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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SAS - Metallomics

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Outline

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

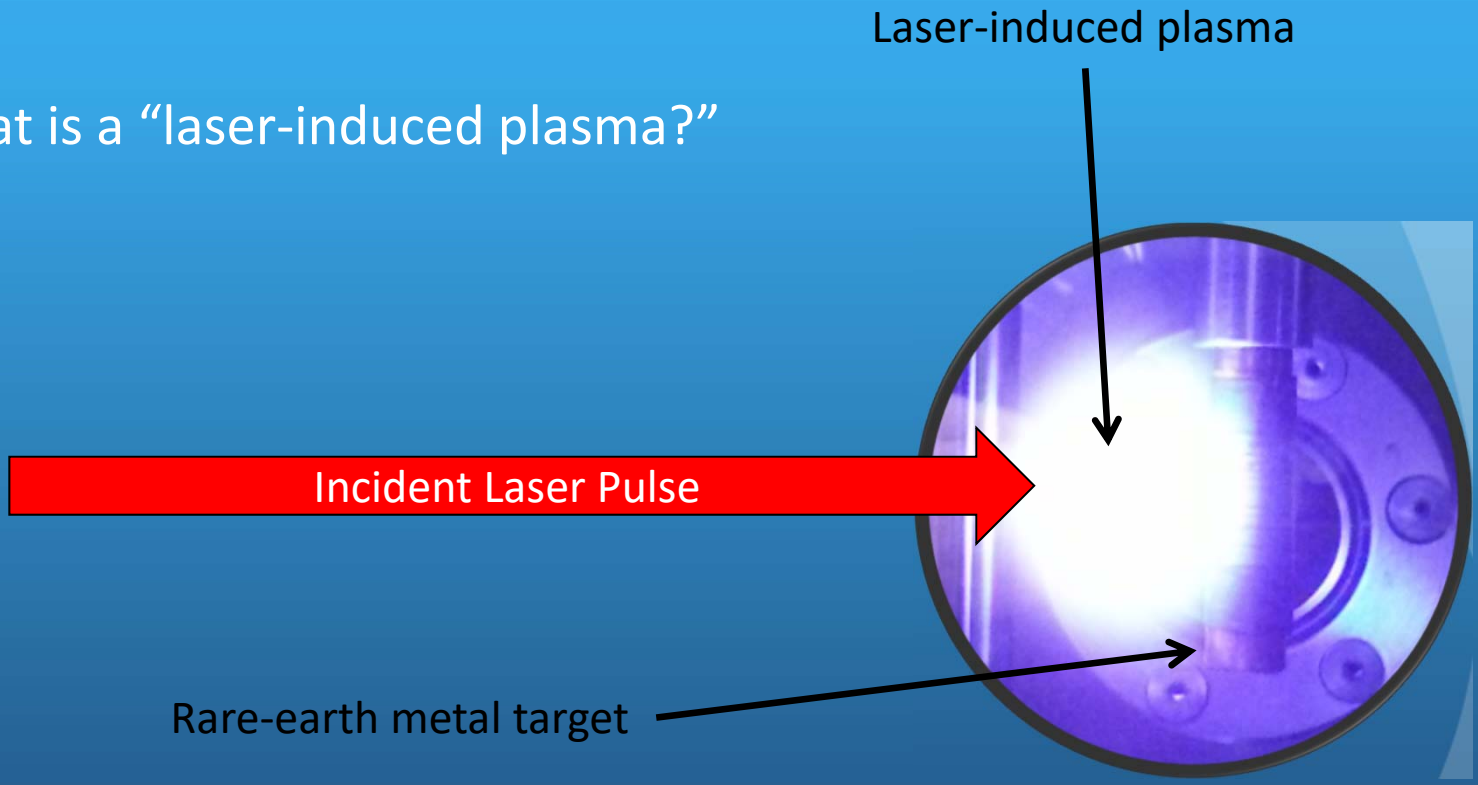
2. Advantages of LIBS over other analytic methods

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

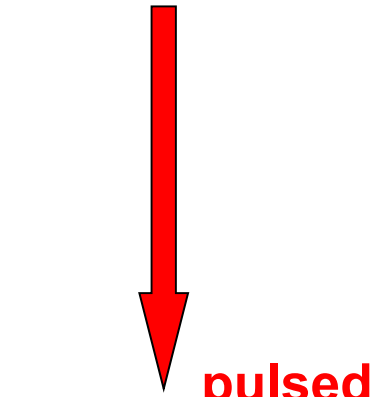
Laser-Induced Plasmas

- What is a “laser-induced plasma?”



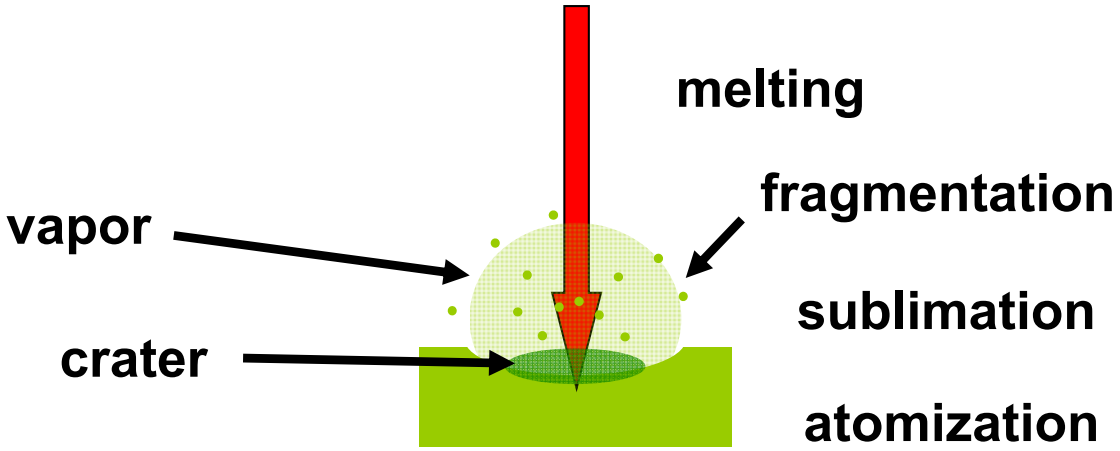
- Can be done with ns, ps, or fs lasers
- Threshold irradiance: $\sim 10^{10}$ - 10^{11} W/cm^2

1) laser interaction with the target



- initiated by absorption of energy by the target from a pulsed radiation field.
- pulse durations are on the order of nanoseconds, but can be performed with pico- and femto-second laser pulses.

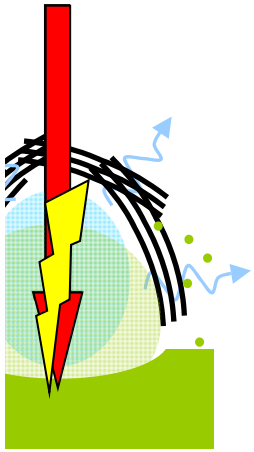
2) removal of samples mass (ablation)



- absorbed energy is rapidly converted into heating, resulting in vaporization of the sample (ablation) when the temperature reaches the boiling point of the material.

- removal of particulate matter from the surface leads to the formation of a vapor above the surface.

3) plasma formation (breakdown)



absorption of the laser radiation by the vapor

continuum emission

electrical breakdown and plasma formation

shock wave

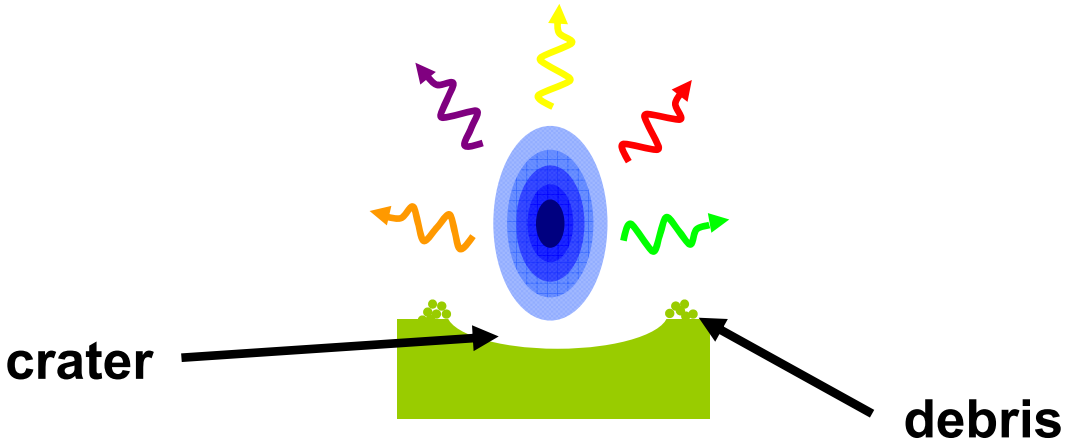
bremsstrahlung

to illuminate the vapor plume.

sub-micrometer droplets that attering of the laser beam, nization, and plasma formation.

4) expansion and element specific emission (atomic or ionic)

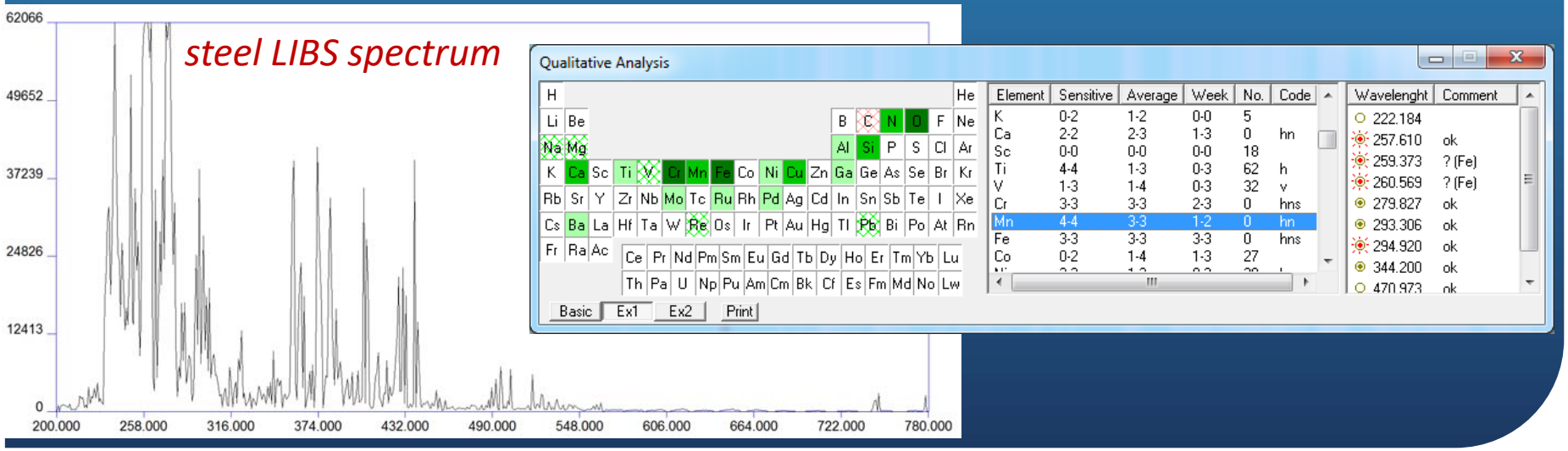
spontaneous emission as atoms/ions decay to ground state



- The dynamic evolution of the plasma plume is then characterized by a fast expansion and subsequent cooling.
- Approximately 1 microsecond after the ablation pulse, spectroscopically narrow atomic/ionic emissions may be identified in the spectrum.

The Goal of LIBS Plasma Creation

- to create an optically thin plasma which is in thermodynamic equilibrium (or LTE) and whose elemental composition is the same as that of the target/sample
  - if achieved, **atomic emission spectral line intensities** can be related to **relative concentrations** of elements (sometimes absolute concentrations)
  - typically these conditions are only met *approximately*.

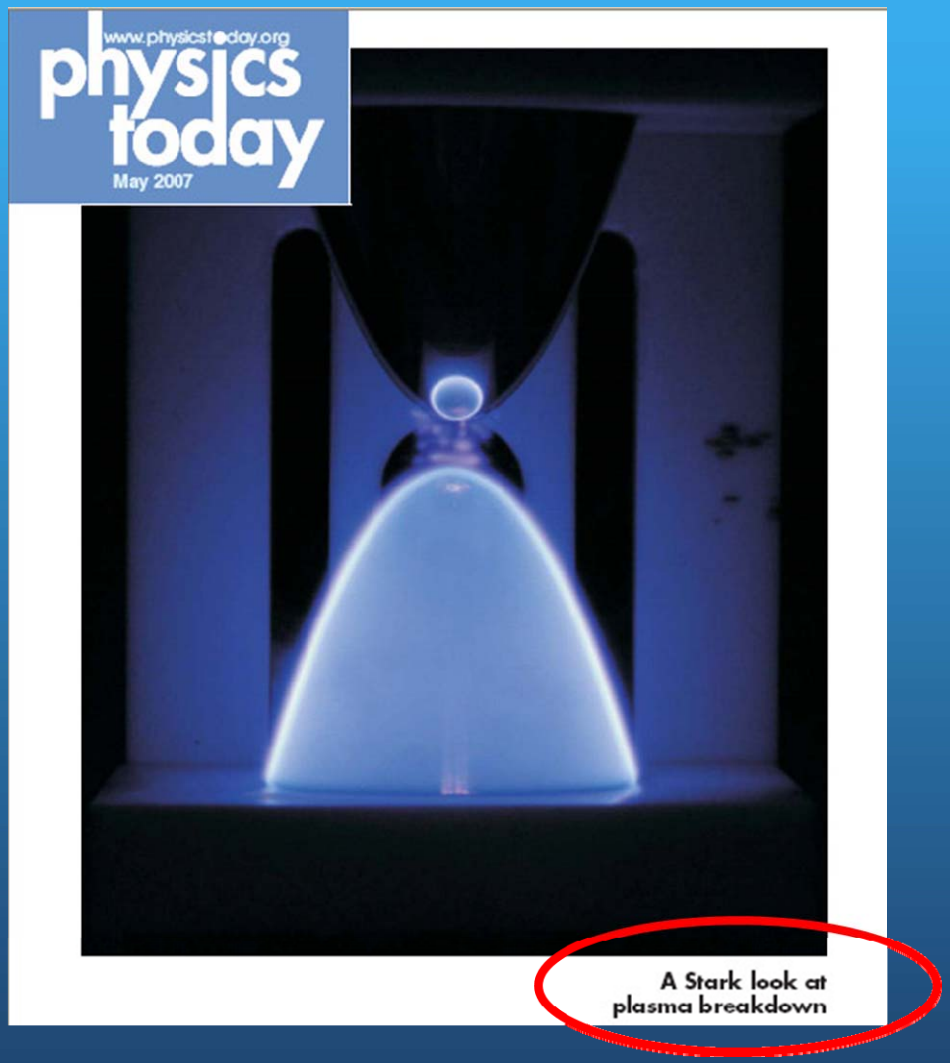


When we do a time-resolved spectroscopy of the plasma, we call it:

"Laser-induced breakdown spectroscopy"

or

LIBS



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Keeping Track of the Elemental Inventory
(underlined elements reported in literature)

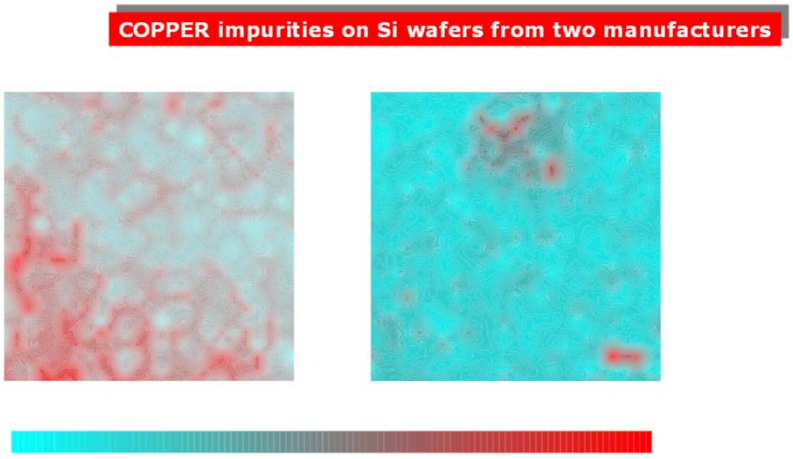
(■ Solids ■ Liquids ■ Gases ■ Artificially prepared)

