Progress and Challenges in Using LIBS for Bacteriological Identification

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LIBS FOR PHARMACEUTICAL AND BIOLOGICAL DIAGNOSTICS

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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

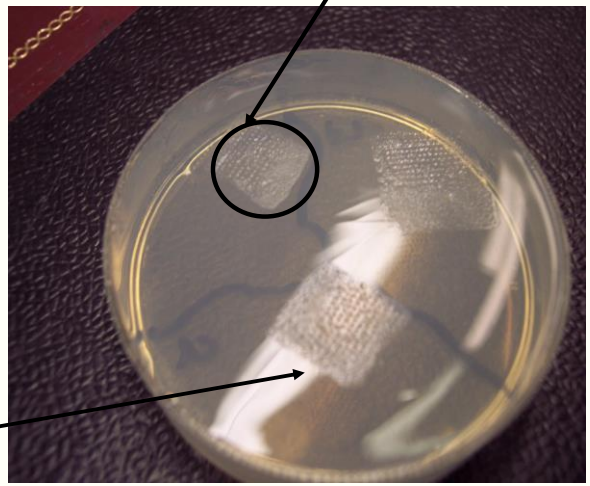
Heat killed bacteria are differentiable (*Multari et al., ARA*)

2015

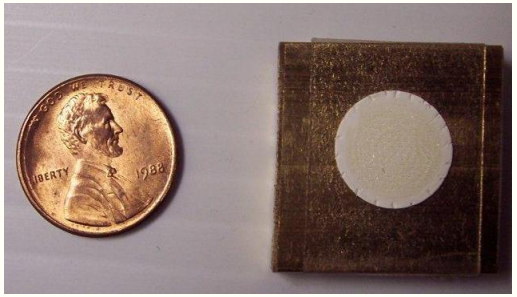
Inactivation by sonication / autoclaving is differentiable (*Sivakumar et al., Delaware State*) with fs-LIBS & ns-LIBS. Changes in spectral intensity were observed.

New Bacteria Testing Procedure

Previously: mounted on agar



Currently: mounting on nitrocellulose bacteriological filter

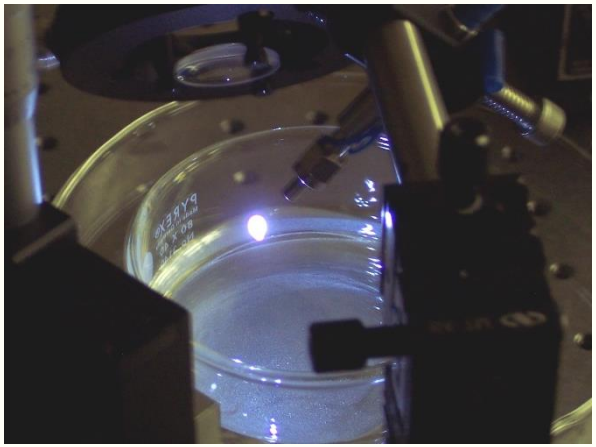


✓ *Advantages: Flatter. Easier to do. More reproducible. Less time.*

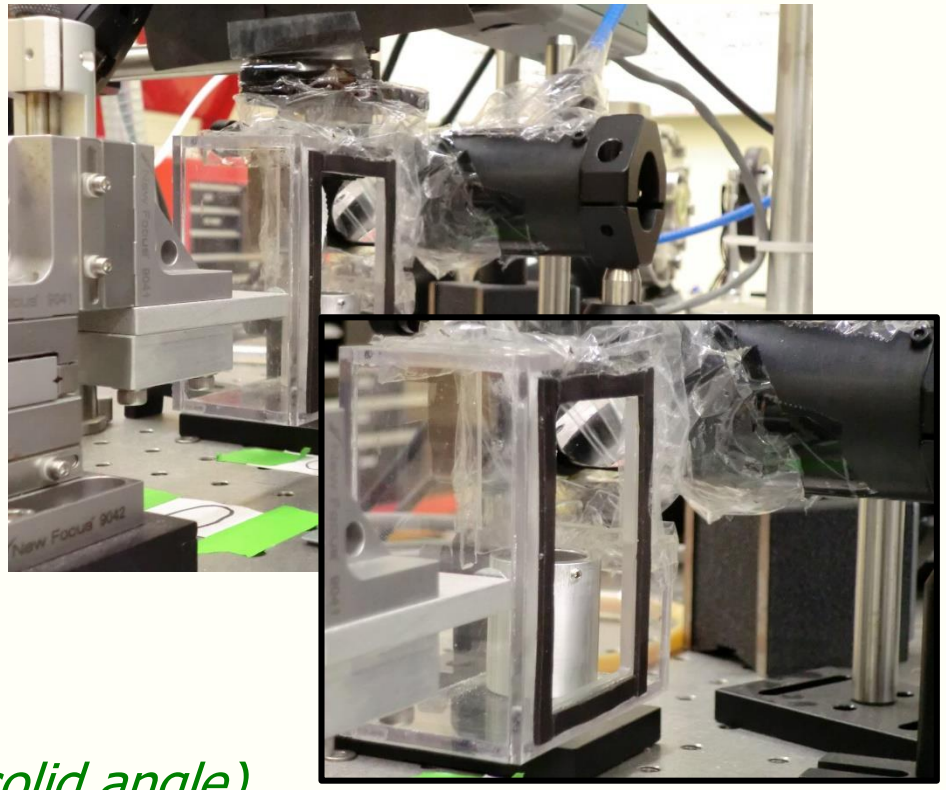
x *Disadvantages: Carbon background.*

New Bacteria Testing Procedure

Previously: light collected by fiber



Currently: using matched parabolic reflectors into fiber



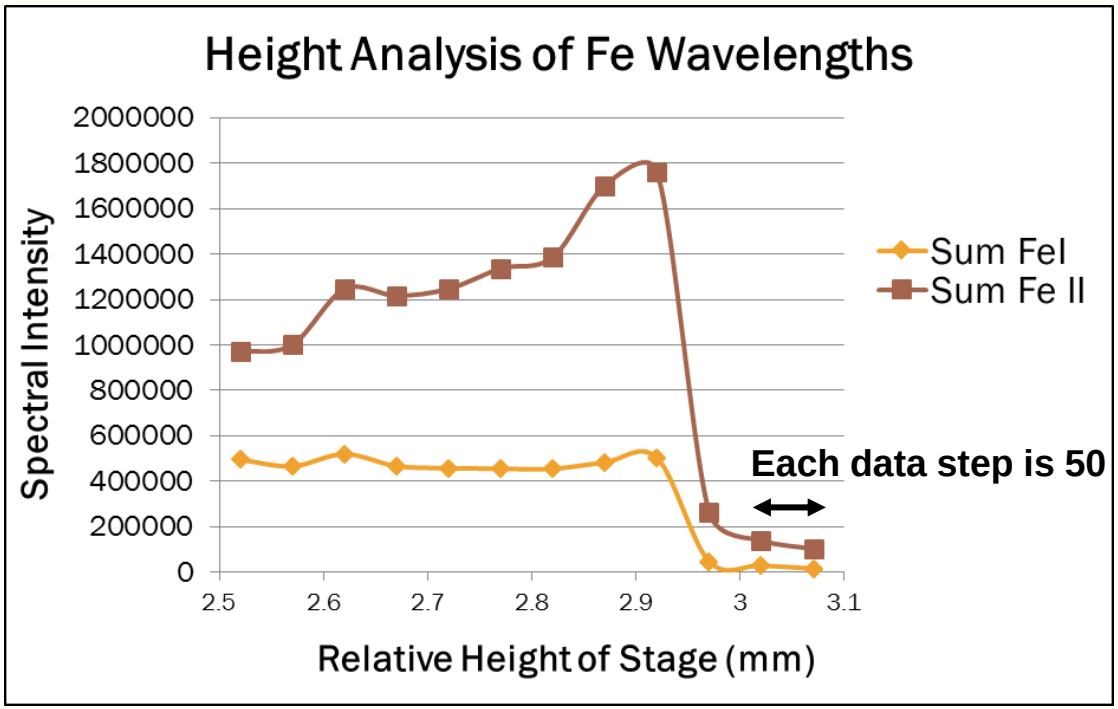
✓ *Advantages: More light collected (solid angle)*

x *Disadvantages: More sensitive to vertical position*

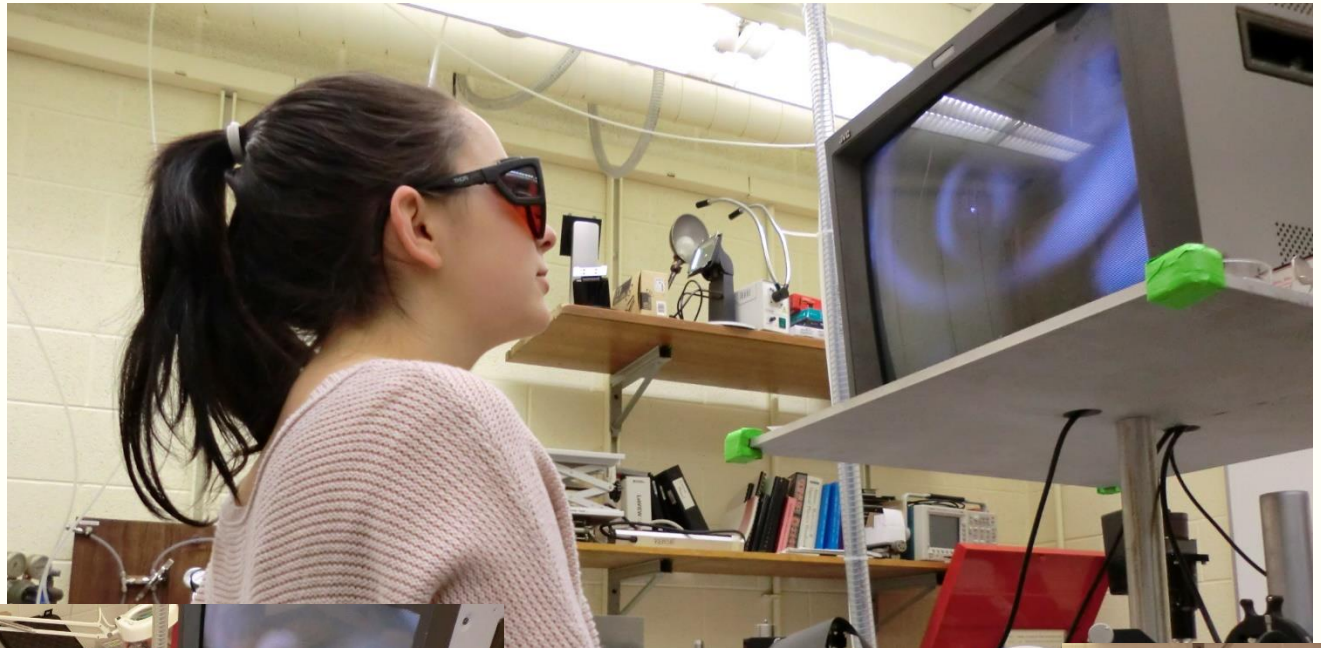
New Bacteria Testing Procedure

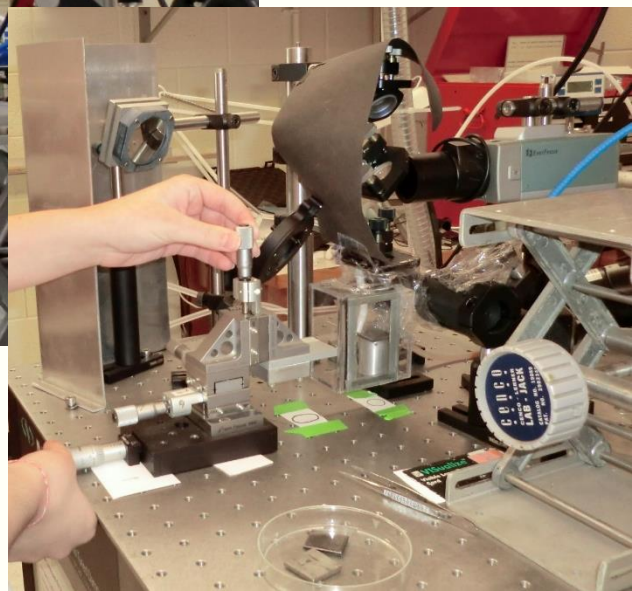
Previously: proper LTSD found by trial and error

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.

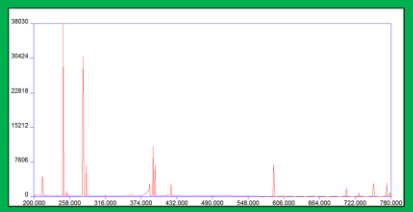
30 µL of vortexed sample are deposited on a standard 0.22 µm cellulose filter in contained wells.

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

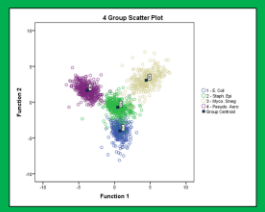
Filter is placed in an argon environment and ablated using a pulsed 1064 nm Nd: YAG laser.

