A New Opportunity Using Elemental Microbiological Multi-variate Analysis for the In Situ Identification of Astrobiological Materials

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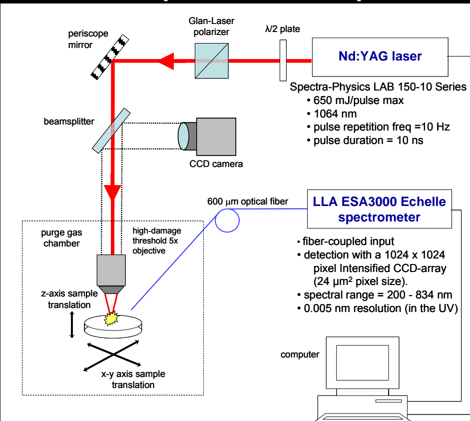
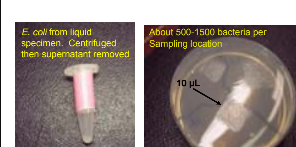
Introduction

It is known that LIBS is a sensitive spectrochemical technique that can rapidly determine a target's elemental composition with excellent spatial resolution and minimal sample preparation. It has only recently begun to be used for characterizing biological samples such as microorganisms (i.e. bacteria) and tissues. On the basis of a bacterium's unique elemental (mostly inorganic) composition, LIBS can be utilized to provide a potentially faster, more portable, and more robust technology than many other methods to perform rapid measurements which are useful for the detection and identification of harmful pathogens in real-time at the point-of-care.

We have already demonstrated that bacteria do possess unique atomic signatures that are robust through time and environment that can be measured via LIBS and that these signatures can be used to rapidly identify an unknown bacterial specimen – Elemental Multi-variate Microbiological Analysis. Extensive studies are now underway to determine the biological variability of these signatures, the effects of sample contamination and mixing, the limits of detection, the ultimate specificity, and to answer numerous other important questions to transition from a laboratory technique to a fieldable, clinical technology.

Experimental Setup





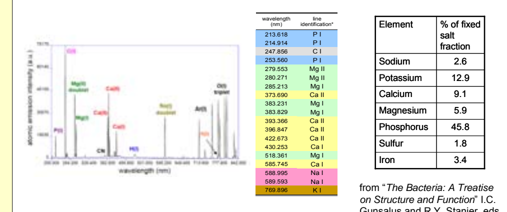
Testing Ablation Substrate

- Bacteria are grown overnight at 37°C in nutrient broth. Then they are streaked on plates for single colony isolation. Bacteria are incubated for 12 hours at 37 °C in an incubator. Samples are centrifuged and the super-natant discarded.
- 10 μL of pellet are trans-ferred to 1.4% nutrient–free bacto-agar.

Filters

- Alternatively, 10 mL of a bacteria containing sus-pension are pushed through a 0.22 μm Millipore microbiological filter.

LIBS Spectrum/Elemental Composition



wavelength (nm)	line identification*
213.618	P I
214.914	P I
247.856	C I
253.560	P I
279.553	Mg II
280.271	Mg II
285.213	Mg I
373.690	Ca II
383.231	Mg I
383.829	Mg I
393.366	Ca II
396.847	Ca II
422.673	Ca II
430.253	Ca I
518.361	Mg I
585.745	Ca I
588.995	Na I
589.593	Na I
769.896	K I

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

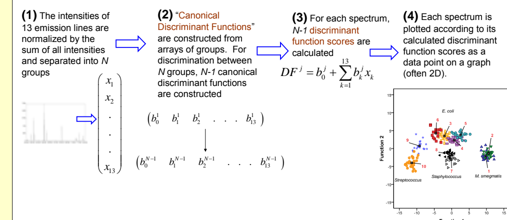
LIBS spectra obtained from bacteria prepared identically over the span of months demonstrate high reproducibility. When classified by EMMA the results are consistent over months/years. This suggests that inherent repeatability is not a limiting factor.

Chemometric Analysis

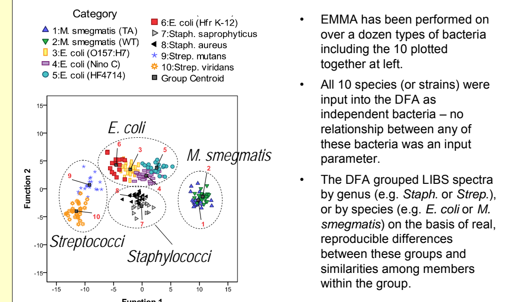
Numerous multi-variate chemometric routines have been applied to classify LIBS spectra.

- discriminant function analysis (DFA) / linear discriminant analysis (LDA)
- principal least squares–discriminant analysis (PLS-DA)
- principal component analysis (PCA)
- artificial neural networks (ANN)

How a DFA is Performed

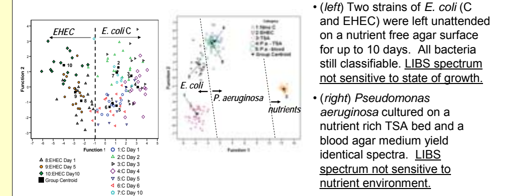


Specificity of LIBS Spectra



- EMMA has been performed on over a dozen types of bacteria including the 10 plotted together at left.
- All 10 species (or strains) were input into the DFA as independent bacteria – no relationship between any of these bacteria was an input parameter.
- The DFA grouped LIBS spectra by genus (e.g. *Staph.* or *Strep.*), or by species (e.g. *E. coli* or *M. smegmatis*) on the basis of real, reproducible differences between these groups and similarities among members within the group.