1 H																2 He
3 Li	4 Be											5 B	6 C	7 N	8 O	9 F	10 Ne
11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
19 K	20 Ca	21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
37 Rb	38 Sr	39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe
55 Cs	56 Ba	57 La	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn
87 Fr	88 Ra	89 Ac	104 Rf	105 Db	106 Sg	107 Bh	108 Hs	109 Mt	110 Uun	111 Uuu	112 Uub		114 Uuq		116 Uuh		
			58 Ce	59 Pr	60 Nd	61 Pm	62 Sm	63 Eu	64 Gd	65 Tb	66 Dy	67 Ho	68 Er	69 Tm	70 Yb	71 Lu	
			90 Th	91 Pa	92 U	93 Np	94 Pu	95 Am	96 Cm	97 Bk	98 Cf	99 Es	100 Fm	101 Md	102 No	103 Lr	

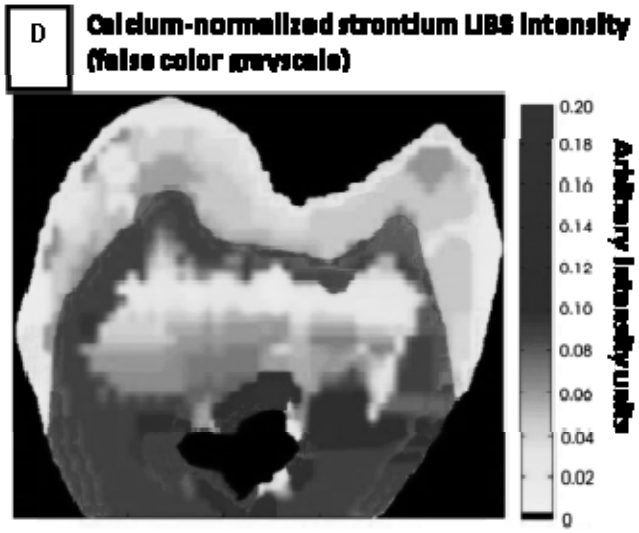
www.arl.army.mil/wmrd/LIBS

Advantages of LIBS - spatial resolution

- Laser allows point sampling (1-100 micron)
- Elemental "surface maps" can then be created



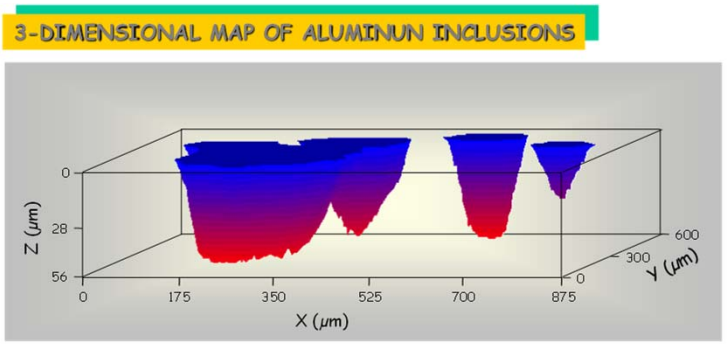
Courtesy of Ben Smith, Javier Laserna



Courtesy of F.C. Alvira et al.

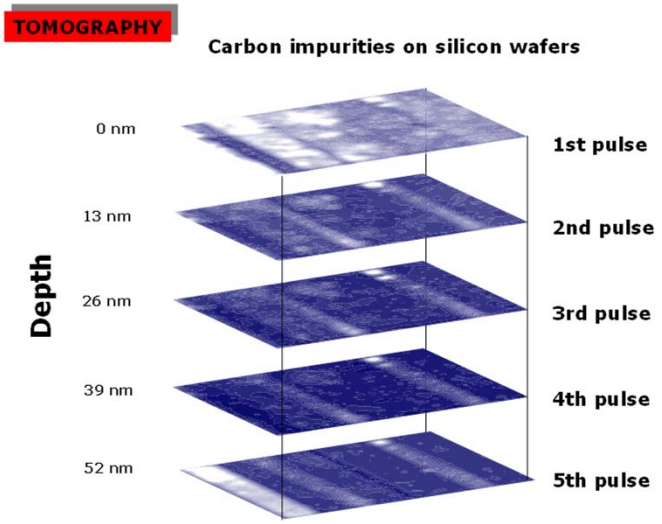
Advantages of LIBS - depth profiling

- Because laser only removes μg to ng of material, ablation crater only microns deep
- Subsequent shots thus sample progressively deeper layers



Courtesy of Ben Smith

- 13 positions
- 100 laser shots in depth
- Mapped volume = 600 x 875 x 56 μm3
- Repetition rate = 10 Hz
- Analysis time = 2 min and 30 s

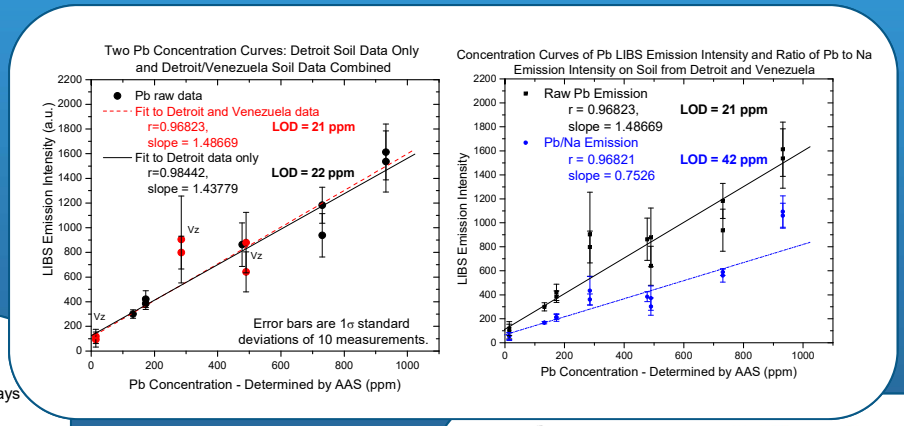


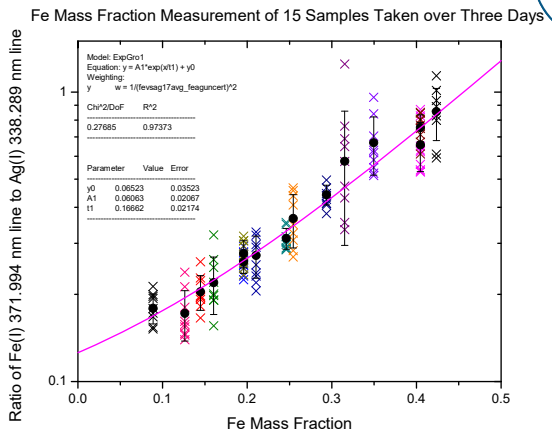
Nitrogen laser; Lateral resolution 15 μm, sampling depth 13 nm

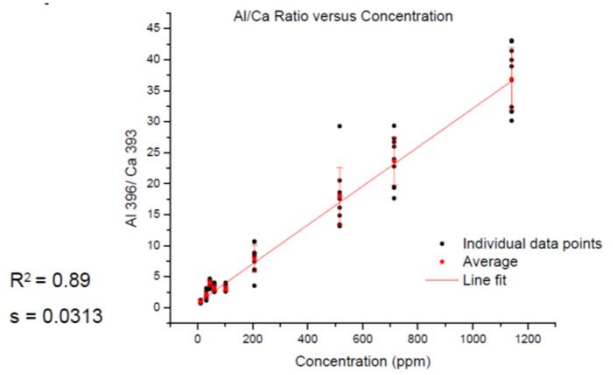
Courtesy of Ben Smith

Advantages of LIBS – sensitivity & speed

- Concentrations of 1-100 ppm usually detectable in seconds using a standard LIBS apparatus







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So why?

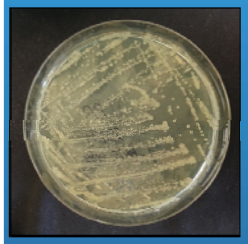
"It is well-accepted that the microbiological expertise and cost required to perform these identifications preclude their common use as a screening mechanism to prevent human infection."[1]

[1]Tarr, P.I. 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. 20, 1-8.

Current Method of Bacterial identification

Our Method of Bacteria Classification

Bacteria is cultured using trypticase soy agar (TSA).

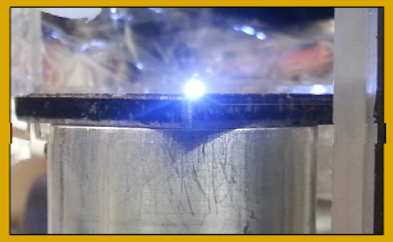


Colonies are removed and placed in 1.5 mL distilled water.

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.

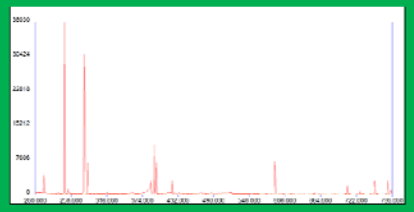
Filter is placed in an argon environment and ablated using a pulsed 1064 nm Nd: YAG laser.



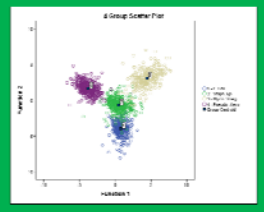
Average time to complete bacterial classification = 1 hour

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

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

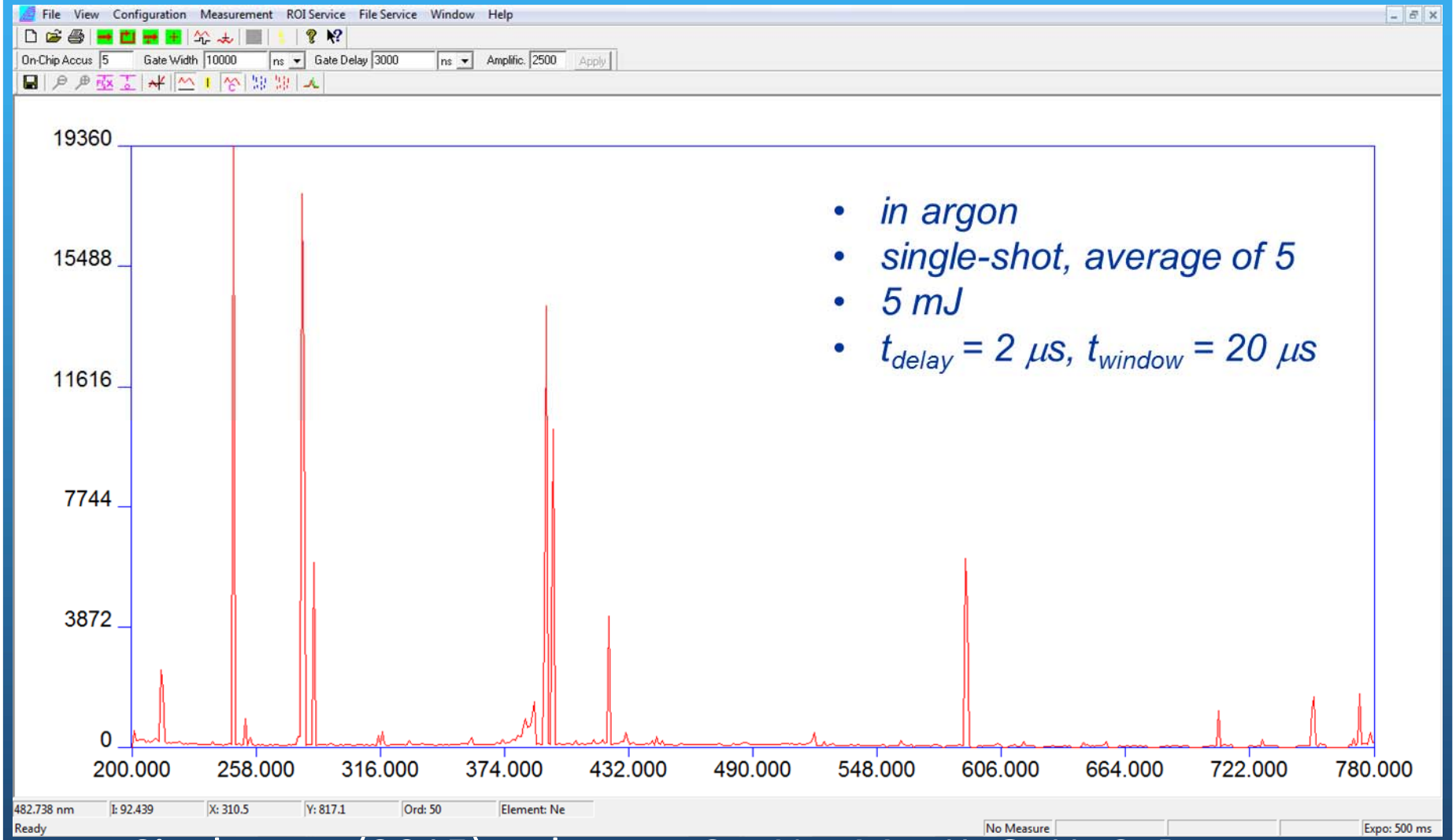


Atomic composition is used to discriminate bacteria against pre-existing library.



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

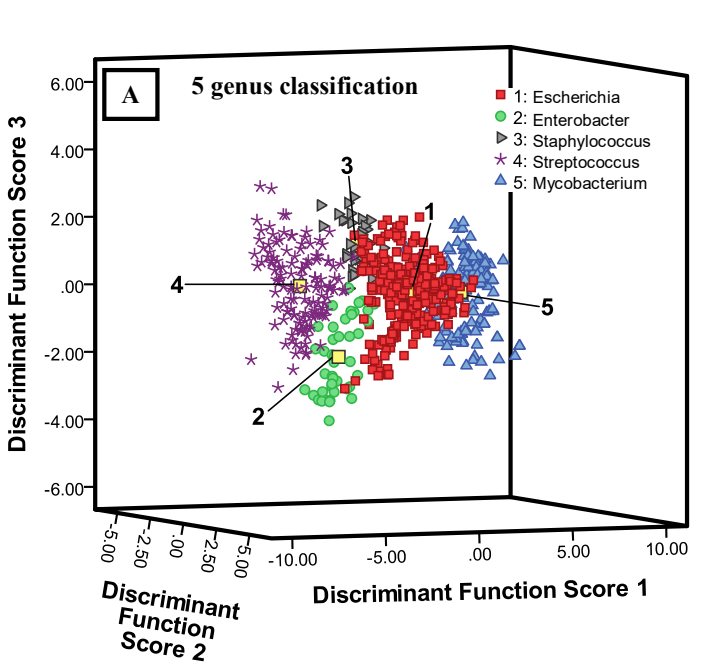
Variable Down Selection



- Sivakumar (2015) only sees Ca, Na, Mg, K, O, H, C, P
- We see Ni, Fe, Ti only when contaminated!

How unique is "unique"?

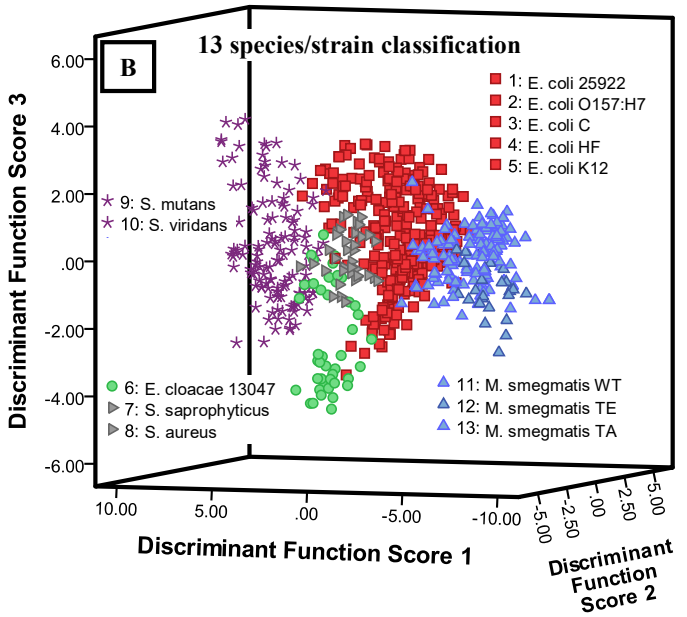
- ✓ We can identify a bacterial species, certainly its genus, with high sensitivity and specificity (confirmed by others).
- ✓ We can differentiate strains of *E. coli* (demonstrated by others in MRSA).
- ✓ Multiple multivariate techniques effective at discriminating spectra.