Average time to complete bacterial classification = 1 hour

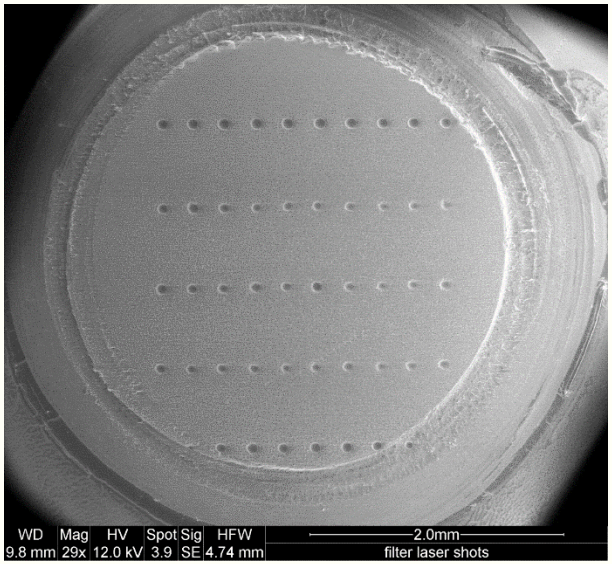
Échelle diffraction grating spectrometer is used to obtain the atomic spectrum and composition of sample.

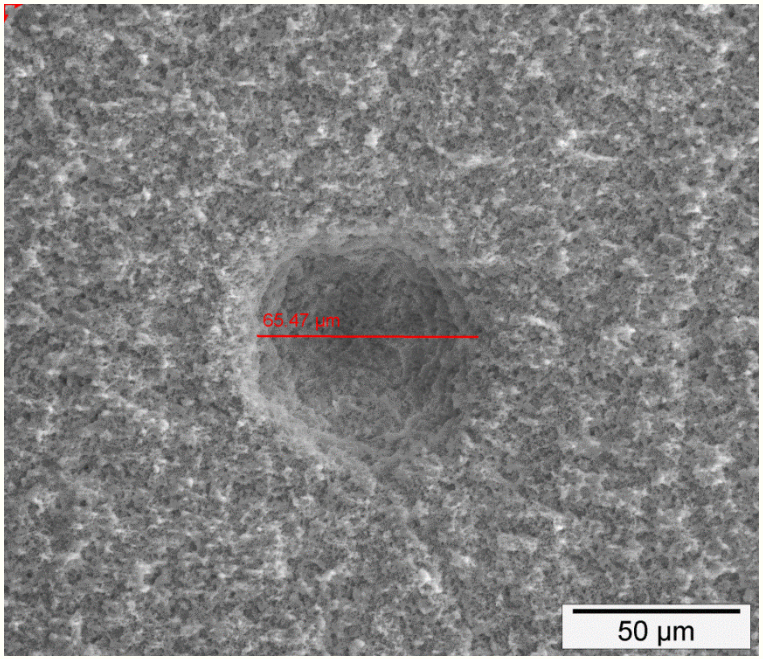


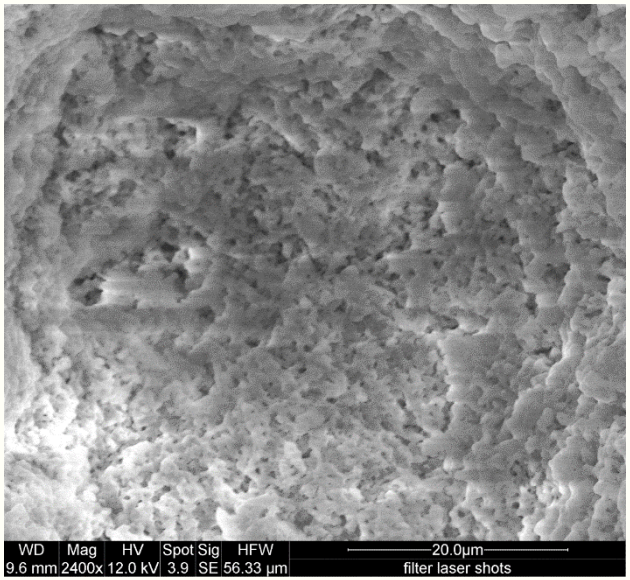
Atomic composition is used to discriminate bacteria against pre-existing library.



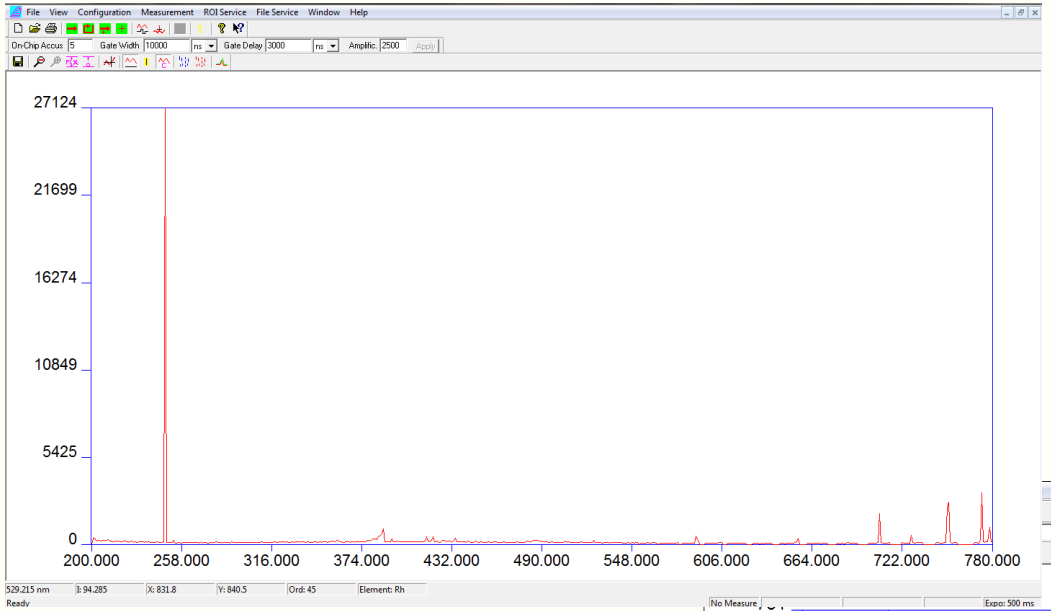
Filter


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


65.47 µm
50 µm


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

Filter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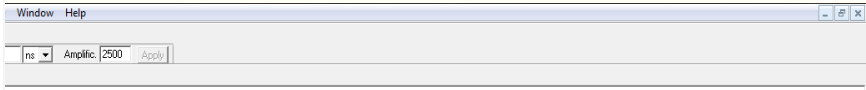
Element: Rh
Ready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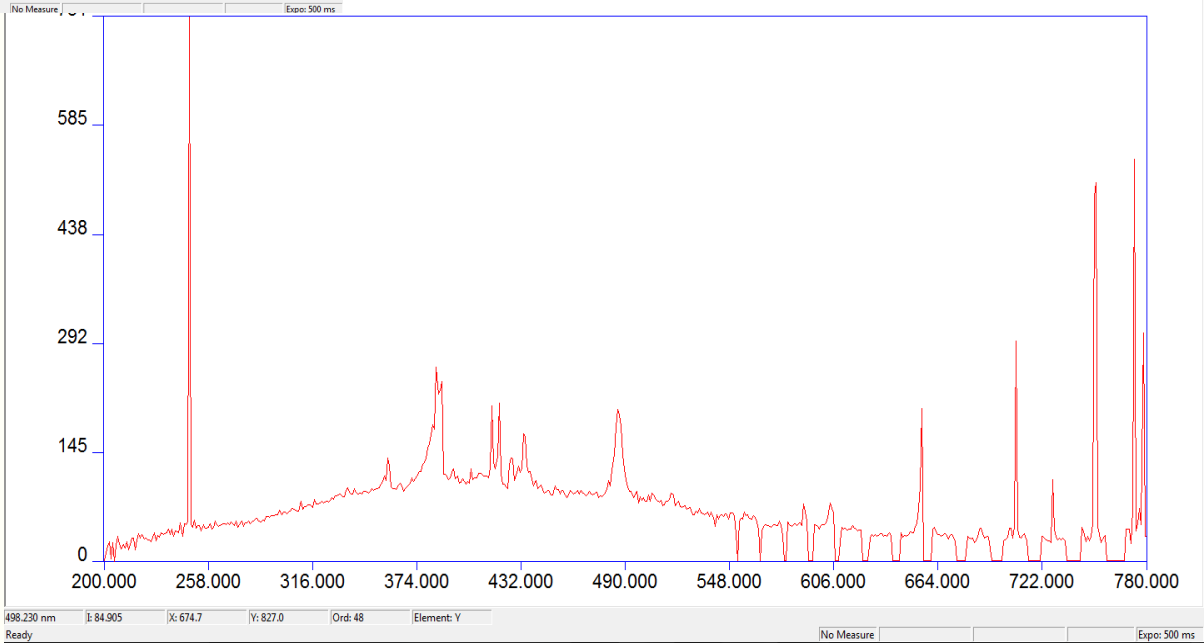
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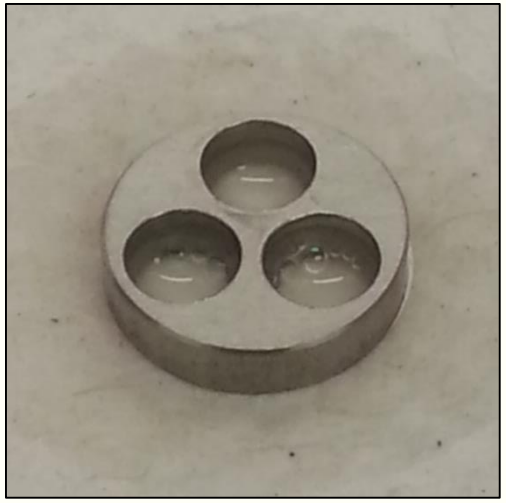
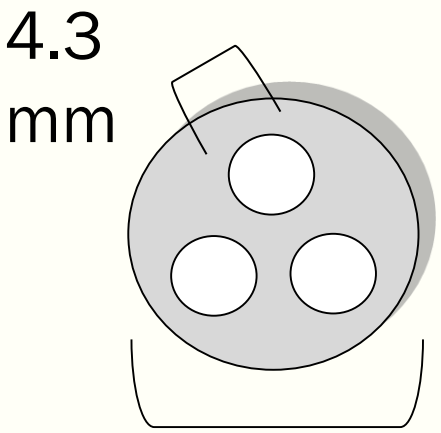
498.230 nm
I: 84.905
X: 674.7
Y: 827.0
Ord: 48
Element: Y
Ready
No Measure
Expo: 500 ms

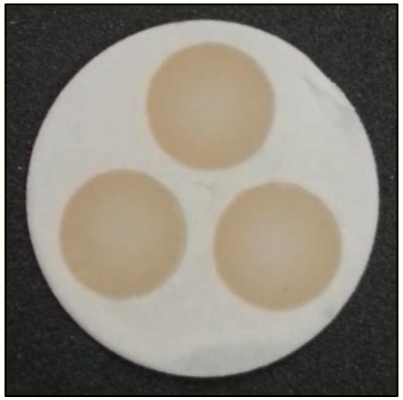
Can bacteria be deposited in a controlled manner?

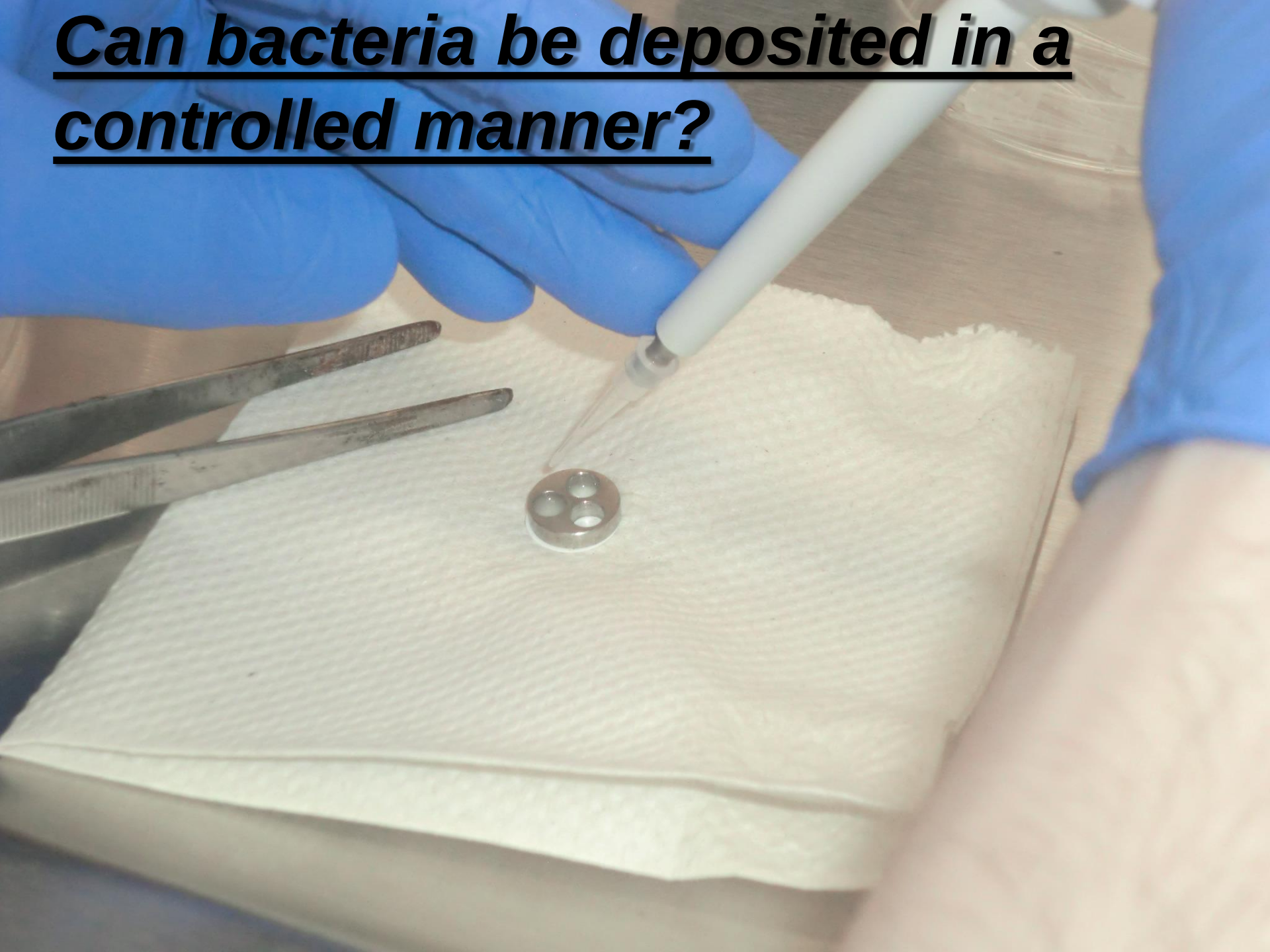
A steel disk was designed in order to create a reproducible area for bacteria to be placed on.



