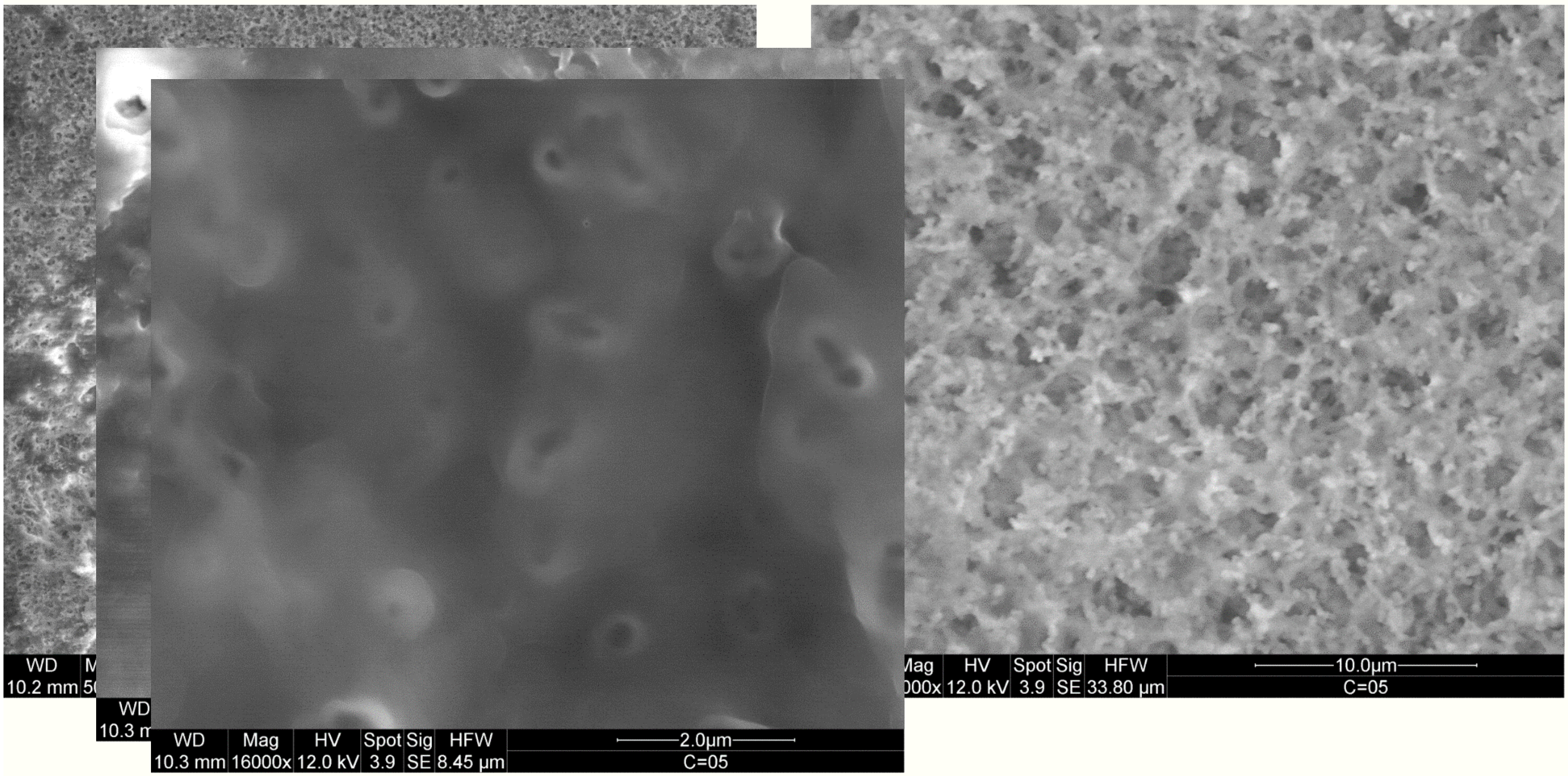
Previous result





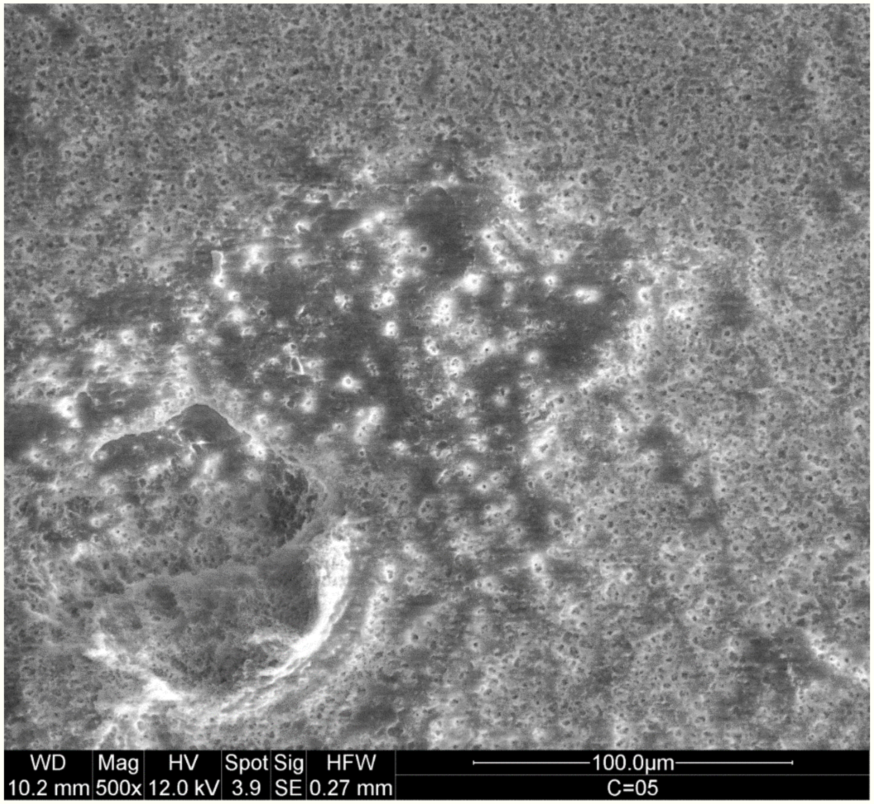
Viability study - Sonication

- Cells were placed in 1 mL distilled water (microcentrifuge tube) sonicated at a setting of 3 W for 10 seconds.
- Deposited on the filter in the same procedure after vortex mixing (obtaining solution close to the bottom of the tube).
- When done the second time, 0.8 W for 10 5s-pulses



Viability study - Sonication

- Sonicated *E. coli* tested at multiple concentration (c=0.05, =0.025). Both classified as autoclaved *E. coli* over live.
- Sonicated cells were clearly disrupted and rather than assisting in sample homogeneity, showed increased clumping/heterogeneity



Summary of Viability Study

Q: So…are live bacteria differentiable from "non-viable" bacteria (autoclaved, UV, sonicated)?

A: Do you want them to be, or don't you?

We see some differences between them (not quantified yet) but they can classify as "live" when comparing against the live species library.

Multari et al. and Sivakumar et al. both DO SEE differences in autoclave (or "heat killed") and live; and also in sonicated vs. live. Claim is that cell lysis leads to a "leaking out" of material used for discrimination.

Q: If that is true, does our UV result confirm this?

Summary of Viability Study

A: Do you want them to be, or don't you?

Q: Could it be because they are looking for them to be different?

- This leads to more sterilization tests (ongoing), asking the questions:
  - Why are they different (from live)?
  - If they are different from live, are they like each other?
  - If they are like each other, why? Should they be? (structure dependence, nuclear loss, deposition on filter – do they lay the same? Clumping?)

Q: Are the results consistent for all species? Results are just shown for *E. coli* but tests are in progress.

Summary of Viability Study

Q: Lastly, is everyone taking exceptional care to insure it is not the deposition of the bacteria on their substrates leading to perceived “differences?”

- Bacteria are definitely NOT inorganic microparticles that can be uniformly deposited easily, yielding background-independent LIBS spectra.
- Great care must be taken.

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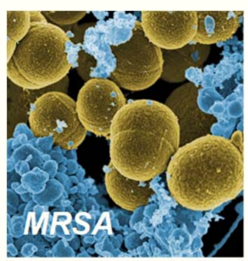

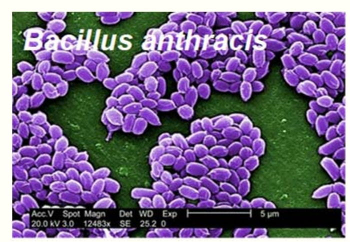

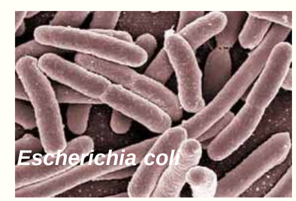

So many questions…

…but all tests to date have proven the possibility of using LIBS for a rapid pathogen diagnostic, as well as numerous other biomedical applications.

Work continues, with generous help from:

- University of Windsor
- NSERC Discovery Grant
- CFI-LOF grant

University of Windsor
thinking forward

Natural Sciences and Engineering Research Council of Canada
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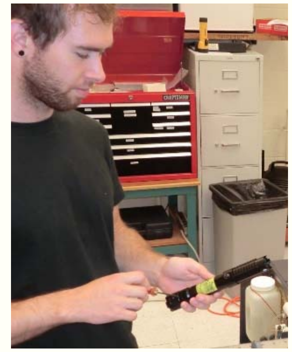
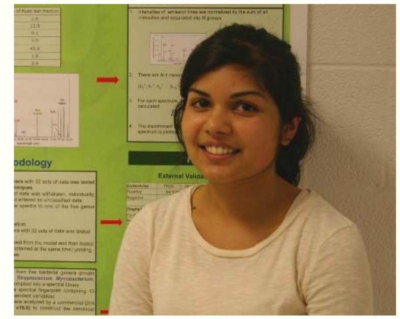
All Credit to the Students

Dan Trojand





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Vlora Riberdy