PLSDA			DFA		
E. COLI	True	False	*E. COLI*	True	False
Positive	95.65%	9.17%	Positive	89.63%	15.95%
Negative	90.83%	4.35%	Negative	84.05%	10.37%
STAPHYLOCOCCUS	True	False	*STAPHYLOCOCCUS*	True	False
Positive	54.05%	0.51%	Positive	86.49%	5.85%
Negative	99.49%	45.95%	Negative	94.15%	13.51%
STREPTOCOCCUS	True	False	*STREPTOCOCCUS*	True	False
Positive	95.59%	1.02%	Positive	99.26%	13.32%
Negative	98.98%	4.41%	Negative	88.68%	0.74%
MYCOBACTERIUM	True	False	*MYCOBACTERIUM*	True	False
Positive	88.31%	1.06%	Positive	96.10%	4.08%
Negative	98.94%	11.69%	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…

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

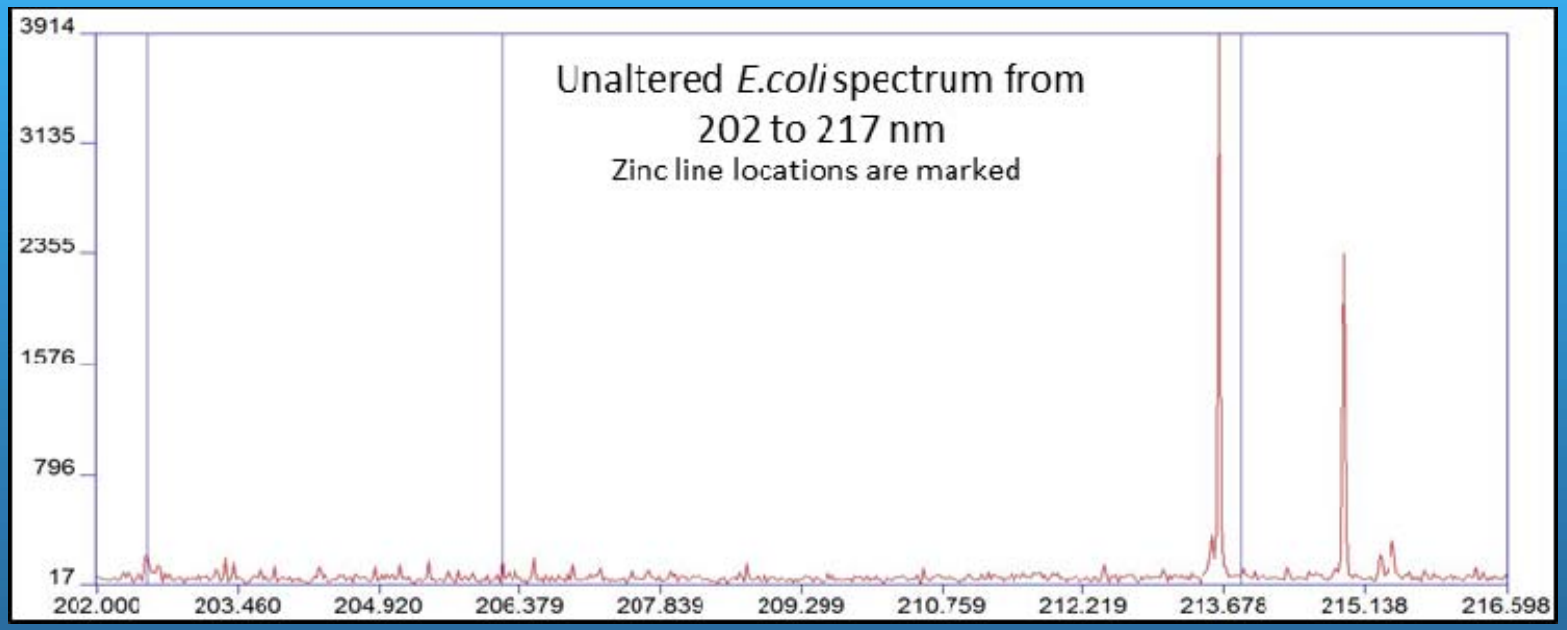
Results: We have already demonstrated…

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

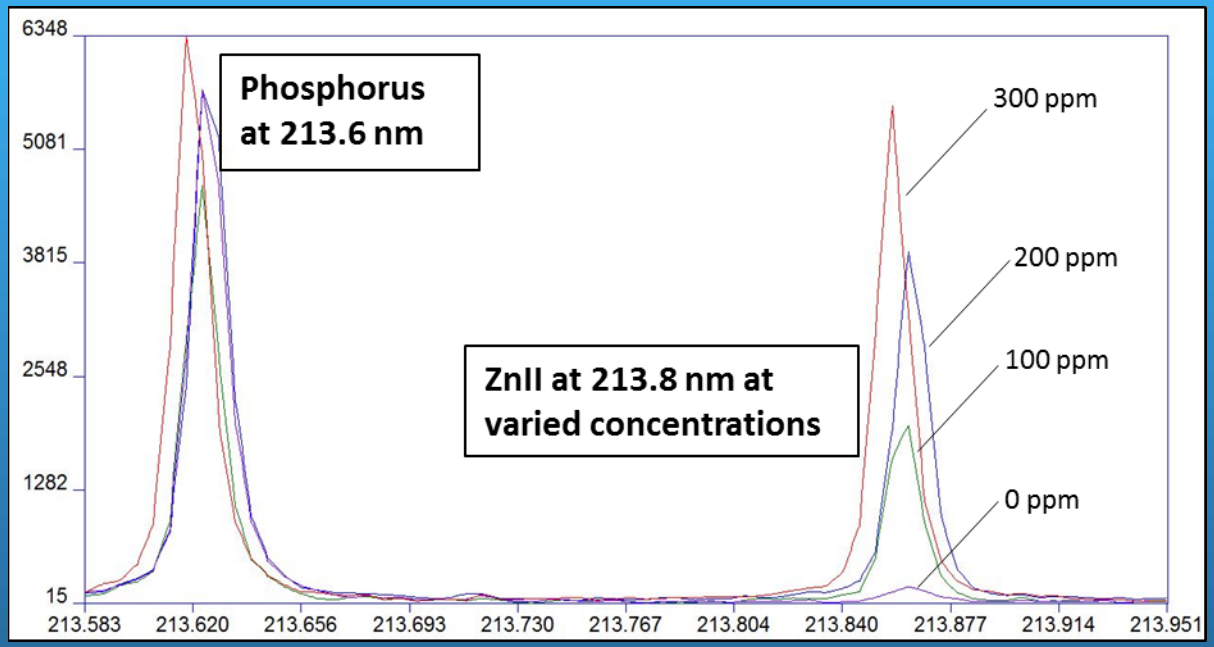
8 publications in Applied Physics Letters, Journal of Applied Physics, Applied Optics, Applied Spectroscopy, Spectrochimica Acta B, and others – confirmed by multiple other groups

Altering Cell Metal Content: Zinc



Zinc lines are not distinguishable from noise at normal growth conditions

Altering Cell Metal Content: Zinc



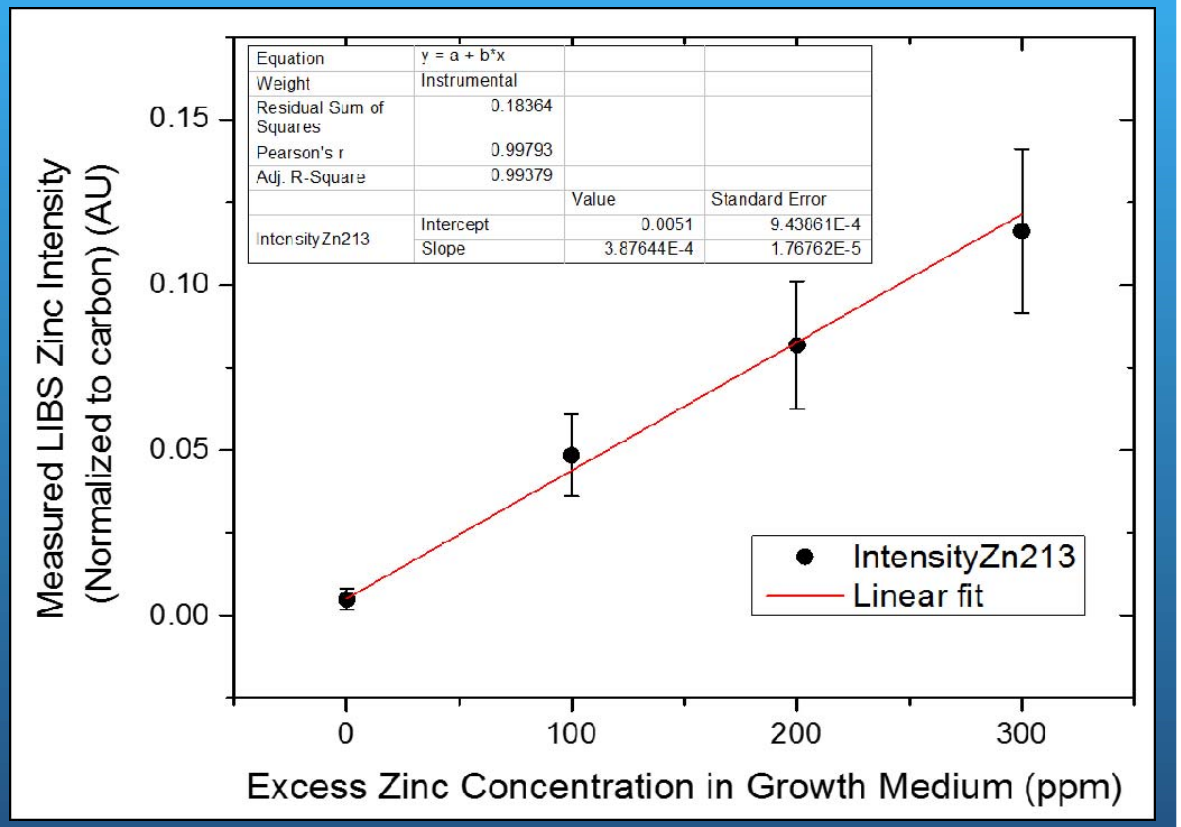
When zinc is added to the *E. coli* growth medium, cellular zinc is observed

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

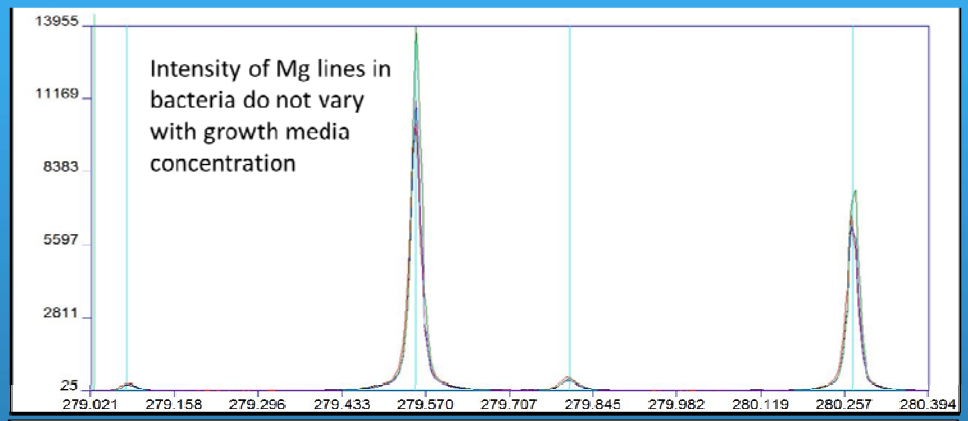
Since bacterial species take their nutrients from their environment, bacteria have been used as an indicator of environmental health, with trace metals in the cells being indicative of contamination of a water supply.

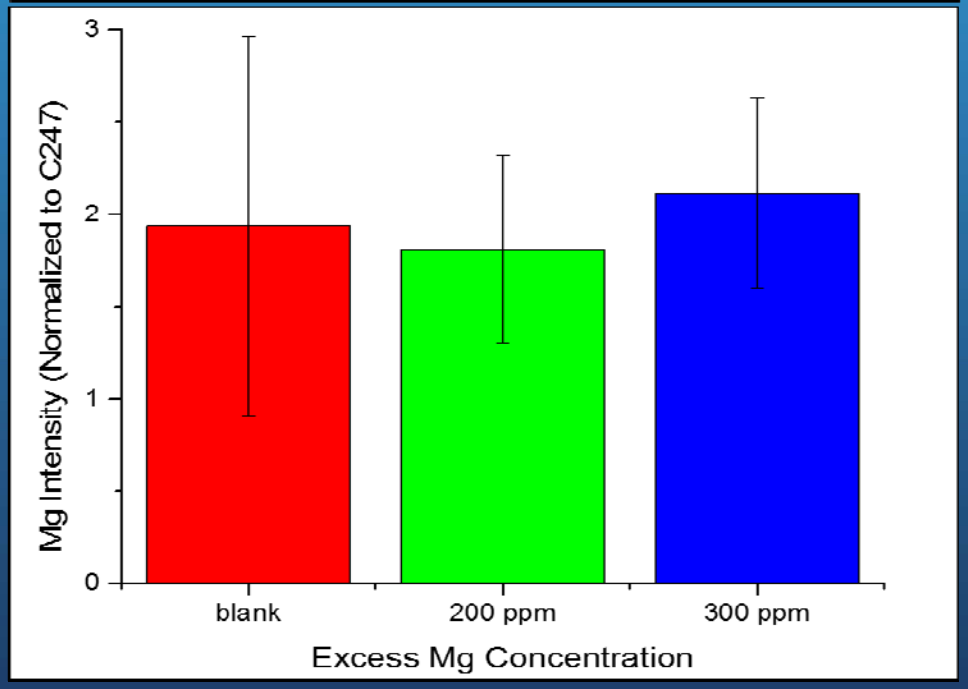
Altering Cell Metal Content: Magnesium

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

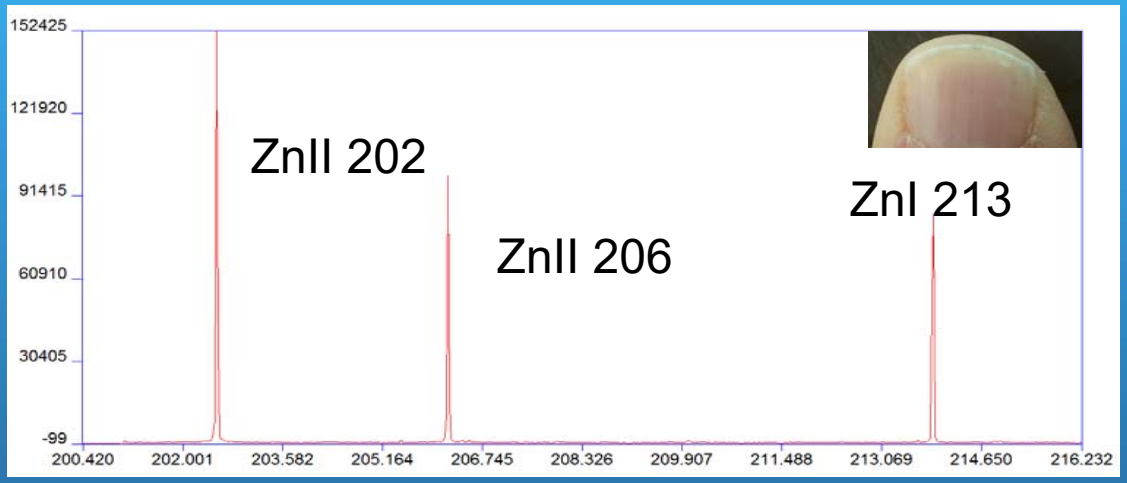
The deviation in intensity reduced as the surplus increased.