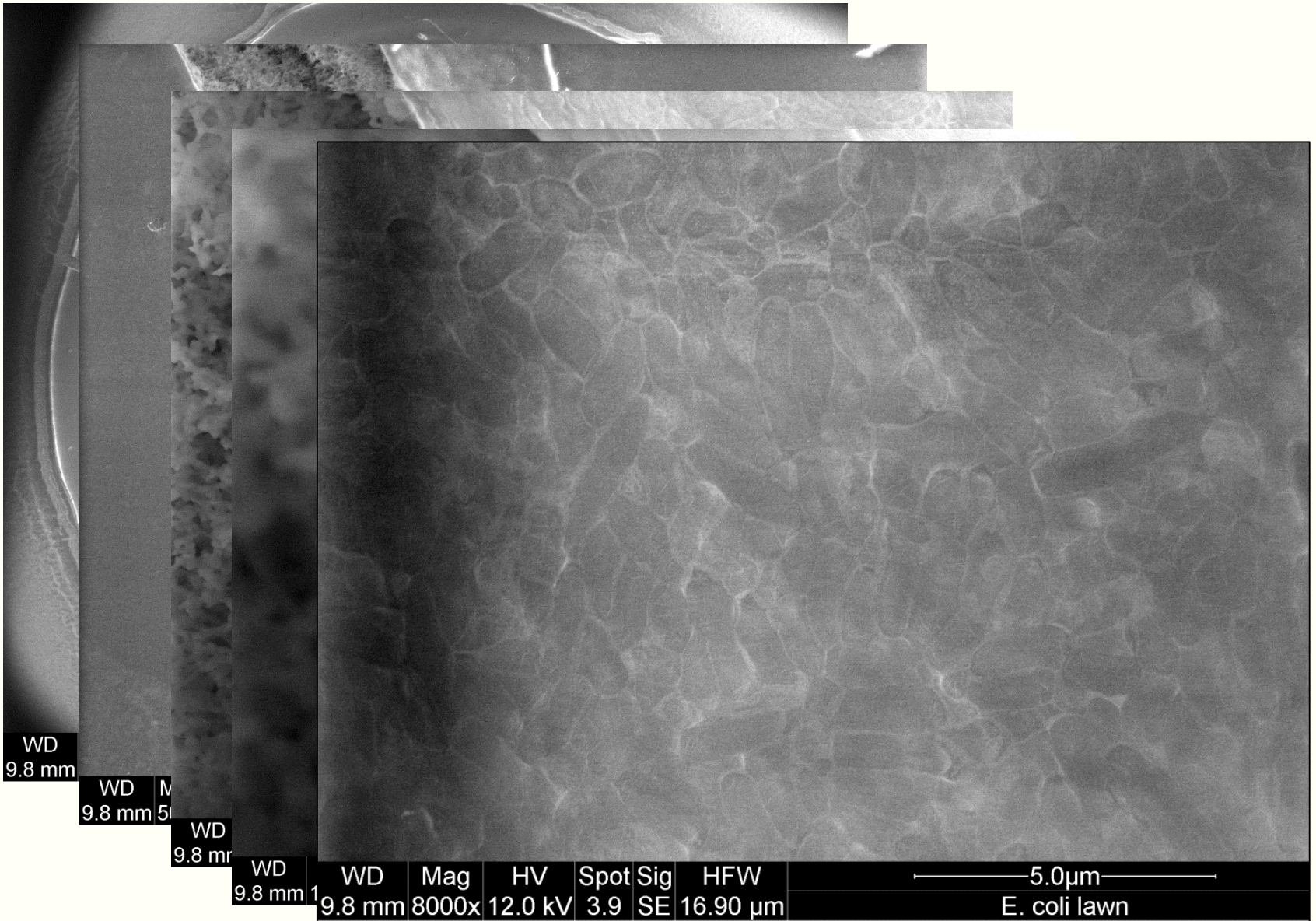


Scanning electron microscopy images verified complete coverage of the deposited bacterial lawn.

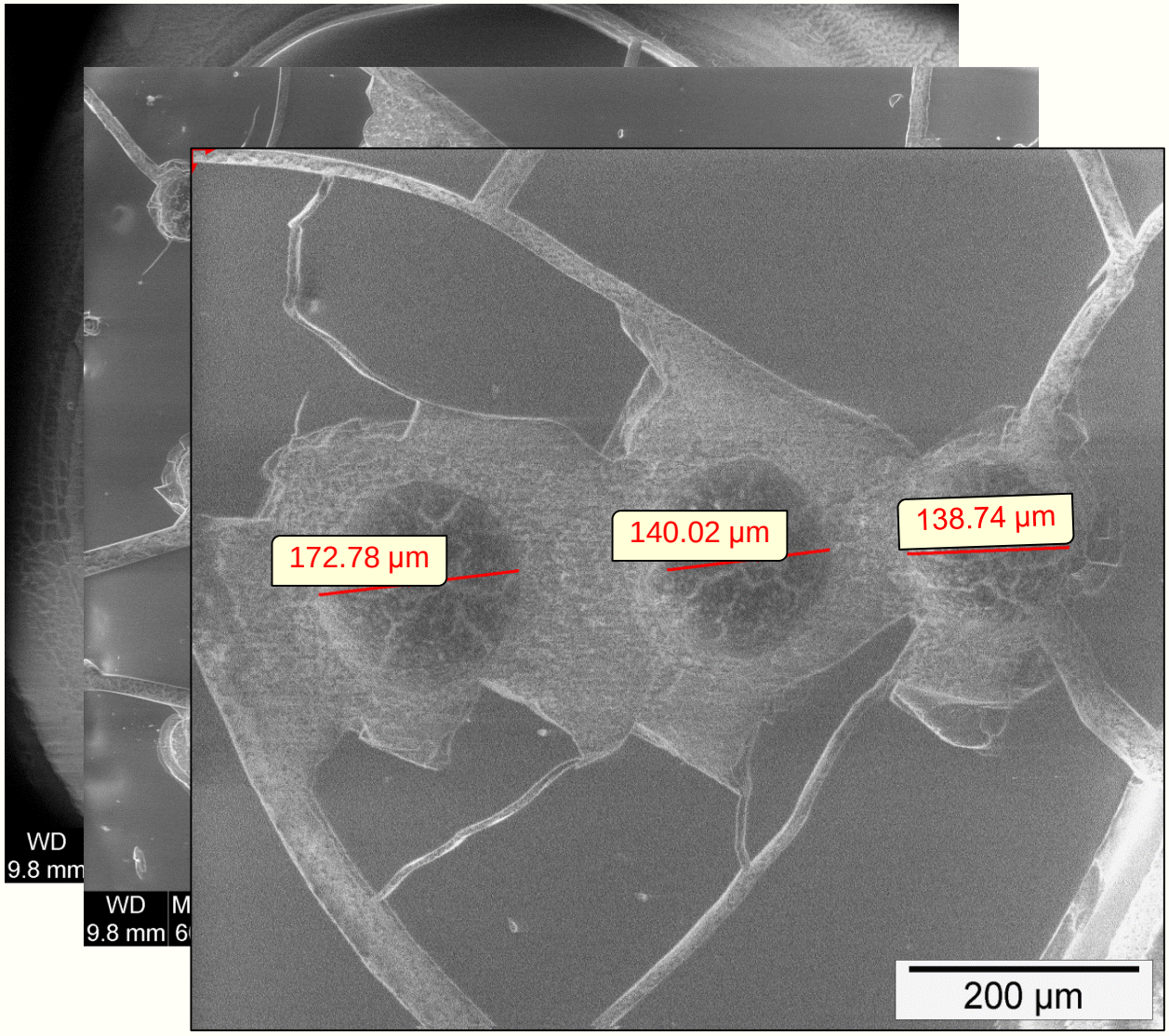



Can bacteria be deposited in a controlled manner?

Can bacteria be deposited in a controlled manner?

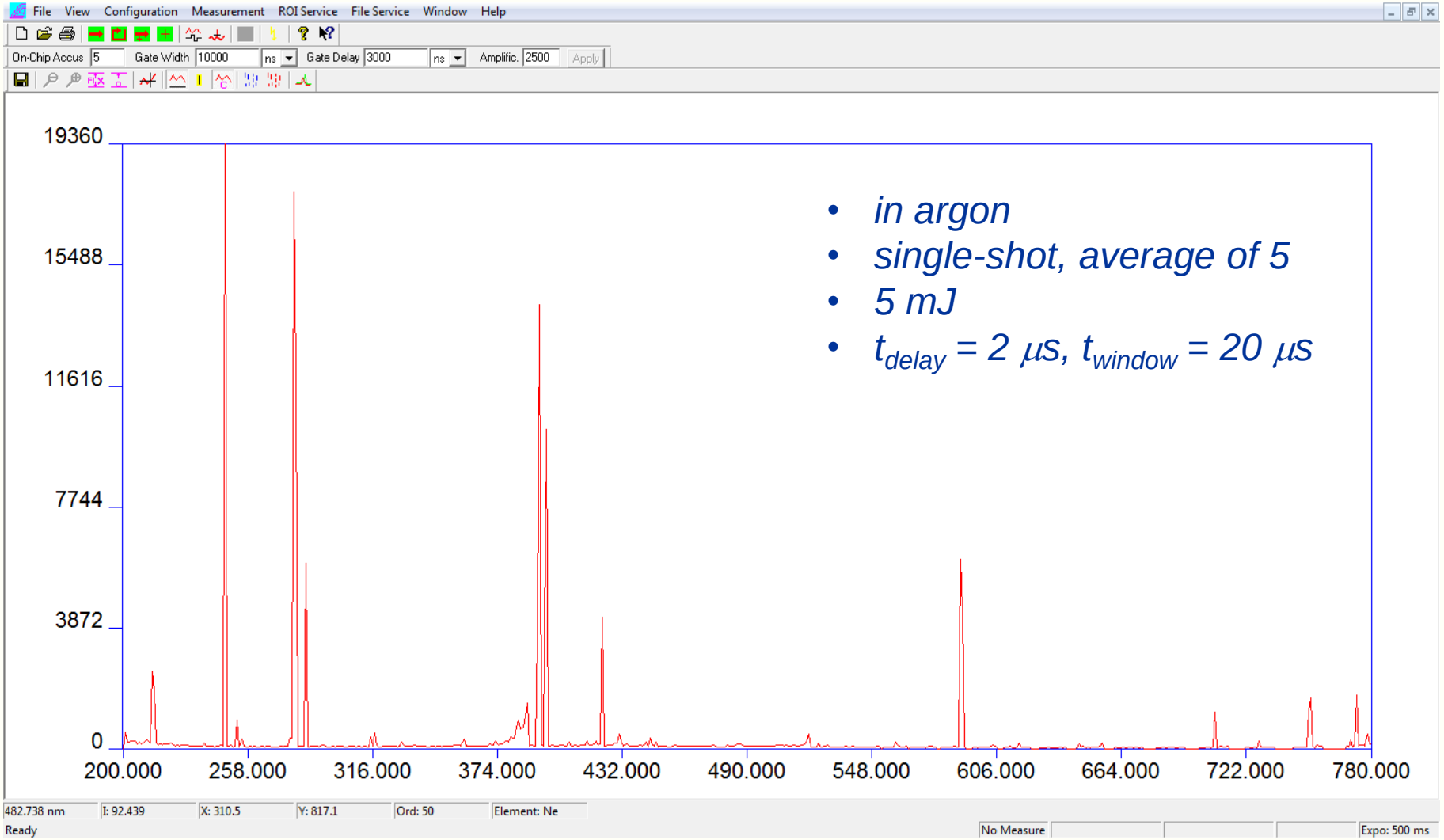


Can bacteria be ablated in a controlled manner?