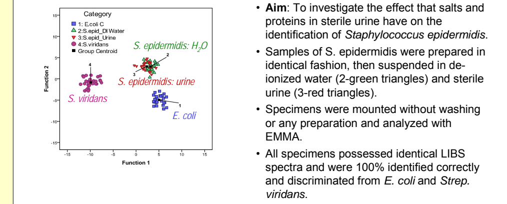
State of Growth / Nutrient Media



- (*left*) Two strains of *E. coli* (C and EHEC) were left unattended on a nutrient free agar surface for up to 10 days. All bacteria still classifiable. LIBS spectrum not sensitive to state of growth.
- (*right*) *Pseudomonas aeruginosa* cultured on a nutrient rich TSA bed and a blood agar medium yield identical spectra. LIBS spectrum not sensitive to nutrient environment.

Presence of Biochemicals

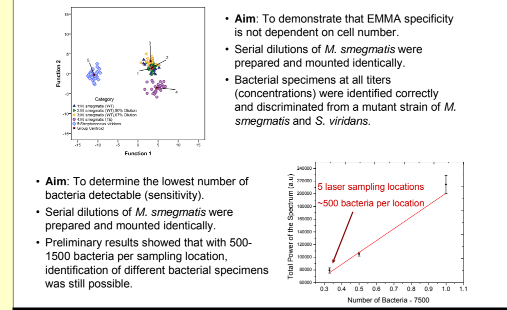
Along with other bacteria, biological samples may contain other biochemicals and/or inorganic salts which may obscure the bacterial signal.



- **Aim**: To investigate the effect that salts and proteins in sterile urine have on the identification of *Staphylococcus epidermidis*.
- Samples of S. epidermidis were prepared in identical fashion, then suspended in de-ionized water (2-green triangles) and sterile urine (3-red triangles).
- Specimens were mounted without washing or any preparation and analyzed with EMMA.
- All specimens possessed identical LIBS spectra and were 100% identified correctly and discriminated from *E. coli* and *Strep. viridans*.

Dilution Experiment / Linearity

Some microbiological tests are cell number-dependent. The LIBS total spectral power should be linearly dependent on cell-number, but specificity should not depend on cell number.

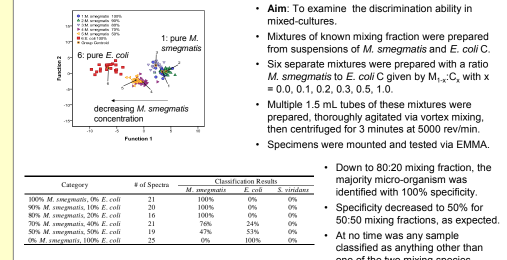


- **Aim**: To demonstrate that EMMA specificity is not dependent on cell number.
- Serial dilutions of *M. smegmatis* were prepared and mounted identically.
- Bacterial specimens at all titers (concentrations) were identified correctly and discriminated from a mutant strain of *M. smegmatis* and *S. viridans*.

- **Aim**: To determine the lowest number of bacteria detectable (sensitivity).
- Serial dilutions of *M. smegmatis* were prepared and mounted identically.
- Preliminary results showed that with 500-1500 bacteria per sampling location, identification of different bacterial specimens was still possible.

Mixing Experiment

Non-sterile samples may contain other types of bacteria which may contribute to the LIBS signature and obscure bacterial identification.



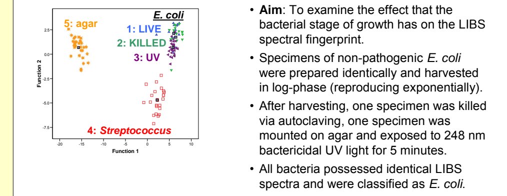
Category	# of Spectra	Classification Results		
		M. smegmatis	*E. coli*	*S. viridans*
100% *M. smegmatis*, 0% *E. coli*	21	100%	0%	0%
90% *M. smegmatis*, 10% *E. coli*	20	100%	0%	0%
80% *M. smegmatis*, 20% *E. coli*	16	100%	0%	0%
70% *M. smegmatis*, 40% *E. coli*	21	76%	24%	0%
50% *M. smegmatis*, 50% *E. coli*	19	47%	53%	0%
0% *M. smegmatis*, 100% *E. coli*	25	0%	100%	0%

- **Aim**: To examine the discrimination ability in mixed-cultures.
- Mixtures of known mixing fraction were prepared from suspensions of *M. smegmatis* and *E. coli* C.
- Six separate mixtures were prepared with a ratio *M. smegmatis* to *E. coli* C given by M_{1-x}:C_x with x = 0.0, 0.1, 0.2, 0.3, 0.5, 1.0.
- Multiple 1.5 mL tubes of these mixtures were prepared, thoroughly agitated via vortex mixing, then centrifuged for 3 minutes at 5000 rev/min.
- Specimens were mounted and tested via EMMA.

- Down to 80:20 mixing fraction, the majority micro-organism was identified with 100% specificity.
- Specificity decreased to 50% for 50:50 mixing fractions, as expected.
- At no time was any sample classified as anything other than one of the two mixing species.

Alive / Dead / Inactivated

In the environment or in vivo the bacteria may be in various stages of growth: actively reproducing (log phase), in stasis (lag phase), or may have been inactivated due to exposure to antimicrobials, UV light, starvation, etc.



- **Aim**: To examine the effect that the bacterial stage of growth has on the LIBS spectral fingerprint.
- Specimens of non-pathogenic *E. coli* were prepared identically and harvested in log-phase (reproducing exponentially).
- After harvesting, one specimen was killed via autoclaving, one specimen was mounted on agar and exposed to 248 nm bactericidal UV light for 5 minutes.
- All bacteria possessed identical LIBS spectra and were classified as *E. coli.*