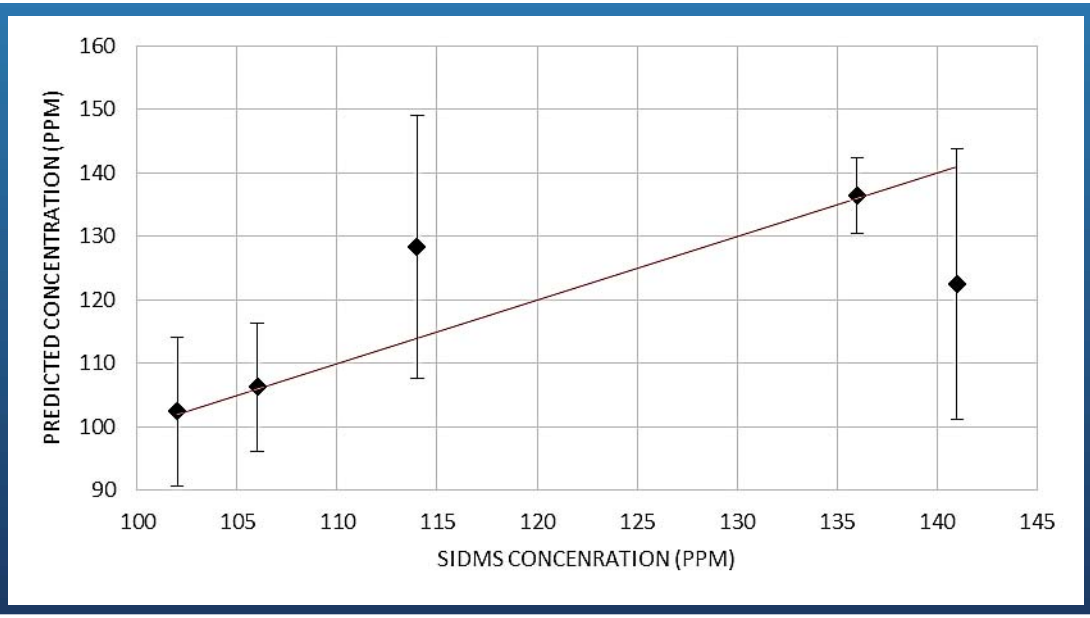
A sample was prepared wherein Mg was precipitated out of the agar solution using HCl prior to autoclaving. This plate provided no bacterial growth.





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.

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

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- *University of Windsor* Faculty of Science

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Allie Paulick

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Vlora Riberdy

Siddharth Doshi

Derek Gillies

Chris Heath

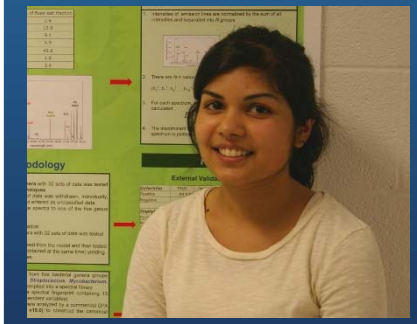
Beau Greaves

Erica Rustico

Courtney Jones

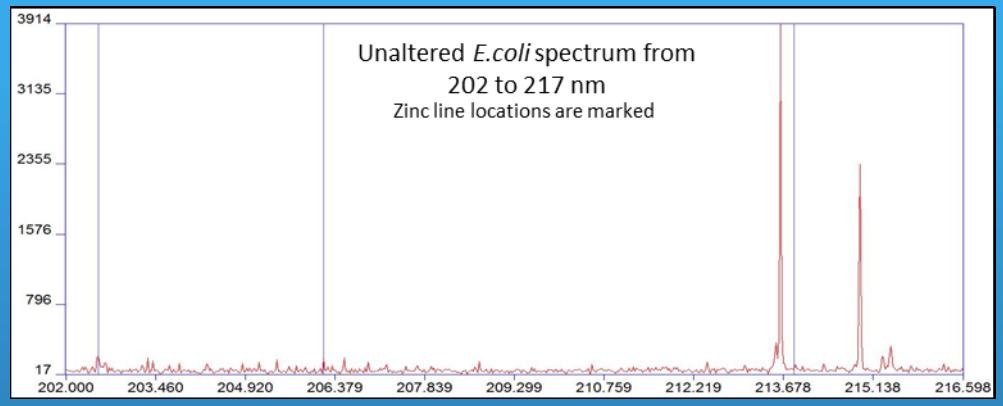
Russell Putnam

Khadijia Sheikh



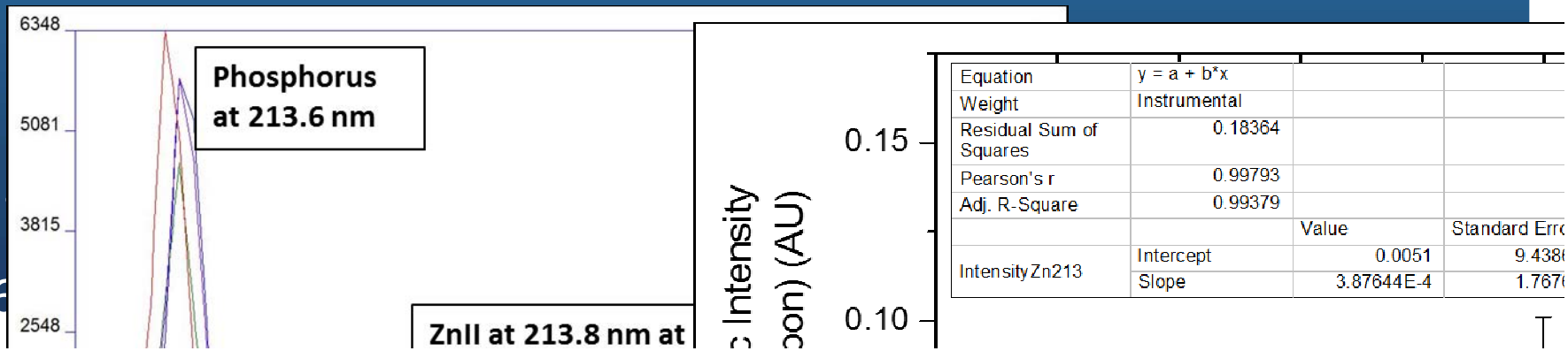
Paul Dubovan

Altering Cell Metal Content: Zinc



Zinc lines are not distinguishable from noise at normal growth conditions

- Cl
- 16



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- 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.

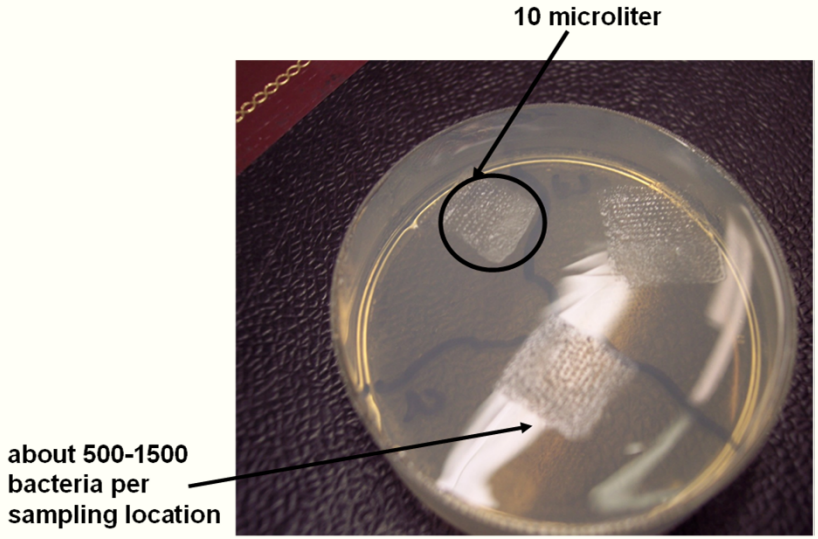
2011 Bacterial LIBS spectra can be obtained from killed (via autoclaving) or inactivated (via UV light) specimens, and such treatment (which renders the specimen completely safe for handling) does not decrease identification specificity and does not decrease LIBS spectral intensity.

2013 Heat killed bacteria are differentiable (*Multari et al., ARA*)

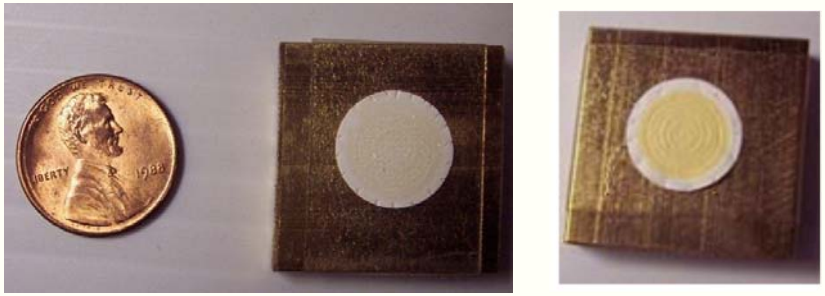
2015 Inactivation by sonication / autoclaving is differentiable (*Sivakumar et al., Delaware State*) with fs-LIBS & ns-LIBS. Changes in spectral intensity were observed.

New Bacteria Testing Procedure

Previously: mounted on agar



Currently: mounting on nitrocellulose bacteriological filter

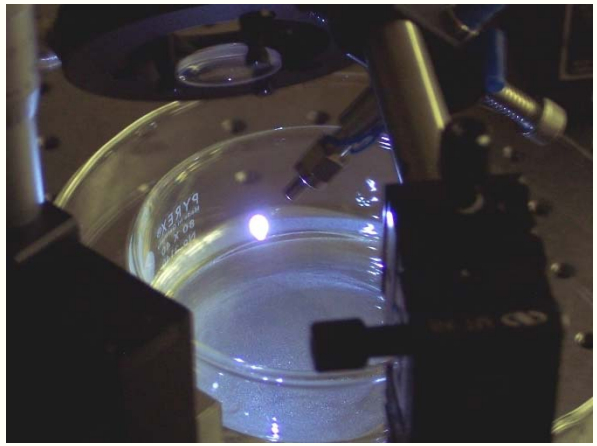


✓ Advantages: Flatter. Easier to do. More reproducible. Less time.

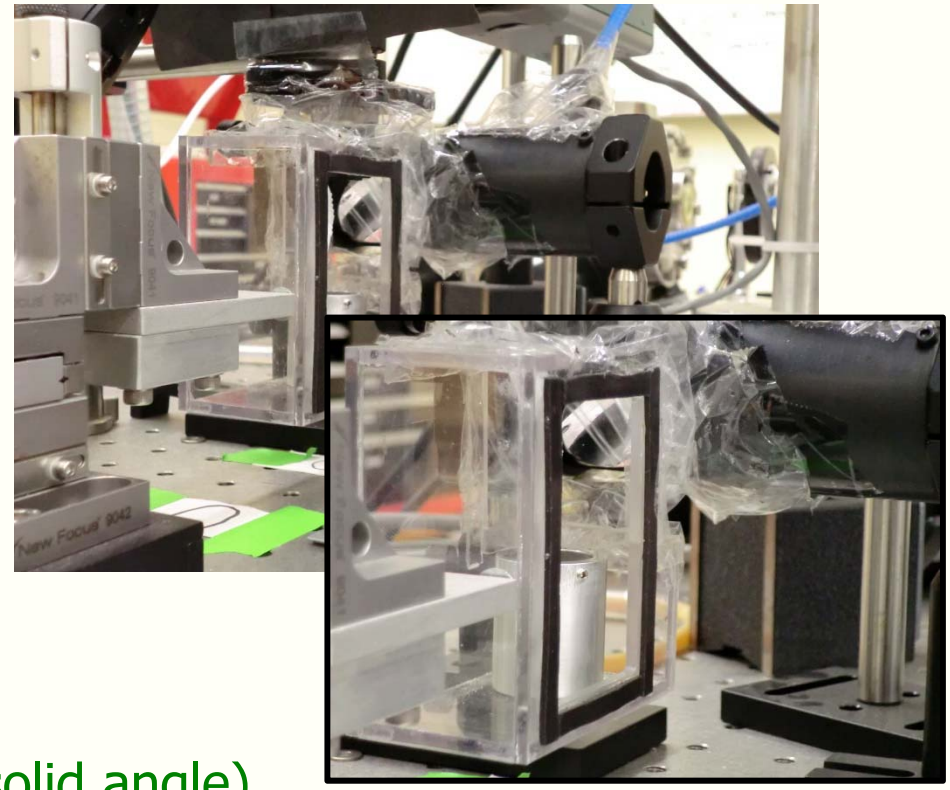
x Disadvantages: Carbon background.

New Bacteria Testing Procedure

Previously: light collected by fiber



Currently: using matched parabolic reflectors into fiber



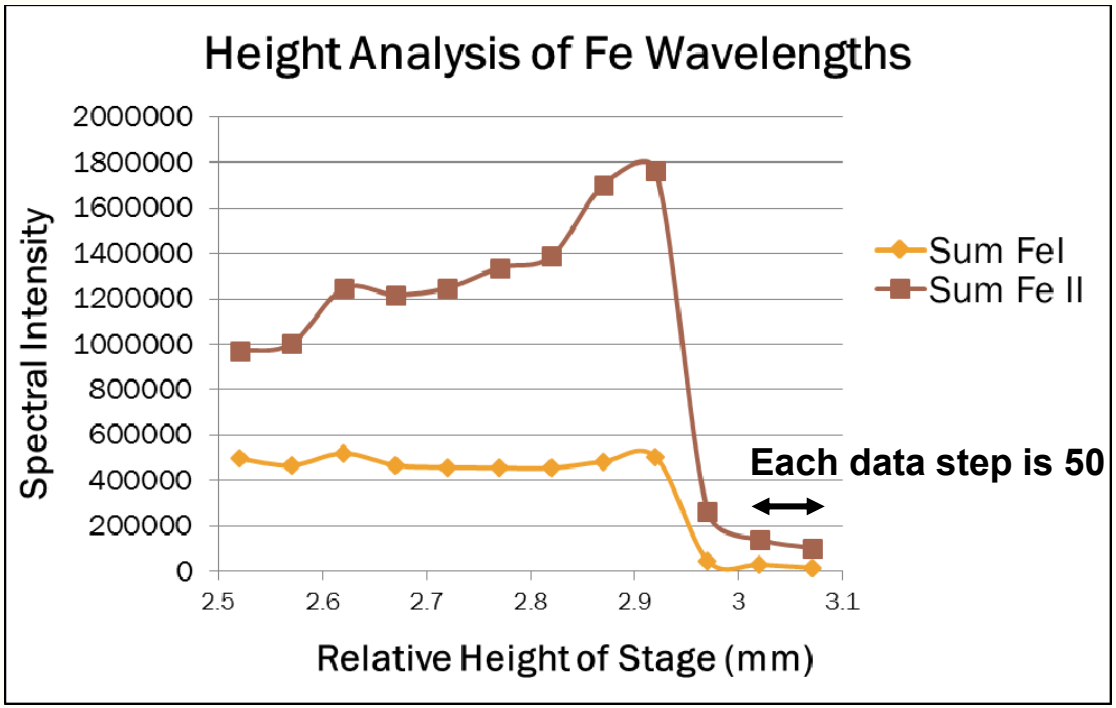
✓ Advantages: More light collected (solid angle)

x Disadvantages: More sensitive to vertical position

New Bacteria Testing Procedure

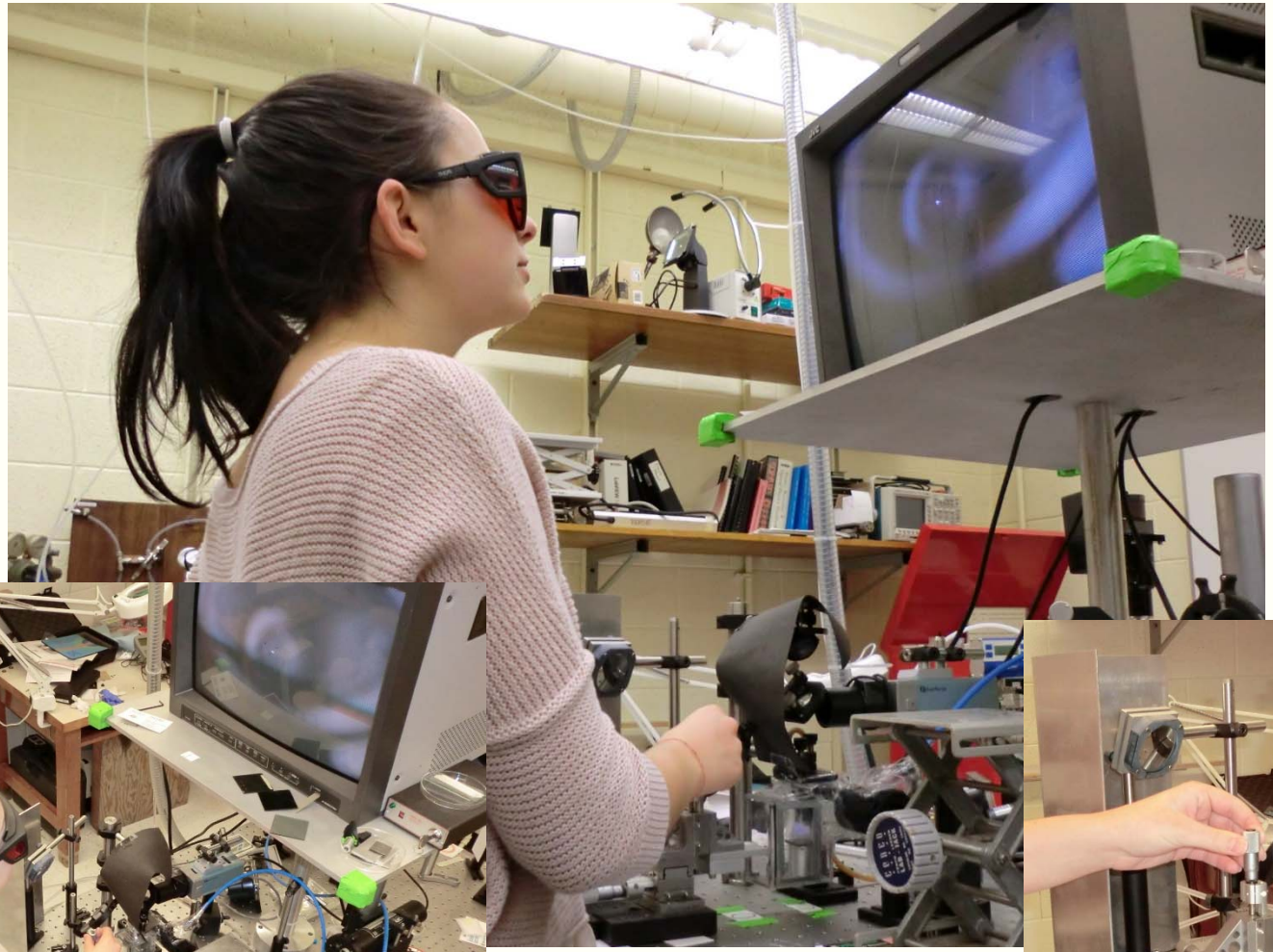
Previously: proper LTSD found by trial and error