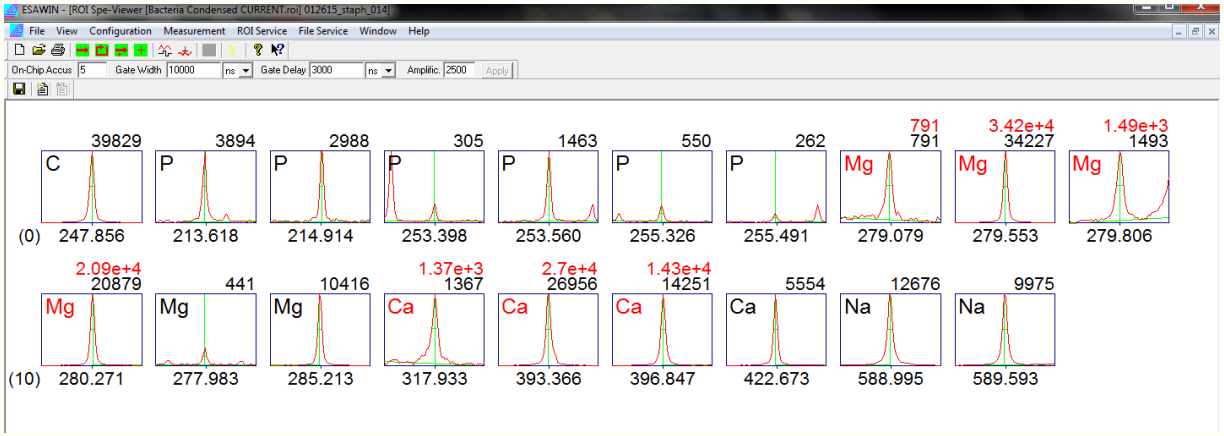


With crater diameters of about 150 µm, the quantity of cells vaporized for each spectrum was estimated at 10^6 (verified using optical densitometry.

Typical Bacterial Spectrum

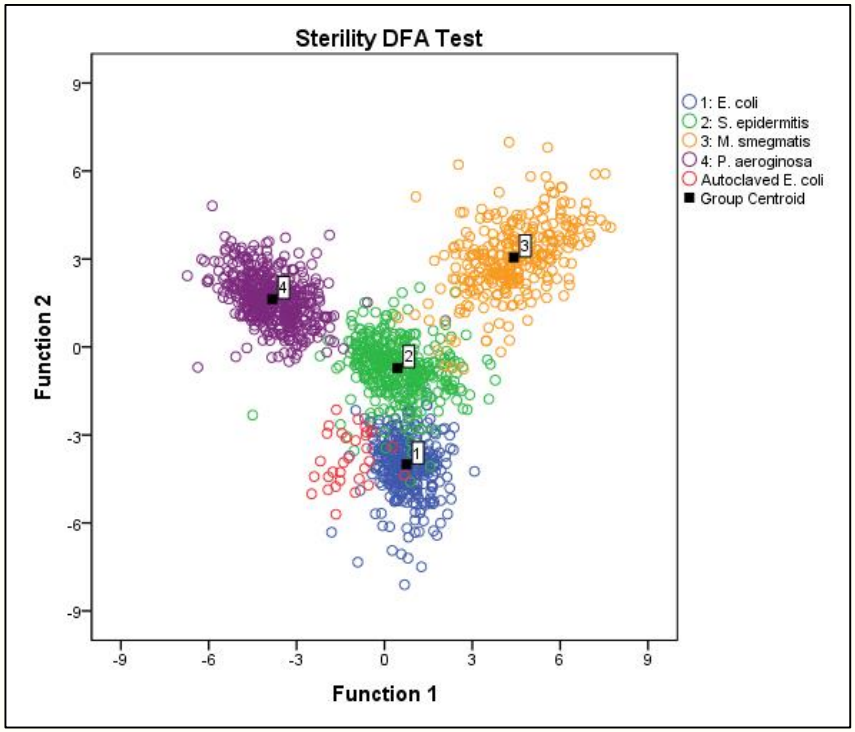


Variable Down-Selection



- New classification model
- 164 independent variable
  - 19 line intensities (all divided by sum)
  - 145 ratios of intensities
- No other metals
  - Farooq (2014) sees S, Cl, Mn, Fe, Al, Cu, etc.
  - Sivakumar (2015) only sees Ca, Na, Mg, K, O, H, C, P
  - We can see other metals when appreciable number of files averaged (to eliminate noise).

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%

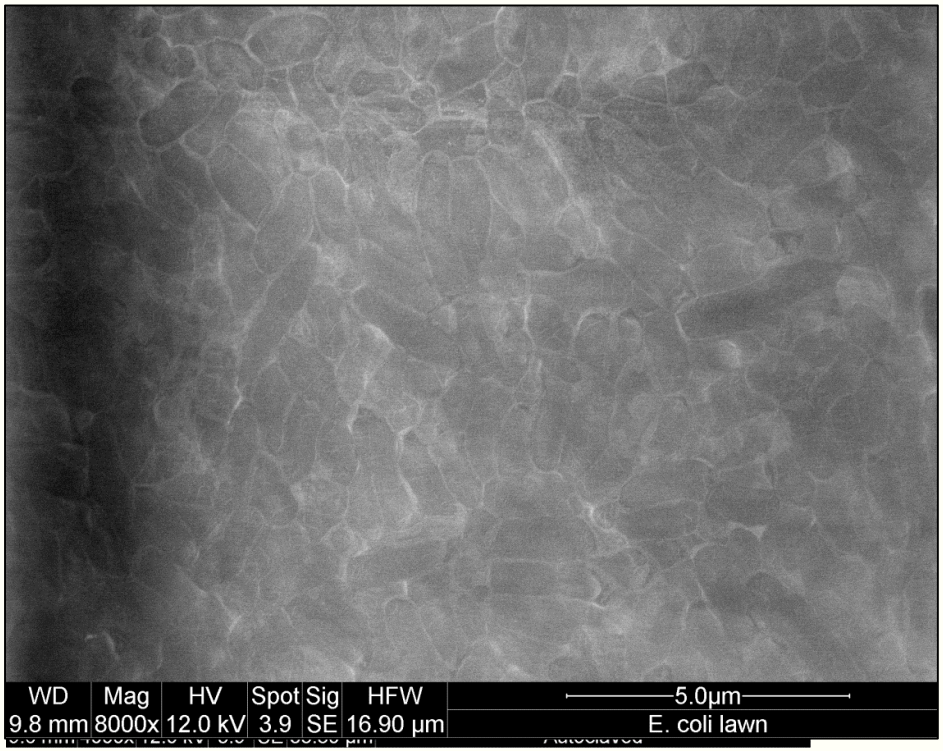
All external validation results

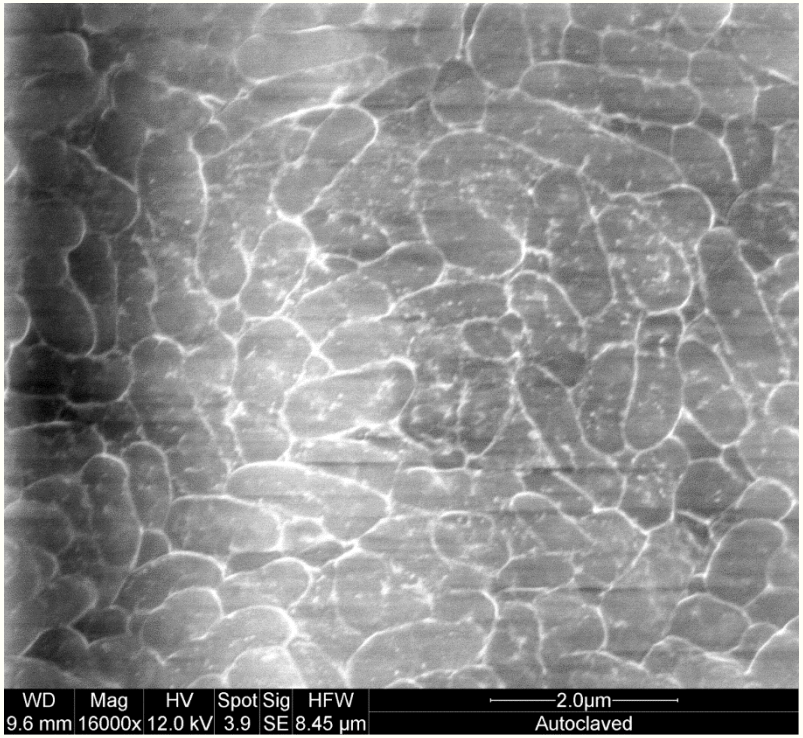
	DFA (by filter)	DFA (by species) above	PLSDA (by species) above
Sensitivity	0.93±0.07	0.98±0.02	0.97±0.03
Specificity	0.98±0.03	0.99±0.01	0.99±0.02

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

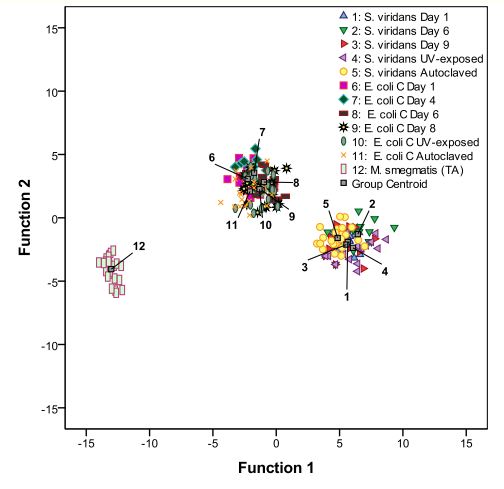
Viability study - Autoclaved

- 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.



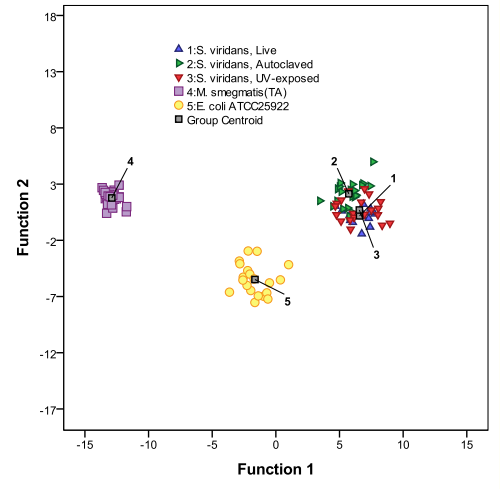


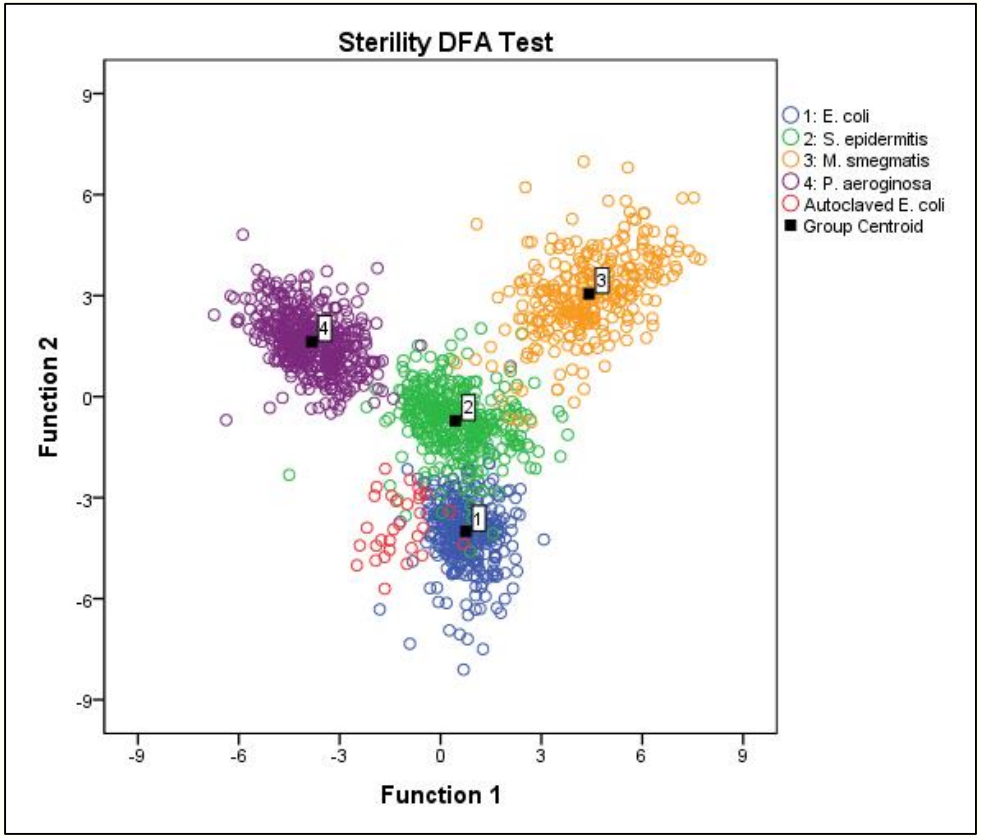
Viability study - Autoclaved



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

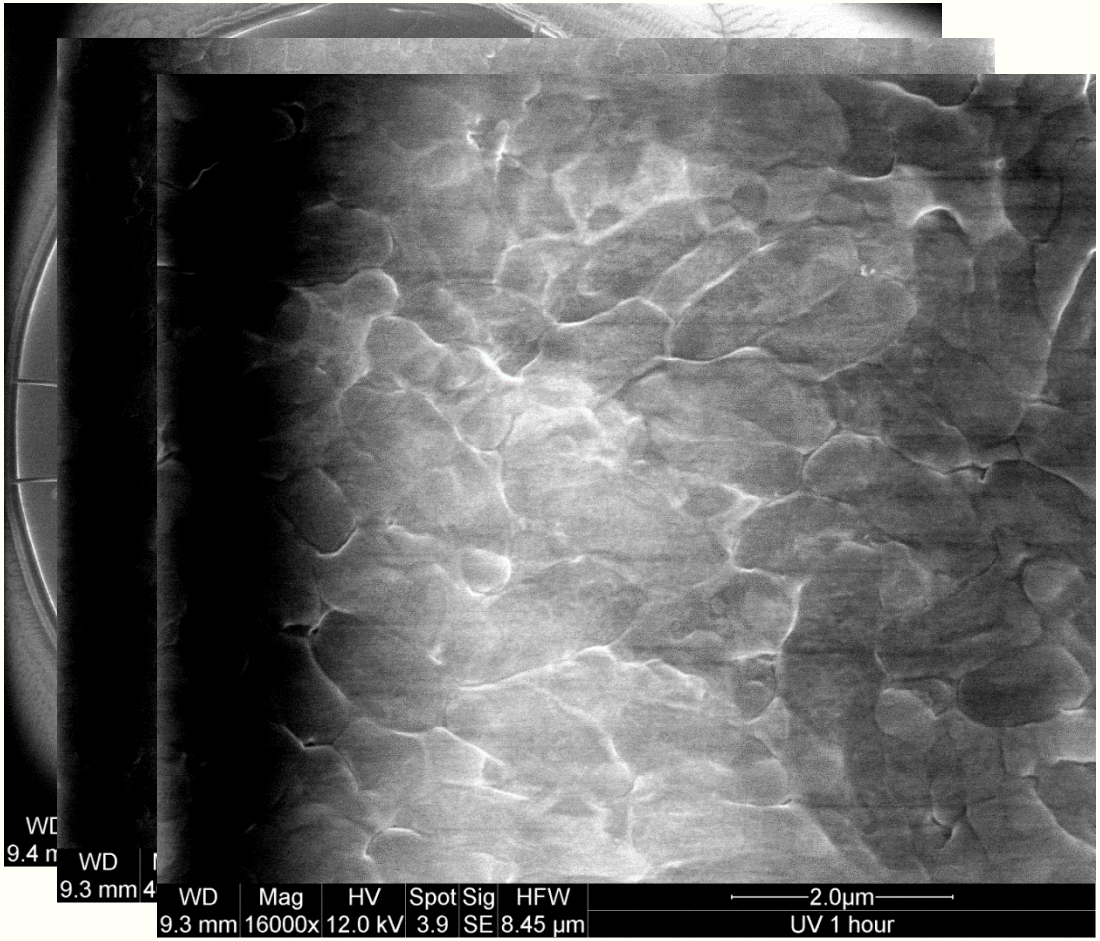
Previous result



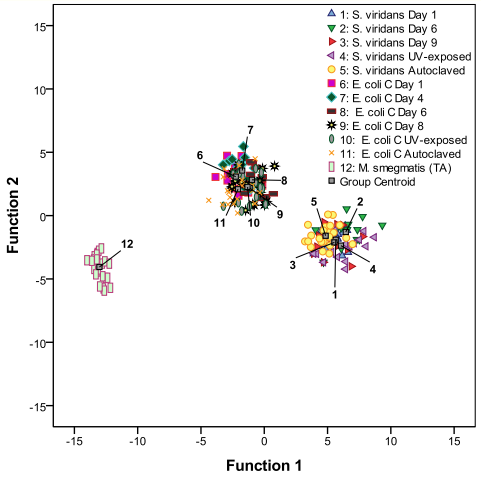


Viability study - UV

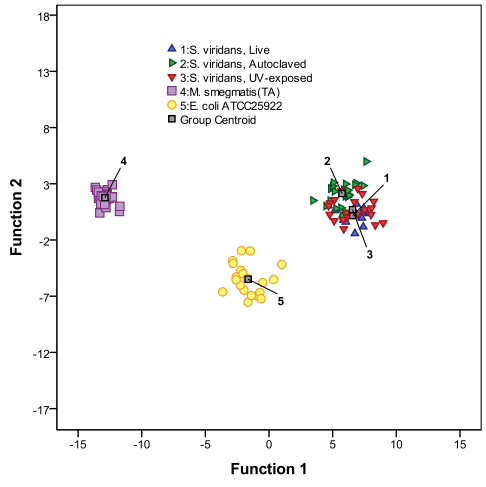
- Live cells were deposited on the filter, the whole filter was then placed under UV light (4 W, 254 nm at about 15 cm from the source) for 1 hr, 30 min, 15 min.
- Saw no difference in signal and they classified as live.
- 30 min was chosen for future experiments.
- UV non-viability confirmed via pressing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



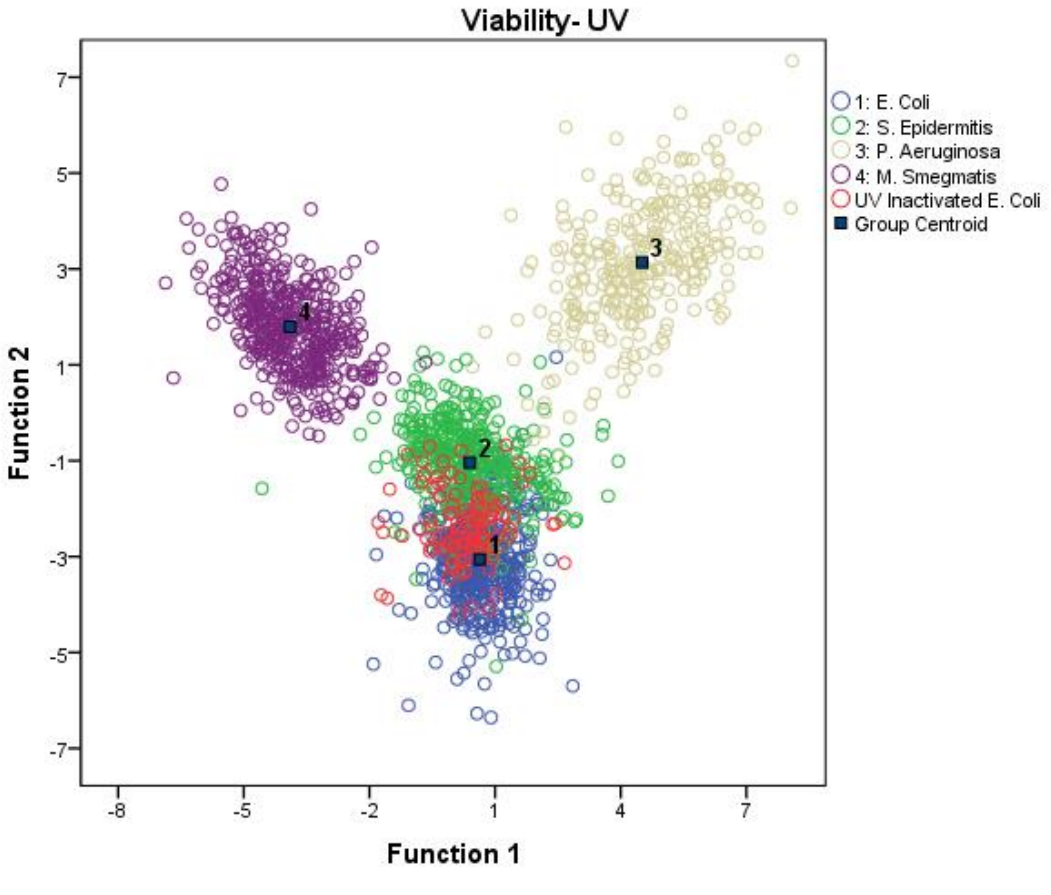
Viability study - UV



Previous result



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



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?

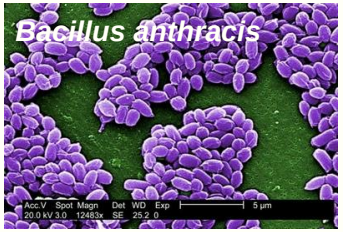
- This leads to more sterilization tests (ongoing), asking the questions:
  - Why are they different (from live)?
  - If they are different from live, are they like each other?
  - If they are like each other, why? Should they be? (structure dependence, nuclear loss, deposition on filter – do they lay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

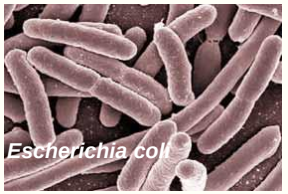
Q: Are the results consistent for all species? Results are just shown for *E. coli* but tests are in progress.

Summary of Viability Study

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

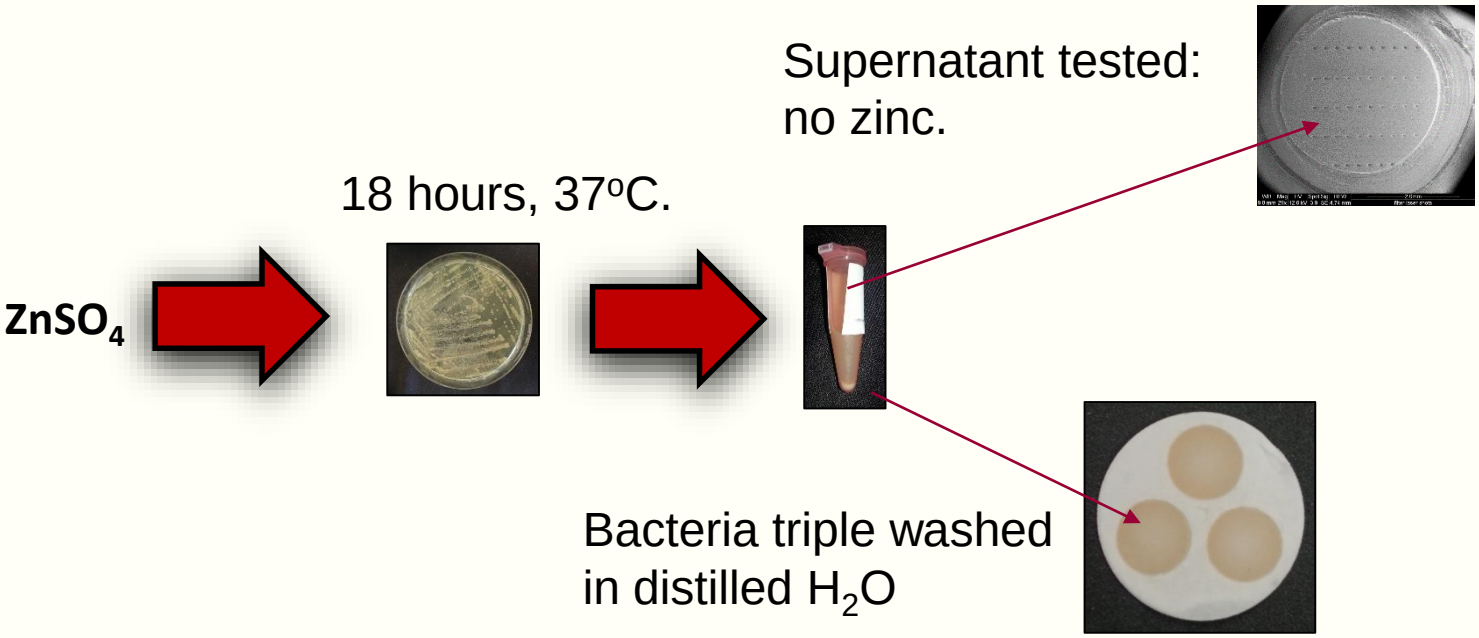
- Bacteria are definitely NOT inorganic microparticles that can be uniformly deposited easily, yielding background-independent LIBS spectra.
- Great care must be taken.