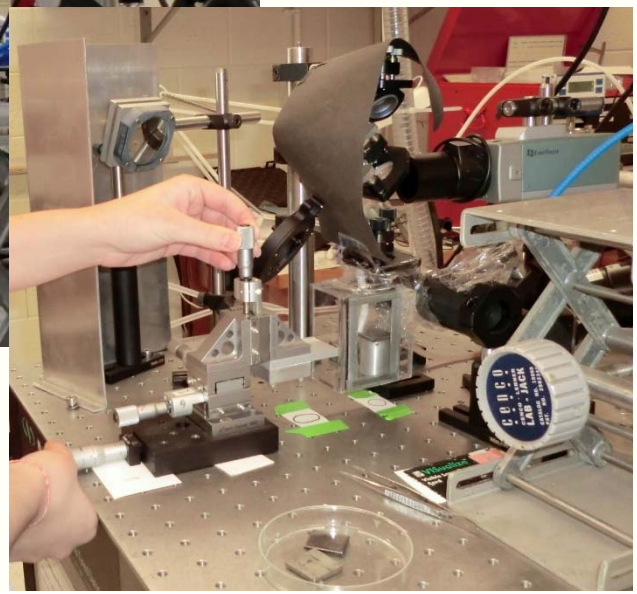
Currently: Appropriate LTSD/focus found with laser indicator.
Calibrated steel sample tested every day for intensity & spot size.
Unnormalized intensities very reproducible and controlled.



Increasing LTSD

Student-Actuated Focus Finder

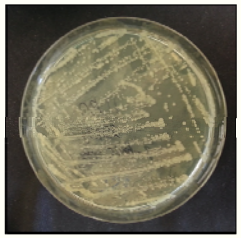




Current Method

Our Method of Bacteria Classification

Bacteria is cultured using trypticase soy agar (TSA).

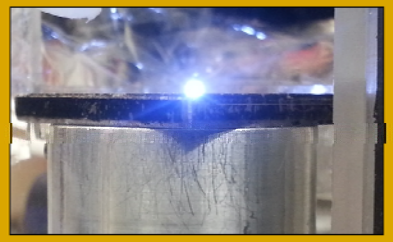


Colonies are removed and placed in 1.5 mL distilled water.

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

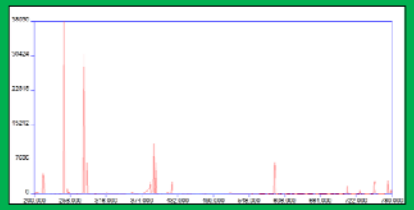
Colloidal solution is dried forming a bacteria lawn on the clinician-friendly filter.

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

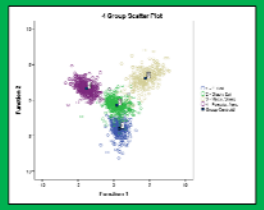


Average time to complete bacterial classification = 1 hour

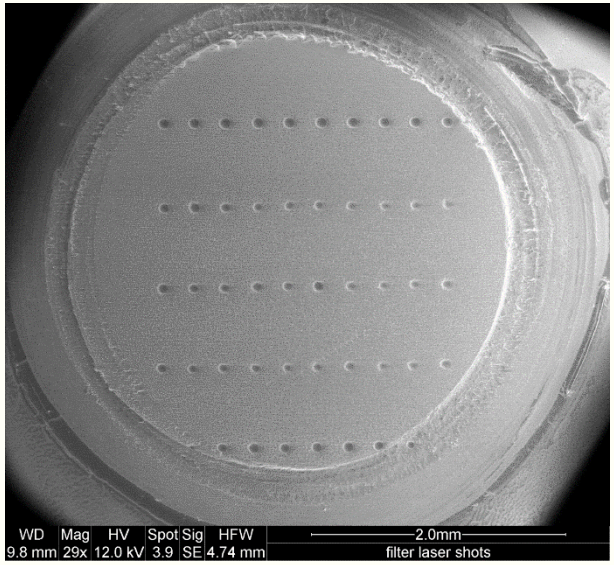
Échelle diffraction grating spectrometer is used to obtain the atomic spectrum and composition of sample.

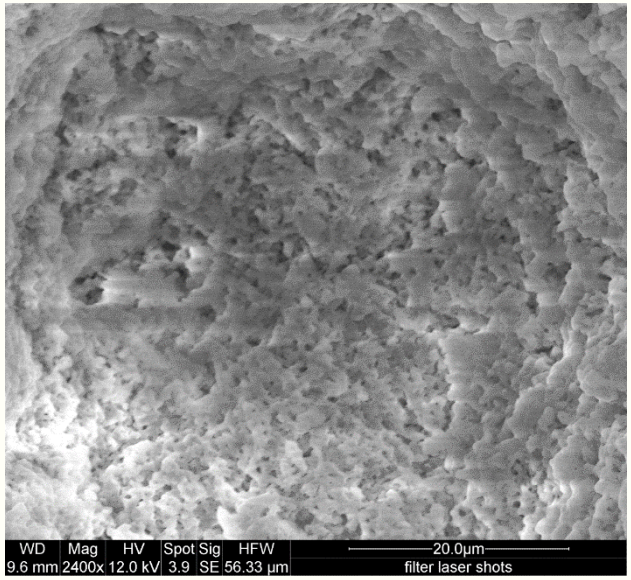


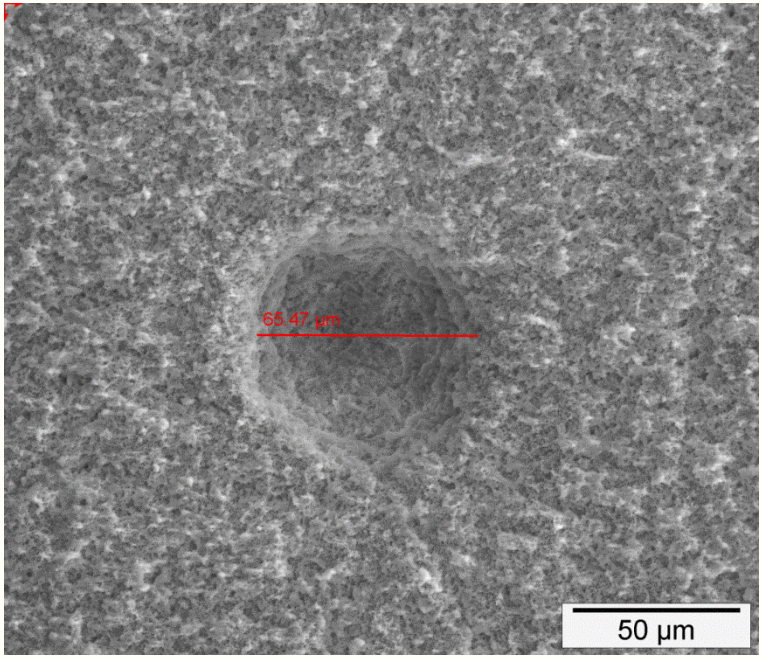
Atomic composition is used to discriminate bacteria against pre-existing library.



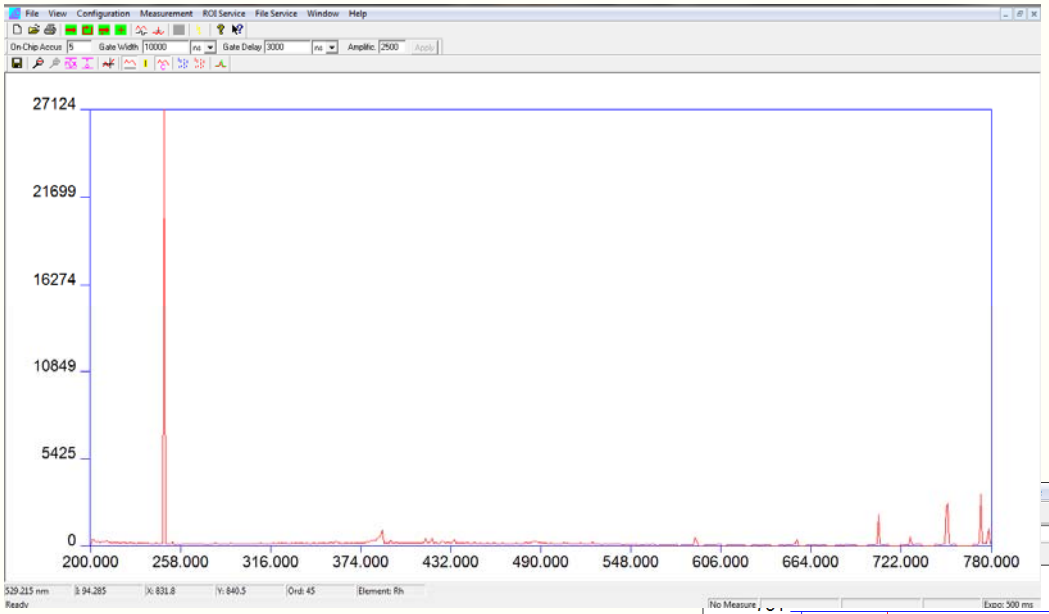
Filter

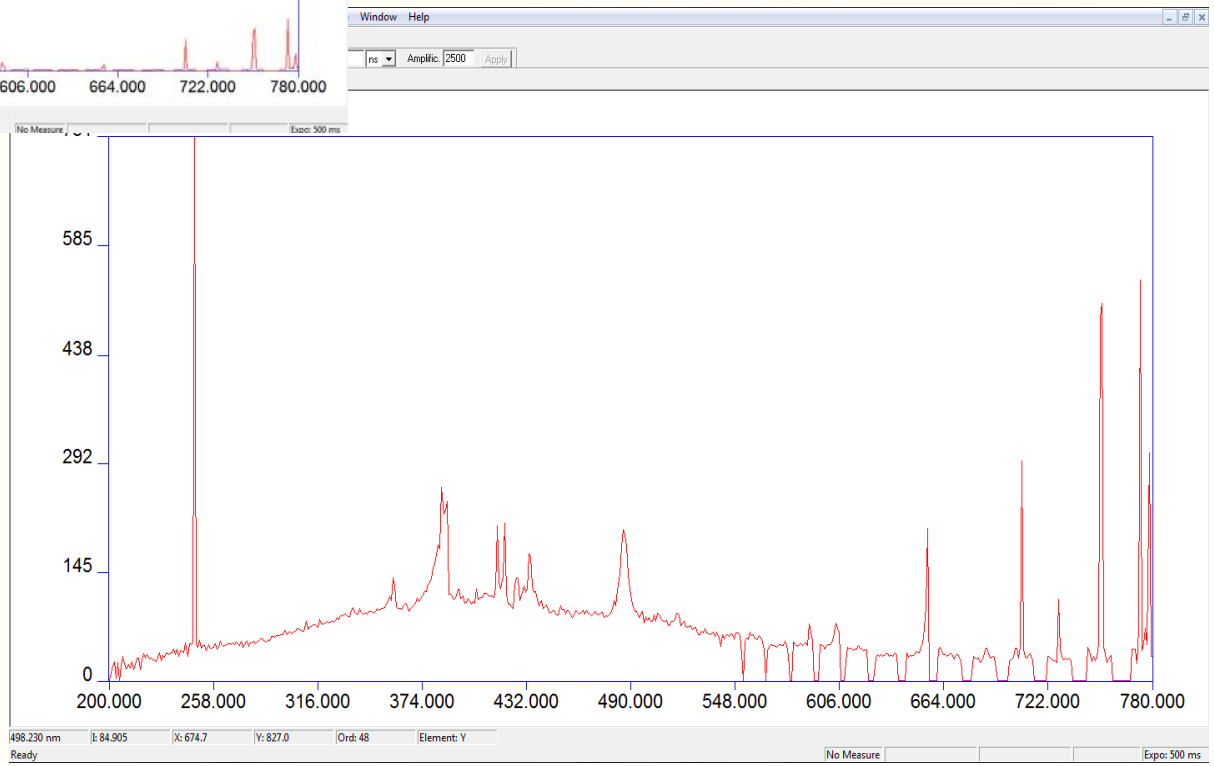

WD Mag HV Spot Sig HFW
9.8 mm 29x 12.0 kV 3.9 SE 4.74 mm
2.0mm
filter laser shots


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

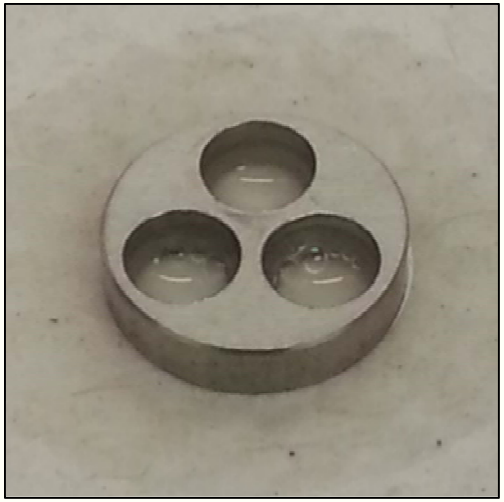
Filter

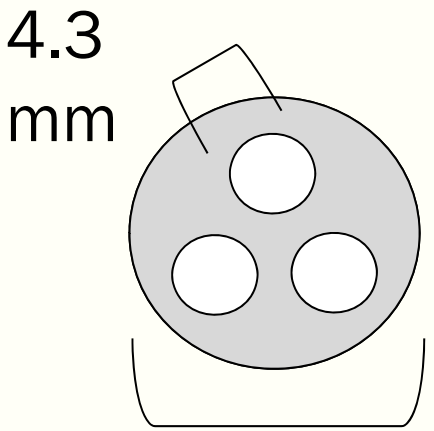

27124
21699
16274
10849
5425
0
200.000
258.000
316.000
374.000
432.000
490.000
548.000
606.000
664.000
722.000
780.000


585
438
292
145
0
200.000
258.000
316.000
374.000
432.000
490.000
548.000
606.000
664.000
722.000
780.000
498.230 nm
I: 84.905
X: 674.7
Y: 827.0
Ord: 48
Element: Y
Ready
No Measure
Expo: 500 ms

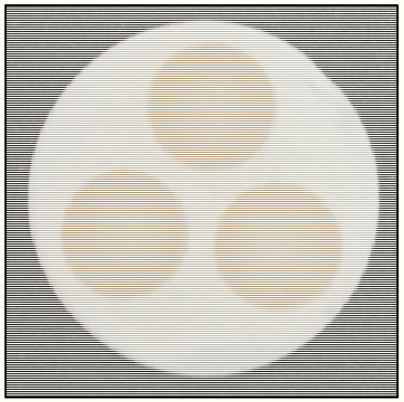
Can bacteria be deposited in a controlled manner?