Intentional Doping of Cells: Zinc

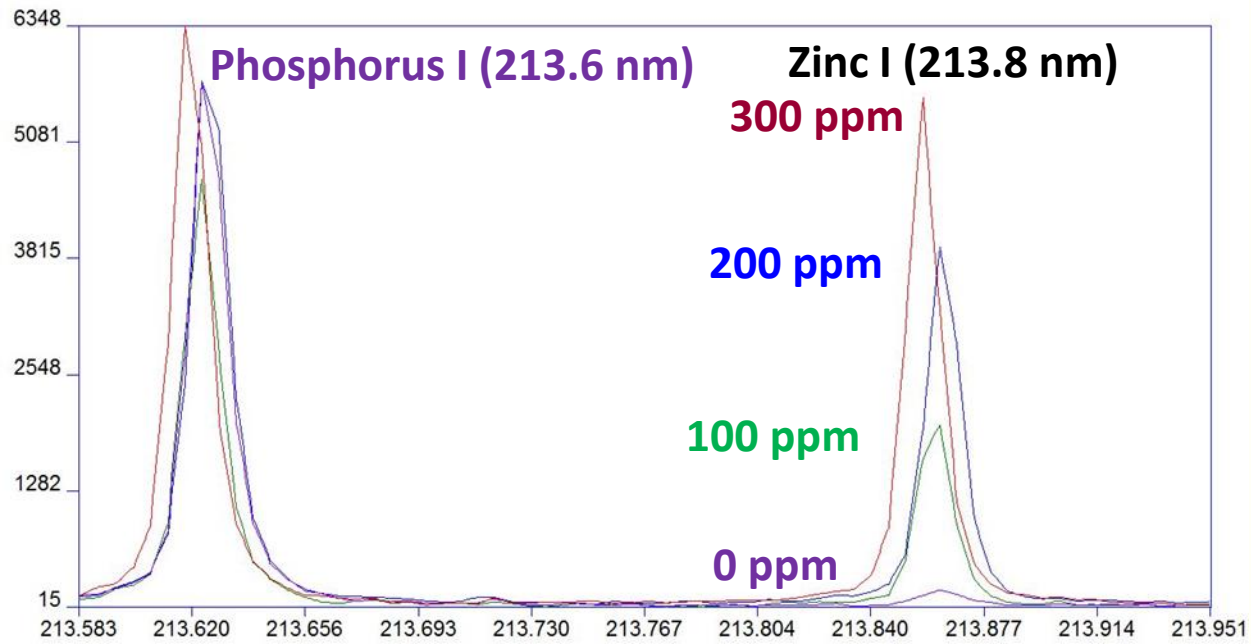
Q: Can metallic elements in the growth environment alter the LIBS spectrum?

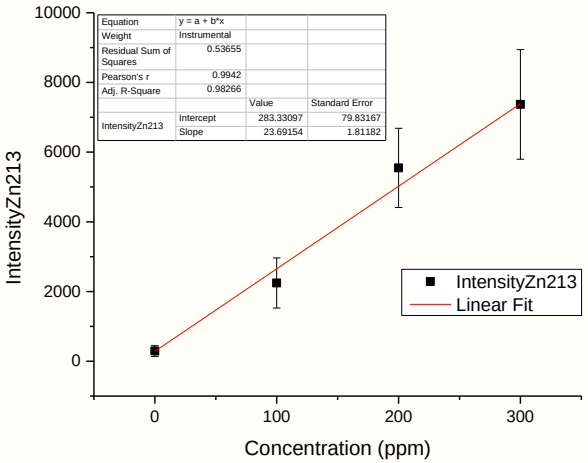


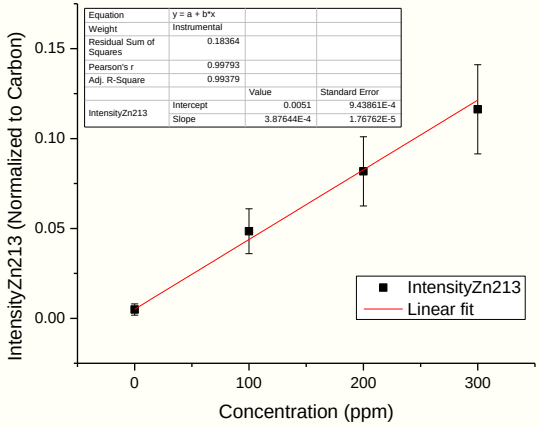
Intentional Doping of Cells: Zinc

Q: Can metallic elements in the growth environment alter the LIBS spectrum?

Zinc-doped *E. coli* LIBS spectrum



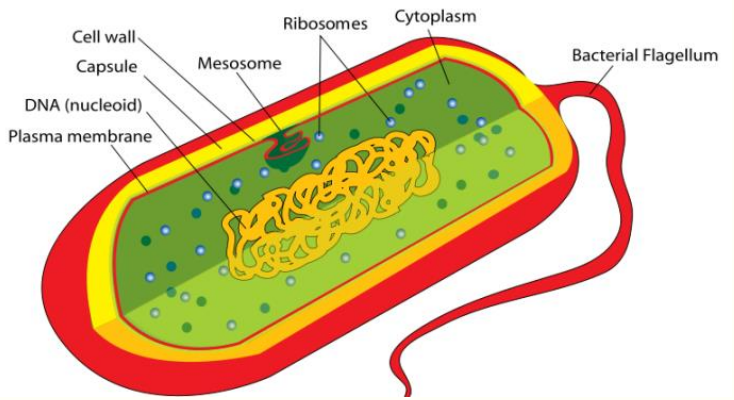




Intentional Doping of Cells: Zinc

Q: Can metallic elements in the growth environment alter the LIBS spectrum?

A: Yes.

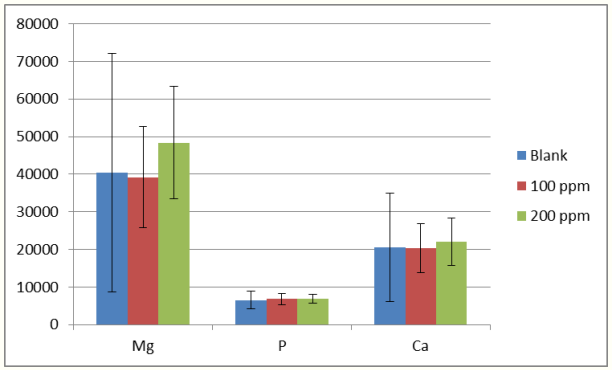


- After rinsing, the zinc signal looked diminished. Is the zinc present inside the cell (in the cytoplasm), or is it merely adsorbed onto the surface?
- Magnesium lines seemed diminished. Zinc and magnesium are comparable in size, and are both divalent cations, and the cell possibly substitutes Zn for Mg when Zn is present in abundance in the growth medium.

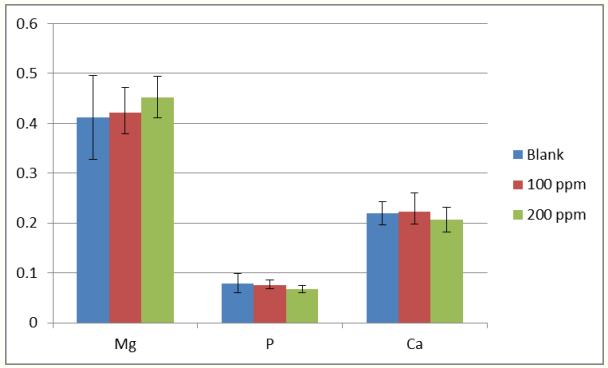
Intentional Doping of Cells: Mg

To test whether significant changes in magnesium concentrations could be achieved within limits of extremely hard water (high magnesium concentrations), we carried out the following experiment:

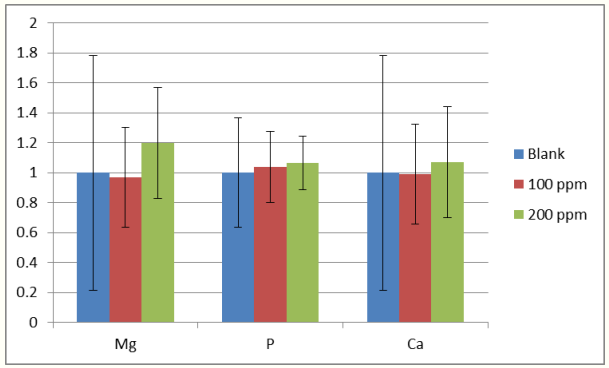
Label	Concentration of Magnesium (ppm)	Magnesium Lines in Spectra
B	0	Normal Mg lines
S1	<0	No bacterial growth
S2	100	Very slightly elevated peaks
S3	200	Very slightly elevated peaks



Raw LIBS intensities



As fraction of total spectrum



Normalized to blank

- 200 ppm cultures classified as control *E. coli*.
- Physiologic concentration is 18-30 ppm.

Impact of blood glucose/metal uptake

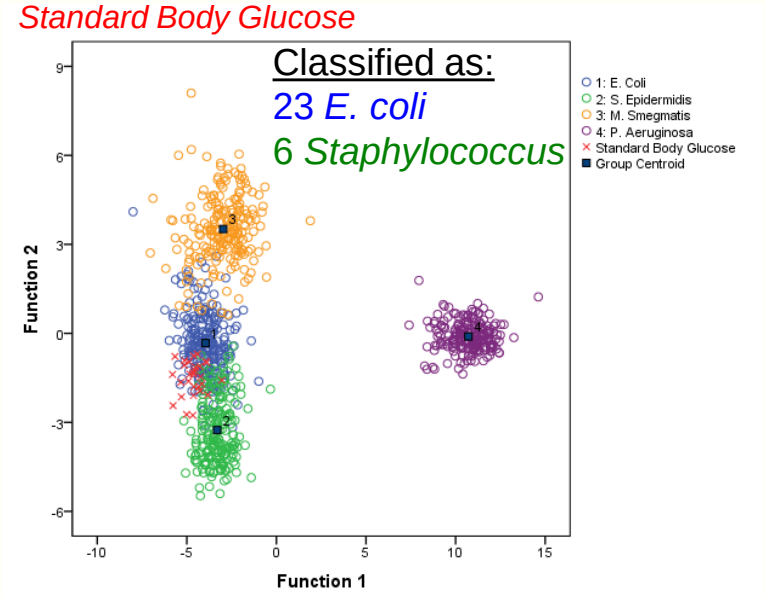
- Bacterial uptake of metal ions is a function of their metabolism, since most metals need active transport to be imbibed.
- Bacteria that grow faster/more rapidly should theoretically show higher metal levels than slower ones (of the same species).
- Perhaps the slight difference in osmotic pressure caused by high sugar content in the environment could cause a change in metal ion concentrations in the bacterial cells?
- In diabetics, blood sugar levels fluctuate rapidly, ranging from very high to very low within a few hours of eating.

We designed an experiment to verify the robustness of DFA classification of bacteria grown in various blood sugar concentrations by doping the growth medium with excess glucose and checking the change in metal ion signals in the LIBS spectra.

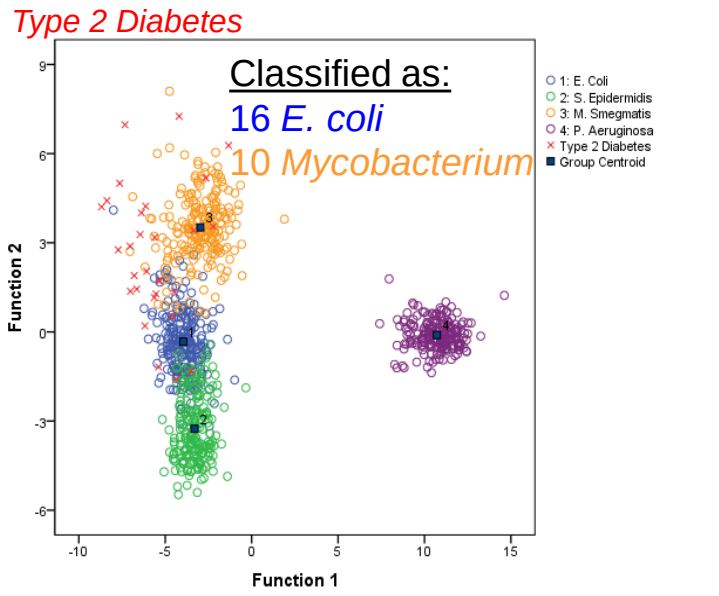
Label	Concentration of Glucose (g/L)	
B	1.35	Control
S1	1.44	Type II diabetes
S2	1.62	Type I diabetes
S3	1.8	Children's type I diabetes