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



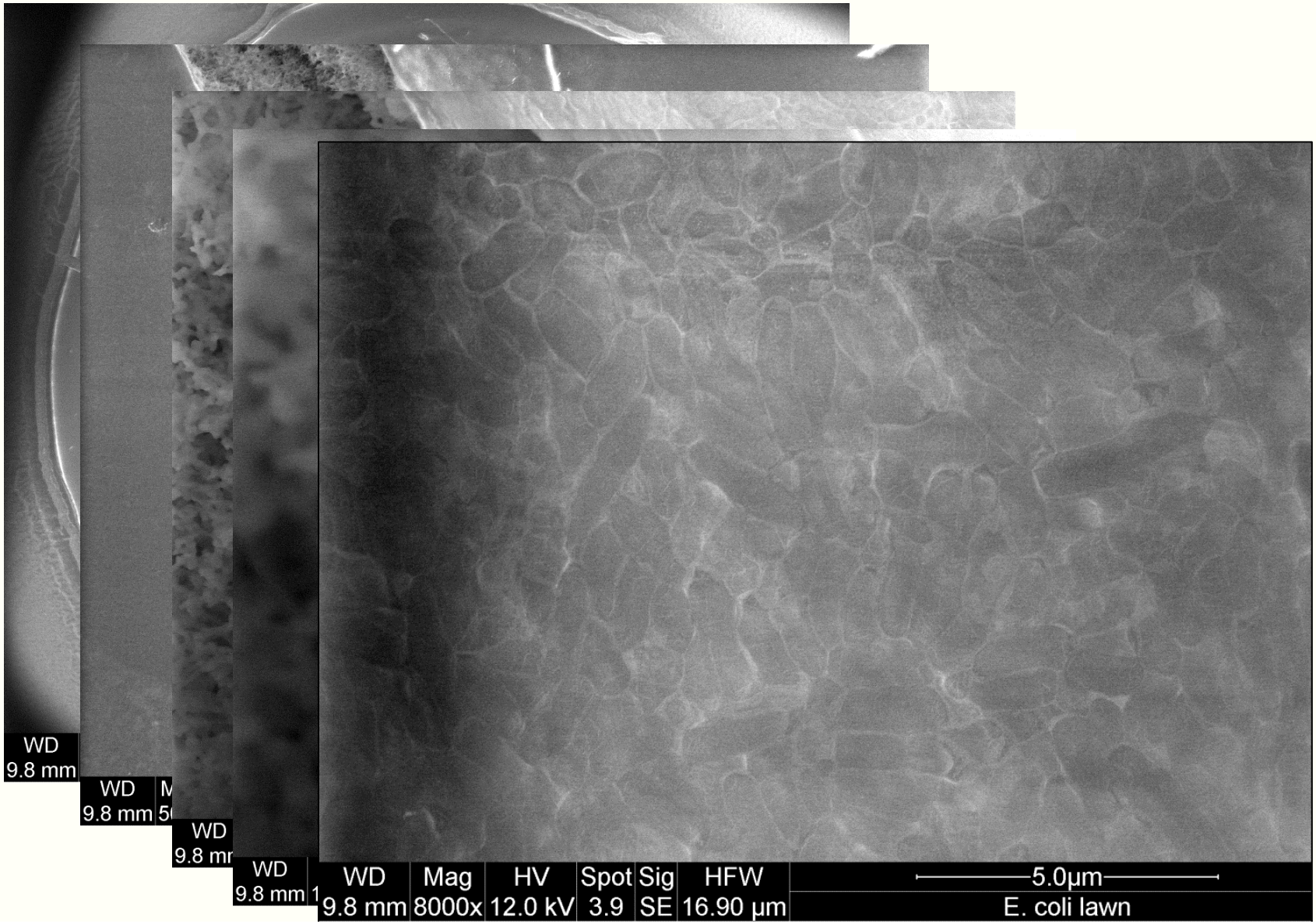


Scanning electron microscopy images verified complete coverage of the deposited bacterial lawn.

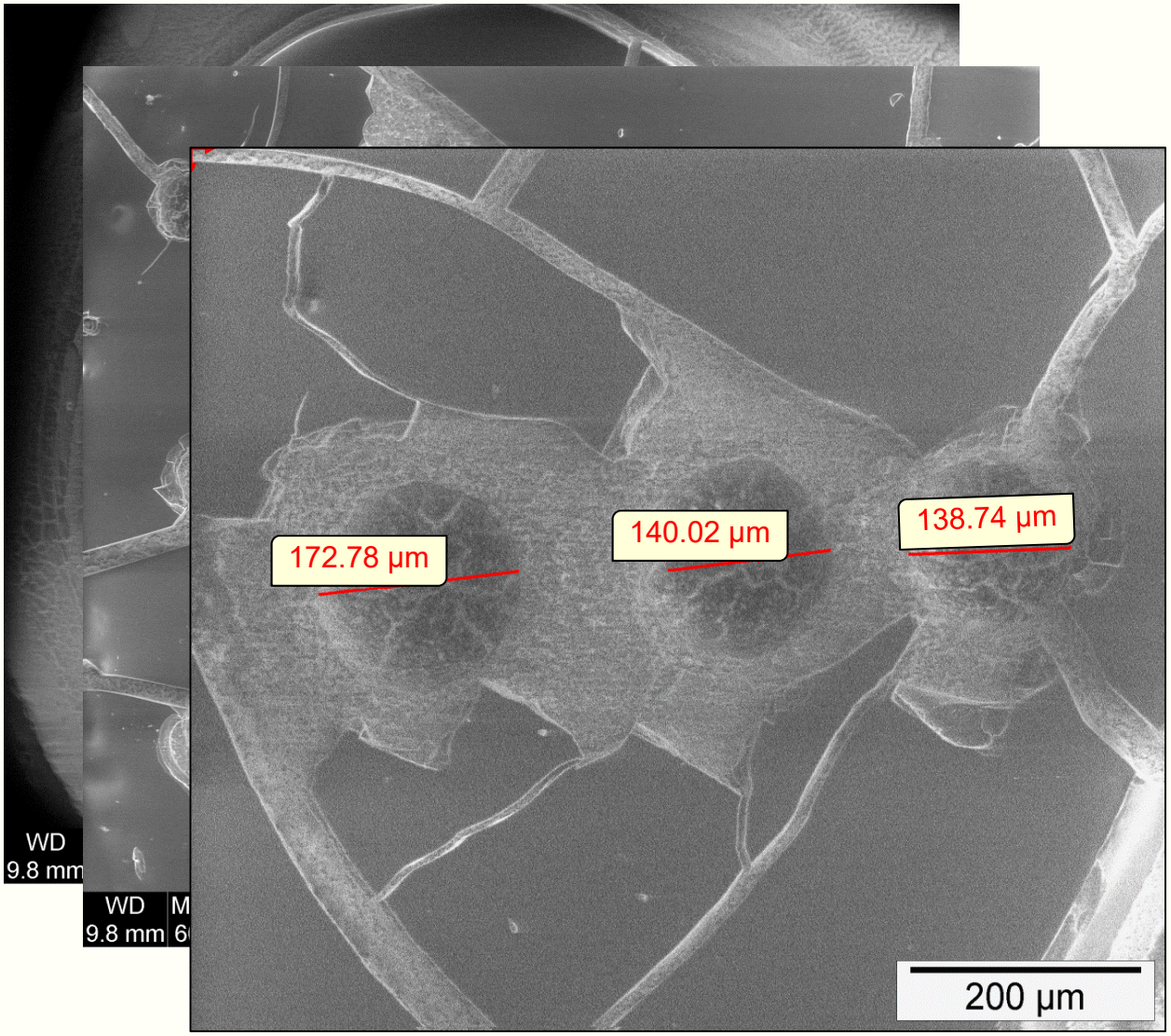


Can bacteria be deposited in a controlled manner?

Can bacteria be deposited in a controlled manner?

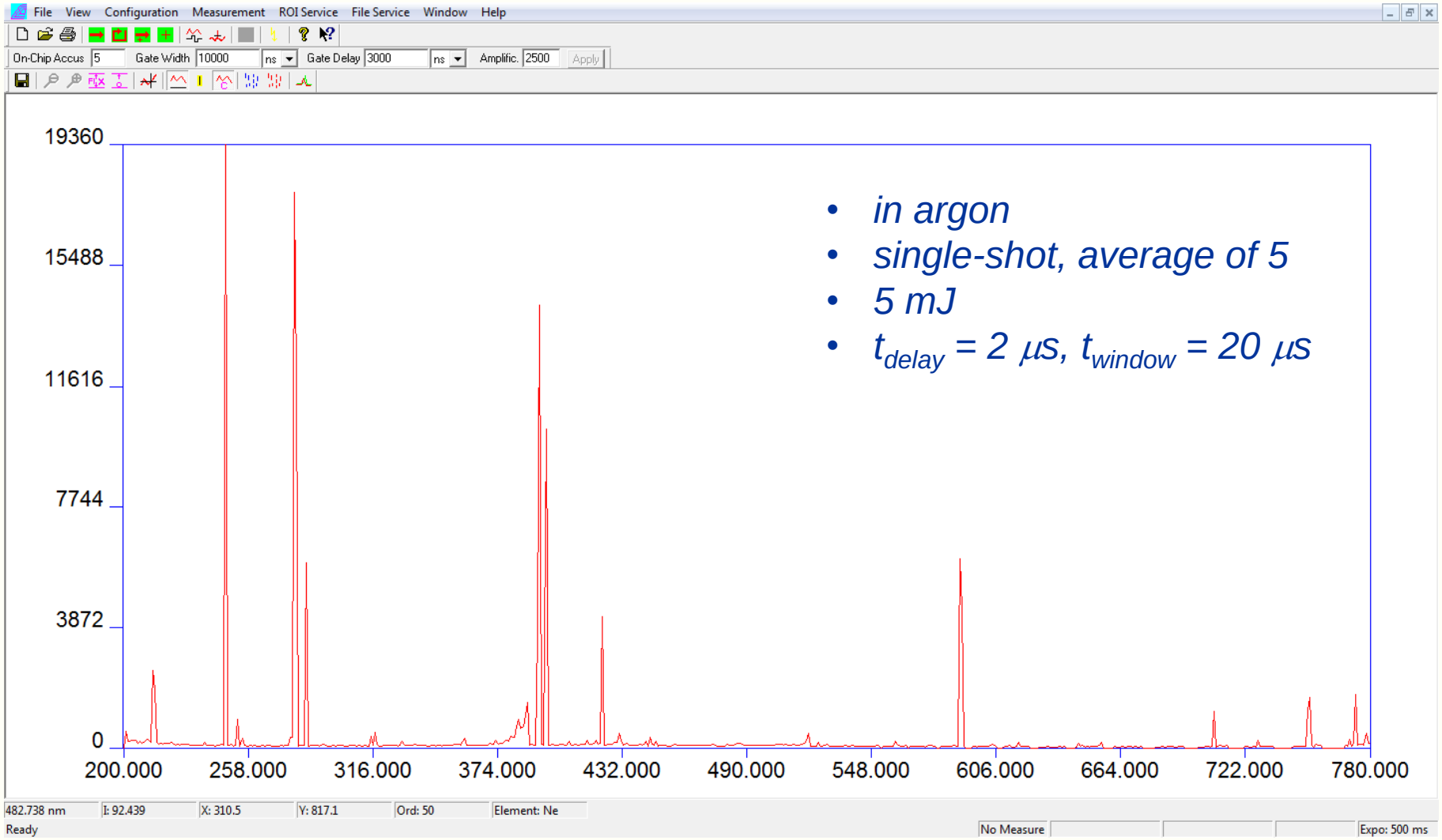


Can laser ablate in a controlled manner?

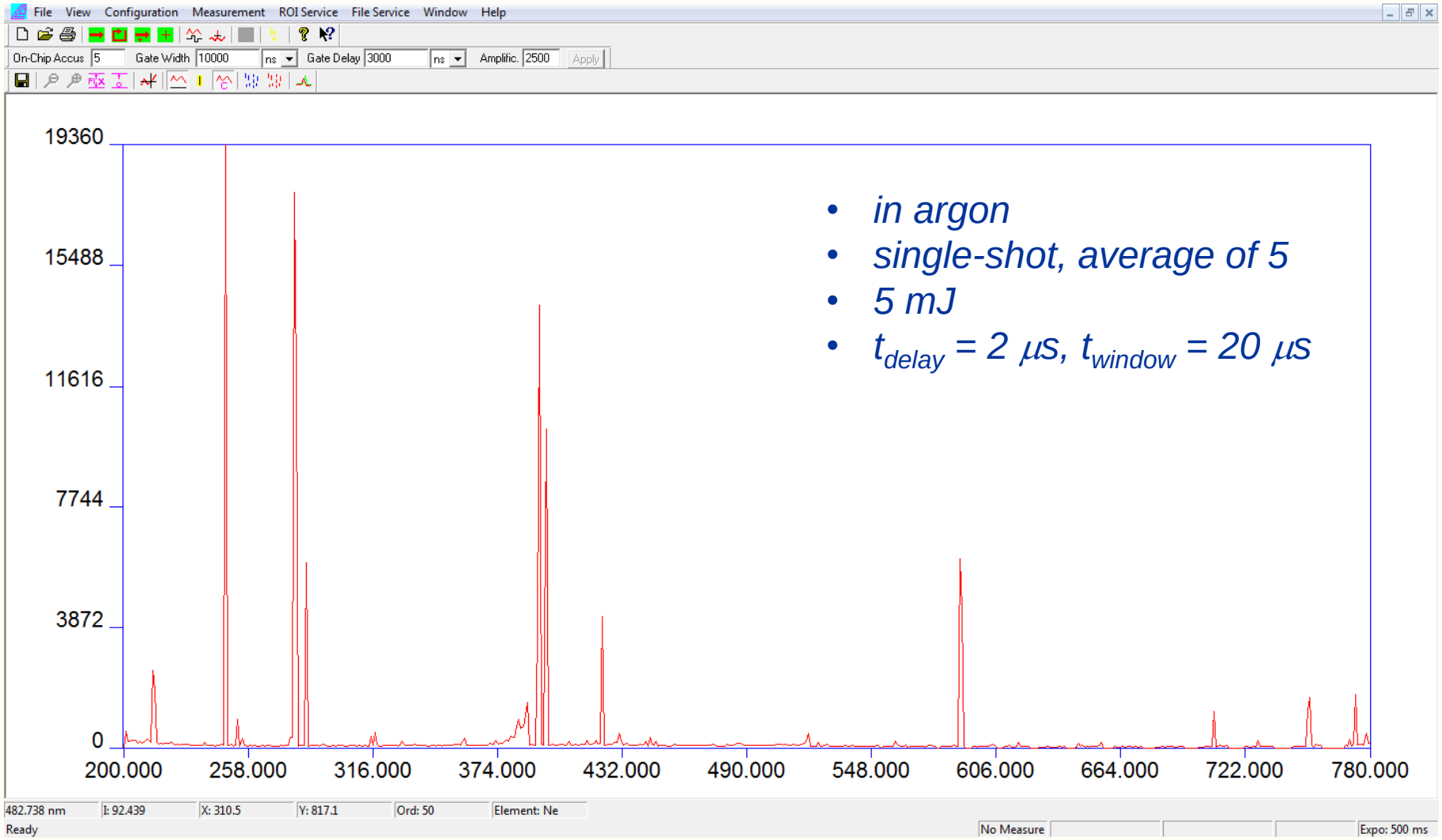


With crater diameters of about 150 µm, the quantity of cells vaporized for each spectrum was estimated at 10^6 (verified using optical densitometry.