Impact of blood glucose/metal uptake

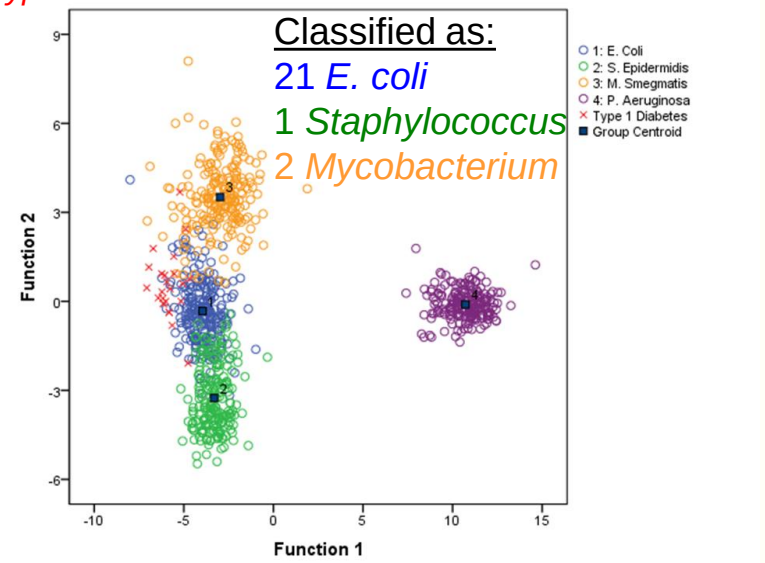
Standard Body Glucose



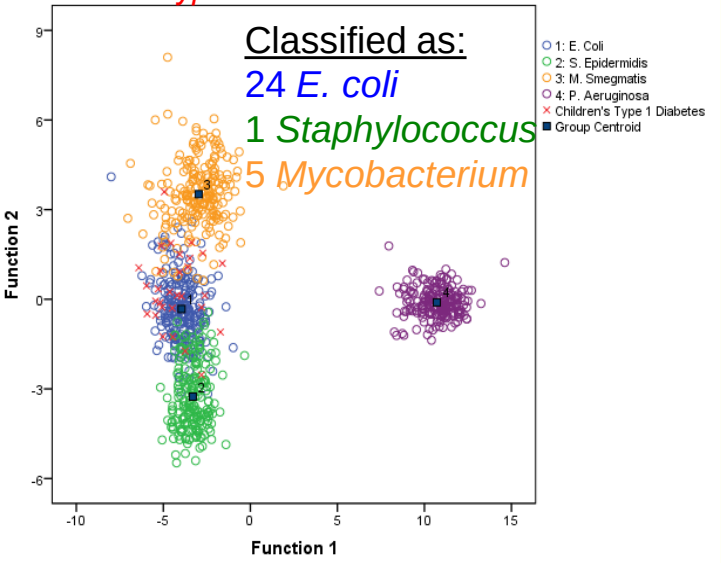
Type 2 Diabetes



Type 1 Diabetes



Children's Type 1 Diabetes



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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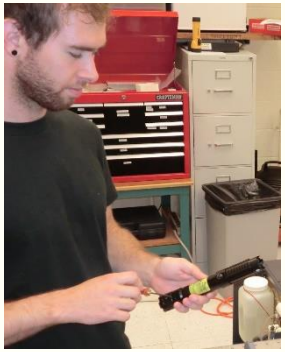
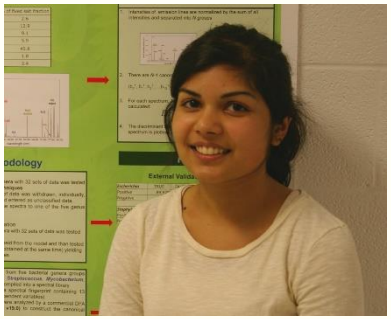
All Credit to the Students

Russell Putnam

Dan Trojand





Khadijia Sheikh

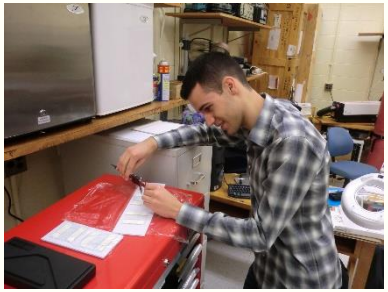
Siddharth Doshi

Anthony Piazza

Dylan Malenfant

Derek Gillies

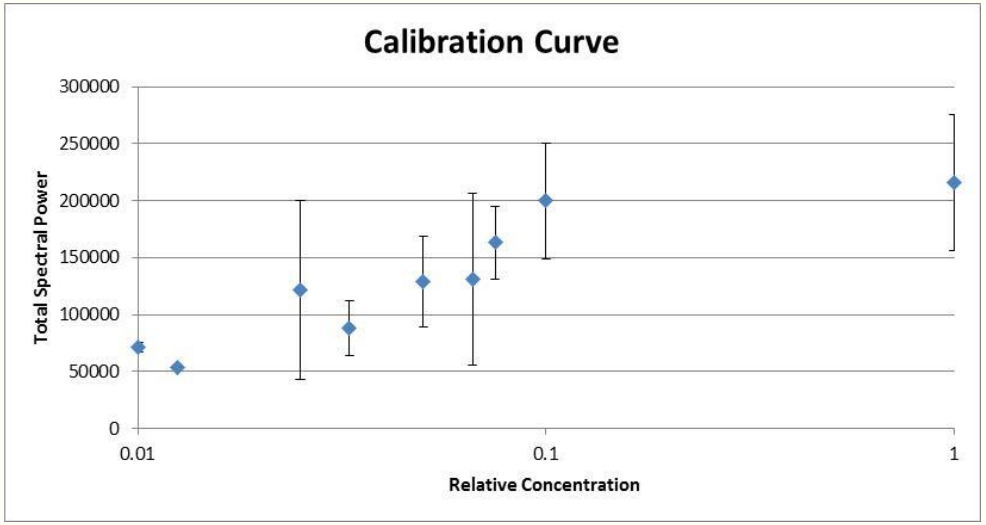
Allie Paulick

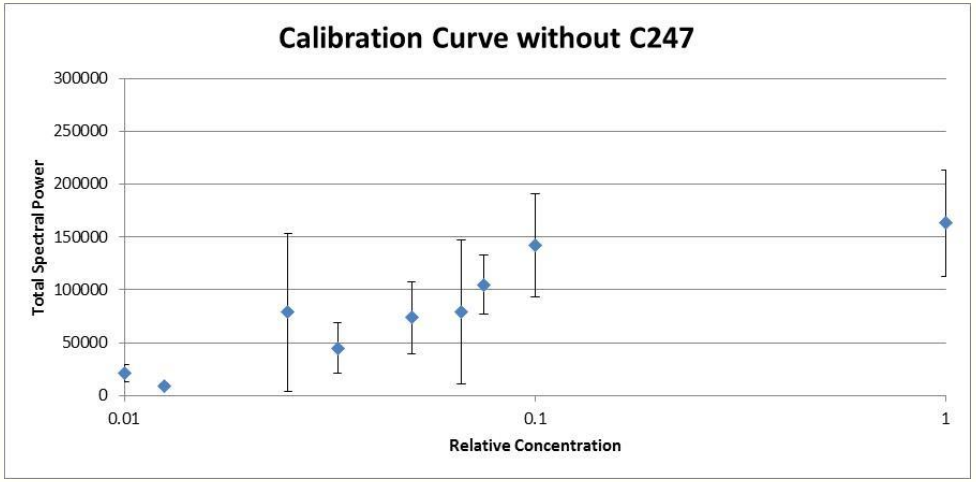


Vlora Riberdy

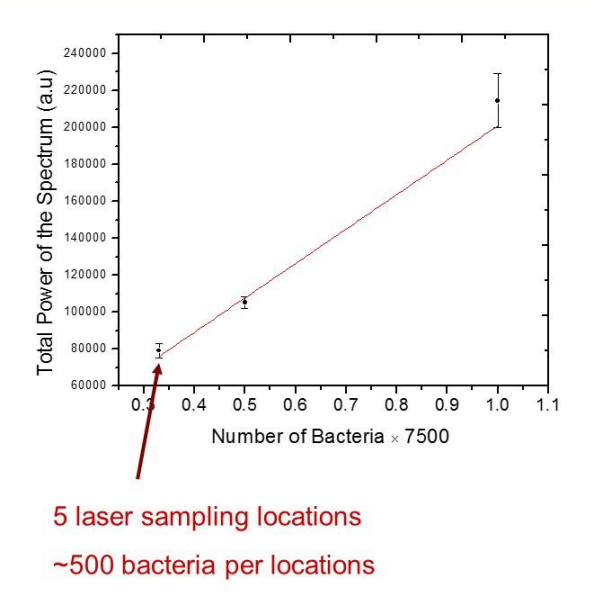
Andrew Daabous

New Concentration Study





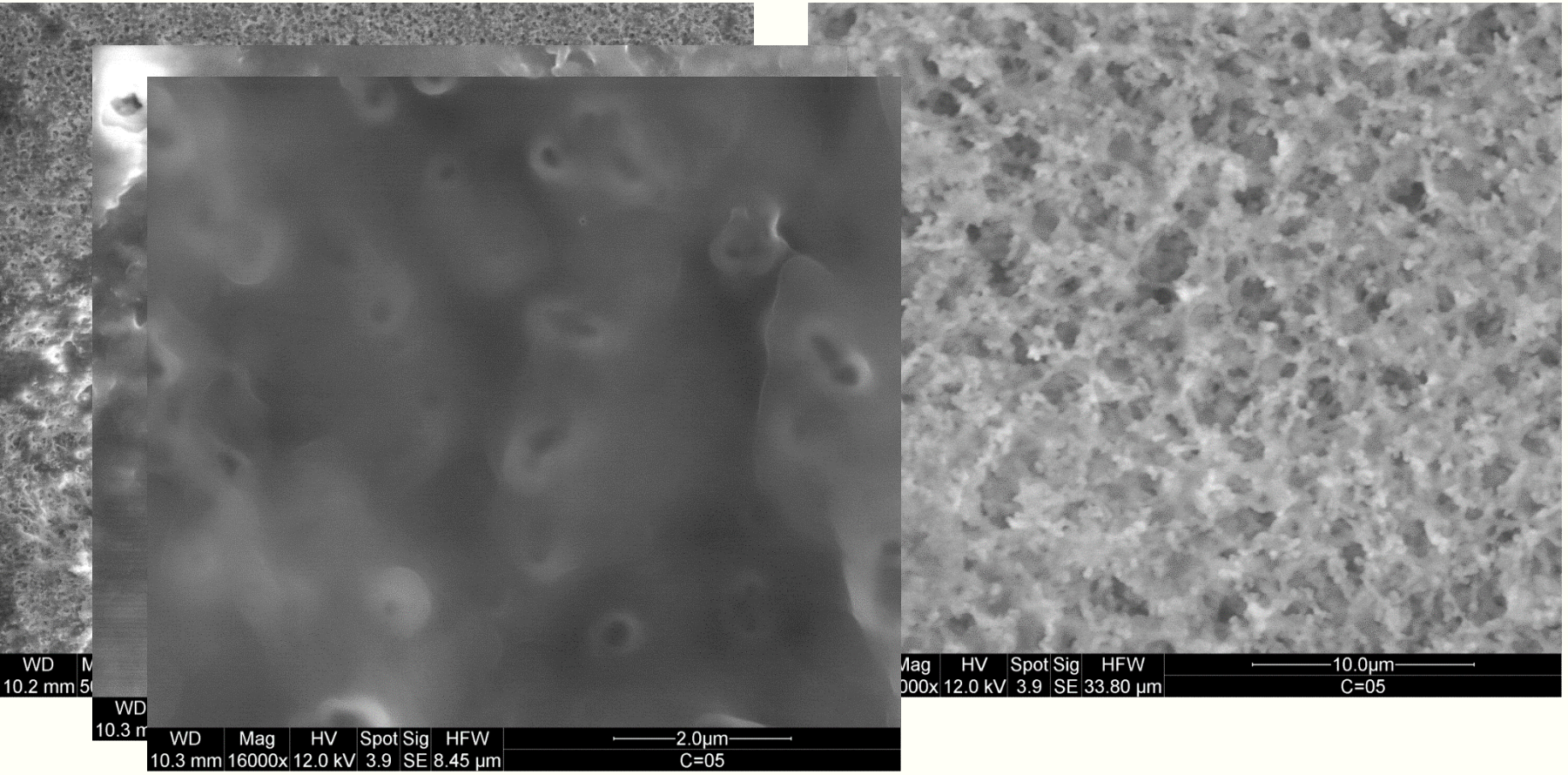
Previous result



- 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

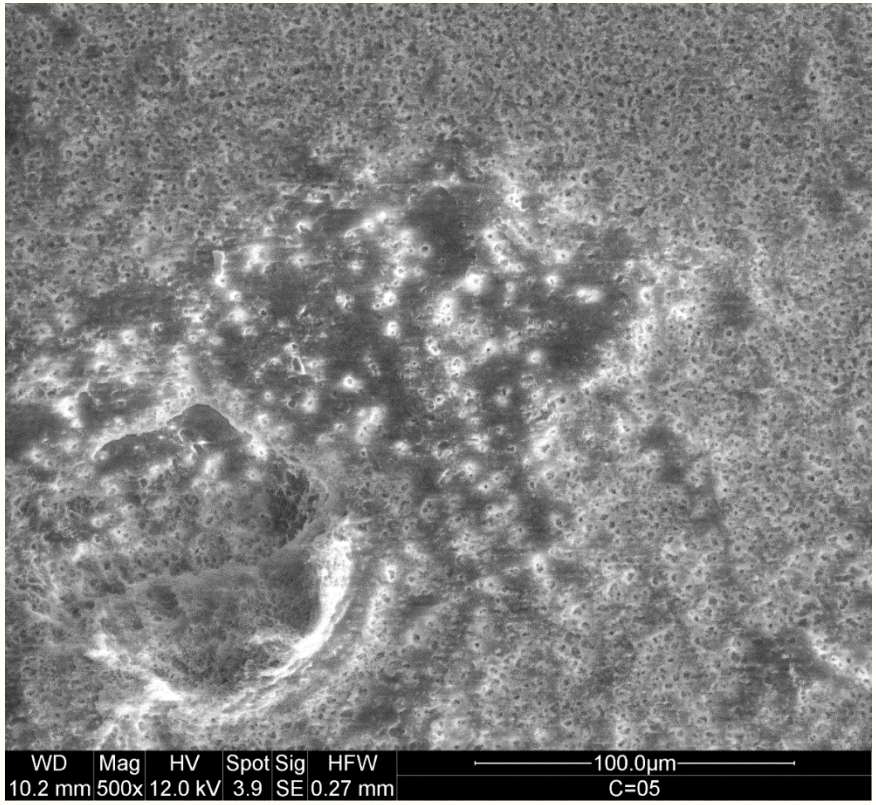
Viability study - Sonication

- 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



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The North American Society for Laser-Induced Breakdown Spectroscopy
Society for Applied Spectroscopy
The Spectroscopical Society of Japan (SPSJ)