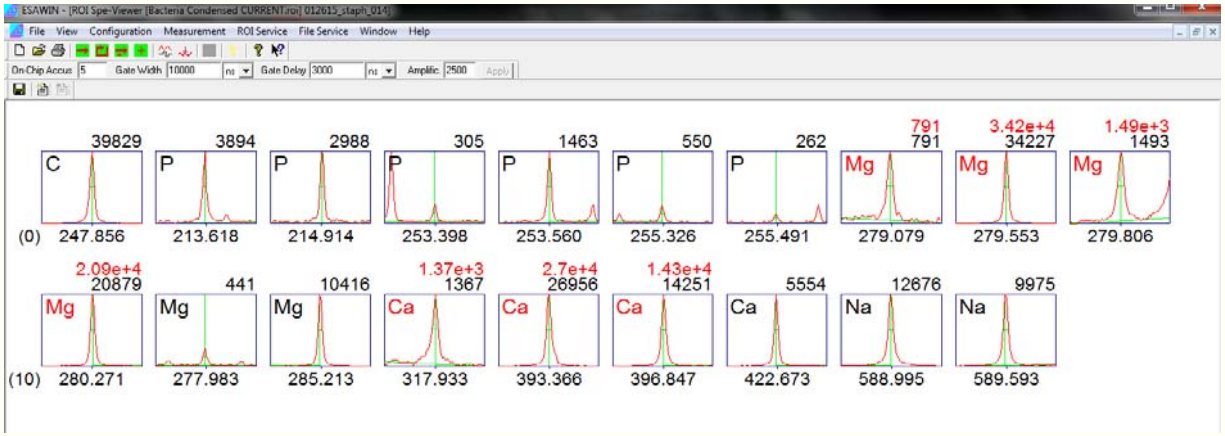
Typical Bacterial Spectrum



Typical Bacterial Spectrum

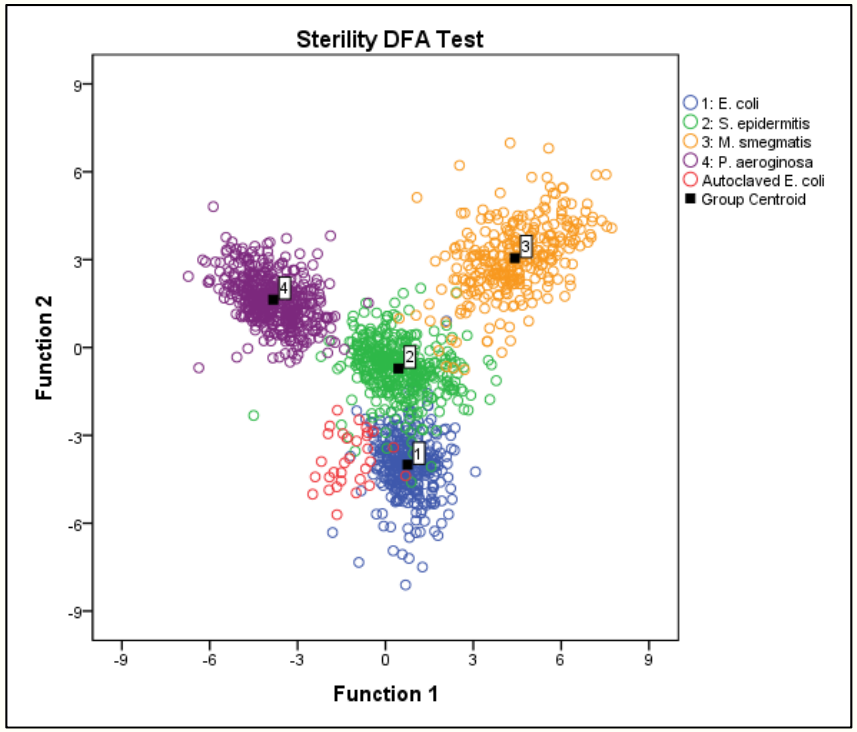


Variable Down-Selection



- 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%

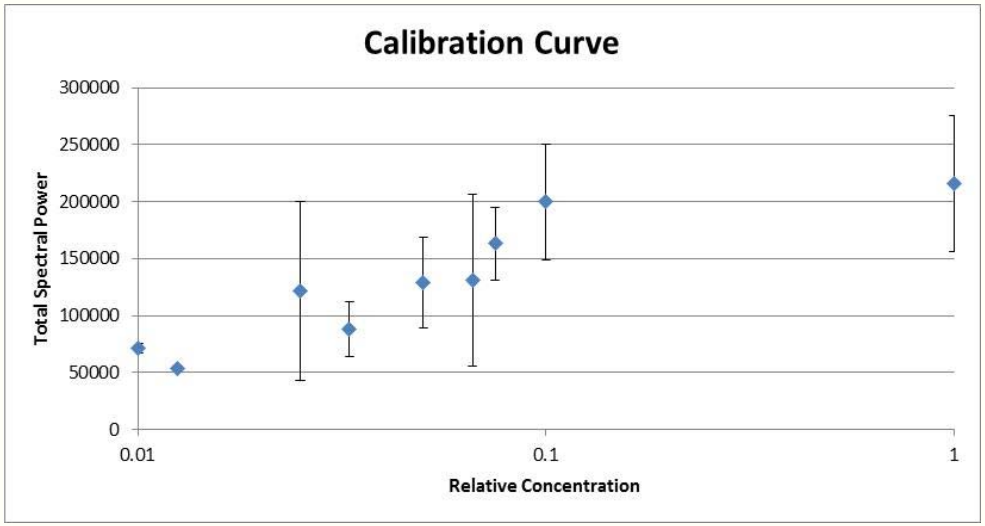
Sensitivity: 97 ± 3% Specificity: 99 ± 2%

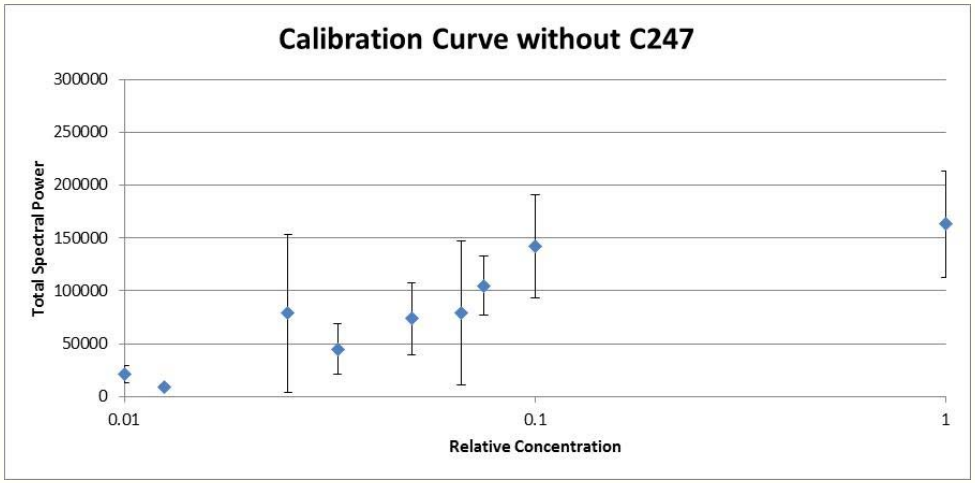
All external validation results

	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

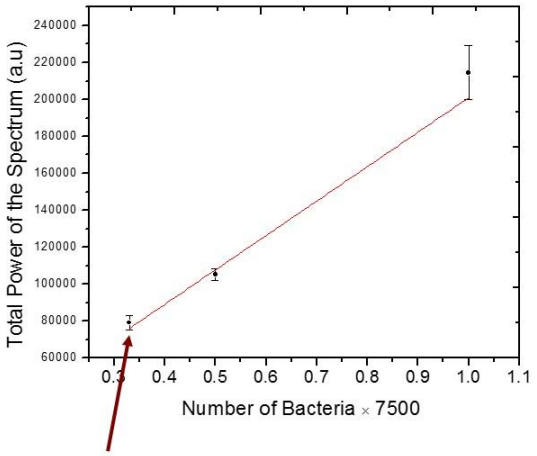
"by filter" means approximately 30 groups in DFA, no relationships between groups assumed

New Concentration Study





Previous result



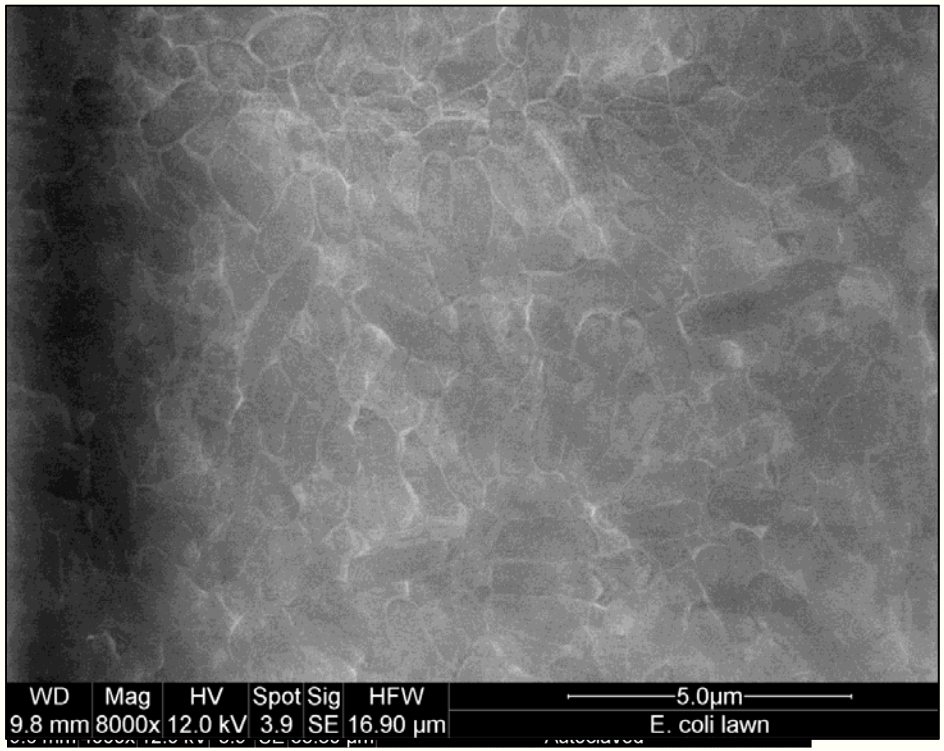
5 laser sampling locations

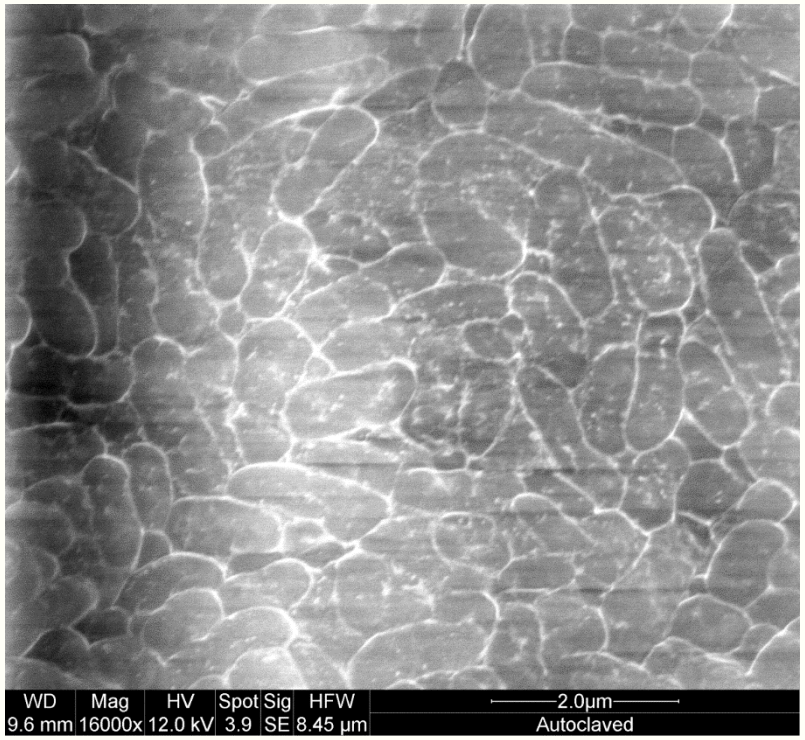
~500 bacteria per locations

- 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

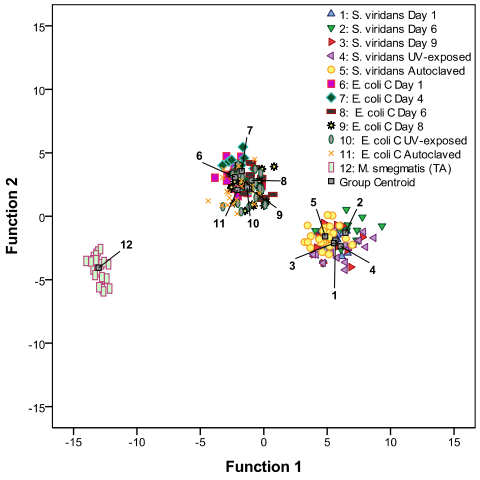
Viability study - Autoclaved

- 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.