Monday Afternoon, *Room 551A*
NUCLEAR FORENSICS
Organizer and Presider: Andrew Duffin

3:50 (181) **Applications of a New Single Stage Accelerator Mass Spectrometer to Trace Detection and Nuclear Forensics**; Albert Fahey[1], Kamron Fazel[1], Kenneth Grabowski[1], Evan Groopman[1]; [1]Naval Research Laboratory

4:10 (182) **X-Ray Microscopy of Nuclear Materials**; Jesse Ward[1], Greg Eiden[1], Andrew Duffin[1]; [1]Pacific Northwest National Laboratory

4:30 (183) **Advances in Analysis of Samples for Nuclear Non-Proliferation at CEA/DIF**; Bruno Bernard-Michel[1], Fabien Pointurier[1], Maxime Bridoux[1], Anne-Laure Faure[1], Amélie Hubert[1], Olivier Marie[1],Anne-Claire Pottin[1]; [1]CEA-DIF, Bruyères le Châtel

4:50 (184) **Discrimination of Uranium ore Concentrates from Several Countries by Chemometric Data Analysis**; Josette El Haddad[1], Aissa Harhira[1], Alain Blouin[1], Mohamad Sabsabi[1], Marvin Zaluski[2], Chunsheng Yang[2],Christopher Drummond[2], Slobodan Jovanovic[3], Tanya Hinton[3], Ali El-Jaby[3]; [1]National Research Council Canada - Energy, Mining and Environment; [2]National Research Council Canada - Information and Communications Technologies; [3]Canadian Nuclear Safety Commission

5:10 (185) **DC Arc Spectroscopy – Plasma Characterization for Direct Solid Analysis of Nuclear Materials**; Benjamin T. Manard[1], John Matonic[1], Robert Jump[1], Dennis Montoya[1], Alonso Castro[1], Ning Xu[1]; [1]Los Alamos National Laboratory

Monday Afternoon, *Room 556B*
NEAR IR
Organizer and Presider: Franklin E. (Woody) Barton

3:50 (186) **NIR With Problem Data Sets**; Franklin Barton[1], James de Haseth[1]; [1]Light Light Solutions Instruments, Inc.

4:10 (187) **A New Look at the Derivative Quotient Method in Regression**; David Hopkins[1], Karl Norris[2]; [1]NIR Consultant, Battle Creek, MI; [2]NIR Consultant, Beltsville, MD

4:30 (188) **Ultra-Compact Smart Spectrometers For Food, Agriculture, and Pharmaceutical Applications**; Nada OBrien[1], Christopher Pederson[1], Peng Zou[1]; [1]JDSU Corporation

4:50 (189) **A Novel Configuration for Near-Infrared Analysis of LPG Composition and Quality Control in a Refinery Setting**; Susan Foulk[1], Shashi Mistry[2], Terry Todd[1], Nate Peters[2], Dian Wang[2]; [1]Guided Wave, Inc.; [2]Suncor Energy

5:10 (190) **Field Analysis of Fuel using a Portable Near-Infrared Spectrometer**; Wayne Smith[1], Carl Brouillette[1], Chetan Shende[1], Stuart Farquharson[1]; [1]Real-Time Analyzers, Inc.

Monday Afternoon, *Ballroom E*
LIBS FOR PHARMACEUTICAL AND BIOLOGICAL DIAGNOSTICS
Organizer and Presider: Lydia Breckenridge

3:50 (191) **Recent Progress and Current Challenges in Using LIBS for Bacteriological Identification**; Steven Rehse[1], Dylan Malenfant[1], Derek Gillies[1], Vlora Riberdy[1], Anthony Piazza[1]; [1]University of Windsor

4:30 (192) **Laser-Induced Breakdown Spectroscopy for the Evaluation of Residual Catalysts in Pharmaceuticals**; Lydia Breckenridge[1]; [1]Bristol-Myers Squibb

4:50 (193) **Identification of Meat Species by using Laser Induced Breakdown Spectroscopy**; Gonca Bilge[1], Banu Sezer[1], Hasan Murat Velioğlu[2], Kemal Efe Eseller[3], Halil Berberoğlu[4], İsmail Hakkı Boyacı[1]; [1]Hacettepe University, Department of Food Engineering; [2]Namık Kemal University, Department of Agricultural Biotechnology; [3]Atılım University, Department of Electrical & Electronics Engineering; [4]Gazi University, Department of Physics

5:10 (194) **Study of Plasma and Identification of Hazardous Elements in the Polystyrene using Laser Induced Breakdown Spectroscopy**; W. Aslam Farooq[1]; [1]King Saud University

Monday Afternoon, *Room 552B*
PHARMACEUTICAL APPLICATIONS OF LOW WAVENUMBER SPECTROSCOPY
Organizer and Presider: James Carriere

3:50 (195) **Application of Low Frequency Raman During the Crystallization Process**; John Wasylyk[1], Ming Huang[1], Robert Wethman[1]; [1]Bristol-Myers Squibb Co.

4:10 (196) **The Contribution of the Low-Frequency Raman Spectroscopy to the Structural Description of Disordered Molecular Systems and Their Transformations: Application to Pharmaceuticals**; Alain Hedoux[1], Laurent Paccou[1], Yannick Guinet[1]; [1]University Lille 1, UMET - UMR CNRS 8207

4:30 (197) **Chemical Imaging of Crystalline Components in Pharmaceutical Dosage Forms by Using Low Frequency Raman Spectroscopy**; Toshiro Fukami[1], Motoki Inoue[1], Hiroshi Hisada[1], Tatsuo Koide[2]; [1]Meiji Pharmaceutical University; [2]National Institute of Health Sciences

4:50 (198) **Calibration of a Terahertz Analyzer for Predicting Solid Fraction in Roller-Compacted Ribbons and Tablets in a Small-Scale Piloting Study to Facilitate Pharmaceutical Formulation Development**; Mark Sullivan[1], Elaine Harrop Stone[2], Monwara Hoque[2], Xiao Hua Zhou[1], Richard McKay[1]; [1]Advantest America Inc; [2]Merlin Powder Characterisation Ltd

5:10 (199) **Low Wavenumber Raman Spectroscopy Applications in API Phase Discovery and Characterization**; Courtney Maguire[1], Andrew Brunskill[1]; [1]Merck Research Laboratories

Monday Afternoon, *Room 555B*
HOT TOPIC DISCUSSION SESSION – TERS RESOLUTION
Organizers: Duncan Graham, Pavel Matousek, and Ian Lewis;
Presider: Duncan Graham

3:50 (200) **Recent Advances in Tip-Enhanced Raman Spectroscopy**; Richard Van Duyne[1]; [1]Northwestern University

4:10 (201) **Resolution and Enhancement in TERS Microscopy**; Satoshi Kawata[1], Atsushi Taguchi[1]; [1]Osaka University

4:30 (202) **Molecular Structure Changes on the Nanometre Scale Investigated and Induced by TERS**; Volker Deckert[1,2]; [1]University of Jena; [2]Leibnitz Institute of Photonic Technology

4:50 **Discussion**

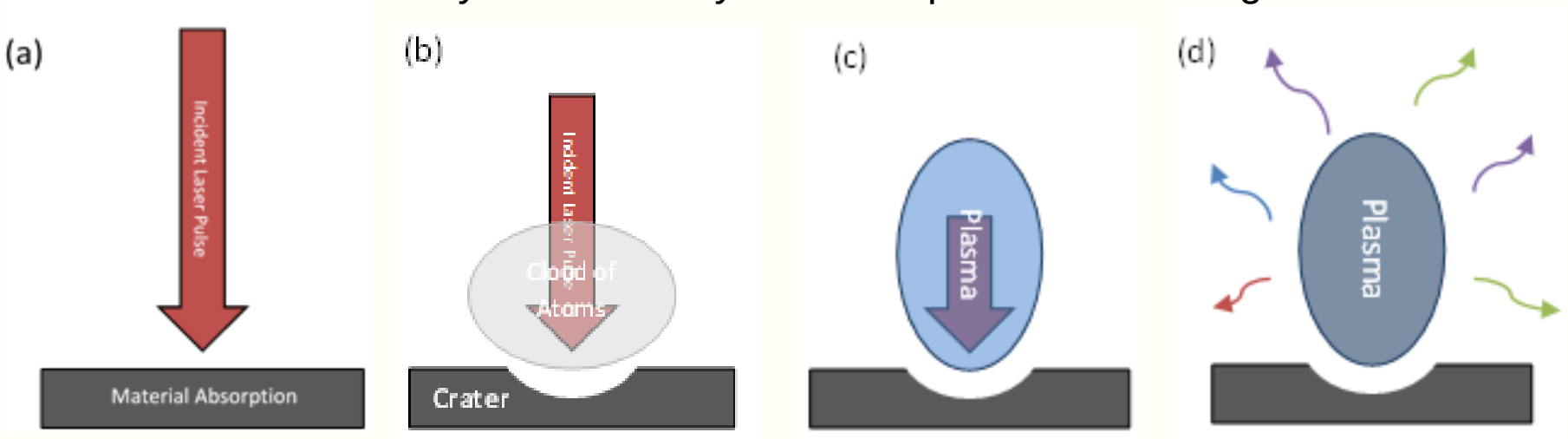
What is LIBS?

a) Intense laser pulse interacts with the target material and energy is absorbed.

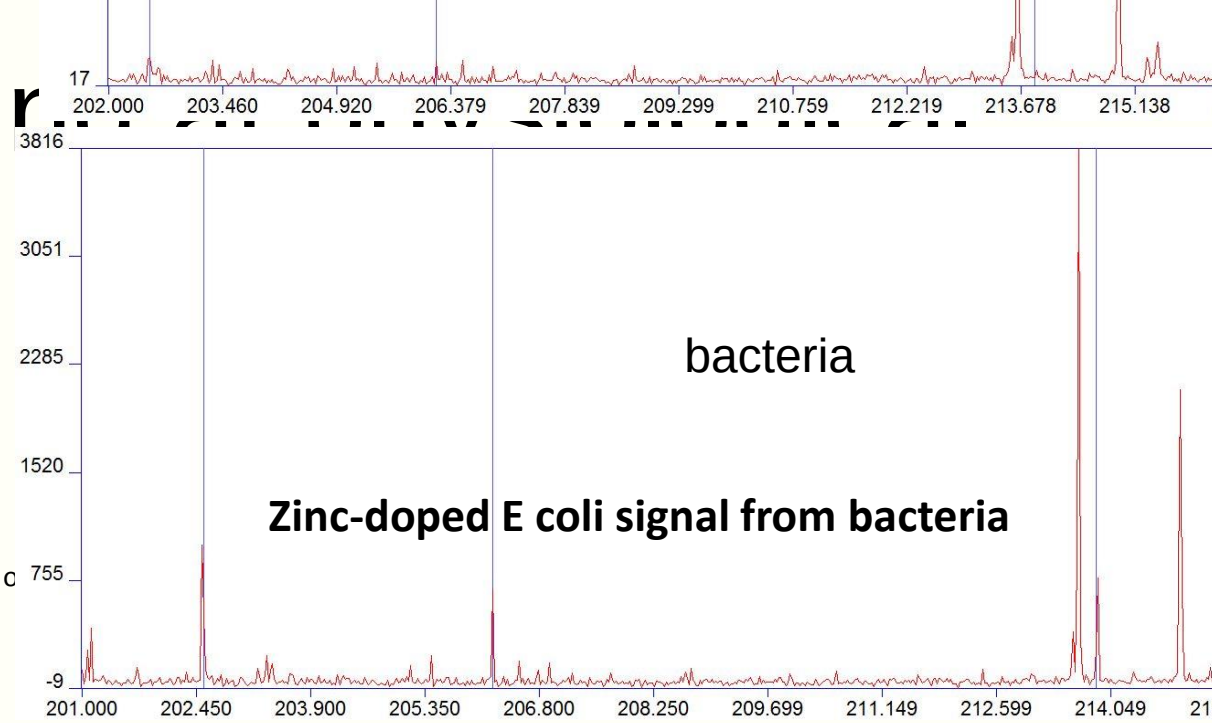
b) Energy absorbed results in heating and vaporization of the material. Matter from the surface is removed and a vapor is formed.

c) Laser pulse is still incident on the vapor. Energy is absorbed, inducing heating and plasma formation (with temperature of ~50,000 K).

d) As the plasma cools, atoms/ions/molecules decay by spontaneous emission. Light is collected and dispersed by the spectrometer. Resulting spectrum is analyzed to identify elements present in the target material.



Zinc dopi[illegible]



Label	Concentration c	
B		
S1		
S2		
S3	0.4	Not seen