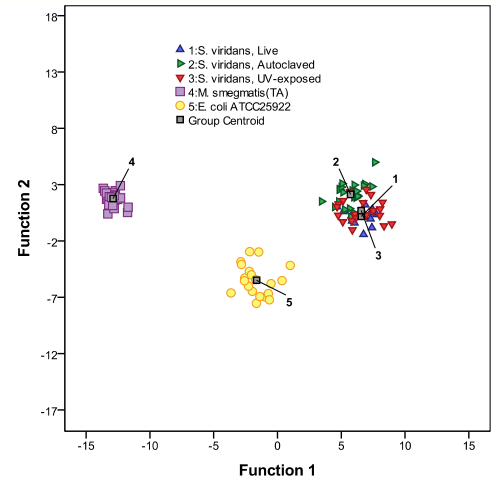




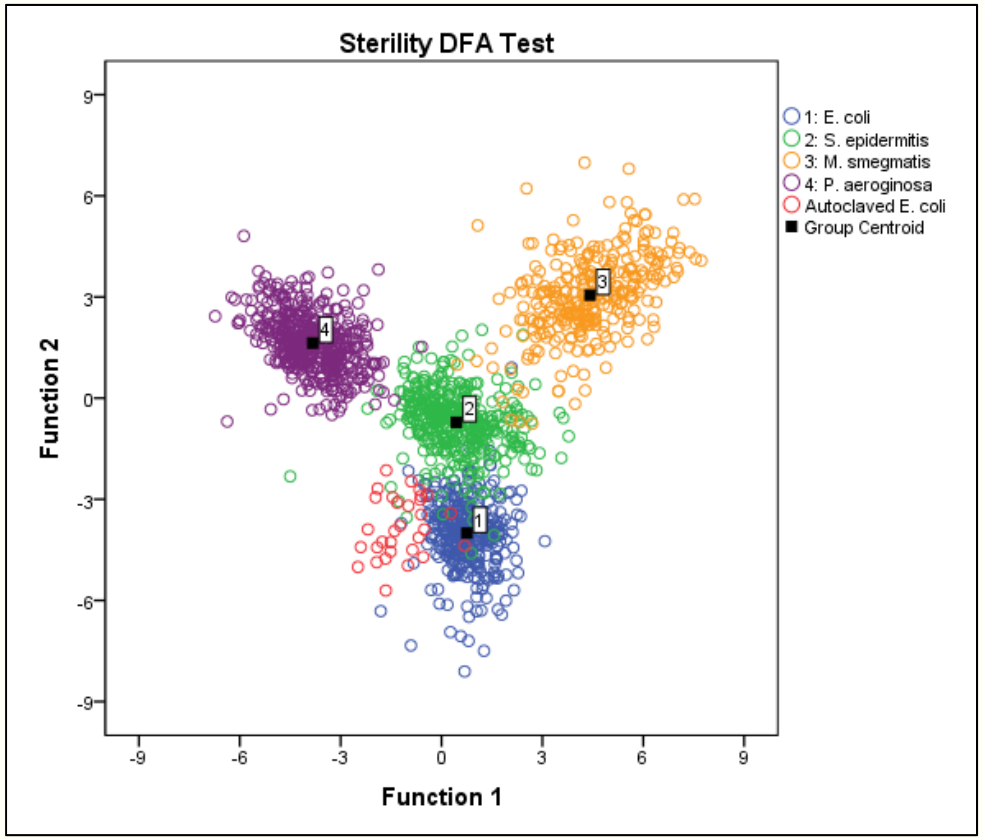
Viability study - Autoclaved



Previous result

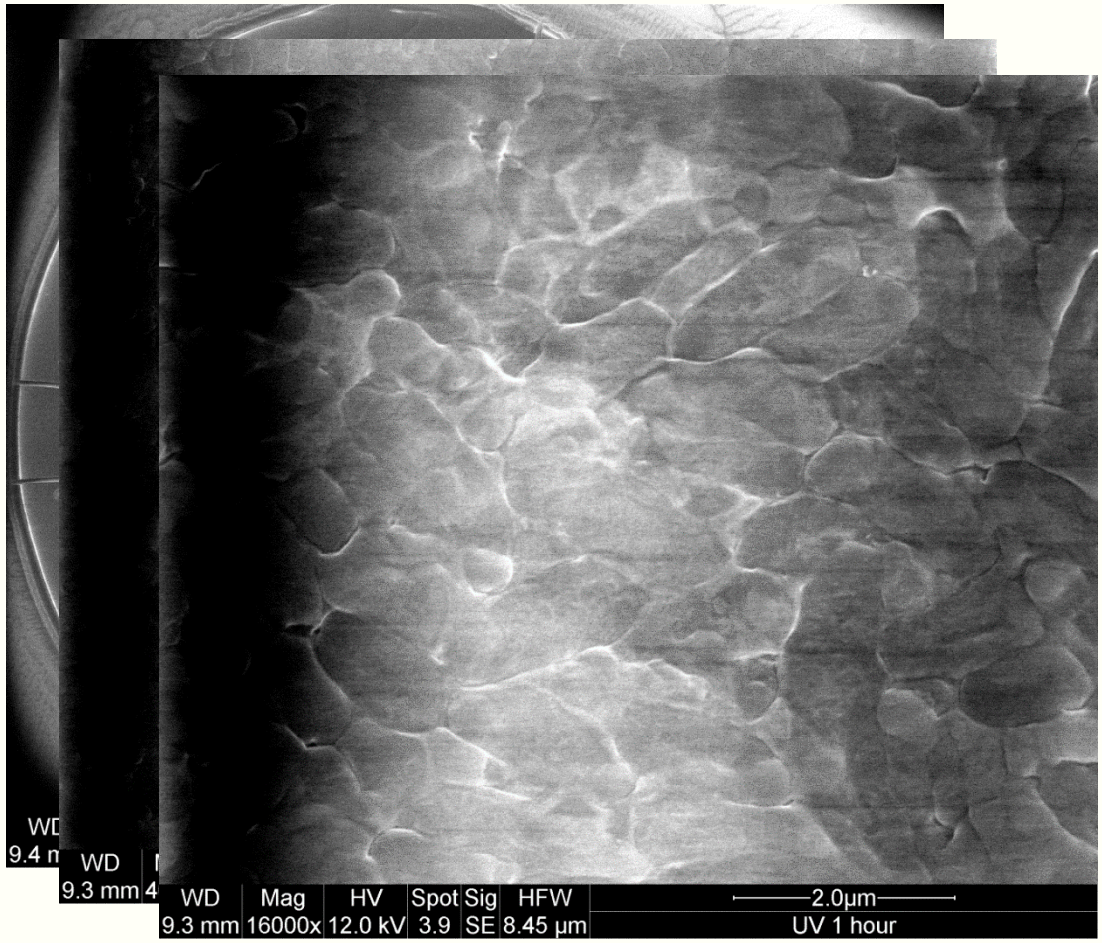


Autoclaved *E. coli* classify >98% as live *E. coli*, yet spectrum seems to be slightly distinct

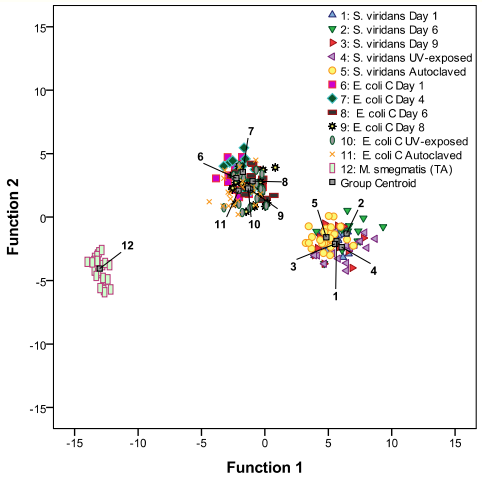


Viability study - UV

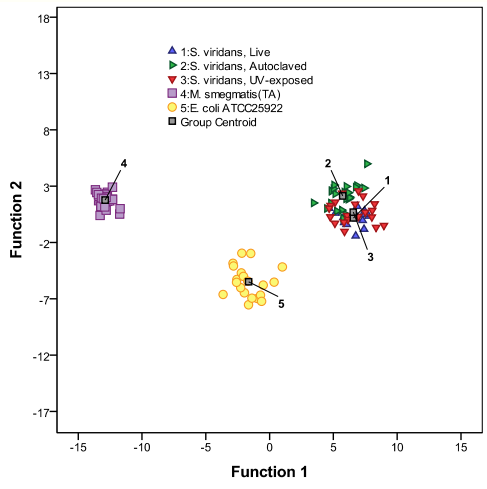
- Live cells were deposited on the filter, the whole filter was then placed under UV light (4 W, 254 nm at about 15 cm from the source) for 1 hr, 30 min, 15 min.
- Saw no difference in signal and they classified as live.
- 30 min was chosen for future experiments.
- UV non-viability confirmed via pressing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



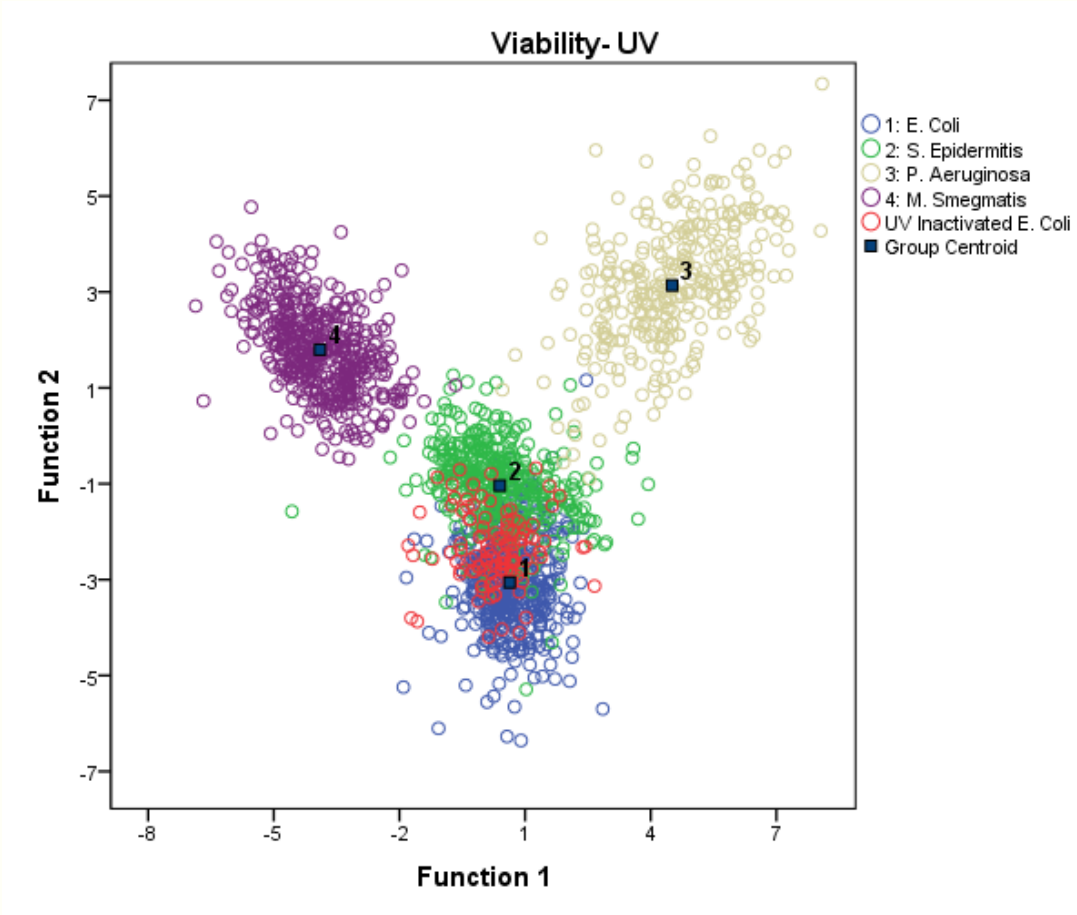
Viability study - UV



Previous result

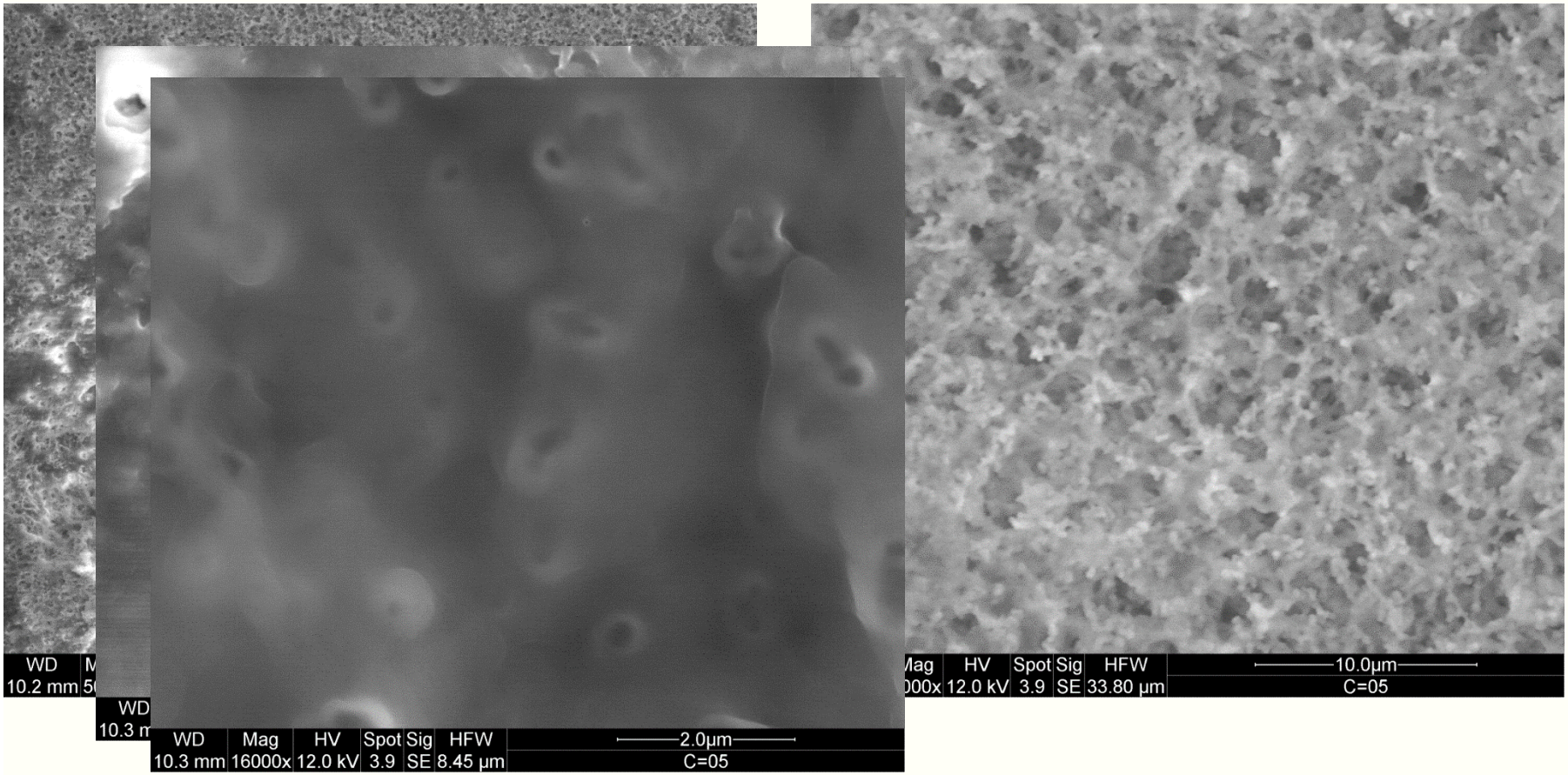


Of 118 UV'd *E. coli* spectra, 100% classified as live *E. coli*



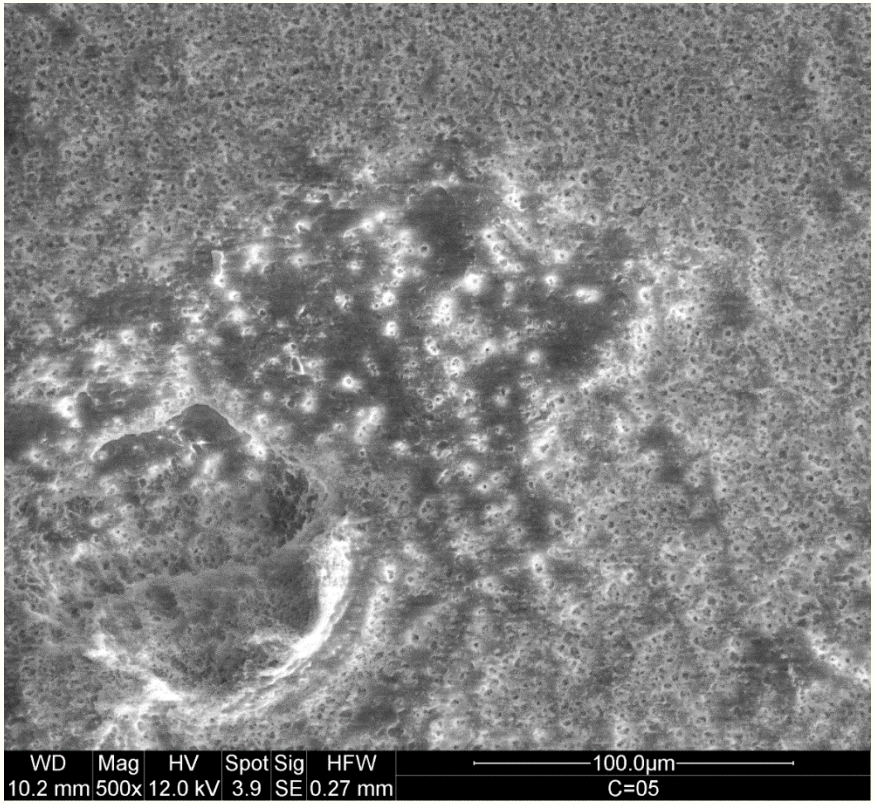
Viability study - Sonication

- 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?

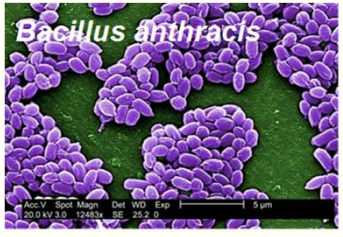
- This leads to more sterilization tests (ongoing), asking the questions:
  - Why are they different (from live)?
  - If they are different from live, are they like each other?
  - If they are like each other, why? Should they be? (structure dependence, nuclear loss, deposition on filter – do they lay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

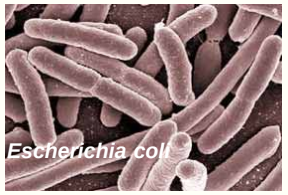
Q: Are the results consistent for all species? Results are just shown for *E. coli* but tests are in progress.

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.





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

University of Windsor
thinking forward

- 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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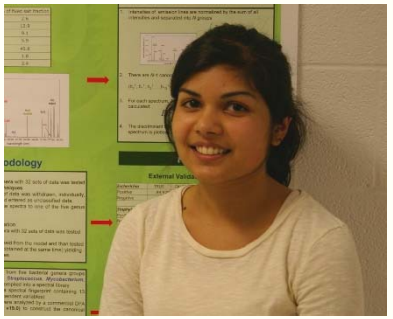
All Credit to the Students

Dan Trojand



Khadijia Sheikh

Russell Putnam

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Derek Gillies

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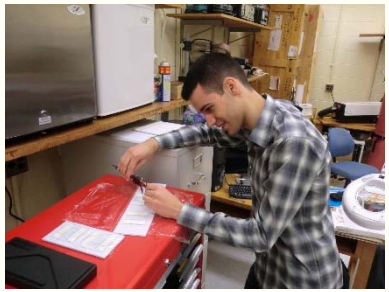
Andrew Daabous



Vlora Riberdy